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Original article

HAEMATOPOIETIC POTENTIAL OF TENCH (*TINCA TINCA*) PRONEPHROS IN RELATION TO AMBIENT TEMPERATURE AND RELATIVE CONDITION FACTOR

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Summary

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The aim of this study was to detect the correlation between the change in the ambient temperature and adaptation of the pronephros – the main haematopoietic organ in fish, which so far has not been subject of research. Compared with other teleosts and their number of blood cells in peripheral blood, tench (*Tinca tinca* L. 1758) showed the corresponding trend of changes in number of pronephros haematopoietic cells after exposure to increased water temperature. The number of all haematopoietic cell lines was changed during the conditions of thermal stress ($P<0.01$; $P<0.05$). The number of erythroblasts was significantly reduced due to shift of haemoglobin curve of dissociation to the right. Leukopoietic cells were increased significantly as a response of pronephros to stress and all endocrine mediators of the stress (cortisol, leukine, etc.). The number of prothrombocytes was slightly decreased due to mobilisation into peripheral blood as in the case of cell precursor of monocytes. Eosinophilic and basophilic granuloblasts were not found in tench pronephros. Correlation (R^2) between body mass and total length was 0.526. Sex-specific variation of haematopoietic cells number in the same group was not observed ($P>0.05$), but it was significant between males or females among different groups ($P<0.01$; $P<0.05$ respectively).

Key words: fish haematopoesis, pronephros, temperature adaptation, tench, touch slides

INTRODUCTION

Almost all environmental factors, beyond the optimum range can be stressful to fish. External factors include temperature, salinity, time of day, the wavelength of light, even the background colour of the aquarium. Indoor environmental factors include the nutritional state of the fish and the possible existence of the disease (Barton *et al.*, 1986). When fish are exposed to a stressor, the physiological response is initiated by identifying threats by the central nervous system. Sympathetic nerve fibres, which innervate the chromaffin cells, stimulate the release of catechola-

mines via cholinergic receptors (Reid *et al.*, 1996). Chromaffin tissue is located in the anterior region of the kidney in bony fish. Since catecholamines, especially adrenaline, are stored in chromaffin cells, their loosening rapidly increases their concentration in the blood (Mazaeud *et al.*, 1977).

Earlier studies have shown changes in the number of cells in peripheral circulation – the number of red blood cells in *Carssius gibelio* (Bloch, 1782) as well as the values of red cell parameters in *Channa punctatus* (Bloch & Schneider, 1802) was still growing (Ravichandra, 2012), as is the case with the number of leukocytes in the tench (Haskovic *et al.*, 2013). On the basis of histological studies of pronephros in Antarctic fish (Romano *et al.*, 2002), changes in the shape of erythrocytes and the number of granulocytes and lymphocytes have been determined. Such a phenomenon is considered to be an adaptation of the pronephros, as the main immune organ at lower temperatures. Sex-specific adaptation of fish pronephros in relation to thermal stress was not studied.

The length-weight relationship of cyprinids has been studied by many researchers (Sarkar *et al.*, 1999; Sunil, 2000). The exact relationship between length and weight differs among fish species according to their inherited body shape, and within a species according to the condition (robustness) of individual fish (Schneider *et al.*, 2000).

The aim of this study was to detect the correlation between the change in the ambient temperature, relative condition factor and haematopoietic potential of pronephros – the main haematopoietic site in tench.

MATERIALS AND METHODS

Site

The research area was Jablanicko reservoir on the Neretva river (Bosnia and Herzegovina). It spreads from the Konjic municipality to municipality Jablanica (43° 41′ N, 17° 51′ E, altitude 263 m asl) with an area of 13 km^2.

Sampling and experimental design

Fishnets (Attwod Fold-N-Stow) were used in the sampling. The sample consisted of 35 specimens – 21 female and 14 males, all specimens were weighed with WBW digital scale and length measured. Total length was measured from the tip of snout to tip of the longest ray of caudal fin (Jayaram, 1999). They were transported to the laboratory in containers supplied with constant aeration of the water. The adaptation of fish in the laboratory environment lasted 20 days (Laboratory of Physiology, Faculty of Science, Sarajevo, Bosnia and Herzegovina) and included daily monitoring of water exchange, oxygen concentration (Winkler method) and ammonia concentration (Nessler method). Aerators (CHAMPIONCX-0098) were used for water aeration. Fish were fed with Eco FeedEx C 48/10 (Eco Feed Ltd, Serbia). Experimental fish (19 individuals) were exposed to increase of water temperature or thermal stress, precisely to 28 ºC for 30 minutes. Sixteen fish were used as controls.

Kidney biopsy and haematological methods

Fish were euthanised with 0.2% tricaine and ice water on 4 to 5 min and then dissected, the digestive tract was removed and the kidney was carefully separated from the body wall. Approximately 0.5 cm^3 of tissue was taken from head kidney

or pronephros with biopsy tweezers. The sample was used for preparation of touch slides, created by a slight zig-zag rolling movements of tissue over the slide using a glass rod or pin. Smearing the sample over the slide by the method of imprint allowed for easy determination of tissue cellularity. After drying the tissue smears, staining was preformed according to Pappenheim (Penta Chemicals). Euthanasia and all procedures with animals were conducted in accordance to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Microscopic analysis

Haematopoietic cells counting was preformed using light microscope Olympus BX41 and Olympus DP12 camera. All photos were imported into Olympus DP Software for further observation and analysis. Morphological analysis, identification and counting included 300 cells of each lineage, exceptions were rare cells, where was taken average value for all found cells.

Statistical analysis

Statistical analysis were done by SPSS (Version 20.0, SPSS, Inc., Chicago, IL, USA), P-values lower than 0.05 ($P<0.05$) considered significant and those lower than 0.01 ($P<0.01$) – highly significant. The relative condition factor (Kn) was calculated from ratio of body mass (g) and total length (cm). All length and weight data were subjected to statistical analysis by fitting length-weight relationship as per Le Cren (1951). Length – weight relationship can be expressed as $W = a.L^b$, the logarithmic transformation of which gives the linear equation $LogW = a + b.logL$, where W = weight in grammes, L= length in mm, a = a constant being the initial growth index, and b = growth coefficient. The constant a represents the point at which the regression line intercepts the y-axis and b – the slope of the regression line. (Le Cren, 1951; Sarkar *et al.*; 1999; Sunil, 2000). The relative condition factor (Kn) as per Le Cren (1951) was expressed as Kn = W/^W where W = observed weight; ^W = calculated weight derived from length-weight relationship.

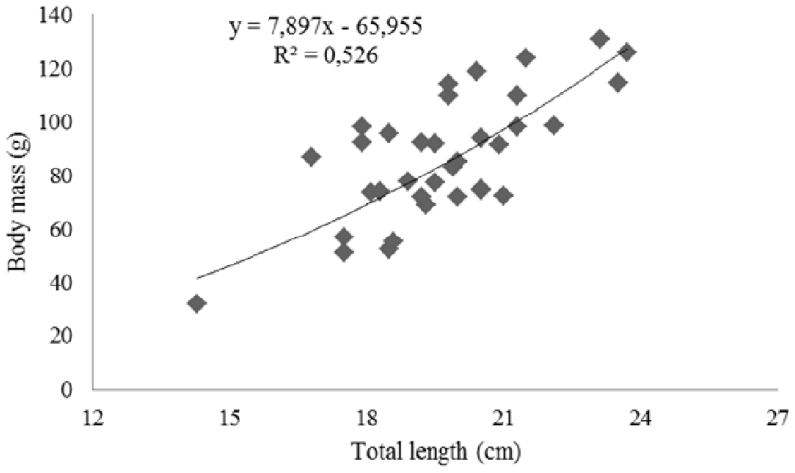

Fig. 1. Weight-length relationship in *Tinca tinca* (n=35) with the condition factor (Kn=1.139).

RESULTS

Body parameters and relative condition factor

Average weight of all males and females was 86.69 ± 23.26 g and average length was 19.76 ± 1.93 cm. Relative condition factor (Kn) was 1.139 ± 0.266 (mean ± standard deviation) and correlation (R^2) between body mass and total length was equal to 0.526. Fig. 1 shows the growth of *Tinca tinca* from the Jablanicko lake with equation $y = 7.807x - 65.955$, where y = body mass (g) and x = total length (cm).

Changes in cell number

The cells that were identified, based on morphological characteristics were classified into one of the following groups: erythroblasts, granuloblasts, lymphoblasts, neutrophilic granuloblasts, pseudoeosinophilic granuloblasts, prothrombocytes, and precursors of monocyte cells (Table 1). There were statistically significant changes in all types of haematopoietic cells. The temperature reduction led to increase in the number of red blood cells, and thus the number of erythroblasts, while causing a highly significant reduction in the number of erythroblasts in the experimental group of fish ($P<0.05$). Granuloblasts number under thermal stress conditions was significantly higher ($P<0.05$) in the front part of the experimental fish kidney. Number of lymphoblasts as the most common leukocyte type in *Tinca tinca* pronephros was significantly increased ($P<0.05$). The number of prothrombocytes

Table 1. Haematopoietic cell types (means±standard deviation, minimum-maximum range, coefficient of variation: CV) in control and experimental *Tinca tinca* from the Jablanicko reservoir

Haemopoietic cell types		Experimental group (n=19)	Control group (n=16)
Erythroblasts	mean± SD	79.85±4.29*	104.71±5.56
	min-max range	70–86	90–128
	CV (%)	5.34	11.46
Granuloblasts	mean± SD	26.14±6.04*	3.14±1.12
	min-max range	17–37	1–5
	CV (%)	25.17	33.54
Neutrophilic granuloblasts	mean± SD	54.42±4.17*	37.86±4.52
	min-max range	46–60	27–47
	CV (%)	7.83	19.22
Pseudoeosinophilic granuloblasts	mean± SD	27.85±5.09*	48.86±4.81
	min-max range	23–38	28–58
	CV (%)	16.82	18.28
Lymphoblasts	mean± SD	42.28±4.11*	31.43±3.31
	min-max range	36–51	22–39
	CV (%)	9.72	12.80
Prothrombocytes	mean± SD	64.00±4.59*	69.86±6.44
	min-max range	54–69	58–79
	CV (%)	7.13	8.53
Monocyte precursor cells	mean± SD	5.42±1.51*	4.23±1.09
	min-max range	3–8	3–6
	CV (%)	27.58	25.81

Note: *P<0.05 between groups.

Table 2. Sex-specific differences between hematopoietic cell counts (means±standard deviation) in control and experimental *Tinca tinca* from the Jablanicko reservoir related to pronephros adaptation to temperature change

Haemopoietic cell types	Experimental group (n=19)		Control group (n=16)	
	♀♀ (n=12)	♂♂ (n=7)	♀♀ (n=9)	♂♂ (n=7)
Erythroblasts	80.83±3.06*	79.85±5.33*	107±12.61	104.71±12.57
Granuloblasts	21.50±3.20*	26.14±7.20*	3.50±1.51	3.14±0.69
Lymphoblasts	51.83±4.02*	54.41±4.19*	36.66±7.58	37.81±7.37
Neutrophilic granuloblasts	33.00±4.00	27.85±4.91*	43.50±10.05	48.84±6.64
Pseudoeosinophilic granuloblasts	42.16±4.07*	42.28±3.90*	34.00±3.57	31.43±4.54
Prothrombocytes	65.00±4.00*	64.00±5.32*	70.00±5.79	69.87±6.56
Monocyte precursor cells	5.50±1.04	5.42±1.90	4.50±1.04	4.00±1.15

Note: *p<0.05 between the same gender of the two groups.

has significantly changed (P<0.05). Number of mononuclear phagocytes was increased (P<0.05).

The widest range of variation was demonstrated in the number of granuloblasts (control group 33.54% and experimental group 25.17%) and monocyte precursor cells (control group 25.81% and experimental group 27.58%) in both groups. Eosinophilic and basophilic granuloblasts were not found in the pronephros of *Tinca tinca*.

Sex-specific variations

Within-group sex-specific differences in abundance of haematopoietic cells were not expressed between males and females. However, sex-specific variations in haematopoietic cell counts were significant between males (P<0.01) and females (P<0.05) of the different groups (Table 2).

DISCUSSION

A small number of scientific papers are published on the topic of changes in the number of haematopoietic cells in haematopoietic centers of fish in response to any type of stress. Most of them are related to the number of cells in the peripheral circulation. Based on value of relative condition factor (Kn=1.139), we concluded that their weigth does not really reflect total body length. In particular, individuals were much more heavier than it was predicted by their total length (Schneider *et al.*, 2000).

A large number of erythroblasts suggests potential effects of different physical, chemical and biological agents on haematologic status. Changes were recorded in the number of red blood cells due to changes in environmental temperature. This phenomenon can be explained through the abundance of mature cells of the erythroid lineage. The reason for the reduced number of erythroblasts could be attributed to the fact that increased environmental temperature caused a reduction in the affinity of haemoglobin for oxygen. The haemoglobin dissociation curve shift to the right, can ultimately lead to the mobilisation of erythroblasts from the pronephros of tench, which enter the circulation in order to compensate for body's needs for oxygen or haemoglobin. The

temperature increase leads to an increase in haemoglobin concentration (Ravichandra, 2012), as well as in other red blood cell parameters (De Souza & Bonilla-Rodriguez, 2007; Dekic *et al.*, 2013). It's a dilemma that will surely remain unresolved until the embryonic marking (fluorescent or radioactive) of erythroid cells and exposure of fish to elevated temperatures in order to determine whether the higher temperature would require mobilisation of erythroblasts or diminishes their number.

Cells with high division and self-renewal potential are granuloblasts. In the control group of fish, their number was very small, but statistically significant difference in granuloblast counts is noticed in fish exposed to change of environmental temperature (Romano *et al.*, 2002). Similar finding was determined with respect to granulocytes in the peripheral circulation (Engelsma *et al.*, 2003). Increased number of granuloblasts resulted from catecholamines and cortisol effects, which stimulate maturation process. Under the influence of chromaffin, interrenal tissue hormones catecholamine and cortisol (Cortes *et al.*, 2013), start the secretion of numerous interleukins (IL3, IL3R, IL5) (Harris & Bird, 2000; Secombes *et al.*, 2001), colony stimulating factors (CSF), growth factors and finally, maturation of haematopoietic cells of white lineage (granulocyte-agranulocytes – CSF) leads to a significant increase in the number of granuloblasts.

Reticular-epithelial cells of stromal pronephros provide mechanical support to haematopoietic cells, but they also create a specific microenvironment and in particular, haematopoietic "niche" for lymphoblasts and pseudoeosinophilic granuloblasts because their numbers were growing under thermal stress. In addition, lymphocytes, as cells are involved in the humoral and cellular immune response, generally do not leave the front and middle parts of the kidney. For this reason, certainly the most common leukoblasts in the pronephros of tench were lymphoblast cells. Their number was significantly increased, while other leukocytes mainly speeded up the process of maturation and expelled all cell reserves into circulation, in order to get to the point of contact with the pathogen and to perform phagocytosis. In doing so, the most important are neutrophils. Basophilic and eosinophilic granuloblasts were absent in the pronephros but they were described in peripheral blood circulation of *Tinca tinca* as basophilic and eosinophilic granulocytes (Haskovic *et al.*, 2013). The number of prothrombocytes was reduced to a significant extent, probably because of the mobilisation of prothrombocytes, as part of the immune response.

Mononuclear phagocyte and cell precursors of tissue macrophages counts were increased, but they were generally kept in the pronephros, probably because thermal stress did not provoke secretion of very complex and heterogeneous fish proinflammatory cytokines (Harris & Bird, 2000; Secombes *et al.*, 2001), which would enhance their mobilisation. The very sensitive immune system of tench, when it comes to change of the ambient temperature, probably provides an insight into the harmful effects of changes in water temperature on the physiological state of tench. From the economic aspect, this can be a very helpful suggestion although tench is not an economically interesting species. Based on the data about the differential blood counts of tench (Haskovic *et al.*, 2013), it can be concluded that the hypothesis on the mobilisation of leukocytes (pseudoeosinophilic granuloblasts,

neutrophilic granuloblasts, and lymphoblasts) is actually true.

Thermal stress leads to significant changes in the number of cells in all haematopoietic cell lines. Based on sex-specific analysis of the haematopoietic cells number, we concluded that there was no sex-related adaptive reaction of the pronephros as main haematopoietic organ, on higher values of water temperature or short period of thermal stress. Our study provides new scientific view about the adaptation answer of main haematopoietic and immunological organs in almost all teleosts. Freshwater fish are more resistant to temperature changes than we previously thought, thus, this study also brings new and applicable knowledge about long-term consequences of climate changes on freshwater fish population.

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