Volume 60(2):113-118, 2016
Acta Biologica Szegediensis
http://www2.sci.u-szeged.hu/ABS

ARTICLE

Morphological identification of haematopoietic cells in pronephros of common carp (*Cyprinus carpio* Linnaeus, 1758)

Damir Suljević, Erna Islamagić, Andi Alijagić*, Muhamed Fočak, Maja Mitrašinović-Brulić

Department of Biology (Biochemistry and Physiology), Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina

ABSTRACT Haemopoietic tissue of the common carp pronephros (*Cyprinus carpio* Linnaeus, 1758) was studied in regard of morphometric analysis of the erythropoietic, leukopoietic and thrombopoietic cell populations. The aim of this study was to perform evaluation of immature precursor cells in head kidney of common carp (sampled from Bardaca lake) because there is no well known data regarding this issue. Microscopic identification of haematopoietic cells included measurement of cell and nuclear size, nuclear-cytoplasmic ratio, determination of the cell and nuclear shape, cytosol coloration and presence of specific granules in the cytosol. The frequency of immature cells and their cell area was also analyzed. Erythroblasts were the most abundant among all observed haemopoietic cell lineages and were the most variable in the size. The largest area was characteristic of monocyte precursors and no significant differences were observed regarding the cell area between prothrombocytes and lymphoblasts, which makes difficult in cell characterization. High number of emerged cells in short time also makes difficult to identify particular stages of maturation in some bloodlines. Rapid maturation of granuloid cells observed within the haemopoietic tissue indicates their functional significance in adaptation to the changeable microenvironment. **Acta Biol Szeged 60(2):113-118 (2016)**

KEY WORDS

common carp
haematopoiesis
pronephros
cell-lineage
cell area

Introduction

The common crap, *Cyprinus carpio* Linnaeus, 1758 is a freshwater cyprinid fish of eutrophic waters in Europe. Although tolerant to most environmental changes, especially low oxigen levels, common carp prefers slow waters and vegetative sediments as their habitat (Rey et al. 2016). In such conditions they are exposed to attacks of pathogens such as bacteria and parasites, they ought to have well-developed defence mechanisms. In many fish species blood cells play a major role in providing immunity and therefore the process of haematopoiesis is very crucial (El-Saydeh et al. 2010; Prajeena et al. 2014). Similarly to most teleost fish (Homechaudhuri and Jah 2001; Huang and Zon 2008; Chen and Zon 2009; Paik and Zon 2015; Alijagic and Suljevic 2016b), pronephros has major role in carp haematopoiesis, in spite of other organs which may also show hematopoietic activity such as mesonephros (Stosik and Deptula 1993), thymus, spleen (Patel et al. 2002) and serosa of the middle part of the intestine (Stosik and Deptula 1993). However, pronephros did not show only hematopoietic activity, it is also the reservoir of cells, a lymphoid and endocrine organ (Wendelaar Bonga 1997; Weyts et al. 1999).

Haemopoietic stem cells are placed along the urinary tubules in kidney marrow. Directed stem cells derive from hemogenic endothelium and differentiate in several haemopoietic lineages (Willett et al. 1999). The pronephric kidney functions as main erythropoietic, lymphomyeloid and thrombopoietic organ in common carp (Kobayashi et al. 2007; Kondera 2011). In juvenile common carp pronephros the blood cell production is consisted of the following cell lines: unidentified blast cells, erythroid, granuloid, lymphoid, monocytoid and thrombocytoid cells (Kondera 2011). Haematopoiesis in common carp consists of 22 blood cell types, which can be easily identified and counted. They can be observed in different stages of maturation and the differences between them are based on various nuclei size, ratio between nucleus and cytoplasm or chromatine condensation (Alijagic and Suljevic 2016b). A combination of quantitative and morphological methods is needed thus it can be done by flow cytometry and microscopically. The combined use of flow-cytometry and electron microscopy makes it possible

Submitted July 23, 2016; Accepted September 24, 2016
*Corresponding author. E-mail: andialijagic@gmail.com

to characterize different cell types and to monitor changes in blood cell populations (El-Saydah et al. 2010).

All haematopoietic cell types in fish are similar to those in mammals, even though mammalian myeloid cell-line proliferation and differentiation predominantly occurs in bone marrow (Kobayashi et al. 2007; Chen and Zon 2009; Ivanovski et al. 2009) unlike haematopoiesis in fish that occurs in several different organs. Basic mechanisms found in vertebrates are also active and functional in channel catfish (Fijan 2002a; Fijan 2002b).

The analogous expression of different markers in zebra fish and other vertebrate models suggests that the molecular mechanisms that regulates haematopoiesis are highly conserved. Consequently maturation of hemangioblast occurs accompanied with the interaction of factors like Scl and Lmo2 (Patterson et al. 2007). Definitive haematopoiesis include Fli1-A, Hhex and Tbx16 transcription factors. Transcription factor with ETS-domain (Fil-A) is implicated in proliferation or differentiation of haematopoietic precursors (Brown et al. 2000). Once the haematopoietic precursors have been specified, additional haematopoietic transcription factors such as Gata1, Pu.1, and Ikaros direct the lineage-specific differentiation of these progenitors into erythroid, myeloid, and lymphoid cell types, respectively (Huang and Zon 2008).

Several researches showed quantitative data on the proportions between blood cell lineages in haematopoietic organs of teleosts (Peters and Schwarzer 1985; Wlasow and Dabrowska 1989; Fijan 2002a; Fijan 2002b; Kondera 2011; Alijagic and Suljevic 2016a; Alijagic and Suljevic 2016b). The aim of the presented study was to perform quantitative and qualitative evaluations of developing blood cells in the pronephros of common carp, *Cyprinus carpio* Linnaeus, 1758.

Materials and Methods

Site

Fish sampling was done on Bardaca lake, which is located in nothern part of Bosnia and Herzegovina. Hydrological status of lake mainly depends upon three rivers: Matura, Stublaja and Brzaja. Lake is also part of Barda a reservoir (45°06 33 N 17°26 07 E / 45.10917°N 17.43528°E), which consists of eleven lakes and it is used as a fishpond.

Sampling and experimental design

The sample consisted of 20 specimens (10 females and 10 males) with an average weight of 18.05 ± 4.105 g (WBW digital scale) and lenght of 12.22 ± 1.299 cm. Fishnets (Attwod Fold-N-Stow) were used in sampling. After sampling, fish were transported from Bardaca lake to the Laboratory of Physiology (Faculty of Science, Sarajevo, Bosnia and Herzegovina) in containers supplied with pure oxigen (water aerators, CHAMPIONCX- 0098) and allowed to acclimate to the laboratory tank for 15 days. Water temperature was 20° C, pH 7.1, oxygen saturation level was 80%. Water monitoring included daily water changes, measurement of oxygen (Winkler method) and ammonia concentration (Nessler method). The fish were fed twice a day formulated Eco FeedEx C 48/10 (Eco Feed Ltd, Serbia).

Kidney biopsy and haematological methods

Fish were euthanized with 0.2% tricaine (Penta Chemicals) prior kidney biopsy and placed in ice-cold water for 4 to 5 minutes. After euthanisation they were dissected using sharp scissors, internal organs were removed from abdominal cavity and the head kidney was collected (approximately 0.5 cm^3) for the preparation, using biopsy tweezers. The surface of isolated fresh organs was smeared gently (slight zig-zag movements with pin) on fatfree slides. After being dried for 24 h, tissue smears were treated by the Leders method (Penta Chemicals) to prove the presence of peroxidase activity in granuloblasts. Smears were stained using May-Grünwald and Giemsa solutions (Pappenhein staining method).

Microscopic analysis

Identification were performed using a light microscope Olympus BX41 and all measurments (number and area) of haemopoietic cells were performed using the Olympus DP12 camera, all photos were imported into Olympus DP Software. The results were presented as percentages of the total number of blood cells. Area of cells occupied by the nucleus was estimated visually. Morphological characterization of haematopoietic cell-lines included determination of cell and nuclear shape, measurement of cell and nuclear size, level of chromatine condensation, nuclear-cytoplasmic ratio, cytosol coloration and presence of specific granules in cytosol. Measurements for all individuals were determined as the mean value of each cell type in a sample of 300 cells.

Statistical analysis

Analysis were performed using SPSS (Version 20.0, SPSS, Inc., Chicago, IL, USA), and data are presented as means ± 1 SD accompanied by range and coefficient of variation (%).

Results

In this research 20 juvenile healthy specimens of common carp *Cyprinus carpio* (10 males and 10 females) were exam-

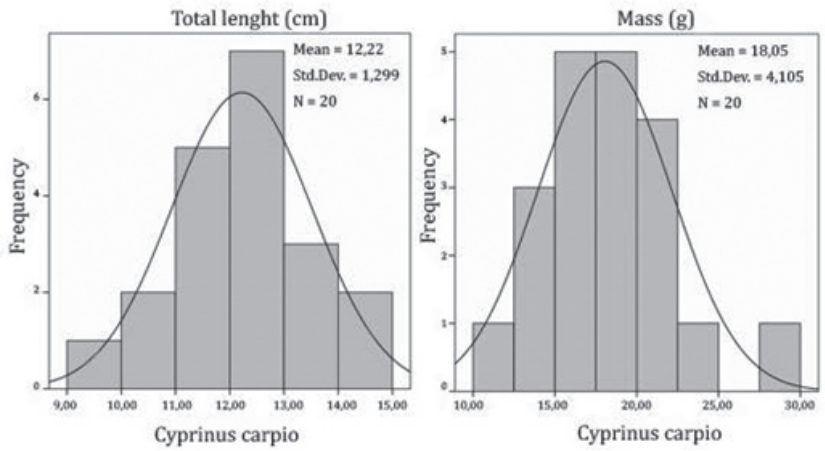

Figure 1. Total lenght and body mass of common carp (*Cyprinus carpio* Linnaeus, 1758).

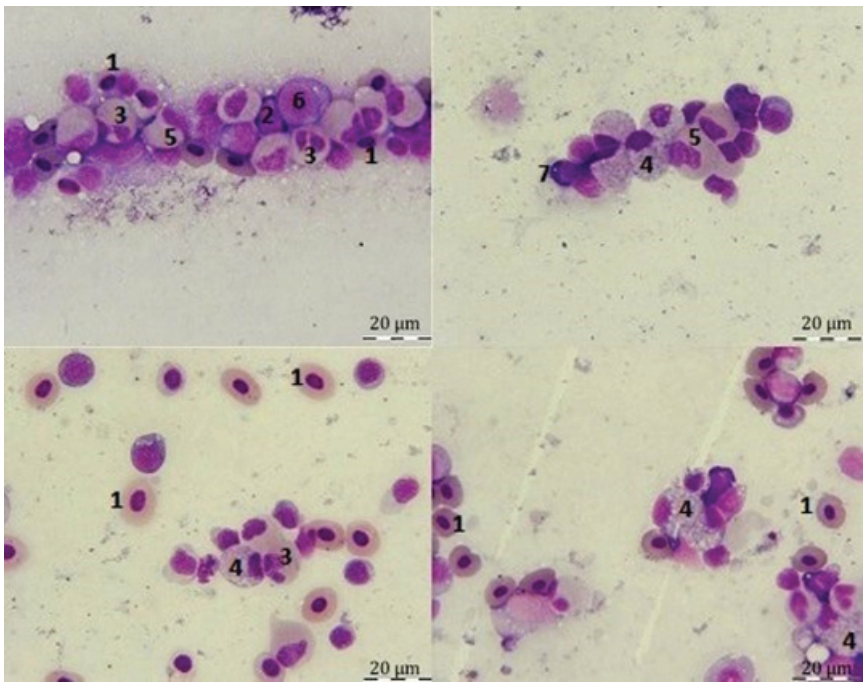

Figure 2. Immature cells in pronephros of common carp: erythroblast (1), lymphoblast (2), neutrophilic metagranulocyte (3), heterophilic metagranulocyte (4), granuloblast (5),monocyte precursore (6), prothrombocyte (7).

ined. The average total length of the specimens was 12.22 ± 1.29 cm and in most of them (seven) amounted value was 12 cm, while the average body mass was 18.05 ± 4.10 g, in which ten specimens had weight in range of 15 to 20 g (Fig. 1).

We characterized the immature elements of the haematopoietic hierarchy formed by haematopoietic stem cells: erythroblast, lymphoblast, neutrophilic metagranulocyte, heterophilic metagranulocyte, granuloblast, monocyte precursore and prothrombocyte.

Hematopoietic cells and their characteristics identified by using light microscopy and cytochemical POX method are presented in Figure 2. and Table 1.

Measures of central tendency, range and coefficient of variation (CV) for all analyzed immature cells were presented in Table 2. The most prevalent were erythroblasts with highest SD. The following were heterophilic metagranulocytes, lym-

Table 1. Appearance (shape and staining property) of the haematopoietic cells in the pronephros of common carp (Pappenhein staining method).

Cell type	Shape		Staining property	
	Cell	Nucleus	Cytoplasm	Nucleus
Erythroblasts	oval	oval	red	deep blue
Lymphoblasts	round	round	blue	deep blue
Neutrophilic metagranulocytes	irregular	irregular or band	light red	violet
Heterophilic metagranulocytes	spherical	eccentric; round, elongated, irregular	light violet with granules	violet
Granuloblasts	irregular	irregular or band	light violet	violet
Monocyte precursores	spherical	spherical or elongated	light blue	violet
Prothrombocytes	round	round	blue	deep blue

Table 2. Frequency (%) and values (number and area) of determined haematopoietic cells in pronephros of common carp.

Cell type	Number				Area (μm²)		
	%	Mean±SD	Range	CV	Mean±SD	Range	CV
Erythroblasts	35.26	105.80±19.08	72-150	18.03	82.32±18.22	57.33-125-58	23.14
Lymphoblasts	11.40	34.20±7.54	21-51	22.04	77.11±11.94	56.57-96.96	15.48
Neutrophilic metagranulocytes	9.91	29.75±7.90	12-42	26.55	116.10±15.60	78.68-134.35	13.44
Heterophilic metagranulocytes	22.28	66.85±9.79	54-99	14.64	120.29±11.44	109.12-154.8	9.51
Granuloblasts	7.21	21.65±6.23	12-33	28.80	110.40±8.08	90.97-123.25	7.23
Monocyte precursores	6.00	18.00±4.57	9-24	25.63	155.10±8.57	143.30-177.64	5.52
Prothrombocytes	7.91	23.75±6.05	15-36	25.48	44.63±9.13	24.73-55.06	20.46

phoblasts, neutrophilic metagranulocytes and granuloblasts, respectively. Prothrombocytes and monocyte precursors were less presented. Table 2. presents the area (μm²) of various hematopoietic cells including the mean, range and CV.

Granuloblast characterized the smallest, whereas erythroblasts had the highest SD, which indicates that the erythroblasts were the most variable in their size. According to the range and CV, the largest variations in size were obtained in erythroblasts and prothrombocytes. Otherwise, the smallest variation in area of hematopoietic precursors were characteristic of monocyte precursors and granuloblasts. Therefore identification of immature cells was based on differences in morphological characteristics (Fig. 1).

Discussion

Maturation of haematopoietic cells in fish differs compared to haematopoiesis in other organisms. Head kidney is a main organ forming the blood elements in fish and its role in the blood elements formation differs among teleost fish (Willett et al. 1999; Rombout et al. 2005). It can be organ-forming erythroid lineages only, or it produces all types of blood cells (Meseguer et al. 1990; Willett et al. 1999; Esteban et al. 2000; Stephens et al. 2004; Kondera 2011). Large cell production in short time make difficult to identify immature cells, especially the number of particular stages of maturation in some lineages (Kondera 2011). In the present study, developed stages were not analyzed but only immature precursors.

Erythroblasts were the most abundant among all observed haemopoietic cell lineages in this study (13.14 ± 1.00%). The percentage of erythroid lineage cells was similar to the results obtained by (Wlasow and Dabrowska 1989) for common carp and (Peters and Schwarzer 1985) for rainbow trout (near 37% and near 45%, respectively). Results were considerably lower in relation to the results obtained by Kondera (2011) for common carp and Fijan (2002a) for channel catfish (13.14 ± 1.00% and 13.0 ± 5.1%, respectively). Lymphoblasts were highly present among all observed haemopoietic cell lineages, after heterophilic metagranulocytes. The percentage of lymphoid cells in the head kidney of the same fish species often differs. In the present study, the frequency of lymphoblasts (11.4%) in common carp was similar to the percentage of lymphoid cells (8.5%) in research observed by Wlasow & Dabrowska (1989), but a smaller amount of lymphoblasts was reported in pronephros for channel catfish and common carp (Fijan 1961; Fijan 2002b; Kondera 2011).

Cells with high selfrenewal potential and high division rate are granuloblasts. The heterophilic metagranulocytes were the most frequent granuloid cell (22.28%). In tench pronephros, pseudoeosinophilic granuloblasts are the most

numerous leukocytes (Alijagic and Suljevic 2016b). Neutrophilic metagranulocytes (9.91%) were more abundant than shown by Fijan (2002b) in channel catfish (4.53 ± 1.53%, neutrophilic progranulocyte and neutrophilic metagranulocyte were counted together) by Wlasow and Dabrowska (1989) and Kondera (2011) in common carp (only neutrophilic progranulocytes were counted 3.40 ± 1.62% and 8.40 ± 0.97%, respectively). The small number of granuloblasts (7.21%) probably was a result of their rapid maturation that have impact on their characterization.

The frequency of monocyte precursors (6%) differs compared to other reports (Fijan 2002a; Kondera 2011). In the head kidney of carp 0.98 ± 0.45% of monocytoid cells were observed and it similarly for channel catfish 0.91 ± 0.82%.

The prothrombocytes were round, with round nucleus which occupies the entire cell, and size of average lymphoblasts. In the present study, number od prothrombocytes found in the head kidney (7.91%) was similar to number of trombocytes in head kidney of channel catfish (Fijan 2002a), but higher compared to results observed in common carp (Kondera 2011).

A small number of scientific papers are published on the topic of size of haematopoietic precursors. Based on the results it can be concluded that the area and thus the size of haematopoietic cells varies a lot. The variability in erythroblasts area was probably because of presence of the different maturation stages, in which ratio between longer and shorter axis of the cell is changeable, as well as nuclear-cytoplasmic ratio. Based on the color of the cytoplasm it is difficult to observe each stage of erythroblast maturation. Cell-nuclear ratio, nuclear shape and size, as the key morphological changes during the maturation of erythroblasts should be included in cell characterisation, both for erythroide and other haematopoietic cell lineages.

No significant differences were observed regarding the cell area between prothrombocytes and lymphoblasts (they also were the smallest identified cells) which make difficult in distinguishing these cells (Kondera 2011). Therefore, prothrombocytes could be classified as leukocyte precursors.

The largest area was characteristic of monocyte precursors (155.10 ± 8.57 μm^2). The myelocyte and metamyelocyte as observed by Diago et al. (1998), and the promyelocyte and myelocyte reported by Wlasow and Dabrowska (1989) are similar to heterophilic metagranulocytes and neutrophilic metagranulocytes as the largest cells in myeloid cell lineage (120.29 ± 11.44 μm^2 and 116.10 ± 15.60 μm^2, respectively). Granuloblasts as myeloid precursors had smaller area; also CV showed no significant individual variations.

In fish, analysis of cell associations in the haemopoietic tissues is of great importance in the study of haemopoiesis and the formation of haemopoietic microenvironments (Gangopadhyay and Homechaudhuri 2011). Romano et al. (2002) studied the histology of the head kidney in two Antarctic fish species and observed a difference in the shape of erythrocytes, increased number of granular and lymphatic cells, and this is considered an adaptation to the function of the pronephros at low temperatures.

The complete blood count is the good indicator of environmental or stress impacts in common carp (Kondera and Witeska 2013). The presence of mature cells in pronephros occurs due to the lack of oxygen in water and it is related with good adaptive mechanisms. This phenomenon would occur much slower if the morphological differentiation followed separate stages of erythropoiesis (Alijagic and Suljevic 2016b). However, little data are available on the effects of environmental factors on haematopoiesis in common carp, which is sensitive to various impacts due to its high production rate of blood cells.

In conclusion, the cellular composition of hematopoietic tissue in same and evolutionary close fish species is similar, but there are quantitative differences. Morphological parameters like cell and nuclear size, nuclear-cytoplasmic ratio and shape, cytosol coloration and presence of specific granules in cytosol are good indicators for microscopic identification of immature cells. However, unification of nomenclature and the cell differentiation criteria is essential in the study of fish haematopoiesis.

The presence of various immature cells in carp is a very unstable parameter that depends on many factors, including environmental factors like physiscal and chemical water parameters, water contamination, parasites as biological stressors and diseases. Frequency of various haematopoietic cells that vary even in the same fish species and rapid cell production in specific environmental conditions reflects the adaptation of organisms to variable environmental conditions. Surely, further researches are needed as an additional confirmation of these assumptions.

References

Alijagic A, Suljevic D (2016a) Haematopoietic potential of tench (*Tinca tinca*) pronephros in relation to ambient temperature and relative condition factor. Bulg J Vet Med DOI: 10.15547/bjvm.985.

Alijagic A, Suljevic D (2016b) Haemopoiesis in the pronephros of tench, *Tinca tinca*, Linnaeus 1758 (Teleostei, Cyprinidae): cytochemical identification and cell morphology. Turk J Vet Anim Sci DOI: 10.3906vet-1512-1534.

Brown LA, Rodaway AR, Schilling TF, Jowett T, Ingham PW, Patient RK, Sharrocks AD (2000) Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. Mech Dev 90:237-252.

Chen AT, Zon LI (2009) Zebrafish blood stem cells. J Cell

Biochem 8:35-42.
Diago ML, Lopez-Fierro P, Razquin B, Villena A (1998) In vitro haemopoiesis induced in rainbow trout pronephric stromal cell line TPS. Fish Shellfish Immunol 8:101-119.
El-Saydah HAA, Abdu SBS, Tamer ESA, Huda FF (2010) Haemopoiesis in the head kidney of tilapia, *Oreochromis niloticus* (Teleostei: Cichlidae): a morphological (optical and ultrastructural) study. Fish Physiol Biochem 36:323-336.
Esteban MÁ, Muñoz J, Meseguer J (2000) Blood cells of sea bass *Dicentrarchus labrax* L. flow cytometric and microscopic studies. Anat Rec 258:80-89.
Fijan N (1961) Haemopoietic function of kidneys in some species of freshwater fish. Biol Glas 14:167-208.
Fijan N (2002a) Morphogenesis of blood cell lineages in channel catfish. J Fish Biol 999-1014.
Fijan N (2002b) Composition of main haematopoietic compartments in normal and bled channel catfish. J Fish Biol 60:1142-1154.
Gangopadhyay K, Homechaudhuri S (2011) Descriptive characteristics of haemopoietic cell lineages in a facultative air breathing fish *Clarias batrachus* (L.). Turk J Zool 35:737-746.
Homechaudhuri S, Jah A (2001) A technique to evaluate the erythropoietic efficiency in fish. Asian Fish Sci 14:453-455.
Huang HT, Zon LI (2008) Regulation of stem cells in the zebra fish hematopoietic system. Cold Spring Harb. Symp Quant Biol 2008 73:111-118.
Ivanovski O, Kulkeaw K, Nakagawa M, Sasaki T, Mizuochi Ch, Horio Y, Ishitani T, Sugiyama D (2009) Characterization of kidney marrow in zebrafish (*Danio rerio*) by using a new surgical technique. Contributions. Sec Biol Med Sci 2:71-80.
Kobayashi I, Moritomo T, Ototake M, Nakanishi T (2007) Isolation of side population cells from ginbuna carp (*Carassius auratus langsdorfii*) kidney hematopoietic tissues. Dev Comp Immunol 31:696-707.
Kondera E (2011) Haematopoiesis in the head kidney of common carp (*Cyprinus carpio* L.): a morphological study. Fish Physiol Biochem 37:355-362.
Kondera E, Witeska M (2013) Cadmium and copper reduce hematopoietic potential in common carp (*Cyprinus carpio* L.) head kidney. Fish Physiol Biochem 39:755-764.
Meseguer J, Esteban MA, Garcia Ayala A, Lopez Ruiz A, Agulleiro B (1990) Granulopoiesis in the head kidney of the sea bass *Dicentrarchus labrax* L. An ultrastructural study. Arch Histol Cytol 53:287-296.
Paik EJ, Zon LI (2010) Hematopoietic development in the zebrafish. Int J Dev Biol 54:1127-1137.
Patel S, Sørhus E, Uglenes FI, Gunna, EP, Bergh Ø, Magne RO, Morton HC, Helge N A (2002) Ontogeny of lymphoid organs and development of IgM-bearing cells in Atlantic halibut (*Hippoglossus hippoglossus* L.). Fish Shellfish Immunol 26:385-395.
Patterson LJ, Gering M, Eckfeldt CE, Green AR, Verfaillie CM, Ekker SC, Patient R (2007) The transcription factors Scl and Lmo2 act together during development of the hemangioblast in zebrafish. Blood 109:2389-2398.
Peters G, Schwarzer R (1985) Changes in hemopoietic tissue of rainbow trout under influence of stress. Dis Aquat Org 1:1-10.
Prajeena KP, Prasad G, Sindhu MI, Deivasigamani B (2014) Haematopoiesis in the head kidney of freshwater eel *Anguilla bicolor bicolor* (McClelland, 1844). J Aquat Biol & Fish 2:187-193.
Rey S, Ribas L, Morera CD, Callol A, Huntingford FA, Pilarczyk M, Kadri S, MacKenzie S (2016) Differential responses to environmental challenge by common carp *Cyprinus carpio* highlight the importance of coping style in integrative physiology. J Fish Biol 88:1056-1069.
Romano N, Ceccariglia S, Mastrolia L, Mazzini M (2002) Cytology of lympho-myeloid head kidney of Antarctic fishes *Trematomus bernacchii* (Nototheniidae) and *Chionodraco hamatus* (Channicthyidae). Cell Tissue 34:63-72.
Rombout JHWM, Huttenhuis HBT, Picchietti S, Scapigliati G (2005) Phylogeny and ontogeny of fish leucocytes. Fish Shellfish Immunol 19:441-455.
Stephens FJ, Raidal SR, Jones B (2004) Haematopoietic necrosis in a goldfish *Carassius auratus* associated with an agent morphologically similar to herpesvirus. J Aust Vet 82:167-169.
Stosik M, Deptuła W (1993) Narza˛dy limfoidalne ryb. Med Weter 49:22-23.
Wendelaar Bonga SE (1997) The stress response in fish. Physiol Rev 77:591-625.
Weyts FAA, Cohen N, Flik GB, Verburg-Van Kemenade ML (1999) Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. Fish Shellfish Immunol 9:1-20.
Willett C, Cortes A, Zuasti A, Zapata A (1999) Early hematopoiesis and developing lymphoid organs in zebrafish. Dev Dynam 214:323-336.
Wlasow T, Dabrowska H (1989) Cellular changes in the blood and haemopoietic tissues of common carp exposed to sublethal concentration of ammonia. Aquat Living Resour 2:169-174.