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Seasonal Immune Rhythms of head kidney and spleen cells in the freshwater Teleost, *Channa punctatus*

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ABSTRACT

Annual rhythms in immune function are the reflection of a crucial physiological strategy to deal with environmental stressors. The fish are pivotal animal models to study the annual rhythm and to understand the evolution of the vertebrate biological system. The current research was planned to assess the annual changes in the innate immune functions of immune cells in a teleost, *Channa punctatus*. Head kidney and splenic macrophage phagocytosis, superoxide generation, and nitrite release were evaluated to assess innate immunity. Cell-mediated immunity was measured through head kidney and splenic lymphocyte proliferation in presence of mitogens. The superoxide anion generation by the cells of head kidney and spleen was maximum in October. A bimodal pattern in nitrite production was observed with the first peak in November and the second in March. Cosinor analysis revealed a statistically significant annual rhythm in nitrite production. Similarly, phagocytosis and lymphocyte proliferation also showed statistically significant annual rhythms. It was concluded that animals maintain an optimum immune response in seasonally changing environments. Elevated immunity during certain times of the year might assist animals deal with seasonal environmental stressors. Further research may be focused upon measuring survival rate and reproductive success after season induced elevated immunity.

Introduction

Most animals use the day length as a marker of switch in the season as photoperiod provides accurate temporal sign of changing season [1,2]. Animals have evolved a strategy to synchronize the internal rhythm with the help of photoperiodic cues. Seasonal adjustment in the internal rhythm is important to maintain the homeostasis. The animals adapt to the changing environmental conditions by changing behavior and physiology and tune to the seasonal changes by adjusting metabolic energy. These changes are essential for survival of animals during the stressful periods of the year. The favorable environmental conditions are essential for life continuing activities like reproduction which is energetically costly and is possible when energy supply is optimum. The physiological processes are energetically costly [3,4] and a significant amount of energy is devoted in reproduction, migration, protection of habitat etc., hence it is important to predict the season when local environmental conditions are favorable. Failure to buffer-up with the adverse conditions of challenging environment leads to sickness and death in non-tropical animals. Similarly, many animals die of sporadic diseases which emerge at certain times of year when the immunity is low and/or due to pathogenic microorganism grow [5–7]. Enhanced immune function is a prerequisite to increase survival before the beginning of the extreme environmental conditions.

Seasonal variation in the immune function is often observed in mammals [8–10], but is relatively poorly understood in fishes. Animal can either start or stop the season induced adaptation depending upon change in the photoperiod. Literatures reveal that the photoperiod can stimulate the feeding, growth and can prepare for reproduction. In parallel, photoperiod can alter the immune functions. Photoperiod stimulated alteration in the immune functions has been worked out mainly in the mammals and birds and studies in fishes are sporadic. Seasonal variation in blood immune functions helps the fresh water reptile, *Natrix piscator* adapt to the changing weather conditions (Singh et al., 2020). Seasonality in immune functions and its possible correlation with melatonin have been worked out in aves (Majewski et al., 2005; Schultz et al., 2017). Photoperiodic control of reproductive and

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non-reproductive functions has been described in reptiles and amphibians (Weil and Crews, 2009). Annual weather changes are known to alter the reproductive function and affect various other functions like food intake, growth performance, locomotor activity, and immune status of teleosts [11–13]. Photoperiod manipulations are performed in aquaculture to regulate the sexual maturation and growth of different fish species [14], including *Perca fluviatilis* and *Sander lucioperca* [15]. However, the literature related to the effect of photoperiod on the fish immune system is very scarce. The role of melatonin in the regulation of fish immunity has been proposed as secretion of melatonin hormone shows circadian and circannual rhythms and it is postulated that alterations in the duration of light affect fish immunity through alterations in melatonin secretion. Bi-directional communication has been worked out in teleosts where the hypothalamus-pituitary-interrenal (HPI) axis affects the immune system and melatonin secretion.

Season induced immune alteration in mammals is an adaptive strategy to help cope with harsh conditions of winter [16]. Morgan et al. [17] found that certain immune functions of *Oncorhynchus mykiss* vary according to change in the photoperiod. Serum lysozyme activity, production of reactive oxygen species, leucocyte counts, and immunoglobulins, were found to be related with the change in the season. It has been observed that erythrocyte count, hematocrit and hemoglobin levels are elevated during summer while mean corpuscular volume is declined [13,18–20]. In contrast, photo-refractoriness has been observed in *Clarias batrachus* and *Esox Lucius* [21,22]. There is a general agreement that extreme winter conditions inhibit immune functions and divert the energy towards other important physiological processes to survive while immune responses increase at higher temperatures [11,23,13,24]. Leucocytes are elevated during summer [25–28] which may be an important adaptation to fight long-photoperiod induced pathogens. No considerable study is available in fishes which prove the presence of multi-frequency rhythms. Fishes are the first vertebrate group to have an innate and adaptive immunity and hence are crucial group study the photoperiod-induced alteration in immune functions. We hypothesize that annual variation in immunity is an important survival strategy and to test the hypothesis we selected the snakehead teleost *Channa punctatus* as a model organism.

Materials and methods

Animals

The male snakehead teleost, *Channa punctatus* (Bloch.) were found from a local person of Bilaspur (22.0796^0N; 82.1391^0E), India and brought to the laboratory. Fish (weighing 40–60 g and S-V length 15–20 cm) were acclimated to the laboratory conditions for 14 days. Fish were fed with fish food, purchased from a local vendor. Fish showing any sign of infection were immediately discarded. Four different reproductive phases, resting, preparatory, spawning and postspawning were identified (Basak et al., 2016). The research work on the fish *Channa punctatus* was approved by Institutional Animal Ethics Committee of the University (Approval Number: 994/GO/Re/S/06CPCSEA). The guiding principle of the committee for control and supervision of experiments on animals (CPCSEA), Ministry of Statistics & Programme Implementation, Government of India, was strictly followed in the care of fish.

Chemicals and reagents

NBT (Nitroblue Terazolium), Mitogens [Con A (Concanavalin A), PHA (Phytohemaglutnin) and LPS (Lipopolysaccharide)] and Culture medium (RPMI 1640) were bought from Sigma chemicals, USA. Dimethyl sulfoxide (DMSO), lymphocyte separation media (HiSep), Gentamycin, L-glutamine, Streptomycin, PBS (Phosphate Buffer Saline), FBS (Fetal Bovine Serum), and other chemicals were bought from Himedia Lab. Pvt. Ltd. (India). Culture medium (100 ml) was added with 1000 μl of 200 mM L- glutamine, 200 μl of gentamycin, 500 μl antibiotic-antimycotic (Gibco), and 5% FBS and called as complete culture medium.

Experimental procedures

To study the seasonal alterations in the immune status, six fish ($N = 6$) were mildly anesthetized and sacrificed during mid of each month for 18 months. The head kidney and the spleen were excised aseptically, weighed immediately, and the splenosomatic and the head kidney somatic indices (spleen and head kidney weight per 100 g body weight) were determined. Isolated spleen and head kidney from each fish were processes separately, kept in cool (4 °C) culture medium and utilized to study the phagocytosis, quantitative NBT reduction assay, nitrite assay and splenic and head kidney lymphocyte proliferation.

Phagocytosis

The splenic and the head kidney cell suspensions were made and flooded onto the individual glass slides and the slides were incubated for 90 min for the preparation of the macrophage monolayer. The non-adherent cells were washed-off with PBS (pH 7.2). After the preparation of the macrophage monolayer, the slides were flooded with the suspension of yeast cell (400 μl) and the phagocytosis was allowed to occur at 25 °C in a 5% humidified CO_2 atmosphere. After 90 min of incubation the slides were rinsed 3 times in PBS. After fixation in methanol, the slides were stained in a mixture of Giemsa and Leishman. From each slide, 100 macrophages were counted from the different areas of the slide randomly. Determination of phagocytic index was done by finding out the average number of the yeast cell phagocytosed by a single macrophage. Another value, called the percent phagocytosis, was obtained by counting the macrophages showing phagocytosis out of 100 macrophages.

Nitro blue tetrazolium (NBT) assay

The oxidative burst by the phagocytes was measured as the reduction of NBT (Nitro Blue Tetrazolium). NBT is a yellow-colored, water-soluble dye that can cross the plasma membrane of the cell. It is bio-reduced into a purple color NBT formazan by the superoxide. NBT assay was executed by following the protocols of Berger and Slapnickova [29]. The spleen and the head kidney cells were maintained at 2×10^6 cells ml^{-1} in the RPMI. The viable cells were checked through the trypan blue exclusion test which exceeded 95%. Fifty microliters of the head kidney and the spleen cell suspension were taken in 96 well-cultured plates in triplicates from each animal. Further, 50 μl of NBT solution (2 mg/ml) was added to each well. For blank, 50 μl RPMI solution and 50 μl NBT solutions were mixed in separate wells. The culture plates were kept in a humid CO_2 atmosphere at 25 °C for 2 h. The plates were centrifuged after incubation and the supernatant was discarded. The wells containing the cells were washed twice with PBS and then fixed in the methanol. Again, 20 μl of 0.1% Triton X-100 was added to each well. The crystals of formazan were dissolved by adding 120 μl KOH (2 M) and 140 μl DMSO. The absorbance was recorded with the help of a microplate reader at 620 nm.

Nitrite assay

Nitric oxide (NO) is an important effector molecule of cellular cytotoxicity. It is very volatile compound formed from the L-arginine by the enzyme Nitric oxide synthase (NOS). NO decomposes to its metabolites such as nitrite (NO^{2-}) and nitrate (NO^{3-}), which are called the Reactive Nitrogen Intermediate (RNI) [30]. NO production was assayed by the method of Ding et al. [31]. Fifty microlitres of the head kidney and the spleen cell suspension (1×10^5 cells ml^{-1}) were taken in 96 well-cultured plates in triplicates. Additionally, 50 μl of RPMI was added to each well. The culture plates were incubated for 24 h in a CO_2

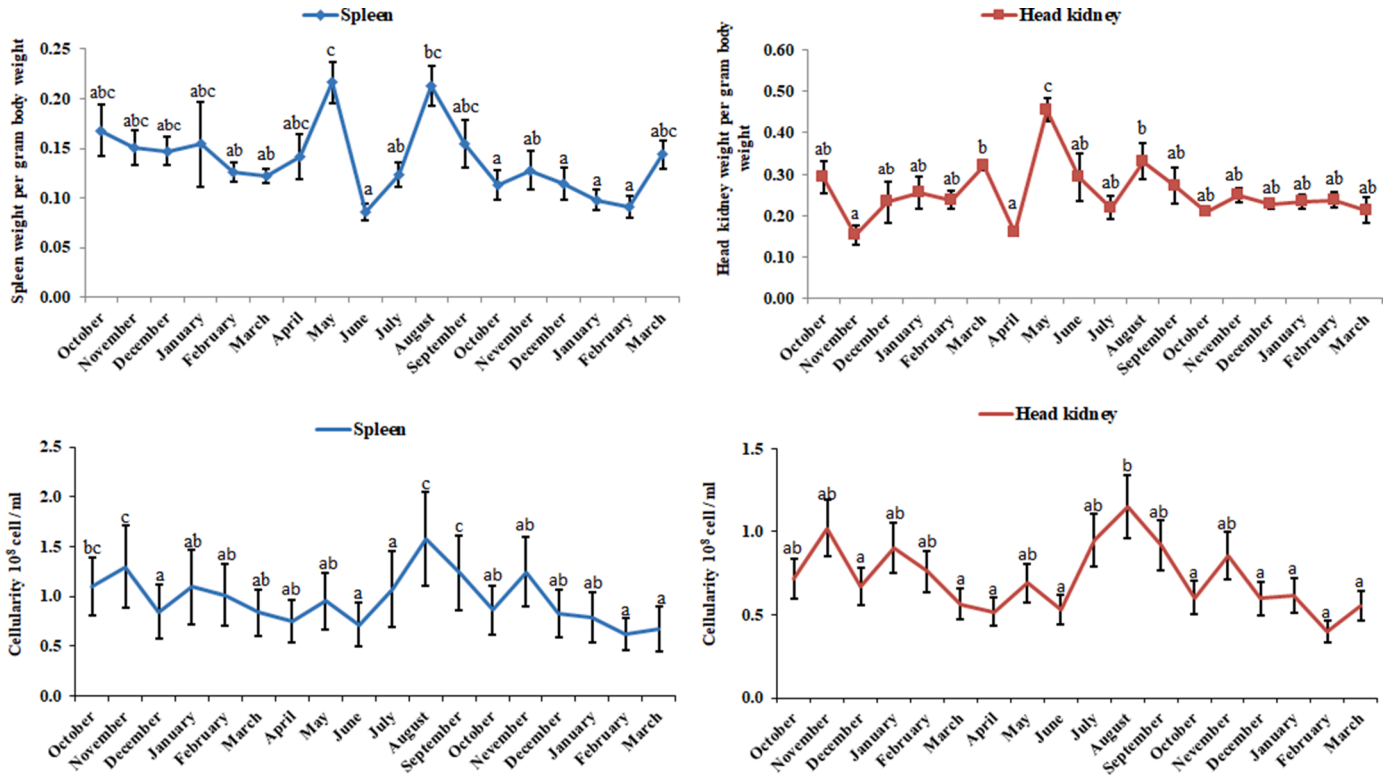

Fig. 1. Seasonal variation in the weight (upper panel) and number of cells (lower panel) in the spleen and the head kidney in the fresh water teleost, *C. punctatus*. The error bars bearing the same superscript do not differ significantly (Newman-Keul's multiple-range test, $p < 0.05$).

atmosphere at 25 °C. In a separate well, 100 μl of the culture medium was taken which served as the blank. After incubation, the plate was centrifuged at 200 x g for 10 min and 50 μl of the supernatant was collected in the separate well. Fifty microlitres of the Griess reagent (0.1% naphthalene-diamine-dihydrochloride in distilled water and 1% sulphanilamide in 3 N HCL) was mixed in the supernatant. The nitrite standard was prepared using $NaNO_2$ and PBS. The absorbance of the colored solution was taken at 540 nm with the help of a microplate reader. The separate wells containing the culture medium served as the blank.

Lymphocyte proliferation assay

Proliferation of lymphocyte was measured utilizing the colorimetric assay based on the tetrazolium salt (MTT) after the methods of Berridge et al. [32]. The colorimetric protocol is an alternative method to radioactive protocol of measuring lymphocyte metabolic activity [33]. In the active cells, the MTT salts are degraded into dark blue formazan crystals by the activity of mitochondrial dehydrogenase enzyme. The head kidney and the spleen cell suspension were prepared, as above, in the culture medium. The head kidney and the splenic lymphocytes were separated from the tissues by centrifugation involving a density gradient (HiSep, density 1.077 g ml^{-1}). The cell suspension was vertexed on the equal volume of gradient and centrifuged at 1500 rpm at 27 °c for 30 min. After centrifugation, the white fraction of lymphocytes at the interface between the medium and HiSep was carefully aspirated. The lymphocyte fraction was washed twice with PBS and resuspended in the culture medium. The trypan blue exclusion test was carried out to check the viability of cells that exceeded 95%. The viable cells were adjusted at 2×10^6 cells ml^{-1} in the RPMI. Basal and the mitogen (Con A at the concentrations of 5 and 10 μg ml^{-1}; LPS at the concentrations of 10 and 20 μg ml^{-1} and PHA at the concentrations of 5 and 10 μg ml^{-1}) stimulated splenic and head kidney lymphocyte proliferation were assessed. The mitogens were dissolved in 0.2 M PBS (pH 7.2) at a concentration of 1 mg ml^{-1}, and further diluted with the culture medium (RPMI 1640). To study the spontaneous or the basal proliferation, 50 μl cell suspension having 1×10^5 cells was put into the well of the culture plate along with 50 μl of the culture medium (RPMI), while to study the induced one, 50 μl of different concentrations of the mitogens (Con A, LPS and PHA) and 50 μl of the cell suspension were added in the wells of the culture plate to make final volume 100 μl per well. The additional well containing only 100 μl of the culture medium served as the blank. All the assays were done in triplicates. The flat bottom 96 well culture plates were used for the lymphocytes proliferation assay. The plates were incubated in the humidified CO_2 atmosphere for 48 h 25 °C. After incubation, 10 μl of MTT reagent (5 mg ml^{-1}) was mixed to each well, and the plates were further incubated in the humidified CO_2 atmosphere for 4 h at 25 °C. The supernatant was aspirated after incubation is over and 100 μl of DMSO was mixed into each well to dissolve the reaction product blue formazan crystals. The absorbance was calculated at 570 nm in the microplate reader.

Statistical analysis

The data were processed by one-way Analysis of Variance (ANOVA) using SPSS and *Post hoc* comparisons were made using the Boneferroni corrections in order to adjust the P values. The significance of the difference was considered when $P < 0.05$. The seasonal data were also evaluated by the Cosinor analysis to find the annual rhythm. The annual rhythm was characterized by various parameters as mesor (rhythm adjusted mean), amplitude (A), acrophase (ϕ), and p denoting the significance of the rhythm.

Results

The splenosomatic index was found maximum during August and minimum during June while the head kidney somatic index was maximum during May and minimum during November ($F_{17,90}$= 3.482,

Table 1
Characteristics of the annual rhythm for the spleen and the head kidney somatic index during 12, 06 and 03 months. Six fish were taken in experiment every month (6 × 18=108 data points).

Variable	Period in months	Period in days (τ)	Data points[a]	Rhythm detection[b]	Mesor	Amplitude	Acrophase
Splenosomatix index	12	365.25	108	0.099	0.14±0.005	0.02(0.025,0.015)	205.35(205.355,205.345)
	06	182.625	108	0.187	0.14±0.005	0.01(0.015,0.005)	226.30(226.5,226.295)
	03	91.312	108	0.01	0.14±0.005	0.02(0.04,0)	201.06(250.62,151.5)
Head kidney Somatic index	12	365.25	108	0.004	0.27±0.009	0.04(0.07,0.01)	164.45(213.83,115.07)
	06	182.625	108	0.834	0.26±0.009	0.01(0.019,0.001)	312.08(312.089,312.071)
	03	91.312	108	0.036	0.27±0.009	0.04(0.049,0.031)	232.14(302.06,162.22)

[a] Number of observations
[b] rejection of the null amplitude hypothesis. Single Cosinor rhythmometry was performed to get the rhythm parameters (based on log-transformed data).

Table 2
Characteristics of the annual rhythm for the cell number in the spleen and the head kidney during 12, 06 and 03 months. Six fish were taken in experiment every month (6 × 18=108 data points).

Variable	Period in months	Period in days (τ)	Data points[a]	Rhythm detection[b]	Mesor	Amplitude	Acrophase
Cellularity Spleen	12	365.25	108	<0.001	1.59 ± 0.07	0.60 (0.86, 0.34)	267.2 (290.59, 243.81)
	06	182.625	108	0.05	1.60 ± 0.08	0.27 (0.35, 0.19)	224.6 (224.69, 224.53)
	03	91.312	108	0.003	1.53 ± 0.08	0.39 (0.67, 0.11)	221.5 (264.21, 178.79)
Cellularity Head Kidney	12	365.25	108	<0.001	1.35 ± 0.05	0.27 (0.45, 0,09)	263.02 (301.7, 224.2)
	06	182.625	108	NS	1.34 ± 0.05	0.06 (0.11, 0.01)	302.35 (302.4, 302.3)
	03	91.312	108	0.003	1.31 ± 0.05	0.19 (0.37, 0.01)	201.05 (266.6, 135.55)

[a] Number of observations.
[b] rejection of the null amplitude hypothesis. Single Cosinor rhythmometry was performed to get the rhythm parameters (based on log-transformed data).

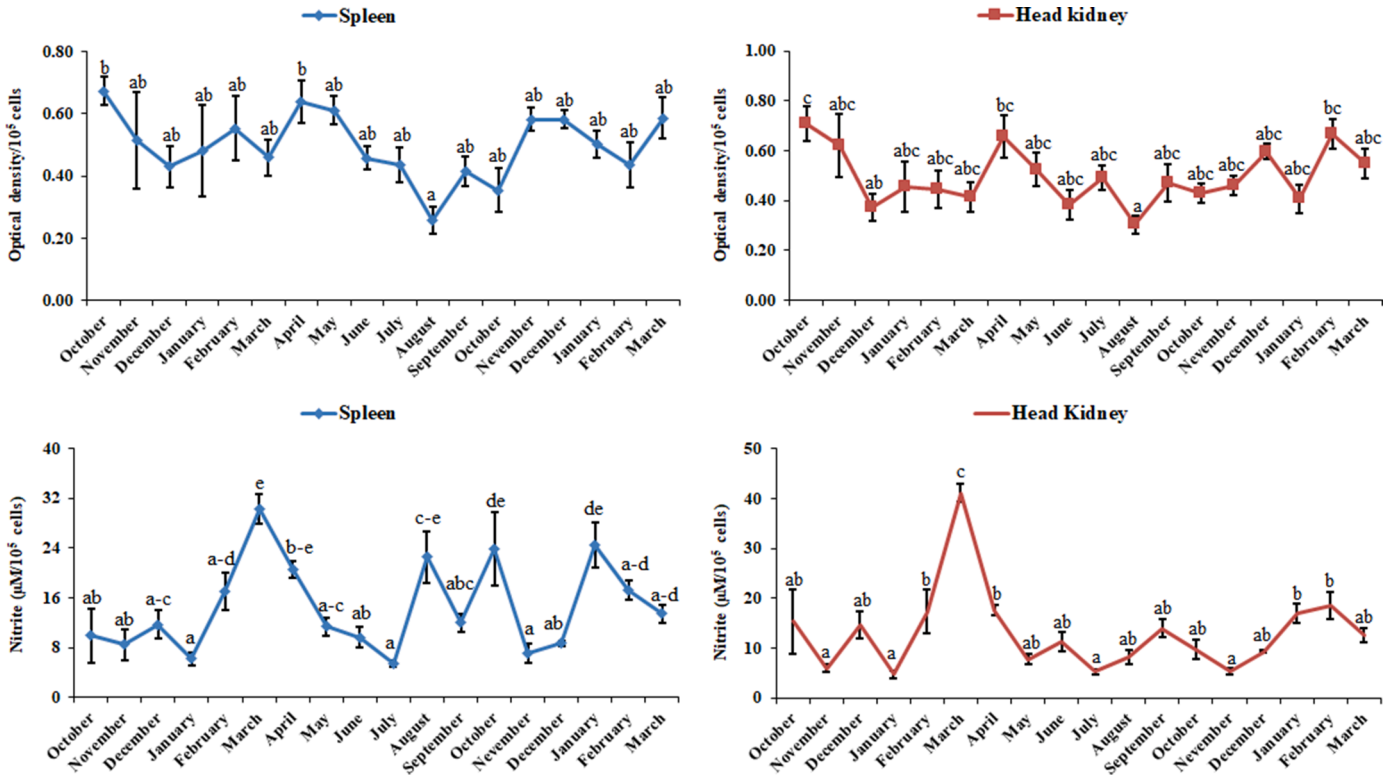

Fig. 2. Seasonal changes in the superoxide anion (upper panel) and nitrite release (lower panel) by the spleen and head kidney cells in *C. punctatus*. The error bars bearing different superscript differ significantly (Newman-Keul's multiple-range test, $p < 0.05$).

$p < 0.001$) (Fig. 1). The acrophase, harmonic mean and amplitude for the 12 months, 06 months, and 03 months periods are given in Table 1. The cosinor analysis of the splenosomatic index revealed that there were no significant annual rhythms in 12 months and 06 months data but the significant annual rhythm was found when the 03 months data were analyzed. The head kidney somatic index showed significant annual rhythm in 12 and 03 months period but not in 06 months period ($F_{17,90}$= 4.897, $p < 0.001$). The cellularity in the spleen and the head kidney were maximum and minimum in August and February-March respectively (Fig. 1). A statistically significant annual rhythm (τ = 365.25 days) was validated in the number of cells for both the lymphoid organs. The spleen cell shows rhythm ($F_{17,90}$= 6.188, $p < 0.001$) for all months of the

Table 3
Characteristics of the annual rhythm for immune activity of the spleen and the head kidney during 12, 06 and 03 months. Six fish were taken in experiment every month. Cells from each animal were cultured in duplicate (12 × 18=216 data points).

Variable	Period in months	Period in days(τ)	Data points[a]	Rhythm detection[b]	Mesor	Amplitude	Acrophase
NBT Spleen	12	365.25	216	0.058	0.49 ± 0.02	0.06(0.08,0.04)	57.86(57.88,57.84)
	06	182.625	216	0.014	0.51 ± 0.02	0.07(0.13,0.01)	269.79(329.07,210.51)
	03	91.312	216	0.891	0.50 ± 0.02	0.01(0.03,−0.01)	299.05(299.07,299.03)
NBT Head Kidney	12	365.25	216	0.316	0.49 ± 0.02	0.04(0.06,0.02)	17.06(17.08,17.04)
	06	182.625	216	0.012	0.51 ± 0.02	0.07(0.13,0.01)	238.93(297.75,180.11)
	03	91.312	216	0.273	0.49 ± 0.02	0.04(0.06,0.02)	153.84(153.86,153.82)

[a] Number of observations.
[b] rejection of the null amplitude hypothesis. Single Cosinor rhythmometry was performed to get the rhythm parameters (based on log-transformed data).

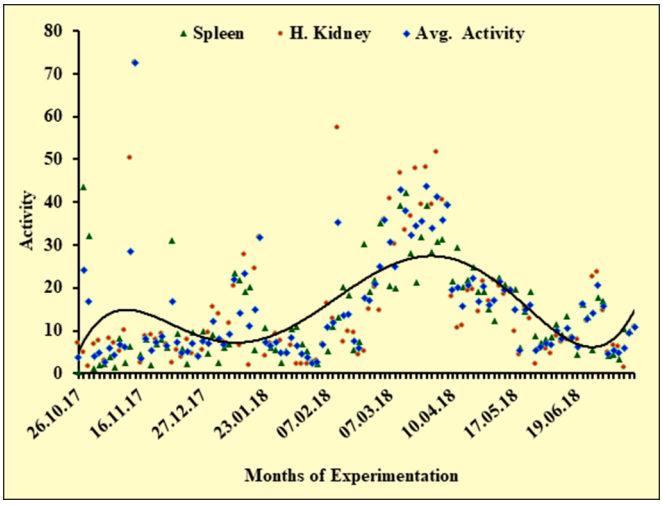

Fig. 3. The modality pattern of rhythm in nitrite assay. A bimodal pattern was observed in nitric oxide production.

experimentation viz., the three months, the six months, and the twelve months. In the case of the head kidney, the significant rhythm ($F_{17,90}$= 2.795, $p < 0.01$) was recorded only during the twelve months and the three months of the experimentation while the six months of experimentation shows an insignificant rhythm (Table 2). The superoxide anion production by the cells of the spleen and the head kidney was maximum in October, and minimum in August month (Fig. 2). The Cosinor results for superoxide anion production are summarized in Table 3. The superoxide anion production by the spleen ($F_{17,198}$= 1.999, $p < 0.05$) and the head kidney cell ($F_{17,198}$= 2.875, $p < 0.001$) were significant in 06 months but not in 12 and 03 months. The nitrite release by the cell of the spleen and the head kidney was maximum in March and minimum in July (Fig. 2). A bimodal pattern in the nitrite release by the spleen and head kidney was observed during the course of the experimentation. Further, the results revealed that the first peak occurred in the month of November while the second peak during the month of March-April (Fig. 3). The Cosinor results for nitrite release are summarized in Table 4 which revealed that the nitrite release by the spleen cells was significant ($F_{17,180}$= 7.533, $p < 0.001$) in 12 and 06-month duration whereas in the case of the head kidney the nitrite release showed significant ($F_{17,198}$= 12.450, $p < 0.001$) annual rhythms in all 12, 06 and 03 month periods. The percentage phagocytosis and the phagocytic index of the splenocyte revealed that the highest peak was found in April and a minimum in February. In the head kidney, the percentage of phagocytosis and the phagocytic index was maximum in May and June and minimum in March (Fig. 4). The cosinor results for phagocytosis are summarized in Table 5. Annual rhythm in the splenic ($F_{17,90}$= 2.136, $p < 0.05$) and the head kidney ($F_{17,90}$= 6.698, $p < 0.001$) percentage phagocytosis was significant in the 12 and 06-month period. Annual rhythm in the phagocytic index of the splenocyte was not significant in 12, 06, and 03 months whereas annual rhythm in the head kidney phagocytic index was significant ($F_{17,90}$= 5.665, $p < 0.001$) in the 12 months. The basal splenic and head kidney lymphocyte proliferations werehighest in March and December and lowest in April (Fig. 5).

The mitogen Conconavalin A (CON A 5 and 10 μg ml^{-1}) induced splenic lymphocyte proliferation was highest in October and lowest in June while head kidney lymphocyte proliferation was highest in January and lowest in April (Fig. 6). The mitogen phytohemaglutanin (PHA 5 and 10 μg ml^{-1}) induced cell proliferation was maximum in March-April and minimum in June (Fig. 7). The B cell mitogen Lipopolysaccharide (LPS 10 and 20 μg ml^{-1}) induced splenic cell proliferation was highest in May and lowest in November while head kidney cell proliferation was highest in January and lowest in April (Fig. 8). The acrophase, harmonic mean and amplitude for the 12 months, 06 months, and 03 months periods are given in Table 6 which showed that the basal splenic lymphocyte proliferation was significant ($F_{17,221}$= 4.805, $p <$ 0.001) in 12 and 03 months but not in 06 month period. The T cell mitogen CON A (5 and 10 μg ml^{-1}) ($F_{17,221}$= 5.117, $p < 0.001$ and ($F_{17,221}$= 5.237, $p < 0.001$) induced lymphocyte proliferation revealed a significant rhythm in 06 month period. The B cell mitogen LPS (10 and 20 μg ml^{-1}) ($F_{17,221}$= 5.494, $p < 0.001$ and ($F_{17,221}$= 4.81, $p < 0.001$) induced lymphocyte proliferation was also statistically significant in 06 month period. Another T cell mitogen PHA (5 and 10 μg ml^{-1}) ($F_{17,221}$= 7.959, $p < 0.001$ and ($F_{17,221}$= 11.879, $p < 0.001$) induced lymphocyte proliferation was significant in the 12, 06, and 03 month periods except

Table 4
Characteristics of the annual rhythm for the immune activity of the spleen and the head kidney during 12, 06 and 03 months. For experimentation on head kidney cells, 6 fish were taken in experiment every month. Cells from each animal were cultured in duplicate (12 × 18=216 data points). For experimentation on spleen cells, 6 fish were taken and cells were cultured in duplicate but some cultures were contaminated and hence not included in analysis.

Variable	Period in months	Period in days (τ)	Data points[a]	Rhythm detection[b]	Mesor	Amplitude	Acrophase
Nitrite Spleen	12	365.25	198	0.033	14.48 ± 0.82	3.12(6.06,0.18)	104.37 (166.14,42.6)
	06	182.625	198	<0.001	14.48±0.75	5.95 (8.61,3.29)	156.18 (180.91,131.45)
	03	91.312	198	0.449	14.52 ± 0.79	1.46(2.5,0.67)	46.01 (46.8, 45.22)
Nitrite Head Kidney	12	365.25	216	<0.001	12.70 ± 0.77	5.61(6.38,4.84)	84.75(85.52,83.98)
	06	182.625	216	0.001	13.15±0.75	5.81(6.56,5.06)	156.48(157.23,155.73)
	03	91.312	216	0.039	13.35±0.77	2.69(3.46,1.92)	327.82(328.59,327.05)

[a] Number of observations.
[b] rejection of the null amplitude hypothesis. Single Cosinor rhythmometry was performed to get the rhythm parameters (based on log-transformed data).

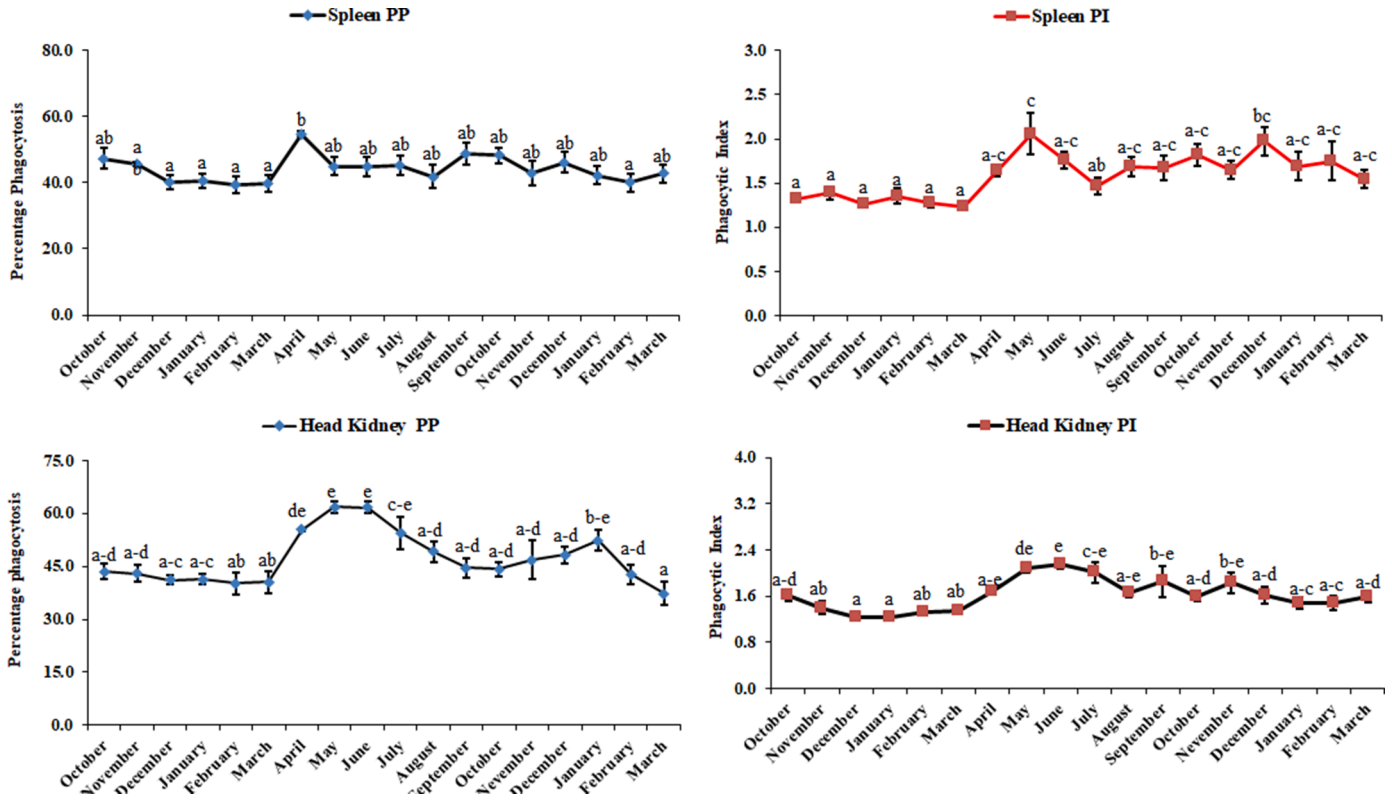

Fig. 4. Annual changes in percentage phagocytosis (PP) and phagocytic index (PI) by the splenic the head kidney macrophages in *C. punctatus*. The error bars bearing the same superscript do not differ significantly (Newman-Keul's multiple-range test, $p < 0.05$).

Table 5
Characteristics of the annual rhythm for the immune activity of the spleen and the head kidney during 12, 06 and 03 months. Six fish were taken in experiment every month (6 × 18=108 data points).

Variable	Period in months	Period in days (τ)	Data points[a]	Rhythm detection[b]	Mesor	Amplitude	Acrophase
Spleen PP	12	365.25	108	0.045	44.66±0.72	2.36 (4.68,0.04)	219.12(300.24,138)
	06	182.625	108	<0.001	44.79±0.67	3.78(6.11,1.45)	232.77(273.03,192.51)
	03	91.312	108	0.121	44.28±0.70	2.10(2.8,1.4)	34.46(35.16,33.76)
Spleen PI	12	365.25	108	0.063	1.62±0.04	0.12 (0.16,0.08)	187.76(186.8,186.72)
	06	182.625	108	0.177	1.59±0.04	0.09(0.13,0.05)	301.69(301.73,301.65)
	03	91.312	108	0.177	1.59±0.04	0.09(0.13,0.05)	301.69(301.73,301.65)
H. kidney PP	12	365.25	108	<0.001	48.86±0.84	7.24(10.17,4.31)	172.66(197.49,147.83)
	06	182.625	108	0.002	47.31±0.90	4.62(7.75,1.49)	317.58(357.83,277.33)
	03	91.312	108	0.319	47.19±0.92	2.06(2.98,1.14)	58.38(59.3,57.46)
H. kidney PI	12	365.25	108	<0.001	1.69±0.03	0.30(0.40,0.20)	192.63(215.86,169.4)
	06	182.625	108	0.062	1.63±0.03	0.12(0.15,0.09)	284.61(284.64,284.58)
	03	91.312	108	0.319	1.6 ± 0.04	0.08(0.12,0.04)	286.55(286.59,286.51)

[a] Number of observations.
[b] rejection of the null amplitude hypothesis. Single Cosinor rhythmometry was performed to get the rhythm parameters (based on log-transformed data).

for PHA (10 μg ml^{-1}) for 12 month period. The harmonic means of the mesor, amplitude, and acrophase obtained separately for the 12 months, 06 months, and 03 month periods are summarized in Table 7 which showed that the basal proliferation of the head kidney lymphocytes was significant ($F_{17,223}$= 7.685, $p < 0.001$) in 12 and 03 months and but not in 06 month period. The Con A (5 μg ml^{-1}) induced lymphocyte proliferation revealed a significant annual rhythm ($F_{17,223}$= 3.117, $p < 0.001$) in 06 month period and the Con A (10 μg ml^{-1}) induced lymphocyte proliferation revealed a significant annual rhythm ($F_{17,223}$= 3.117, $p < 0.001$) in 12 and 03 month period. The LPS (10 μg ml^{-1}) induced lymphocyte proliferation revealed a significant annual rhythm ($F_{17,223}$= 10.71, $p < 0.001$) in the 12 and 06 month period whereas the LPS (20 μg ml^{-1}) induced lymphocyte proliferation showed a significant annual rhythm ($F_{17,223}$= 13.872, $p < 0.001$) in 06 and 03 month period. Further, the T cell mitogen PHA (5 μg ml^{-1}) induced lymphocyte proliferation was significant ($F_{17,223}$= 6.778, $p < 0.001$) in 12 and 03 months but the PHA (10 μg ml^{-1}) induced lymphocyte proliferation was significant ($F_{17,223}$= 8.904, $p < 0.001$) in 12 and 06 months. Table 8 shows the variations in the environmental factors (photoperiod, relative humidity and temperature) during different months of study in Bilaspur, India.

Discussion

Earlier works have elucidated that the internal and external cues like age, diet, feeding, frequency, migration, ration, seasons, sex, starvation, and temperature affect the fish health [34,35]. Seasonality, mainly characterized by the variations in the photoperiod and the temperature, has been explained to influence the immune functions of several vertebrates, including fish, reptiles, birds, and mammals [11,9,13,36]. The

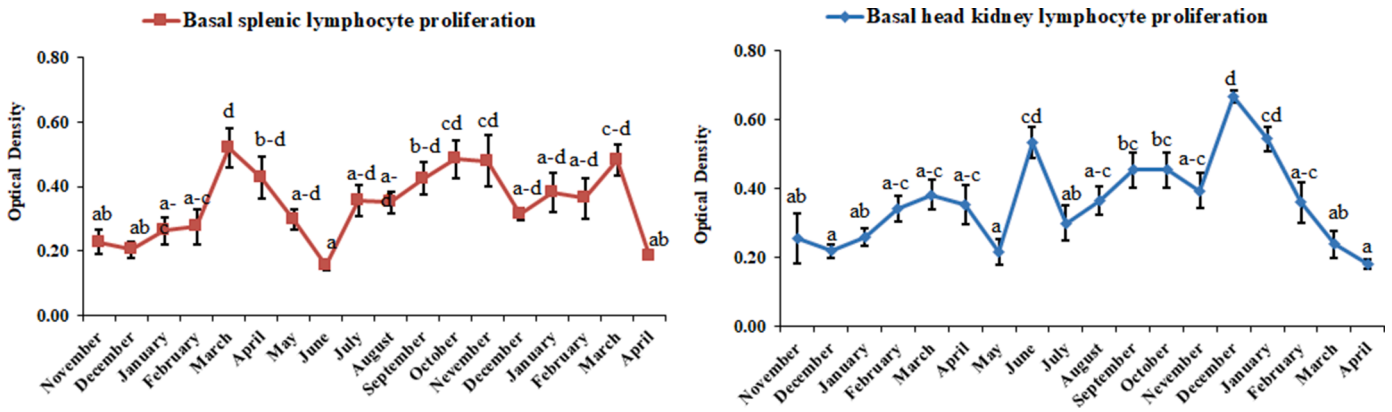

Fig. 5. Seasonal changes in the basal splenic and head kidney lymphocyte proliferation in *C. punctatus.*The error bars bearing the same superscript do not differ significantly (Newman-Keul's multiple-range test, $p < 0.05$).

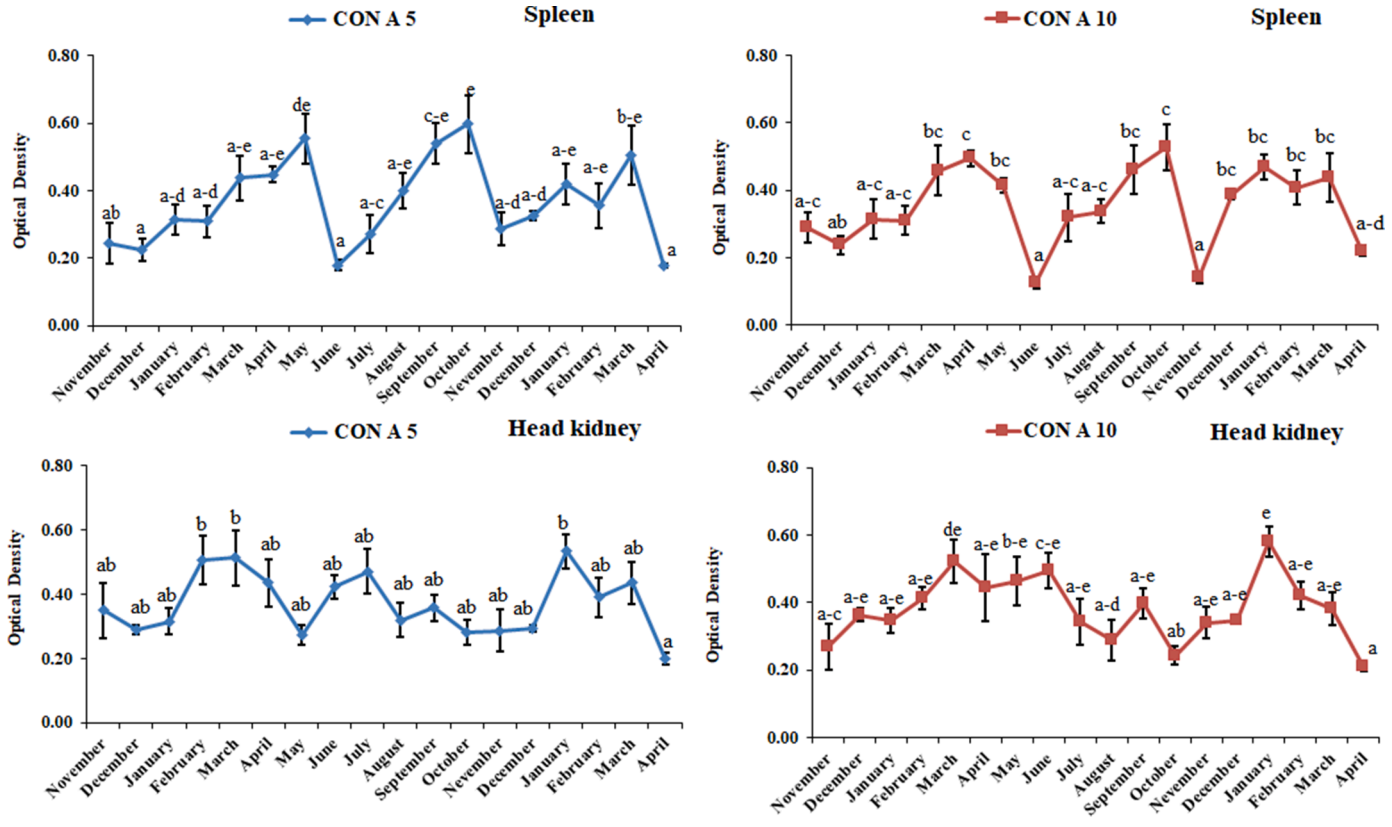

Fig. 6. Seasonal changes in the mitogen induced splenic (upper panel) and head kidney (lower panel) lymphocyte proliferation in *C. punctatus.* The error bars bearing the same superscript do not differ significantly (Newman-Keul's multiple-range test, $p < 0.05$). Mitogen: Con A- Concanavalin A, 5 and 10 μg ml^{-1}.

fish also show the circadian rhythms of various parameters like body color, food intake, oxygen consumption, and physiological parameters [37,38]. Falcon et al. [37] have suggested that the main feature of rhythms is the regularity of their oscillations for a certain period, even when the photoperiodic signals are absent. The existence of the central and the peripheral clocks has been reported in the zebrafish and these clocks were proved to be directly influenced by the light [39]. Baekelandt et al. [40] showed seasonal and daily variations of the photoperiod affecting the innate immune response in a teleost fish.

Since fish are poikilotherms, a decrease in the temperature affects the pace of their physiological responses [41]. It has been postulated that decline in temperature induces innate immune responses in the fish, while elevated temperatures may cause the activation of specific immunity [42,11]. A similar observation was found in a study on the perch (*Perca fluviatilis*) which showed that the pattern identification by the glucan binding proteins was more common in the fish reared at lower temperatures and that opsonin (specific recognition) was elevated at higher temperatures [43]. Xu et al. [44] have shown that IgG and IgM levels were at dip in the fall which may be because of low splenosomatic index as we have found the highest splenosomatic index in May and August and minimum in January-February. The number and state of activation of the leucocytes in the blood, kidney, and spleen are important indicators of the immunocompetence of an organism. The correlation between ROS production and temperature has been demonstrated in earlier works which have suggested that the adaptation at declined temperature led to a rise in the superoxide production [45–47]. Le Morvan et al. [48] reported that superoxide production and phagocytic activity of macrophage are elevated with a fall in the

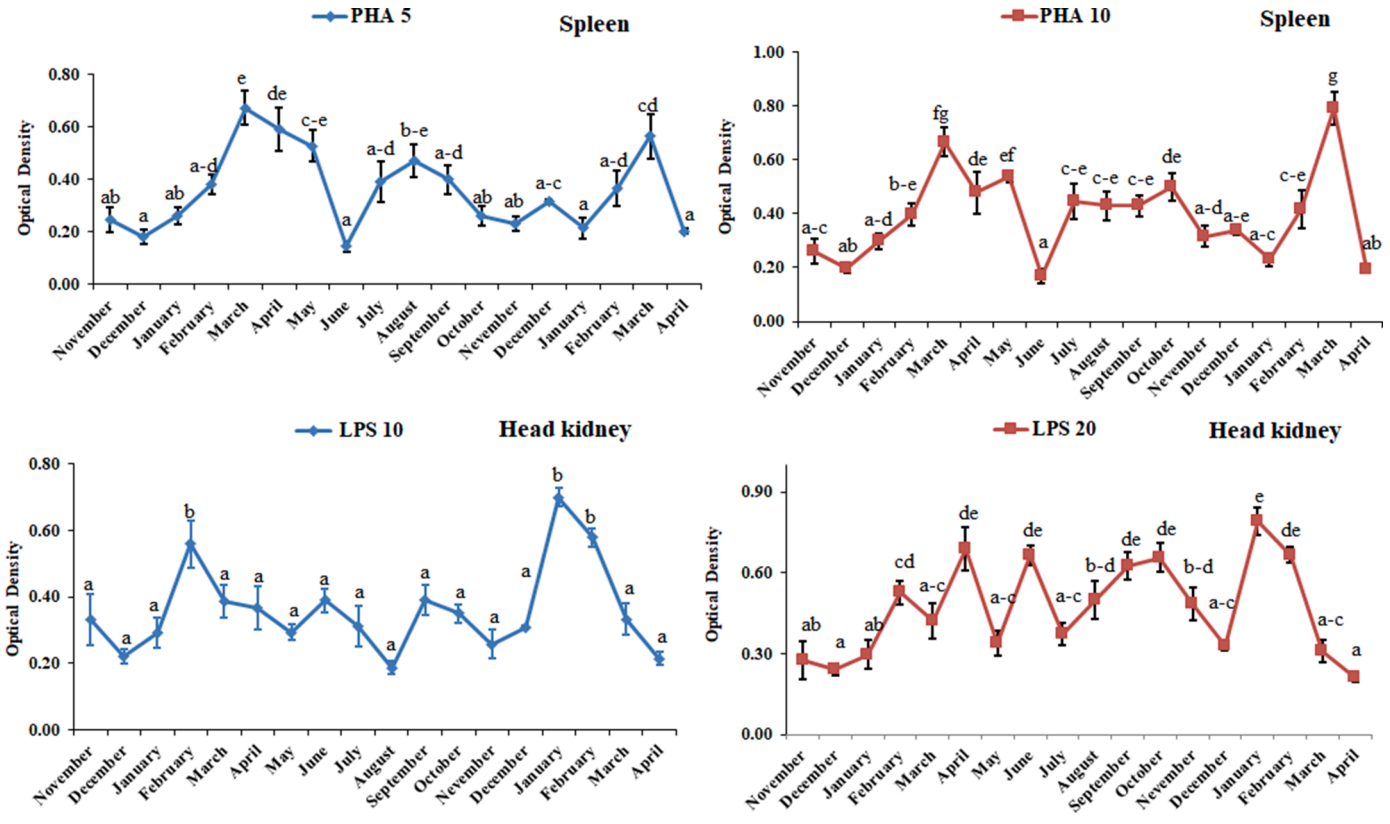

Fig. 7. Seasonal changes in the mitogen induced splenic (upper panel) and head kidney (lower panel) lymphocyte proliferation in *C. punctatus*. The error bars bearing the same superscript do not differ significantly (Newman-Keul's multiple-range test, $p < 0.05$). Mitogen: PHA- Phytohemagglutinin, 5 and 10 μg ml^{-1}.

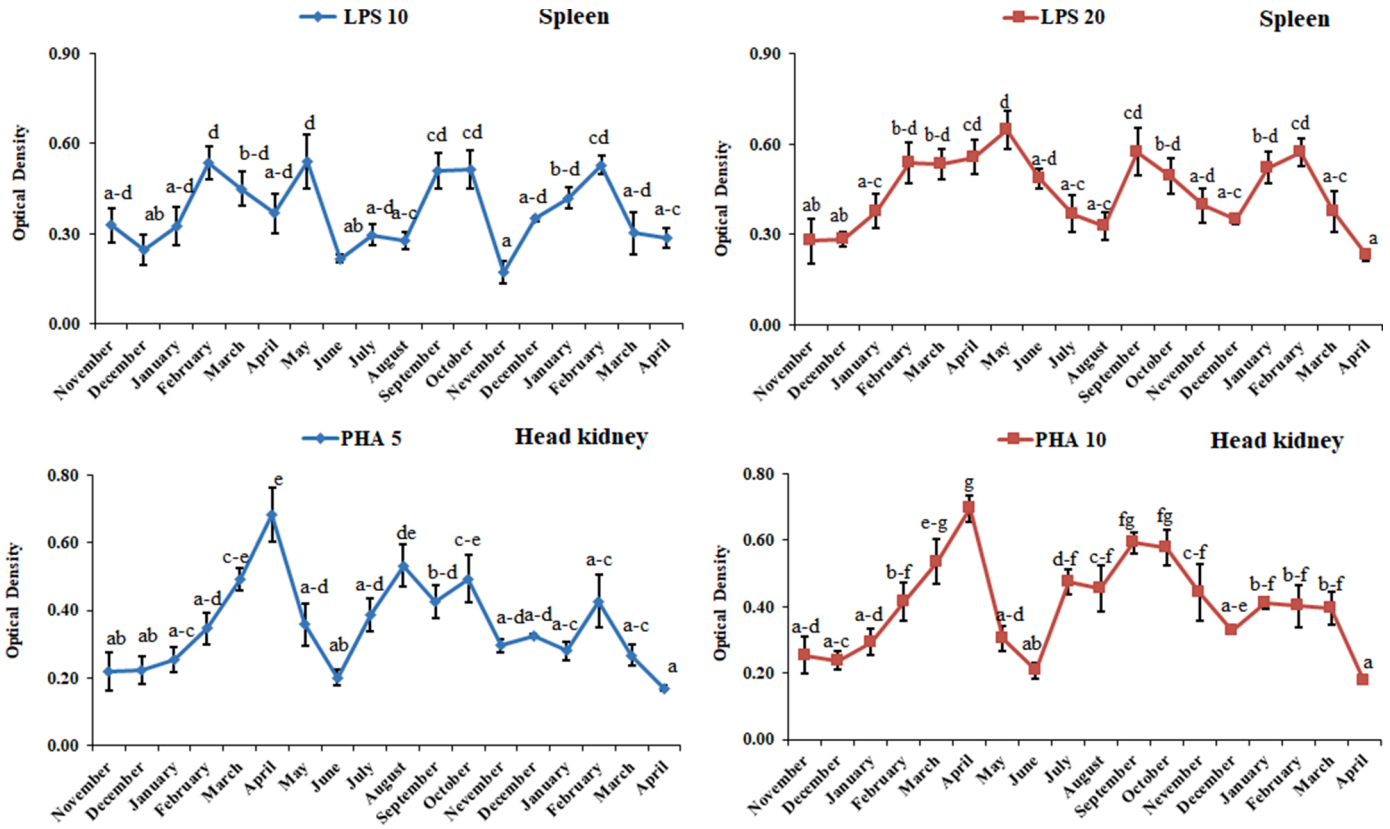

Fig. 8. Seasonal changes in the mitogen induced splenic (upper panel) and head kidney (lower panel) lymphocyte proliferation in *C. punctatus*. The error bars bearing the same superscript do not differ significantly (Newman-Keul's multiple-range test, $p < 0.05$). Mitogen: LPS- Lipopolysaccharide, 10 and 20 μg ml^{-1}.

Table 6
Characteristics of the annual rhythm for the immune activity of the spleen during 12, 06 and 03 months. Six fish were taken in each month and cells were cultured in triplicate from each fish. But in some months a few culture wells were contaminated and hence were not included in data analysis.

Variable of splenic lymphocyte cell proliferation	Period in months	Period in days (τ)	Data points[a]	Rhythm detection[b]	Mesor	Amplitude	Acrophase
Basal	12	365.25	239	0.202	0.34 ±0.01	0.04 (0.05,0.03)	312.46 (312.49,312.43)
	06	182.625	239	<0.001	0.34 ±0.01	0.10 (0.15,0.05)	159.38 (185.94,132.82)
	03	91.312	239	0.054	0.35 ±0.01	0.04 (0.05,0.04)	307.79 (307.80,307.78)
Con A 5	12	365.25	239	0.198	0.37 ±0.02	0.04 (0.06,0.02)	280.58 (280.60,280.56)
	06	182.625	239	<0.001	0.36 ±0.02	0.12 (0.18,0.06)	168.6(195.06,142.14)
	03	91.312	239	0.120	0.37 ±0.02	0.04 (0.06,0.02)	314.97 (314.99,314.95)
Con A 10	12	365.25	239	0.443	0.36 ±0.01	0.03 (0.04,0.02)	338.89 (338.90,338.88)
	06	182.625	239	<0.001	0.35 ±0.01	0.11 (0.16,0.06)	158.69 (183.00,134.38)
	03	91.312	239	0.451	0.36 ±0.01	0.03 (0.04,0.02)	40.88(40.89,40.88)
LPS 10	12	365.25	239	0.344	0.37 ±0.01	0.03 (0.04,0.02)	9.93(9.94,9.92)
	06	182.625	239	<0.001	0.37 ±0.01	0.09 (0.14,0.04)	153.69 (184.41,122.97)
	03	91.312	239	0.351	0.38 ±0.01	0.03 (0.04,0.02)	201.37 (201.38,201.36)
LPS 20	12	365.25	239	0.683	0.44 ±0.01	0.02 (0.03,0.01)	96.21(96.22,96.20)
	06	182.625	239	0.030	0.43 ±0.01	0.06(0.12,0)	138.57(200.14,77)
	03	91.312	239	0.370	0.45 ±0.02	0.03 (0.05,0.01)	285.44 (285.46,285.42)
PHA 5	12	365.25	239	0.002	0.35 ±0.02	0.09 (0.15,0.03)	132.73(169.61,95.85)
	06	182.625	239	<0.001	0.34 ±0.01	0.14 (0.19,0.09)	156.96 (177.17,136.75)
	03	91.312	239	<0.001	0.35 ±0.02	0.10 (0.16,0.04)	249.43 (282.71,216.71)
PHA 10	12	365.25	239	0.223	0.39 ±0.01	0.04 (0.05,0.03)	137.76 (137.77,135.75)
	06	182.625	239	<0.001	0.38 ±0.01	0.17 (0.22,0.12)	159.17 (174.61,143.73)
	03	91.312	239	<0.001	0.40 ±0.01	0.10 (0.15,0.05)	264(294.78,233.88)

[a] Number of observations.
[b] rejection of the null amplitude hypothesis.

environmental temperature. The temperature dependent impairment of the production of reactive oxygen species (ROS) was found in *Ictalurus punctatus, Epinephelus coioides, Oreochromis mossambicus* and *Oncorhynchus mykiss* [49–52]. It was postulated that winter (acute and chronic) jeopardized the phagocytic and oxidative burst activities leading to compromised innate and cell mediated immune status. In vitro experiments demonstrated that low winter like temperature caused insignificant change in the innate immune functions of macrophage in *Oncorhynchus mykiss* as the respiratory burst activity was comparable to control animals [46]. The respiratory burst activity and the phagocytic functions of the macrophages were elevated in an in vivo study where *Cyprinus carpio* were cultured at 12 °C for 28 days when compared to the fish kept at 20 °C [48]. Similarly, the granulocytes from the blood of *Tinca tinca*, kept at 12 °C (winter like conditions) showed increased phagocytic capacity and the production of ROS when compared with the fish kept at 22 °C [53]. These researches implicate the enhancement of innate immune functions in long-term winter exposure leading to an optimal adaptive survival.

The fish may utilize NO as an effector of the hormonal activity [54]. The presence of two NO synthase (NOS) isoforms have been reported in the fish [55]. Treatment of zebrafish (*Danio rerio*) with resulted in an elevation of NO [56,57]. Bryan et al. [58] have explored that nitrite has dual effect as it acts as a signaling factor in endocrine system and it is also involved in the generation of NO. In our study nitrite release by the splenic and the head kidney cells was greater in March and lowest in the month of July and November. The reason for this differential immune activity may be attributed to the energy trade-off for different components of immune system or season-dependent annual cycle of immune stressors (viruses, bacteria, and parasites). The literature survey reveals differential activities of phagocytes as phagocytic functions were increased in summer [23,59,24] but declined in winter [60,13]. The percentage phagocytosis of the spleen cell was maximum in April and September and minimum in February-March. The phagocytic index of the splenocyte macrophages was observed higher in May and December and lowest in March. The phagocytic index of the head kidney macrophages cell reached the maximum value in May-June, and during February and in March the PI value was minimal.

The data of the present study documents statistically significant rhythms in the activity of splenic lymphocytes after stimulation with ConA 5 and 10 μg ml^{-1}, LPS 10 and 20 μg ml^{-1}. The peak responses were located in October and May. Another mitogen, PHA showed a statistically significant annual rhythm in the responsiveness of the splenic lymphocyte. The peak responses were located in March-April. The present results also document three-monthly rhythms both in PHA and LPS

Table 7
Characteristics of the annual rhythm for immune activity of head kidney during 12, 06 and 03 months. Six fish were taken in each month and cells were cultured in triplicate from each fish. But in some months a few culture wells were contaminated and hence were not included in data analysis.

Variable of Head kidney lymphocyte proliferation	Period in months	Period in days (τ)	Data points[a]	Rhythm detection[b]	Mesor	Amplitude	Acrophase
Basal	12	365.25	241	0.001	0.36 ±0.01	0.08(0.13,0.03)	302.24 (336.55,267.93)
	06	182.625	241	0.065	0.36 ±0.01	0.04(0.05,0.03)	34.52
	03	91.312	241	<0.001	0.38 ±0.01	0.09(0.14,0.04)	8.59(34.78,17.60)
Con A 5	12	365.25	241	0.222	0.37 ±0.02	0.03(0.05,0.01)	69.30(69.28,69.32)
	06	182.625	241	<0.001	0.36 ±0.01	0.80(0.85,0.75)	91.82(129.62,54.02)
	03	91.312	241	0.879	0.37 ±0.01	0.01(0.02,0.00)	238.13 (238.14,238.12)
Con A 10	12	365.25	241	0.010	0.37 ±0.01	0.05(0.10,0.00)	77.24(127.68,26.80)
	06	182.625	241	0.004	0.37 ±0.01	0.06(0.10,0.02)	57.80(111.22,4.38)
	03	91.312	241	0.287	0.38 ±0.01	0.03(0.04,0.02)	328.54 (328.55,328.53)
LPS 10	12	365.25	241	<0.001	0.35 ±0.01	0.09(0.13,0.05)	26.34(61.74,9.06)
	06	182.625	241	<0.001	0.35 ±0.01	0.08(0.12,0.04)	83.24(117.73,48.75)
	03	91.312	241	0.265	0.37 ±0.01	0.03(0.04,0.02)	123.90 (123.91,123.89)
LPS 20	12	365.25	241	0.134	0.48 ±0.02	0.05(0.07,0.03)	293.59(293.61,)
	06	182.625	241	0.008	0.47 ±0.02	0.07 (0.13,0.0.01)	115.81(163.00,68.62)
	03	91.312	241	0.013	0.48 ±0.02	0.07(0.13,0.01)	43.32(94.68,8.04)
PHA 5	12	365.25	241	0.073	0.37 ±0.01	0.04(0.05,0.03)	222.27 (222.28,222.26)
	06	182.625	241	<0.001	0.35 ±0.01	0.11(0.16,0.06)	160.04 (184.19,135.89)
	03	91.312	241	0.906	0.36 ±0.01	0.01(0.02,0.00)	38.34(38.35,38.33)
PHA 10	12	365.25	241	0.018	0.41 ±0.01	0.06(0.11,0.01)	269.71 (322.10,217.32)
	06	182.625	241	<0.001	0.39 ±0.01	0.14(0.19,0.09)	158.37 (176.74,140.00)
	03	91.312	241	0.152	0.41 ±0.01	0.04(0.05,0.03)	357.33 (357.34,357.32)

[a] Number of observations.
[b] rejection of the null amplitude hypothesis. Single Cosinor rhythmometry was performed to get the rhythm parameters (based on log-transformed data).

Table 8
Variation in the environmental variables (photoperiod, relative humidity and temperature and) during different months of study.

S. N	Month	Relative Humidity (%)	Temperature (°C)	Photoperiod (h)
1.	October	62.28	27.53	8.43
2.	November	45.17	24.05	9.73
3.	December	34.81	21.79	10.64
4.	January	29.65	20.18	10.98
5.	February	27.35	25.82	9.07
6.	March	17.84	30.53	11.53
7.	April	18.28	34.54	10.57
8.	May	22.84	37.68	11.21
9.	June	44.04	34.75	8.04
10.	July	69.18	29.85	2.52
11.	August	80.03	27.59	1.55
12.	September	71.75	27.88	6.12
13.	October	46.31	27.79	10.52
14.	November	39.54	25.02	10.38
15.	December	41.14	20.54	8.52
16.	January	35.75	20.44	10.22
17.	February	29.79	25.10	9.36
18.	March	24.73	29.81	11.13

induced proliferation of lymphocyte. Further, a six-monthly rhythm in the stimulation of splenic B lymphocytes was observed. It is remarkable that the activities of lymphocytes from the anterior kidney and spleen to all three mitogens were exemplified by a three-monthly rhythm with peaks exactly in the same months. Low winter-like temperatures seem to slow-down or delay the generation immune factors in response to the pathogen mimics or the pathogens themselves, implicating the suppression of a pro-inflammatory response. The specific immune system of vertebrates is stimulated by specific pathogen and results in memory [61,62]. Antigen presenting cells express MHC I and II which are identified by T-lymphocytes and after identification the latter secrete cytokines which then stimulate other cells of the immune system to mount antigen-specific action [63].

While the innate immune functions are crucial clearing initial pathogen invasion, specific immune components are evenly pivotal and needed for elimination of pathogens. It is imperative to perform more intensive studies including the test of virulence of the pathogen [64] in order to strengthen our knowledge of the impact of environmental conditions on host-pathogen systems, specifically in verifying the underlying immune mechanisms that result in host mortality. Duration of sunlight is the main environmental signal which is taken by the most animals to anticipate the season. Season dependent rhythm in the

immune functions help the animal cope with environmental stressors such as pathogens. For the perpetuation of a species, it is important to use the energetic budget appropriately in various physiological responses. Further work is essentially needed to study the involvement of circadian rhythm in adaptive immune functions.

Data availability

The data are available in the manuscript. Any further query may be addressed to corresponding author.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data are available in the manuscript. Any additional query may be addressed to corresponding author.

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