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Annual changes in hematological parameters of common carp juveniles under laboratory conditions

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Abstract: *Annual changes in hematological parameters of common carp juveniles under laboratory conditions.* The aim of the study was to evaluate the changes of the values of hematological parameters of carp juveniles in annual cycle under stable laboratory conditions. Some parameters showed distinct rhythms of changes, e.g. 2 peaks of hematocrit occurred in II and VIII, of hemoglobin concentration in I and VI, while erythrocyte count showed maximum in II. The largest erythrocytes were observed in VIII, and the smallest in XII. Leukocyte count showed two peaks in XII and III. Maximum lymphocyte frequency occurred in III and minimum in XI, while percentage of neutrophils showed the reverse pattern. Oxidative metabolic activity of phagocytes peaked in III, while minimum occurred in XI–XII and VI–VII. Thrombocyte count was highest in XII, and lowest in VII. The obtained results revealed that the values of hematological parameters in carp considerably changed during the year despite little alterations in environmental factors. Some of these changes, e.g. increase in oxidative activity of phagocytes in spring and increase in hemoglobin level in summer were similar to those that occur in fish under natural conditions. Another changes, such as increase in erythrocyte size or decrease in leukocyte count suggest long-term adjustment to the laboratory environment.

Key words: blood, erythrocytes, fish, leukocytes, season, thrombocytes

INTRODUCTION

Hematological parameters are sensitive indicators of fish health and physiological status. However, their values may be affected by various environmental and intrinsic factors, e.g. temperature, photoperiod, water quality, fish age and phase of reproductive cycle. Therefore, determination of the reference ranges of fish blood parameters is more difficult than in homoiotherm animals (Luskova 1997). According to Leard et al. (1998), for parameters that do not considerably vary a single baseline range is appropriate, while for parameters that show distinct seasonal fluctuations it is necessary to develop seasonal or monthly reference ranges.

Most studies concerning seasonal changes in values of fish hematological parameters have been conducted under natural conditions. Their results usually show distinct patterns in seasonal changes of most indices. Hematocrit, hemoglobin concentration and erythrocyte count tend to increase in summer and to decrease in winter, while mean corpuscular volume usually shows a reverse pattern (e.g.

Morgan et al. 2008, Jeronimo et al. 2011, Golemi et al. 2013, Langer et al. 2013, Pradhan et al. 2014). However, some authors reported no significant season-related alterations in the values of red blood parameters (Abdel-Hameid 2011, Fallah et al. 2014).

Also the values of white blood indices usually show seasonal patterns. According to Bowden et al. (2007), Morgan et al. (2008), Santos et al. (2009) and Das et al. (2012), in winter immune functions decrease, while at higher temperatures immune response is enhanced. Leukocyte count is usually higher in summer compared to winter (e.g. Orun et al. 2003, Gupta et al. 2013, Kohanestani et al. 2013, Seriani et al. 2013). On the other hand Buchtikova et al. (2011) observed the lowest phagocyte respiratory burst activity in summer and the highest in winter, accompanied by the highest percentage of phagocytes in blood.

There is little information about seasonal variation in thrombocyte count in fish, and the data obtained by various authors are contradictory: increase in summer was reported by Orun et al. (2003) and Vigliano et al. (2014), Jeronimo et al. (2011), Gupta et al. (2013), while Langer et al. (2013) observed maximum thrombocyte abundance in winter. Santos et al. (2009) and Seriani et al. (2013) found no significant season-related alterations.

However, many research projects on fish are held in laboratory but information about the changes in values of hematological parameters in annual cycle under constant conditions is lacking and one could expect that in little variable environment hematological values (at least in juvenile fish that do not undergo physiological changes related to spawning) are stable. The aim of the present study was to evaluate annual changes in hematological parameters of common carp juveniles kept under controlled laboratory conditions.

MATERIALS AND METHODS

Six months old common carp *Cyprinus carpio* L. juveniles (36.7 ±8.0 g) harvested from the extensive rearing pond of the Inland Fisheries Institute in Żabieniec at the beginning of October were transported to the laboratory of the Department of Animal Physiology, Siedlce University of Natural Sciences and Humanities in plastic bags with pond water filled with pure oxygen. The fish were placed in a 300 L flow-through aerated tank and acclimated to the laboratory conditions for 3 weeks prior to the beginning of the experiment. During this time water temperature was 17–18°C, pH 6.5, O_2 concentration 6.1–8.0 mg/l, NO_2^- – 0.00–0.06 mg/l, NH_4^+ – 0.0 mg/l. During acclimation period the fish were fed commercial carp feed Aller Aqua Classic 4.5 mm at the rate of 1% of body mass. Water quality parameters were monitored once a week using pH meter (PRL TN 5123, Elwro, Poland), DO meter HI 9143 (Hanna Instruments, USA), and colorimetric kits Visocolor nitrite and Visocolor ammonia (Macherey-Nagel, Germany).

After acclimation period, 30 fish were transferred to another 300 l aerated flow-through tank where they were kept for 12 months at natural photoperiod. Water quality parameters were measured once a week. Water temperature was 18 ±2°C, pH 7.2–7.6, dissolved oxygen concentra-

tion 8.5 ±0.3 mg/l, NO_2^- < 0.1 mg/l, NH_4^+ < 1 mg/l. The fish were fed the same feed as during acclimation period every morning to satiation (only on blood sampling days fish were fed after blood collection). Every month (between the 19 and 29 day) blood was collected from 10 randomly harvested fish, always between 9 and 10 a.m. About 150 µl of blood (4 drops) were sampled by heart puncture from each fish using heparinized chilled needles and heparinized chilled plastic Eppendorf tubes. No anesthesia was applied, the procedure lasted no longer than 30 s and the fish were returned into the tank. Hematological procedures were performed according to Svobodova et al. (1991). Hematocrit (Ht) values were measured using microhematocrit method. Erythrocyte (RBC) and leukocyte (WBC) counts were evaluated in blood diluted 100× with Hayem solution, using microscope Nikon Eclipse E600 (under magnification 400×). Hemoglobin concentration (Hb) was measured using cyanmethemoglobin method (10 µl of blood was mixed with 1 ml of Drabkin solution) with UV-Vis spectrophotometer Helios Gamma (Thermo Electron Corporation, USA) at 540 nm wavelength. Then, Hb was calculated according to the relationship between the extinction and concentrations of standard hemoglobin solutions. Other red blood cell parameters: mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to the formulas: MCV = (Ht × 10) / RBC, MCH = Hb / RBC, MCHC = (Hb × 100) / Ht. Unstimulated oxidative activity of phagocytes (NBT) was measured using nitrotetrazolium blue reduction method. Fifty µl of blood was mixed with an equal amount of 0.2% nitrotetrazolium blue solution and incubated for 1 hour at 28°C, and then 1 ml of dimethyloformamide (DMF) was added and the samples were vortexed for 5 minutes and centrifuged. Extinction of the supernatant was read at a 546 nm wavelength and concentration of formazan (a product of nitrotetrazolium blue reduction) was calculated according to the relationship between the extinction and concentrations of standard formazan solutions. Blood smears were also made (2 for each blood sample), stained with May-Grunwald and Giemsa solutions and fixed with Histokitt (Glaswarenfabrik Karl Hecht, Germany). In each preparation 300 erythrocytes were evaluated and percentage of erythroblasts was calculated. Both cell diameters and both nucleus diameters of 50 mature erythrocytes in each smear were measured using calibrated eyepiece reticule. Cell and nucleus perimeter areas were calculated (using the formula for ellipse area Πrl, where r and l are shorter and longer radii, respectively). The ratio of longer to shorter diameter was also calculated as "elongation index" and the ratio of cell to nucleus area as "nuclear index". In the smears 100 leukocytes were also viewed and classified to calculate differential leukocyte count: percentage of lymphocytes, neutrophils, monocytes, basophils and eosinophils. Thrombocytes were counted per 100 leukocytes in each smear, and then thrombocyte count (PLT) was calculated using this proportion and WBC values.

All fish survived the experiment and did not exhibit any signs of disease. Their final body mass was 107.9 ±18.0 g.

The average values of hematological parameters were calculated for each month and for statistical comparison of differences – per season (autumn: X–XII, winter: I–III, spring: IV–VI, summer: VII–IX). The results were subjected to Shapiro-Wilk's test to evaluate normality of distribution of the variables. As most of them showed normal or close-to-normal distribution, one-way ANOVA and Duncan's test were used to evaluate significance of differences (at $p \leq 0.05$) using Statistica 9.1 (StatSoft, Inc., USA).

RESULTS

Red blood cell parameters of carp juveniles reared under laboratory conditions showed distinct seasonal patterns (Table 1). Maximum hematocrit values occurred in II and VIII, both preceded by gradual increase. Seasonal statistical comparison revealed the lowest mean Ht value in autumn and the highest in winter and summer. Hemoglobin concentration also showed two peaks: in I and VI. The highest average seasonal Hb level occurred in winter and the lowest in autumn. Erythrocyte count showed one peak in II that coincided with maximum Ht level. The highest RBC occurred in winter, and the lowest in autumn. The average frequency of erythroblasts ranged from 7.3 ±1.5% in VIII to 11.4 ±3.8% in XI but the highest seasonal average value occurred in spring, while the lowest in summer. The MCV, MCH and MCHC values showed fluctuations without any distinct seasonal pattern or significant seasonal differences.

Although, the only significant differences in calculated MCV occurred between autumn and summer, direct measurements of erythrocytes revealed significant seasonal differences in their size (Table 2). The largest erythrocytes (in terms of area) were in summer, while the smallest in autumn. Similar changes were observed in the area of erythrocyte nuclei. The changes in cell and nucleus size were related mainly to the significant increase in their long diameters. An increase in erythrocyte elongation was also observed and a decrease in area of cell occupied by the nucleus.

The changes in the values of white blood cells parameters of carp also showed seasonal patterns (Table 3). Two peaks of leukocyte count occurred: in XII and III. The highest seasonal average WBC values were observed in autumn and winter, while the lowest in summer. Differential leukocyte count also showed significant seasonal alterations although no distinct monthly patterns were observed. The highest lymphocyte frequency occurred in winter and the lowest in summer, while percentage of neutrophils showed the reverse pattern. In all seasons immature neutrophils predominated in carp blood (myelocytes and metamyelocytes) comprising from 66.1 ±30.7% of neutrophils (in summer) to 83.7 ±30.5% (in autumn), while mature cells (band and segmented) were less abundant. The maximum oxidative metabolic activity of phagocytes (NBT) occurred in III, while minimum in XI–XII and VI–VII. Seasonal average NBT values significantly differed showing the maximum in winter and minimum in summer and autumn. Thrombocyte count did not show any distinct monthly pattern, but reached the maximum in winter, while the lowest value occurred in summer.

TABLE 1. Seasonal changes in the values of red blood parameters in common carp (different letter superscripts indicate statistically significant differences, Duncan test, $P \leq 0.05$, $N = 30$)

Season	Ht (%)	Hb (g/l)	RBC (10^6·µl)	MCV (fl)	MCH (pg)	MCHC (g/l)	Erythroblasts (%)
Autumn	24.5 ±3.7 ^{a}	54.4 ±21.8 ^{a}	1.44 ±0.33 ^{a}	182.9 ±50.2 ^{a}	40.5 ±20.5 ^{a}	221.5 ±79.9 ^{ab}	9.6 ±3.3 ^{a}
Winter	32.6 ±3.7 ^{b}	82.7 ±17.4 ^{b}	1.67 ±0.31 ^{b}	200.3 ±37.4 ^{ab}	51.6 ±16.0 ^{b}	255.9 ±57.9 ^{b}	9.5 ±2.1 ^{ab}
Spring	29.2 ±4.9 ^{c}	66.2 ±23.6 ^{c}	1.52 ±0.31 ^{abc}	198.8 ±49.4 ^{ab}	44.5 ±15.3 ^{ab}	222.7 ±55.1 ^{ac}	10.5 ±2.2 ^{a}
Summer	31.2 ±4.7 ^{bc}	69.7 ±23.0 ^{c}	1.47 ±0.26 ^{ac}	217.6 ±41.2 ^{b}	47.5 ±14.0 ^{ab}	220.1 ±58.2 ^{ac}	8.3 ±1.7 ^{b}

TABLE 2. Seasonal changes in erythrocyte morphometric parameters in common carp (different letter superscripts indicate statistically significant differences, Duncan test, $P \leq 0.05$, $N = 1500$)

Season	Long cell axis (µm)	Short cell axis (µm)	Long nucleus axis (µm)	Short nucleus axis (µm)	Cell area (µm^2)	Nucleus area (µm^2)	Elongation index	Nuclear index (%)
Autumn	9.8 ±0.7 ^{a}	6.0 ±0.5 ^{a}	4.3 ±0.4 ^{a}	2.6 ±0.1 ^{a}	45.9 ±5.4 ^{a}	8.7 ±0.9 ^{a}	1.65 ±0.15 ^{a}	19 ±2 ^{a}
Winter	10.0 ±0.6 ^{b}	6.0 ±0.4 ^{a}	4.4 ±0.3 ^{b}	2.6 ±0.1 ^{a}	47.2 ±5.2 ^{b}	8.9 ±0.8 ^{b}	1.68 ±0.13 ^{b}	19 ±2 ^{a}
Spring	10.3 ±0.6 ^{c}	6.2 ±0.5 ^{b}	4.4 ±0.4 ^{b}	2.6 ±0.1 ^{a}	50.2 ±5.7 ^{c}	9.0 ±0.7 ^{c}	1.66 ±0.12 ^{a}	18 ±2 ^{b}
Summer	10.6 ±0.5 ^{d}	6.2 ±0.5 ^{b}	4.5 ±0.3 ^{c}	2.6 ±0.0 ^{a}	51.8 ±5.4 ^{d}	9.1 ±0.6 ^{d}	1.71 ±0.27 ^{c}	18 ±3 ^{b}

TABLE 3. Seasonal changes in white blood parameters in common carp (different letter superscripts indicate statistically significant differences, Duncan test, $P \leq 0.05$, $N = 30$)

Season	WBC (10^3·µl^{-1})	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)	NBT (g/l formazan)	Thrombocytes (10^3·µl^{-1})
Autumn	94.8 ±50.2 ^{a}	94.9 ±4.6 ^{ab}	1.0 ±1.2 ^{a}	3.6 ±3.8 ^{ab}	0.90 ±0.47 ^{a}	13.7 ±9.3 ^{ab}
Winter	92.0 ±38.8 ^{a}	96.7 ±3.6 ^{a}	0.8 ±1.3 ^{a}	2.4 ±3.0 ^{a}	1.62 ±0.63 ^{b}	15.2 ±6.9 ^{a}
Spring	83.3 ±36.7 ^{a}	95.0 ±4.2 ^{ab}	1.3 ±1.6 ^{a}	3.3 ±2.9 ^{ab}	1.25 ±0.60 ^{c}	13.0 ±8.3 ^{ab}
Summer	59.8 ±26.8 ^{b}	93.5 ±3.9 ^{b}	1.2 ±1.5 ^{a}	4.4 ±2.7 ^{b}	1.06 ±0.34 ^{ac}	9.9 ±6.7 ^{b}

DISCUSSION

The results of present study revealed significant seasonal changes in the values of most hematological parameters of carp under stable laboratory conditions, some of them showing distinct annual patterns. However, most values (except for slightly higher erythroblast frequency) fit within the reference ranges obtained for common carp juveniles under various environmental conditions by various authors (Witeska et al. 2016). The values of most red blood cell parameters showed minimum values in autumn and maximum in winter. Hematocrit and hemoglobin levels showed two distinct peaks (each peak Hb value took place a month earlier than Ht), while only one RBC peak occurred. The observed hematological changes were different than commonly observed under natural conditions. Most authors reported significant reduction in the values of red blood indices in winter and the increase in summer (Orun et al. 2003, Golemi et al. 2013, Seriani et al. 2013) which indicates hematological adjustment to temperature-related metabolic rate. Autumnal decreases in red blood cell parameters were observed by Santos et al. (2009) and Kohanestani et al. (2013) and they were followed by similar or lower values in winter. In the present study low values of RBC, Ht and Hb observed in autumn were followed by increase in winter which suggests that under laboratory conditions process of winter adjustment was interrupted. This was probably because water temperature did not decrease to normal winter level. Second peak of Ht and Hb observed in summer was similar as observed by other authors and was probably related to natural changes in photoperiod.

Direct measurement of erythrocytes revealed that their size and shape significantly changed: the smallest and least elongated cells with proportionally large nuclei were observed in autumn, while the largest and most elongated ones occurred in summer. Similar pattern is visible in MCV values (despite no significant differences). Size of fish erythrocytes was proved to be inversely related to metabolic rate (Maciak et al. 2011) and aerobic swimming ability (Lay and Baldwin 1999). Many authors observed the highest MCV in winter and the lowest in summer (Golemi et al. 2013, Langer et al. 2013). In our study gradual increase in erythrocyte size with time might have been related to long-term adaptation to laboratory conditions: reduced swimming activity due to limited tank space and high feed availability accompanied by stable and high level of dissolved oxygen.

Leukocyte count showed two peaks (in XII and III), and the average seasonal value of WBC gradually decreased from autumn to summer. This pattern was contrary to the data obtained under natural conditions by authors who observed WBC peak in summer (Lamkova et al. 2007, Morgan et al. 2008, Gupta et al. 2013, Kohanestani et al. 2013, Fallah et al. 2014). The NBT value reached one distinct maximum in III which coincided with one of the WBC peaks. Various authors reported different seasonal patterns of innate immune parameters in fish. Some data indicate the maximum activity of phagocytic cells in summer (Lamkova et al. 2007, Santos et al. 2009, Das et al. 2012), while another show increase in this parameter in other seasons (Morgan et al. 2008, Buchtikova et

al. 2011). Cuesta et al. (2008) reported that oxidative activity of phagocytes and other immune indices may be positively affected by melatonin and thus probably related to photoperiod.

In this study thrombocyte count showed the maximum in winter and minimum in summer, similarly as WBC and activity of phagocytes (NBT). Similarly maximum thrombocyte count was also observed by Gupta et al. (2013) and Langer et al. (2013), contrary to the findings by Orun et al. (2003) and Vigliano et al. (2014). Stosik et al. (2019) noted that thrombocytes in fish display strong defence potential which is expressed in their phagocytic activity and ability to neutralise and degrade the bacteria absorbed. There is evidence that they make up nearly a half of the population of phagocytising cells among peripheral blood phagocytes (Nagasawa et al. 2014). Kozińska et all. (1999 a, b) observed that role of thrombocytes in phagocytosis increases at low temperatures, when the activity of granulocytes and monocytes declines.

According to Bowden et al. (2007), seasonality is a complex event made up of many potential cues, among which the changes of temperature and day length are the most important. The results of present study showed significant changes in the values of most hematological parameters of common carp juveniles in annual cycle but independent of water temperature or other environmental factors most of which (except for photoperiod) were stable or varied little throughout the experimental period. Some of the changes, e.g increase in oxidative metabolic activity of phagocytes in spring and increase in hemoglobin level in summer were similar to those that occur in fish under natural conditions which indicates the possibility of endogenous control. Therefore, the causes of these changes still are to be elucidated, with particular attention paid to photoperiod.

CONCLUSIONS

Most of hematological values of common carp juveniles kept under stable laboratory conditions showed significant seasonal variability but they were in accordance with the reference ranges. However, variability of hematological parameters in common carp juveniles irrespective of environmental conditions makes it necessary to include reference groups sampled simultaneously with experimental groups in the laboratory experiments.

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Streszczenie: *Sezonowe zmiany parametrów hematologicznych u młodocianych karpi w warunkach laboratoryjnych.* Celem badań była ocena zmian wartości parametrów hematologicznych młodocianych karpi w rocznym cyklu, w stabilnych warunkach laboratoryjnych. Niektóre parametry wykazywały wyraźne rytmy zmian, np. dwa piki hematokrytu wystąpiły w II i VIII, stężenie hemoglobiny w I i VI, podczas gdy liczba erytrocytów osiągnęła maksymalny poziom w II. Największe erytrocyty zaobserwowano w VIII, a najmniejsze w XII. Liczba leukocytów wykazała dwa piki w XII i III. Maksymalny procent limfocytów wystąpił w III, a minimalny w XI, podczas gdy odsetek neutrofilów wykazywał odwrotną prawidłowość. Aktywność metaboliczna fagocytów (NBT) osiągnęła maksimum w III, natomiast minimum wystąpiło w XI–XII i VI–VII. Liczba trombocytów była największa w XII, a najmniejsza w VII. Uzyskane wyniki wykazały, że wartości parametrów hematologicznych u karpi uległy znacznym zmianom w ciągu roku, mimo niewielkich zmian parametrów środowiskowych. Niektóre z tych zmian, np. wzrost aktywności oksydacyjnej fagocytów wiosną i wzrost poziomu hemoglobiny latem były podobne do tych, które występują u ryb w warunkach naturalnych. Inne, takie jak zwiększenie wielkości erytrocytów lub zmniejszenie liczby leukocytów sugerują długoterminowe przystosowanie do środowiska laboratoryjnego.

Słowa kluczowe: krew, erytrocyty, ryby, leukocyty, pora roku, trombocyty

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