Indian J. Fish., 61(1) : 57-62, 2014

Reference ranges and seasonal variations in innate immune responses of kalbasu, *Labeo calbasu* (Hamilton)

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ABSTRACT

Kalbasu, *Labeo calbasu* (Hamilton) reared under natural conditions were examined for variation in selected innate immune parameters during three different seasons of a year. The reference ranges for the innate immune parameters *viz*.. lysozyme, peroxidase, ceruloplasmin, antiprotease and alternative complement haemolytic activitiy, superoxide production by phagocytes, total protein level of serum, haemolysin, haemagglutination and bacterial agglutination titre and blood glucose level of this species were established. The seasonal and sex-related variations in some of the parameters in this fish were also evaluated. Significant seasonal variations in peroxidase activity, superoxide production by phagocytes, alternative complement activity and haemolysin titre were observed. In contrast, serum lysozyme activity and blood glucose level did not show significant seasonal variations. Males had higher haemagglutination titre in the breeding season when compared to females.

Keywords: Innate immunity, *Labeo calbasu*, Reference ranges, Seasonal variation, Sex

Introduction

Innate immune factors play major role in defence mechanism of fish to a wide array of pathogens. Many cells particularly phagocytes and humoral factors *viz.,* lysozyme, complement components, proteases, transferrin and lectins contribute to this innate defence. These innate immune parameters vary among species, even when living within a similar environment (Sahoo *et al*., 2005). The establishment of the reference ranges is important to know the health status of particular fish species as well in studying their response to infections or immunomodulation strategy. Fish are poikilotherms, which live in close association with their environment. Thus, the changes in temperature and seasonality dominate their life cycle, especially with regard to their reproductive activities, food intake, locomotion and immune response (Slater and Schreck, 1998; Bromage *et al*., 2001; Hernandez and Tort, 2003). Generalised depression in the immune status of fish during colder conditions is often seen in the form of winter syndrome when fish become prone to several diseases. Such an immunocompromised state is compensated by various other factors. Seasonal variation in immune parameters has been studied in several fish species including tench *Tinca tinca* (Collazos *et al*., 1995 a, b, 1996), dab *Limanda limanda* (Hutchinson and Manning, 1996), halibut *Hippoglossus hippoglossus* (Bowden *et al*., 2004), gilthead sea bream *Sparus aurata* (Hernandez and Tort, 2003), plaice *Pleuronectes platessa* (Fletcher and White, 1976), rohu *Labeo rohita* (Swain *et al*., 2007), common carp *Cyprinus carpio* (Saha *et al*., 2002 and Asian catfish *Clarias batrachus* (Kumari *et al*., 2006). However, seasonal variations in immune parameters of *Labeo calbasu* (Hamilton) have not so far been investigated.

Seasonal changes in temperature remains the predominant factor influencing the immune status as well as other physiological processes in fish. They show higher metabolic activity at higher temperature and adjust their food intake as per the environmental temperature. Thus, their rate of anabolism also depends on temperature of their surrounding (Hernandez and Tort, 2003). Several workers have noted temperature-dependent changes in both specific as well as innate immune parameters in fish. Bly and Clem (1992) observed suppression of both B and T cell functions in channel catfish when water temperature was lowered from 23 to 11 °C for 24 h. Elevation in blood glucocorticoids level is marked in winter conditions, which help fish adapt to physiological stress generated by lower ambient temperature and decreased food availability (Nelson and Demas, 1996). Lysozyme helps in bacterial cell lysis by hydrolysing the bond between N-acetylmuramic acid and N-acetylglucosamine of the bacterial cell wall. Increase in serum lysozyme level in summer when compared to that in winter was observed in Atlantic halibut (Bowden *et al*., 2004). However, Hernandez and Tort, (2003) observed no significant correlation between temperature and serum lysozyme levels in gilthead sea bream. They also noted seasonal pattern in complement and agglutination activity, which showed lowest values in the coldest months. Kumari *et al*. (2006) observed that though the various immune parameters like superoxide production, peroxidase activity, alternative complement activity and haemagglutination titre showed seasonal fluctuations in Asian catfish, there was no seasonal pattern. Seasonal changes in phagocytic activity and superoxide production in blood were also noted for tench (Collazos *et al*., 1995a, b).

Apart from temperature, photoperiod also controls seasonality. Changes in photoperiod induce changes in the levels of melatonin produced by the pineal gland, which has many physiological consequences such as changes in hormonal, neural and immune functions (Bowden *et al*., 2004).

Labeo calbasu (Hamilton) is one of the most popular carp species after the major carps (rohu, *Labeo rohita*, catla, *Catla catla* and mrigal, *Cirrhinus mrigala*) in Indian aquaculture. It is widely distributed in various river systems and preferred for culture due to its growth potential (Chondar, 1999; Gurumayam and Goswami, 2002; Mahapatra *et al*., 2005). Though, few studies regarding its growth and reproduction in different conditions have been undertaken (Khumar and Siddiqui, 1991; Sahu *et al*., 2007), no attempt has been made so far to measure the immune status of this species. This study aims at establishing base line values for few innate immune parameters of this species, which are indispensable for any disease diagnosis, immunomodulation, toxicological and other such studies. This paper also presents the seasonal and sex-related variations in some of the immune parameters in this fish.

Materials and methods

Fish

Labeo calbasu fingerlings were reared in three different earthen ponds (0.4 ha each) for one year along with other Indian major carp species in polyculture system. They were fed with rice bran and groundnut oil cake in 1:1 proportion, during the experimental period. Adequate water level was maintained in the ponds round the year from a constant water source. The physico-chemical parameters of water were analysed during each sampling time following standard methods (APHA, 1989). The pH, total alkalinity ($CaCO_3$), nitrite nitrogen (NO_2-N), ammoniacal nitrogen and nitrate nitrogen (NO_3-N) ranged from 6.91 to 7.75, 68 to 120 mg l^{-1}, 0.11 to 0.16 mg l^{-1}, 0.36 to 2.83 mg l^{-1} and 0 to 0.11 mg l^{-1}, respectively.

Sampling

Blood samples were collected (10-42 samples for each season) randomly from adult fish (700-1000 g) reared in the ponds during the second week of April, August and December months of the year, representing summer, rainy and winter seasons, respectively. Fish were bled by caudal venipuncture with plastic syringe after anesthetising them with MS222 (Sigma, USA). An aliquot of the blood was heparinised (50 IU m l^{-1}) and the remaining part was allowed to clot at room temperature and then kept at 4 °C. The separated serum samples were stored at -70 °C until analysis. The blood and serum samples could be collected sex-wise only in the rainy (breeding) season as it was not possible to morphologically differentiate males from females during the other two seasons.

Immune parameters

Superoxide production by blood phagocytes during respiratory burst activity was assayed by the reduction of nitroblue tetrazolium (NBT) to formazan (Anderson and Siwicki, 1995). Briefly, blood was mixed with 0.2% NBT in equal proportion (1:1) and incubated for 30 min at 25 °C. One ml of dimethyl formamide (DMF) (SRL, India) was added to fifty microliters of this mixture to solubilise the reduced formazan product. The optical density (at 540 nm) of the supernatant was measured after centrifuging the above mixture at 2000 *g* for 5 min, against DMF blank to measure the reduced amount of NBT.

The total peroxidase content of serum was determined as described by Quade and Roth (1997) and partially modified by Sahoo *et al*. (2005). Briefly, 10 µl of serum was diluted with 90 µl of HBSS without Ca^{2+} or Mg^{2+} in 96-well microtitre plate to which 35 µl of 20 mM 3,3', 5, 5'-tetramethyl benzidine hydrochloride (TMB) (Genei, India) and 5 mM H_2O_2 were added. After 2 min of incubation, 35 µl of 4 M sulphuric acid was added to stop the reaction. The optical density was read at 450 nm in a microtitre plate reader (Anthos 2010, Austria).

The lysozyme activity of serum was determined by turbidimetric assay according to Sankaran and Gurnani (1972) with partial modifications. A suspension of 150 µl of *Micrococcus lysodeikticus* (0.2 mg ml^{-1} in 0.02 M sodium acetate buffer, pH 5.5) was added to previously dispensed test serum (15 µl) in a 96-well U-bottom microtitre plate and initial O.D was measured at 450 nm. The final O.D was mesured 1 h after incubation at 24 °C (Sahoo *et al*., 2005). A standard curve was prepared using lyophilised hen egg white lysozyme, HEWL (Sigma). Serum lysozyme values were expressed as µg ml^{-1} equivalent to hen egg white lysozyme activity.

Plasma glucose was determined by enzymatic colorimetric method with GLUCOSE FL kit (Chema Diagnostica, Italy). Ceruloplasmin activity in serum was measured as p-phenylene diamine (PPD) oxidase activity (Sigma) as described by Pelgrom *et al*. (1995) and Dautremepuits *et al*. (2004) with minor modification. Serum (50 µl) or standard of ceruloplasmin was mixed with 1 ml of acetate buffer (1.2 M, pH 5.0) containing 0.1% PPD as substrate. Further, each sample was incubated in the presence of 1ml NaN_3 (0.5%) (azide blank). The mixtures were incubated for 30 min at 37 ºC. The reaction was stopped by the addition of 1 ml of NaN_3. One unit of ceruloplasmin was defined as the amount of oxidase that catalysed a decrease in absorbance of 0.001 per min at 550 nm.

The total protein level of the serum was determined following Bradford (1976) method using a standard curve prepared with bovine serum albumin. Serum antiprotease assay was done as described in Ellis (1990). The alternative complement haemolytic activity (ACH_{50}) and total haemolysin titre were also evaluated. The ACH_{50} activity was determined as described by Yano (1992) and modified by Kumari and Sahoo (2005). The results were expressed as ACH_{50} (units ml^{-1}) for the reciprocal serum dilution giving 50% haemolysis. The total haemolysin titre of serum was assayed by incubating sera with rabbit RBC (RaRBC) for 1 h at 37 °C after serial dilution. The haemolysin titre was defined as the last dilution showing complete lysis of RaRBC. Values are expressed as reciprocal of haemolysin titre.

Haemagglutination assay (HA) was performed as described by Kumari and Sahoo (2005). Double serial dilution of the inactivated sera (56 °C for 20 min) were made in PBS (with Ca^{2+} and Mg^{2+}), and then 50 µl of 1% rabbit RBC (RaRBC) was added to each well of the microtitre plate and incubated for 1 h at 37 °C. The HA titre was defined as the last dilution of serum showing minimal positive agglutinin. Values are expressed as reciprocal of HA titre.

Natural agglutinin levels in the serum of individual fish were determined by plate agglutination technique (Plumb and Areechon, 1990). Briefly, inactivated sera were diluted two-fold serially in PBS (with Ca^{2+} and Mg^{2+}) in a 96-well microtitre plate and then 50 µl of formalin-killed *E. tarda* (adjusted to Mac Farland's Standard No. 9) was added to each well. The bacterial agglutination titre was defined as the last dilution of serum showing minimal positive agglutination. Values were expressed as reciprocal of the agglutination titre.

Statistical analysis

The data were represented as mean ± S.E. As most of the data had a non-Gaussian distribution (except total protein content, lysozyme, peroxidase, antiprotease, alternative complement and ceruloplasmin activities) as shown by Kolmogorov-Smirnov test, thus the reference ranges for the parameters were calculated by non-parametric methods. Significant difference ($p<0.5$) between the parameters of male and those of female fish was found by Student's t-test. One-way analysis of variance was used to calculate seasonal differences within each parameter.

Results

The temperatures of summer, rainy and winter seasons were noted to be 33, 31 and 22.5 °C, respectively during the sample collection weeks. The reference ranges for each immune parameter were calculated as 25^{th}-75^{th} percentile by taking all the seasons into consideration (Table 1). There was no significant difference in the immune parameters of males and females except for haemagglutination titre, where males exhibited higher titre than the females (Table 2). Seasonal variation was observed in few immune parameters *viz.,* peroxidase activity, superoxide production and haemolytic activity (Table 3). Lysozyme activity and blood glucose content did not show any seasonal variation. Fish exhibited significantly ($p < 0.05$) lower haemolytic activity in summer compared to those in winter. In contrast, the peroxidase activity and superoxide production by phagocytes appeared higher in summer than in winter though the difference was not significant. The highest peroxidase activity and the lowest superoxide production levels were evident in rainy season.

Discussion

Seasons affect the physiology and behaviour of an individual due to broad variations in photoperiod and temperature. Like other animals, fish also adapt to seasonal changes in the environment by changing their feeding pattern, reproductive behaviour and immune response to pathogens (Bowden *et al*., 2007). Adverse conditions may help bacterial and other pathogens to cause several diseases in fish. Low environmental temperature and decreased availability of food may cause mortality due to hypothermia, starvation, or shock in fish (Nelson and Demas, 1996). Higher prevalence of epidermal papilloma and lymphocystis in dab was reported during spawning period when temperature was low (Wolthaus, 1984). Thus, it is necessary

Table 1. Reference and mean values of various immune parameters of *Labeo calbasu* irrespective of season or sex

Immune parameter (unit)	N	Mean	SE	Median	Min	Max	25^{th} -75^{th} Percentile
Lysozyme activity (µg ml^{-1})	35	6.13	0.59	5.90	0.69	13.87	3.29-8.50
Peroxidase activity ($OD_{450\ nm}$)	45	1.20	0.06	1.13	0.40	1.97	0.86-1.52
Total protein content (g dl^{-1})	24	2.01	0.23	1.93	0.10	5.08	1.38-2.58
Antiprotease activity (% inhibition)	10	69.87	4.23	68.14	43.51	88.39	62.98-79.75
Superoxide production ($OD_{540\ nm}$)	38	0.43	0.03	0.37	0.13	0.80	0.27-0.58
Blood glucose level (mg dl^{-1})	35	48.15	3.25	43.67	15.45	109.86	37.07-56.17
Haemolysin titre	51	8.06	0.71	8	1	32	4-8
Haemagglutination titre	48	12.94	2.11	8	1	64	2-16
Bacterial agglutination titre	40	79.60	11.56	64	8	256	16-128
ACH_{50} activity (units ml^{-1})	44	28.68	2.32	26.54	3.37	83.03	19.30-35.77
Ceruloplasmin activity (units 50 μl^{-1})	37	1.91	0.28	1.63	0.06	7.12	0.50-2.60

Table 2. Sex-related variation in innate immune parameters of *Labeo calbasu*

Immune parameter	Sex	N	Mean	SE
Lysozyme activity (µg ml^{-1})	Male	9	5.51	1.42
	Female	14	7.52	0.89
Peroxidase activity ($OD_{450\ nm}$)	Male	9	1.25	0.17
	Female	10	1.38	0.13
Superoxide production ($OD_{540\ nm}$)	Male	8	0.33	0.05
	Female	5	0.37	0.04
Blood glucose level (mg dl^{-1})	Male	7	53.59	12.74
	Female	5	45.83	7.34
Haemagglutination titre	Male	11	23.27*	5.31
	Female	10	9.50	2.25
Bacterial agglutination titre	Male	11	41.45	6.89
	Female	12	24.67	5.78
ACH_{50} activity (units ml^{-1})	Male	10	31.84	6.50
	Female	7	34.63	6.73
Haemolysin titre	Male	12	8.41	1.35
	Female	10	8.11	0.92
Ceruloplasmin activity (units 50 μl^{-1})	Male	10	3.48	0.64
	Female	11	2.19	0.28

Samples are collected during breeding season. Asterisk indicates significant difference ($p<0.05$) between male and female values

Table 3. Seasonal variation in the innate immune parameters of *Labeo calbasu*.

Immune parameters	Season	N	Mean	S. E.	Min	Max
Lysozyme activity ($\mu g\ ml^{-1}$)	Summer	10	5.32[a]	0.80	1.61	9.88
	Rainy	23	6.75[a]	0.79	0.69	13.87
	Winter	10	4.21[a]	0.46	2.22	7.43
Peroxidase activity ($OD_{450\ nm}$)	Summer	10	1.21[ab]	0.06	0.87	1.54
	Rainy	19	1.35[b]	0.11	0.40	1.97
	Winter	18	1.03[a]	0.09	0.59	1.85
Superoxide production ($OD_{540\ nm}$)	Summer	10	0.55[b]	0.05	0.32	0.80
	Rainy	13	0.35[a]	0.03	0.21	0.61
	Winter	18	0.44[ab]	0.05	0.13	0.80
Blood glucose level ($mg\ dl^{-1}$)	Summer	10	54.29[a]	5.53	32.48	96.14
	Rainy	12	50.35[a]	7.82	15.45	109.86
	Winter	17	44.43[a]	2.13	29.33	57.67
ACH_{50} activity (units ml^{-1})	Summer	10	15.26[a]	3.35	3.37	39.18
	Rainy	17	32.99[b]	4.59	13.09	83.03
	Winter	18	31.32[b]	2.12	13.71	47.47
Haemolysin titre	Summer	10	7.20[a]	1.16	4.00	16.00
	Rainy	22	8.12[a]	0.83	1.00	32.00
	Winter	19	14.11[b]	1.74	4.00	32.00

Same superscripts indicate that there is no significant difference between seasonal values for an immune parameter ($p<0.05$).

to study seasonal variation in immune parameters to know the health status of the fish.

Previously, several studies noted fluctuations in immune parameters with seasonality. Lowering of water temperature in winter was reported to cause immunodeficiency in channel catfish and gilthead sea bream associated with 'winter-kill' syndrome (Bly and Chem, 1992; Tort *et al.,* 1998). Sexual activity during breeding season influenced the IgM levels, which remained very high in this season, was found in common carp (Saha *et al*., 2002). They also reported increase in cortisol levels with increase in the water temperature. Scapigliati *et al*. (1999) noted higher immunological level in seabass during winter with respect to other seasons. Significant seasonal fluctuations in peroxidase activity, superoxide production by phagocytes, alternative complement activity and haemolysin titres were observed in *L. calbasu*. On the other hand, serum lysozyme activity and blood glucose level did not show any significant seasonal variation.

Lysozyme is an important bactericidal enzyme that cleaves the bond between N-acetylmuramic acid and N-acetyl glucosamine present in the bacterial cell walls. Results of earlier workers with regard to seasonality in lysozyme activity in fish were not consistent. Significant decrease in this activity in wild caught dab and Atlantic halibut was noticed in winter than in summer (Hutchinson and Manning, 1996; Bowden *et al*., 2004). In contrast, no significant correlation was observed between temperature and serum lysozyme levels in gilthead sea bream (Hernandez and Tort, 2003). In this study also, lysozyme activity did not show any seasonal trend, which suggested that lysozyme activity would be less sensitive to seasonal or temperature changes as mentioned by Hernandez and Tort (2003).

Complement pathways in vertebrates help in killing various pathogens by degrading their cell membranes. The alternative complement pathway is regarded as more prominent than other complement pathways in fish (Swain *et al*., 2006). In the present study, significantly lower ($p<0.5$) alternative complement activity was recorded in summer than in winter season. The haemolysin titres of serum in *L. calbasu* were also found to be lower in summer than in winter. In contrast, lower complement activity was observed in winter as compared to summer in gilthead seabream (Hernandez and Tort, 2003). Further, Swain *et al*. (2007) found no significant seasonal difference in haemolytic activity in Indian major carp, *L. rohita*.

Phagocytes play important role in killing pathogens by producing oxygen free radicals and hypohalides. Seasonal variation in superoxide production by phagocytes was noticed in *L. calbasu*. It was found lower in rainy and winter seasons than in summer. Collazos *et al*. (1995a, b) also noted seasonal variation in phagocytic activity in tench (*Tinca tinca*) and marked higher phagocytic activity at 22 °C during the spring and summer compared to that in winter. Myeloperoxide in neutrophils produces highly bactericidal hypohalides from peroxides. The peroxidase activity in this study was found to be lower in winter as compared to rainy and summer seasons as noted in *L. rohita* (Swain *et al*., 2007).

Rise in blood glucose level indicates stressful conditions in fish. This is due to rapid increase in catecholamines followed

by cortisol-dependent gluconeogenesis. No significant seasonal variation in blood glucose levels in *L. calbasu* was found during the present study probably indicating the absence of physiological stress during period of study. This study also indicated that fish is able to fight against infections equally over different seasons by maintaining a balance between various innate immune factors/ pathways as observed here.

Based on small sample sizes (available only from the rainy season), no statistically significant differences in the immune parameters were observed between males and females of *L. calbasu*, except for haemagglutination titre, in which males showed higher titre than females. Hutchinson and Manning (1996) also observed no significant sex-wise differences in serum lysozyme activity and total protein levels in the dab (*L. limanda*). While significant decrease in lysozyme activity was observed in male lumpsucker (*Cyclopterus lumpus*) as compared to that in females during the breeding season (Fletcher *et al*., 1977). Results of the study indicate that both males and female of *L. calbasu* are equally immunocompetent during breeding season.

Establishing baseline and reference ranges of the innate immune parameters for a species is necessary in order to use those parameters as markers for disease diagnostics/health status. In this study we established reference ranges for various innate immune parameters *viz*., lysozyme, peroxidase, ceruloplasmin, antiprotease and alternative complement haemolytic activities, superoxide production by phagocytes, blood glucose level, total protein content of serum, haemolysin, haemagglutination and bacterial agglutination titres for *L. calbasu*. Significant seasonal variations in peroxidase activity, superoxide production by phagocytes, alternative complement activity and haemolysin titre were observed in kalbasu. However, serum lysozyme activity and blood glucose level did not show significant seasonal variations. The reference ranges as well as seasonal and sex-wise variations in innate immune parameters of *L. calbasu* elucidated in the present study would provide basic information for further immunological studies in this species.

Acknowledgements

Thanks are due to the Director, Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, India for providing the necessary facilities for the work.

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Date of Receipt : 12.01.2012
Date of Acceptence : 12.09.2013