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SPLEEN HISTOLOGY IN THE FEMALE OHRID TROUT, *SALMO LETNICA* (KAR.) (TELEOSTEI, SALMONIDAE) DURING THE REPRODUCTIVE CYCLE

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Abstract – The Ohrid trout is an endemic autochthonous species dating from the Tertiary period with an unique ecological and commercial value. We still lack the basic knowledge of normal histology of many visceral organs in this species, including the spleen. To tackle this limitation, the investigation was focused on a histological description of the spleen and the influence of the breeding cycle on some aspects of the spleen (the relative volumes of the red and white pulp). Examination by light microscopy showed the same basic structural features of the salmonid spleen: red and white pulp with randomly distributed melanomacrophage centers, surrounded by a fibrous capsule. White pulp comprises about 35-40% of the volume of the parenchyma, 2-fold less than red pulp. During the breeding cycle, we observed a significant increase of white pulp and decrease of red pulp in the spawning stage compared to earlier stages. After spawning, the relative volumes of white and red pulp exhibit a tendency to decrease and increase, respectively. Based on correlations between the amount of white and red pulp and the ovary somatic ratio, we assumed there could be a connection between the sex steroid status and the pulp content in the spleen.

Keywords: Ohrid trout, Salmo letnica, spleen, white and red pulp, stereology

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INTRODUCTION

The Ohrid trout, *Salmo letnica* (Karaman, 1924) is an endemic representative species from the family of trout and salmons, naturally distributed only in Lake Ohrid, a refuge for numerous freshwater organisms from the Tertiary period whose close representatives can be found only as fossil remains. This species is important not only as a "living fossil", but also from a local ecological as well as commercial point of view which is proving to be unsustainable on the long term unless proper scientifically based pollution regulation and fish management policies are enforced in Lake Ohrid. So it is surprising that we still lack the basic knowledge of numerous general aspects of these species. Despite recent research on the ontogenetic development of the skeleton (Ristovska et al., 2006; Ristovska, 2007), spermiogenesis (Tavciovska-Vasileva, 1999), and liver microanatomy (Jordanova, 2004; Jordanova et al., 2007; 2008; 2009), the Ohrid trout continues to be a largely unstudied species at the a histological level. Studies of the spleen and many other visceral organs in the Ohrid trout are unfortunately lacking and this is an important research area which the present study hopes to initiate.

The spleen is the only lymph-node organ in teleost fish (Roberts, 2001). The histological investigations of the spleen in teleosts have been mainly focused on the compartments that are important for the defense systems of the fishes; the lymphocytes and the macrophages (Montero et al., 1999; Schmitt and Dethloff, 2000; Fournie et al., 2001; Kurtovic et al., 2008), and/or alterations

in this organ caused by the presence of different toxicants in the environment (Gogal et al., 1999; Garcia-Abiado et al., 2004). Nowadays much less attention is dedicated to the spleen structure and microanatomy despite the fact that recent evidence indicates that for the detection and interpretation of histopathological changes in fish, prior knowledge of the normal histological state of each species is important (Hinton and Lauren, 1990; Hinton et al., 1992; Hinton, 1993). More specifically, spleen size and weight in fish varies according to several natural factors such as age and gender (Tischendorf, 1985). Also, in the Atlantic salmon (*Salmo salar*) and some other salmonids great seasonal changes in spleen size can occur (reviewed by Fänge and Nilsson, 1985). Moreover, the study by Press et al. (1994) indicated that the proportion of the spleen occupied by red and white pulp varied widely between Atlantic salmon individuals. However, despite such seasonal-related and inter-individual changes (especially in salmonids) hypothesized in literature, they have not yet been satisfactory (quantitatively) demonstrated.

Since the teleosts number is more than 20,000 fresh and salt-water species, it is not surprising that there can be large morphological differences between fish species. Such variations between species, even in the same family, have indicated the importance of investigations into the organ structure for individual fish species (Rocha and Monteiro, 1999). Description of the normal structure and normal ranges of values for a particular population of fish are even more crucial for commercial and ecologically important species, as emphasized by Rocha et al. (1995).

Taking the aforementioned into account, our primary objective was to describe, for the first time, the microscopic anatomy of the spleen of the Ohrid trout, and to identify the general alterations in organ compartments (red and white pulp), if any, following gonadal development. All new knowledge will help to draw attention to the Ohrid trout species and ongoing efforts to better understand and thus better handle and protect the Ohrid trout. As this trout is a local top predator, it is particularly vulnerable to the impacts of bioaccumulating toxicants. This study offers baseline knowledge for future studies of the eventual negative effects of water pollution or other stressors on the spleen, as a unique organ in the immune defense system of the fish body.

MATERIAL AND METHODS

Fish collection

The adult female Ohrid trout (*Salmo letnica* Karaman, 1924) used in this study were collected in Lake Ohrid ($41^0$05'N, $20^0$45'E), located between the southwest part of Macedonia and the northwest part of Albania. All the specimens (n = 25) were randomly selected from legally obtained professional fisheries catches. Collections were carried out at two month intervals, from October 2001 until September 2002. The weight and total length of the Ohrid trout were 470-1,290 g and 300-505 mm, respectively. Five fish per group were sampled for each of the 5 key gonad stages (pre-vitellogenesis, early vitellogenesis, late vitellogenesis, spawning, and post-spawning). Necropsy and material collection were carried out on board and as soon as possible after fishing. To prevent artifacts of handling, the specimens were kept alive until processing (generally less than one hour), with frequent replenishment of water from the Lake.

Tissue collection and processing

All the animals were inspected externally and internally for gross abnormalities. Fork length (FL) and body weight (BW) were recorded and used to calculate the condition factor, CF = 100 x BW (g) x FL $(mm)^{-3}$, as an indicator of the general health status or "well being" of the specimens. The fish were sacrificed by severing the spinal cord and dissected immediately after. The spleen and ovary were removed, and then cut into 3-4mm thick slides and fixed in 10% neutral buffered formalin. For light microscopy (LM) analysis the sampled pieces were routinely dehydrated through ascending series of ethanol, cleared in xylene, infiltrated and embedded into paraffin. Sections were stained with hematoxylin and eosin (H&E).

Quantitative analyses

From the spleen piece we took serial sections (5 μm thick), picking some for analysis by a systematic random sampling approach, so as to obtain a representative final set of slides (about 5 per spleen). From each section, 15-18 systematically sampled fields were observed and quantified at a final magnification of 100 x (the only magnification which can ensure good visualization of the white and red pulp) with the first field being randomly selected. In average, 100 fields per fish were systematically selected and studied. Unbiased stereological techniques based on manual direct point counting (Feere and Weibel, 1967) were used to estimate the relative volume of red [V_V (red pulp/spleen)] and white pulp [V_V (white pulp/spleen)] expressed as percentages, according to the following general formula:

$$V_V \text{ (structure, reference)} = V_V \text{ (s, r)} = [P(s) \times 100] \div P(r),$$

where V_V (structure, reference) is the percentage of the total volume of a defined reference space occupied by the investigated structure; P(r) is number of points which lie over the whole referent space; P(s) is number of points falling over the inspected structure.

In this study, parenchyma was determined as the reference space. For point counting, a square lattice grid with 180 points was inserted into the ocular of the microscopy.

Statistical analyses

The data are presented as group means from individual fish values, accompanied with the respective coefficient of variation (CV = SD/Mean), thus allowing an immediate assessment of the inter-individual variability. For statistical analyses, the software STATISTICA 7.0 for Windows was used. After checking the normality and homogeneity of variances of the data sets, they were analyzed by one-way ANOVA. Whenever the ANOVA disclosed significant results, comparisons among the breeding stages were made using the post-hoc Tukey's test. Spearman's rank correlation analyses were used to find linear associations between the amounts of red and white pulp on the one hand, and, on the other hand, the ovary somatic index (GSI). Differences were considered significant when $p \leq 0.05$.

RESULTS

Condition factor

The CF gradually decreased from 1.4 (CV = 0.08) in pre-vitellogenesis and early vitellogenesis to 1.3 (CV = 0.05) at late vitellogenesis and 1.2 (CV = 0.19) in spawning, and showed the lowest value in post-spawning 1.1 (CV = 0.08), but statistical significant differences were not observed. The CF values indicated that the investigated Ohrid trout, although we used wild specimens, were in good general health.

Qualitative findings

In the Ohrid trout the spleen is dark red in color and has sharply defined edges. The organ consists of two general components, the white pulp and the red pulp, enclosed by a capsule (Fig. 1.). The splenic capsule is composed of connective tissue forming a thin outer wall, and does not project any trabeculae into the parenchyma. Differences in the thickness of the capsule were observed in the examined Ohrid trout individuals. Namely, the extent of the spleen capsule became thicker in the spawning and post-spawning individuals - average 29.7μm, regarding the fish that are in vitellogenesis – average 10.9μm (pre-vitellogenesis and advanced vitellogenesis).

The main elements of the spleen parenchyma are white and red pulp (Fig. 1). In the Ohrid trout a distinction between them is possible, although demarcation between red and white pulp is poorly defined. In particular, demarcation of the pulps is more easily visually detected visually in the spawning stage in comparison with the other gonad stages (before and after spawning). The white pulp is composed of lymphoid tissue, surrounding small arteries and diffusely intermeshing with the red pulp. The red pulp is

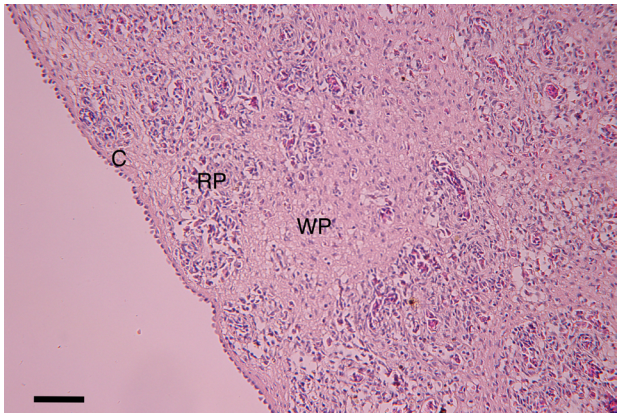

Fig. 1. Light micrograph of the spleen of the Ohrid trout illustrating components of the parenchyma, red (RP) and white pulp (WP) enclosed by thin capsule (C). H&E. Bar = 60 μm.

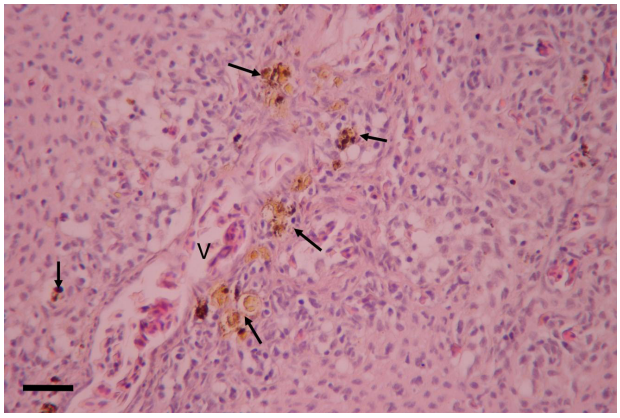

Fig. 2. Light micrograph of the spleen of the Ohrid trout. Note the melanomacrophage centers (arrows) adjacent to the blood vessel. V, vein. H&E. Bar = 30 μm.

composed of a reticular cell network and supporting blood-filled sinusoids that hold diverse cell populations, including macrophages and lymphocytes.

Scattered through the parenchyma are numerous accumulations of the pigmented macrophages, i.e. melanomacrophage centers (MMC) were found (Fig. 2). They appear as poorly organized, irregular cell clusters which may be located in the white, as well as in the red pulp, usually concentrated in a large amount around the blood vessels. In the H&E stained sections, the MMC were packed with dark brown-black or brown-yellow deposits.

Quantitative findings

The relative volumes (V_V) of the white and red pulp in relation to spleen parenchyma are presented in Table 1. The CVs showed that V_V of the bout pulps had a more or less identical and low inter-animal variability. Further analyses showed that the white pulp occupied approximately 35-40% of the volume of the parenchyma. The relative volume of red pulp was around 60%. Along the breeding cycle, the relative volume (amount) of red pulp is decreasing from the pre-vitellogenesis to the spawning stage, and shows a tendency to increase to the post-spawning stage. In contrast with the red pulp, the relative volume of white pulp displays the opposite patterns of change during the reproductive cycle: a significant increase in the spawning as compared with the previous gonad stages and a tendency to decrease in the post-spawning stage.

The correlation analyses further showed that the relative volume of white and red pulp changes as the ovary matures, as positive correlations existed between the GSI and the relative volume of white pulp ($r = 0.43$; $p < 0.05$), and negative correlations existed between GSI and relative volume of red pulp ($r = -0.43$; $p < 0.05$).

DISCUSSION

The first description of spleen structure was presented by Theodor Billroth in 1857 (citated by Zapata et al., 1996). Over the years, spleen structure and function in many vertebrate species, including fish, has been precisely described. However, in the Ohrid trout, our knowledge about the cytology and histology of the spleen and other visceral organs is scarce. Since no histological studies of the spleen of the Ohrid trout have been reported previously, here we used wild feral fish, which, according to the condition factor and necropsy-based analyses (through visual assessment of external/internal abnormality), were in good health. These basic studies are important for comparative morphological analyses and

Table 1. Relative volume (V_V), expressed in %, of the red and white pulp in the spleen of the Ohrid trout during different reproductive (ovary) stages*.

Gonadal Stage	V_V (red pulp, spleen)	V_V (white pulp, spleen)
Pre-vitellogenesis	65.07 (0.01)[a]	34.93 (0.02)[a]
Early vitellogenesis	64.56 (0.02)[a]	35.44 (0.03)[a]
Late vitellogenesis	64.96 (0.04)[a]	35.03 (0.07)[a]
Spawning	59.67 (0.05)[b]	40.32 (0.08)[b]
Post-spawning	62.15 (0.04)[ab]	37.85 (0.07)[ab]

* Data are expressed as the mean (coefficient of variation). Within a column, values with different superscript letters are significantly different ($p < 0.05$), according to the post-hoc Tukey's test.

also for understanding of the pathological or physiological alterations of the organs, either related to infections or environmental disease.

The basic structure of the spleen in the Ohrid trout, the structure of the capsule and of the two general parenchyma components (the lymphopoietic white pulp and the hematopoietic red pulp) are similar to that described for other fish species (Zapata, 1980, 1982; Fänge, 1987; Press et al., 1994; Espenes et al., 1995; Atsuta et al., 1999; Fournier-Betz et al., 2000; Lin et al., 2005). From our qualitative description of spleen pulps, one detail deserves notice. Although in several teleosts, including some salmonids, the white pulp contains ellipsoids (Espenes et al., 1995, 1995a; Furukawa et al., 2002), it was not possible to detect them in the examined trout. This suggests that the spleen in the Ohrid trout probably has indistinct ellipsoids or they are absent as in some non-salmonid fish (Yoffey, 1929, citated by Zapata et al., 1996).

In the Ohrid trout, separation of the red and white pulp is much less distinct than in other fish species (Zapata et al., 1996; Powell, 2000). This is a morphological characteristic of the spleen in salmonids (Powell, 2000). Besides this, in our trout demarcation of the pulps is much less distinct during previtellogenesis and vitellogenesis in contrast with the spawning and the post-spawning period. The only references to a study where the relations between season and demarcation of the pulps are mentioned were made with frogs (Bassiouni, 1997) and snakes (Bassiouni, 1991). In frogs the author noted a less distinct demarcation in the spleen in the animals during winter hibernation. In contrast, in snakes a poor demarcation was evident in the spring and autumn. Our results in general did not agree with this trend since demarcation is much more distinct in the spawning period, and spawning of the Ohrid trout occurs in winter (from December to March). This suggests species-specific effects of the season on pulp demarcations.

As to the quantification of red and white pulp, our data showed that red pulp in the spleen of the Ohrid trout is twice as large as white pulp. The same proportions of the two pulps were detected in anurans (Tischendorf, 1985). Unfortunately, in the literature we did not find data for the relative volumes of white and red pulp in any fish species. The only quantitative study which, according to our knowledge, contains such data, was done in mammals (van Krieken et al., 1983; Jensen and Kristensen, 1986; Vojdani et al., 2010). The relative volume of white and red pulp, respectively 8.4-13.4% and 87.7-82.4% of the spleen parenchyma in mammals (Jensen and Kristensen, 1986), is lower and higher when compared with data from the Ohrid trout. We can only conclude that without quantitative data for other fish species a more precise comparison of the degree to which the white and red pulp amount in Ohrid trout is similar or different cannot be made.

It is known that changes in spleen size occur along the breeding cycle in fish (Fänge and Nilsson, 1985; Kortet et al., 2003), which in some salmonids is accompanied by a drastic change of spleen form

in the phase of maximal food intake or exclusively sexual activity (Tischendorf, 1985). Concerning the effects of season and/or gonadal maturation on the spleen pulp, we found that the white pulp significantly increased and the red pulp decreased from pre-vitellogenesis to spawning, and then has a tendency to decrease or increase respectively towards post-spawning. These changes in the spleen pulps in the Ohrid trout may be connected to physiological alterations of the organ during the reproductive cycle. For example, as fish mature sexually the proportion of circulating lymphocytes declines (Alcorn et al., 2002), and right after spawning their number increases (Kortet et al., 2003). Moreover, in this study correlations were disclosed between GSI and both the red and white pulp. This strongly suggests connections between gonadal maturation, sex steroids status and both pulp relative volumes. In accordance with this, some reports on frogs and mammals have shown that hormones can affect red and white pulp volume. For example, a synthetic corticosteroid dexamethasone can decrease the white pulp of the spleen (Garrido et al., 1987). Estrogen also caused a reduction of the spleen's white pulp volume (Tchernitchin et al., 1990).

Irrespective of seasonal changes, in fish white pulp proliferation, lymphocyte depletion (Schwaiger et al., 1996; Garcia-Abiado et al., 2004), as well as an increase in the spleen size (Gogal et al., 1999), has often been associated with environmental contamination. Different experimentally induced viral and bacterial infections can also provoke splenomegaly (Powell, 2000; Decostere et al., 2001) as well as many diverse lesions in the spleen pulp, such as congestion (Falk et al., 1995; Simko et al., 2000), anemia (Decostere et al., 2001), hemorrhages and necrosis (Ekman and Norrgren, 2003). Thus, it cannot be excluded that changes in the pulp volumes detected in Ohrid trout were associated with a toxicant present in the Lake's ecosystem. However, taking into account that in our study the wild trout displayed apparent good health according to CF and the absence of signs of disease, the more likely cause for the increased and decreased volume of white and red pulp, respectively, in spawning are natural breeding-related events.

In conclusion, this paper presents a first study of the histological organization of the spleen of the Ohrid trout. Our contribution is unique in its scope because it is the first quantitative assessment of spleen red and white pulp in one salmonid species during an entire gonadal cycle. Because alterations of the spleen can occur in some pathological conditions (Kinnunen and Johnson, 1985; Schwaiger et al., 1996; Gogal et al., 1999; Garcia-Abiado et al., 2004), the qualitative and quantitative data obtained in this study can be used in the future as standards for normal structure.

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