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Changes in the THC and DHC of *Poekilocerus pictus* during infection of *Candida albicans*

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Abstract
Many studies have documented in insect cellular immune responses to bacteria. The use of the fungal pathogen *Candida albicans* extends the principle to fungi. Haemocytes are involved in conferring a cellular or haemocytic immunity in insect. Injection of *Candida albicans* conidia into *P. pictus* resulted in changes in the total hemocyte count and differential hemocytes counts. Total hemocyte 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 *C. albicans*. 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 *Candida albicans.*

1. Introduction

Insects have been remarkably successful in evolution. Current estimates are that they account for 90% of all known species within the animal kingdom. With exception of the oceans, insects colonize all ecological niches on earth. Consequently, they are confronted with an extremely large variety of potentially harmful microorganisms and parasites. The evolutionary success of the insects can be attributed to various reasons, among which is undoubtedly the presence of a highly efficient immune system. In contrast to vertebrates, insects lack true antibodies and, hence, also an adaptive immune response. They rely solely on a well-developed innate immune system to defend themselves against microbial infections. This immune system detects microbes through a limited set of receptors encoded by the germinal lineage, and this system does not keep a record of former microbes encountered, as is observed in the adaptive immune system of vertebrates. The innate immune system can be subdivided in two major categories, i.e. humoral and cellular immunity. Humoral responses require several hours for their full expression and involve the induced synthesis of antimicrobial proteins and the activation of the prophenoloxidase cascade. The cellular defense reactions are typically induced within minutes of infection and include phagocytosis, nodulation and encapsulation. The separation of the innate immune system in a humoral and cellular component is somewhat arbitrary, since both components work in a more or less coordinated way to create a general inflammatory response to microbial infection.

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 (Au *et al*., 2004, Dean *et al*., 2004b, Lackie, 1988) [21]. Tauber and Yeager (1935, 1936) [28, 29] 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 hemocytes during bacterial infections (Kostritsky *et al* 1924; Babers, 1938; Wittig, 1965, Krishnan and Chaudhuri, 1998, 1999; Krishnan *et al.,* 2000) [17, 2, 18-20]. In bacterial- infected *Prodenia* larvae, hemocytes counts either remained unchanged or declined markedly; thus Rosenberger and Jones (1960) [24] concluded that the hemocytes 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) [30]. High doses of infection in *Pseudoletia* larvae resulted in drastic reduction in plasmatocytes and a great increase in spherulocytes and prohemocytes, with no change in the number of granulocytes (Wittig, 1966) [32]. Low doses, on the other hand, had a different effect on the blood picture because the granulocytes and plasmatocytes decreased while the spherulocytes and prohemocytes increased significantly (Wittig, 1966) [32]. Krishnan and Chaudhuri (1998; 1999) [18, 19] and Krishnan *et al* (2000) [20] 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 fungal infection was elucidated. As not much work has been done on *P. pictus* regarding insect immunity against bacterial and fungal 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 fungal infection with respect to the chief haemocytes involved in defense response and the insight into basis and mechanism of defense reactions in *Poekilocerus pictus.*

2. Materials and methods

2.1 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 (25-30 ºC) 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.

2.2 Fungal Culture

Culture of a Candida albicans were purchased from ATCC (American type culture collection) and sabourad dextrose agar from Himedia. Fungus *Candida albicans* were grown in sabourad dextrose agar media at 37 ^{0}C in bacteriological incubator. 10^5 colony forming units (CFUs)/ml were used in the all experiments (Fig. 3).

2.3 Method of Injection

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 *Candida albicans* were injected in 10µl aliquots, using a 25 µl Hamilton syringe

2.4 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). THC was done at 2h, 4h, 8h and 24h post infection in 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 were 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) [25] was used to count relative number of different types of haemocytes. DHC was done at 2h, 4h, 8h and 24h post infection in 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.

2.5 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 *** $P \leq 0.0001$

3. Results

3.1 Total Hemocyte Count (THC)

Total Hemocyte count (THC) was observed at 2h, 4h, 8h and 24h post infection in fungal infected insects and in control 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.

3.1.1 Total haemocyte count (THC) in control and *Candida albicans* 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 6.60±0.16*×10^3 per mm^3 at 2h p.i.
- The mean ± Standard error of total haemocyte count was observed to be 6.45±0.23*×10^3 per mm^3 at 4h p.i.
- The mean ± Standard error of total haemocyte count was observed to be 4.24±0.08**×10^3 per mm^3 at 8h p.i.
- The mean ± Standard error of total haemocyte count was observed to be 4.18±0.14*×10^3 per mm^3 at 24h p.i.

Table 1: Total haemocyte count at different time points post injection in control and 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
Candida albicans (fungus)	5	5.17±0.08	6.60±0.16*	6.45±0.23*	4.24±0.08**	4.18±0.14*

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

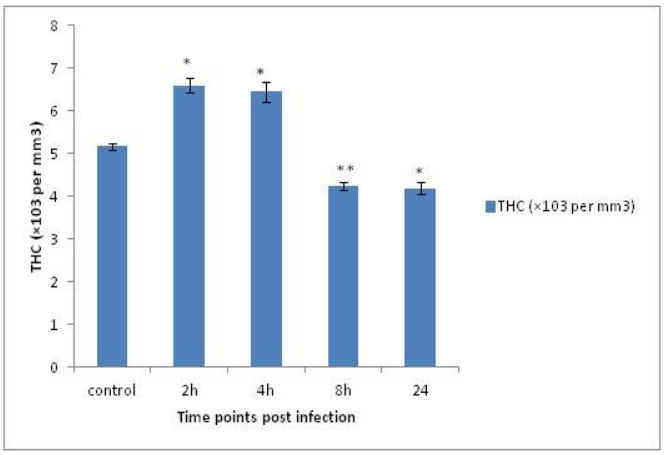

Fig 1: Total haemocyte count in control and *Candida albicans* treated *P. pictus*

3.2 Differential Hemocyte 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. 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.

3.2.1 The relative percentage of different types of haemocytes in control and *Candida albicans* 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 5.40%, 8.80%, 7.40% and 10.60% respectively.
- The relative percentage of plasmatocytes in control insects is 60.00% and treated insects at 2h, 4h, 8h and 24h p.i. are 29.40%, 24.40%, 20.40% and 22.00% respectively.
- The relative percentage of granulocytes in control insects is 25.40% and treated insects at 2h, 4h, 8h and 24h p.i. are 53.80%, 66.00%, 64.00% and 65.60% respectively.
- The relative percentage of spherulocytes in control insects is 10.40% and treated insects at 2h, 4h, 8h and 24h p.i. are 8.00%, 8.20%, 6.20% and 7.40% respectively.

Table 2: The relative percentage of different types of haemocytes in control and *Candida albicans* 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	5.40±1.07	8.80±1.28	7.40±0.87	10.60±1.72*
PL	5	60.00±3.53	29.40±2.48**	24.40±1.47**	20.40±2.31**	22.00±2.00**
GR	5	25.40±1.72	53.80±2.80**	66.00±3.76**	64.00.±2.02**	65.60±3.31**
SP	5	10.40±1.32	8.00±1.41	8.20±1.31	6.20±1.15	7.40±1.28
AD	5	1.33±0.33	1.00±0.00	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
* $P \leq 0.01$
** $P \leq 0.001$

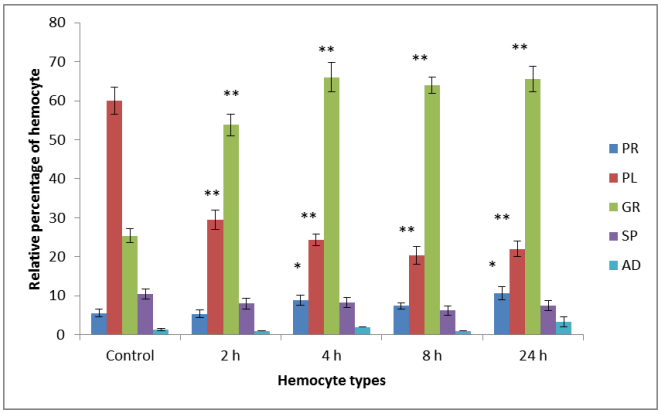

Fig 2: Bar diagram showing the relative percentage of different types of haemocytes in control and *Candida albicans* treated *P. pictus*

4. Discussion

In the present study Cellular changes such as THC (Total Hemocyte counts), DHC (Differential Hemocyte 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) [26] that total hemocytes 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) [10] 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) [31] who observed that 30 min after injection of latex beads THC was decreased in *Galleria mellonella*.

Wiesner (1991) [31] 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) 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) [27] 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 haemocyte count (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; Gunnarsson, 1988; Pech & Strand, 1996. 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) [11]; Pech & Strand (1996) [23] and Gillespie *et al*., (1997) 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) [4] 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) [11, 5, 13, 9]. 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) [7] 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) [26] showed that the relative proportion of plasmatocytes was higher and, concomitantly, the proportion of granular cells was lower in differential hemocyte counts from *Culex quinquefasciatus* (Diptera: Culicidae) against *Candida albicans* infection at 3, 6, and 18 h after inoculation.

Faraldo and Lello (2003) observed that after 5 min post-injection of china ink solution in *Dermatobia hominis*, no cellular alterations were observed. 5 hours post-injection, although not counted, the number of granular cells in the haemolymph was visibly increased. This is in confirmation with the present findings that GRs increased at 15 min to 24 h p.i.

Borges *et al*., (2008) [4] 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 Bidochka and Khachatourians, (1987) Gunnarsson, (1988) [12] and Hung and Boucias, (1992) [15]. 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.

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