Chapter VII. INDIRECT ELISA

VII.1. Introduction

Use of ELISA in nutritional studies was suggested by Hagler (1998). This enables the rapid screening of predators to obtained accurate data on gut content (reviewed by Van Weeman and Schuurs, 1971a, b; Sundarland, 1988; Greenstone, 1996). The most important factors which considered in the development of antibody-based assay was the level of sensitivity and specificity achieved (Sheppard and Harwood, 2005). Laboratory optimisation is necessary to quantify the rates of antigen decay, the effects of temperature on decay rates, the consequence of alternative prey consumption on detection periods and difference is detection limits between the predators. All these factors can influence the detection of prey material (Sunderland, 1996).

Furthermore, the mass collection of arthropods for gut content analysis can yield false-positive data due to surface level contamination with target prey or increased interaction between the predators and prey due to inappropriate sampling protocols (Harwood and Obyckii, 2005a and 2005b). The simplicity of screening protocols associated with antigen-antibody based assays has been allowed large-scale field analyses of predators prey interactions (eg. Harwood et al., 2004; Hagler and Naranjo, 2005). This technique of using pest specific antibodies was pioneered in the early 1998 (e.g. Hagler et al., 1992; Hagler and Naranjo, 1994 a, b). In the largest gut-content study reported (Hagler and Naranjo, 2005) assayed predators by indirect ELISA. Most investigators employing gut content
immunoassay have used whole body homogenates for their assays (Fitcher and Stephen, 1984; Hagler et al., 1992; Hagler and Naranjo, 1997). Microscopic gut content analyses are easy and affordable, but they are ineffective for most predators because the prey was liquefied (or) chewed into tiny unrecognizable pieces (Miles, 1972).

Visual identification of gut content revealed the feeding preferences of coleopteran predators (Forbes, 1983) of coccinellid feeding on pollen or aphids. Further more the additional investigation had been developed an indirect enzyme – linked immunosorbert assay (ELISA) which employs a species and antibody specific for examining predators of the Pectinophora gossypiella (Saunders) on the pinkbooworm eggs (Hagler et al., 1994; Hagler and Naranjo, 1997). Valuable information can be gathered and gut dissection has enable the identification of prey remains from museum specimen. Though reduviids are good biological control agents (Schaefer, 1988; Ambrose, 1999; Sahayaraj, 2007a), till now no information was available about the usage of gut content analyses using ELISA. Hence, this study was proposed to examine the effect of total protein content on the efficacy of R. marginatus and R. fuscipes maintained with six temperatures and fed with three chosen pests using indirect ELISA.

VII.2. Materials and Methods

20 to 25 adults of R marginatus and R. fuscipes (> 15 days old) were maintained separately in environmental chambers at 10, 15, 20, 25, 30 and 35°C on C. cephalonica, S. litura and D. cingulatus separately. Predators were removed from the environmental chambers after 15 days and immediately frozen at -20°C.
Each predator were homogenised in 500 µl of PBS and assayed for *S. litura*, *C. cephalonica* and *D. cingulatus* remains in the gut and haemolymph of the predators. Antibody was determined through immunoassays performed into round-bottom wells of polystyrene microtiter plates coated with 500 ng of *S. litura*, *C. cephalonica* and *D. cingulatus* purified protein separately. After antigen sensitisation, the free reactive sites of the wells were blocked with 1 % BSA.

**VII.2.1. Haemolymph collection**

Haemolymph was collected from 6 to 10 adults of *R. marginatus* and 10 *R. fuscipes* by puncturing the Antennae region with a 0.33 mm diameter needle attached to a 1 ml syringe and withdrawing haemolymph from the antennae taking care to avoid contamination of haemolymph with body fluid. Saturated phenylthiourea (2 µl) was added to the pooled haemolymph sample to prevent coagulation. Samples were centrifuged at 12000 g for 3 hours and the supernatant used for ELISA.

**VII.2.2. Preparation of Pest Antigen**

In a small beaker, take 25 ml of distilled water and placed a dialysis membrane for 10 to 20 minutes and then replaced in to boiled water for 10 minutes inorder to remove impurities, unwanted proteins and any inactive enzymes. Make sure the dialysis membrane did not touch the wall of the beaker. Check the leakage of the membrane using squeeze bottle. Desired length was selected and both inner and outer sides were rinsed with distilled water. One end of the membrane was tied securely with the thread. Whole body of three pests (*S. litura, C. cephalonica* and
D. cingulatus) were homogenised with 500 µl to 1 ml of cold PBS solution then passed the crude content through the dialysis membrane (29 mm) and placed inside a beaker containing 500 ml of PBS. Open end of the membrane was closed securely by cotton thread. The membrane was then placed in 500 ml of PBS buffer solution and dialysed for over night at 4°C. During this process PBS buffer was changed 3 to 4 hours in order to remove the impurities. Finally the purified protein adhered on the inner surface of the membrane was used as pest antigen.

**VII.2.3. Indirect Gut content ELISA**

Samples were prepared for ELISA by homogenising individual predators using 500µl phosphate buffered saline in 96-well assay plate were coated separately with a 100µl of aliquot for each antigen sample and incubated at 4°C overnight. The unbound antigen was discarded from the assay plate and 300 µl of 1.0% (10.0 mg/ µl) BSA in distilled H₂O was added. Allow it for 30 minutes at room temperature to block any unoccupied protein binding sites in the wells. Wells were rinsed three times with PBS – Tween 20 (0.05%) and twice with PBS. Fifty micro litters of the pest antigen was then added separately to each well (Hagler et al., 1994). Then the plates were incubated for 1hr for at room temperature then rinsed by the above said manner. Aliquots (50 µl) of anti-rabbit’s IgG conjugated to alkaline phosphates diluted 1:500 in 1.05% BSA was added to each well of the plates and incubated for 1 hour. Plate contents were discarded and again plates were rinsed three times as described above. A 50 µl aliquot of substrate solution was added to each well using the regents supplied in are alkaline phosphate substrate kit (Nune, Mexisorp, UK) following the addition of 50µl of 2N H₂SO₄
Then absorbance of each well was measured using a SLR 36 ELISA strip reader (Glaxo, Mumbai) at 450nm. Each pest’s antigen was considered as positive sample separately.

**VII.2.4. Effect of predator total protein content in Indirect ELISA sensitivity**

Predators devoid of the pest’s antigen were frozen at -40°C for 3 days. Separately stock solutions were prepared by homogenising 10 to 15 (D. cingulatus), 9 to 17 (C. cephalonica) and 3 to 10 (S. litura) with to 5 to 10 ml of PBS. 50 µl aliquot of this stock solution was equivalent to a single pest’s antigen. 50 µl aliquot of stock pest antigen solution was added to each predator sample for total volume of 500 µl. Six to ten predators were homogenised in 500 µl PBS and treated as a negative controls. The total protein content of each individual was determined method (Bragdon, 1976). Then the samples were assayed for indirect ELISA as described in session VII.2.2. The mean ELISA absorbance value was recorded for each temperature treatment separately using ELISA reader (Glaxo, India).

**VII.3. Result**

Protein content of *Rhynocoris marginatus* in relation to three pests such as *S. litura*, *D. cingulatus* and *C. cephalonica* were significantly different. For instance the overall mean protein concentration was 400, 330, 400, 475 and 425 µg/insect for *R. marginatus*, *R. fuscipes*, *S. litura*, *D. cingulatus* and *C. cephalonica* respectively. We have not maintained any negative control. The
standardized ELISA consisted of homogenate each predator, regardless of its total protein content, in 500 µl of PBS with *S. litura, D. cingulatus* and *C. cephalonica* as pest antigen. Pest antigen was detected in every *R. marginatus* sample that was spiked with a single pest yielding a mean ELISA absorbance value (Figures 21 and 22). The *R. marginatus* samples values were increased with pest antigen was immuno reactive. We are used a standardized indirect gut and haemolymph content ELISA for all temperature reared predators, because we would like to find out the qualitative feeding behavior of these predator in relation to various pests.

We standardized the first step by coating the pest antigen predator/ 500 µl of PBS in the indirect ELISA plates. However, it reveals from the result that irrespective of the prey consumption, 500-µl dilution yielded maximum protein content both in *R. marginatus* and *R. fuscipes*. To minimise the high frequency of the ELISA false-negative reactions, first we added equivalent amount of pest antigen to *R. marginatus* and *R. fuscipes* samples that were homogenised in with 500 µl (500 µg/well) to 1500 µl PBS. Then we analysed each sample by an indirect ELISA. From the observations we understood that a single well which contain 100 µl homogenate is required for this study.

**VII.3.1. Effect of predator protein content on ELISA sensitivity**

Mean protein content of *R. marginatus* fed with three pests is presented in figure 21. Results revealed that protein content was gradually increased up to 20°C, and then declined to 25 and 30°C when *R. marginauts* was fed with *C. cephalonica* and *S. litura*. However, protein content was gradually increased up to 30°C in *R. marginatus* and *R. fuscipes* fed with *D. cingulatus*. Similar trend was also
observed in *R. fuscipes* fed with *S. litura, D. cingulatus* and *C. cephalonica*. The indirect ELISA was unreliable of these detecting immune response for two heteropteran predator (Figures 21and 22).

**VII.3.2. ELISA response on gut of both predators.**

*R. marginatus* was maintained at lower temperature threshold ($< 25^\circ$C) after *S. litura* feeding *R. marginatus* showed more immune response than the individual reared at 35$^\circ$C (Fig. 20). There was a irregular manner of positive response recorded at all temperatures on the tested pests. But declined level was visibly found between 25 to 35$^\circ$C (Fig. 23). In *D. cingulatus* fed individuals more immune response was recorded between 20 to 30$^\circ$C, and then it was declined at 35$^\circ$C. However, in *R. marginatus* fed with *C. cingulatus* adults revealed that immune response was increased linearly from 10 to 30$^\circ$C and then the response declined at 35$^\circ$C. As in the case of *R. fuscipes* fed with *S. litura*, gut immune response did not have a more variation between 10 to 20$^\circ$C, eventhough suddenly increased up to 20 to 30$^\circ$C, again slowly declined towards at 35$^\circ$C, this kind of similar immune response observed when fed with other two pests (Fig.24). Among the three pests, *S. litura* provide more gut immune response to these two reduviid predators than *D. cingulatus* and *C. cephalonica*.

**VII.3.3. ELISA response on Haemolymph**

In another study, immune response was recorded using predators haemolymph. It was shown that the antigenic protein replied immunoreactive absorbant value was higher in *R. marginatus* (0.72 ± 0.02) on *S. litura* followed by
C. cephalonica (0.63 ± 0.03) and D. cingulatus (0.62 ± 0.03) at 30°C. Similarly in R. fuscipes, higher response was also noted at 30°C for all the three pests (0.75 ± 0.01, 0.68 ± 0.02, 0.54 ± 0.02 for S. litura, C. cephalonica and D. cingulatus respectively). In all other temperatures revealed the immune response was more or less equal for all the pests of these two predators. Among the three pests, maximum response was observed on S. litura followed by C. cephalonica and D. cingulatus (Table 21a and 21b).

VII.4. Discussion

The results of this study suggested that selective prey consumption of reduviid predators with optimum temperature reflect that these reduviids preferred S. litura. Result also confirmed that haemolymph possessed more immune response which immune reactive property (or) tendency normally decay (or) disrupt at higher (< 35°C) and lower temperature (> 15°C). Results also revealed that immune response of both predators fluctuated according to the temperature regimes quoted by Hagler and Naranjo (1997). In other findings of indirect ELISA revealed that the predator gut immune response was decreased and increased based upon meal size, suitable prey consumed, temperature regimes and prey detective interval (Sunderland et al., 1987; Sopp and Sunderland, 1989). Most of the studies attributed interspecies differences in the prey detection to variable metabolic rate as a function of time and temperature (Engval and Perlman, 1972 a, b; Fitcher and Stephen, 1981; Sopp et al., 1992; Greenstone and Hunt, 1993; Hagler, 1998). From this results it was clear that ELISA study can also be considered to known the temperature depend immune reaction of both R. marginatus and R. fuscipes. It was
also noticed that there was a rapid decline with preys like *S. litura* and *D. cingulatus*. In addition, Naranjo and Hagler (1997) recorded considerable species gut content immune response variation present on predators gut by immunoassays performance. Previous studies of Sahayaraj, (2000); Sahayaraj *et al.* (2004) showed that when *R. marginatus* was provided with *H. armigona* and *S. litura*, reduviid preferred *S. litura*. Prey preference studied also revealed that *R. marginatus* preferred mostly *S. litura* followed by *D. cingulatus* (Sahayaraj, 1994).

From the present experiment it was noted that both reduviid predators possessed haemolymph and gut content by ELISA detected immune response was adversely affected depends upon the lower as well as higher temperatures. These results are also in conformation with the report of Sopp and Sunderland (1989). This indirect ELISA study clearly indicated that between the two predators, reduviid *R. fuscipes* (145 mg) (0.072) gut content revealed more immune response than the larger predator of *R. marginatus* (310 mg) (0.068). A similar result was also recorded by Hagler and Naranjo (1996). Stuart *et al.*, (1990) reported that optimal concentrations of reagent were determined through sequential check board of primary antibodies followed by primary antibody versus standard antigen dilutions, its leads to standard curves generated in an indirect ELISA. The results revealed that the marked differences of pest specific with higher immune response explained mainly depends upon the given temperature regimes. For instance the response was in favour at 25°C for *S. litura*, 30°C for *C. cephalonica* and at 35°C for *D. cingulatus* which concordant observation was made by Ma *et al.*, 1984. Because, the prey consumed by less protein contain small predators have a greater
chance for attached or adhered to the ELISA microplate matrix than the prey consumed by large protein-rich predators. The total protein concentration present in the ELISA samples should not exceed 125 μg / samples to minimize the probability of ELISA false negative reaction. This relationship suggests that there is a rapid initial decay of ELISA sensitivity occurs as protein content increases (Sundarland et al., 1978). In effect, the extraneous, non-target proteins associated with large predators “block” the targeted prey proteins from binding on to the ELISA matrix (Pickel, 1981). The net result is a higher frequency of false-negative reactions with large predators (Ma et al., 1984).

In summary, factors such as variable predator digestive rates (Symondson and Liddel, 1993), prey sizes (Sopp and Sunderland, 1989; Symondson and Liddell, 1996), temperature (Hagler and Narganjo 1999a, b), predator metabolic status and developmental stage of the prey (Hagler et al., 1992) can all effect the quantitative outcome of immunoassays (Sunderland 1996). However very few investigations have considered the total protein content present in the samples as an important variable which affecting the qualitative and quantitative outcome of indirect immunoassays.

VII.5. Conclusion

*R. marginatus* and *R. fuscipes* subjected to various constant temperature and fed with *C. cephalonica*, *S. litura* and *D. cingulatus*. Of these 3 pests, *S. litura* fed individuals were more immuno reactive at both 25 (0.61) and 30°C (0.63) in *R. marginatus* and *R. fuscipes* (0.69 and 0.71 at for 25 and 30°C) than *C. cephalonica* and *D. cingulatus*. The results showed that remaining temperature
had an immune response positive but lower than that of 25°C, 35°C. It was also concluded that *R. fuscipes* haemolymph was more immunoreponse than *R. marginatus*. 