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Quantitative analysis of the pigment composition of macrophage aggregates in the spleen of chub (*Squalius cephalus* L., 1758) from the river Crn Drim. Influence of sex and season

Квантитативна анализа на пигментниот состав на макрофагните агрегати во слезината на клен (*Squalius cephalus* L., 1758) од реката Црн Дрим. Влијание на пол и сезона

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Abstract

The research examined the quantity of total macrophage aggregates, as well as the quantity of macrophage aggregates with different pigment composition, in the spleen of chub from the river Crn Drim. In total, 156 fish were collected for qualitative and quantitative assay. In addition to the relative and total volume, and the number of macrophage aggregates per unit area, the fish were measured for body weight and total length, condition factor, spleen weight and spleen-somatic index. All examined parameters were interpreted in relation to season and sex. In males collected in the spring-summer season, general relative and total volume increase, as well as the relative volume of macrophage aggregates that contained lipofuscin/ceroid and the combination of lipofuscin/ceroid and melanin was noted, compared to values measured for males collected in the autumn-winter. In relation to sex, values measured for males were also higher when compared to values measured for females, although insignificantly. Since the fish were collected from relatively unpolluted sites, the results from this research can be used as reference point for the use of macrophage aggregates in the studies for biomonitoring of the aquatic ecosystems.

Key words: chub, Crn Drim, spleen, macrophage aggregates, pigment composition, stereological measurements.

Апстракт

Ова истражување го одреди квантитетот на тотални макрофагни агрегати, како и квантитетот на макрофагни агрегати со различен пигментен состав во слезината на клен од реката Црн

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Дрим. Вкупно 156 риби беа колекционирани за квалитативна и квантитативна анализа. Покрај релативниот волумен, тоталниот волумен и бројот на макрофагни агрегати на единица површина, мерени беа телесната тежина, должината на телото, кондицискиот фактор, тежината на слезината и сплиносоматскиот индекс на рибите. Сите измерени параметри беа толкувани во однос на сезоната и полот. Кај мажјаците колекционирани во сезоната пролет-лето, беше забележан раст на вредостите за вкупниот релативен и тотален волумен, како и релативниот волумен на макрофагните агрегати кои содржат липофусцин/цероид и комбинација од липофусцин/цероид и меланин, наспроти вредностите за мажјаците колекционирани во сезоната есен-зима. Во однос на полот, повторно мажјаците покажаа повисоки вредности за овие параметри во однос на женките, но сепак статистички незначително. Имајќи предвид дека рибите се уловени од релативно чиста животна средина, резултатите од ова истражување можат да се искористат како референтни вредности во студиите за биомониторинг на акватични екосистеми.

Клучни зборови: клен, Црн Дрим, слезина, макрофагни агрегати, пигментен состав, стереолошки мерења.

Introduction

The environmental pollution is growing rapidly and aquatic ecosystems are subjected to extensive degradation owing to the fact that untreated industrial, agricultural and waste waters from the households are directly discharged into the natural waters (Agius 1985; Rebok et al. 2015). Although the pollutants in the water might be within allowed concentrations, their effect can be harmful to organisms due to the effect of bioconcentration and biomagnification through the food chain (Katagi, 2010). Also, the accumulation of the pollutants can be conditioned by many factors (Katagi 2010; Jordanova et al. 2016a, 2016b). These types of environmental threats are the reason for the need of establishing biomarkers sensitive to the early signs of aquatic pollution (Agius 1985; Rebok et al. 2015). The use of macrophage aggregates (MACs) as potential biomarkers for health monitoring of wild fish populations was suggested based on the idea that the increase in the number, area and the pigment content in the MACs, may be caused by changes in the environment (Wolke et al. 1985). Researches had shown that the number of MACs can vary in relation to the size, nutritional, reproductive status or the health of the fish (Wolke et al. 1985; Agius & Roberts 2003; Rebok et al. 2015; Jordanova et al. 2016b). For example, it is confirmed that the number and size of MACs in fish is growing with the aging and starving, as well as with increased susceptibility to disease (Wolke et al. 1985; Agius & Roberts 2003). Also, the pigment content can vary depending on the fish condition. The three notable pigment groups found the MACs have the ability to absorb and neutralize free radicals, positively charged ions and other potentially toxic agents (Evans & Nowak 2016). Hemosiderin is a brown, granular, relatively insoluble pigment containing a protein and an iron (ferric) component (Agius & Roberts 2003). The iron in ferric state that is derived from the decomposed hemoglobin, and thus hemosiderin is a breakdown product of red blood cells, is commonly present in the fish spleen, but in low concentrations (Blazer et al. 1987). Overall, hemosiderin is the storage form of iron and this pigment originates in dietary sources and from the hemolysis of erythrocytes (Wolke et al. 1985). Lipofuscin/ceroid results from the oxidative polymerization of polyunsaturated fatty acids and proteins, and since this pigment is accumulating in the cells with age and tissue destruction the same has been called 'wear and tear' pigment (Wolke et al. 1985; Agius & Roberts 2003). The lipofuscin/ceroid is a waste material in one cell, though this pigment can't be decomposed or removed by exocytosis (Terman & Brunk 1998). Melanin is a complex polymer that can absorb and neutralize free radicals, cations and other potentially toxic agents, which are derived from the degradative processes occurring in macrophages after these cells phagocytose discarded material (Zuasti et al. 1989). Melanin is thought to have a major bactericidal role in fish at low environmental temperatures (Wolke et al. 1985; Agius & Roberts 2003).

According to the above, the reason why MACs are still considered as "future biomarkers" is owing to the shortage of quantitative studies for the normal variations of the MACs due to natural factors (Agius 1985; Rebok et al. 2015). Furthermore, the

values of the studied parameters can be easily altered with the methods of capturing and transporting of the fish (Agius 1985).

In bony fish, MACs are widely distributed in the spleen, kidney and liver (Agius & Roberts 1981; Wolke et al. 1985; Fournie et al. 2001; Agius & Roberts 2003; Jordanova et al. 2006). There is a heterogeneous MACs population in the spleen, whose differences most likely reflect the different functions they perform: from capturing antigens and specialized communication with T and B cells, to phagocytosis and degradation of erythrocytes in the red pulp (Ross & Auger 2002). The spleen is a lymphoid organ with many functions among which the defense of the organism, making it a frequent subject of research not only in histology, but in immunological fields as well (Rebok 2006; Rebok et al. 2011). Considering that the immune system is the first who reacts to changes in the environment (Skouras 2002), in addition to the spleen being a compact organ suitable for histological analysis with light microscopy, this organ is an ideal subject in the studies for biomonitoring based on MACs changes. In order MACs to be used successfully as biomarkers for the health condition of fishes and early environmental pollution, it is vital to begin with determination of the normal variations of their morphological variables.

Despite the fact that these types of research are a significant method of evaluating the effects from environmental pollution, data for splenic MACs in fish from the Adriatic basin is scarce, and there is a lack of histological studies of the visceral organs of fish in the Republic of North Macedonia. Moreover, the data about the histology of the visceral organs and the health status of fishes native to the river Crn Drim are scarce. To our knowledge, there are studies only for histopathology of the liver of *Chondrostoma ochridanus* (Velkova-Jordanoska et al. 2012), hepatic capillariasis in barbel (*Barbus rebeli* Koller, 1926) (Jordanova et al. 2018a) and accumulation of heavy metals in some organs in barbell and chub (*Squalius squalus* Bonaparte, 1837) (Jordanova et al. 2018b). However, there are extremely scarce amount of studies which examined quantitative influence of endogenous factors as sex and season on spleen MACs (Rebok 2006; Jordanova et al. 2006, 2016b; Rebok et al. 2015). Therefore, this study represents the first attempt to quantify the splenic MACs from the chub inhabiting the river Crn Drim in order to establish the normal variations of amount of MACs as well as their pigment composition.

Materials and methods

Investigated area

The fish were captured from the river Crn Drim, which is part of the Adriatic basin. This river is 120 km long and springs out from the Ohrid Lake, flows through the western parts of the North Macedonia and the northern parts of the Albania. The river Crn Drim has many tributaries passing through urban and agricultural areas, collecting pollution from different sources along the way (Musa et al. 2012).

Due to the human activity, the river Crn Drim as well as other riverine systems, is threatened by unauthorized waste disposal in the river bed, erosion as a result of deforestation, building hydropower plants that don't fulfill the requirements for biological minimum and so on (Poci 2012). Regardless, the water of the river Crn Drim was safe for drinking less than 50 years ago, but today the situation is changed, judging from the decrease of the riverine biodiversity (Musa et al. 2012). Nonetheless, the assessment of heavy metals in the river Crn Drim indicates that this river is relatively unpolluted. In other words, the concentration of trace elements in the water and sediment of the river was in the permissible limit, with lower values measured in spring, due to the raised water levels of the river (Jordanova et al. 2018b; Hristovski et al. 2019).

Collection of the fish

Model of research in this study is the chub (*Squalius cephalus*, syn. *Leuciscus cephalus* Linnaeus, 1758). This fish is quite suitable species for studies related to the biomonitoring of riverine systems due to its wide distribution on the Balkan Peninsula (Economidis & Banarescu 1991; Durand 1999a, 1999b). The chubs were collected from the river Crn Drim during two seasons: spring-summer (S-S) (n=78) and autumn-winter (A-W) (n=78). Even though all 4 seasons are covered, as there were no significant differences between fish collected in autumn and winter, as well as between fish collected in spring and summer, we separated the fish in two groups: autumn-winter (A-W) and spring-summer (S-S). In total 156 fish were captured: 121 males and 35 females. Electrofishing was the method of choice for capturing the fish, conducted in accordance with standardized electric fishing

procedures as emphasized in the CEN directive (CEN EN 14011 : 2003). After capturing, the fish were transported in vital condition to the laboratory, with minimized stress in oxygenized containers. Subsequently, the fish were prepared for dissection with anesthetic Clove oil (Sigma). After the euthanasia, the total length (TL) was measured in centimeters with accuracy of 0.1 cm, in addition to their body weight (BW) in grams with accuracy of 0.01 g. These data were used in estimating the general health state of the fish, with the condition factor (CF) formula:

$$CF = BWx100/TL^3$$

Collection of organs

In the laboratory, fish were dissected and spleens were removed. Gonads were also isolated in order to determine the sex and gonadal stage of the fish. After isolation, the organ's weight was measured on a digital scale and the both organs were fixed in Bouin's fixative. The data for spleen weight (SW) were used for calculating the spleen-somatic index (SSI):

$$SSI = SWx100/BW$$

Tissue processing

After the spleen and the gonads were fixed in Bouin's fixative for 48 hours, the organs were subjected to dehydration in a series of alcohols, and were embedded in paraffin. Paraffin sections of 5 μm were made with manual rotating microtome. The tissue samples from the gonads were stained with Hematoxylin and Eosin, while sections from the spleen were stained with the Perls method. This method stains the intracellular pigment hemosiderin in blue, the cell's nucleuses are colored red and the spleen parenchyma pink. The other pigments do not require specific staining methods, because even though lipofuscin/ceroid gives positive results in neutral-lipid and acid-phosphatase staining, this pigment is characterized with autofluorescence (Mochizuki et al. 1995) and when subjected to Perl's staining it shows as yellow coloration; while melanin is a non-reflective pigment that during a microscopic observation can be noticed as black or brown granules.

Classical stereological methods based on counting points, with ocular grid with 180 points according to the Freere & Weibel (1967) methods were used, in order to determine the relative (Vv) and total (V) volume of the MACs. On average 50 visual fields per slide chosen with the systematic random sampling (SRS) method were analyzed, using a magnification of 400x. The tissue samples were analyzed with research microscope Nikon Eclipse 80i equipped with a digital camera Nikon Coolpix P600. MACs were counted separately in respect of pigment composition (hemosiderin, lipofuscin/ceroid, melanin), along with their combinations (hemosiderin and lipofuscin/ceroid; hemosiderin and melanin; lipofuscin/ceroid and melanin; hemosiderin, lipofuscin/ceroid and melanin). These measurements were used for calculating the relative volume (Vv) of the MACs containing different pigments and/or their combinations, with the following formula:

$$Vv(MACs, spleen) = [P(s) \times 100] / P(r)$$

where, P(s) is the number of points that overlay the MACs, and P(r) is the number of points that overlay the spleen parenchyma.

The total volume (V) was calculated by multiplying Vv(MACs, spleen) with the volume of the spleen V(spleen) which was determined with the displacement method. The V was calculated with the following formula:

$$V(MACs, spleen) = Vv(MACs, spleen) \times V(spleen)$$

The same ocular net, with square surface of $21{,}025 \times 10^{-3}$ mm^2, was used for determining the number of MACs per unit area (No. of MACs/mm^2):

$$\text{No. of MACs/mm}^2 = \text{No.of MACs}/(AF \times 21{,}025 \times 10^{-3})$$

where, No. of MACs represents the number of MACs counted for every individual fish, and AF represents the number of analyzed visual fields.

Statistical analysis

Results from the microscopic analysis, as well as the morphometric data were processed with the software Statistica 7.0 for Windows (StatSoft), while the graphs that present the pigment composition of the MACs based on the average values for the parameters, were made with Excel for Windows. After checking the normality (with the Kolmogorov-Smirnov test) and the homogeneity (with the Levene's test) of variances of our data, a logarithm transformation was necessary since the data sets were neither normal nor homogenous. In order to compare different groups in relation of sex and season, the log10-transformed data for BW and TL, CF, SW, SSI, Vv and V of the total MACs and their No, were analyzed

with two-way ANOVA, followed by post-hoc Tukey test. The results are presented as mean values followed by standard deviation and the above were considered significant for $p<0,05$.

Results

The results obtained by processing of the morphometric data with two-way ANOVA are shown in Table 1. Some trends are noteworthy in this table, although they are statistically insignificant. Therefore, in relation to BW and TL the fish from both sexes have higher values for the S-S season. CF shows nearly equal values between sexes in the S-S season, by contrast to season A-W when males have slightly higher CF values opposed to the females. Regardless, this increase is statistically inconsequential. Respectively to the increase of BW and TL values, the values for SW exhibit faintly higher increase in both sexes during S-S. Contrary, the values for SSI are slightly higher for A-W.

Regarding the qualitative analysis, MACs with different pigment composition were noted: hemosiderin, lipofuscin/ceroid and melanin; MACs that contain combinations of: hemosiderin and melanin, hemosiderin and lipofuscin/ceroid, melanin and lipofuscin/ceroid; as well as MACs that contain a combination of the three pigments mentioned above. The ratio of the pigment quantity differed among different MACs, although lipofuscin/ceroid stood out as the dominant pigment. The shape of the MACs was irregular (Fig. 1a), rarely spherical (Fig. 1b). Whether as singular cells, or in association with other cells in the form of aggregates, the macrophages were scattered through the white and the red pulp of the spleen parenchyma, often near the blood vessels (Fig. 1).

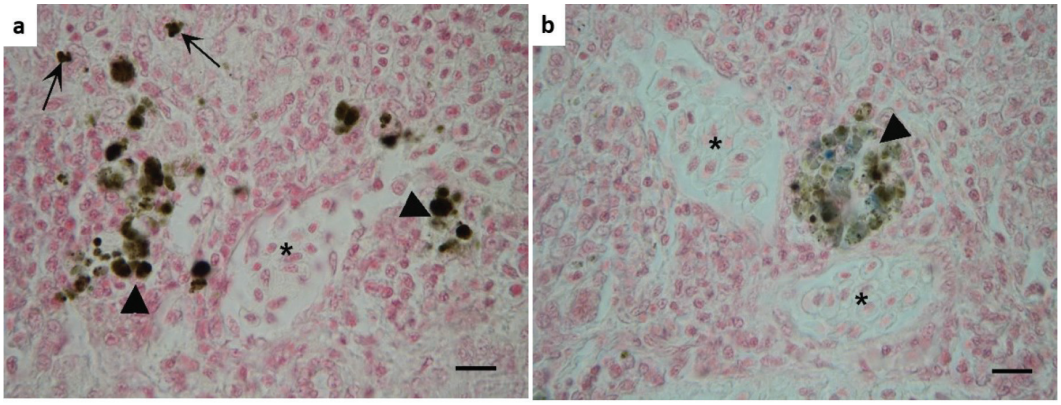

Fig. 1. Singular pigmented macrophages (→) and MACs (▶) with irregular (a) and spherical (b) shape near blood vessels (*) in the spleen of chub from the river Crn Drim. The tissues are stained with Perls. Bar=10μm. Magnification of 400x

Table 1. Body weight[1] (BW), total length[1] (TL), condition factor (CF), spleen weight (SW), spleen somatic index (SSI), relative volume (Vv) of MACs, total volume (V) of MACs and number of MACs per unit area (No. of MACs/mm^2). The results are shown for both sexes separately, in two seasons: spring-summer (S-S) and autumn-winter (A-W).

Parameters	**S-S**				**A-W**			
	F		**M**		**F**		**M**	
N	2		76		33		45	
BW (g)	164.50	(12.02)	223.73	(112.42)	122.45	(41.89)	113.40	(69.32)
TL (cm)	23.40	(0.14)	26.15	(4.54)	22.55	(2.54)	21.02	(3.62)
CF (%)	1.51	(0.17)	1.52	(0.58)	1.31	(0.25)	1.57	(0.76)
SW(g)	0.18	(0.06)	0.26	(0.39)	0.17	(0.08)	0.21	(0.19)
SSI (%)	0.11	(0.04)	0.14	(0.16)	0.14	(0.05)	0.22	(0.30)
Vv(%)	0.93	(0.49)	1.44	(1.08)^A	0.89	(0.62)	1.11	(1.67)^B
V (cm^3)	0.15	(0.04)	0.35	(0.68)	0.14	(0.08)	0.32	(0.88)
No. of MACs/mm^2	0.03	(0.02)	0.024	(0.02)^A	0.02	(0.01)	0.018	(0.01)^B

[1]The results are shown as mean values, followed by standard deviation.

[2]For each value, the different capital letters A and B indicate statistically significant differences between seasons, for one sex (read horizontally); in respect of two-way ANOVA, followed by Tukey test.

V(MACs) has insignificantly higher values in season S-S in both sexes, whereas in both seasons the V(MACs) values are more than twice higher in males. Statistically significant data is found only in the values for Vv(MACs) and No. of MACs/mm^2 ($p<0.05$ for both), in relation to season. Specifically, higher values for these parameters have been measured in males from the S-S season, opposed to the males from the A-W season.

Results for the Vv(MACs) with reference to pigment composition are shown in Table 2. In this table statistically significant variations can be noticed between males in relation to season. The Vv of MACs filled with lipofuscin/ceroid ($p<0.01$) and MACs that contain the combination of hemosiderin and lipofuscin/ceroid ($p<0.05$), shows statistically significant higher values in males from A-W, opposed to males from S-S. In contrast, the Vv values of MACs that contain the pigment combination of melanin and lipofuscin/ceroid are statistically higher in males from season S-S ($p<0.001$).

Results for the V(MACs) with reference to pigment composition are shown in Table 3. Statistically significant differences in these results can be seen between the males in different seasons. The values for V of MACs filled with lipofuscin/ceroid are higher in males from A-W ($p<0.05$), whereas the values for V of MACs whose pigment composition is made up by both lipofuscin/ceroid and melanin are higher in males from S-S ($p<0.01$).

Results for the No. of MACs/mm^2 with reference to pigment composition are shown in Table 4. Much like the results above, statistically significant data for the No. of MACs/mm^2 can be found only in males regarding different seasons. The values for the No. of MACs/mm^2 filled with lipofuscin/ceroid ($p<0.001$) and melanin ($p<0.05$) separately, are higher in males from A-W. In contrast, the No. of MACs/mm^2 rich with the pigment combination of lipofuscin/ceroid and melanin is significantly higher in males from S-S, opposed to males from the A-W season ($p<0.001$).

Table 2. Relative volume (Vv) of MACs regarding pigment composition. The results are shown for both sexes separately, in two seasons: spring-summer (S-S) and autumn-winter (A-W).

Vv relative volume	**S-S**		**A-W**	
	F	**M**	**F**	**M**
N	2	76	33	45
Vv (MACs-hemosiderin) %	0	0.003 (0.02)	0.007 (0.02)	0.006 (0.02)
Vv (MACs-lipofuscin) %	0.1 (0.02)	0.02 (0.09)^A	0.12 (0.26)	0.15 (0.32)^B
Vv (MACs-melanin) %	0	0.003 (0.01)	0.002 (0.005)	0.002 (0.01)
Vv (MACs-hemosiderin,melanin) %	0	0.004 (0.01)	0.007 (0.02)	0.006 (0.02)
Vv (MACs-hemosiderin,lipofuscin) %	0	0.02 (0.08)^A	0.06 (0.16)	0.25 (1.14)^B
Vv (MACs-melanin,lipofuscin) %	0.42 (0.08)	0.98 (0.75)^A	0.50 (0.33)	0.39 (0.32)^B
Vv (MACs-hemosiderin,melanin,lipofuscin) %	0.50 (0.59)	0.41 (0.93)	0.19 (0.55)	0.30 (0.67)

[1]The results are shown as mean values, followed by standard deviation.

[2]For each value, the different capital letters A and B indicate statistically significant differences between seasons, for one sex (read horizontally); in respect of two-way ANOVA, followed by Tukey test.

Table 3. Total volume (V) of MACs regarding pigment composition. The results are shown for both sexes separately, in two seasons: spring-summer (S-S) and autumn-winter (A-W).

Vtotal volume	**S-S**		**A-W**	
	F	**M**	**F**	**M**
N	2	76	33	45
V (MACs-hemosiderin) cm^3	0	0.0005 (0.003)	0.001 (0.002)	0.002 (0.01)
V (MACs-lipofuscin) cm^3	0.003 (0.004)	0.01 (0.02)^A	0.03 (0.07)	0.03 (0.07)^B
V (MACs-melanin) cm^3	0	0.0004 (0.002)	0.0002 (0.001)	0.0005 (0.002)
V (MACs-hemosiderin,melanin) cm^3	0	0.001 (0.002)	0.001 (0.002)	0.002 (0.005)
V (MACs-hemosiderin,lipofuscin) cm^3	0	0.003 (0.01)	0.01 (0.02)	0.11 (0.59)
V (MACs-melanin,lipofuscin) cm^3	0.08 (0.04)	0.25 (0.50)^A	0.08 (0.06)	0.08 (0.15)^B
V (MACs-hemosiderin,melanin,lipofuscin) cm^3	0.07 (0.08)	0.09 (0.29)	0.02 (0.05)	0.10 (0.29)

[1]The results are shown as mean values, followed by standard deviation.

[2]For each value, the different capital letters A and B indicate statistically significant differences between seasons, for one sex (read horizontally); in respect of two-way ANOVA, followed by Tukey test.

Table 4. Number[1] (No. of MACs/mm^2) of MACs per unit area regarding pigment composition. The results are shown for both sexes separately, in two seasons: spring-summer (S-S) and autumn-winter (A-W).

Number of MACs	S-S		A-W	
	F	M	F	M
N	2	76	33	45
No. of MACs-hemosiderin/mm^2	0	0.0005 (0.002)	0.001 (0.002)	0.001 (0.001)
No. of MACs-lipofuscin/mm^2	0.001 (0.001)	0.001 (0.002)^A	0.003 (0.004)	0.004 (0.005)^B
No. of MACs-melanin/mm^2	0	0.0003 (0.001)^A	0.0003 (0.0004)	0.001 (0.001)^B
No. of MACs-hemosiderin,melanin/mm^2	0.002 (0.001)	0.0003 (0.001)	0.001 (0.002)	0.001 (0.002)
No. of MACs-hemosiderin,lipofuscin/mm^2	0	0.001 (0.01)	0.001 (0.001)	0.002 (0.004)
No. of MACs-melanin,ipofuscin/mm^2	0.01 (0.01)	0.02 (0.01)^A	0.01 (0.01)	0.01 (0.01)^B
No. of MACs-hemosiderin,melanin,lipofuscin/mm^2	0.02 (0.02)	0.005 (0.01)	0.002 (0.01)	0.003 (0.01)

[1]The results are shown as mean values, followed by standard deviation.

[2]For each value, the different capital letters A and B indicate statistically significant differences between seasons, for one sex (read horizontally); in respect of two-way ANOVA, followed by Tukey test.

Discussion

Recently, besides the CF researchers often use the BW of fishes as indicator for their wellbeing i.e. fitness (Blazer et al. 1987). For cyprinid fish, BW and TL are mainly in a positive correlation (Krüger et al. 1996; Jordanova et al. 2016a, 2016b). The results for BW and TL in this research are leaning towards higher values for the S-S season which corresponds with the spawning season of the chub. This is in accordance with the results from the research conducted on *Leuciscus cephalus*, where fish were heavier in spawning season due to the increase of the gonads (Poisot et al. 2009). Furthermore, it has been observed that the CF of the Ohrid trout has gradually declined during the reproductive cycle, specifically from the previtellogenesis period till the postspawning period (Jordanova 2004; Rebok 2006). The behavior of increased feeding during summer season of *Squalius cephalus* was also noted, especially in younger fish (Hellawell 1971). In recent time, humans affect the condition of fishes indirectly, through pollution. This has been proven with various studies of fishes from different polluted sites (Jordanova et al. 2016a, 2017; Rebok et al. 2017; Ivanova et al. 2020). The river Crn Drim is relatively unpolluted (Jordanova et al. 2018b; Hristovski et al. 2019), therefore the anthropogenic pressure did not affect the CF of the chub to a large extent. In regard to the CF in our study, the fish in both seasons are in relatively good condition (1,31 to 1,57) (Treer et al. 1999), as the heavier the fish is, in relation to its length, the better its condition is (Lamková et al. 2007).

To a great extent, the SW depends from the animal species, sex, BW and nutritional status (Tischendorf 1985; Jordanova et al. 2016b). It is established that the reproductive cycle and the season change cause significant variations in the SW (Jordanova at al. 2016b). Specifically, the spleen enlarges or shrinks depending on the spawning period (Fänge & Nilsson 1985; Rebok et al. 2011; Jordanova et al. 2016b). In our study slight increase of SW and SSI values were noted in male chubs. Our results are dissimilar from the data in a study conducted on *Leuciscus cephalus* which indicated higher investment in spleen size in females (Lamková et al. 2007). The differences in the SW can be ascribed to the natural variations in the immune system between the two sexes of fish (Jordanova et al. 2016b).

The first expected response would be on a cellular level, considering that the cells of the immune system are one of the first cells that respond to changes in the environment (Skouras 2002); therefore, MACs can be expected to respond promptly to changes in the environment. The histological inspection of the spleen proved the presence of pigmented MACs in its parenchyma. MACs that had mostly irregular or nodular shape were scattered throughout the red and the white pulp of the spleen, usually concentrated around blood vessels (Agius 1979a). However, MACs with more regular conformation i.e. spherical shape were noted as well, similarly to studies focused on other species (Jordanova et al. 2016b). Furthermore, our findings overlap with those from other studies of chub focusing on different organs (Jordanova et al. 2017) and studies of spleen from a different species (Jordanova et al. 2006; Rebok et al. 2011; Rebok et al. 2015), as well as studies focus on both different species and different

organs (Jordanova et al. 2006). In addition to the observed MACs, singular macrophage cells were noted as well. This trait is distinctive for salmonids where pigmented macrophages are randomly distributed throughout their lymphoid tissues rather than forming compact aggregates (Agius 1979b). Nonetheless, mature macrophages are known to be present in the red and white pulp of the spleen, as well as lining the ellipsoids of the spleen (Agius 1979a).

The splenic MACs are a normal occurrence in teleost fish, and their number, volume, size, as well as pigment content can vary due to some natural factors or various environmental stimuli (Agius 1980; Wolke et al. 1985; Wolke 1992; Agius & Roberts 2003; Rebok 2006; Rebok et al. 2015; Jordanova et al. 2016b; Ivanova et al. 2020). Our investigation showed that results about the No. of MACs/mm^2 are roughly balanced between the sexes and solely males in S-S display increase in the number of MACs, which consequently reflects on the values for Vv. Taken in consideration that the spawning season of chub starts in April and ends in May (Koç et al. 2007), a possible explanation for the increased No. of MACs/mm^2 in S-S is the stress that males undergo during reproduction. Related to the beginning of the reproductive cycle, there has been noted increased activity in chub (*Squalius carolitertii* Doadrio, 1988) from April onwards (Maia et al. 2006). The increased activity has been triggered from rise in the temperatures and was especially prominent in males since they invest the majority of their energy supplies in search of partner. This may lead the males to starvation. Starvation boosts the catabolism of tissues whose outcome is increased No. of MACs/mm^2 in the fish tissues (Micale & Perdichizzi 1990). There is a negative correlation between CF and the concentration of MACs on account of nutritional imbalance, specifically starvation results with increased No. of MACs/mm^2 and decreased values for CF (Schwindt et al. 2006). In our study fish display a good condition, therefore it is more likely that stress from the reproductive is responsible for the increase of the MACs parameters activities (Maia et al. 2006; Koç et al. 2007). Also, the increased No. of MACs/mm^2 can be ascribed to the lower immunity typical for fish in spawning season, which makes them more vulnerable to infection and it has been explained as a consequence of increased activity of the non-specific immune response due to low water temperatures (Rebok 2006). Aside from physiological changes, pollutants can also alter the presence of MACs (Fournie et al. 2001; Rebok 2006; Jordanova et al. 2016b; Ivanova et al. 2020). For example, greater No. of MACs/mm^2 in chub (*Squalius vardarensis* Karaman, 1928) caught in spring has been found in fish exposed to heavy metals and waste waters (Ivanova et al. 2020). The increased values for the No. of MACs/mm^2 in our males cannot be explained with pollution of the water, by virtue of low concentration of heavy metals in the river Crn Drim (Jordanova et al. 2018b; Hristovski et al. 2019).

Seeing that hemosiderin is a byproduct of the erythrocyte degradation, its concentration is growing in starving or ill fish and fish exposed to pollutants (Wolke 1992; Haaparanta et al. 1996; Ivanova et al. 2020). According to the above, the low values that the chub from the river Crn Drim exhibits for splenic MACs rich with hemosiderin, may correlate to the low concentration of heavy metals measured in the river (Jordanova et al. 2018b; Hristovski et al. 2019). Moreover, the presence of parasites in the river Crn Drim (Jordanova et al. 2018a) does not affect the hemosiderin levels to a significant extent, which makes sense if we take in consideration that diseases caused by parasites are much more frequent in cultured fishes, which undergo artificial conditions and numerous stress factors that alter their ability to successfully protect themselves against parasitic infections (De Vico et al. 2008).

Statistically significant differences can be noted in the values for Vv(MACs) ($p<0.01$), V(MACs)($p<0.05$) and No. of MACs/mm^2($p<0.001$), for the MACs that contain lipofuscin/ceroid. Moreover, because tissue catabolism is the main factor that contributes to the lipofuscin/ceroid formation, starvation may be a possible explanation for the increased No. of MACs/mm^2 (Blazer et al. 1987). Seeing that our chubs are in good condition, starvation as a reason for the increased concentration of lipofuscin/ceroid in the MACs is very unlikely. Despite that, the accumulation of lipofuscin/ceroid in the splenic MACs could be as a result of tissue damage due to pollution or bacterial, viral (Agius & Roberts 2003) or parasitic infection (De Vico et al. 2008). A significant increase of lipofuscin/ceroid in splenic MACs of gilt-head bream (*Sparus aurata*, Linnaeus 1758) infected by a flatworm (*Sparicotyle chrisophrii*, Van Beneden & Hesse 1863) was recorded. The increase in this study was ascribed to tissue and cell damage that occur in the parasitic infection. Nonetheless, lipofuscin/ceroid was also found in adequate amounts in the MACs of healthy sea breams, which was

explained as a normal constituent of the splenic MACs (De Vico et al. 2008). Furthermore, a study conducted on Swiss mice, an infection by a parasite (*Toxoplasma gondii* Nicolle & Manceaux, 1909) provided early ageing of the spleen which was proven by the increase of lipofuscin accumulations (Pereira et al. 2020). Considering that there is an abundance of parasites in the river Crn Drim (Jordanova et al. 2018a), the above can be taken into account as reasonable explanation for the higher values for lipofuscin/ceroid laden splenic MACs of the chub. Nonetheless, this increase might be a result of normal physiological accumulation of the 'wear and tear' pigment due to ageing (Terman & Brunk 1998).

Melanin is the second most common pigment in the MACs (Agius & Roberts 2003) and its higher concentrations can be a result from MACs activity at low temperatures MACs (Agius & Agbede 1984; Agius & Roberts 2003). Reminiscent of the above, our analysis displays melanin as second most common pigment, led by lipofuscin/ceroid. Statistically significant differences in the No. of MACs/mm^2 rich in melanin are prominent in males from A-W ($p<0.05$).

The pigment hemosiderin is most frequently found in combination with lipofuscin/ceroid (Agius & Roberts 2003). As statistically significant, only the value for Vv in males from A-W prevails, but the visual inspection proved that lipofuscin/ceroid was the dominant pigment in this type of MACs (Agius & Agbede 1984; Agius 1985; Agius & Roberts 2003; Jordanova et al. 2016b; Ivanova et al. 2020). In relation to the pigment composition, there is almost no variation in the results for Vv(MACs), V(MACs) and No. of MACs/mm^2, in MACs that contain only hemosiderin, which is in accordance with our observation. Higher No. of MACs/mm^2 that contain the pigment combination of hemosiderin and lipofuscin/ceroid was evident in *Squalius vardarensis* collected from waters polluted with heavy metals (Ivanova et al. 2020). However, our data does not comply with the explanation above because the river Crn Drim is not polluted with heavy metals (Jordanova et al. 2018b; Hristovski et al. 2019). However, the presence of hemosiderin might be due to parasitic infections (Dezfuli et al. 2017) especially since there are studies that prove the abundance of parasites in *Barbus rebeli* (68% of the examined fish) from the same river (Jordanova et al. 2018a).

MACs that contain combination of the pigments melanin and lipofuscin/ceroid stand out with high values for all measured parameters. Such values are evident for Vv(MACs) ($p<0.001$), V(MACs) ($p<0.01$) and No. of MACs/mm^2 ($p<0.001$) in males, explicitly with higher values in S-S. Taking into account the major bactericidal role attributed to melanin (Agius & Roberts 2003), a bacterial infection may be a likely interpretation for the increase of MACs that contain the pigment composition mentioned before. Melanin which is accompanied by lipofuscin/ceroid accumulation is associated with rivers burdened with organic matter (Ivanova et al. 2020) probably due to combination of factors including water temperatures, reproductive status etc. This is in accordance with the finding that the river Crn Drim is subjected to organic pollution as a consequence of urban and agricultural runoff (Musa et al. 2012). Another possible explanation might indicate that the increased concentration of these pigments is owing to combination of factors which takes in consideration the sex differences (Jordanova et al., 2016b) given that only values for males showed significant increase. Besides, an increased concentration of lipofuscin/ceroid and melanin infused MACs has been recorded in *Leuciscus cephalus* infected with parasitic spores from *Myxoboluscyprini* (Doflein 1898) (Holzer & Schachner 2001).

The values for the MACs that contain hemosiderin, lipofuscin/ceroid and melanin display consistency among the investigated groups, however without statistical significance. There are multiple factors that can result in increasing values for MACs that contain inclusions with the three pigments, among which: aging, starvation, combined pollutants, bacterial, viral and fungal infections, pesticides, as well as the water quality (Agius 1979a, 1979b; Wolke et al. 1985; Blazer et al. 1987; Krüger et al. 1996; Fournie et al. 2001; Agius & Roberts 2003; De Vico et al. 2008; Dezfuli et al. 2017; Ivanova et al. 2020). The insignificant variations of the chub in our research are owing to the relatively unpolluted environment.

Conclusions

The collected chubs were in good condition in both seasons, and contain normal spleen histology as well as SSI. MACs values were season dependent with slight variations between sexes. Generally, an increase of No. of MACs/mm^2 containing a combination of melanin and lipofuscin/ceroid in males

from S-S was noted, which reflects upon the Vv(MACs), V(MACs) and the total No. of MACs/mm^2. Similarly, the Vv and V of melanin and lipofuscin/ceroid containing MACs were increased in males in this season. In A-W an increase of the No. of MACs/mm^2 of lipofuscin/ceroid laden MACs contributes to the increase of values for Vv(MACs) and V(MACs) of lipofuscin/ceroid containing MACs. Although there is an increase of values for hemosiderin and lipofuscin/ceroid laden MACs, the same is most likely a result of the increase of lipofuscin/ceroid rather than hemosiderin. In conclusion, lipofuscin/ceroid is the most prominent pigment and in fishes that inhabit relatively unpolluted waters, this pigment is often accompanied by melanin inclusions, notably during the S-S season. The results from this paper can be used as preliminary guide for the studies dedicated to biomonitoring of freshwater ecosystems in the Republic of North Macedonia.

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