Chapter 3

NON-SPECIFIC IMMUNE RESPONSE OF PENAEUS MONODON TO IVP ADMINISTRATION
3.1. Introduction

Successful shrimp production requires the use of effective disease prevention strategies and a good understanding of the basic immune functions. Several factors such as water quality, disease, toxins, trace nutrients (astaxanthin, vitamins and minerals), probiotics, immune stimulants ($\beta$-glucan, peptidoglycan and lipopolysaccharide) and genetic make up have been shown to influence the immune mechanisms in Penaeus monodon (Supamattaya et al., 2006). Shrimp immune system involves actions and reactions against its pathogens by shrimps. The state of protection from infectious disease in shrimps includes the non-specific as well as the specific component which cannot be equated to a vertebrate immune mechanism.

It is observed that unlike vertebrate immunity which is composed of innate and adaptive responses, invertebrates rely solely on an efficient multiple innate defense reaction mechanism to combat infections. The innate immune system in shrimp, characterized by the lack of immunoglobulin, is efficient to protect and preserve them from all intruding pathogens or environmental antigens. The target recognition of innate immunity is the so-called pattern recognition molecules (PRMs) shared among groups of pathogens. Host organisms have developed the response to these PRMs by a set of receptors referred to as pattern recognition proteins or receptors (PRPs or PRRs) (Janeway, 1989). These patterns include the lipopolysaccharides (LPS) of Gram negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gram positive bacteria, the mannans of yeasts, the $\beta$-1, 3-glucan of fungi and double-stranded RNAs of viruses (Hoffmann et al., 1999). Non-specific or the innate immune mechanisms in P. monodon involves the fixed and the mobile
defence mechanisms. The fixed non-specific immune mechanism involves the structural barriers which act as the first line of defence against pathogens. These include: hard cuticle, tegumental glands, epithelial immunity, branchial podocytes, autotomy of appendages, regeneration of appendages and rapid wound healing to prevent loss of haemolymph.

The mobile non-specific immune system has two main components, the humoral and cellular systems both of which are activated upon immune challenge (Liu., 2009). The cellular component involves those mediated by haemocytes and the humoral component involves those mediated by cell free haemolymph. The cellular and humoral immune mechanisms of the shrimp function synergistically to protect the shrimp and eliminate foreign particles and pathogens.

The cellular immune response in *Penaeus monodon* involves a number of different cell types including haemocytes and the fixed phagocytes (Supamattaya et al, 2006). *Penaeus monodon* possesses three different types of blood cells. According to ultrastructural features seen with electron microscopy (EM), *P. monodon* haemocytes can be divided into granular, semigranular and hyaline (or agranular) haemocytes (van de Braak, 2002).

The various cellular immune response include phagocytosis (Bachere et al., 1995; Itami et al., 1998; van de Braak et al., 2002b; He et al., 2004), apoptosis (Sahtout et al., 2001; Wang et al., 2008b), encapsulation (Sung et al., 2003; Bian and Egusa, 1981), nodule formation (Bian and Egusa, 1981; Nash et al., 1988), melanisation (Bian and Egusa, 1981; Nash et al., 1988) and cell adhesion (Sritunyalucksana et al., 2001; Lin et al., 2006).

The humoral response in shrimp is favoured by the different biological compounds in the haemolymph that inhibit or eliminate foreign bodies and pathogens. These include the pro Phenol Oxidase (pro PO) activating system
(Hernandez-Lopez et al., 1996; Vargas-Albores et al., 1996); the clotting cascade (Yeh et al., 1998; 1999); soluble pattern recognition proteins (PRPs) (He et al., 2004; Vargas-Albores and Yepiz-Plascencia, 2000) lectins (Luo et al., 2006; Rittidach et al., 2007; Yang et al., 2007; Ma et al., 2008), anti LPS factors (ALF) (Somboonwiwat et al., 2005; de la Vega et al., 2008), peptidoglycan-binding proteins, lipopolysaccharide (LPS)-binding proteins, beta 1,3-glucan-binding proteins (Romo-Figueroa et al., 2004; Jimenez-Vega et al., 2002; Cheng et al., 2005) etc.; the enzymes involved in the antioxidant defense mechanism - superoxide dismutase, peroxidase, catalase, nitric oxide synthase (Rameshthangam and Ramasamy, 2006; Jiang et al., 2006, Mathew et al., 2007); defensive enzymes like lysozyme (Sotelo-Mundo et al., 2003), acid phosphatase (Chen et al., 1999), alkaline phosphatase (Jiang et al., 2004; Joseph and Philip, 2007), other molecules like haemocyanin (Adachi et al., 2003); reactive oxygen intermediates (ROI) (Munoz et al., 2000; Wang et al., 2006b); reactive nitrogen intermediates (RNI) (Jiang et al., 2006); alpha 2 macroglobulin (Gollas-Galvan et al., 2003; Rattanachai et al., 2004) and antimicrobial peptides (Destoumieux et al., 1997; Bachere et al., 2000; Munoz et al., 2002; Hu et al., 2006). Studies on the response of shrimps to pathogens at the gene and molecular level reveal that more immune related mechanisms are involved in pathogen exclusion.

Studies on the immunological responses of _Penaeus monodon_ to DNA vaccine by analyzing the immune indices such as proPO, SOD, Respiratory burst, lysozyme and alkaline phosphatase activity showed a high level of proPO, SOD, lysozyme and alkaline phosphatase activity in the haemolymph of vaccinated group (without WSSV challenge) (Rajeshkumar et al., 2008; Li et al., 2010).

Various non-specific humoral immune mechanisms present in the hemolymph of experimental animals (_Penaeus monodon_) were analysed by
employing the following non-specific immune response indices, and the results are presented in this Chapter.

1. Total Haemocyte Count
2. Respiratory burst / ROI
3. pro PO activity
4. Lysozyme activity
5. $\alpha$-2 macroglobulin activity
6. Transglutaminase activity
7. Superoxide dismutase
8. Catalase
9. Glutathione peroxidase
10. Glutathione s transferase
11. Acid phosphatase
12. Alkaline phosphatase
13. Lipid peroxide
14. Aspartate aminotransferase
15. Alanine aminotransferase
16. Nitric oxide synthase

3.2. Materials and Methods

3.2.1. Sample collection for non-specific immune assays

Haemolymph sample was collected using capillary tube rinsed with the anticoagulant (0.01M Tris-HCl, 0.25M Sucrose, 0.1M Tri Sodium Citrate, pH-7) from a set of experimental animals (6 animals of 10±1g) administered with the feed types (normal feed/ IVP) as described in Chapter 1. The samples were collected on the 3rd day post challenge after the 7 day administration of the test and control feeds, and the non-specific immune parameters such as total haemocyte count, phenol oxidase activity and reactive oxygen intermediate production / respiratory burst activity were determined within 8hrs. Aliquots of haemolymph were also stored at -80ºC for analyzing the remaining non-specific immune response parameters.

3.2.2. Non-Specific Immune Assays

3.2.2.1. Total Haemocyte Count

Total haemocyte count (THC) was determined by using a Neubaeur's haemocytometer. A drop of anticoagulant-haemolymph mixture was placed on
the haemocytometer and haemocytes were counted by observing under a bright field microscope and expressed as cells mL⁻¹ haemolymph.

3.2.2.2. Phenoloxidase (PO) activity

Phenol oxidase activity was determined by incubating 100µL of haemolymph with 100µL of 10% SDS for 30 min. at 25°C and by the addition of 2.0 mL of substrate (0.19% L-DOPA in Tris-HCl buffer). The dopachrome formed was measured in a UV-Visible spectrophotometer at 490 nm, at every 30s for 3 mins. and the activity expressed as increase in absorbance minute⁻¹ 100 µL⁻¹ haemolymph (Soderhall et al., 1981).

3.2.2.3. Respiratory Burst Activity

Intracellular superoxide anion (O₂⁻) or the reactive oxygen intermediates (ROI) or the respiratory burst activity was measured by the reduction of Nitro blue tetrazolium (NBT) (Song and Hsieh, 1994) as described by Chang et al. (2000). For this assay, a sample of 100µL haemolymph was incubated with 100µL 0.2% NBT for 30 mins. at 10°C. The cells were separated by centrifugation (1200rpm, 4°C, 10 mins.) and fixed in 100% methanol. It was then incubated for 10mins. at room temperature (RT, 28°C) and subjected to centrifugation (300rpm, 4°C, 10 mins.). The supernatant was removed after centrifugation and the cells were dried and then rinsed in 50% methanol and solubilised in 140µL DMSO and 120µL 2M KOH. The absorbance at 620nm was recorded and the activity expressed as O.D. 100µL⁻¹ haemolymph.

3.2.2.4. Lysozyme activity

Lysozyme activity was determined by a turbidometric assay (Acharya et al., 2004) where in the bacterium - Micrococcus luteus (lysodeikticus)- exquisitely sensitive to lysozyme, when exposed to the enzyme, lyses it rapidly. Lysozyme activity was monitored by a decrease in absorbance at 450nm, as organisms lyse in the cuvette and the transmitted light is directly proportional to
the lysozyme concentration. Briefly, a 50µL haemolymph sample was mixed with 150µL suspension of *Micrococcus lysodeikticus* (0.2mg/mL) in a microplate and absorbance measured at 450nm, every 2 min. for 30 min. and the activity expressed as difference of absorbance at initial and at 10min. 100µL⁻¹ haemolymph.

### 3.2.2.5. α-2 macroglobulin activity

The α-2 macroglobulin activity was determined (Gollas-Galvan et al., 2003) based on its ability to inhibit trypsin’s ability to hydrolyse proteins but allowing trypsin’s ability to hydrolyze low molecular mass ‘amide substrates’ such as BAPNA (N-benzoyl-DL-arginine-p-nitroanilide). For the assay, a 50µL sample was mixed with 10µL trypsin (1mg/mL) and incubated for 15 min. at 37°C. It was then mixed with 10µL soybean trypsin inhibitor and again incubated for 10 min. at 37°C, and 100µL BAPNA (N-benzoyl-DL-arginine-p-nitroanilide) (1mg/mL) in 50mM Tris having pH8 was added to the reaction mixture and incubated for 2hr at 37°C. The absorbance was measured at 415nm in a microplate reader (TECAN Infinite™, Austria) and expressed as OD 100µL⁻¹ haemolymph.

### 3.2.2.6. Transglutaminase activity

Transglutaminase activity was determined by the colorimetric hydroxamate assay according to Folk and Cole (1965) method as described in Montero et al. (2005). Briefly, a 100µL haemolymph sample was mixed with a solution containing 100µL 1M Tris-acetate buffer (pH-6), 100µL 0.1M CBZ-L-glutaminyl glycine, 25µL 0.1M CaCl₂, 25µL 2M hydroxylamine and 25µL 0.02M Na₂EDTA. The mixture was then incubated at 37°C for 10min. and 0.75mL of ferric chloride – trichloroacetic acid added to stop the reaction. It was then centrifuged at 4000g for 15 min. and the absorbance of the supernatant taken at 525nm and expressed as O.D. 100µL⁻¹ haemolymph.
3.2.2.7. Superoxide dismutase

Superoxide dismutase activity was determined according to Marklund and Marklund (1974) as described in Rajeshwari et al. (2007) where the rate of autooxidation of pyrogallol (benze 1-2-3–triol) is inhibited by the presence of superoxide dismutase. Briefly, a 0.1mL haemolymph sample was mixed with 0.05mL ethanol and 0.03mL chloroform and shaken for 15 min. Centrifugation was carried out at 12,000xg for 15 mins. and the supernatant taken for the assay. The absorbance of a mixture of 25µL 50mM Tris–HCl (pH 8.2), 25µL distilled water, 75µL supernatant and 2mM pyrogallol was taken at 470nm at 1 min interval for 3 min. in a microplate reader (TECAN Infinite™, Austria) and expressed as change in absorbance/min. 100µL⁻¹ haemolymph.

3.2.2.8. Catalase

The absorbance of hydrogen peroxide at 240nm is measured directly to calculate the reaction rate of catalase (Rajeshwari et al., 2007). In the presence of catalase, the reaction rate is proportionally (linearly) enhanced. Briefly, 125µL potassium phosphate buffer was mixed with 75µL 15mM H₂O₂ and 10µL haemolymph and the absorbance was taken at 240nm in 30sec. interval for 3mins. and expressed as change in absorbance/min. 100µL⁻¹ haemolymph.

3.2.2.9. Glutathione peroxidase

Glutathione peroxidase activity was determined by the amount of enzyme that converted reduced glutathione to oxidized glutathione in the presence of hydrogen peroxide which was detected by the reduction of 5,5-dithiobis 2 nitro benzoic acid (DTNB). The assay was done as described in Rajeshwari et al. (2007). Briefly, 100µL sodium phosphate buffer (pH 7), 20µL 10mM Sodium azide, 200µL 4mM reduced glutathione, 20 µL 2.5mM H₂O₂, 100 µL haemolymph and 280 µL distilled water were mixed and incubated at 37°C for 3min. One hundred µL 10% TCA was added to terminate the reaction and the mixture was centrifuged at 8000rpm for 10mins. One hundred µL of the
supernatant was taken in a microplate and mixed with 75 µL 0.3M disodium hydrogen phosphate and 25 µL 10mM DTNB and the absorbance was taken at 412nm at 30s interval for 3mins. and expressed as change in absorbance min\(^{-1}\) 100 µL\(^{-1}\) haemolymph.

### 3.2.2.10. Glutathione-s-transferase

Glutathione-s-transferase (GST) activity was determined by following the method described in Rajeshwari et al. (2007). 1-chloro-2, 4-dinitrobenzene (CDNB) is a synthetic GST substrate and the GST assay measures the conjugation of CDNB with reduced glutathione. This conjugation increases the wavelength of maximum absorption to 340nm and the increase in absorbance correlates to the amount of glutathione in the sample. 100µL potassium phosphate buffer (pH-7) was mixed with 50µL haemolymph, 20µL distilled water, 25µL CDNB and incubated at 37ºC for 10mins 25µl of 30mM reduced glutathione was added to the reaction mixture and the absorbance read at 340nm in 30sec interval for 3mins. and expressed as change in absorbance/min 100µL\(^{-1}\) haemolymph.

### 3.2.2.11. Acid phosphatase

Acid phosphatase activity of the haemolymph was determined as described by Bisswanger (2004) with o-carboxyphenyl phosphate, under acid conditions. Briefly, a 20µL haemolymph sample was mixed with 140µL 0.15M Acetate (pH-5) and 40µL 3.65mM o-carboxyphenyl phosphate in a UV microplate and incubated at 25ºC for 3 min. The absorbance was taken at 300nm at 1min interval for 6min. and expressed as change in absorbance/min 100µL\(^{-1}\) haemolymph.

### 3.2.2.12. Alkaline phosphatase

Alkaline phosphatase activity was determined by the hydrolysis of the chromogenic substrate p-nitrophenyl phosphate to p-nitrophenol by the enzyme as described by Bisswanger (2004). Briefly, 170 µL 0.1M Glycine-KOH (pH-
10.5) was mixed with 10µL 0.5M p-nitrophenyl phosphate and 2µL haemolymph in a microplate and the absorbance taken at 405 nm at 1min interval for 6 min and expressed as change in absorbance/min. 100µL⁻¹ haemolymph.

3.2.2.13. Lipid peroxide

Lipid peroxide in haemolymph was determined as per Ohkawa et al. (1979) in which the lipoprotein was precipitated by trichloroacetic acid, and only the water-soluble malondialdehyde present in the supernatant estimated by its reaction with thiobarbituric acid (TBA) and the resultant thiobarbituric acid reactive substances (TBARS) were measured. Briefly, a 100µL haemolymph sample was mixed with 1mL 20%TCA and incubated at 25°C for 20 min. The mixture was centrifuged at 500xg for 10min. An aliquot of 0.75mL of the supernatant was mixed with 0.25mL 0.8% Thiobarbituric acid (TBA) and heated in a boiling water bath for 1 hr. 1mL n-butanol was added to the above mixture and centrifuged at 4000rpm for 10 min. 100µL of the organic layer was taken in a microplate and the absorbance was measured at 532nm and expressed as absorbance 100µL⁻¹ haemolymph.

3.2.2.14. Aspartate aminotransferase and alanine aminotransferase

Haemolymph aspartate aminotransferase and alanine aminotransferase activity levels were determined as per Reitman and Frankel (1957). The reaction mixture containing 93µL 0.25M α-ketoglutarate, 890µL 0.1M L-Aspartic acid / 0.1M DL-Alanine (for aspartate aminotransferase and alanine aminotransferase respectively), 55.4µL 0.277mM pyridoxal, 44µL 0.01M Arsenate, 106.6µL potassium phosphate buffer (pH-7.4) and 30µL haemolymph were incubated for 30mins. The reaction was stopped by adding 2µL of 0.1% 2, 4 dinitrophenyl hydrazine and centrifuged at 5000xg for 3mins. Fifty µL of the supernatant was mixed with 150µL 1.3M NaOH and the absorbance measured at 440nm and expressed as O.D. 100µL⁻¹ haemolymph.
3.2.2.15. Nitric oxide synthase activity

As nitric oxide synthase (NOS) causes the conversion of arginine to L-citrulline and NO, NOS activity was examined by determining the L-citrulline and total nitrite in the sample. L-citrulline was determined according to Jiang et al. (2006). Briefly, a 100µL haemolymph sample was mixed with 50µL 165 U mL⁻¹ urease and incubated at 37ºC for 30mins. The reaction was stopped by adding 50µL 2.45M TCA and centrifuged at 13,000xg for 15mins. at room temperature. 50µL of the supernatant was mixed with 150µL ADMS reagent, incubated in boiling water bath for 25mins, cooled to room temperature (28ºC) in dark, centrifuged at 13,000xg for 5 mins. and the absorbance was read at 450nm and expressed as O.D. 100µL⁻¹ haemolymph.

Nitrite was determined by formation of reddish purple azo dye produced at pH 2 to pH 2.5 by coupling diazotized sulphanilamide and N-(1-naphthyl)-ethylene diamine dihydrochloride (NED). For the reaction, a 100µL haemolymph sample was mixed with 900µL Milli Q water, 20µL sulphanilamide and 20µL NED and incubated for 10mins. The absorbance was taken at 543nm and expressed as O.D. 100µL⁻¹ haemolymph.

3.2.3. Statistical Analysis

The data collected were analyzed statistically by one way and two way ANOVA. Wherever the treatment mean was found to be significant at Least Significant Difference (LSD) at 5% level, significant difference between the groups were calculated using the formula,

\[
\text{LSD} = \sqrt{\frac{2 \times \text{Ve} \times r}{5\% \ \text{table t value}}}
\]

where, \( r = \text{no. of samples / treatment} \)

\( \text{Ve} = \text{Mean Square within the group} \)

Differences were considered statistically significant at \( p < 0.05 \).
Chapter 3

3.3. Results

Level of significance in the variation of non-specific immune parameters of *Penaeus monodon* with in the groups (unchallenged and challenged) was obtained by one-way ANOVA and between the groups (unchallenged and challenged on 5th and 10th day post administration) was obtained by two way ANOVA as tabulated in Table 1 and Table 2, respectively.

3.3.1. Within the groups - Unchallenged group comprising normal and IVP (C0); normal and IVP challenged on 5th day post administration (C5) and normal and IVP challenged on 10th day post administration (C10)

3.3.1.1. Unchallenged group (Normal- N0, IVP- I0)

   Non-specific immune parameters such as reactive oxygen intermediates (ROI) and lipid peroxide showed significant differences within the group (one way ANOVA at $p<0.05$). ROI, and lipid peroxide levels were higher in the IVP administered group without challenge than in the group of animals fed on normal diet, (Table 1 and 3)

3.3.1.2. Challenged group

3.3.1.2.1. Challenged on 5th day post administration (Normal- NC5, IVP- IC5)

   In the group which was challenged on 5th day post administration and sampled on 3rd day post challenge (Normal- NC5, IVP- IC5), total haemocyte count and phenol oxidase activity showed significant differences within the group (one way ANOVA at $p<0.05$). In this case total haemocyte count and phenol oxidase activity were higher in the IVP administered and challenged group on 5th day than that of the normal (Table 1 and 3)
3.3.1.2.2. Challenged on 10\textsuperscript{th} day post administration (Normal- NC10, IVP-IC10)

In the group which was challenged on 10\textsuperscript{th} day post administration and sampled on 3\textsuperscript{rd} day post challenge (Normal- NC10, IC10), no significant differences in non-specific immune parameters were observed between the treatments at \(p<0.05\) (Table 1 and 3).

3.3.2. Between the groups, unchallenged group comprising normal and IVP (C0); normal and IVP challenged on 5\textsuperscript{th} day post administration (C5) and normal and IVP challenged on 10\textsuperscript{th} day post administration (C10)

The analysis of non-specific immune parameters between the groups (C0, C5, C10), by two way ANOVA showed significant difference \((p<0.05)\) only in total haemocyte count (THC). The THC was higher in the group C0, unchallenged, followed by 5\textsuperscript{th} post administration challenged and least on 10th day post administration challenged. The THC did not show significant difference between the unchallenged group (C0) and the group challenged on 5\textsuperscript{th} day post administration (C5). Same is the situation between the THC in the unchallenged group (C0) and the group challenged on 10\textsuperscript{th} day post administration (C10) (Table 2 and 3).

3.3.3. Between the groups (Normal (N) and IVP (I) each comprising unchallenged group and challenged group on 5\textsuperscript{th} day and 10\textsuperscript{th} day post administration)

Non-specific immune parameters like ROI and transglutaminase showed significant difference \((p<0.05)\) between the groups N and I in statistical two way ANOVA. IVP administered group showed significantly higher ROI and transglutaminase than the normal feed administered group.
3.3.4. Non-specific immune parameters which do not show significant difference ($p<0.05$) within the group and between the groups.

Non-specific immune parameters like superoxide dismutase, catalase activity, glutathione s transferase, nitric oxide synthase, glutathione peroxidase activity, alpha 2 macroglobulin activity, lysozyme activity, acid phosphatase activity, alkaline phosphatase activity, aspartate aminotransferase activity and alanine aminotransferase activity did not show significant difference in the different groups (N0, I0; NC5, IC5; NC10, IC10; C0, C5, C10; N, I) analyzed.

3.4. Discussion

Research on shrimps has shown that the basic mechanisms such as direct sequestration and killing of infectious agents which involve synthesis and exocytosis of a battery of bioactive molecules operate through the open circulating system (circulating haemolymph comprising different types of haemocytes) play extremely important roles in the immune defence mechanism. Essentially, the haemocytes execute inflammatory-type reactions such as phagocytosis, haemocyte clumping, production of reactive oxygen metabolites and the release of microbicidal proteins (Smith et al., 2003). Hence, haemolymph of the experimental animals were analyzed for the non-specific immune response in *P. monodon* IVP administration and challenge with WSSV.

3.4.1. Within the groups of animals unchallenged under normal diet and IVP administered (N0, I0); challenged on 5th day post administration (NC5, IC5); challenged on 10th day post administration (NC10, IC10)

3.4.1.1. Unchallenged under normal diet and IVP administered (N0, I0)

In the present study, the reactive oxygen intermediates (ROI) and lipid peroxide in the experimental animals (*P. monodon*) after the administration of
IVP (10) (before challenge) was significantly higher than in the normal feed administered animals.

Song and Hsieh (1994) demonstrated in vitro the phenomenon known as respiratory burst which could elicit a respiratory burst in *P. monodon* haemocytes, thought to be related to phagocytosis, when shrimp responded to immunostimulants such as PMA, zymosan and β-glucan. The amount of microbicidal substances generated by shrimp haemocytes were similar to those observed in fish macrophages (Lee and Shiau 2004). The stimulation of the phagocytic cell membrane leads to increased consumption of oxygen, the reduction of which, catalysed by a membrane-bound enzyme, NAD(P)H oxidase, gives rise to O$_2^-$ (Lee and Shiau, 2004). Since O$_2^-$ is the first product to be released from the respiratory burst, the measurement of O$_2^-$ has been accepted as a direct and accurate way of measuring respiratory burst activity (Citarasu et al., 2006). An increase in ROI has been observed in *P. monodon* administered with specific amount (lesser / higher amount caused immune defect) of feed incorporated with β 1,3 glucan, certain Indian herbs, Vitamin C with its derivatives, copper and Vitamin E (Chang et al., 2000, 2003; Citarasu et al., 2006; Lee and Shiau, 2002a, 2002b, 2003, 2004). Liu et al. (2006c) has observed that the administration of sodium alginate has decreased its ROI.

Lipid peroxidation has been reported to inactivate membrane bound enzymes because oxidation of ‘SH’ groups present in the active sites leads to conformational alteration in the enzymes (Kako et.al., 1988). Lipid peroxidation and consequent tissue damage are the major problems associated with failure of the antioxidant system. Lipid peroxides are themselves free radicals with large reaction constants and will therefore lead to cell death. Kidd (1991) indicate that peroxides, hydroxyradicals etc. can attack molecules like DNA, RNA, enzyme, protein, phospholipids etc. and then damage membrane integrity. Increased lipid peroxidation can lead to production of malondialdehyde (MDA) that enhances
the formation of free radicals from polyunsaturated fatty acids in cell membranes. Viral infection seems to result in an alteration in the cellular activity, which leads to the dysfunction of the complex antioxidant system. Pathogenic stress such as bacterial, fungal and viral infection can induce peroxidation of membrane lipids (Chih-Hong et al., 2003). WSSV infection in *P. monodon* caused a significant increase in the activity of lipid peroxidation in all tissues and a substantial decrease in the activity of antioxidants (superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPx) and glutathione-s-transferase (GST)) (Mohankumar and Ramasamy, 2006a, 2006b; Mathew et al., 2007). Enhanced levels of lipid peroxidation in WSSV infected animals indicate that they are under high oxidative stress. Mathew et al. (2007) has found a sharp increase in the concentration of lipid peroxides in hemolymph of WSSV infected *P. monodon*.

The significant difference observed in *P. monodon* administered with test feed IVP and those studies where the animals were administered with other feed additives, is the activation of non-specific immune parameters like THS, PO activity and SOD activity along with the ROI. Though the lipid peroxide was significantly higher in IVP administered group, the antioxidant activity (involving SOD, catalase, glutathione peroxidase, and glutathione-s-transferase) of the same did not show significant differences from the normal feed administered group. However, the significant activation of ROI and lipid peroxide in IVP administered group (unchallenged) than in control, unlike in other feed additive studies, shows that the IVP administered had a different mechanism of immune system activation in these animals.

### 3.4.1.2. Challenged on 5th day post administration (NC5, IC5)

In the group which was challenged on 5th day post administration of different feeds (normal-NC5, IVP-IC5) and sampled on 3rd day after challenge, THC and phenol oxidase activity showed significant difference between NC5 and IC5.
The THC and phenol oxidase activity in the IVP administered group (IC5) was significantly higher than in the normal feed administered (NC5) group.

Total Haemocyte Count (THC) refers to the generally accepted three major categories of blood cells (haemocytes) in decapod crustaceans such as hyaline cells, semi-granular cells and granular cells. Each has distinctive morphological features and physiological functions (Johannson et al., 2000). Haemocytes are responsible for clotting, exoskeleton hardening and elimination of foreign materials (Song and Hsieh, 1994). Mean THCs of healthy penaeid shrimps ranged from 20 to 40 x 10^6 cells ml^-1 (Chang et al., 1999). Molting, development of organs, reproductive status, nutritional condition and disease have been shown to influence haemocyte abundance (Cheng and Chen, 2001). A low circulating haemocyte count is strongly correlated with a greater sensitivity to pathogens (Persson et al., 1987). It has been reported by Chang et al. (2003) and Citarasu et al. (2006) that the WSSV infection by injection has caused a decrease in the THC.

The phenol oxidase system is the prime immune defence of invertebrates and any reductions in the activity of phenol oxidase enzyme might lead to the failure of phagocytosis, which is the common process in the cellular defence of crustaceans. The prophenoloxidase activating system (proPO system) has an important role as a non-self recognition system and participates in the innate immune response through melanization, cytotoxic reactions, cell adhesion, encapsulation, nodule and capsule formation, hemocyte locomotion and phagocytosis (Soderhall and Cerenius, 1998; Lee and Soderhall, 2002; Cerenius and Soderhall, 2004; Jiravanichpaisal et al. 2006; Rajeshkumar, 2008). Phenol oxidase (PO), the key enzyme in the synthesis of melanin, occurs in haemolymph as an inactive pro-enzyme pro phenol oxidase (proPO). proPO is activated to form PO when it reacts with zymosan (carbohydrates from yeast cell walls), bacterial lipopolysaccharide (LPS), urea, calcium ion, trypsin or heat.
(Chang et al., 2003). Though the exact mechanism by which WSSV affects phenol oxidase enzyme system in \textit{P. monodon} is still unknown, studies in other crustaceans suggest that viral infection mediated oxidative stress is a major factor responsible for the reduction of phenol oxidase activity (Le Moullac et. al., 1998; Cheng et. al., 2002). A drastic reduction in phenol oxidase activity was noticed in WSSV infected animals by Mathew et. al. (2007). Increase in PO activity has been reported in \textit{P. monodon} (after challenge) after the administration of beta glucan (Chang et. al., 2003) immunostimulant herbs (Citarasu et. al., 2006) and ‘DNA vaccination’ (Rajeshkumar et. al., 2008). From the activation of phenol oxidases generating highly cytotoxic quinines that might inactivate viral pathogens (Ourth and Renis, 1993), and the significant high THC observed on 5\textsuperscript{th} day post administration challenge group, it could be inferred that the IVP administered animals showed lower WSSV infection rate as compared to the infection rate in the normal feed administered group.

\textbf{3.4.1.3. Challenged on 10\textsuperscript{th} day post administration (NC10, IC10)}

In the group which was challenged on 10\textsuperscript{th} day post administration and sampled on 3\textsuperscript{rd} day post challenge (Normal- NC10, IVP- IC10), the absence of significant difference in the non-specific immune parameters showed that there was no significant variation between the treatments (normal feed administered and IVP administered) on the 10\textsuperscript{th} day. This suggests the subsiding efficacy of IVP over a period of time.

\textbf{3.4.2. Between the unchallenged (C0) and challenged groups on 5\textsuperscript{th} day post administration (C5) and 10\textsuperscript{th} day post administration C10)}

Significant difference was observed in \textit{P. monodon} (comprising IVP and normal feed administered) before and after WSSV challenge only in total haemocyte count.
Among them, the total haemocyte count (THC) was the highest in unchallenged group (C0) followed by the 5th day post administration challenge (C5) group and the 10th day post administration challenge (C10) group. Significant higher THC was observed in the unchallenged group than in 10th day post administration challenge (C10) group but with 5th day post administration challenge (C5) the difference was not significant. This observation parallel to the findings of Chang et al. (2003) and Citarasu et al. (2006) that a decrease in the THC could be caused by WSSV infection. The lack of significant difference between the unchallenged group and the group challenged on 5th day post administration may be because of the oral route of challenge adopted in the present study which in-turn might have taken a prolonged time for infection compared to injection challenge. However, the 5th day post administration challenged group did not show significant difference from 10th day post administration group. Possibly, a reduction in THC subsequent to challenge both in normal and IVP administered group suggests that WSSV may be acting on the heamocytes inspite of IVP administration reducing their count.

3.4.3. Between the normal feed (N) and IVP (I) administered

Between the groups, IVP (I) and normal (N) feed administered (comprising unchallenged and challenged), ROI and transglutaminase showed significant difference. ROI and transglutaminase was significantly higher in IVP administered *P. monodon* than in the normal feed administered group which was similar to the response shown to feed additives (Chang et al., 2000; Chang et al., 2003; Lee and Shiau, 2002a; 2002b; 2003; 2004; Citarasu et al., 2006).

Coagulation of haemolymph is part of the innate immune response of crustaceans which prevents loss of body fluids and entry of opportunistic pathogens. In shrimps, coagulation is initiated by activation of hyaline cells which releases its contents including the clotting enzymes. Transglutaminase (TGase) in the haemocytes effectively polymerizes shrimp clottable proteins to
form stabilized gel (Yeh et al., 2006). A decrease in the transglutaminase activity in WSSV infected *Fenneropenaeus chinensis* by RT-PCR analysis (Liu et al., 2007b) and delay in clotting time in *F. indicus* has been reported (Yoganadhan et al., 2003b; Sarathi et al., 2007). Song et al. (2003) has observed that the decrease in TGase activity coincided with poor hemolymph coagulation in *Litopenaeus vannamei* infected with Taura syndrome virus. The present study shows that in *P. monodon*, administered with IVP, there was significantly higher transglutaminase activity there by causing an effect on the coagulation mechanism.

The non-specific immune response of *P. monodon* after the challenge cannot be compared to previous studies related to feed additives followed by a WSSV challenge. This is due to the fact that in these studies, the route of challenge has been different i.e., injection (Chang et al., 2003; Citarasu et al., 2006). Moreover, a single defined nature of WSSV challenge (eg: virus titre / strain, type of infected tissue, dose of infected tissue used, nature / frequency / time / route of challenge etc.) has not been used in studies analyzing the non-specific immune parameters in response to WSSV infection in *P. monodon* (Chang et al., 2003; Citarasu et al., 2006; Rameshthangam and Ramasamy, 2006; Mathew et al., 2007). Rajeshkumar et al. (2008) has not mentioned a defined nature of challenge in their report of non-specific immune parameters in ‘DNA vaccinated’ *P. monodon*. In these experiments using *P. monodon*, another factor which varies is the day of sampling after the challenge.

However, in the present study significant difference was not observed in the non-specific immune parameters like SOD activity, catalase activity, glutathione-s-transferase, glutathione peroxidase activity, nitric oxide synthase, alpha 2 macroglobulin activity, acid phosphatase activity, alkaline phosphatase activity, lysozyme activity, aspartate aminotransferase activity and alanine aminotransferase activity. These immune indices which are reported to be active
in their respective studies may not be functional in a significant way for the immune response of *P. monodon* against WSSV as seen in the present study.

All together, it is inferred that *P. monodon* when administered with IVP, adopts a unique immune mechanism with an inter play of various pathways which has to be explored in a deeper and wider perspective.
Table 1. Level of significance of non-specific immune parameters of *Penaeus monodon* within the groups determined by one-way ANOVA (*p*-value < 0.05)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Phenotype</th>
<th>Unchallenged (N₀, I₀)</th>
<th>Challenged on 5DPA (NC₅, IC₅)</th>
<th>Challenged on 10DPA (NC₁₀, IC₁₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total Haemocyte Count</td>
<td>0.077697</td>
<td>*0.047688</td>
<td>0.436918</td>
</tr>
<tr>
<td>2.</td>
<td>Phenol oxidase activity</td>
<td>0.900577</td>
<td>*0.025084</td>
<td>0.330355</td>
</tr>
<tr>
<td>3.</td>
<td>Reactive oxygen intermediates (ROI)</td>
<td>*0.010231</td>
<td>0.324749</td>
<td>0.953653</td>
</tr>
<tr>
<td>4.</td>
<td>Transglutaminase</td>
<td>0.112157</td>
<td>0.591145</td>
<td>0.346203</td>
</tr>
<tr>
<td>5.</td>
<td>Superoxide dismutase</td>
<td>0.373255</td>
<td>0.355232</td>
<td>0.771850</td>
</tr>
<tr>
<td>6.</td>
<td>Glutathione-s-transferase</td>
<td>0.366706</td>
<td>0.928043</td>
<td>0.315266</td>
</tr>
<tr>
<td>7.</td>
<td>Nitric oxide synthase</td>
<td>0.196407</td>
<td>0.288193</td>
<td>0.526846</td>
</tr>
<tr>
<td>8.</td>
<td>Catalase</td>
<td>0.473069</td>
<td>0.099648</td>
<td>0.511446</td>
</tr>
<tr>
<td>9.</td>
<td>Glutathione peroxidase</td>
<td>0.772011</td>
<td>0.227407</td>
<td>0.888205</td>
</tr>
<tr>
<td>10.</td>
<td>Lysozyme activity</td>
<td>0.821316</td>
<td>0.358730</td>
<td>0.256660</td>
</tr>
<tr>
<td>11.</td>
<td>Alpha 2 macroglobulin</td>
<td>0.745041</td>
<td>0.078217</td>
<td>0.481408</td>
</tr>
<tr>
<td>12.</td>
<td>Acid phosphatase</td>
<td>0.635311</td>
<td>0.100192</td>
<td>0.390267</td>
</tr>
<tr>
<td>13.</td>
<td>Alkaline phosphatase</td>
<td>0.537119</td>
<td>0.057566</td>
<td>0.127934</td>
</tr>
<tr>
<td>14.</td>
<td>Lipid peroxide</td>
<td>*0.041367</td>
<td>0.988517</td>
<td>0.584691</td>
</tr>
<tr>
<td>15.</td>
<td>Aspartate aminotransferase</td>
<td>0.668668</td>
<td>0.172116</td>
<td>0.393692</td>
</tr>
<tr>
<td>16.</td>
<td>Alanine aminotransferase</td>
<td>0.278359</td>
<td>0.371495</td>
<td>0.326886</td>
</tr>
</tbody>
</table>

* - significant difference (*p*-value < 0.05)
Table 2. *p*-value of non-specific immune parameters of *Penaeus monodon* (between the groups) determined by two-way ANOVA

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Phenotype</th>
<th>Unchallenged (C0) and Challenged (C5, C10)</th>
<th>N, I</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total Haemocyte Count</td>
<td><em>0.023990</em></td>
<td>0.745891</td>
</tr>
<tr>
<td>2.</td>
<td>Phenol oxidase activity</td>
<td>0.142214</td>
<td>0.064373</td>
</tr>
<tr>
<td>3.</td>
<td>Reactive oxygen intermediates (ROI)</td>
<td><em>0.056990</em></td>
<td><em>0.017629</em></td>
</tr>
<tr>
<td>4.</td>
<td>Transglutaminase</td>
<td>0.078330</td>
<td><em>0.048097</em></td>
</tr>
<tr>
<td>5.</td>
<td>Superoxide dismutase</td>
<td>0.312304</td>
<td>0.337821</td>
</tr>
<tr>
<td>6.</td>
<td>Glutathione-s-transferase</td>
<td>0.425096</td>
<td>0.283200</td>
</tr>
<tr>
<td>7.</td>
<td>Nitric oxide synthase</td>
<td>0.364233</td>
<td>0.359184</td>
</tr>
<tr>
<td>8.</td>
<td>Catalase</td>
<td>0.340420</td>
<td>0.349084</td>
</tr>
<tr>
<td>9.</td>
<td>Glutathione peroxidase</td>
<td>0.687518</td>
<td>0.287790</td>
</tr>
<tr>
<td>10.</td>
<td>Lysozyme activity</td>
<td>0.399615</td>
<td>0.298218</td>
</tr>
<tr>
<td>11.</td>
<td>Alpha 2 macroglobulin</td>
<td>0.787786</td>
<td>0.306189</td>
</tr>
<tr>
<td>12.</td>
<td>Acid phosphatase</td>
<td>0.546694</td>
<td>0.191602</td>
</tr>
<tr>
<td>13.</td>
<td>Alkaline phosphatase</td>
<td>0.237333</td>
<td>0.392147</td>
</tr>
<tr>
<td>14.</td>
<td>Lipid peroxide</td>
<td>0.491364</td>
<td>0.225949</td>
</tr>
<tr>
<td>15.</td>
<td>Aspartate aminotransferase</td>
<td>0.145564</td>
<td>0.097107</td>
</tr>
<tr>
<td>16.</td>
<td>Alanine aminotransferase</td>
<td>0.290019</td>
<td>0.076295</td>
</tr>
</tbody>
</table>

* - significant difference (*p*-value < 0.05)
Table 3. The order of significance in the experimental groups

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Phenotype</th>
<th>Order of Significance (as obtained from Least Significant Difference – LSD at 5% level)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Within the groups</strong> (N0, I0)</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Reactive Oxygen Intermediates (ROI)</td>
<td>I0 &gt; N0</td>
</tr>
<tr>
<td>2.</td>
<td>Lipid peroxide</td>
<td>I0 &gt; N0</td>
</tr>
<tr>
<td></td>
<td><strong>(NC5, IC5)</strong></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Total Haemocyte Count (THC)</td>
<td>IC5 &gt; NC5</td>
</tr>
<tr>
<td>4.</td>
<td>Phenol oxidase activity</td>
<td>IC5 &gt; NC5</td>
</tr>
<tr>
<td></td>
<td><strong>Between the groups - (C0, C5, C10)</strong></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Total Haemocyte Count (THC)</td>
<td>C0 &gt; C10, {C0 = C5, C5 = C10}</td>
</tr>
<tr>
<td></td>
<td><strong>Between the groups - (N, I)</strong></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Reactive oxygen intermediates (ROI)</td>
<td>I &gt; N</td>
</tr>
<tr>
<td>2.</td>
<td>Transglutaminase</td>
<td>I &gt; N</td>
</tr>
</tbody>
</table>

N0 – Normal feed administered, unchallenged
I0 – IVP administered, unchallenged
NC5 – normal feed administered, IC 5- IVP administered - animals which were challenged with WSSV on 5th day post administration and sampled on 3rd day post challenge
NC10 – normal feed administered, IC10- IVP administered - animals which were challenged with WSSV on 10th day post administration and sampled on 3rd day post challenge
C0 – Unchallenged animals
C5 – Challenged with WSSV on 5th day post administration and sampled on 3rd day post challenge
C10 – Challenged with WSSV on 10th day post administration and sampled on 3rd day post challenge
N – Normal feed administered animals
I – IVP administered animals

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