KnE Life Sciences

BRDEM-2019
International applied research conference
«Biological Resources Development and Environmental Management»
Volume 2020

Knowledge E
enriching | engaging | empowering

Conference Paper

Muscle Protease Activities in *Salmo Trutta* L. Inhabiting the Krivoi Ruchey River

Nadezhda Kantserova, Liudmila Lysenko, Marina Krupnova, Ekaterina Tushina, and Nina Nemova

Institute of Biology, Karelian Research Centre of Russian Academy of Sciences, Petrozavodsk, Russian Federation

Abstract

The effects of size, age, and smoltification on muscle protein breakdown systems were studied in wild brown trout *Salmo trutta* L. inhabiting the Krivoi Ruchey River of the White Sea basin, Kola Peninsula. Activities of autophagy-related proteases including cathepsin B and D, calpains, and proteasome were assayed in the skeletal muscle of brown trout parr and smolt of different age group. Youngest fish group consisted of the most actively growing individuals possessed the higher rate of protein breakdown compared to older groups. Different patterns of muscle protein breakdown inherent to brown trout parr and smolts were shown to be associated with calpain system and cathepsin D activities. Thus, increased activity of these proteases in smoltifying individuals obviously results in amino acid accumulation that could be a mechanism of seawater tolerance required for seaward migration. This study is the first to show the age- and stage-related dynamics in protease activities in skeletal muscle of brown trout inhabiting the Krivoi Ruchey River. Growth- and smoltification-related patterns of protease activities were quite similar in brown trout from the Krivoi Ruchey River and previously studied rivers of the White Sea basin, however, some habitat-related differences were observed.

Keywords: brown trout, growth, smoltification, skeletal muscle, protein breakdown, Krivoi Ruchey River

Corresponding Author:
Nadezhda Kantserova
nkantserova@yandex.ru

Received: 24 December 2019
Accepted: 9 January 2020
Published: 15 January 2020

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the BRDEM-2019 Conference Committee.

1. Introduction

Brown trout *Salmo trutta* L. is a widespread salmonid with a great diversity in life history strategies compared with other more obligatory anadromous salmonid species. There are numerous studies describing brown trout variability in size and growth rate as well as in feeding niche and habitat use and consequently high ecological plasticity of this species [1]. For example, brown trout at the age of 4+ may vary in size from 20 g for fish in small brooks to between 0.5 and 1 kg in fast-growing piscivorous and anadromous populations. Age and size of brown trout smolts preparing to migrate to the sea vary significantly too [1].

Individual growth in teleosts has an indeterminate nature and depends on the opposing metabolic processes of protein synthesis and breakdown [2]. Protein breakdown occurs mainly through the action of three distinct pathways: intralysosomal digestion by cathepsins, calcium-dependent proteolysis by calpains, and the ubiquitin-proteasome system. In the skeletal muscles, calpains digest large structural proteins initiating myofibril disintegration while cathepsins and the ubiquitin-proteasome system are responsible for processing of released proteins to short peptides and amino acids [3--5]. Besides, calpains regulate muscle physiology contributing to muscle cell differentiation and myofibril formation [3]. It has been reported that protein breakdown mediating by proteolytic enzymes corresponds with protein turnover and fish growth rates [6, 7]. The individual roles of muscle proteases in total protein turnover has been partially characterized in Atlantic halibut [8], channel catfish [9], grass carp [10], and salmonids including arctic charr [11], Atlantic salmon [6, 12, 13], and rainbow trout [7, 14, 15]. These studies expanded the knowledge on the role of intracellular proteases in the mechanisms of growth in fish species exhibiting various life-history strategies and tactics.

However, there is a few data characterizing physiological and biochemical differences between brown trout individuals at distinct phases of growth and life stage, as well as a contribution of environmental factors affecting growth. So, plasma thyroxine concentrations and seawater tolerance were described in wild and hatchery-reared brown trout [16].The contribution of the non-lysosomal protein breakdown systems to the muscle growth of brown trout parr and smolts inhabiting the Indera river was described [12].

Thus, this study aimed to evaluate associations of fish size, age, and life stage (parr vs smolts) with the activities of the main proteolytic enzymes involved in the muscle protein breakdown, such as proteasome, calcium-dependent proteases (calpains), and cathepsins B and D, in the skeletal muscles of brown trout *Salmo trutta* L. collected in the Krivoi Ruchey river of the White Sea basin (Kola Peninsula, Russia).

2. Methods and Equipment

2.1. The study area

Wild brown trout *Salmo trutta* L. 1758 sampling was conducted in June on the spawning river Krivoi Ruchey, the White Sea basin, Kola Peninsula, Russia.

2.2. Sampling

Brown trout collection and study were conducted in accordance with a resolution no. 51 2015 03 0119 by Barents-Belomorsk territorial department of the Federal Agency for Fisheries. Brown trout parr were captured by electrofishing; to avoid possible effects of electrofishing, parr were kept for a day in cages located in the mainstream portion of the river. Brown trout smolts were captured during their natural seaward migration at a smolt trap located in the river about half a kilometer upstream to an estuary. Smolt status was evaluated using both morphological and physiological variables based on visual assessments. Mean body weights and fork lengths of fish are presented in Table 1. For each individual, a small piece of the white muscle was taken from a point below the first dorsal fin and well above the lateral line. Tissue pieces were frozen in liquid nitrogen and kept at −80 ˚C prior to analyses. Skeletal muscles protease activities were determined for pooled parr 0+ (8-10 individuals), in remaining fish groups protease activities were measured in individual animals (4-7 individuals for each group).

2.3. Reagents and equipment

Chemical reagents, protease inhibitors, and protein substrates were purchased from Sigma-Aldrich (St Louis, MO, USA) and of analytical grade. Technical equipment of the Core Facility of the Karelian Research Centre of the Russian Academy of Sciences was used.

2.4. Extraction of intracellular proteases

Muscle samples were homogenized for 1 min at 50 Hz with 10 volumes of 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM EDTA, 20 mM dithiothreitol, 1 mM ATP, 5 mM $MgCl_2$, 0.1% Triton X-100, as well as protease inhibitor cocktail (0.5 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL aprotinin, and 1 mM PMSF) to prevent self-digestion of proteases [7]. Homogenates were centrifuged at 15,000 rpm for 30 min to obtain a pooled fraction of cytoplasmic and organelle proteins referred as the enzyme-containing fraction. All procedures were carried out in ice or at 4 ˚C.

2.5. Proteasome activity assay

The chymotrypsin-like activity of the proteasome was determined in the enzyme-containing fraction using a fluorescence assay [17]. Peptidase activity against a synthetic oligopeptide substrate was measured in a reaction mixture containing 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ATP, 30 μM Suc-LLVY-AMC as the substrate, and 20 mM Tris-HCl (pH 7.5) in the absence or presence of 5 μM specific inhibitor MG132. Following incubation at 37°C for 30 min, proteasome activity was calculated as the difference in fluorescence intensity between the samples with and without inhibitor at excitation and emission wavelengths 380 nm and 440 nm, respectively. Resulting proteasome activity was normalized to sample protein concentration and expressed as relative fluorescence fold change (FU).

2.6. Calpain activity assay

Calcium-dependent proteolytic activity was quantified using a microplate assay and casein as a substrate [18]. A reaction mixture with 500 mL total volume was composed of the following: 0.4% alkali-denatured casein, 20 mM dithiothreitol, 50 mM Tris-HCl (pH 7.5), 5.0 mM Ca^{2+} (as $CaCl_2$) or 5.0 mM EDTA (negative control) and the enzyme-containing fraction. Following incubation at 28°C for 30 min, the remaining protein was quantified by Bradford assay [19]. Enzymatic activity was expressed in activity units (AU) defined as the amount of the enzyme that causes an increase of 0.1 in absorbance at 595 nm per hour. Specific calpain activity was normalized to sample protein concentration.

2.7. Cathepsin B activity assay

Cathepsin B activity was assayed in the enzyme-containing fraction by Barrett technique [20]. The enzyme-containing fraction was pre-activated with 4 mM cysteine and incubated with Z-Arg-Arg-AMC synthetic substrate in the reaction mixture containing the following components: 0.0625 mM substrate, 100 μL of the enzymatic fraction, 2 mM Na-EDTA, 4 mM DTT, 0.2% CHAPS in the sodium phosphate buffer (pH 6.0). After incubation for 5 min at 37°C, the reaction was stopped by the addition of 1 mL of 3% SDS. The concentration of free AMC was determined at excitation and emission wavelengths 380 nm and 440 nm, respectively. The unit of activity (AU) was defined as the enzyme quantity in a gram of tissue able to degrade 1 μmol of the substrate in 1 min at 37°C.

2.8. Cathepsin D activity assay

The haemoglobinolytic activity of cathepsin D was tested in the enzyme-containing fraction by the Anson's technique. The reaction mixture of the total volume of 1000 µL contained 250 µL of the 2.5% bovine albumin solution (w/v), 250 µL of the enzymatic fraction, and 500 µL of 0.2 M acetate buffer (pH 2.8) with 10 mM β-mercaptoethanol and 1 mM EDTA. After the incubation at 37°C for 20 min, the reaction was stopped by the addition of trichloroacetic acid in the final concentration of 5%. The acid-soluble peptides content was determined by absorbance at 280 nm after centrifugation at 18000 g for 15 min. Cathepsin D activity was expressed in units (AU) of trichloroacetic acid-soluble product absorbance per g tissue per hour.

2.9. Determination of protein content

Water-soluble protein concentration (mg of protein per g of wet weight) in an enzyme-containing fraction was determined according to Bradford technique [19] using bovine serum albumin as a standard.

2.10. Statistical analysis

The results are expressed as means ± S.D. Initially, the raw data were checked for normality of distribution and homogeneity of variances using Kolmogorov-Smirnov and Levene's tests, respectively. Multiple analysis of variance (MANOVA) was used to evaluate the overall effect of age, and life stage (parr vs smolt) on protease data. Differences among means were detected using Tukey's honest significant difference test ($p \leq 0.05$).

3. Results

It was shown that the structure of brown trout population from the Krivoi Ruchey river was quite complex, and the catch contained both parr and smolts of different age groups. The morphometric indices of brown trout parr and smolts of different year-classes showed age-related significant difference. The growth increments per time unit decreased with increasing age or size; for instance, the body weight of brown trout parr 0+ differed more than nine times from that of the parr 1+ and the body weight gain during the third year was substantially lower showing a 4.7-fold increase. Length and

weight parameters of parr and smolts at the same age were significantly different (Table 1).

Proteasome activity in brown trout skeletal muscle depended on age (F=30.16, p=0.0001), while effect of life stage (parr vs smolt) (F=0.42, p=0.5207) had not been found. Parr 2+ had significantly lower proteasome activity in comparison with parr 0+ (Table 2).

Calpain activity in brown trout skeletal muscle depended on age (F=6.88, p=0.0013) and stage (parr vs smolt; F=6.46, p=0.0168). Significant differences in calpain activity in parr 0+ vs parr 2+ were shown (Table 2).

Cathepsin B activity in brown trout skeletal muscle depended on age (F=2.85, p=0.0338), while the effect of stage (F=3.15, p=0.0822) had not been found. Parr 2+ had the significantly lower cathepsin B activity if compared with parr 0+ (Table 2).

Cathepsin D activity depended on fish age (F=18.21, p=0.0001) and stage (F=4.62, p=0.048). Cathepsin D activity in parr 0+ was higher than that of individuals of the other year-classes. Cathepsin D activity was significantly higher in smolts 2+ compared to that in parr of the same age group (Table 2).

TABLE 1: Length-weight parameters of brown trout from the Krivoi Ruchey river.

Group	Length, cm	Weight, g
0+ (parr)	2.49±0.1	0.17±0.05
1+ (parr)	5.54 ± 0.79^{a}	1.64 ± 0.66^{a}
2+ (parr)	9.3 ± 0.83^{ab}	7.77 ± 1.69^{ab}
2+(smolt, female)	16.25 ± 0.77^{abc}	35.49 ± 7.87^{abc}
3+ (smolt, female)	18.03 ± 1.74^{abc}	52.39 ± 12.58^{abc}
3+(smolt, male)	17.98 ± 1.77^{abc}	51.96 ± 15.62^{abc}
4+(smolt, female)	20.88 ± 1.29^{abcd}	77.85 ± 12.32^{abcd}
4+ (smolt, male)	20.73 ± 0.5^{abce}	71.25 ± 7.55^{abce}

Alphabet letters indicate significant differences: ``a'' in comparison with parr 0+, ``b'' in comparison with parr 1+, ``c'' in comparison with parr 2+,``d'' in comparison with 3+ (smolt, female), ``e'' in comparison with 3+ (smolt, male).

Alphabet letters indicate significant differences: ``a'' in comparison with parr 0+, ``b'' in comparison with parr 1+, ``c'' in comparison with parr 2+.

4. Discussion

Protein breakdown in fish skeletal muscle appears to rely on autophagic-lysosome and calcium-dependent systems primarily [15]. Calcium-dependent proteases cleave

Table 2: Intracellular protease activities in the skeletal muscle of brown trout from the Krivoi Ruchey river.

Group	Proteasome activity, FU	Calpain activity,AU	Cathepsin B activity, AU	Cathepsin D activity, AU
0+ (parr)	128.2±11.9	168.1±43.1	6.9±0.2	0.42±0.03
1+ (parr)	83.2±11.8	91.9±20.4	4.8±0.8	0.03 ± 0.01^{a}
2+ (parr)	59.8 ± 8.4^{a}	60.3 ± 24.2^{a}	3.9 ± 1.2^{a}	0.04 ± 0.01^{a}
2+(smolt, female)	52.1 ± 7.9^{a}	111.4±21.4	4.2±0.3	0.12 ± 0.01^{abc}
3+(smolt, female)	35.9 ± 5.2^{ab}	103.6±30.2	5.3±0.6	0.13 ± 0.01^{abc}
3+(smolt, male)	34.1±5.7	115.7 ± 17.6^{c}	5.7±0.8	0.16 ± 0.02^{abc}
4+(smolt, female)	40.4 ± 6.1^{a}	136.6 ± 28.3^{c}	6.8±0.14	0.14 ± 0.01^{abc}
4+ (smolt, male)	46.3±7.13	118.4 ± 40.2^{c}	5.9±0.7	0.15 ± 0.01^{abc}

key myofibrillar proteins maintaining muscle architecture and function, including titin, tropomyosin, troponin, etc. [3], for subsequent degradation via cathepsins or proteasomes. Among numerous lysosomal proteases, cathepsin D has the principal role in protein breakdown by autophagy in fish [13]. The ubiquitin-proteasome system in fish skeletal muscle appears to contribute to only 4-17% total protein breakdown [15], whereas protein turnover in mammal skeletal muscle relies on proteasomal digestion of ubiquitin targeted proteins predominantly [21]. So, protein breakdown mechanisms in fish skeletal muscle are distinct from those in mammals.

Muscle growth in juvenile fish is associated with high rates of muscle protein synthesis, accretion, and new muscle fiber formation exceeding the rate of protein breakdown. Our results indicate different muscle protein breakdown rates throughout studied enzyme systems between brown trout parr of different age groups. It was shown that calpain, proteasome, and cathepsins B and D activities decreased significantly in the muscle of brown trout parr from studied river as fish age and size increased. So, the youngest fish group which consists of the most intensively growing individuals possessed the higher rate of protein breakdown compared to older groups of parr. High proteolytic activities of calpains, proteasomes, and cathepsins in the skeletal muscle of parr 0+ are apparently associated with the role of studied enzymes in the metabolism of de novo synthesized myofibrillar proteins through their turnover and elimination by protein quality control machinery with intracellular proteases as the key executors. Obtained results correspond with literature data. So, it was shown that protease activity in the skeletal muscle of rainbow trout [7] and Atlantic salmon [6] decreased as the animals became older and bigger. Besides protein turnover, calpains regulate some events in muscle fibre formation and hyperplastic growth. So, increased calcium-dependent activity in the skeletal muscle of youngest parr may indicate specific contribution of the calpains at early stages of myogenesis [3].

Smoltification is a complex of biochemical, physiological, morphological, and behavioral modifications which prepare freshwater-dwelling parr for survival and growth in the marine environment [22]. Smoltification is controlled by environmental factors, such as photoperiod, water temperature, streamflow, and water quality as well as by endogenous ones, including endocrine signals. Of the wide range of hormones known to be involved in smoltification, growth hormone (GH), thyroid hormones, and cortisol levels increase in circulating blood and they act individually or synergistically to regulate food behavior, osmoregulation, metabolism, and growth during smoltification [22]. In teleosts, cortisol has an overall role in the regulation of energy and lipid metabolism, as well as protein turnover in particular by increasing protein catabolism [23]. Obtained results indicate the difference in muscle protein breakdown rates between brown trout parr and smolts principally through regulation of the calpain system and cathepsin D. So, increased activity of these proteases during smoltification could be a part of enhanced muscle catabolism in response to cortisol.

It should be mentioned that brown trout smoltification is more flexible in comparison to this process in Atlantic salmon [16]. Unlike Atlantic salmon decision to smoltify depending on size reached in aprevious year [22], brown trout decides in the current spring whether to smoltify. Brown trout smoltification and migration are triggered by environmental conditions and food accessibility [24]. The system of osmoregulation of brown trout smolt is often immature until seaward migration, in contrast to Atlantic salmon. Thus, smoltification of brown trout is not as complete as that of Atlantic salmon [16]. Our results showing that brown trout smoltification is associated with increased protein turnover estimated by calpain and cathepsin D activity correspond to the previous observations [12]. Due to impulsive smoltification, brown trout have to develop hypoosmoregulatory mechanisms promptly. Usually, the development of seawater tolerance in salmonid smolts is correlated to an increase in gill Na+/K+-ATPase activity, a key mechanism of osmoregulation in fish [25]. We can hypothesize that besides Na+/K+-ATPase upregulation, an increase in protein breakdown may contribute to brown trout salinity tolerance due to hyperosmotic properties of free amino acids resulting from protein hydrolysis. The osmolyte role of free amino acids in the cytoplasm has been demonstrated for some euryhaline species including salmonids such as rainbow trout [26]. This protease-mediated mechanism of cell osmolality maintenance seems to be common for anadromous fish, such as brown trout, and some euryhaline invertebrates [26].

Growth and smoltification-related patterns of protease activities are quite similar in brown trout from the rivers of the White Sea basin such as the Krivoi Ruchey

and previously studied Indera river [12], however, some habitat-related differences were observed. These two rivers -- the Krivoi Ruchey and the Indera -- are of the same geographical zone and characterized by the similar annual temperatures and biodiversity, though the Krivoi Ruchey is more abandoned with organic drifting food resources, macrozoobenthic mass and biodiversity than the Indera. Brown trout individuals inhabiting more favorable biotope, such as the Krivoi Ruchey, achieve larger size to the same age and possess lower activities of muscle proteases including cathepsin D, cathepsin B, calpain, and proteasome (the latter two tended to be lower) at stages parr 0+ and smolts of any age. Results indicate that suppressed muscle protein breakdown throughout proteolytic systems corresponds with intense muscle growth in brown trout from the Krivoi Ruchey and confirm that fish growth regulation involves not only protein synthesis but also protein breakdown.

5. Conclusion

Thus, our study on brown trout inhabiting the Krivoi Ruchey river demonstrated age and stage-related differences in muscle protein breakdown rates developing coordinately throughout the calpain, proteasome, and cathepsin systems. Maximal protease activities observed in the youngest (0+) brown trout parr are associated with maximal individual growth increments in the group and expectedly decrease with fish age. Growth and smoltification-related patterns of protease activities were quite similar in brown trout populations from the Krivoi Ruchey river and previously studied rivers of the White Sea basin, however, some habitat-related differences were observed. This study indicates unique features of *S. trutta* life cycle and strategy and confirms an interspecies distance of smoltification process in brown trout and other salmonids. Smoltification-induced physiological changes affect fish growth and muscle physiology specifically regulated by proteases and protein breakdown.

Funding

The research was supported by the Russian Science Foundation, project no. 14-24-00102 (field experiments, sampling, and cytosolic protease activity assays) and budgetary theme № 0218-2019-0076 (lysosomal protease activity assays).

Acknowledgement

The authors deeply appreciate DSc. Alexey Veselov, Ph.D. Denis Efremov, and Mikhail Ruchyov (IB KarRC RAS, Lab. for Fish and Water Invertebrate Ecology) for their assistance in field sampling.

Conflict of Interest

The authors have no conflict of interest to declare.

References

[1] Klemetsen, A., Amundsen, P-A., Dempson, J.B., Jonsson, B., Jonsson, N., O'Connell, M.F., Mortensen, E. (2003). Atlantic salmon Salmosalar L., brown trout Salmotrutta L. and Arctic charrSalvelinusalpinus (L.): a review of aspects of their life histories. *Ecology of Freshwater Fish*,vol. 12, pp. 1--59.

[2] Johnston, I.A., Bower, N.I., Macqueen, D.J. (2011). Growth and the regulation of myotomal muscle mass in teleost fish. *Journal of Experimental Biology,* vol. 214, pp. 1617--1628.

[3] Goll, D.E., Thompson, V.F., Li, H., Wei, W., Cong, J. (2003). The calpain system. *Physiological Reviews*, vol. 83, pp. 731--801.

[4] Glickman, M.H., Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiological Reviews*, vol. 82, pp. 373--428.

[5] Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B., Turk, D. (2012). Cysteine cathepsins: from structure, function and regulation to new frontiers. *Biochimica etBiophysicaActa*,vol. 1824, pp. 68--88.

[6] Lysenko, L.A., Kantserova, N.P., Kaivarainen, E.I., Krupnova,M.Yu., Nemova, N.N. (2017). Skeletal muscle protease activities in the early growth and development of wild Atlantic salmon (Salmosalar L.). *Comparative Biochemistry and Physiology, Part B*, vol. 211C, pp. 22--28.

[7] Overturf, K., Gaylord, T.G. (2009). Determination of relative protein degradation activity at different life stages in rainbow trout (Oncorhynchus mykiss).*Comparative Biochemistry and Physiology, Part B*, vol. 152(2), pp. 150--160.

[8] Macqueen, D.J., Meischke, L., Manthri, S., Anwar, A., Solberg, C., Johnston, I.A. (2010).Characterisation of capn1, capn2-like, capn3 and capn11 genes in Atlantic

halibut (Hippoglossushippoglossus L.): Transcriptional regulation across tissues and in skeletal muscle at distinct nutritional states. *Gene*, vol. 453, pp. 45--58.

[9] Preziosa, E., Liu, S., Terova, G., Gao, X., Liu, H. (2013). Effect of nutrient restriction and re-feeding on calpain family genes in skeletal muscle of channel catfish (Ictaluruspunctatus). *PLoS ONE*, vol. 8(3), pp. e59404.

[10] Sun, Y., Liang, X., Chen, J., Tang, R., Li, L., Li, D. (2018). Change in ubiquitin proteasome system of grass carp Ctenopharyngodonidellusreared in the different stocking densities. *Frontiers in Physiology*, vol. 9, pp. 837.

[11] Cassidy, A.A., Saulnier, R.J., Lamarre, SG. (2016). Adjustments of protein metabolism in fasting arctic charr, Salvelinusalpinus. *PLos One*, vol. 11(4), pp. e0153364.

[12] Kantserova, N.P., Lysenko, L.A., Veselov, A.E., Nemova, N.N. (2017). Protein degradation systems in the skeletal muscles of parr and smolt Atlantic salmon Salmosalar L. and brown trout Salmotrutta L. *Fish Physiology and Biochemistry*, vol. 43(4), pp. 1187--1194.

[13] Mommsen, T.P. (2004). Salmon spawning migration and muscle protein metabolism: the August Krogh principle at work.*Comparative Biochemistry and Physiology, Part B*, vol. 139, pp. 383--400.

[14] Salem, M., Silverstein, J., Rexroad, C., Yao, J. (2007). Effect of starvation on global gene expression and proteolysis in rainbow trout (Oncorhynchus mykiss). *BMC Genomics*, vol. 8, p. 328.

[15] Seiliez, I., Dias, K., Cleveland, B.M. (2014). Contribution of the autophagy-lysosomal and ubiquitin-proteasomal proteolytic systems to total proteolysis in rainbow trout (Oncorhynchus mykiss) myotubes. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, vol. 307, pp. R1330--R1337.

[16] Quigley, D.T.G., Harvey, M.J., Hayden, T.J., Dowling, C., O' Keane, M.P. (2006). A comparative study of smoltification in sea trout (Salmotrutta L.) and Atlantic salmon (Salmosalar L.): seawater tolerance and thyroid hormone titres. *Proceedings of the Royal Irish Academy*, vol. B106B(1), pp. 35--47.

[17] Rodgers, K.J., Dean, R.T. (2003). Assessment of proteasome activity in cell lysates and tissue homogenates using peptide substrates. *International Journal of Biochemistryand Cell Biology*, vol. 35, pp. 716--727.

[18] Enns, D.L., Belcastro, A.N. (2006). Early activation and redistribution of calpain activity in skeletal muscle during hindlimb unweighting and reweighting. *Canadian Journal of Physiologyand Pharmacology*, vol. 84, pp. 601--609.

[19] Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analitical Biochemistry*, vol. 72, pp. 248--254.

[20] Barrett, A.J. (1980). Fluorimetric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates. *Biochemical Journal*, vol. 187, pp. 909--912.

[21] Zhao, J., Brault, J.J., Schild, A., Cao, P., Sandri, M., Schiaffino, S., Lecker, S.H., Goldberg, A.L, (2007). FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metabolism*, vol. 6, pp. 472--483.

[22] Stefansson, S.O., Björnsson, B.T., Ebbesson, L.O.E., McCormick, S.D. (2008). Smoltification. In: Finn RN, Kapoor BG (eds) Fish larval physiology. Science Publishers, Enfield, pp 639--681.

[23] Mommsen, T.P., Vijayan, M.M., Moon, T.W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries*, vol. 9, pp. 211--268.

[24] Jones, D.A., Bergman, E., Greenberg, L. (2015). Food availability in spring affects smolting in brown trout (Salmotrutta). *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 72, pp. 1694--1699.

[25] Marshall, W.S. (2002). Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. *Journal of Experimental Zoology*, vol. 293, pp. 264--283.

[26] Somero, G.N., Yancey, P.H. (2011). Osmolytes and cell-volume regulation: physiological and evolutionary principles. *Comparative Physiology,*vol. 31, pp. 441--484.