# Seasonal changes of pronephros lymphoid tissue in grass carp (*Ctenopharingodon idella*): a histometrical and histological study

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## Summary

The major lymphoid tissues in teleost fish are the kidneys, thymus, spleen and mucosa-associated lymphoid tissue including the skin, gills and intestine. The head of kidney (pronephros) is an important haematopoietic organ and has morphological similarities with the bone marrow in higher vertebrates. In this study, during 12 months from August 2002 to July 2003, 120 grass carp (10 fish/month) were harvested from 3 fish culture ponds in Ahvaz. The water, weather temperatures and light duration of days (photoperiod) were recorded. After biometrical examination of age, weight and the length of the fishes, the whole kidneys were removed from abdominal cavity and samples were fixed in bouin's solution for light microscopy and in glutaraldehyde for electron microscopy. The results showed that the lymphoid tissue distribution has significant changes during different seasons so that, the mean ± SEM of lymphoid tissue distribution in hot seasons (33.53 ± 0.59 $mm^2/cm^2$) was more than cold seasons (19.20 ± 0.65 $mm^2/cm^2$). Also the statistical results showed that water temperature has more significant ($P<0.05$) effect on lymphoid tissue. Degenerated lymphoid cells with pale and vacuolated cytoplasm were observed. A reversed correlation was found between the number of normal and degenerated lymphocytes. Electron microscopy revealed that degenerated lymphocytes were devoid of membranous organelles, their cytoplasms were vacuolated and the nuclear envelope had some alterations.

**Key words:** Seasonal changes, Pronephros, Lymphoid tissue, Grass carp

## Introduction

In teleost fish, the kidney is a multifunctional organ. The kidney not only fulfills excretory and osmoregulatory function, but also contains high numbers of haematopoietic and phagocytic cell (Ostrander, 2000).

As in all vertebrates, the kidney in fish is located retroperitoneal, exterior to the dorsal wall of the body cavity. The kidney is a paired organ that has been described as having various anatomical and functional compartments. The kidney in teleost fish, consists of an anterior aglomerular and a middle and posterior glomerular compartments. The anterior part of the kidney in teleost, lacks excretory tissue and is often referred to as the "head kidney" (Perss and Evensen, 1999). At the meantime, head kidney also serves as secondary lymphoid organ; analogous to a lymph node. That is important in the induction and elaboration of immune response (Kaattari and Irwin, 1985). It is predominantly a lympho-myeloid compartment. The form of the head kidney varies between species; in some species there are two separate extensions in the most anterior part of the organ (Perss and Evensen, 1999). The anterior kidney is primarily composed of haematopoietic tissue and the posterior portion contains the tubules and glomeruli of the excretory system. Evidence of lymphopoiesis in the anterior section comes from observations of multiple mitotic figures in the anterior kidney (Ostrander, 2000).

In recent years, the literature describing the morphological and functional organization of fish immune systems has dramatically increased. The reasons for this may derive from common immunological antecedents, since fish, similar to other vertebrates, possess an efficient and developed immune defense mechanism. Furthermore, fish farming of the teleost species at high densities offers an increased opportunity for spreading of infectious diseases at all stages of production (Scapigliati *et al*., 1999).

Seasonal changes affect both the structure and function of the immune system in ectothermic vertebrates (Zapata *et al*., 1983, 1992; Zapata, 1996). In teleost fish, apart from a few reports concerning seasonal variations in the number of circulating blood cells (Zapata and Cooper, 1990), there are a few histometrical studies on cell content of various lymphoid organs. Alvarez *et al*., (1998) have performed some experiments on seasonal changes in the lymphoid organs of wild brown trout, S*almo trutta*.

In the present study, we have analysed the seasonal changes of pronephros lymphoid tissue in grass carp (*Cetenopharingodon idella*) histometrically and histologically in Ahvaz, southwestern Iran.

## Materials and Methods

Between August (2002) and July (2003), 120 (10 fishes/month) grass carp (C*etenopharingodon idella*) were harvested monthly by net fishing from three considered fish culture ponds in Ahvaz, southwestern Iran. Special care was taken to include healthy grass carp in our study. Fishes were anaesthetized with stroke to head. They then were weighed; their body lengths were measured and their age determined by accomplished history of hatching time. A total of 120 male and female fishes, were collected with an age of 1–2 years, a length of 93–672 mm and a weight of 98–1300 gr. Their anterior kidneys (pronephros) were removed and fixed by bouin and glutaraldehyde for light and electron microscopic studies, respectively. Sections for light microscopic study were prepared by routine paraffin embedding method and were stained with haematoxylin-eosin (Bancroft and Gamble, 2002). For electron microscopy, small tissue pieces were fixed in ice-cold 4% glutaraldehyde in 0.1 M phosphate buffer (pH=7.4) for 4 hrs, washed in the same buffer (7.5% sucrose), post-fixed in 1% osmium tetra-oxide in the same buffer, dehydrated in acetone and embedded in Epon 812 (Bozzola and Russell, 1999). Semi-thin sections (1-µm thick) were stained with an alkaline solution of toluidine blue. Ultra-thin sections were mounted on copper grids, double-stained with uranyl acetate and lead citrate, and examined under a Philips 400 electron microscope at 60-80 kV in Razi Vaccine and Serum Research Institute. The graticule lens and calibrated slide were used for the estimation of lymphoid areas, as well as the area occupied by lymphoid tissue. The structure of pale lymphocyte and presence of renal corpuscles and excretory ducts were studied histologically. The pale lymphocyte index (PI) was calculated using the following formula (Alvarez *et al*., 1998):

$$PI = \frac{\text{Number of pale lymphocytes}}{\text{Number of pale lymphocytes + Number of normal lymphocytes}} \times 100$$

The measurements for each sample were made on five fields of five slides. (Briefly: 5 fields×5 sections×10 samples×12 month).

To monitor seasonal and monthly variations and to consider the effects of weight and length, a two-stage nested analysis of variance (ANOVA, $P<0.05$) with weight and length as covariates was made on each parameter. Differences in means were determined by using Tukey multiple comparison tests. Furthermore, the regression analysis was used to determine the effects of age, water temperature, weather temperature and photoperiod on each parameter. Quadratic transformation of the age was used to improve the correlation between the age and other measured parameters.

The correlation between distinct variables was estimated using Pearson's correlation coefficient. The above procedures were performed using Minitab R13 software.

## Results

Macroscopic observation showed that

the kidney of grass carp, like other species of fishes, consisted of three parts; head, body and tail (Fig. 1). The head of the kidney is mainly consisted of lymphoid and haematopoietic tissues (Fig. 2). In this compartment a few number of excretory tissue and glomeruli were observed. Lymphoid tissue distribution (LTD) in pronephros was significantly different in various months and seasons.

The results showed that the LTD reached its maximum value in summer (35 ± 1 in August) and its minimum in winter (16 ± 0.7746 in January). The LTD in spring (33 ± 1.1832 in June) was similar to that in summer, whereas the LTD in autumn had different pattern so that, in October (30 ± 0.8165) it was similar to summer, and in December (20 ± 0.8563) it was similar to winter (Table 1). A significant difference

**Table 1: The mean±SEM lymphoid tissue distribution and percentage of pale lymphocytes in each month**

| Month | Lymphoid tissue distribution | %Pale lymphocytes |
|---|---|---|
| January (a) | 16 ±0.7746$^{b, j, k}$ | 20 ±0.8433$^{I, j, k, l}$ |
| February (b) | 19 ±0.7454$^{a, I, j, k, l}$ | 19 ±0.3651$^{I, j, k, l}$ |
| March (c) | 22.6 ±0.7483$^{I, j, k, l}$ | 17 ±0.5164$^{h, I, j, k, l}$ |
| April (d) | 25 ±1.1180$^{j, l}$ | 14.1 ±0.4231$^{I, j, k, l}$ |
| May (e) | 28.7 ±0.7873$^{I, j, k, l}$ | 13 ±0.3303$^{h, I, j, k, l}$ |
| June (f) | 33 ±1.1832$^{I, j, k, l}$ | 12 ±0.2582$^{h, I, j, k, l}$ |
| July (g) | 33.8 ±1.0520$^{I, j, k}$ | 12 ±0.3651$^{h, I, j, k, l}$ |
| August (h) | 35 ±1$^{j, k}$ | 9 ±0.4472$^{c, e, f, g, I, j, k}$ |
| September (I) | 31.8 ±0.8667$^{b, c, e, f, g}$ | 10 ±0.6325$^{a, b, c, d, e, f, g, h}$ |
| October (j) | 30 ±0.8165$^{a, b, c, d, e, f, g, h}$ | 10 ±0.3651$^{a, b, c, d, e, f, g, h, l}$ |
| November (k) | 24.2 ±0.9286$^{a, b, c, e, f, g, h}$ | 15 ±0.5164$^{a, b, c, d, e, f, g, h}$ |
| December (l)* | 20 ±0.8563$^{b, c, d, e, f}$ | 18 ±0.4944$^{a, b, c, d, e, f, g, k}$ |

*The letters *a* to *l* in column 1 are abbreviated characters for showing each month. Existence of each letter in each cell of columns 2 and 3 shows significant difference between these months (P<0.05)

was observed in LTD between spring and winter (P<0.05), summer and either autumn or winter (P<0.05), autumn and either summer or winter (P<0.05), and winter and all other seasons (P<0.05; Table 2). The LTD differed significantly in different months (P<0.05; Table 1). Among the

**Table 2: The mean±SEM lymphoid tissue distribution and percentage of pale lymphocytes in each season**

| Season | Lymphoid tissue distribution | %Pale lymphocytes |
|---|---|---|
| Spring (a)* | 29±0.8228$^{d}$ | 13±0.2445$^{d}$ |
| Summer (b) | 33.5±0.5964$^{c, d}$ | 10±0.3599$^{c, d}$ |
| Autumn (c) | 24.7±0.9020$^{b, d}$ | 14±0.6649$^{b, d}$ |
| Winter (d) | 19.2±0.6546$^{a, b, c}$ | 18±0.4106$^{a, b, c}$ |

*The letters *a* to *d* are abbreviated characters for showing each season. Existence of each letter in each cell shows that there is significant difference between different seasons (P<0.05)

studied factors, the water temperature, weather temperature, photoperiod and age square had each directly correlated with LTD (Fig. 3). However, the body length and weight did not have any significant effects on LTD. Evaluation of the total effective factors on the ATLA, showed that water temperature had the maximum effect on lymphoid tissue (Table 3).

Degenerated lymphocytes (pale lymphocytes) were observed in all seasons. The cytoplasm of these cells was pale and the nucleus was condensed and fragmented. (Fig. 4). The average of degenerated lymphocyte percentage (ADLP) was maximum in winter (20 ± 0.8433 in January) and minimum in summer (9 ± 0.4472 in August). In spring, the maximum of ADLP was absorbed in April (14.1 ± 0.4231) while the minimum was in June (12 ± 0.2582). In autumn, the maximum of ADLP was observed in December (18 ± 0.4944) and the minimum in October (10 ± 0.3651; Table 2). No significant changes were observed in spring and autumn, however, there were significant differences between spring and winter, summer and either autumn or winter, autumn and either summer or winter, and winter and all other seasons (P<0.05).

There were significant differences in ADLP in different months (P<0.05; Table 2). Water and weather temperature, photoperiod and age square, each had reverse correlation with the ADLP (Fig. 5). The body length and weight, however, had no effects on the ADLP. Evaluation of all factors on the ADLP revealed that water

**Fig. 1: The three parts of grass carp kidney; head (H), body (B) and tail (T)**

**Fig. 2: The "head kidney" structure; tubule (T), lymphoid tissue (L) and haematopoietic tissue (H) (H&E, ×530)**

temperature and age square had the maximum effect on ADLP (Table 3).

## Discussion

The head kidney is an important haemopoietic organ and serves as secondary lymphoid organ (Perss and Evensen, 1999). The head of kidney contains a variety of tissues that have no function in the urinary system. Lymphoid tissue is quite prominent in this portion of the fish kidney, as is haemopoietic blast cells (Stoskopf, 1993).

The present histometrical and histological results showed that the head kidney tissue in grass carp has a few number of excretory tissue and glomeruli. The seasonal variations in the lymphoid organs have been studied in wild brown trout, *Salmo trutta*, (Alvarez *et al*., 1998). They showed that the number of lymphocytes maximize in spring and autumn and that there are two periods of lymphoid involution in winter and summer. In the present study, the LTD was maximum in summer (33.5 ± 0.5964 $mm^2/cm^2$) and was minimum in winter (19.2 ± 0.6546 $mm^2/cm^2$). However, the LTD was high in spring (29 ± 0.8228 $mm^2/cm^2$) and autumn

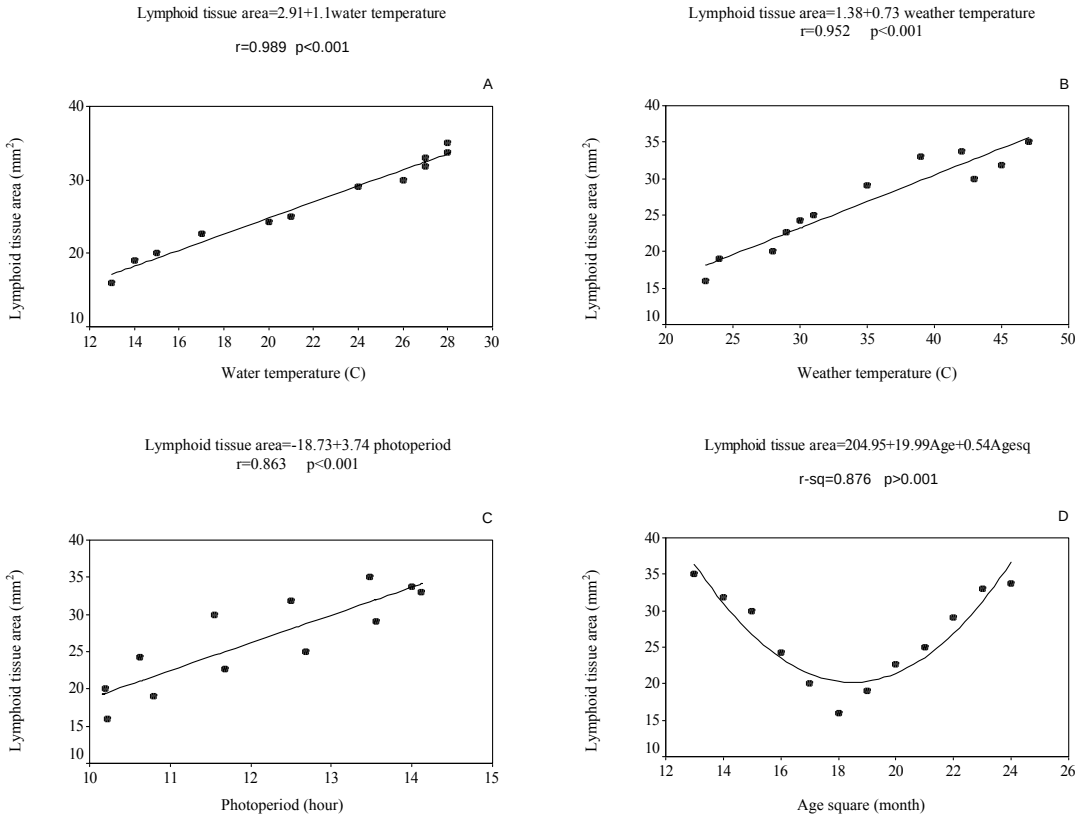


**Fig. 3: The regression analysis of lymphoid tissue area (distribution) vs water temperature (A) and weather temperature (B) photoperiod (C) and age square (D)**

**Table 3: The regression analysis of lymphoid tissue distribution and percentage of pale lymphocyte vs age, water temperature, weather temperature and photoperiod**

| Response variable | Independent variable | Coefficients | Std. error | P-value | Correlation |
|---|---|---|---|---|---|
| Lymphoid tissue distribution | Intercept | 19.622 | 27.842 | 0.507 | 0.995 |
| | Water temperature | 0.648 | 0.253 | 0.043 | |
| | Weather temperature | 0.123 | 0.192 | 0.547 | |
| | Photoperiod | 0.798 | 0.565 | 0.208 | |
| | Age | -2.300 | 2.590 | 0.409 | |
| | $Age^2$ | 6.093E-02 | 0.070 | 0.415 | |
| Percentage of pale lymphocyte | Intercept | 68.045 | 11.662 | 0.001 | 0.997 |
| | Water temperature | -0.592 | 0.106 | 0.001 | |
| | Weather temperature | -9.130E-02 | 0.080 | 0.300 | |
| | Photoperiod | -0.482 | 0.237 | 0.088 | |
| | Age | -3.867 | 1.085 | 0.012 | |
| | $Age^2$ | 0.111 | 0.029 | 0.009 | |

(24.7 ± 0.9020 $mm^2/cm^2$). Tamura and Honma (1974, 1975) reported a minimal development of the lymphoid tissue in two gobiid species in winter, for both of which the number of lymphocytes reached the maximum value in August and September. Presumably, this and other divergent results reflect species-specific differences (Zapata *et al*., 1992). On the other hand, it is assumed that fishes are always farmed at location with a water temperature range appropriate for the species under cultivation and any shift in temperature towards the upper or lower thermal limits for the species will result in destructive consequences on performance (Black and Pickering, 1998). According to our results, among the studied factors (i. e., water temperature, weather

**Fig. 4: Head kidney structure in spring season; pale lymphocyte (P); normal lymphocyte (Lc) and lymphoblast (Lb), (H&E, ×1320)**

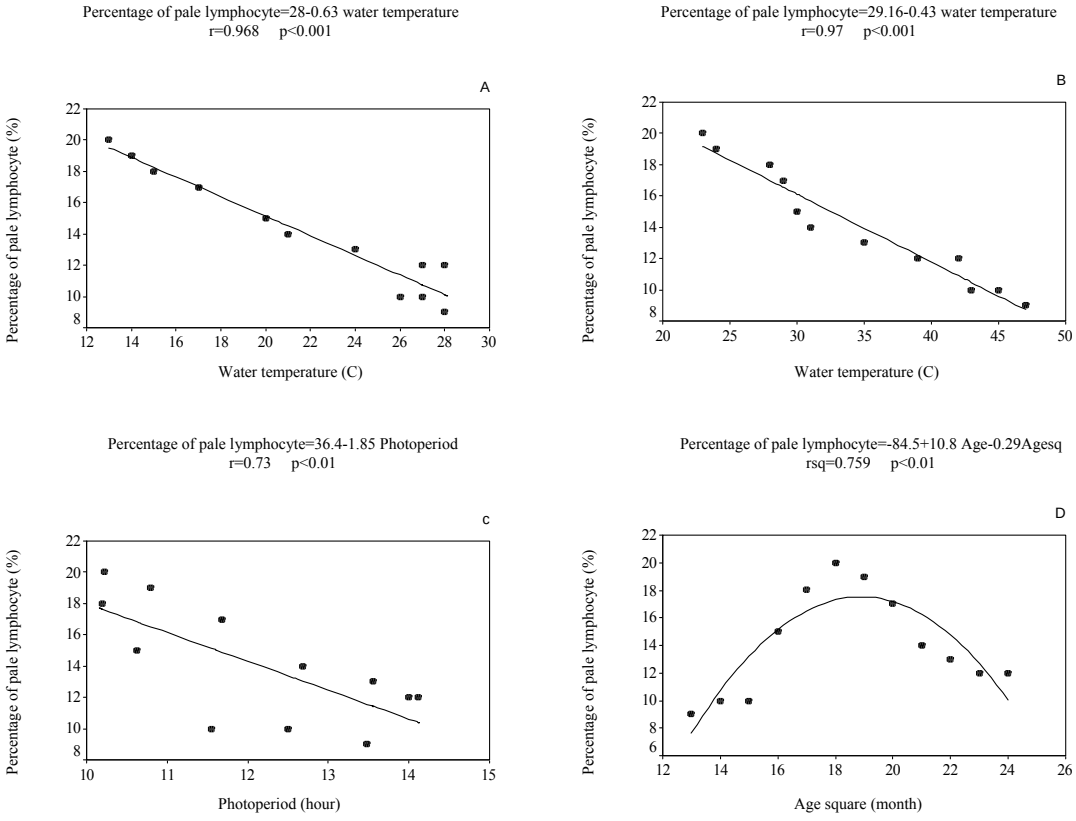


**Fig. 5: The regression analysis of pale lymphocyte percentage vs water temperature (A) and weather temperature (B) photoperiod (C) and age square (D)**

temperature, photoperiod and age square), water temperature had the most influential effect on the LTD. Considering the climatic conditions in Ahvaz city (Khuzestan province, Iran), where hot seasons extended from May to October, which including, spring and summer and even the first month of autumn, the presence of high values of LTD in spring and autumn like summer is not unexpected.

Results of this research revealed that the ADLP had its maximum value (18 ± 0.4106) in winter and its minimum value (10 ± 0.3599) in summer. Among the studied factors, water temperature and age square had the most influential effect on the ADLP. Alvarez *et al*., (1998) found that the PI in pronephros reached the highest values during winter and summer. Morphologically, pale lymphocytes seem to correspond to dead cells (Zapata and Cooper, 1990). It may be due to apoptosis, in which the cell shrinks and condenses, the cytoskeleton collapses, the nuclear envelope disassembles, and the nuclear DNA breaks up into fragments (Alberts *et al*., 1998). We observed that pale lymphocytes had electron-lucent cytoplasm, devoid of cell organelles and showed signs of degeneration and in some cases, necrotic nuclei, that corresponded with the findings of Alvarez *et al*., (1998) on wild brown trout.

It is concluded that there is significant seasonal variations in pale lymphocytes; an inverse correlation was found between normal and pale lymphocytes in the lymphoid organs of grass carp, indicating, in general, a high correlation between high number of pale lymphocytes and a poor development of the lymphoid tissue and also poor function of immune system.

Regarding to the causative agents involved in the seasonal changes affecting fish lymphoid tissues, environmental factors, mainly temperature (Wright and Cooper, 1981), photoperiod (Tamura *et al*., 1981) and endogenous neuroendocrine rhythms have been proposed (Zapata and Cooper, 1990; Zapata *et al*., 1992; Zapata, 1996). Most authors assume, however, an indirect influence of environmental factors on endogenous rhythms governing the seasonal changes of the immune system.

In fact, the changes observed in the lymphoid organs of grass carp correlate with the circannual variations in water temperature, weather temperature, photoperiod and age square. It is speculated that the causes of involution of the lymphoid organs on fish diseases are still unknown; however, it is clear that seasonal changes in the pronephros may unveil a transitional condition of immunosuppression. This condition could explain at least in part the seasonal incidence of some diseases in fish culture ponds.

## Acknowledgement

This study was supported by a research grant from Shahid Chamran University, Ahvaz, Iran. The authors are grateful to fishery organization of Khouzestan province.

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