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Cent. Eur. J. Biol. • 7(3) • 2012 • 460-469
DOI: 10.2478/s11535-012-0026-3

Central European Journal of **Biology**

Haematological and innate immune responses in *Puntius sarana*: normal range and seasonal variation

Research Article

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Received 15 September 2011; Accepted 02 February 2012

Abstract: A study was conducted to measure the normal ranges, seasonal and annual variations in haematological and immune parameters of juvenile healthy *Puntius sarana* (weighing 75-100 g) during three major seasons over two consecutive years. Significantly ($P<0.05$) lower serum myeloperoxidase and ceruloplasmin activities, superoxide production, plasma glucose level, packed cell volume and haemoglobin concentration were detected in winter as compared to the summer season. However, serum lysozyme activity, antiprotease activity, total erythrocyte count (TEC) and TLC profiles were consistent during all seasons. The reference intervals (25^{th}-75^{th}) of each parameter were estimated and a range was established. The annual changes in immune parameters with minimum and maximum values were measured and a significant variation was noticed in myeloperoxidase, antiprotease, total protein levels and TEC over two consecutive years. The lower levels in haematological and innate immune status of fish during winter possibly indicate a higher disease risk period for the species.

Keywords: *Annual variation • Haematology • Humoral immunity • Puntius sarana • Reference intervals*

1. Introduction

Like human beings, fish and other aquatic animals also enjoy a good environment and exhibit an intimate relationship with their environment with respect to their growth, physiology and development. It is well documented that these important environmental conditions are directly or indirectly regulated by the season. However, the aquatic environment of fish is mainly affected by temperature, pH and photoperiod and these factors are also responsible for the behaviour, physiology and breeding cycles of these species. Moreover, the non-specific as well as the specific immune system of fish are also directly coordinated by the change in the environment [1].

The aquatic habitat of fish with variable temperature is the most favourable condition for the growth and multiplication of different pathogens causing various fish diseases. Among the different potential environmental factors, temperature is the main factor that affects both the innate and adaptive immune response of fish. The non-specific immunity like phagocytosis, cytotoxicity and production of specific immune factors are also adversely influenced by the lower temperature [2] whereas ambient temperatures have marked influence on immune reactivity in fish, including the kinetics of specific cell-mediated responses [3,4]. In many fish species the specific defence substance (immunoglobulin M, IgM) fluctuated with water temperature [5-9]. In general, the best responses are obtained at the normal summer temperatures which of course vary considerably according to the nature of the species, whether the fish is coldwater, temperate, or warm water species [10]. The temperature at which the optimum immune response attained by the species is termed immunologically "permissive" and temperatures below this, but still within the normal physiological range, is found to be immunosuppressive (non-permissive temperatures) [11]. The immunologically permissive and non-permissive temperature changes

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depending on the species of fish. The non-permissive temperature is reported to be 4°C in salmonid, 14°C in carp and 22°C in channel catfish [12]. Moreover, there is a strong correlation between seasonal fluctuations and susceptibility to various infectious diseases [13,14]. Thus, it is crucial to know whether it is due to increase in susceptibility of the host or prevalence of the pathogen in a culture system during a particular period. It will be interesting to note how seasonal changes affect the defence system of each species for further development of intervention strategies to improve the health of particular species. Haematological and immunological measurements and peripheral blood parameters are important in evaluating health status of many organisms, including fish. Normal ranges for such parameters and their seasonal fluctuations have been studied for many fish species, *i.e.*, *Tinca tinca, Clarias batrachus* and *Ictalurus punctatus* [15-17].

The Olive barb, *Puntius sarana* (Hamilton) is an important cultured and widely distributed medium carp species of south-east Asian regions [18]. Because of its enriched nutritional value, in some parts of the world the market demand and price of this carp are much higher than for other fish species. Despite improved aquaculture practices for the breeding and culture of the species, urgent attention is needed from a conservation view point as the species is reported to be endangered [19,20]. The genus *Puntius* of subfamily Cyprininae (Family: Cyprinidae) has several species, *P. sarana* being one of them. This fish has two subspecies - *P. sarana sarana* (Hamilton-Buchanan) and *P. sarana subnasutus* (Valenciennes). Although the induced breeding and other improved cultured practices have been standardized for this species, little research has been completed to explore the health indicators. The present study was envisaged to establish a physiological normal range in various haematological and innate immune parameters for *P. sarana* and their seasonal and annual fluctuations.

2. Experimental Procedures

2.1 Fish

Puntius sarana juveniles (75-100 g) used for the study were captured from the mono-culture ponds of the Central Institute of Freshwater Aquaculture, Bhubaneswar, India. The experiments were conducted following the approved guidelines of the Institute's ethical committee. During the yearlong culture period the temperature, pH and dissolved oxygen content of the pond water were checked, monitored and found to be within the suitable limit. Earthen ponds of 0.08 ha (40×20 m) with water inlet-outlet system were prepared for the fish culture with intermittent supply of water from feeder canals as detailed in Jena *et al*. [21]. The ponds (0.04 ha) were stocked with fingerlings (4,000 nos./ha) and the culture continued for a period of one year prior to harvest [22] following the standard protocol for stocking of medium carps. Similar stocking culture practices were repeated during the next year. Variation was studied by taking into consideration three prime seasons, summer (April-June), rainy (July-September), and winter (November-January). In order to make the study more appropriate we observed the annual variation in two consecutive years. For each season, the bleeding was done thrice at the pond site, during the consecutive months taking 15-20 fish at a time from alternate ponds. Before the sampling, representative fish samples were checked for the presence of any pathogen and only healthy fish were utilized for the experiment. The experiment was done in varied temperature ranges with little fluctuations in a particular season. The details of the water quality parameters during the entire experimental period are given in Table 1. During this period the fish were fed with groundnut oil cake:rice bran: 50:50 in dough form at 2.0-1.5% of their biomass once a day with routine management practices.

Season		Temperature (°C)		pH		Dissolved oxygen (mg/l)		Alkalinity (mg $CaCO^3$/l)	
	Year	2007	2008	2007	2008	2007	2008	2007	2008
Summer	Min	33	36	7.2	7.0	4.1	4.5	120	121
	Max	37	39.3	7.5	7.3	5.3	5.1	123	124
Rainy	Min	30	30	7.2	7.0	4.2	4.5	121	123
	Max	33.5	33.4	7.6	7.5	5.1	5.3	125	124
Winter	Min	20	25	6.5	7.4	4.2	4.1	121	120
	Max	25.5	29.4	7.5	7.5	5.0	5.2	125	123

Table 1. The minimum and maximum values of the physiochemical parameters measured during the experimental period.

2.2 Sampling

During each netting operation, five fish were randomly sampled for presence of any disease in the pond. To check the presence of parasites, external and internal parts of the fish were examined under microscope besides squash examination of gills and kidney. The kidney samples were aseptically processed for presence of any bacterial pathogen following Thoesen [23]. As the fish in general did not show any fungal or skin lesions and as there was no history of virus infection in those ponds or species reported previously, the samples were not processed for virus or fungus infections. Finally, apparently healthy juveniles caught by drag net were only utilized in this study. Before bleeding the fish was anaesthetized in MS222 and blood was collected *via* caudal puncture. The sampling was done during the morning hours before supplementary feeding. Maximum precautions were taken to avoid any handling stress. For haematological parameters, 200 µl of blood was collected in a tube containing heparin (50 $IUmL^{-1}$ of blood) to prevent clotting. The remaining blood was allowed to clot and kept at 4°C for 3 h. Thereafter, serum was collected after centrifugation at 4000×*g* for 5 min and kept at -30°C for further analysis.

2.3 Haematological parameters

2.3.1 Total erythrocyte and leukocyte count

To determine the total erythrocyte count (TEC), 50 µl heparinised blood was diluted 200 times and for total leukocyte count (TLC), 20 times with Hendrick's solution [24]. Counting was performed in Neubauer's chamber and the values expressed as cells per mm^3.

2.3.2 Total haemoglobin level, haematocrit content and plasma glucose level

The total haemoglobin concentration was estimated by cyanomethemoglobin method using Drabkin's reagent [25] whereas the haematocrit value was determined following microcapillary method and expressed as a percentage [26]. All the haematological parameters were assayed within one hour of bleeding. The plasma glucose content was quantified by enzymatic colorimetric method with GLUCOSE FL kit (Chema Diagnostics, Italy).

2.4 Non-specific immune parameters

2.4.1 Superoxide production assay

The total production of superoxide radicals during respiratory burst activity was measured by the nitroblue tetrazolium (NBT) assay [27]. Heparinised blood of 50 µl and 0.2% of NBT was mixed in 1:1 ratio and incubated for 30 minutes at 30°C. To the mixture, 1 ml of dimethyl formamide was added to solubilize the insoluble formazan granule formed by the blood and NBT dye. The mixture solution was centrifuged at 5000×*g* for 5 min. Optical density was taken at 540 nm to measure the concentration of superoxide radicals.

2.4.2 Myeloperoxidase assay

The assay measures the exocytosis of myeloperoxidase (MPO) using 3,3',5,5'-tetramethylbenzidine as a substrate with a partial modified technique of Quade and Roth [28]. 10 µl of serum was diluted with Hank's Balanced Salt solution containing Ca^{2+} and Mg^{2+} in duplicate wells of a 96-multiwell plate. To each well, 35 µl of diluted stock of 20x tetramethylbenzidine (TMB)/H_2O_2 was added which act as chromogenic substrate for the peroxidase activity. After 5 min of incubation the reaction was stopped by adding 35 µl of 4 M H_2SO_4 and the optical density was read at 450 nm.

2.4.3 Antiprotease assay

Antiprotease activity can be detected utilising an assay which relies on the inhibition of trypsin activity by serum samples following Zuo and Woo [29]. Briefly, 10 µl of test serum was mixed with 100 µl of trypsin (bovine pancreas type I, Sigma) and incubated at 25°C for 30 min along with 110 µl PBS as control. It was further incubated with 1 ml of casein dissolved in PBS (2.5 mg/ml) for 15 min at 25°C. The reaction was terminated with the addition of 500 µl of 10% trichloroacetic acid (TCA). The sample was centrifuged at 10000x*g* for 5 min to remove protein precipitates. The OD of the supernatant was measured at 280 nm and the percentage trypsin inhibition was calculated comparing it to the control and reference samples.

2.4.4 Ceruloplasmin assay

Ceruloplasmin activity in serum samples was measured as *p*-phenylene diamine (PPD) oxidase activity (Sigma, France) as described by Pelgröm *et al.* [30] with a slight modification. Briefly, 50 µl of serum sample or standard of ceruloplasmin (Sigma) was mixed with 1 ml acetate buffer (1.2 M, pH 5) containing 0.1% PPD as substrate. Concurrently, each sample was incubated in the presence of 1 ml 0.5% sodium azide (NaN_3). The mixtures were incubated for 30 min at 37°C and thereafter stopped by the addition of 1 ml NaN_3 (0.5%). One unit of ceruloplasmin was defined as the amount of oxidase that catalyses a decrease in absorbance of 0.001/min at 550 nm.

2.4.5 Total protein level

Total serum protein levels of the fish was measured following the protocol of Bradford [31].

2.4.6 Alternative complement haemolytic (ACH_{50}) activity

The alternative complement activity was performed for the determination of haemolytic activity of blood complement factor following the technique described by Matsuyama *et al*. [32] with partial modifications by using rabbit red blood cells (RaRBC). The results are expressed as ACH_{50} (U/ml), the reciprocal serum dilution giving 50% haemolysis.

2.5 Statistical analysis

The seasonal variation among the species was estimated by one-way ANOVA followed by Duncan's Multiple Range Test using SPSS 13.0 package (SPSS Inc., Chicago, USA). Non-parametric method was used to find out the normal range of all the immune parameters and the reference intervals (25^{th} and 75^{th} percentiles). Student's T-test was performed to study the annual variation taking mean values and data was expressed as mean ± SE. To determine any possible correlation existing between the major fluctuating physicochemical parameter such as temperature and various innate immune parameters, Pearson's correlation analysis was carried out.

3. Results

There was no record of any disease incidence or mass mortality during the period under experiment in the studied ponds. The mean values, lower (25^{th}) and upper (75^{th}) percentiles and range of each parameter obtained from samples collected during all three major seasons over two years duration are presented in Table 2. A wide variation of ranges were found in the immune parameters. The variation of immune parameters recorded during different seasons and two consecutive years are presented in Tables 3 and 5, respectively.

A comparative study was carried out for two years to establish the seasonal changes in the immune factors and significant ($P<0.05$) seasonal variation was also observed in some of the immune parameters. The complement activity was significantly high in summer and winter compared to rainy season. Myeloperoxidase content was significantly lower in winter in comparison to summer and rainy seasons. In summer, the ceruloplasmin level showed significantly higher activity compared to other seasons. A significant reduction was noticed in the value of haemoglobin concentration in winter season and the higher values in the rest of the periods. Similar was the trend in respiratory burst activity keeping its production significantly higher in the hottest months followed by rainy and winter. Conversely, significantly higher total protein level were found in the rainy season compared to summer and winter. A significantly higher glucose level was noticed with a mean value of 92.35 in summer as compared to the other seasons. Similar was the case of PCV percentage, with a highest value recorded in summer. However, there was not any significant seasonal impact on serum lysozyme activity, antiprotease activity, TEC and TLC in *P. sarana*. Further, a strong positive correlation between myeloperoxidase activity and TEC with temperature was noticed. An increase

Immune parameters	N	MEAN	SE	MIN	MAX	25^{th}-75^{th} PERCENTILE
ACH_{50} (units/ml)	61	40.34	1.98	12.10	80.40	28.00-52.00
Myeloperoxidase activity (OD at 450 nm)	141	0.93	0.03	0.11	1.70	0.64-1.17
Lysozyme activity (μg/ml)	69	3.69	0.13	2.22	6.51	3.14-4.06
Antiprotease (% inhibition)	133	81.48	0.43	58.46	96.71	78.68-84.33
Total protein level (g/dl)	124	6.56	0.26	2.42	14.65	4.54-7.35
Ceruloplasmin (units/50 μl)	133	0.61	0.03	0.05	2.40	0.38-0.71
Nitroblue tetrazolium assay (OD at 540 nm)	90	0.26	0.01	0.01	0.49	0.21-0.32
Packed cell volume (%)	85	29.14	0.76	11.00	46.00	25.00-34.00
Glucose (mg/dl)	86	74.42	3.13	13.81	135.45	53.47-93.86
Haemoglobin (g/dl of blood)	81	7.09	0.27	1.69	12.90	5.60-8.90
TEC (10^6 cells cubic $mm^{-1}$of blood)	66	1.83	0.09	0.50	3.56	1.31-2.14
TLC (10^3 cells cubic $mm^{-1}$of blood)	82	26.45	1.17	7.54	54.80	18.87-31.63

Table 2. Estimated normal range of haematological and immune parameters in *P. sarana*, based on the observations made over two years.

TEC-Total Erythrocyte count; TLC- Total leukocyte count; Cu mm-cubic millimeter

PARAMETERS (UNIT)	SUMMER	RAINY	WINTER
ACH_{50} (units/ml)	28.57 ± 3.02^{b}	21.57 ± 1.4^{a}	26.89 ± 1.08^{b}
Lysozyme activity (μg/ml)	3.46 ± 0.14	3.94 ± 0.36	3.86 ± 0.26
Myeloperoxidase activity (OD at 450 nm)	1.12 ± 0.07^{b}	1.06 ± 0.04^{b}	0.65 ± 0.04^{a}
Antiprotease activity (% inhibition)	82.57 ± 0.66	81.04 ± 0.77	81.56 ± 0.62
Total protein level (g/dl)	5.42 ± 0.31^{a}	8.61 ± 0.59^{b}	5.23 ± 0.19^{a}
Ceruloplasmin (unit/50 μl)	0.81 ± 0.1^{b}	0.61 ± 0.05^{a}	0.52 ± 0.03^{a}
Nitroblue tetrazolium assay (OD at 540 nm)	0.36 ± 0.01^{c}	0.27 ± 0.01^{b}	0.17 ± 0.02^{a}
Glucose (mg/dl)	92.35 ± 4.99^{b}	62.04 ± 3.56^{a}	70.13 ± 5.69^{a}
Packed cell volume (%)	31.24 ± 1.26^{b}	27.41 ± 1.45^{a}	27.25 ± 1^{a}
Haemoglobin (g/dl)	8.71 ± 0.38^{b}	7.66 ± 0.33^{b}	5.43 ± 0.39^{a}
TEC (10^{6} cells cu $mm^{-1}$of blood)	1.97 ± 0.26	1.9 ± 0.16	1.6 ± 0.11
TLC (10^{3} cells cu $mm^{-1}$of blood)	45.01 ± 4.12	34.89 ± 2.95	41.47 ± 2.98

Table 3. Effect of the season on various haematological and innate immune parameters measured in Olive barb, *P. sarana*. Data are presented as mean ± S.E. Means bearing different alphabetical superscripts in a row are significantly ($P<0.05$) different.

TEC- Total erythrocyte count; TLC- Total leukocyte count

Sl. no.	Parameters (unit)	Correlation with temperature
1	ACH_{50} (units/ml)	-0.14
2	Lysozyme activity (μg/ml)	-0.50
3	Myeloperoxidase activity (OD at 450 nm)	1.00
4	Antiprotease activity (% inhibition)	0.33
5	Total protein level (g/dl)	0.41
6	Ceruloplasmin (unit/ 50 μl)	0.83
7	Nitroblue tetrazolium assay (OD at 540 nm)	0.94
8	Glucose (mg/dl)	0.41
9	Packed cell volume (%)	0.66
10	Haemoglobin (g/dl)	0.99
11	TEC (10^{6} cells/cubic mm of blood)	1.00
12	TLC (10^{3} cells/cubic mm of blood)	-0.02

Table 4. Correlation of the immune parameters with temperature.

in temperature was found to be directly associated with the natural increase of ceruloplasmin, NBT, PCV and haemoglobin levels during the various seasons. However, ACH_{50}, lysozyme activity and TLC were found to be negatively correlated with water temperature as summarized in Table 4.

The annual variation in the immune parameters between the two years were calculated using student's T-test. A significant variation in myeloperoxidase, antiprotease activity, total protein level, total erythrocyte count was observed whereas other parameters showed consistency in their values over these two years.

4. Discussion

Numerous studies have supported the close relationship between the immune function of teleosts with their environment [33]. Until a fish adapts its specific immune system, it tends to rely on the non-

PARAMETERS (UNIT)	Year	Mean ± SE	Max	Min	T-test	25th-75th percentile
ACH_{50} (units/ml)	2007	27.02 ± 1.49	54.80	7.54	0.08	18.93-34.82
	2008	24.12 ± 0.7	35.71	15.01		20.00-26.62
Myeloperoxidase activity (OD at 450 nm)	2007	0.92 ± 0.04	1.70	0.11	0.00*	0.71-1.10
	2008	1.11 ± 0.05	1.99	0.24		0.75-1.52
Antiprotease (% inhibition)	2007	82.02 ± 0.57	96.71	58.46	0.00*	80.46-85.08
	2008	75.3 ± 1.05	91.60	46.35		66.77-82.41
Ceruloplasmin (units/50 μl)	2007	0.66 ± 0.03	2.19	0.19	0.29	0.51-0.74
	2008	0.7 ± 0.04	2.40	0.05		0.40-0.89
Total protein level (g/dl)	2007	7.11 ± 0.38	14.65	2.42	0.00*	4.77-8.38
	2008	4.43 ± 0.2	8.75	1.20		3.15-5.77
Nitroblue tetrazolium assay (OD at 540 nm)	2007	0.27 ± 0.02	0.49	0.01	0.41	0.20-0.38
	2008	0.28 ± 0.01	0.50	0.10		0.22-0.31
Glucose (mg/dl)	2007	72.75 ± 4.51	135.45	13.81	0.09	48.73-100.95
	2008	82.86 ± 3.82	132.17	26.25		63.96-103.56
Packed cell volume (%)	2007	28.7 ± 1.17	46.00	11.00	0.63	24.00-34.75
	2008	28.03 ± 0.8	42.00	11.00		25.00-32.00
Haemoglobin (g/dl)	2007	7.12 ± 0.43	12.90	1.69	0.08	4.46-9.38
	2008	6.25 ± 0.27	9.53	3.35		4.60-7.90
TEC (10^6 cells cubic $mm^{-1}$of blood)	2007	1.58 ± 0.15	3.56	0.50	0.01*	1.02-1.93
	2008	1.98 ± 0.08	3.47	0.90		1.68-2.19
TLC (10^3 cells cubic $mm^{-1}$of blood)	2007	35.18 ± 2.94	62.40	16.50	0.46	22.10-48.50
	2008	38.03 ± 2.26	80.40	12.10		24.80-51.45

Table 5. Variation in annual average of several blood parameters observed in *P. sarana* over two consecutive years. T-test values with superscript (*) is significantly different.

specific immunity in lower ambient temperature. Moreover, changes in the haematological and immune parameters provide substantial diagnostic information of the species reflecting the disease and stress signal. Hence, in the present study, considerable efforts have been made to measure the changes in immune and haematological parameters over a period of two consecutive years in medium carp *Puntius sarana.*

Variations in haematological and immune parameters have been reported in Tench that exhibited greater alternative complement activity in winter in both male and female [34,35]. A positive correlation between season and immune parameters like in serum lysozyme level, respiratory burst activity, blood cell count and antibody titre was found in rainbow trout, *Oncorhynchus mykiss*, while the haematocrit value was negative [36]. Atlantic halibut also showed significantly different lysozyme levels between summer and winter [37].

The complement system is an important component of both innate and adaptive immunity which helps in resistance to infections in mammals as well as fish. Fish complement displays its optimum activity in lower temperature (15-20°C) that can be well-correlated with the ambient water temperature to which the fish encounter. In the present study although no significant annual difference in ACH_{50} value was observed, the summer and winter seasons show higher ACH_{50} activity. Similarly, Alcorn *et al*. [38] reported the highest activity of complement in winter in *Oncorhynchus nerka*. In another study, Ndong *et al*. [39] reported elevated level of complement activity in Nile tilapia when it was transferred to higher temperature (31-35°C). The reason for low activity of complement during rainy season compared to other seasons and a negative correlation between the temperature and complement activity in *P. sarana* might be due to the role of other environmental factors.

During stress, the oxygen consumption of a cell increases and they produce reactive oxygen species through NADPH-oxidase system. Although free

radicals implicate their beneficial effect on pathogen clearance, signal transduction, gene regulation and angiogenesis, over production of these radicals may cause cytotoxic death of the cell due to oxidative stress. Hence, enzyme like myeloperoxidase acts as enzymatic defender of reactive oxygen species, removing excess ROS, thus helps in detoxification [40]. Significantly higher myeloperoxidase activity in *Puntius sarana* during summer and rainy seasons was noticed during the study. Similarly, superoxide production during the respiratory burst activity was significantly higher in summer compared to other seasons. This implies that the oxygen-dependent killing mechanism including phagocytosis and myeloperoxidase activity was higher in summer than other seasons. Moreover, a strong positive correlation in myeloperoxidase activity and superoxide production with respect to temperature noticed during this study might feasibly suggest a direct association between temperature and the peroxidase activity during aestivation and breeding periods. Wang *et al.* [40] reported that myeloperoxide activity and levels were quite high during July to September in *Apostichopus japonicas*. Although the reasons are not properly known, but the same trend in phagocytic activity was reported in rainbow trout, which proves that immune suppression occurs in winter [35]. Moreover, the variation in myeloperoxidase activity during the year 2008 clearly depicts the increase in activity with respect to a rise in temperature.

Lysozyme, the antibacterial enzyme is an acute phase protein reported to be insensitive to temperature or seasonal changes [41]. Although a positive correlation between water temperature and lysozyme activity have been marked in some fish species [42], there was no significant change in its activity observed among different seasons during this study. Our finding was in agreement with Hernandez and Tort [41], where they noticed the lysozyme activity of *Sparus aurata* and *Oreochromis niloticus* was less sensitive to seasonal or temperature changes. Being an antimicrobial peptide, it shows an acute phase response during infection or disease, with insensitivity to temperature or seasonal changes. Although the lysozyme activity in different seasons was found to be insignificant, the fluctuation in its mean values showed a negative correlation of the parameter to temperature. This finding casts some doubt on the involvement of temperature in influencing serum lysozyme activity.

In a broad sense, acute phase proteins are the macromolecules which released during acute stress, injury, trauma or infections and normally maintain the homeostasis of the internal environment of an animal [43]. The, acute phase protein ceruloplasmin is a regulator for hepatic iron mobilization as well as a scavenger of free radicals and superoxides [44,45]. A higher activity of ceruloplasmin level was found in summer season. Thus, the higher values of all the immune parameters obtained during the aestivation period and a positive correlation with temperature suggests the possible role of temperature in this species. However, no significant difference was evident in serum antiprotease activity indicates that the fish was free from any infection during the whole experimental period and healthy from a defence point of view.

The increase in plasma glucose levels is correlated with the production of catecholamine during stress. Although higher levels of glucose were marked during summer, the level seems to be within the normal range and the same is the case with haemoglobin. The blood cell counts (RBC and WBC) are regarded as an indicator of health status of the animal and often used for disease diagnosis such as anaemia, leucopoenia or various immune dysfunctions [17]. This study revealed no effect of seasonal changes on these haematological parameters. Although effect of seasonality was not detected in TEC, but varying effects were observed while measuring the annual variation. The secondary indices like haematocrit values decreased during rainy and winter compared to summer which is similar to the findings of Mahajan and Dheer [46] in *Channa punctatus*. A positive correlation between TEC, haemoglobin content, haematocrit values and temperature was established. The physiological changes taking place during the breeding seasons may be influencing the haematological parameters in this species. However, the minor variations observed in TLC during all the seasons indicated that this narrow range of fluctuations in water temperature might have poor effect on this parameter.

The present study has established baseline information regarding the innate immune function during a particular season in a new culturable fish species that may aid in conservation of this endangered fish. Furthermore, any wide variations in the normal range data will be able to aid in revealing major environmental fluctuations that might affect the health status of fish. A marked variation among all the parameters was observed and overall, most of the parameters were high in summer and low in winter except lysozyme which might be insensitive to the temperature fluctuation. Most of the parameters showed either lower or equal in level or activity during

the rainy season as compared to the winter season, thus indicating a clear influence of temperature in modulating the immune status of fish. Generally, immune function decreases during winter and is enhanced at higher temperatures, which was confirmed from this study. However, the response to stress in different species varies seasonally. The fluctuations in different seasons may be correlated with photoperiod, energy availability and consumption, temperature or length of the day. To date, no advanced efforts have been taken up to resolve the questions related to the physiological and immune response underlying seasonality. Thus, in *P. sarana* further work is needed to provide a novel insight into the mechanisms of immune functions during different seasons.

Acknowledgements

We thank the Director, Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, India for providing necessary facilities during this study.

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