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Morphology of the epaxial musculature and osteological development of the early developmental stages of softmouth trout (*Salmothymus obtusirostris*, Heckel, 1851)

Srebrenka Nejedli[1]*, Zvonimir Kozarić[1], Ivan Katavić[2], Ivona Žura Žaja[3], and Ivana Tlak Gajger[4]

[1]*Department of Anatomy, Histology and Embryology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

[2]*Institute of Oceanography and Fisheries, Split, Croatia*

[3]*Department of Physiology and Radiobiology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

[4]*Department of Biology and Pathology of Fish and Bees, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

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ABSTRACT

Investigations were carried out on the early developmental stages of softmouth trout (*Salmothymus obtusirostris*, Heckel, 1851), taken from a hatchery in Proložac near the River Vrljika, Croatia. Samples were collected every two days 10-56 days post-hatching (dph), fixed in 10% buffered formalin, embedded in paraffin, cut into 10 µm thick serial longitudinal sections and stained with Hematoxylin and Eosin, Toluidin Blue, Alcian Blue-specific (pH = 2.5) and Alcian Blue and Alizarin Red. The number of myomeres in the dorsal epaxial musculature of the softmouth trout increased between 10-18 dph and 50-56 dph to either 42 or 59 myomeres, respectively. Total body length increased from a minimal value of 19.57 mm to a maximum value of 25.80 mm between 10-56 dph. The number of vertebrae in all investigated groups was 59. Through this follow-up of the period of development of softmouth trout to the stage of complete yolk sac absorption it was established that it takes about 24 to 26 dph when they start opening their mouth and when all the bones of the head and all fins are visible. In the period of 26-56 dph the number of myomeres was 55-59, and this could be used for taxonomic identification of the early developmental stages of softmouth trout. Throughout whole investigation period no signs of ossification in the vertebral column, fins and head bones were observed or any skeletal malformations.

Key words: softmouth trout; *Salmothymus obtusirostris,* Heckel, 1851; early developmental stages; epaxial muscles; osteological development

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*Corresponding author:

Srebrenka Nejedli, DVM, Department of Anatomy, Histology and Embryology, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia, Phone: + 385 1 2390 242; E-mail: snejedli@vef.hr

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Introduction

The softmouth trout (*S. obtusirosis*) is an endemic species of the Adriatic Basin (ĆALETA et al., 2015) assigned to the family of salmonids on the Balkan peninsula KOTTELAT and FREYHOF (2007). Its natural distribution is limited to a few Adriatic rivers in Croatia, Bosnia and Herzegovina and Montenegro. There are five natural populations of this subspecies, found in the rivers Neretva, Vrljika, Jadro, Krka and its confluents, and the Zeta (ĆALETA et al., 2015). It has been noted that the softmouth trout was previously considered as a separate genus of *Salmothymus*, and its populations in different rivers and their confluents sometimes have different subspecies (ROGLIĆ, 2012).

The River Vrljika has rich biological diversity and endemic species of freshwater fish, including *S. obtusirostris,* which are the only salmonid known to inhabit this river (SNOJ et al., 2008). The body is spindle-shaped with a short head and the teeth are small or non-existent. The scales on the body of the fish are small, and field of the lateral line is between 100 and 120 (ROGLIĆ, 2012). They have an adipose fin situated behind the dorsal fin, at the beginning of the caudal peduncle, body coloration varies and the *S. obtusirostris* from the River Vrljika is yellowish-green to golden yellow (ĆALETA et al., 2015). Usually, in *S. obtusirostris* the final development of gonads occurs in the spring, mainly from February to April (ĆALETA et al., 2015).

Aquaculture enables rapid production of good quality fish meat. Growth of farmed fish, especially of the larvae in the developing stages (as is the case in nature) depends on many environmental factors, such as water temperature, the type, amount and composition of feed, the feeding system, etc. (GALLOWAY et al., 1999; AYALA et al., 2001; LOPEZ-ALBORS et al., 2003). Selective breeding programs, as well as advances in understanding of the nutritional requirements, have succeeded in dramatically increasing muscle growth rates in salmonid fish (JOHNSTON, 2001).

A great deal of fish cultured products come from salmonid farms (BOGLIONE et al., 2014) and studies of muscle growth are therefore important for the future development of fish farming. Fish muscles are known to be suitable for study because they are not distinctly separated, as is the case with the muscles of higher vertebrates. In addition, they have less connective tissue and segmented structure. Muscle segments (myomeres) are separated from each other by connective tissue septa (myosepts). The number of myomeres and vertebrae are considered to be a good indication in the taxonomic identification of fish species (ARAÚJO-LIMA and DONALD, 1988; OCHOA et al., 2010). Muscles consist of muscle fibres and some connective tissue. Body and tail muscles support the backbone on the left and right sides, beneath and above the vertebrae, both epaxially and hypaxially. There are separate muscles along the fins and head bones. The major part of the trunk and tail muscles consists of white muscle fibres used for forceful and rapid contractions during hunting of prey or escaping from predators, and they quickly show fatigue (VAN

LEEUWEN, 1995), in comparison to the red muscles which comprise a markedly smaller portion of the muscles (COUGHLIN, 2002). Skeletal muscles in fish develop hypertrophy and hyperplasia (GALLOWAY et al., 1999). The myomeres are separated by miosepta collagenous sheets with complex fibre patterns (VAN LEEUWEN, 1999) and white muscle comprises more than 90% of the myomeres in most species (JOHNSTON et al., 2000). Knowledge of the morphological aspects of skeletal development provides valuable information to prevent early incidences of malformations in farmed fishes (ÇOBAN et al., 2009).

Some species such as *S. obtusirostris* are endemic and threatened, and therefore controlled production is important to repopulate their natural habitat (TOMLJANOVIĆ, 2014). Since studies on the *S. obtusirostris* are lacking, we investigated the number of myomeres in the epaxial musculature, vertebral quantification and ossification of bones as well as the opening of the mouth of *S. obtusirostris*.

Materials and methods

Investigations were carried out on one hundred softmouth trout in early developmental stages (*S. obtusirostris*) at age 10-56 days post-hatching (dph) taken from a pilot scale hatchery located in Proložac near the River Vrljika in Croatia, as part of the restocking programme of the River Vrljika. Spawning and incubation were carried out at environmental temperature, which ranged from 10-11 °C corresponding to the River Vrljika environment. Fish were first fed with *Artemia salina* and later with commercial starter feed (EWOS micro crumble feeds). Fish were sampled every two days from 10-56 dph and fixed in 10% buffered formalin. Juvenile fish were embedded in paraffin and cut longitudinally in series sections of 10 μm in thickness and were stained with Hematoxylin and Eosin (HE) (ROMEIS, 1968), Toluidin Blue specific staining (PEARSE, 1968) Alcian Blue-specific (pH = 2.5) (MOWRY, 1956) and Alcian Blue and Alizarin Red (DINGERKUS and UHLER, 1977) staining. The described procedure was applied in order to show the tissue structure (muscles, cartilage and bones), to measure myomeres in the epaxial musculature, to show the possibility of bone ossification, to count the vertebrae and determine the time of the opening of the mouth. The cartilage stained blue when Alcian Blue and Alizarin Red were used, while bone tissue stained red. Total body length (TL) was measured using a Stereomicroscope, Olympus S761 with the use of a microscopic camera, PROMICRA. Basic statistical analyses were obtained using GraphPad Prism software (USA).

Results

The average number of myomeres increased during the investigated period, and it was between 10-18 dph 42.17, 20-28 dph 50.40, 30-38 dph 55.65, 40-48 dph 56.92, 50-56 dph 58.90 myomeres, respectively (Table 1). The total body length (TL) increased from the minimal body length of 19.57 mm between 10-18 dph to the maximum 25.80 mm between 50-56 dph (Table 1). The number of vertebrae in all investigated groups was 59.

Table 1. Total body length, number of myomeres and vertebrae of softmouth trout (*S. obtusirostris*, Heckel, 1851).

Age of softmouth trout in days	Parameters of description statistics	Total body length (mm)	Number of myomeres	Number of vertebrae
10-18 (n = 17) (n´ = 12)	M	20.28	42.17	59
	Min	19.57	37	
	Max	20.83	47	
	SD	0.63	5.00	
20-28 (n = 22) (n´ = 15)	M	21.63	50.40	59
	Min	21.30	47	
	Max	21.89	55	
	SD	0.30	4.01	
30-38 (n = 25) (n´ = 20)	M	22.38	55.65	59
	Min	22.26	55	
	Max	22.50	57	
	SD	0.12	1.02	
40-48 (n = 21) (n´ = 13)	Mean	23.55	56.92	59
	Min	22.65	56	
	Max	24.15	58	
	SD	0.75	1.00	
50-56 (n = 15) (n´ = 10)	Mean	25.42	58.90	59
	Min	25.07	58	
	Max	25.80	59	
	SD	0.37	0.55	

M - mean value, SD - standard deviation, Min - minimum, Max - maximum, n - number of fish used for body length measurements, n´- number of fish used for counted of myomers and vertebrae

At the age of 10 dph the head could be seen in the *S. obtusirostris* (Fig. 1). The eye could be seen with the developing retina at the age of 12 dph (Fig. 2) while retinal layers were completely developed at the age of 18 dph (Fig. 3).

The myomeres counted below the head (Fig. 4), on the trunk (Fig. 5) and tail parts of body (Fig. 6) were clearly visible. The period of larval development to the stage of complete yolk sac absorption took about 24 to 26 dph when they started opening their mouth and all the fins and head bones were formed (Fig. 7). In all examined specimens, no signs of ossification in the spine, fins or head bones were seen, or any skeletal malformations (Fig. 7).

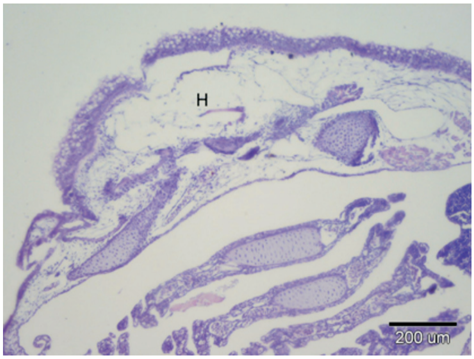

Fig. 1. The head (H) of a softmouth trout (*S. obtusirostris*, Heckel, 1851) at 10 dph, transverse section, H&E, ×10.

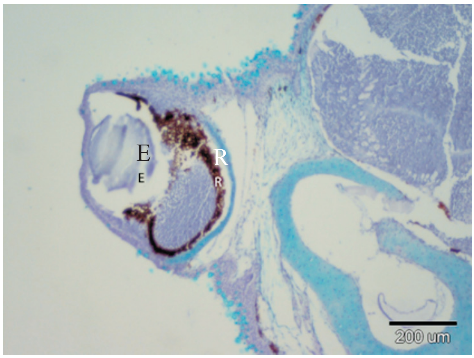

Fig. 2. The eye (E) with developing retina (R) of a softmouth trout (*S. obtusirostris*, Heckel, 1851) at 12 dph, transverse section, Alcian Blue, ×10.

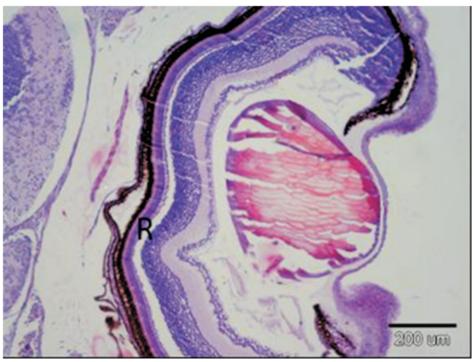

Fig. 3. The eye with completely developed retinal layers (R) of a softmouth trout (*S. obtusirostris*, Heckel, 1851) at 18 dph, transverse section, H&E, ×10.

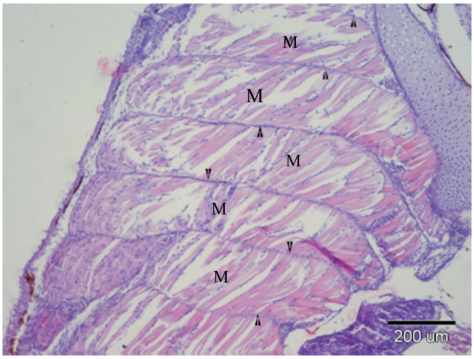

Fig. 4. Myomeres (M) and myosepta (arrowhead) in the epaxial musculature below the head of A softmouth trout (*S. obtusirostris*, Heckel, 1851) seen at 18 dph, transverse section, H&E, ×10.

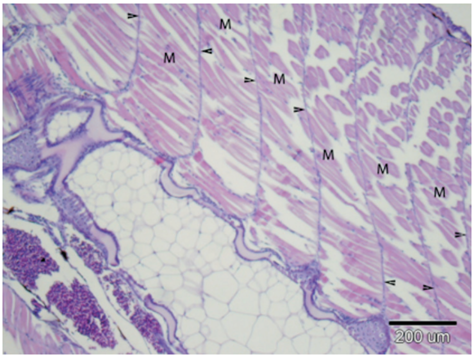

Fig. 5. Myomeres (M) and myosepta (arrowhead) in the epaxial musculature on the trunk of A softmouth trout (*S. obtusirostris*, Heckel, 1851) seen at 56 dph, transverse section, H&E, ×10.

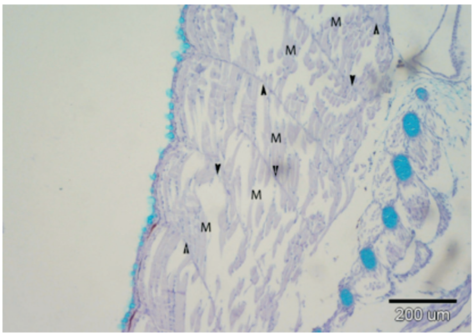

Fig. 6. Myomeres (M) and myosepta (arrowhead) in the epaxial musculature on the tail of A softmouth trout (*S. obtusirostris*, Heckel, 1851) seen at 18 dph, transverse section, Alcian Blue, ×10.

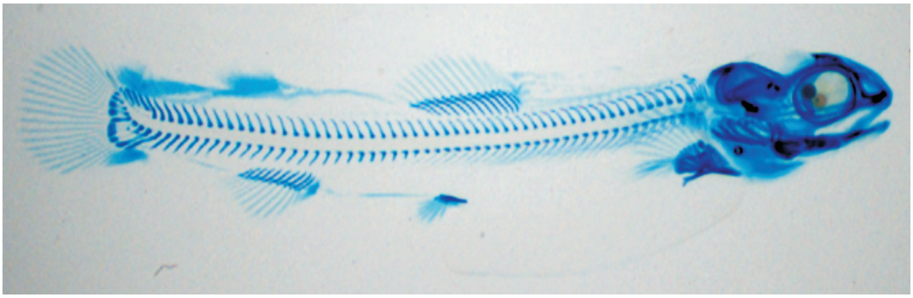

Fig. 7. Skeletal system of the softmouth trout (*S. obtusirostris*, Heckel, 1851), at 24 dph, Alcian Blue and Alizarin Red, ×4.

Discussion

ĆALETA et al. (2015) described the distribution and reduced habitat of all four subspecies of the *S. obtusirostris*: the Neretva softmouth trout (*S. o. oxyrthynchus*), Krka softmouth trout (*S. o. krkensis*), Solin softmouth trout (*S. o. salonitana*) and Zeta softmouth trout (*S. o. zetensis*). In the Red Book of Freshwater Fish in Croatia (MRAKOVČIĆ et al., 2006) Vrljika softmouth trout have been equated with the Jadro softmouth trout, but the genetic results of mitochondrial DNA haplotypes (control region, partial cytochrome b and ATPase 6 genes) indicate a sister relationship between the Vrljika and Neretva softmouth (Adriatic) trout *S. obtusirostris* (SNOJ et al., 2008). The Vrljika softmouth trout can be identified by an array of derived phenotypic and molecular character states. Therefore, for the conservation of this population it should be formally recognized at the same taxonomic level as other geographically separated populations of softmouth trout (SNOJ et al., 2008).

Accurate identification of fish species at all stages from larvae to adult is justified, to support the ichthyological studies which provide basic information for management (TERMVIDCHAKORN and HORTLE, 2013). In our investigations, the development of the *S. obtusirostris* juvenile fish was analysed, primarily the number of myomeres. In fish at the age of 10-56 dph, the average number of myomeres increased and was between 10-18 dph 42.17, 20-28 dph 50.40, 30-38 dph 55.65, 40-48 dph 56.92 and 50-56 dph 58.90 myomeres. As compared to our results KANG et al. (2014) also found in *Acanthopagrus schlegeli* an increasing trend in the number of myomeres from 22 to 26 at TL from 2.04 to 3.24 mm. The number of vertebrae in all investigated groups was 59. In adult *S. obtusirostris* ROGLIĆ (2012) determined 53-70 vertebrae. TREMBLAY et al. (1984) found approximately 54 vertebras in the larvae of haddock at TL 8-41 mm and 45-220 mm. Myomere counts in older specimens are often equivalent to vertebrae counts (TERMVIDCHAKORN and HORTLE, 2013).

The TL of *S. obtusirostris* increased from 19.57 to 25.80 mm between 10-56 dph. Considering the fact that the larva's ability to move is related to its size (SPIERTS, 2001), it should be noted that there is also a relationship to feeding (GALLOWAY et al., 1999; COUGHLIN, 2002).

Salmonidae are teleostei and the study of the osteological development is important in fisheries, biology and aquaculture (HASANPOUR et al., 2015). From the fisheries' perspective, it helps in identifying fish in early developmental stages (FRITZSCHE and JOHNSON, 1980; SAKA et al., 2008). The development of osteological structure in teleostei is of crucial value in phylogenetic studies (KOUMONDOUROUS et al., 2001). According to the present study no data about development and ossification processes in the *S. obtusirostris* were obtained. In our investigations of *S. obtusirostris* no signs of ossification were observed in the vertebral column or in the fins or head bones during period of 56 dph. A completely ossified vertebral column in *Salmo letnica,* Karaman, 1924 (Teleostei: *Salmonidae*) was observed in 92 day-old trout (RISTOVSKA et al., 2006).

Knowledge about the normal development of the skeleton is crucial (HASANPOUR et al., 2015) in addressing when and where abnormalities occur under rearing conditions. It can be used as an early bio-indicator of non-optimal rearing conditions (LEWIS and LALL, 2006) and also as a parameter of a proper diet (CAHU et al., 2003). Fish-like trout cannot swim efficiently with spinal deformities so they have greater risk from predators and are less capable of taking food (SILVESTRONE and HAMMELL, 2002). Farm fish are affected by skeletal malformations with the incidence depending on the species and developmental stage (BOGLIONE et al., 2014). In all investigated *S. obtusirostris* we did not notice any skeletal malformations. On the other hand, ROGLIĆ (2012) reported that out of the 88 *S. obtusirostris*, sampled from hatcheries in Proložac, only two (2.27%) had deformation of the spine.

The transition from endogenous to exogenous feeding which follows hatching is a critical stage in larval development, and is reflected in the overall degree of muscle development at that time (KOUMANS and AKSTER, 1995). The period of development of *S. obtusirostris* to stage of complete yolk sac absorption when they started opening their mouths was about 24 to 26 dph, which is similar to the results obtained by ROGLIĆ (2012), who stated that in *S. obtusirostris* complete yolk sac absorption takes about 25 dph.

The results of the present study of endemic species *S. obtusirosis* indicate that the number of myomeres was 55-59 in the period of 26-56 dph, which could be used for taxonomic identification of the early developmental stages of *S. obtusirosis*. The maximum number of myomeres on 56 dph was 59, which is the same as the number of vertebras. After the period of 24 to 26 dph when *S. obtusirostris* started opening their mouths, all head bones and fins were visible. During the whole investigation period no signs of ossification in the vertebral column, fins and head bones were visible. Also, no skeletal malformations were visible which is a good indicator of optimal rearing conditions and proper diet.

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SAŽETAK

Istraživanja su provedena na ranim razvojnim stadijima mekousne pastrve (*Salmothymus obtusirostris,* Heckel, 1851). Uzorci su prikupljeni u mrijestilištu u Prološcu u blizini rijeke Vrljike, u Hrvatskoj, i to svaki drugi dan od 10. do 56. dana poslije valenja (dpv). Uzorci su fiksirani u 10 %-tnom formalinu i uklopljeni u parafin te izrezani u serijama na 10 µm tanke rezove koji su potom obojeni hematoksilinom i eozinom, toluidinskim modrilom te alcijan plavo-specifičnom (pH = 2,5), alcijan plavom i alcijan crvenom metodom bojenja. Broj miomera u dorzalnoj epaksijalnoj muskulaturi mekousnih pastrva povećavao se s povećanjem njihove dobi te je između 10. i 18. dpv iznosio 42, a između 50. i 56. dpv 59 miomera. Dužina tijela u razdoblju od 10. do 50. dpv povećavala se od minimalno 19,57 do maksimalno izmjerene vrijednosti od 25,80 mm. Broj kralježaka u svih istraživanih uzoraka bio je 59. Praćenjem razvoja mekousnih pastrva do stadija potpune apsorpcije žumančane vrećice 24. - 26. dpv, kada su počele otvarati usta, utvrđeno je da su vidljive sve kosti glave i sve peraje. U razdoblju od 26. do 56. dpv broj miomera mekousne pastrve iznosio je 55 - 59 i taj bi podatak mogao poslužiti za taksonomsku identifikaciju ranih razvojnih stadija mekousnih pastrva. Tijekom cijelog istraživanja nisu utvrđeni vidljivi znakovi osifikacije na kralješcima, kostima glave ili na perajama niti su utvrđene vidljive malformacije kostura.

Ključne riječi: mekousna pastrva; (*Salmothymus obtusirostris*, Heckel, 1851); rani razvojni stadiji; epaksijalna muskulatura; osteološki razvoj