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COMPARATIVE HISTOLOGY OF TESTES OF BROWN (*SALMO TRUTTA M. FARIO)* AND CALIFORNIA *(ONCORHYNCHUS MYKISS)* TROUT DURING THE SPAWNING PERIOD

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Abstract: The testes of fish are paired organs, of a variable shape in different species of fish. Their structure in the salmonid species is lobular. With the histological assays, we established that the lobes were separated by the connective tissue septa, which, given the intensity of spermatogenesis in the studied groups of fish (*Salmo trutta m. Fario; Oncorhynchus mykiss*) sporadically disappear, in fact, they break. In the space between the lobes there are also cross-sections of blood vessels with visible erythrocytes. During the spermatogenesis, in the interstitium there are clearly observable interstitial endocrine (Leydig) cells that excrete steroid hormones. The intensity of the spermatogenesis in the studied fish varies, which is concluded on the basis of the presence of the spermatogenesis cells. In nature, the reproductive cycle in fish is mostly based on an annual cycle, and that is why different stages of reproduction take place at a different temperature and during a different photoperiod. Hence, regardless of the same time period, the spawning time in November, different types of breeding, and finally salmon farming, point to the very important factors that influence reproduction - diet and microclimatic conditions.

Key words: Brown trout, California trout, histology of testes, spawning

Introduction

Bosnia and Herzegovina is a country with high potentials for fish production, considering that it has natural resources of unpolluted water, which is the main pre-condition for the development of fishing industry. Natural water in Bosnia and Herzegovina consists of 20,000 km of rivers and streams, 1,400 ha of coastal line and 4,000 ha of lakes. Fish producers often note that fish is the only food of animal origin that has made it to the tables in the EU countries. According to the data from

the Bosnia and Herzegovina Agency for Statistics for 2012, Bosnia and Herzegovina produced 3,584.2 t of fish, by 11.5 % less than in 2011. According to the agency's data, carp accounts for 16% of the total amount of consumer fish, trout for 78.6% and other fish for 5.4 %. There are many EU regulations and frequently their changes, amendments or replacements are in line with the new scientific findings, including this area of animal production. According to the EC 2013 Progress Report on Bosnia and Herzegovina - European Commission (Brussels, October 16, 2013, SWD (2013) 700 FINAL), "Regarding fisheries, the Republika Srpska and the Federation of Bosnia and Herzegovina adopted legislations on freshwater fisheries that are partially aligned with the *acquis.* Bosnia and Herzegovina needs to step up efforts to implement the *acquis* for this area in order to facilitate an increase in exports of fish and fishery products to EU." All of the above stated facts point to Bosnia and Herzegovina's great opportunities to increase production of consumer fish, fish progeny and ikra, as the main or additional source of income for population that would be sufficient not only for domestic but for the demanding international market as well. Given that many experts from different scientific aspects invest great efforts to realize these ideas, keeping in mind that this branch of production is very demanding and complicated, both from the aspects of production and placement - uncertainty of placement, a special attention is directed at increasing the reproductive capacity of fish cultured in our fish ponds. With histological assays of the testes of Brown *(Salmo trutta m. Fario*) and California trout (*Oncorhynchus mykiss*) taken from different sites during the spawning time, we attempted to study the intensity of spermatogenesis in these species.

Material and methods

The material required for the histological assays was sampled from different sites of the rivers Vrbas and Neretva in November. The total of 20 males was taken, 10 from different locations and of different breeding. Brown trout was taken from the free watercourse of the river Vrbas, while California trout was cultured in cages on the river Neretva.

Upon fishing, testes were carefully removed from the surrounding tissue and placed in powdered solution of 10% formaldehyde until the moment of their embedding in the paraffin blocks. The testes were placed in 70% alcohol for two days, then in 96% alcohol for one day, and in the end in 100% alcohol for one day. Later, they were transferred into a mixture of 100% alcohol and toluol for two hours and at the end in toluol for four hours. Such prepared testes were placed in paraffin I for five hours and paraffin II for twelve hours, completing the embedding procedure in the paraffin blocks. The processing of the testes from fixation to paraffin embedding was performed on a rotational tissue processor (MICROM

model STP 120). After the embedding, the testes were cut using digital microtome (LEICA RM 2145), several serial cuts from 0.5 to 1.5 micrometer thick. The pieces were placed on glass slides and then they were deparaffinized by being taken through a set of alcohol ranging from lower to higher concentrations. After that, they were stained with hematoxylin-eosine, covered by glass cover and glued with Canada balsam. The histological assays were done with a light microscope under magnification of 100, 200 and 400 times. The results of the histological assays were descriptively displayed in the form of microphotographs.

Results and discussion

It should be emphasized that in fish with external fertilization, spermatozoids are excreted together with semen into their immediate surroundings during the spawning period, where they are momentarily activated by flagella motions and they engage in different physical interactions: osmotic pressure appears on the membrane of a spermatozoid; the surface-to volume-ratio of the fish spermatozoids is much larger than in the majority of other species, as well as the physical connection between the flagella and the surfaces they react with (such is egg shell), (*Cosson et al., 2008*). The same authors note the effect of the bioenergetics aspects, due to which spermatozoids swim fast, reaching a high frequency of flagellum (from 70-100 Hz), implying a large consumption of ATP. It is a known fact that the fish reproductive system, especially that of the salmonid species, is significantly affected by climate factors, especially by water temperature, saturation with oxygen, pH of water, altitude, breeding method, diet and so on. Photoperiod and temperature have different impact on different species of fish, and they can even have different impact on an individual within one species of fish, which depends on the reproductive cycle of the individual itself – there is no universal rule. Of course, all of it is regulated primarily by neuroendocrine system, i.e. the pituitary gland, its hormones that directly act on gonads. The quality and morphology of semen and sperm, i.e. anatomic and functional parameters of the reproductive system in male fish, as well as the content and the quantitative and qualitative composition of sperm are influenced by ambient conditions, water temperature (*Krol et al., 2006*). Early studies of cyclic gonads in male fish also bring into the connection the factors mentioned, as well as the connection of reproductive physiology and activity of the pituitary (*Khannai et al., 1966*). The pituitary gland in fish is built of two basic parts, adenohypophysis and neurohypophysis. Adenohypophysis is divided on three areas marked as proadenohypophysis, mesoadenohypophysis and metaadenohypophysis (*Kozaric, 2001*). All three parts of the pituitary gland are built of different types of cells excreting different hormones with different activity. The cells of mesoadenohypophysis are particularly interesting for the salmonid species. They are divided in three groups,

and the highest in number are those that excrete growth hormone, which, among other things, act on the reproductive organs of fish, and gonadotroph cells, which are basophile cells, and their number varies depending on the sexual cycle of fish (*Bone et al., 1995*).

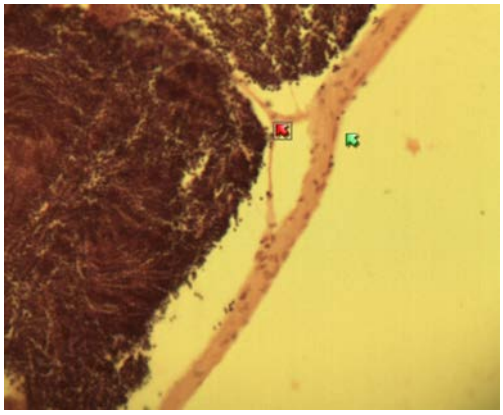

Figure 1. Connective-tissue lining with visible septa (200x, hematoxylin-eosine), Neretva

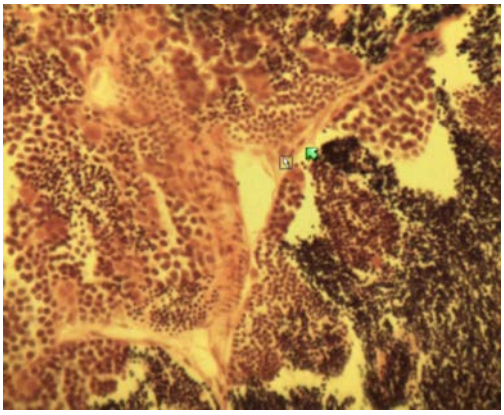

Figure 2. Arrows indicate Myoepithelial and Leydig cells in the interstitum (200x, hematoxylin-eosine), Vrbas

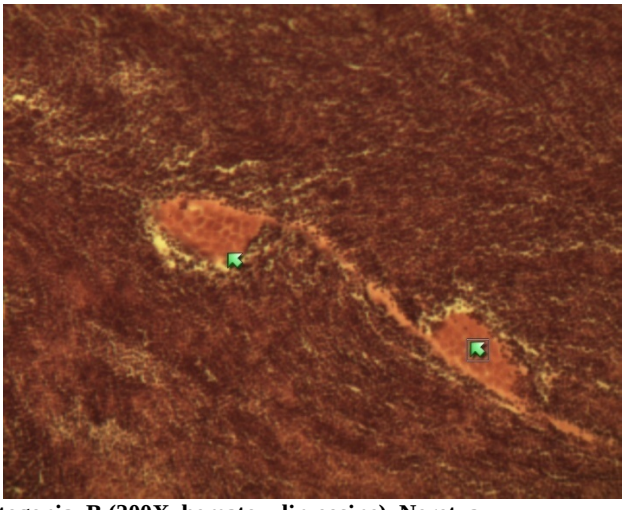

Figure 3. Spermatogonia B (200X, hematoxylin-eosine), Neretva

The process of spermatogenesis is generally a seasonal phenomenon in fish that begins several months before the start of the spawning period, depending on the fish species. Spermatogenesis is the process that occurs throughout the year, but in male fish it is active once a year.Spermatogonia A and B make the first phase of

spermatogenesis, and they contain a diploid number of the chromosome, 2nxy. Mitosis of each spermatogonium B creates two spermatocytes of the first order, 2nxy. A further stage of meiosis I forms spermatocytes of the II order with a haploid number of chromosomes, either nx or ny. Spermatocytes of the II order are short lived; they are divided through meiosis II, forming the spermatids. They are not being divided but rather transformed into mature sexual cells - spermatozoids (*Vergilio et al., 2012*), and it should also be noted that the endocrine system of vertebrates is involved as the main controlling system of spermatogenesis. Males reach maturity usually before females, which is usually around age two in the salmonid species, while females become sexually mature at age three. The spawning period for the salmonid species in our climate conditions (moderate-continental climate) is from November until March. The histological assays of testes in Brown trout (*Salmo trutta m. Fario*) and California trout (*Oncorhynchus mykiss*) that were sampled at different locations in Bosnia and Herzegovina, established discrepancies in the micro-structure and the presence of cells of spermatogenesis during the same time period, but under different microclimatic conditions and diet. The testes of Brown trout, fished from Vrbas, in Central Bosnia, show more intensive picture of spermatogenesis. The water temperature at the time of fishing was 6 °C, while oxygen saturation was 11 ppma. On the surface of testes, there is a very thin connective tissue lining, the starting place of the connective tissue septa, which are broken and disappear in an abundant, active part of testes – parenchyma (Figure 7). Along with the visible connective tissue septa, which imply lobularity of testes, there are also elongated, spindle-like myoepithelial cells and clearly observable polymorphic Leydig cells with spherical nucleus, which are the endocrine cells that excrete steroid hormones (Figure 2). In the parenchyma of testes, there are numerous spermatogonia A, large, round cells with clearly visible, large, light colored basophilic nucleus, and spermatogonia B, which are somewhat smaller and with darker colored nucleus (Figure 4). Division of spermatogonia creates primary spermatocytes that following the meiotic division create the secondary ones. On the histological preparations, spermatocytes of the first order are somewhat bigger than the spermatocytes of the second order, and with somewhat larger, dark colored nuclei. Both types of spermatocytes are grouped in irregular groups within the parenchyma of testes (Figure 4). In the parenchyma of Brown trout, there are Sertoli cells, predominantly located beside spermatogonia A and B, of irregular, pear-like shape, with somewhat darker colored nucleus in relation to spermatogonia. Sertoli cells have a nutrient role, and they also excrete steroid hormones in fish. They show cyclical changes that are connected with spermatogenesis, and they enable differentiation of spermatogonia into sperm (*Katarzyna Dzewulska et al., 2002).* In semen channels, there are also Sertoli cells, supportive, nutrient cells, light colored, inserted among the cells of spermatogenesis. During the spermatogenesis, they are subjected to cytomorphological changes, vacuolization, degeneration, i.e. disorganization of the

ultra-structure, even the necrotic material of Sertoli cells (*Tavchiovska-Vasileva et al., 2012*). The secondary spermatocytes are divided by another meiotic division and they create spermatids that are stained intensively basophilic and have extremely scarce cytoplasm. Each spermatid will develop into spermatozoa (Figure 6).

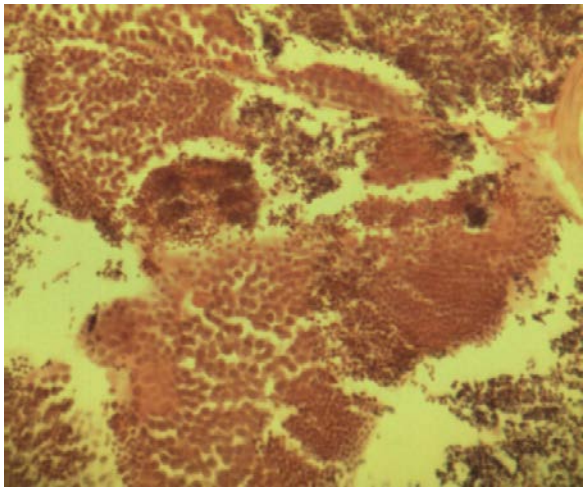

Figure 4. Cells of spermatogenesis; Spermatid, Sertoli cells, spermatozoa and spermatocytes of the I and II order, Spermatogonia A and B (200x, hematoxylin-eosine), Vrbas

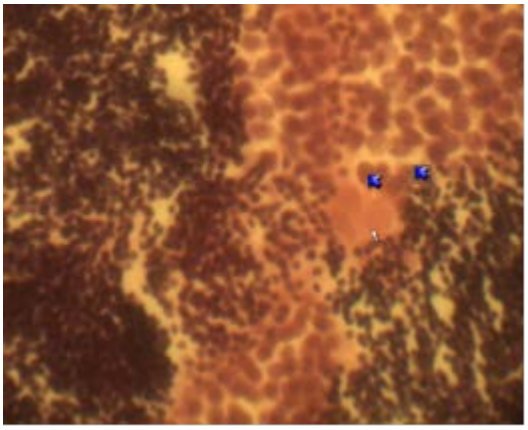

Figure 5. Spermatogonia A and B, Sertoli cells (400x, hematoxylin-eosine), Neretva

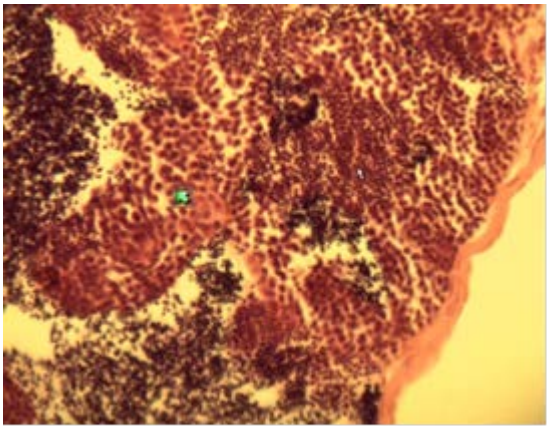

Figure 6. Spermatozoa, spermatid (200x, hematoxylin-eosine), Vrbas

The histological characteristics of California trout fished from the river Neretva in November, previously cultured in the cage system and fed with pelleted, commercial food, show discrepancies in the microstructure in relation to the previously described species. The temperature of the river Neretva at the time of fishing was 8.5 °C, and oxygen saturation was 10 ppm. The surface of testes shows

clearly differentiated connective-tissue lining, where there are noticeable smooth muscle and spindle cells and a few intersections of capillaries, the starting place of the connective tissue septum that separates testes into lobes, therefore, the lobularity is more clearly expressed than in Brown trout (Figure 1). Within the lobes, there is a large number of present spermatozoa, the cells of smaller dimension and with darkly colored nuclei, and on the lobes, where the connective tissue septum is not in continuity, there are sporadically observable Spermatogonia A, the largest spermatogenesis cells, with clearly visible nucleus and cytoplasm (Figure 5), and among which one can notice the nuclei of the nutrient oval Sertoli cells. Spermatogonia B (Figure 3) are smaller cells with darkly colored nuclei and more scarce cytoplasm. In adult, sexually more mature salmonid species, spermatogenesis occurs seasonally, which is closely associated with climate conditions - microclimatic conditions *(Genten et al., 2009*). The continuing changes take place at the cellular level, starting with germinative cells located in the parenchyma of testes, representing the largest cells of spermatogenesis, which are going through the mitotic division, the first division, and then the second division, followed by the first meiotic division of the cells that produce the secondary spermatocytes with a haploid number of chromosomes. These then go through the second meiotic division and they produce spermatids with extremely basophilic nucleus and scarce cytoplasm. Each spermatid will then give a spermatozoon (*Ando et al., 2000*).

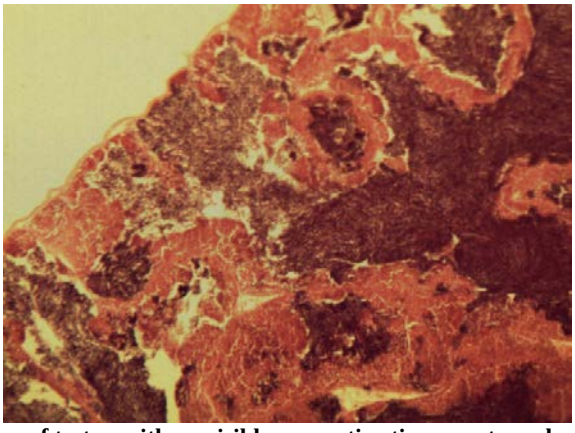

Figure 7. Parenchyma of testes, with no visible connective tissue septa and very thin connective tissue capsules (200x, hematoxylin-eosine) Vrbas

Conclusions

- The testes of Brown trout and California trout are lobular structures; reproduction cycle is based on an annual cycle and

different reproduction stages – spermatogenesis is conditioned by different exogenous and endogenous factors.

- The intensity of spermatogenesis in these species during the spawning time point to the diversities of the microstructure and spermatogenesis cells under variable microclimatic conditions and diet.
- The presence of Sertoli cells within the lobes and their morphs confirm their supportive and nutrient functions to the cells of spermatogenesis
- Leydig cells, their presence point to their neuroendocrine function and participation in the regulation of reproductive cycle, from the aspect of endogenous factors.
- The intensity of spermatogenesis, based on the presence of the spermatogenesis cells varies, i.e. spermatogenesis in Brown trout in relation to California trout is intensified, clear and advanced spermatogenesis and presence of all spermatogenesis cells in Brown trout.

Uporedna histologija testesa potočne (*salmo trutta m. fario)* i kalifornijske *(oncorhynchus mykiss)* pastrmke u periodu mresta

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Rezime

Testesi-semenici riba su parni organi, različitih oblika kod različitih vrsta riba. Kod pastrmskih vrsta su režnjevite građe. Histološkim istraživanjima smo utvrdili da su režnjići odeljeni vezivno-tkivnim pregradama, koje se obzirom na intenzitet spermatogeneze u istraživanim skupinama riba (*Salmo trutta m. Fario; Oncorhynchus mykiss*) mestimično gube, zapravo pucaju. U prostorima između režnjića se mogu zapaziti i preseci krvnih sudova sa vidljivim eritrocitima. Tokom spermatogeneze u intersticijumu su jasno uočljive endokrine, intersticijske (Leydigove) ćelije koje luče steroidne hormone. Intenzitet spermatogeneze u istraživanim grupama riba je različit, što se zaključuje na osnovu prisustva ćelija spermatogeneze. U prirodi, reproduktivni ciklus riba je uglavnom baziran na godišnjem ciklusu i zato se različite faze reprodukcije odigravaju na različitoj temperaturi i fotoperiodu. Dakle, bez obzira na isti vremenski period, vreme mresta u mesecu novembru, ali različite načine uzgoja, te na koncu i salmonikulture,

upućuju na veoma bitne činioce koji utiču na reprodukciju - ishrana i mikroklimatski uslovi.

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