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Hematological parameters in relation to sex, morhometric characters and incidence of white spot syndrome virus in tiger shrimp *Penaeus monodon* Fabricius, 1798 from Sunderban, West Bengal

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ABSTRACT

Seasonal influence on the hemato-immunological parameters and prevalence of white spot disease (WSD) in wild-caught tiger shrimp, *Penaeus monodon* was studied. A total of 210 specimens of *P. monodon* were collected from the Sunderban estuarine mangrove ecosystem during pre-monsoon (March to May) and monsoon (June to September) seasons in 2010. The animals were sexed, measured (total length and carapace length) and weighed. The hemato-immunological parameters such as total hemocyte count (THC), granular hemocyte (GH) and nongranular hemocyte (NGH) count, phenoloxidase activity and serum protein levels were analysed. The animals were diagnosed for WSSV by nested polymerase chain reaction (PCR). Significant difference ($p<0.01$) in THC, GH and NGH count was observed between seasons in both sexes. However, no significant difference was noticed in serum phenoloxidase activity and serum protein level in both sexes between seasons. None of the animals were diagnosed positive for WSSV in first step PCR throughout the study period. However, few samples were diagnosed positive in the second step PCR in the month of September. No significant difference was observed in any of the hemato-immunological parameters studied, between second step positive and negative samples, which suggests that level of infection in the second step PCR positive samples might not be sufficient to alter the immunological parameters.

Keywords: Hemato-immunological parameters, *Penaeus monodon*, Seasonal variation, Shrimp, Sunderbans, White spot disease

Introduction

The tiger shrimp, *Penaeus monodon* is naturally distributed in water bodies throughout the Sunderban area which is the primary penaeid shrimp species currently being cultured in the region Environmental changes have been reported to be an important cause for increased prevalence of shrimp disease leading to the marked reduction in shrimp production (Pan Lu-Qing *et al.,* 2007). Shrimps possess only innate (non-specific) immune mechanisms comprising anatomical barriers, proteolytic cascades, clotting mechanism and cellular elements. Haemocytes play major role in non-specific immune response by phagocytosis, melanisation, encapsulation, cytotoxicity, clotting and release of clotting proteins, agglutinins, hydrolytic enzymes and antimicrobial peptides upon their lysis (Peraza-Gomez *et al.,* 2011). In shrimps, the hematological parameters are expected to be affected primarily on exposure to infectious agents including the dreaded white spot virus (WSV) . There is very limited information available on seasonal influence on the hematological parameters and prevalence of WSD among wild caught tiger shrimp and therefore the present study was undertaken to investigate the same in *P. monodon* collected from Sunderban area.

Materials and methods

A total of 210 specimens of *P. monodon* were collected randomly between March and September, 2010 from the wild source in Sunderbanss and transported to the laboratory in high-density polythene bags with aeration. The period from March to May was categorised as pre-monsoon and the period from June to September as monsoon for statistical analysis of the data obtained. The morphological parameters such as sex, weight, total length and carapace length were recorded (Owens and O' Neill. 1997). The water quality parameters such as temperature, dissolved oxygen (DO by Winkler's method) and salinity were recorded at the time of sample collection. The data collected were analysed using SPSS for Windows v.17.0 programme (SPSS Inc., Chicago, IL, USA).

Analysis of hemato-immunological parameters

Phenoloxidase activity

The serum collected from each animal was used for measuring the phenoloxidase (PO) activity spectrophoto-metrically (Techcomp, China) from the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) as

described by Pan Lu-Qing *et al.* (2007). Briefly, 0.01 M solution of L-DOPA was prepared in 0.1 M phosphate buffer (12.3 ml 0.2 M Na_2HPO_4, 87.7 ml 0.2 M NaH_2PO_4, pH 6.0). Equal volume of serum and L-DOPA (100 µl each) were added to a spectrophotometer cuvette (10 mm) containing 3 ml of phosphate buffer. The mixture was agitated and the optical density (OD) was measured at 490 nm, at 2 min intervals for 60 min. Phenoloxidase activity was determined from the increase in OD per min. Total haemolymph protein was determined spectrophotometrically as per Lowry *et al.* (1951) using bovine serum albumin as a standard.

Total haemocyte count

Haemolymph (0.1 ml) was withdrawn from the ventral sinus of the first abdominal segment into a syringe containing equal volume of fixative (10% formalin in 0.45 M NaCl) and transferred to an eppendorf tube for total haemocyte count (THC) as well as for granular haemocyte (GH) and nongranular haemocyte (NGH) counts adopting the method of Sritunyalucksana *et al.* (2005). After 10 min, 20 µl of the fixed haemocyte suspension was mixed with same volume of Rose Bengal solution (1.2% Rose Bengal in 50% ethanol) and incubated at ambient temperature (27-35 °C) for 20 min. before being used for haemocyte counts. Haemocyte counts were made in a haemocytometer (improved Neubauer, Marienfeld, Germany) in 5/25 squares (vol. of one square = 0.2 x 0.2 x 0.1 mm^3). THC was calculated as: THC ml^{-1} of haemolymph = 5 × count × 10^4 × dilution factor.

Granular and nongranular hemocyte count

For GH and NGH counts, smears were prepared on clean, greese free glass slides, from the fixed and Rose Bengal stained haemocyte suspension. The smears were completely dried before counterstaining with haematoxylin solution (50 g aluminium or potassium alum, 1g haematoxylin crystals, 0.2 g sodium iodate, 1g citric acid, 50 g chloral hydrate and distilled water to 1l) for 7 to 10 min. The slides were then rinsed with tap water for 10 min, dehydrated in ascending grades of ethanol (10 dips each), cleared in xylene (3 times for 3 min each) and mounted using DPX mountant (Merck) with a cover glass. The proportions of GH that included both large-granular and small-granular/semigranular hemocytes in 200 total hemocytes were recorded and these proportions were used to calculate the total number of GH (*i.e.*, GH count/200 × THC). NGH counts were also calculated in the same manner.

Detection of prevalence of white spot disease by PCR

DNA extraction

Gill/pleopods were collected, fixed in 95% ethanol and subsequently, 25 mg of gill tissue was homogenised with 500 µl of digestion buffer (50 mM Tris-Hcl, pH 8; 1 mM EDTA, pH 8; 500 mM NaCl; 1% SDS; proteinase K 5 µg) and incubated for one hour at room temperature followed by boiling at 100 °C for 10 min in water bath. Supernatant was collected after centrifuging at 10000 *g* for 10 min at room temperature, 2.2 volume of 70% ice-cold ethanol was added and kept for 30 min. for precipitation of DNA. The sample was then centrifuged at 10000 *g* for 10 min at 4 °C (Eppendorf, Germany). The pellet was collected, air dried and suspended in 100 µl of 1x TE buffer (50 mM Tris-Hcl; 1 mM EDTA; pH 8). The extracted DNA sample was stored at -40 °C until further analysis.

Polymerase chain reaction (PCR)

For two-step PCR amplification, four DNA oligonucleotide primers reported by Kimura *et al.* (1996) were used. PCR was carried out in 25 µl of reaction mixture that consisted of 2.5 µl (10x) of buffer with 2.5 mM of $MgCl_2$, 1.0 µl (10 pmol) of each first step primers, 0.05 µl (100 mM) of each dNTPs, 1.0 µl of DNA template, 0.2 µl (1U) of *Taq* DNA polymerase (Genei, Bangalore) and 19.1 µl of autoclaved Milli Q water. PCR was performed in a thermocycler (Eppendorf, Germany) with initial denaturation at 95 °C for 3 min and 30 cycles of PCR (denaturing at 95 °C for 30 sec; annealing for 30 sec at 58 °C; and extension at 72 °C for 30 sec), followed by final extension at 72 °C for 5 min. This was followed by nested PCR with a combination of II sets of primers with I step PCR product as the template (same condition as above). The reaction mixture was then cooled down to 4 °C. The PCR product was then electrophoresed on 1% of agarose gel prepared in 1x TBE buffer (0.09 M Tris borate; 0.002 M EDTA; pH 8) having ethidium bromide (0.2 µg per ml) for band visualisation using a UV transilluminator (Syngene, UK). Further, the shrimp samples were also screened by PCR for other viral infections such as infectious hypodermal and hematopoietic necrosis virus (IHHNV) (OIE, 2003), yellow head virus (YHV) and gill associated virus (GAV) using the IQ 2000™ YHV/GAV detection kit (Farming IntelliGene Tech. Corp., Taiwan) (Biswas *et al.*, 2012).

Results and discussion

Morphological parameters such as the total body weight (g), total length (mm) and carapace length (mm) were found to be significantly (p<0.01) different among both the sexes during different months (Table 1). Owens and O' Neill (1997) reported that female *P. monodon* were larger in total length than their male counterparts of same age group after adolescence, however they did not observe any difference in carapace length between the summer and winter populations. But there was difference between the weights of the two populations, with shrimps in the summer

Table 1.Month-wise comparison of morphological and hematological parameters of *Penaeus monodon* from Sunderbans

Months	Total body weight (g)**	Total length (mm) **	Carapace length (mm) **	THC x 10^6**	GH count x 10^6	NGH count x 10^6**	Total serum protein (mg ml^{-1})	Serum PO activity $min^{-1}mg$ $protein^{-1}$
Male								
March	11.89±2.78^{a}	102.88±6.15^{a}	24.71±1.99^{a}	14.83±1.17^{a}	2.85±0.25	11.99±1.06^{a}	81.44±4.86	0.40±0.08
April	13.90±1.44^{a}	120.92±4.77^{ab}	30.82±1.68^{ab}	15.56±0.78^{ab}	3.31±0.17	12.25±0.62^{ab}	75.17±3.82	0.52±0.05
May	27.78±3.92^{bc}	144.86±10.66^{bc}	36.74±3.22^{bc}	16.82±0.78^{abc}	3.20±0.49	13.62±0.30^{abc}	79.59±4.23	0.57±0.03
June	17.23±1.89^{ab}	124.58±4.20^{ab}	29.71±1.34^{ab}	17.25±0.57^{bc}	3.47±0.28	13.78±0.33^{abc}	76.56±5.11	0.55±0.02
July	34.68±5.17^{c}	156.96±7.84^{c}	42.92±3.05^{cd}	20.54±0.22^{d}	3.63±0.17	16.90±0.09^{d}	78.47±3.56	0.59±0.12
August	37.73±5.23^{c}	158.59±10.63^{c}	44.38±3.44^{cd}	19.00±0.86^{cd}	3.68±0.06	15.32±0.86^{cd}	75.16±0.87	0.64±0.08
September	39.36±4.84^{c}	164.89±6.61^{c}	45.44±2.06^{d}	17.89±0.04^{bc}	3.77±0.04	14.12±0.03^{bc}	72.89±7.70	0.39±0.06
Female								
March	16.48±5.66^{ab}	116.70±13.25^{a}	29.86±3.73^{a}	15.39±0.55^{b}	3.07±0.08^{ab}	12.32±0.61^{a}	83.56±4.67	0.58±0.07
April	15.14±1.73^{a}	122.12±3.67^{a}	31.12±1.15^{ab}	13.88±0.45^{a}	2.92±0.11^{a}	10.96±0.35^{a}	87.11±4.29	0.49±0.08
May	28.69±0.38^{bc}	149.90±0.68^{bc}	38.11±0.16^{bc}	17.09±0.22^{c}	3.36±0.18^{abc}	13.73±0.34^{b}	82.69±2.39	0.48±0.02
June	16.87±0.97^{ab}	125.61±2.35^{ab}	29.72±0.95^{a}	17.90±0.47^{cd}	3.41±0.42^{abc}	14.49±0.11^{b}	82.03±6.42	0.45±0.02
July	36.68±6.91^{c}	164.50±11.86^{c}	43.67±3.17^{c}	20.91±0.19^{e}	3.88±0.25^{c}	17.03±0.36^{c}	88.20±7.54	0.58±0.13
August	38.53±3.65^{c}	161.15±7.63^{c}	45.42±2.24^{c}	18.84±0.76^{d}	3.70±0.01^{bc}	15.14±0.76^{b}	82.00±3.33	0.57±0.09
September	38.13±4.75^{c}	163.07±8.18^{c}	44.56±3.28^{c}	18.33±0.39^{cd}	3.84±0.10^{c}	14.50±0.32^{b}	65.93±12.02	0.65±0.20

**$p<0.01$; *$p<0.05$

- Values bearing different superscripts in a column differ significantly

being twice as heavy as those sampled in winter. In the present study, there was a significant difference ($p<0.05$) between two seasons in total body weight, total length and carapace length among the male populations, but among the female populations, significant difference ($p<0.05$) was observed only in total body weight (Table 2). No significant difference was observed in morphological paramentes between sexes during different months and within season. Since the animals were collected from the wild source, the exact age of the animals could not be ascertained. Total serum protein and serum phenoloxidase activity did not vary significantly during different months, between seasons and between sexes.

Owens and O' Neill (1997) reported THC values ranging from 2.10×10^7 (flow cytometry) to 2.33×10^7 (haemocytometer) in *P. monodon.* THC values ranging from $2.67 \pm 0.44 \times 10^7$ (ATP analysis) to $2.72 \pm 0.31 \times 10^7$ (haemocytometer) have been reported among cultured populations of *P. monodon* (Chang *et al.,* 1999). Comparatively lower values reported in the present study could be attributed to various factors as the shrimps were sampled from wild source. THC and NGH ($\times 10^6$) count differed significantly ($p<0.01$) among both the sexes during different months of the year. Moreover, significant difference was observed between two seasons in THC, GH and NGH count ($p<0.01$; $p<0.05$) within sexes. The shrimps used for the present study were found to be mostly healthy and found free from shrimp viruses *viz.*, IHHNV, YHV and GAV, by PCR screening. In addition, all the animals were I step negative for WSSV throughout the study .

Table 2. Seasonal comparison of morphological and hematological parameters of *P. monodon* from Sunderbans

Seasons	Total body weight (g)*	Total length (mm)*	Carapace length (mm)*	THC x 10^6**	GH count x 10^6*	NGH count x 10^6**	Total serum protein (mg ml^{-1})	Serum PO U $min^{-1}mg$ $protein^{-1}$
Male								
Pre-monsoon	17.86±2.89^{a}	122.89±7.17^{a}	30.76±2.11^{a}	15.74±0.55^{a}	3.12±0.18^{a}	12.62±0.44^{a}	78.73±2.35	0.50±0.04
Monsoon	29.88±3.87^{b}	146.71±6.83^{b}	39.00±2.71^{b}	18.93±0.56^{b}	3.60±0.10^{b}	15.33±0.52^{b}	76.73±1.88	0.59±0.04
Female								
Pre-monsoon	20.10±2.75^{a}	129.57±6.50	33.03±1.71	15.45±0.51^{a}	3.12±0.09^{a}	12.33±0.46^{a}	84.45±2.07	0.52±0.04
Monsoon	30.69±4.15^{b}	150.42±7.47	39.60±2.74	19.22±0.52^{b}	3.66±0.16^{b}	15.55±0.45^{b}	84.08±3.19	0.53±0.05

**$p<0.01$; *$p<0.05$

- Values bearing different superscripts in a column differ significantly

Nested PCR positivity was observed for WSSV during the month of September (monsoon) (Fig. 1) when the salinity was least, and DO and water temperature were moderately low (Fig. 2). Ananda Raja *et al.* (2012) also reported nested PCR positivity in apparently healthy shrimps. No significant difference in hematological parameters was observed between sexes and between WSSV nested positive and negative animals. In contrary, Owens and O' Neill (1997) reported that the NGH counts were significantly different between the sexes, with females having higher levels than males while THC and GH counts in male and female prawns were not significantly different. Significant reduction in THC and haemolymph protein was observed in experimentally infected shrimps with WSSV (Yoganandhan *et al.,* 2003; Mathew *et al.,* 2009). The THC and GH counts decreased significantly in TSV-infected Pacific white shrimp, *Litopenaeus vannamei* (Yen-Ling Song *et al.,* 2003) under challenge trials. Lo *et al.* (1997) found a high percentage of wild-caught and healthy looking *P. monodon* brood stock with white spot syndrome virus (WSSV). THC of the blue shrimp, *Litopenaeus stylirostris*, decreased significantly and the phenoloxidase activity increased significantly when the temperature dropped from 27 to 18 °C (Moullac and Haffner, 2000). Short term temperature fluctuations can cause decline of the immune capacity in shrimps (Yu, 1993; Pan Lu-Qing *et al.,* 2007). Pan Lu-Qing *et al.* (2007) reported reduction in THC and increased phenoloxidase activity with change of temperature for a short period. However, after a period of adaptation, all immune parameters tended to be stable. In the present study, the water temperature was between 31 and 34 °C, DO ranged between 3.75 and 6.1 and salinity was between 8 and 21.2 gl^{-1}(Fig. 2). Apart from change in temperature, DO and salinity, there are many more hidden environmental factors which can cause variation in the hematological parameters during different months and seasons (Owens and O' Neill, 1997).

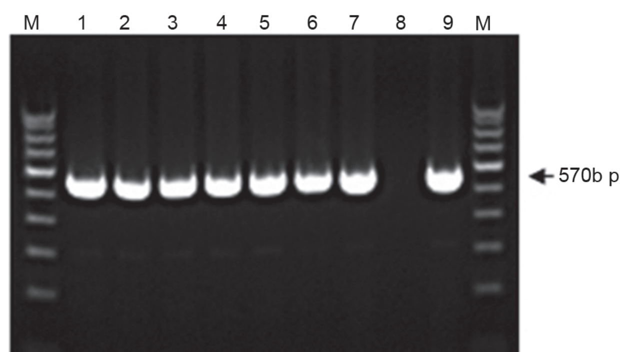

Fig. 1. Wild tiger shrimp, *P. monodon* showing nested PCR positivity for WSSV
Lane M : 100 bp ladder; Lane 1-7 : *P. monodon* nested PCR positive; Lane 8 : nested negative control; Lane 9 : nested positive control

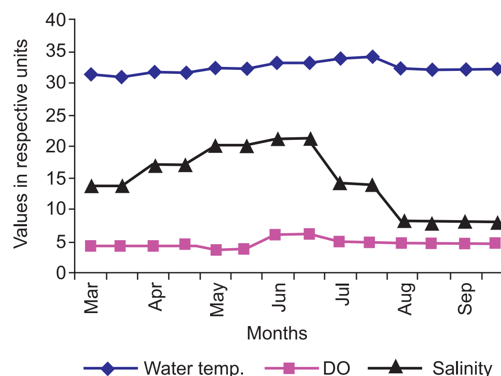

Fig. 2. Water quality parameters recorded from the source at the time of collection of the shrimp samples

The results of the present study revealed that the morphological parameters in wild tiger shrimp populations were significantly different among both the sexes during different months and seasons. But no significant difference was observed between sexes. No significant difference was observed in total serum protein and serum phenoloxidase activity during different months, seasons and between sexes. Hematological parameters differed significantly within both the sexes during different months, but no significant difference was observed between sexes and between WSSSV nested PCR positive and negative animals, which suggests that level of infection in the second step PCR positive samples might not be sufficient to alter the haemato-immunological parameters.

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