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Histological, Histochemical and Ultrastructural Investigations on the Esophagus of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract: The histology of the esophagus of *Oncorhynchus mykiss* was studied. The structure of the esophagus exhibited differences as well as similarities when compared with previous studies. In this study, the histochemical and ultrastructural structure of the tunica muscularis externa in the esophagus of this fish is investigated. The muscle fibers are described as red and intermediate fibers according to the pH stability of their mATPase activities. The red fibers showed intense staining, while intermediate fibers showed moderate staining with succinic dehydrogenase (SDH) and toluidine blue. In addition, small diameter fibres were seen. These indicated to be the continuation of development during the structure and function of the esophagus in the juvenile phase. Ultrastructural organization of sarcomeres, Z lines, mitochondria, triads and myofibrils of red and intermediate fibers were different from each other. The myofibrils of intermediate fibres showed clear differences. As a result, it is thought that the histochemical and ultrastructural characteristics of the tunica muscularis externa optimize the performance of the esophagus according to specific functional requirements.

Key Words: *Oncorhynchus mykiss*, Digestive system, Esophagus (muscle), Histology, Histochemistry, Ultrastructure.

Genç Gökkuşağı Alabalığı (*Oncorhynchus mykiss*) Özofagusunda Histolojik, Histokimyasal ve Ultrastrüktürel Araştırmalar

Özet: *Oncorhynchus mykiss* özofagusunun histolojisi çalışılmıştır. Onun özofagusunun yapısı önceki çalışmalarla karşılaştırıldığında benzerlikler kadar farklılıklar da göstermiştir. Bu çalışmada balığın özofagusundaki tunika muskularis eksterna'nın histokimyasal ve ultrastrüktürel yapısı araştırılmıştır. Kas lifleri mATP az aktivitelerinin pH dayanıklılığına göre kırmızı ve orta lifler olarak tanımlanmıştır. Orta lifler süksinik dehidrojenaz (SDH) ve toluidin mavisi ile orta düzeyde boyanma gösterirken, kırmızı lifler yoğun boyanma göstermiştir. Ayrıca, tunika muskularis eksterna'da küçük çaplı lifler görülmüştür. Bunlar, jüvenil evre süresinde özofagusun yapı ve görev bakımından gelişmesinin devamlı olduğunu göstermiştir. Kırmızı ve orta liflerin sarkomerleri, Z çizgileri, mitokondrileri, triadları ve miyofibrillerinin ultrastrüktürel organizasyonları birbirinden farklı bulunmuştur. Orta liflerin miyofibrilleri belirgin farklılıklar göstermiştir. Sonuç olarak, tunika muskularis eksterna'nın histokimyasal ve ultrastrüktürel özelliklerinin özel fonksiyonel ihtiyaçlara göre özofagusun çalışmasını en iyi düzeye getirdiği düşünülmüştür.

Anahtar Sözcükler: *Oncorhynchus mykiss*, Sindirim sistemi, Özofagus (kas), Histoloji, Histokimya, Ultrastrüktür.

Introduction

Detailed anatomical-histological and physiological descriptions of the digestive tract of the coast rainbow trout have been performed (Bakke-McKellep et al., 2000; Grosell et al., 2000). The ontogeny of the digestive system and the effect of growth conditions on this process have also been studied (Dement'eva, 1976; Sarıeyyüpoğlu et al., 2000). Phylogenetic predetermination of the structure of the esophageal epithelium and the characteristic features of the intestinal morphology have been suggested within the Salmonidae (Dzhumaliev, 1981).

In many reports, fibers from the conventional fish muscles classification have frequently been referred to as white, pink (intermediate) and red according to their coloration and histochemical reactions (Johnston et al.,

1975; Kilarski, 1990; Coughlin and Lawrence, 1999; Dal Pai et al., 2000; Jabarsyah et al., 2000). These also signify fast, intermediate and slow fibers, respectively (Patruno et al., 1998; Fernandez et al., 2000). Previous ultrastructural investigations combined with histochemical analysis showed characteristic differences at the ultrastructural level among the muscle fiber types (Kilarski and Kozlowska, 1985; Stoiber et al., 1998). Particular attention with regard to fish muscle has been also paid to the composition of the myosin filament population, sarcoma length and the width and shape of the Z - band (Kilarski and Kozlowska, 1983; Kilarski and Kozlowska, 1985; Bobe et al., 2000). Recent electron microscopic investigations were generally concerned with the possible source of new muscle fibers (Fauconnea and Paboeuf, 2000; Johnston et al., 1998).

The esophagus wall in fish is very muscular with interweaving skeletal muscle fibers, which may extend as far as the stomach (Brown, 1993). Therefore, a histological comparision of the tunica muscularis externa in the esophagi of fish that consume different types of food was made (Gentile et al., 1993), but transmission electron micrograph studies have not been previously documented. For this reason, the aim of the present study was to histologically examine the different parts of the esophagus of the rainbow trout (*Oncorhynchus mykiss*), a carnivore, and to contribute a more exact classificiation of this species, based on its digestive system, as in the case of mammals and birds. Furthermore, information is provided about histochemical and ultrastructural characteristics of the tunica muscularis externa in the esophagus. It is well known that the contractile properties of a muscle are directly related to the relative proportions of the various fiber types within that muscle (Rosser and George, 1986).

Materials and Methods

The esophagi of *O. mykiss* of age 1+ were obtained from the Department of Fisheries, Faculty of Agriculture, Atatürk University.

Histology: The dissected esophagi (length; 7.75 cm) were divided into 5 parts along their length and fixed in neutral buffered formalin for the histological investigation of the esophagi. The specimens were treated with ethanol and embedded in paraffin. Slices 5-10 µm thick were stained with Heidenhain haematoxylin-eosin. (Specimens were photographed with an Olympus Vanox PM-10-A). In addition, the thickness of the wall of the esophagus and circular muscle layer were measured.

Histochemistry: Esophagus samples were divided into 2 parts. One part was used for histochemical analysis and the other for electron microscopice observations. Frozen transverse sections of the first parts were cut at –20 °C, mounted onto glass slides and air dried at room temperature. Unfixed sections of the tunica muscularis externa were subjected to different acid and alkaline preincubations to compare the results obtained for fish muscles: pH 4.2, 4.4 and 4.6 for 5 and 7 s (acid preincubations); and pH 10.1, 10.2, 10.4 and 10.6 for 2 to 20 min (alkaline preincubations) (Kilarski, 1990; Jabarsyah et al., 1999a). For the histochemical demonstration of myofibrillar adenosine triphosphatase (mATPase), these were stained according to Johnston et al. (1975). In addition, the succinic dehydrogenase method (SDH) was employed on the sections in order to determine the mitochondria content (Pool et al., 1976). The muscle fiber diameters were measured directly from cryosections and stained for mATPase after alkaline preincubation.

Measurements: The measurements were performed with a light microscope using an eye-piece micrometer, previously calibrated for the magnification used, with a stage micrometer (0.01 mm; VEB Carl Zeiss JENA). Estimation of the fiber size was carried out by measuring the minimum diameter of each type of muscle fiber (Kowalewski and Miltzow, 1990) to avoid errors due to fiber obliquity (Benjamin et al., 1992).

Matching the Histochemical Preparations with the Thin Sections: The flat-embedded sections for electron microscopy were traced with a brightfield microscope equipped with a drawing tube; outlines of fascicles and positions of blood vessels and nerves were marked. This outline was superimposed onto the histochemical preparation until matching fascicles were identified. When regions were matched, fibers in several fascicles of the histochemical preparation were traced at x 200 magnification and each fiber was labeled for its ATPase

activity after acid and alkaline preincubation. Fascicles that were matched in the resin-embedded preparation were then trimmed out of the block and mounted onto a blank gelatin capsule filled with polymerized resin. The mounted preparation was then trimmed further and a few thick (0.5 µm) sections were made. These sections were stained with toluidine blue and, after again being matched with the drawings of the histochemical preparations, the fibers were traced onto transparent plastic sheets at x 200 magnifications so that each fiber could be matched according to its histochemical characteristics.

Electron Microscopy: For electron microscopy, the second part of the thin slices was trimmed again to minimize the sample. These were fixed in 3% glutaraldehyde buffered with 0.2 M NaH_2PO_4 + Na_2HPO_4 (pH = 7.2-7.3) and postfixed in 1% OSO_4. After dehydration in increasing concentrations of acetone the samples were embedded in Araldite CY 212. Semithin sections of the tunica muscularis externa were studied with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a JEOL-100 SX electron microscope.

Results

Histology and Histochemistry

When stained by haematoxylin-eosin it was observed that the esophagus parts of *O. mykiss* had a rather thick wall in both the medial and in neighboring parts on the caudal (Table 1).

The cranial esophagus consisted of an epithelial layer, connective tissue and a circular muscle layer from the outside to the inside (Figure 1a). In addition, adipose tissue was developed between the connective tissue of the mucous membrane and muscularis mucosa in the neighboring parts on the cranial esophagus (Figure 1b). Adipose cells were frequently accompanied by longitudinal bundles of striated muscle fibers in the medial esophagus (Figures 1c and d).

The circular muscle layer was thickest in the neighboring parts on the caudal (Figure 2a). Moreover, the histological structure of the caudal esophagus was similar to the cranial part (Figure 2b). However, in the epithelium of the caudal part the numerous mucous cells were displaced by the epithelial cells towards the basal membrane, producing the appearance of a single-layered epithelium (Figures 2a and b).

Table 1. Thickness of tunica muscularis externa layer and wall of the esophagus (in µm).

Sections of Esophagus	Age 1+	
	Thickness of wall	Thickness of tunica muscularis externa layer
Cranial	202 (4.1)	73 (0.7)
Under part of cranial esophagus	227.5 (5.2)	82.5 (1.4)
Medial	358 (3.2)	161 (1.2)
The neighboring parts on caudal	337 (5.1)	178.5 (3.5)
Caudal	281 (3.2)	71 (1.7)

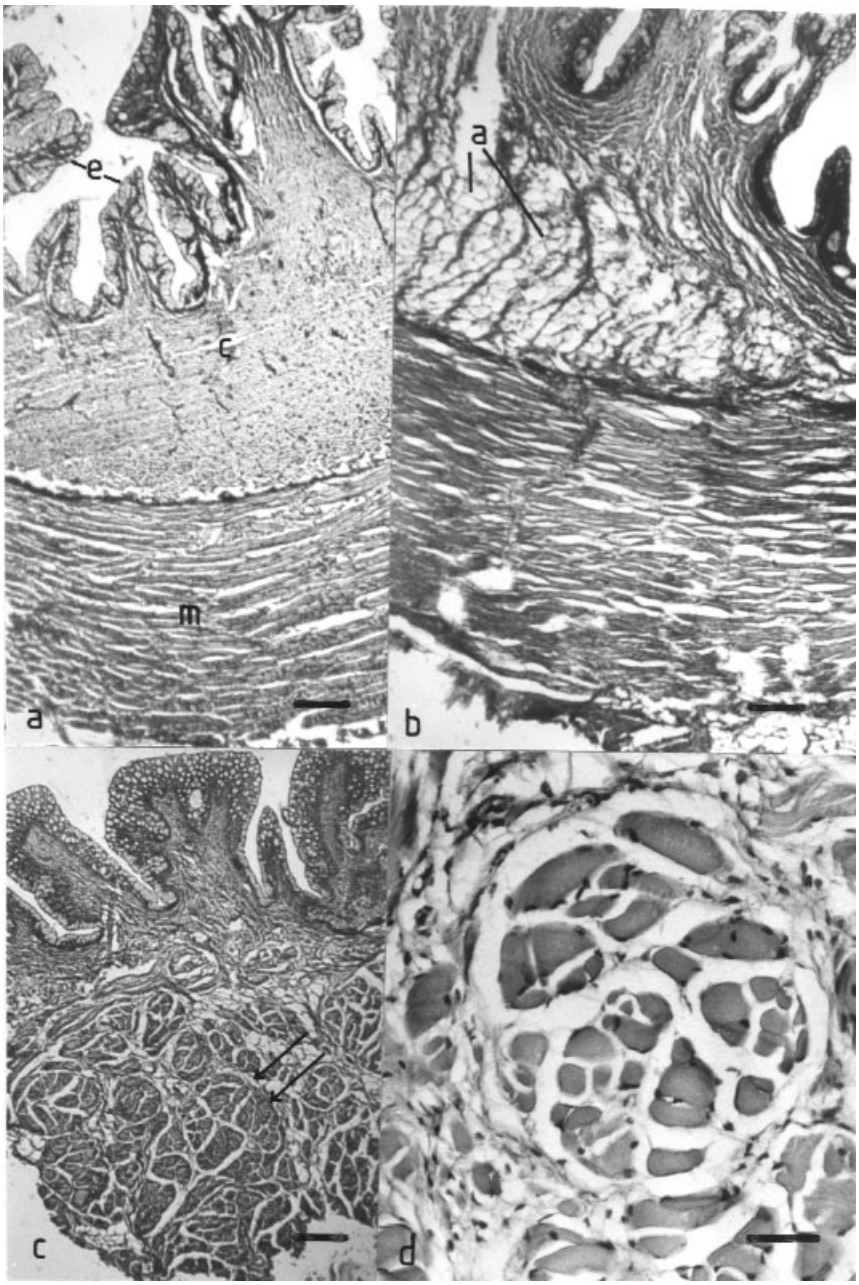
The mean values of the medial and neighboring parts on the caudal esophagus were more than 2 times those of other parts. This difference in mean thickness among these parts was statistically significant ($P < 0.005$).
The figure for each part is an average of 50 measurements, and the standard deviation of each is given in parantheses.

The present investigation was also based on the histochemical analysis of muscle fibers building a circular muscle layer in the esophagus and indicated small (11 ± 3.2) and intermediate diameter (18 ± 5.1) fibers. Table 2 shows that all fibers were low stained with mATPase at pH 4-5 but gave discriminate reactions after preincubation at pH 10.2 and 10.4. The small fibers were stained lightly and most of the intermediate fibers were moderately stained, although a few intermediate fibers were also intensely stained. In each case, the red fibers were stained positively for SDH. When stained for SDH, the intermediate fibers showed a moderate coloration in comparison with the red fibers. Similar results were also observed in semithin sections stained with toluidine blue.

Ultrastructure

The tunica muscularis externa in the rainbow trout esophagus were examined by electron microscopy. A satellite cell occupying a groove within the surface of the fibers typically had a heterochromatic nucleus and scanty cytoplasm (Figure 3). Furthermore, clear cytoplasm adjacent to the satellite cell contained numerous mithochondria with many criastae.

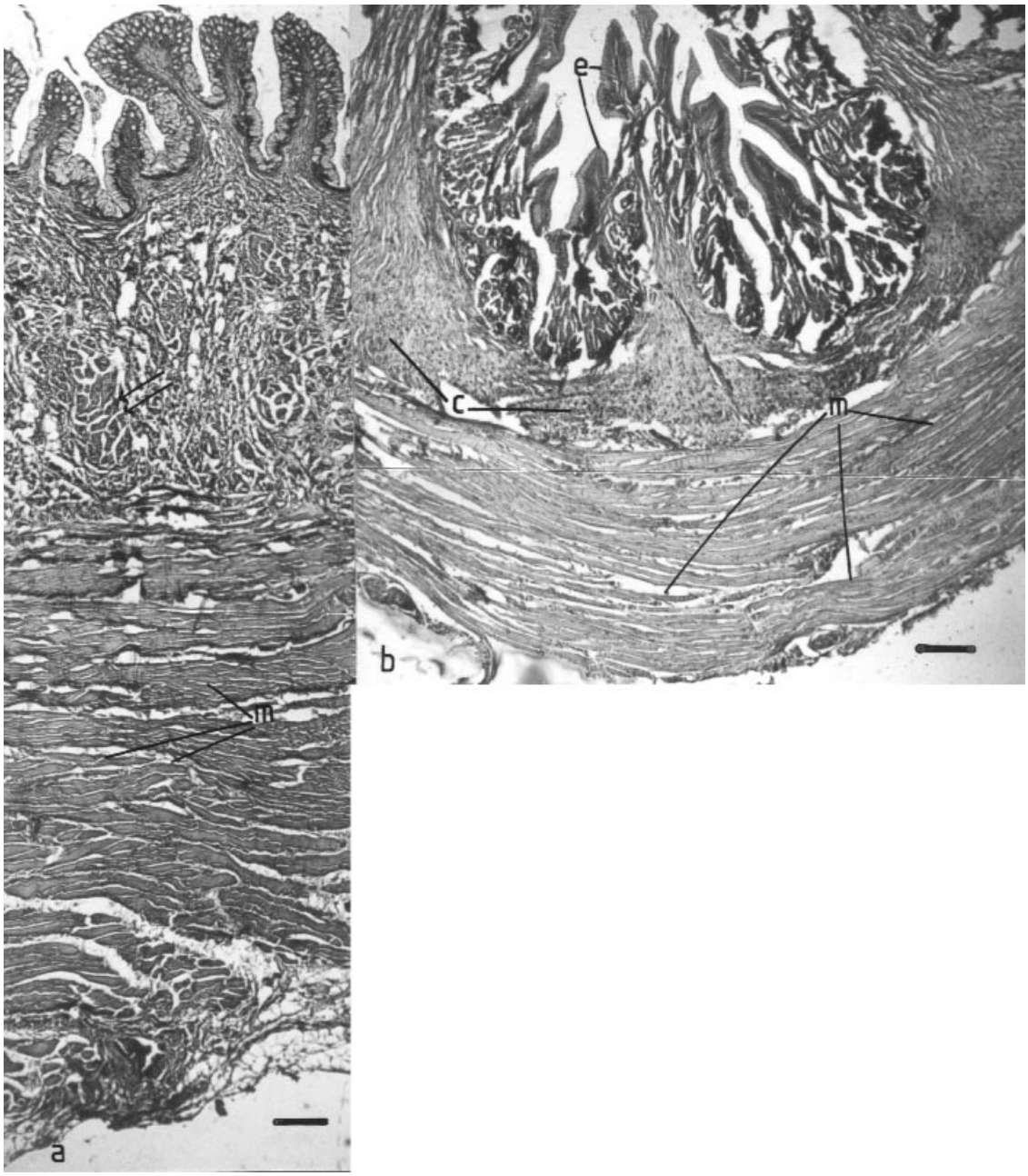
Some of the myofibrils of the red fibers were often confluent and formed a few continuous masses of



Figures 1 a-d. Transversal sections of the esophagus of rainbow trout (a-d); a) Cranial esophagus. Epithelium (e), connective tissue of mucous membrane (c), circular layer of striated muscle fibers (m), x31.25, b) Under part of cranial esophagus. Adipose tissue (a), x31.25, c-d) Medial. Longitudinal layer of striated muscle fibers (double arrow), x40, x200, respectively.

myofilaments in sarcoplasm containing glycogen particles (Figure 4). Z lines in these muscle fibers also had dense material. The sarcoplasmic reticulum was more or less radially oriented and there were small mitochondria clusters in the sarcoplasmic area of these muscle fibers. Triads were located at the level of the Z line (Figure 3).

The myofibrils of intermediate fibers inside ran as independent units (Figure 5a). The T tubules of these



Figures 2 a and b. Transversal sections of the esophagus of rainbow trout; a) The neighboring parts on the caudal, x40. Abbr. as in Figure 1 (a) and (c-d), b) Caudal, x40. Abbr. as in Figure 1 (a).

fibers were located at the A-I band junction and were more or less regular. On the other hand, the M lines of the A bands were not distinct. The sarcomas were short and demarcated by a regular thin Z line, whereas the organization of myofilaments of peripheral intermediate fibers was rather regular (Figure 5b). The sarcoplasmic reticulum was easily discernible. In addition, junctional structures were found between muscle fibers or different types of the same type running in different directions at the site where they intersect. Dense materials were seen on the cytoplasmic surface of the sarcolemma at the junctional sites of each fiber. In addition, basal lamina material existed in the space at the junction between adjoining fibers (Figure 5c).

Table 2. Histochemical reactions and fiber dimensions of the tunica muscularis externa in the *O. mykiss* esophagus.

Fiber types	Average diameter	m-ATPase pH 4-5	m-ATPase pH 10.1	m-ATPase pH 10.2	m-ATPase pH 10.4	m-ATPase pH 0.6	SDH	Toluidine blue
R	11±3.2	-	- (++/+++)	-	-	-	+++	+++
I	18±5.1	-	+++	++ (+++)	++ (+++)	- (++)	++	++

R, red fibers; I, intermediate fibers
(+++) High, (++) moderate, (-) very low.

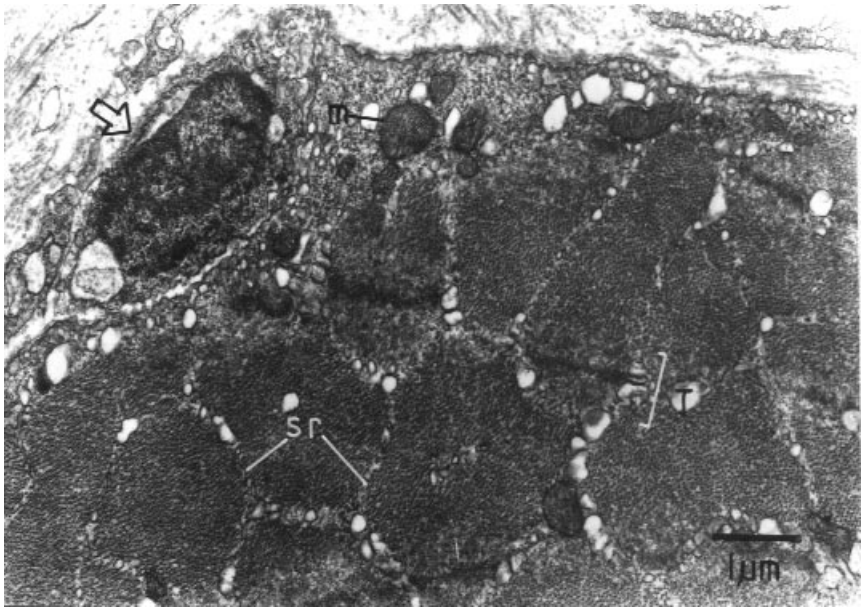


Figure 3. Electron micrograph of the tunica muscularis externa in transverse section. A satellite cell (arrow), mitochondria (m), sr (sarcoplasmic reticulum), triad (T). x10,000.

Discussion

A circular layer of striated muscle was developed in the wall of the esophagus of *O. mykiss* fry (Sarıeyyüpoğlu et al., 2000). It was reported that in carnivore nutrition the esophagus needs to have well developed circular muscles for the mechanical transmission of moving food (Çelikkale, 1991). Our investigations show that the thickness of this layer was different throughout the length of the esophagus. Furthermore, a second longitudinal layer of striated muscle had developed. These findings have not been previously documented in Salmonoidei. On the other hand, a special feature of the fine structure of the esophagus was also the adipose tissue in the wall between the connective tissue of the mucous membrane and the muscularis mucosa, which was noted for the first time by Verigina and Savvaitova (1974) in *Salvelinus*. This layer is not mentioned in studies on the esophagus of other fish species. Verigina and Savvaitova (1974) found that fat deposits in this area are characteristic of *Salmo, Salvelinus* and *Stenodus;* i.e. of fish making prolonged spawning migrations during which feeding is discontinued (Pirozhnikov, 1955). In 1-year-old *Salmo, Salvelinus leucomaenis*, and *S. malma*, individuals the fat deposits are already visible. The fat layer is partially developed in *Salmothymus*. However, the fat layer is completely absent in fishes of the genera *Oncorhynchus, Hucho, Brachymystax* and *Thymallus*. In the species of the genus *Coregonus*, the fat layer is also absent. *Brachymystax lenok, Thymallus* and *Hucho hucho*

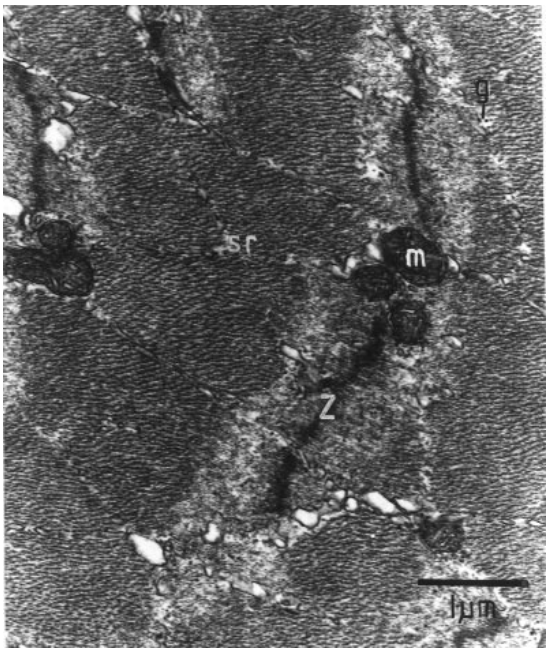


Figure 4. Red fibers of the tunica muscularis externa. Myofilaments (mf), mitochondria (m), sr (sarcoplasmic reticulum), g (glycogen), Z (Z-line). x15,000.

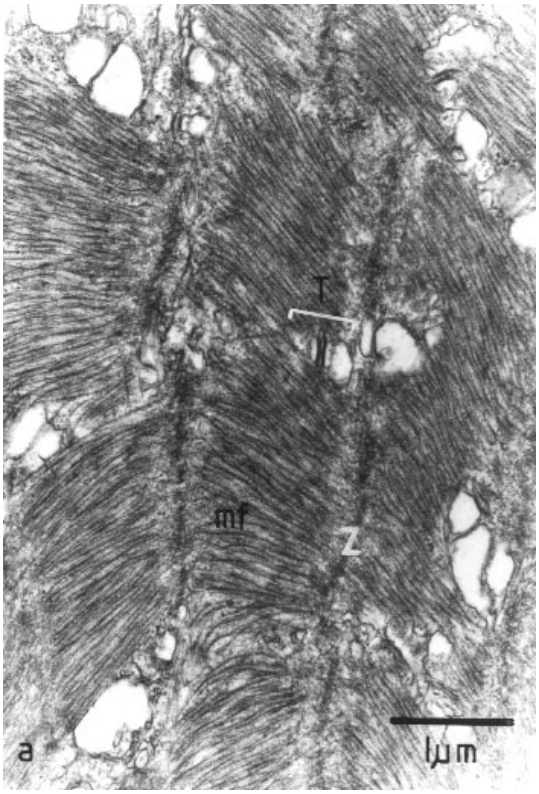


Figure 5a. Internal intermediate fibers of the tunica muscularis externa. Myofilaments (mf), Triad (T), Z (Z-line. x15,000.

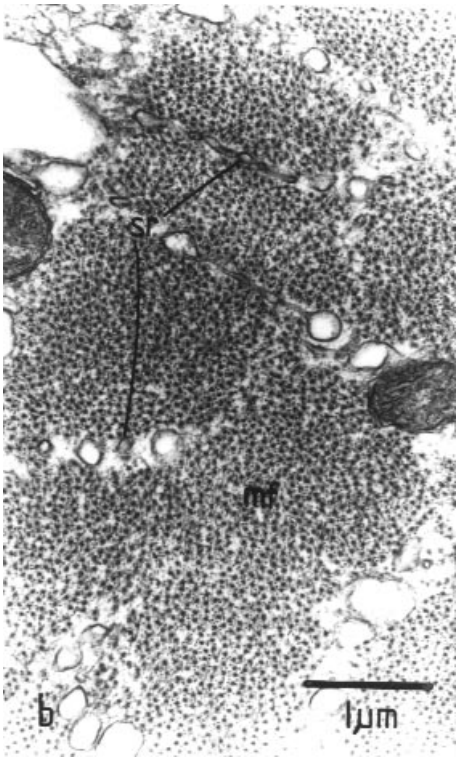


Figure 5b. Peripheral intermediate fibers of the tunica muscularis externa in transverse section. Myofilaments (mf), sr (sarcoplasmic reticulum). x15,000.

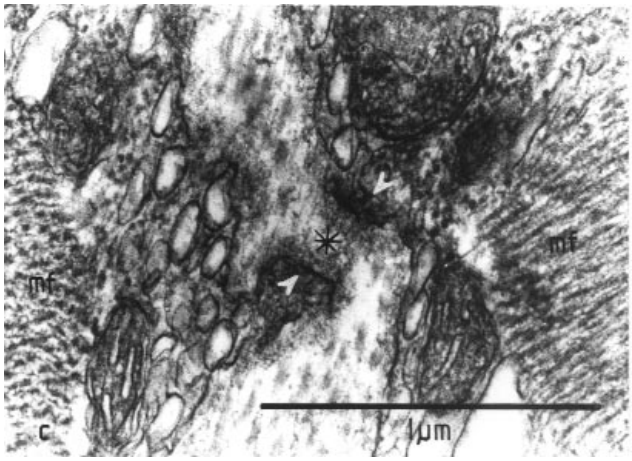


Figure 5c. Intermyofibrous junctions. Dense materials (arrow heads), basal lamina material (asterisk), myofilaments (mf). x50,000.

do not take part in extensive spawning migrations and *Hucho perryi*, when migrating, do not swim far upstream in rivers and continue feeding (Kirillov, 1972). It was thought that the absence of the adipose tissue in the

esophagus wall of *Oncorhynchus* species might depend on the characteristic feature of their biology: their death after spawning makes the energy stock for regenerative processes redundant (Voronina, 1997). In this study, the adipose tissues were observed in the other parts, except for the cranial and caudal parts, of *O. mykiss* esophagi. Thus, we can say that there was a contrast between our findings and what had been suggested.

Again, phylogenetic predetermination of the structure of the esophageal epithelium has been suggested (Dzhumaliev, 1981). However, a difference was noted in the structure of the epithelium of the cranial and caudal parts of the esophagus (Boselova and Meitner, 1977; Meister et al., 1983; Voronina, 1997). Similar changes in the epithelium were also observed in our study.

In general, preincubation at an acid pH was unsuitable for fish muscle because of the indiscriminate inactivation of the fibers (Egginton and Johnston, 1982; Chayen et al., 1987). Similarly, in the present study, the muscle fiber types of the tunica muscularis externa were discriminated by the alkaline stabilities of actomyosin ATPase activity, as stated by Daerolf et al. (2000). In addition, morphometric analysis of the esophagus showed that the mean fiber diameters were smaller than the diameters of muscle fibers of teleost fish. It was reported that the diameters of red and intermediate fibers were 26-41 µm and 42-82 µm respectively, in the ordinary muscle of many sample fish (Nag, 1972; Chayen et al., 1987; Kilarski, 1990; Fernandez et al., 2000; Geyikoğlu and Özkaral, 2000). Furthermore, the existence of fibers < 20 µm diameter was used as a criterion for the continuation of muscle fibre recruitment (Fernandez et al., 2000). Thus, the presence of small caliber fibers in *O. mykiss* may indicate the continuation of development in the structure and function of the esophagus. After preincubation at an alkaline pH (10.4) for 15 min, red fibers were not active. They resembled the red muscle fibers of the other species of fish described earlier (Usher et al., 1994; Ramirez-Zarzosa et al., 1998). Their high SDH activity and the relative abundance of mitochondria in electron micrographs of these fibers was probably related to their resistance to fatigue when activated during slow movements (Kilarski, 1990; Battersby et al., 1998; Devincenti et al. 1998; Rescan et al., 2001). Mitochondrial clusters were the primary mechanism for enhancing the aerobic capacity of muscle in cold-water fish (Johnston et al., 1998). Thus, it was thought that these fibers in the esophagus might be related to slow contraction speed. Pink (intermediate) muscle fibers were found in the muscles of many fish (Jabarsyah et al. 1999b). In the present study, the histochemical properties of the intermediate fibers in the *O. mykiss* esophagus were in accordance with those reported for other fish (Devincenti et al., 1998; Ramirez-Zarzosa et al., 1998). They were stained moderately for SDH and for mATP ase after alkaline preincubation. The intermediate muscle fibers were also significantly different, not only among vertebrates but also among fishes themselves (Kilaraski and Kozlowska, 1983). Similarly, in our ultrastructural investigations, the organization of peripheral and inner intermediate muscle fibers was different in that myofibrils were arranged more or less radially. In general, in the case of fish skeletal muscle thick filaments, the myosin head array in resting muscles is not perfectly helical but contains periodic perturbations (Squire et al., 1998). In addition, these fibers contained shorter sarcomers than red fibers and thinner Z lines than in the intermediate fibers distinguishable in fish (Kilarski and Kozlowska, 1983; Kilarski and Kozlowska, 1985; Ogata ,1988). Moreover, the triads containing T tubules were located at every A-I band level of each myofibril. Therefore, intermediate fibers could be called "A-I fibers" (Nakao et al., 1984). Since intermediate fibers generate force more rapidly than red fibers (Coughlin and Lawrence, 1999) fast activation could be ensured (Nakao et al., 1984).

In this study, desmosome-like junctional structures were determined as crucian carp pharyngeal pads (Nakao et al., 1984). Thus, junctional structures between red and intermediate fiber types might achieve the effective organization of forces produced by the contraction of the esophagus when the rainbow trout swallows.

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