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Haemocytes count of *Poekilocerus pictus* (Fabr.) (Orthoptera: Acrididae) during fungal infection

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Abstract
Insect circulating haemocytes are primarily responsible for the immune defense against parasites and pathogens**.** In this paper THC and DHC after *Aspergillus niger* infection were elucidated**.** Injection of *A. niger* conidia into *P. pictus* resulted in changes in the total haemocyte count and differential haemocytes counts. Total haemocyte counts were higher at 2h and 4h post infection and lower at 8h and 24h post infection than control insects. There was a considerable change in the relative percentage of granulocytes and plasmatocytes in the hemolymph after challenge with *A. niger*. It is observed that plasmatocytes and granulocytes are the principal cell types, which respond the most during the defense.

Keywords: THC, DHC, *Poekilocerus pictus*and *Aspergillus niger.*

Introduction
Insects represent the largest class within the animal kingdom in terms of species number. The resistance of insects to pathogens has certainly contributed to their extreme proliferation and diversity. At present, insects are found in most of the biological niches except for the deep marine environment and the polar regions. More than one million of insect species have been described and it is estimated that an equivalent number of species remains to be identified.
Multicellular animals defend themselves against infectious organisms by two systems known as innate and acquired immunity. The innate immune system relies on germline encoded factors for recognition and killing of foreign invaders, whereas the acquired immune system produces receptors by somatic gene rearrangement that recognize specific antigens and that allow organisms to develop an immunological memory (Fearon, 1997)[11]. Insects lack an acquired immune system but have a well-developed innate response. Initial defences include the physical barriers of the integument or gut, clotting responses by hemolymph, and the production of various cytotoxic molecules at the site of wounding. Foreign entities that pass these barriers and enter the hemocoel must contend with additional cytotoxic molecules as well as an array of different haemocytes. The insect immune system is further subdivided into humoral and cellular defence responses. Humoral defences include the production of antimicrobial peptides (Meister *et al*., 2000; Lowenberger, 2001)[26, 25], reactive intermediates of oxygen or nitrogen (Bogdan *et al*., 2000; Vass and Nappi, 2001)[4], and the complex enzymatic cascades that regulate coagulation or melanisation of hemolymph (Muta and Iwanaga, 1996; Gillespie *et al*., 1997)[27, 13]. In contrast, cellular defense refers to haemocyte-mediated immune responses like phagocytosis, nodulation and encapsulation (Strand and Pech, 1995; Schmidt *et al*., 2001) [35, 31]. While great progress has been made over the last several years in identifying antimicrobial peptides and the signaling pathways that regulate their synthesis, much less is known about control of cellular defense responses. This is due in large part to the small size of many insects, which makes collection of haemocytes and identification of haemocyte-produced effector molecules difficult. It is also often difficult to conduct manipulative experiments on haemocyte-mediated defense responses *in vivo* or to isolate defined populations of haemocytes for use in experiments conducted *in vitro.*
The main effectors of cellular immune responses in insects are the blood cells or haemocytes. Many studies have been made of haemocyte counts, either as the total haemocyte count (THC) or differentially according to type (DHC). It is well attested that both THC and DHC change rapidly during immune responses to infection (Lackie, 1988) [24]. Taubereager (1935,1936)[36, 37] and Beard (1945) [3] noted an increase in THC

in the hemolymph of insects due to bacterial infection. But many authors have observed drastic reduction in the number of haemocytes during bacterial infections (Kostritsky *et al* 1924; Babers, 1938; Wittig, 1965, Krishnan and Chaudhuri, 1998, 1999; Krishnan *et al.,* 2000)[20, 2, 21, 22, 23]. In bacterial-infected *Prodenia* larvae, haemocytes counts either remained unchanged or declined markedly; thus Rosenberger and Jones (1960)[30] concluded that the haemocytes are not very effective in protecting the insect. In *Gallaria*, THC increased upto 48 hours following infection with *Staphylococcus* and decreased rapidly (Werner and Jones, 1969)[38]. High doses of infection in *Pseudoletia* larvae resulted in drastic reduction in plasmatocytes and a great increase in spherulocytes and prohaemocytes, with no change in the number of granulocytes (Wittig, 1966)[40]. Low doses, on the other hand, had a different effect on the blood picture because the granulocytes and plasmatocytes decreased while the spherulocytes and prohaemocytes increased significantly (Wittig, 1966)[40]. Krishnan and Chaudhuri (1998; 1999)[21, 22] and Krishnan *et al* (2000)[23] observed a reduction in both plasmatocytes and granular cells of *B. mori* infected with bacteria.

Poekilocerus pictus (Fabr.) is an orthopteran insect known as a monophagous pest of the medicinal plant ak (*Calotropis* sp.). Its large size makes this insect easy to manipulate physically, enabling easy injection of substances. Another advantage to the large size of *P. pictus* is the ability to collect between 0.5-1 ml of haemolymph from each insect meaning that fewer insects are required for experiments.

In the present study chief defensive cells involved in celluler immune response against *Aspergillus niger* infection was elucidated. As notmuch work has been done on *P. pictus* regarding insect immunity against bacterial and *Aspergillus niger* infection. The more detailed knowledge of *Poekilocerus pictus* defense response will help us to achieve greater success in our efforts for biological control of insect population and to explore insect immunity in general. Infact this study encompasses comprehensive investigation into immune response of *P. pictus* which will help to clarify, some of unresolved issues in insect immunity against *Aspergillus niger* infection with respect to the chief haemocytes involved in defense response and the insight into basis and mechanism of defense reactions in *Poekilocerus pictus.*

Materials and methods

Insect collection and its rearing

Grasshoper, *Poekilocerus pictus* (Orthoptera: Acrididae) were collected from *Calotropis* plants from different locations around Sagar (M.P.). They were maintained under laboratory condition (25-30ºC) and fresh *Calotropis* leaves were provided daily for feeding. Insects were kept in wide mouth bottles in the laboratory and marked for their ages. 4-5 day old adult females of *P. pictus* were used in all experiments.

Culture of a *Aspergillus niger* obtained from thefaecal matter of *P. pictus* was used as natural pathogen and sabourad dextrose agar was purchased from himedia. Fungus *Aspergillus niger* was grown in sabourad dextrose agar media at 28 ^{0}C in bacteriological incubator. 10^5 conidia/ml were used in the all experiments

Method of Injection

Injection of fungus *Aspergillus niger* for THC and DHC studies

Insects were surface sterilized by swabbing their surfaces with 70% ethanol. Control insect were injected with 10 µl of sabouraud dextrose broth and test insects were infected by injecting a standard fungal dosage of 10^5 CFU/insect, fungus *Aspergillus niger* were injected in 10µl aliquots, using a 25 µl Hamilton syringe

Collection of hemolymph for THC and DHC studies

For the counting of the total haemocyte count (THC), the arthropodial membrane of the legs was first swabbed with 70% ethanol, allowed to air dry and then pierced with a sterile needle and hemolymph was collected and diluted 20 times in saline versene (NaCl-.9g KCl-.942g $CaCl_2$ -.082g $NaHCO_3$ -.002g D.W.- 100ml + 2% versene) and transferred immediately to an improved Neubauer haemocytometer. The number of cells were counted under the Olympus light microscope and calculated by the formula suggested by Jones (1962)[19]. THC was done at 2h, 4h, 8h and 24hpostinfection in bacterial infected *Poekilocerus pictus* and fungus infected *Poekilocerus pictus*.

For the counting of the relative number of different types of haemocytes (differential haemocyte count, DHC), the arthropodial membrane of the legs was first swabbed with 70% ethanol, allowed to air dry and then pierced with a sterile needle and haemolymph was collected by bleeding a drop of haemolymph onto a glass microscope slide then expelled onto a glass microscope slide. The slide was then incubated for 10 min at room temperature to allow the haemocytes to adhere to the slide. The glass slide with the air dried film were immersed in Giemsa solution for 20 minutes to 2 hours (1drop of concentrate per milliliter distill water) and then mounted. The haemocytes were observed at 400X magnification using an Olympus light microscope. Each time 100 cells were counted and percentage of various haemocytes was determined. The method of Shapiro (1966) [32] was used to count relative number of different types of haemocytes. DHC was done at 2h, 4h, 8h and 24hpostinfection in bacterial and fungal infected *Poekilocerus pictus.* The experiment was repeated five times using completely random design (CRD). Data were expressed as the mean ± standard error of mean.

Statistical analysis

The data were expressed as mean ± Standard error. Statistical analysis of data obtained from the THC and DHC were analyzed through one way analysis of variance (ANOVA) and Dunnet multiple comparison test. The level for significance was taken as * $P \leq 0.01$, ** $P \leq 0.001$ and *** $P \leq 0.0001$

Results

Total Haemocyte Count (THC)

Total Haemocyte count (THC) was observed at 2h, 4h, 8h and 24h post infection in fungal infected insects (Table 1 and Fig.1). THC increases at 2h and 4h post infection and decreases at 8h and 24 h post infection when compared to control insects in fungal infected insects.

Statistical analysis showed that the value of THC had significantly varied at every time points in fungal infected insects when compared to control insects. (Table 1)

Total haemocyte count (THC) in control and *Aspergillus niger* treated *P. pictus*.

- The mean ± Standard error of total haemocyte count was observed to be 5.17±0.08 ×10^3 per mm^3 in control *P. pictus*
- The mean ± Standard error of total haemocyte count was observed to be 6.21±0.05*×10^3 per mm^3 at 2h p.i.
- The mean ± Standard error of total haemocyte count was observed to be 6.46±0.04*×10^3 per mm^3 at 4h p.i.
- The mean ± Standard error of total haemocyte count was observed to be 4.08±0.05*×10^3 per mm^3 at 8h p.i.
- The mean ± Standard error of total haemocyte count was observed to be 4.09±0.11**×10^3 per mm^3 at 24h p.i.

Table 2: Total haemocyte count at different time points post injection in control and *A. niger* treated *P. pictus*

Treatment	No. of Insect	Total haemocyte count (×10^3 per mm^3)(mean ± S.E.)				
		Control	Treated(Time points post injection)			
			2 h	4 h	8 h	24 h
Aspergillus niger (fungus)	5	5.17±0.08	6.21±0.05*	6.46±0.04*	4.08±0.05*	4.09±0.11**

Values are expressed as means of five different counting ± S.E.
S.E.: Standard error
* P ≤ 0.01
** P≤ 0.001

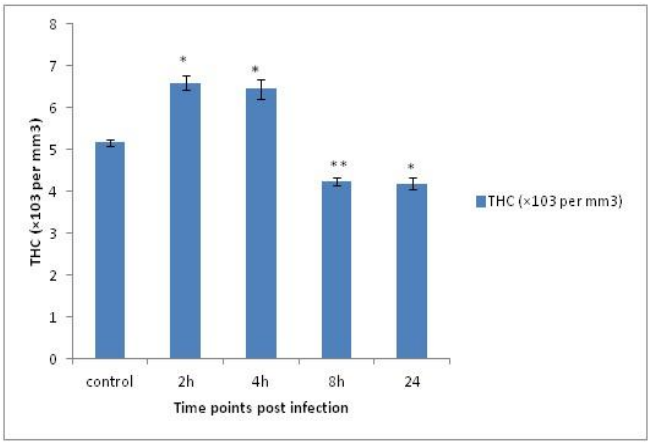

Fig 1: Total haemocyte count in control and *A. niger* treated *P. pictus* Differential Haemocyte Counts (DHC)

In the present study differential haemocyte counts were examined to observe the changes in the population of different haemocyte types in defense response.
The DHC was observed in insects after injection of fungus. It is observed that plasmatocytes and granulocytes are the principal cell types, which respond the most during the defense.

The relative percentage of plasmatocytes show sharp and marked decline in plasmatocytes when compared to controls in treated insects at 2 h to 24 h p.i. The relative percentage of granulocytes in treated insects gradually increases from 2 h to 24 h p.i. when compared to controls. (Figs.2) However the relative percentage of prohaemocytes varies among different time points post injection and shows significant difference at 24h p.i. when compared to controls. The relative percentage of spherulocytes shows significant difference at 24h p.i. when compared to controls. The relative percentage of adipohaemocytes did not show any deviation when compared with controls.

The relative percentage of different types of haemocytes in control and *Aspergillus niger* treated *P. pictus* are as follows (Table 2 and Fig.2)

- The relative percentage of prohaemocytes in control insects is 5.60% and treated insects at 2h, 4h, 8h and 24 h p.i. are 5.20%, 8.80%, 7.20% and 6.80% respectively.
- The relative percentage of plasmatocytes in control insects is 60.00% and treated insects at 2h, 4h, 8h and 24h p.i. are 30.00%, 30.80%, 30.60% and 22.20% respectively.
- The relative percentage of granulocytes in control insects is 25.40% and treated insects at 2h, 4h, 8h and 24h p.i. are 55.20%, 60.80%, 57.80% and 60.80% respectively.
- The relative percentage of spherulocytes in control insects is 10.40% and treated insects at 2h, 4h, 8h and 24h p.i. are 8.80%, 7.60%, 9.80% and 9.80% respectively.

Table 2: The relative percentage of different types of haemocytes in control and *A. niger* treated *P. pictus*

Haemo-cyte type	No. of insects	Relative % of haemocyte types (mean ±S.E.)				
		Control	Treated(Time points post injection)			
			2 h	4 h	8 h	24 h
PR	5	5.60±1.03	5.20±0.86	8.80±1.15	7.20±1.39	6.80±1.65
PL	5	60.00±3.53	30.00±1.70**	30.80±2.41**	30.60±0.92**	22.20±2.49**
GR	5	25.40±1.72	55.20±3.26**	60.80±3.02**	57.80.±3.00**	60.80±2.41**
SP	5	10.40±1.32	8.80±1.39	7.60±1.36	9.80±1.15	9.80±1.42
AD	5	1.00±0.00	1.33±0.33	2.00±0.00	1.00±0.00	3.33±1.20

- PR- Prohaemocytes; PL- Plasmatocytes; GR- Granulocytes; SP- Spherulocytes; and AD- Adipohemcoytes.
- Values are expressed as means of five different counting ± S.E.
- S.E.: Standard error
- * $P \leq 0.01$
- ** $P \leq 0.001$

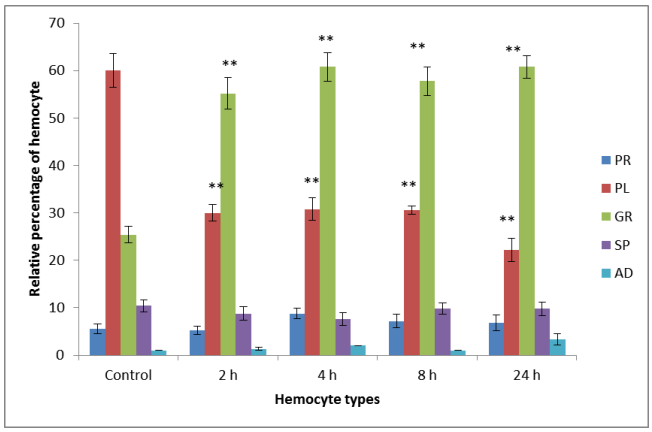

Fig 2: Bar diagram showing the relative percentage of different types of haemocytes in control and *A. niger* treated *P. pictus*

Discussion

In the present study Cellular changes such as THC (Total Haemocyte counts), DHC (Differential Haemocyte Counts) involved in the defense response after fungal infection were elucidated.

The THC seems to be affected in various ways after injection of bacteria and fungi in the haemocoel. It was observed that total number haemocytes differed variably at different time points post injection. THC increases progressively at 2 h to 4 h p.i. and decreases at 8 h to 24 h p.i. This increase in THC was probably due to the requirement of plasmatocytes and granulocytes in defense response. However at 8 h to 24 h p.i. there is a significant decrease with lowest number of THC in adult female when compared to controls. This decrease in THC was probably due to the involvement of plasmatocytes in nodulation response.

The present findings agree with Da silva *et al*., (2000)[9] that total haemocytes counts in mosquitoes inoculated with *C. albicans* increased gradually at 6 h p.i. and then decreased in a similar manner until 24h p.i.

The results of present study are in partial agreement with those of Gillespie *et al*., (2000)[12] that increased THC after injection of *M. anisopliae var acridium* in locust *Schistocerca gregaria* in the first 2 days but declined over the next 2 days due to the formation of nodules in the insect, whereas disagrees with the results of Weisner (1991)[39] who observed that 30 min after injection of latex beads THC was decreased in *Galleria mellonella*.

Wiesner (1991)[39] observed that at 30 minutes after injecting latex beads, total number of free floating haemocytes was reduced because of a great loss in plasmatocytes (PLs) and granular cells (GRs) in *Galleria mellonella*. In contrast to these findings, the present study shows that at 2 h to 4 h p.i. THC increases because of the increase in GRs when compared to PLs, which had decreased in number.

In the present study THC increases progressively at 2 h to 4 h p.i. and decreases at 8 h to 24 h p.i.. These findings are in partial agreement with those of Hoffmann *et al*., (1974)[17] as they showed decrease in THC after injection of *Bacillus thuringiensis* (with the culture medium) in male adults of *Locusta migratoria* and also in partial agreement with Strand and Noda (1991)[34] as they also showed that total haemocyte counts were higher in parasitized larvae than unparasitized larvae in *Pseudoplusia includens* after parasitism by *Microplitis demolitor*.

The decrease in THC in the present study was probably due to the involvement of plasmatocytes in nodulation response. However the results disagree with those of Anandakumar and Michael (2011)[1] as they showed that increase was observed in differential haemocytecount (DHC)of plasmocytes alone in *Bombyx mori* L. inoculated with *Bacillus thurungenses*.

In the present study the largest population of cells were the plasmatocytes followed by granulocytes, prohaemocytes, spherulocytes.

It was observed that plasmatocytes and granulocytes are the principal cell types, which respond during defense. there was a decline in the relative percentage of plasmatocytes in adult females from 2 h to 24 h p.i. when compared with controls there is decline in plasmatocyte percentage as compared to controls. This decrease in PL was probably due to their involvement in defense response such as nodulation. In confirmation with the present findings Chain & Anderson, 1983[7]; Gunnarsson, 1988[15]; Pech & Strand, 1996 [29]. Also noted decline in PL titre in diseased insects.

In the present study the relative percentage of granulocytes increased from 2 h to 24 h p.i. as compared to controls. This increase in GR was probably due to their continuous involvement and their requirement in nodulation. However in contrast to the present study Gotz & Boman (1985) [14]; Pech & Strand (1996)[29] and Gillespie *et al*., (1997)[13] observed decline in GR, which was due to their involvement in nodule formation.

In the present findings GR increases from 2 h to 24 h p.i. which is in partial agreement with Borges *et al*., (2008) [5] who described that prohaemocytes decrease whereas plasmatocytes and granulocytes increase from 30 min to 120 min p.i. in latex beads inoculated *R. prolixus*.

The contribution of both granular cells and plamatocytes in cellular defense reactions in haemolymph are broadly recognized in insects (Gotz and Boman, 1985; Brehaelin and Zachary, 1986; Gupta, 1991; Pathak, 1993; Drif and Brehaelin, 1993)[14, 6, 16, 10, 10]. This is in confirmation with the present results where DHC shows that the plasmatocytes and the granulocytes are the principal cells to be affected.

In the present study plasmatocytes and granulocytes were the principal cell types, which responded during defense. There was a decline in the relative percentage of plasmatocytes in adult females from 2 h to 24 h p.i. when compared to controls. This result is in conformation with Chain and Anderson (1982)[8] as they also noted disappearance of plasmatocytes from the haemolymph after the injection of a suspension of a bacterial pathogen, *Bacillus cereus*, into larvae of the wax moth, *Galleria mellonella*. However it disagree with Papadopoulou-Karabela *et al*., (1992) as they showed that plasmatocytes were higher at 10 h after infection in honeybees infected with *Pseudomonas aeruginosa*.

In the present study the relative percentage of plasmatocytes decreased and relative percentage of granulocytes increased from 2 h to 24 h p.i. when compared to controls. In contrast to the present findings Silva *et al*., (2000)[33] showed that the relative proportion of plasmatocytes was higher and, concomitantly, the proportion of granular cells was lower in differential haemocyte counts from *Culex quinquefasciatus* (Diptera: Culicidae) against *Candida albicans* infection at 3, 6, and 18 h after inoculation.

Borges *et al*., (2008)[5] observed significant increase in the percentage of granulocytes and plasmatocytes at 60 min and

120 min p.i. whereas significant decrease in percentage of prohaemocytes in latex-treated insects was observed. This result is in partial agreement with the present findings where PL decreased but number of granulocytes increased at 2 h p.i. to 24 h p.i.

Decline in the THC of insects during fungal infection have been recorded before Gunnarsson, (1988)[15] and Hung and Boucias, (1992)[18]. However, the initial increase in THC observed in the present work appears to be a novel observation. The subsequent decline in THC observed in this study may result in part from the formation of nodules induced by soluble fungal metabolites since there was a significant inverse correlation between THC and nodule count.

References

1. Anandakumar MD, Michael AS. Haematology and Haemochemistry of Silkworm, *Bombyx Mori* L. Infected with *Bacillus Thurungenses*. Int. J Environ Sci. 2011; 2:452.
2. Babers FH. A septisemia of the southern armyworm caused by *Bacillus cereus*. Ann. Entomol. Soc. Amer. 1938; 31:371-373.
3. Beard RL. Studies on the milky disease of Japanese beetle larvae. Conn. Agric. Exp. Stn. Bull. 1945; 491:505-583.
4. Bogdan C, Rollinghoff M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr. Opin. Immunol. 2000; 12:64-76.
5. Borges AR, Santos PN, Furtado AF, Figueiredo RCBQ. Phagocytosis of latex beads and bacteria by haemocytes of the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduvidae). Micron 2008; 39(4):486-494.
6. Brehelin M, Zachary D. Insect haemocytes: a new classification to rule out controversy. In: Immunity in Invertebrates,ed. Brehelin, M. Springer Verlag, Berlin,1986, 36-48.
7. Chain BM, Anderson RS. Observations on the cytochemistry of the haemocytes of an insect, *Galleria mellonella*. J Histochem Cytochem. 1983; 31:601-607.
8. Chain BM, Anderson RS. Selective depletion of the plasmatocytes in *Galleria mellonella* following injection of bacteria. J Insect Physiol. 1982; 28(4):377-384.
9. Da-Silva JB, De Albuquerque CM, De Araujo EC. Immune defense mechanisms of *Culex quinquefasciatus* (Diptera: Culicidae) against *Candida albicans* infection. J Invertebr Pathol. 2000; 76(4):257-262.
10. Drif L, Brehelin M. Structural, classification and functions of insect haemocytes In: Insect immunity (Ed. Pathak, J.P.N.), Oxford and IBH Pub. Co., New Delhi, 1993, 1-14.
11. Fearon DT.Seeking wisdom in innate immunity. Nature 1997; 388:323-324.
12. Gillespie JP, Burnett C, Charnley AK. The immune response of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae* var *acridum.* J Insect Physiol.2000; 46:429-437.
13. Gillespie JP, Kanost MR, Trenczek T. Biological mediators of insect immunity. Annu. Rev. Entomol. 1997; 42:611-643.
14. Götz P, Boman HG. Insect immunity. In: Comprehensive Insect Physiology, Biochemistry, and Pharmacology, eds. Kerkut, G.A. and Gilbert, L.I. Pergamon Press, Oxford, 1985; 3:453-485.
15. Gunnarsson SGS. Infection of *Schistocerca gregaria* by the fungus *Metarhizium anisopliae:* Cellular reactions in the integument studied by scanning electron and light microscopy. J Inver Pathol. 1988; 52(1):9-17.
16. Gupta AP. Insect immunocytes and other haemocytes: role in cellular and humoral immunity. In: Immunology of Insects and other Arthopods, ed. Gupta, A.P. CRC Press. Boca Raton,1991, 19-118.
17. Hoffmann D, Brehelin M,Hoffmann JA. Modifications of the hemogram and of the hemocytopoietic tissue of male adults of *Locusta migratoria* (Orthoptera) after injection of *Bacillus thuringiensis*. J Inver Pathol. 1974; 24(2):238-247.
18. Hung SY, Boucias DG. Influence of *Beauveria bassiana* on the cellular defense response of the beet armyworm, *Spodoptera exigua*. J Inver Pathol. 1992; 60(2):152-158.
19. Jones JC. Current concepts concerning insect haemocytes. Amer. Zool. 1962; 2:209-246.
20. Kostritsky M, Toumanoff M, Metalnikov S. Bacterium *tumefaciens* chez la chenille de *Galleria mellonella*. C. R. Acad. Sci. 1924; 179:225-227.
21. Krishnan N, Chaudhuri A. Eicosanoids – A mediator of the cellular defense response of silkworm *Bombyx mori* L. to bacterial infection (Abstr. No. 4) Presented on the Current Technology Seminar on Silkworm Disease Management, Silkworm Rearing Technology and Mulberry Pathology held during July 22-23rd 1998, at CSR&TI, Berhampore, West Bengal, India, 1998.
22. Krishnan N, Chaudhuri A. Influence of phospholipase A_2 inhibitor, dexamethasone on nodulation response to bacterial infection in the silkworm *Bombyx mori* L. Abstr No. 6. Presented at the "National symposium on the Modern Trends in Entomological Researches in india" held during February 21-23rd, 1999, at Dr. H. S. Gour University, Sagar, M.P., India, 1999.
23. Krishnan N, Madana Mohanan N, Chaudhuri A, Mitra P, Saratchandra B, Roy AK. Role of 3,4-dihydroxyphenylalanine in the nodulation response of silkworm *Bombyx mori* L. to bacterial infection. Curr. Sci. 2000; 79(7):1011-1016.
24. Lackie AM. Haemocyte behaviour. Adv. Insect Physiol.1988; 21:85-178.
25. Lowenberger C.Innate immune response of *Aedes aegypti*. Insect Biochem Mol Biol. 2001; 31:219-229.
26. Meister M, Hetru C, Hoffmann JA.The antimicrobial host defense of *Drosophila*. In: Du Pasquier, L., Litman, G.W. (Eds.), Origin and Evolution of the Vertebrate Immune System. Current Topics in Microbiology, Springer-Verlag, Berlin, 2000; 248:17-36.
27. Muta T, Iwanaga S. The role of hemolympyh coagulation in innate immunity. Curr. Opin. Immunol. 1996; 8:41-47.
28. Papadopoulou-Karabela K, Iliadis N, Liakos V. Haemocyte changes in honeybee (Apis mellifera L) artificially infected by Pseudomonas aeruginosa. Apidol. 1992; 24:81-86.

29. Pech LL, Strand MR. Granular cells are required for encapsulation of foreign targets by insect haemocytes. J Cell Sci.1996; 109(8):2053-60.
30. Rosenberger CR, Jones JC. Studies on the total blood counts of the southern armyworm larva, *Prodenia eridenia* (Lepidoptera) Ann. Entomol. Soc. Amer. 1960; 53:351-355.
31. Schmidt O, Theopold U, Strand MR. Innate immunity and evasion by insect parasitoids. BioEssays 2001; 23:344-351.
32. Shapiro M. Pathological changes in the blood of the greater wax moth, *Galleria mellonella* (Linnaeus) during the course of starvation and nucleopolyhedrosis. Ph.D. Thesis. University of California, Brkeley, California, 1966.
33. Silva JBD, Albuquerque CMRD, Araujo ECD, Peixoto CA, Hurd H. Immune defense mechanisms of *culex quinquefasciatus* (Diptera: Culicidae) against *Candida albicans* Infection. J Inver Pathol. 2000; 76:257-262.
34. Strand MR, Noda T. Alterations in the haemocytes of *Pseudoplusia includens* after parasitism by *Microplitis demolitor*. J. Insect Physiol. 1991; 37(11):839-850.
35. Strand MR, Pech LL. Immunological basis for compatibility in parasitoid–host relationships. Annu. Rev. Entomol. 1995; 40:31-56.
36. Tauber OE, Yeager JF. on the total blood counts of insects. I. Orthoptera, Odonata, Hemiptera and Homoptera. Ann Entomol Soc Amer. 1935; 28:229-240.
37. Tauber OE, Yeager JF. On the total hemolymph (blood) cell counts of insects. II. Neuroptera, Coleoptera, Lepidoptera and Hymenoptera. Ann. Entomol. Soc. Amer. 1936; 29:12-18.
38. Vass, E., and Nappi, A. J. 2001. Fruit fly immunity. Bioessays 51: 529-535.
39. Werner RA, Jones JC. Phagocytic haemocytes in unfixed *Gallaria mellonella* larvae. J Insect Physiol. 1969; 15:425-437.
40. Wiesner A. Induction of immunity by latex beads and by hemolymph transfer in Galleria mellonella. Deve. Comp. Immunol. 1991; 15(4):241-250.
41. Wittig, G. 1965. A study of the role of blood cells in insect disease. 12th Int. Conger. Entomol. (Lond.) 1964: 743.
42. Wittig G. Phagocytosis by blood cells in healthy and diseased caterpillars. II. A consideration of the methods of making haemocyte counts. J Invertebr Pathol. 1966; 8:461-477.