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DETERMINATION OF SOME HEMATOLOGICAL PARAMETERS AND NON-SPECIFIC IMMUNE RESPONSES IN *Garra rufa* (HECKEL, 1843) LIVING IN KANGAL (SİVAS) BALIKLI ÇERMİK THERMAL HOT SPRING AND TOPARDIÇ STREAM (SİVAS)

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Abstract:

In this study, *Garra rufa* Heckel, 1843 (popularly known as doctor fishes), which is a fatty fish species living in Kangal Balıklı Çermik Thermal Hot Springs and Topardıç Stream mixing with these hot spring waters, was investigated. Hematological and non-specific immune parameters were investigated by comparing each environment in terms of seasons and each season in terms of environments. The amount of erythrocyte *(*RBC) and leukocyte (WBC) in the stream was found to be increased in summer and decreased in winter. On the other hand, no seasonal differences were observed in the pools due to the thermal water. Hb, Hct, monocyte, neutrophil, phagocytic activity values were found to be low in the winters and high in the summers in the stream whereas no changes were observed in the pools. No difference was found between seasons and environments in terms of the amount of eosinophil cells. Lymphocyte and MCV levels in the stream were found to be increased in winter and decreased in summer. No change was observed in the pools. Seasonal and environmental no difference was observed in MCH values of both pools and the stream, whereas MCHC values in the stream were found to be high in summer and low in winter. In the research, differences in winter and summer seasons and effects of different environments in these values were investigated.

Keywords: *Garra rufa*, Kangal Balıklı Çermik Thermal Hot Springs, Hematology, Non-Specific Immunity

Introduction

Hematological parameters are very important in determining health and physiological status of the fish (Clauss et al., 2008; Adeyemo et al., 2009). In addition, these parameters reflect the changes in the organism correctly and play an important role in the detection of disease and metabolism of fish living in different ecological environments (Clarence and Hickey, 1982; Cengizler and Şahan, 2000). Hematological and immunological values are considered important criteria for fish health (Siwicki et al., 1994). Fishes are poikilothermic creatures, in which changes are observed in hematological parameters due to environmental factors such as bacteria, parasites, water temperature, oxygen content, pH and so on. Hematological values in fish changes with the effects of seasonal variations that are associated with changes in water temperature and climatic changes (Atamanalp and Yanık, 2003).

In this study, *Garra rufa* (Heckel, 1843) fish species living in Kangal Balıklı Çermik Thermal Hot Spring (Sivas) and Topardıç Stream (Sivas) and their hematological and non-specific immune parameters were investigated by comparing each environment in terms of seasons and each season in terms of environments. Within the scope of the study, hematological parameters such as red blood cells count (RBC), mean corpuscular volume (MCV), mean hemoglobin concentration (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC), hemoglobin (Hb) and hematocrit (Hct) amounts were analyzed. In addition, non-specific immune parameters such as phagocytic cell activity, monocyte, lymphocyte, eosinophil and neutrophil cells were discussed. The results of the mentioned parameters were evaluated in a comparative manner for summer (June, July and August) and winter (December, January, February) months in terms of the fishes living in Kangal Balıklı Çermik Thermal Hot Spring and Topardıç Stream. This study mainly aims to reveal some unknown hematological parameters and non-specific immune response characteristics of the doctor fish (*Garra rufa*) living in Balıklı Çermik Thermal Hot Spring and Topardıç Stream which are known to host a quest for finding health in the history of humanity.

Materials and Methods

Kangal Balıklı Çermik Thermal Hot Springs is located 13 km in the north-east direction of Kangal district which is 90 km away from Sivas province (Özer et al., 1987). There are pools flowing with hot thermal waters and these waters in pools mix with Topardıç Stream in a distance of 100 meters (Duman and Şahan, 2014). The pH value of the isothermal water is about 7.2 and its temperature ranges between 35 ^{o}C – 36 ^{o}C throughout the year (Özer et al., 1987). In the research, fishes were obtained from thermal pools and 500 meters away from the stream where pools are discharged into the stream. A total of 180 *Garra rufa* fishes were used during the research. Temperature and amount of oxygen in the pools and stream was measured and weight, *t*otal lenght and age of the fishes were determined. These measurements were taken in both winter and summer seasons on a monthly basis and average values are given for these seasons. Peterson method is used for determining the age of the fish in the pools (Akbulut et al., 2008). During sampling, no gender discrimination was made and the fish were taken from each environment randomly. These fishes collected from pools are fatty fish species (*Garra rufa)* adapted to hot environments (Timur et al., 1983; Demirsoy, 1993; Gözükara and Çavaş, 2004). Ectoparasites and bacteriological examination were realized in fish. Blood samples were collected from caudal vein of the fishes with an injector by using an anesthetic agent (MS-222) (Imanpoor et al., 2010).

In the research, Cyanmethemoglobin method was used for determination of hemoglobin amount (Blaxhall and Daisley, 1973; Tanyer, 1985) “Microhematocrit Technique” was employed for determination of Hct (Blaxhall and Daisley, 1973; Konuk, 1981; Şahan and Cengizler, 2002). The blood samples collected from caudal vein of fishes for determination of “leukocyte cell formulas" were spread on a slide with the help of another slide. These samples were examined under a light microscope at x100 magnification by using May-Grünwald and Giemsa staining methods. All areas in each preparation were scanned and percentage of leukocyte cells (monocyte, lymphocyte, neutrophil, eosinophil) were determined by counting a total of 100 leukocyte cells (Şahan and Cengizler, 2002; Dorafshan et al., 2008). The cells were counted on a thoma slide for determination of erythrocyte and leukocyte cells by using Natt-Herrick solution (Arnold, 2005). The erythrocyte indices were calculated according to the following formula:

Mean Corpuscular Volume (MCV) (μ^3) = Hct (%) ÷ RBC (10^6/ mm^3) x 10

Mean Hemoglobin Concentration (MCH) (pg) = Hb (g/100 mL) ÷ RBC (10^6/ mm^3) x 10

Mean Corpuscular Hemoglobin Concentration (MCHC) (%) = Hb (g/ 100mL) ÷ Hct (%) x 10 (Kocabatmaz and Ekingen, 1984).

The phagocytosis experiment is based on spectrophotometric measurement principle of phagocyted levels of yeast cells stained with Congo red by leukocytes. Histopaques are sodium diatrizoate and polysucroz solutions with a density of 1.077 ±0.001 g/mL and 1.119 ±0.001 g/mL, respectively. Histopaque 1.119 (Sigma) and Histopaque 1.077 (Sigma) containing hemagglutination buffer solution that will from layers is added to siliconized tubes. Then, 1 ml blood is carefully added to the tubes in the form of layers. Samples (500 g) were centrifuged for 15 minutes (4°C) and leukocytes were separated carefully. Then cells washed twice in HBSS (Hanks Balanced Salt Solition, Sigma) and adjusted to $2x10^6$ viable cells ml^{-1}. 250 ml leukocyte solution was mixed with 500 ml yeast cell suspension that was autoclaved and stained with Congo red (yeast cells/leukocyte count: 40/1). This mixture was incubated for one hour at room temperature and then 1 mL HBSS was added to the mixture and 1 mL Histopaque 1.077 (Sigma) to the bottom part of the mixture. Samples *were* centrifuged at 850 g for 5 minutes in order to separate leukocytes from yeast cells. The resulting leukocytes were washed twice with HBSS and stored at 37 °C for 12 hours after mixing with 1 ml trypsin EDTA solution (5.0 g/L trypsin and 2.0 g/L EDTA, Sigma). Trypsin-EDTA was measured as 510 nm at the spectrophotometer (Jeney et al., 1997; Şahan and Duman, 2010; Duman and Şahan, 2014).

SPSS 10.0 software was used for statistical analyses. The differences between experimental groups and significance levels of these differences were determined by One-Way ANOVA – Tukey Test ($P<0.05$ $P>0.05$) (Hayran and Özdemir, 1995).

Results and Discussion

Total lenght, weight and age data of fishes collected from pools and the stream are presented in Table 1; oxygen amounts and temperature values are presented in Table *2;* RBC, Hb, Hct, MCV, MCH, MCHC, WBC, *l*ymphocytes, monocytes, neutrophils, eosinophils and phagocytic activity values of *Garra rufa* are given in Table 3 and other properties of thermal hot spring water are presented in Table 4, respectively. In addition, pictures of erythrocyte, lymphocyte, monocyte and neutrophil obtained from this Cyprinidae species are shown in Figure 1, 2 and 3. No findings were found in the bacteriological and parasitological examinations of the examined fish.

Table 1. Total lenght, weight and age *Garra rufa*'s in the stream and pools

Garra rufa	Winter (X ± SD)		Summer (X ± SD)	
	Pool	Stream	Pool	Stream
Total lenght (cm)	11.82 ±0.49	12.19 ±0.72	11.55 ±0.76	11.78 ±1.15
Weight (g)	15.96 ±1.89	16.71 ±1.51	17.33 ±1.29	16.64 ±1.97
Age (year)	3	3	3	3
X ± SD: Mean value ± Standard deviation				

Table 2. Oxygen and temperature levels in stream and pools

	Winter (X ± SD)		Summer (X ± SD)	
	Pool	Stream	Pool	Stream
Oxygen (mg/L)	5.1 ±0.15	11.6 ±0.35	4.9 ±0.16	5.8 ±0.40
Temperature (^oC)	35.3 ±0.36	10.3 ±0.60	35.1 ±0.20	31.6 ±1.45
X ± SD: Mean value ± Standard deviation				

Table 3. RBC, Hb, Hct, MCV, MCH, MCHC, WBC, lymphocyte, monocyte, neutrophil, eosinophil, and phagocytic activity values of *Garra rufa*

	Winter (X ± SD)		Summer (X ± SD)	
	Pool	Stream	Pool	Stream
RBC ($x10^6/mm^3$)	1.97 ±0.34[a]	1.22 ±0.24[b]	1.91 ±0.41[a]	1.88 ±0.36[a]
Hb (g / dL)	7.38 ±0.56[a]	5.58 ±0.70[b]	7.71 ±0.34[a]	7.83 ±0.81[a]
Hct (%)	41.89 ±0.57[a]	34.91 ±0.58[b]	42.12 ±0.93[a]	39.62 ±1.64[c]
MCV (μ^3)	219.8 ±48.7[a]	298.1 ±72.7[b]	231.8 ±62.2[a]	219.3 ±50.7[a]
MCH (pg)	38.7 ±9.1[a]	47.2 ±10.6[b]	42.1 ±10.0[a]	43.3 ±10.9[a]
MCHC (%)	17.6 ±1.31[a]	15.9 ±1.99[b]	18.3 ±0.88[a]	19.8 ±2.20[c]
WBC($x10^3/mm^3$)	3.80 ±0.19[a]	2.39 ±0.07[b]	3.76 ±0.07[a]	3.77 ±0.11[a]
Lymphocyte (%)	64.8 ±0.63[a]	76.0 ±0.62[b]	64.7 ±0.47[a]	67.7 ±0.50[c]
Monocyte (%)	21.9 ±0.51[a]	16.9 ±0.58[b]	23.7 ±0.77[a]	22.9 ±0.59[a]
Neutrophil (%)	10.3 ±0.98[a]	6.5 ±1.18[b]	10.9 ±0.73[a]	11.9 ±0.51[c]
Eosinophil (%)	1.78 ±0.54[a]	1.87 ±0.48[a]	2.03 ±0.55[a]	1.86 ±0.51[a]
Phagocytic activity (O.D. 510 nm)	0.45 ±0.02[a]	0.36 ±0.01[b]	0.44 ±0.02[a]	0.47 ±0.01[c]

X ± SD: Mean value± Standard deviation. Different letters in averages means statistical difference ($p<0.05$).

Table 4. Other properties of thermal hot spring water (Timur et al., 1983)

Properties	Values
Appearance	Limpid
Smell	None
Taste	None
Color	Typical
Turbidity	None
Ph	7.8
Hardness (F.S.º)	26
Organic matter (p.p.m.)	0
Ca+Mg (mg/lt)	80+14
Cl_2	0
NH_3	None
NO_2	None
NO_3	None

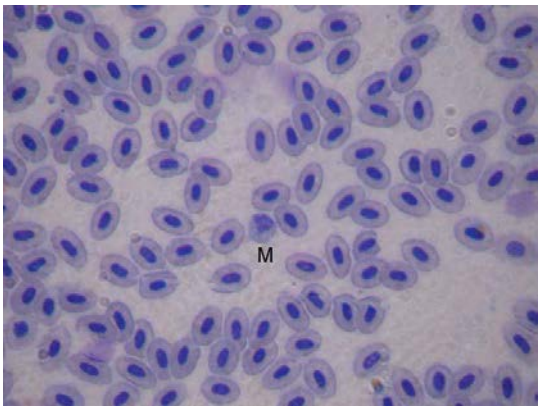

Figure 1. Blood cells of *Garra rufa* (M: monocyte), May-Grunwald Giemsa x100

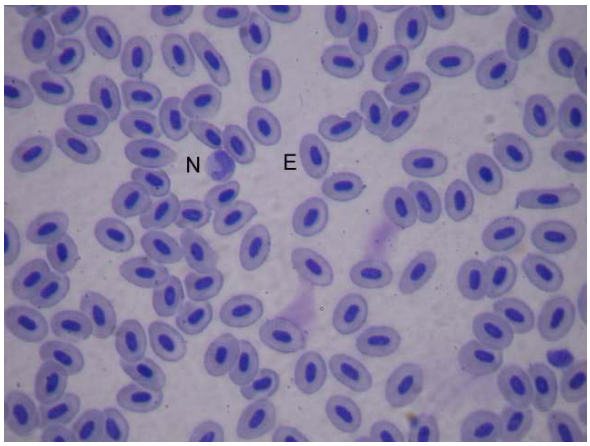

Figure 2. Blood cells of *Garra rufa* (E: Erythrocyte, N: Neutrophil), May-Grunwald Giemsa x100

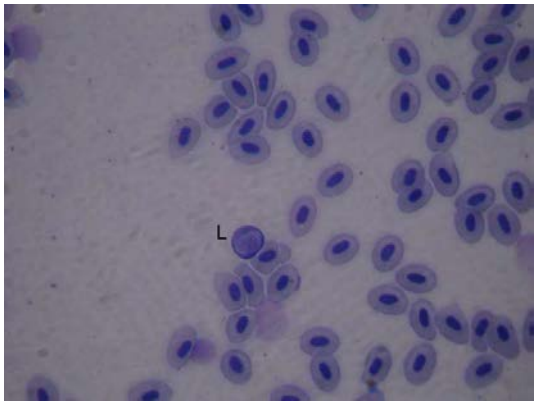

Figure 3. Blood cells of *Garra rufa* (L: L*ymphocyte*), May-Grunwald Giemsa x100

In summer, there was no statistically significant difference between RBC, Hb, MCV, MCH, WBC, monocyte, eosinophil levels of fishes collected from pools and the stream; while there was an increase in the MCHC, lymphocyte, neutrophil and phagocytic activity values and decrease in the Hct values *of* the fishes in the stream compared to those in the pools.

On the other hand, in winter, there was decrease in RBC, Hb, Hct, MCHC, WBC, monocyte, neutrophil and phagocytic activity levels and an increase in MCV, MCH and lymphocyte values of the fishes in the stream compared to those in the pools. No differences were determined in terms of eosinophil.

In the comparison between the fish in the pool in summer and winter seasons; statistically no significant differences were determined in RBC, Hb, Hct, MCV, MCH, MCHC, WBC, lymphocyte, monocyte, neutrophil, eosinophil and phagocytic activity levels.

As a result of the comparisons of fishes living in the stream in summer and winter seasons; RBC, Hb, Hct, MCHC, WBC, *m*onocyte, neutrophil, phagocytic activity levels of *Garra rufa* fishes were found to be increased in summer and decreased in winter; whereas MCV, MCH and lymphocyte values were found to be decreased in summer and increased in winter, respectively. There was no difference in the level of eosinophil.

The presence of thermal water in the pools made the pool water unaffected from the seasonal changes, however the water in the stream being not thermal originated has caused differences on the hematological and immunological parameters of the fish. The difference between water temperatures, the change in oxygen levels, increased metabolic activities and increased energy demand caused an increase in the amount of RBC in the summer. According to the results of earlier studies, these changes are caused by seasonal differences between water parameters, oxygen-carrying capacity of blood depends on water temperature and therefore, changes in water temperature have effects on erythrocyte cells (Nanba et al., 1987; Örün et al., 2003; Aras et al., 2008). Living conditions of the fishes and oxygen levels in different environments as well as water temperature changes are observed to have effects on Hb, Hct, MCV, MCH and MCHC amounts. Similar studies shows a changing trend in MCH and MCHC values with temperature. Researchers have identified higher MCV values and winter and lower values in summer. These differences are considered to be caused by environmental changes such as changes in water temperature and oxygen levels (Siwicki et al., 1994; Yılayaz and Bitmiş, 2002; Ginneken et al., 2007; Dias et al., 2008; Arnaudova et al., 2008). There is a proportionally relationship between RBC and these parameters. These changes are considered to be associated with increases and decreases in the amount of RBC. Hct, Hb, RBC and WBC values were determined to be decreased due to the decline of water temperature and this finding is supported by other researchers (Grigg, 1969; Leard et al., 1998; Rambhaskar and Rao, 2006). In the related studies, it has been reported that seasonal changes in water temperature cause differences in the non-specific immune response parameters of fishes (Yılayaz and Bitmiş, 2002; Örün et al., 2003). These studies suggest that there are different values in different seasons. In our study, the increase in lymphocyte percentages starting in the winter months were followed by the decrease during summer months, it was found that there were differences in monocyte and neutrophil percentages based on the cold and warm seasons and they reached the maximum in the summer. The percentage of eosinophil was found to be almost zero and no seasonal or environmental changes were seen in the percentage of eosinophil for the species included in the study. Although the number of studies related to this field is very limited, researchers reported similar results (Morvan et al., 1998; Swain et al., 2007; Kortet and Vainikka, 2008). In our study, phagocytic cells were found to be increased in spring and summer seasons when the water temperature also increased, and we have found that there was a decline in the phagocytic activities due to the immune suppression of the fishes at lower temperatures. This finding is in line with similar studies and phagocytic activity is found to be at highest in summer and at lowest in winter, respectively. They have determined that this is associated with the response created by immune system of the fish with increased microbial capacity in the water due to the higher water temperature in summer (Collazos et al., 1995; Korter and Vainikka, 2008). In the present research, it was observed that the water temperature has effects on the leukocytes and seasonal changes in the water temperature positively affect WBC. In the earlier studies, increased metabolic activity, reduced oxygen level and increased energy demand caused by increased water temperature are reported to be main reasons of these

changes (Yılayaz and Bitmiş, 2002; Akmirza and Tepecik, 2007).

Conclusion

It was thought that, with the increase in the microbiological activity in the warming waters in the summer and the increase in the microorganism density in the water, the immunological responses by the fish had an effect on these changes. The previous study results on this subject were found to be in line with the results obtained in our study.

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