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Investigation of the physiological stress indicators and immunocompetence status of road side insects in comparison to the insects of domestic gardens of Jabalpur city

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Abstract
The basic aim of the current study was to investigate the physiological stress indicators in haemolymph of insects of domestic gardens and road sides and relative analysis of the immunocompetence status of insects of roadside and domestic gardens of Jabalpur city to keep a check on their biodiversity loss and to assess their role in maintaining ecological balance. During the present study period insect samples were collected from 2 busy roadsides habitat and 10 domestic gardens of Jabalpur city to investigate the immunocompetence status of the insects of road sides and domestic gardens of Jabalpur city from July 2015 to September 2016. Haemolymph of insects were collected in ice cooled 1.5 ml microcentrifuge tubes prefilled with 300µl sodium cacodylate based anticoagulant followed by relative analysis of various commonly used physiological stress indicators in insect haemolymph such as total and differential haemocyte counts, total protein composition (in terms of refractive index),clotting capacity of hemolymph and protein profile to portray the immunocompetence status of the insects of road side in comparison to those found in the domestic gardens. During the current estimation of Heamolymph proteins of insects of all the 05 orders of road side insects by SDS-PAGE followed by Commassic blue and silver staining revealed the presence of the heat shock proteins in the insects which help to increase their resistance against adverse conditions of the roadsides. The present study indicated that the high values of THC of the adult insects of both roadsides and domestic garden correlated with less coagulation time which serve as indicator of good immunocompetence status. The present comparative study of insects of both the habitats indicates that the haematological parameters like DHC%, THC, serum protein content and clotting time of insects of domestic gardens were found to be lesser than that of the road side insects which indicates poor immunocompetence status of insects of domestic garden in comparison to the insects of roadsides of Jabalpur city.

Keywords: Road side insects, domestic gardens, anthropological effects, haemolymph parameters immunocompetence status

Introduction
Native vegetation near the roadsides or inside the domestic gardens may provide suitable habitat for diverse community of insects which serve as pollinators and as natural scavengers helping us in the removal, degradation and management of municipal and domestic wastes usually dumped on the roadsides and within the gardens in urban areas. Most of the researchers usually concentrate on biodiversity studies at a larger level and small habitats are left ignored. In these habitats the micro and macro invertebrates are exposed to extreme conditions like air pollution and thermal pollution from passing vehicles, construction works, bush fire during burning of wastes by municipal workers as well as various other anthropogenic impacts leading to the biodiversity loss of some of the valuable species of insects. This demands an incessant check on their biodiversity loss for sustainable development.

Insects constitute key indicators that enable the monitoring of the impact of urbanization on biodiversity, responding sensitively to changes in habitat extent and quality and to altered management practices associated with urbanization [1, 2]. Insects play key roles in nutrient cycling, organic matter decomposition, pollination and soil aeration in urban ecosystems [3]. Yet, although arthropod taxa, such as, ants and butterflies, are abundant in urban environments, little is known about how these are distributed within a city environment [4, 5].

The present study aims to evaluate changes in the composition of hemolymph to portray the

immunocompetence status of the insects of road side in comparison to those found in the domestic gardens. The quantification of changes in haemolymph chemistry can identify periods of elevated stress, particular stressors and reduced immunocompetence [6, 7]. Commonly used physiological stress indicators includes total and differential haemocyte counts (THC and DHC) [8], clotting capacity [9], total protein (typically measure das refractive index (RI) [10, 11] and ion concentrations (e.g., potassium, magnesium and calcium) [12].

Materials and Methods

During the present study period insect samples were collected from 2 busy roadsides habitat and 10 domestic gardens of Jabalpur city to investigate the immunocompetence status of the insects of road sides and domestic gardens of Jabalpur city from July 2015 to September 2016. Insects were killed in ice by dipping in anticoagulant or by suffocation on application of steam. Haemolymph were collected in ice cooled 1.5 ml microcentrifuge tubes prefilled with 300µl sodium cacodylate based anticoagulant followed by centrifugation at 10,000 rpm for 10 min in cooling microcentrifuge [4^0C]. Differential haemocyte count was determined as per the methods given by Jussila et al.[8], Total haemocyte counts by method given by S.R. Pugazhvendan and M. Soundararajan [13] and Clotting time was immediately assessed by a method given Jussila *et al* [8] with some modifications. Refractive index (for total protein) was determined by using a hand held refractometer, Total haemolymph protein (TP) concentrations was determined by modified Lowry method using a spectrophotometer. Estimation of Haemolymph protein was done by SDS-PAGE followed by silver staining to determine the molecular weight distribution of the insect protein fractions. For SDSPAGE 35-205kD marker [SRL] was used.

Results and Discussion

The present study revealed that the immunocompetence status of insects are directly guided by its haemolymph parameters which may in turn respond differently to various ecological factors. Some of the observations are discussed as follows-

Determination of Hemolymph Parameters

During the current study hemolymph parameters of 5 insect species and 1 larva representing 5 insect orders Ant[Hymenoptera],Grasshopper [Orthoptera],larva & butterfly [Lepidoptera], Beetles [Coleoptera] and cockroach [Blattodea]were determined as follows:-

Differential haemocyte counts

During current morphological examination of haemocytes of insects road sides and domestic gardens of Jabalpur city 4 distinct types of haemocytes were observed as prohemocytes, granulocytes, plasmatocytes, and adipohaemocytes (Fig:1) with significant difference in their differential counts (Table1). Several types of hemocytes have been described in insects by their morphological, cytochemical, and functional characteristics. The most common types of hemocytes are prohemocytes, plasmatocytes, granulocytes, spherulocytes, adipocytes, and oenocytoids. Their characteristics slightly differ in various insect species [14, 15].

Prohemocytes were small, round, oval, or elliptical with nuclei almost filling the entire cell i.e., cells have a high nuclear/cytoplasmic ratio. (Fig.1) Plasmatocytes appeared as elongated or spindle-shaped cells. with a rounded or elongate nucleus. (Fig1).Granulocytes were most easily distinguishable from other hemocytes with round and oval shape and centrally located nucleus surrounded by granules. (Fig1). Adipohaemocytes were found to be spherical, oval with small, round and centrally located nucleus surrounded by fat droplets (using light microscopy- 400X and 1000X magnification (Fig1). All through the differential count prohaemocytes were found to be dominant in most of the insects except grasshopper and larva of both roadsides and domestic gardens. The results are in compliance with the findings of the study of ultrastructure of the haemocytes of the silkworm, *Bombyxmori* L. found that prohaemocytes are spherical in shape, plasmatocytes were fusiform with elongated nucleus, granulocytes to be polymorphic in size and shape, spherulocytes were characterised by their oval shape and oenocytoids were large cells characterized mostly by round shape [16-18]. Plasmatocytes are pleomorphic cells and are accordingly rounded, fusiform or spindle shaped [19] Variable shapes of the plasmatocytes have also been reported with special emphasis on fusiform type [20, 21].

Total haemocyte count

During the present study, total haemocyte count assessed for different insects of road side and domestic gardens were found to be different (Table 2; Fig.3).On comparison of the 05 insect orders the THC of cockroach among the road side insects was found to be highest as 11,200 cells / $mm^3$of haemolymph while that of larva of order Lepidoptera of road side was found to be lowest as 3,200 cells / $mm^3$of haemolymph. Similarly THC of cockroach among the insects of domestic garden was also found to be highest as 11,600 cells / $mm^3$of haemolymph while that of larva of order Lepidoptera of domestic garden was found to be lowest as 3,000 cells / $mm^3$of haemolymph. High values of THC in both cases can be correlated with the less coagulation time. The total haemocyte count estimated for different breeds of silkworm, *Bombyx mori* L. showed significant differences among the breeds [17].

During the present relative study higher THC values were observed in adult insect of both roadsides and domestic garden in comparison to larval stages. The density of haemocytes (total haemocyte count) in insects generally depends upon the blood volume of the insects. [22]. The feeding efficiency of the larvae increases the haemocyte count in insects [23]. In different life stages of Mediterranean flour moth, *Ephestia kukniella* it was reported that the THC may normally vary greatly with amount of haemolymph, stages of development and physiological status of the insect [24]. The quantitative analysis of haemocytes have shown that the total number of haemocytes increase continuously throughout the past the embryonic development stages (from the third nympal instars to adult) reach a peak level in the adult stage, this is increasing number in T,H.C is correlated with an increasing demand for nutrient supply, cellular defense and production of immunologic factor [15].

Clotting time

The hemolymph clotting time was immediately assessed as soon as the legs were pulled off. During the present study, flow of hemolymph ceases and total clotting occurred within 10 seconds in all insects except the larva in case of both the insect types. Generally, clotting times do not exceed 2–3 minutes, and values exceeding this are recorded as "unclotted" [9, 12]. The hemolymph clotting time can be used as an indicator of stress in shrimp [25]. Similar findings with high THC values and lowest clotting time were observed in

the present study too in the insects belonging to both the habitats.

Analysis of Chemical parameters

Total haemolymph protein and refractive index of insect haemolymph

During the present study period, the concentration of total haemolymph protein and refractive index showed wide variation among the different insect species. The results of current study shows that among the roadside insects maximum percentage of protein in terms of refractive index was observed in the haemolymph of larva as 1.9 Brix % RI and total Protein as 8.0 mg/ ml while minimum values were observed as 0.4 Brix % RI and total Protein as 0.3 mg/ ml in the haemolymph of Butterfly. In case of insects of domestic garden insects maximum percentage of protein in terms of refractive index was observed in the haemolymph of Cockroach as 2.0 Brix % RI and total Protein as 3.2 mg/ ml while minimum values were observed similarly as 0.2 Brix % RI and total Protein as 0.2 mg/ ml in the haemolymph of Butterfly which can be correlated with the difference in the amount of haemolymph taken as sample (Table 3). The hemolymph refractive index increases during the prepupal stage [26]. Once the correlation between RI and total protein is known, the equation for the line of best fit can be used to convert future RIs for that species (and co– generics) in the field [27]. Haemolymph proteins from 15 species of brachyuran crabs were studied and among the species maximum protein content of 10.97% was observed in Scylla serrata and minimum protein content of 2.30% in Thalamita chaptali [28]. In the present findings the values of RI of larva of road sides were found to be more than that of the adult insects and in case of domestic garden the values of RI of larva was again found to be more than that of the adult insects except Cockroach which is a good indicator of healthier status of larva as well as cockroach [Table.3].

Analysis of Haemolymph protein

During the current estimation of Heamolymph proteins of insects of all the 05 orders of road side insects by SDS-PAGE followed by Commassic blue and silver staining revealed 4 bands of molecular weight 209,160,75 and 40 kDa in L1 [Grasshopper] representing 5.90 mg/ ml of total protein and 01 band of 30 kDa in L6 [Lepidopteran Larva] representing 8.35mg/ ml of total protein were revealed. Additional Bands of 75 kDa in L4 [Butterfly] representing 1.05 mg/ ml of total protein, 5 bands of 110,75,68,52 and 40 kDa representing 6.15 mg/ ml of total protein in L5[Beetles] and 209 kDa in L 8 [Cockroach] representing 3.05 mg/ ml Haemolymph proteins of total protein were visible after Silver staining. Bands were not observed in other lanes. (Fig.4) Additional SDS –PAGE of Heamolymph proteins of black yellow bug and giant bug collected from road side was also done. The bands reveals the presence of the heat shock proteins in the insects which help to increase their resistance against adverse conditions of the roadsides. Estimation of Heamolymph proteins of insects of domestic gardens by SDS-PAGE followed by Commassic blue and silver staining revealed bands in all the 06 lanes of 220 kDa representing 0.5,3.2,1.6, 1.4, 0.2,& 1.2 mg/ ml of total protein (Fig 5). Additional bands of 14 kDa were also visible in L3[Beetles], L4[Ants] & L6[Larvae] after Silver staining.(Fig.4) Hemolymph protein of P. interpunctella larvae were ectrophoretically separated into 10 different bands using Amido black stain (0.1% Amido black in 30% ethyl alcohol and 0.7% acetic acid) [29]. During study of biochemical changes in the hemolymph of Culex quinquefasciatus four bands with apparent molecular masses of 84, 81, 74 and 72 kDa (named Cq1, Cq2, Cq3, and Cq4, respectively) were observed as the major polypeptides of late fourth-instar larval fat body extracts. Cq1, 2, 3, and 4 are abundant in the hemolymph of early pupae, declining in concentration in the hemolymph of late pupae and adults [30].

Analysis of variance of differential haemocyte counts [DHC%] -

The value of analysis of variance of the DHC % of road side insects was found to be 19.8 and those of insects of domestic garden was found to be15.52. At d.f.(5,12)the table value of F at 5% level of significance equals to 3.11.Therefore the calculated value in case of both the type of insects were found to be more than the table value hence the difference in mean values of sample is highly. However more variations were observed in DHC% of insects of roadsides (Table 4, 5)

Table 1: Differential haemocyte counts [DHC%] of insects of road side and domestic garden

Types of Haemocytes	Ant		Cockroach		Grasshopper		Beetle		Butterfly		Larva	
	Road side	Domestic garden	Road side	Domestic garden	Road side	Domestic garden	Road Side	Domestic garden	Road side	Domestic garden	Road side	Domestic garden
Granulocytes	02	03	34	38	64	55	25	24	32	30	81	85
Prohaematocytes	71	80	57	58	13	24	54	56	43	48	02	04
Plasmatocytes	05	04	09	04	23	20	21	20	25	22	16	10
Adipohaemocytes	22	13	-		-	01	-		-		01	01

Table 2: Total haemocyte counts [THC in mm^3 of haemolymph] of insects of road side and domestic garden

Ant		Cockroach		Grasshopper		Beetle		Butterfly		Larva	
Road side	Domestic garden	Road side	Domestic garden	Road side	Domestic garden	Road side	Domestic garden	Road side	Domestic garden	Road side	Domestic garden
5,600	2,400	11,200	11,600	7,200	6,000	8,800	7,600	12,000	5,200	3200	3,000

Table 3: Table showing comparative values of Refractive index (in Brix %) and Total Protein conc. (µg/ ml) of haemolymph of insects of road side and domestic gardens

S. No.		Road Side Insects			Insects of Domestic Gardens		
		Absorbance at 660 nm	Total Protein conc. in sample(mg/ ml)	Refractive index values (in Brix %)	Absorbance at 660 nm	Total Protein conc. in sample(mg/ ml)	Refractive index values (in Brix %)
1.	Ant	0.432	2.0	0.6	0.173	0.5	0.4
2.	Cockroach	0.456	2.3	1.6	0.528	3.2	2.0
3.	Grasshopper	0.856	5.8	1.2	0.390	1.6	1.5
4.	Beetle	0.909	6.5	1.8	0.352	1.4	1.6
5.	Butterfly	0.185	0.3	0.4	0.147	0.2	0.2
6.	Larva	1.234	8.0	1.9	0.310	1.2	1.7

Table 4: Table showing calculation of Sum of square between the sample and within the sample of road side insects –

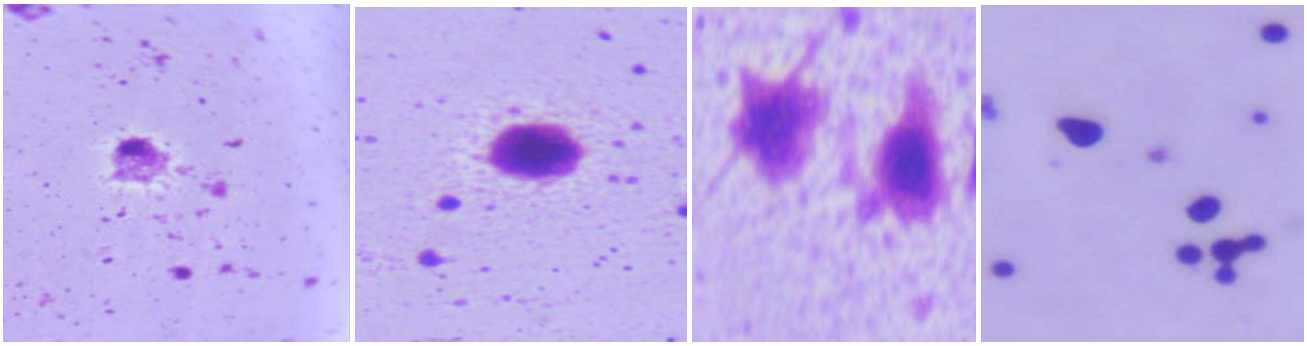
Source of variance	Sum of square	Degree of freedom	Mean square
Between the sample	9008	C-1 = 6-1 =5	9008/5 =1801.6
Within the sample	1088	N-C (18-6) =12	1088 / 12 = 90.6

Table 5: Table showing calculation of Sum of square between the sample and within the sample of insects of domestic gardens

Source of variance	Sum of square	Degree of freedom	Mean square
Between the sample	9153	C-1 = 6-1 =5	9153/5 =1830.6
Within the sample	1415	N-C (18-6) =12	1415 / 12 = 117.9

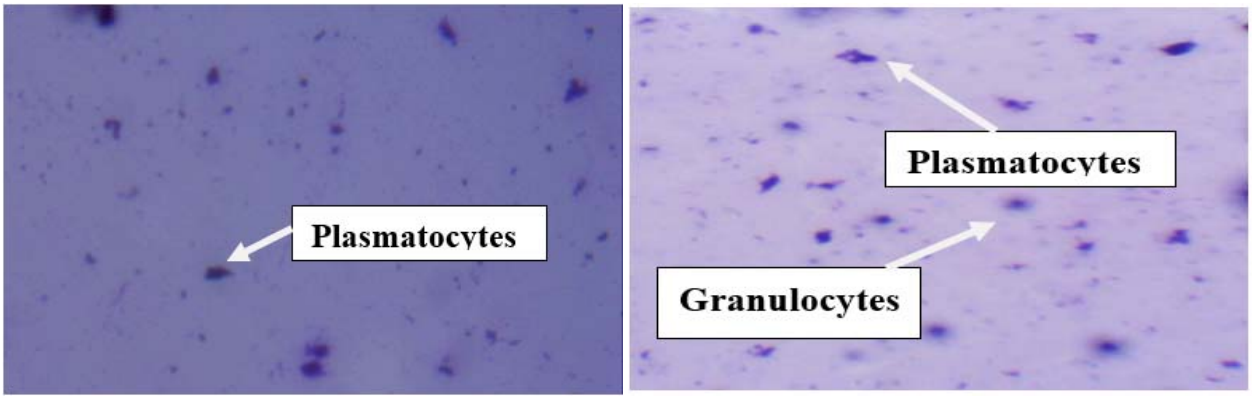
$$F = \frac{\text{Variance between the sample}}{\text{variance within the sample}} = \frac{1801.6}{90.6} = 19.8$$

$$F = \frac{\text{variance between the sample}}{\text{variance within the sample}} = \frac{1830.6}{117.9} = 15.52$$



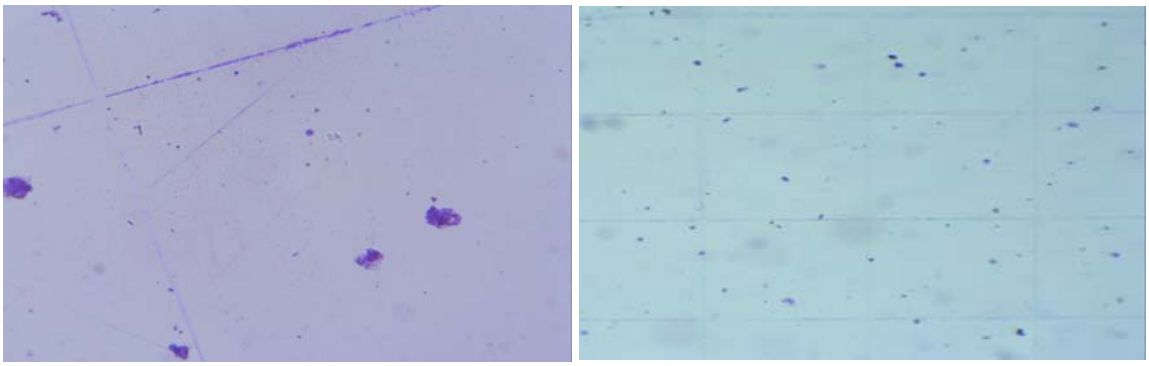
Adipohaemocytes Granulocytes Plasmatocytes Prohaemocytes

Fig 1: Differential Haemocyte count [1000X magnification]



Grasshopper Butterfly

Fig 2: Differential Haemocyte count of Grasshopper [Roadside] and Butterfly [Domestic garden]- [400X magnification]



Cockroach [Domestic garden] Ant[Road side]

Fig 3: Total Haemocyte count of Cockroach & Ant[400X magnification]

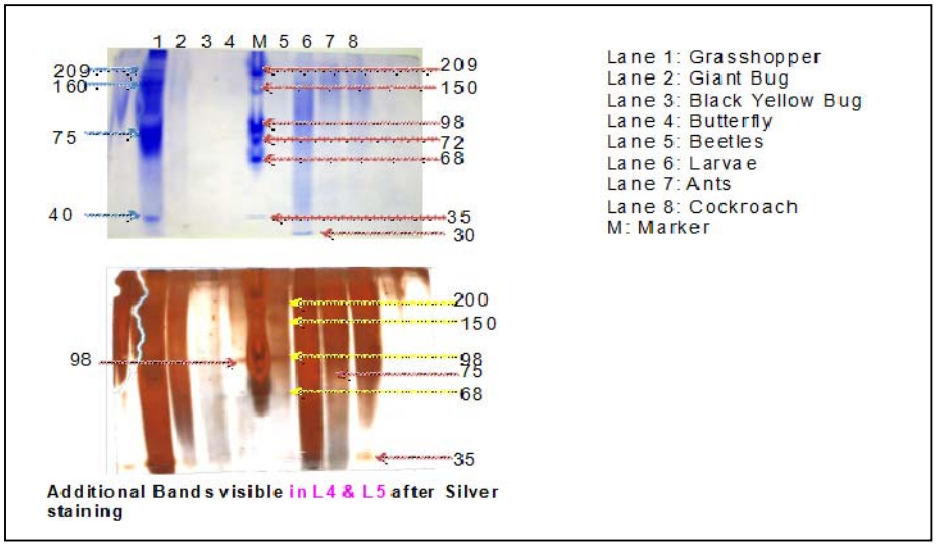


Fig 4: Estimation of Heamolymph protein by SDS-PAGE [Road side insects]

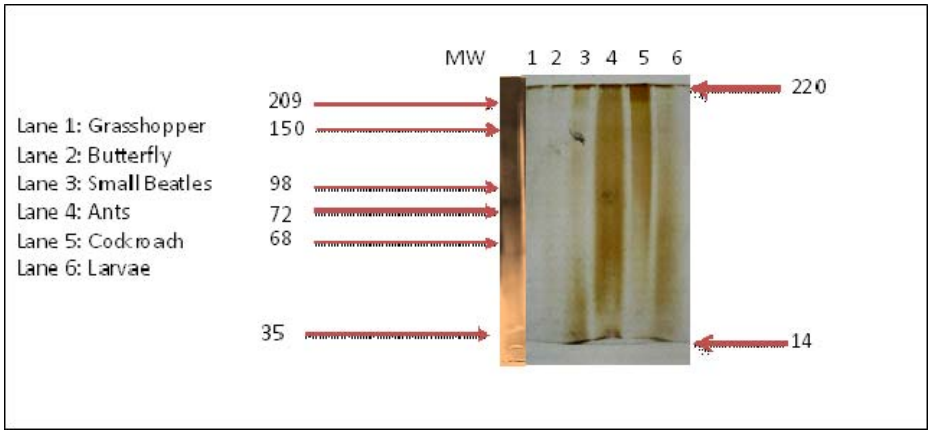


Fig 5: Estimation of Heamolymph protein by SDS-PAGE [Domestic garden insects]

Conclusion

The present study conclude that the high values of THC of the adult insects of both roadsides and domestic garden correlated with less coagulation time can serve as indicator of good immunocompetence status**.** Also the present comparative study of insects of both the habitats indicates that the haematological parameters like DHC%, THC, serum protein content and clotting time of insects of domestic gardens were found to be lesser than that of the road side insects which indicates poor immunocompetence status of insects of domestic garden in comparison to the insects of roadside of Jabalpur city.

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