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Protein degradation systems in the skeletal muscles of parr and smolt Atlantic salmon *Salmo salar* L. and brown trout *Salmo trutta* L.

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Abstract Although protein degradation limits the rate of muscle growth in fish, the role of proteolytic systems responsible for degrading myofibrillar proteins in skeletal muscle is not well defined. The study herein aims to evaluate the role of calpains (calcium-activated proteases) and proteasomes (ATP-dependent proteases) in mediating muscle protein turnover at different life stages in wild salmonids. Protease activities were estimated in Atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.) parr and smolts from the Indera River (Kola Peninsula, Russia). Calpain and proteasome activities in Atlantic salmon skeletal muscles were lower in smolts as compared with parr. Reduced muscle protein degradation accompanying Atlantic salmon parr-smolt transformation appeared to provide intense muscle growth essential for a minimum threshold size achievement that is required for smoltification. Calpain and proteasome activities in brown trout parr and smolts at age 3+ did not significantly differ. However, calpain activity was higher in smolts brown trout 4+ as compared with parr, while proteasome activity was lower. Results suggest that brown trout smoltification does not correspond with intense muscle growth and is more facultative and plastic in comparison with Atlantic salmon smoltification. Obtained data on muscle protein degradation capacity as well as length-weight parameters of fish reflect differences between salmon and trout in growth and smoltification strategies.

Keywords Smoltification · Atlantic salmon · Brown trout · Protein degradation · Calpain · Proteasome

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Introduction

Atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.) exhibit phenotypic plasticity and life-history variations ranging from fully freshwater residents to anadromous forms (Klemetsen et al. 2003). Atlantic salmon and brown trout hatch in fresh water, grow there for one to several years, and can then smoltify. During smoltification or parr-smolt transformation, freshwater-dwelling parr undergo independent but coordinated morphological, behavioral, physiological, and biochemical transformations which preadapt them for survival and growth in the marine environment (Björnsson and Bradley 2007; Stefansson et al. 2008; Björnsson et al. 2011). Smoltification is regulated by environmental factors, such as photoperiod and water temperature, as well as by endogenous ones, such as endocrine system. Growth hormone/insulin-like growth factor (GH/IGF) system, thyroid hormones, cortisol, and prolactin are thought to act individually or synergistically to control food behavior, osmoregulation, metabolism, and growth during smoltification (Björnsson et al. 2011). Endocrine profiles during smoltification have been described quite completely for Atlantic salmon (McCormick et al. 1995, 2000, 2002; Agustsson

et al. 2001; Handeland et al. 2003), coho salmon (Sower et al. 1992; Shrimpton et al. 1994), and, to a lesser extent, brown trout (Quigley et al. 2006).

As mentioned previously, various life-history strategies and tactics, some of which are smoltification-associated, are used by different salmonid species (Klemetsen et al. 2003). Atlantic salmon spend at least 1 year in fresh water, migrate in spring as smolt, and spend at least 1 year at sea (McCormick 1994). Salmon smoltification is a size-dependent phenomenon (Stefansson et al. 2008). Atlantic salmon parr populations in autumn and winter are described by a bimodal size distribution (lower and upper growth modes) due to the different growth rates of individuals (Kristinsson et al. 1985; McCormick 1994). Under favorable growth conditions (food availability, water temperature, etc.) in Atlantic salmon populations, bimodality can be observed already in 0+ parr. Upper mode fish will smoltify during the next spring, whereas lower mode fish delay smoltification and migration for at least one more year (Stefansson et al. 2008). Thus, whether Atlantic salmon will smoltify or not is determined soon after midsummer of the previous year (Metcalfe et al. 1988). Brown trout as a rule spend three or more years in fresh water before migration (McCormik 1994); the decision to migrate is influenced by environmental conditions (Olsson et al. 2006). Additionally, Jones et al. (2015) reported that spring food availability during the current year influences smolt status in brown trout.

Individual growth in teleosts has an indeterminate nature, which is described by a constant increase of body length (skeletal growth) and mass (muscle growth), albeit the rate slows until mortality (Johnston et al. 2011). Skeletal muscle growth depends on a tightly controlled balance between protein synthesis and degradation (Johnston et al. 2011). Protein synthesis driven by hormone regulation is well studied in Atlantic salmon (Bower et al. 2008; Bower and Johnston 2010; Hevrøy et al. 2011), rainbow trout (Cleveland and Weber 2010), and other teleosts (Amaral and Johnston 2011). Protein degradation occurs mainly through the actions of three distinct pathways: intralysosomal digestion by cathepsins, calcium-dependent proteolysis by calpains, and the ubiquitin-proteasome system. The calcium-dependent proteolytic pathway may be a major pathway for regulating muscle turnover in fish (Salem et al. 2004, 2005a, b; Overturf and Gaylord 2009), while ubiquitin-targeted protein digestion by the proteasome is primarily responsible for bulk protein degradation (Seiliez et al. 2008). The role of protein degradation in spawning, other life stages, and distinct growth phases in salmonids has been studied (Mommsen 2004; Salem et al. 2004, 2005a, b; Overturf and Gaylord 2009; Lysenko et al. 2015; Nemova et al. 2016), but very little information on proteolysis in salmonid smoltification is available (Seear et al. 2010). This study aims to estimate the enzymatic activity of proteasomes and calpains, two proteases of muscle protein degradation pathways, in parr and smolts of salmonid species with different smoltification strategies: Atlantic salmon (*S. salar* L.) and brown trout (*S. trutta* L.).

Materials and methods

Sampling

Wild fish sampling was conducted on 20 June 2015 from the Indera River, which is located in the basin of the White Sea in Kola Peninsula, Russia. Water temperature in the Indera River was measured simultaneously with parr and smolts sampling and varied within the range of 11.3–11.5 °C. Atlantic salmon and brown trout parr were captured by electrofishing (Fa-2, Norway). To avoid possible effects of electrofishing, parr were kept for 24 h in cages located in the mainstream portion of the river. Several studies have shown that full physiological recovery of electroshocked fishes takes no more than 24 h (Schreck et al. 1976; VanderKooi et al. 2001; Bracewell et al. 2004; Woolmer et al. 2011). Atlantic salmon and brown trout smolts were captured during their natural seaward migration at a smolt trap located in the river 300 m from an estuary. Fish were not anesthetized. Each fish was killed with a blow to the head prior to body weight (W) and fork length (L) measurement. Fish age was determined by use of scales for Atlantic salmon and sacculus otoliths for brown trout. Fulton's condition factor (CF) was calculated from the formula: $CF = 100 \times W \times L^{-3}$. Mean body weights, fork lengths, and CF are presented in Tables 1 and 2. Fish were then frozen in liquid nitrogen, transported to the laboratory, and maintained at −80 °C. Tissue sampling for enzymatic assay was performed by removing a piece of muscle near the dorsal fin followed by whole-fish thawing at 4 °C for 5 to 20 min.

Table 1 Length-weight parameters and condition factor of Atlantic salmon from the Indera River

Group	Number	Length, cm	Weight, g	CF
2+ (parr)	6	9.8 ± 0.7	8.2 ± 1.8	0.87 ± 0.04
2+ (smolt, female)	9	11.9 ± 0.8a	13.4 ± 3.3a	0.79 ± 0.06a
2+ (smolt, male)	5	12.1 ± 0.9a	14.9 ± 3.7a	0.84 ± 0.06
3+ (smolt, female)	5	14.1 ± 0.5a, b	21.3 ± 3.2a, b	0.76 ± 0.03a
3+ (smolt, male)	6	14.1 ± 0.9a	20.9 ± 3.4a, c	0.75 ± 0.05a

Letters indicate significant differences: "a" in comparison with parr 2+, "b" in comparison with 2+ (smolt, female), "c" in comparison with 2+ (smolt, male)

Enzyme assays

Reagents and equipment

Chemical reagents, protease inhibitors, and protein substrates were purchased from Sigma-Aldrich (St Louis, MO, USA) and of analytical grade. Technical facilities of the Equipment Sharing Centre of the Institute of Biology, KarRC of RAS were used, such as freezing chamber UF 240-86 E (Snijders Scientific, The Netherlands); homogenizer Tissue Lyser LT (Qiagen, Germany); centrifuge Allegra 64R (Beckman Coulter, USA); and microplate reader CLARIOstar (BMG LABTECH, Germany).

Extraction of intracellular proteases

Samples (0.1 g each) were homogenized in 1:10 *w*/*v* 20 mM Tris-HCl (pH 7.5) with 150 mM NaCl, 5 mM EDTA, 20 mM dithiothreitol, 1 mM ATP, 5 mM $MgCl_2$, 0.1% Triton X-100, and a protease inhibitor cocktail (0.5 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL aprotinin, and 1 mM PMSF). Homogenates were centrifuged at 15,000 rpm for 30 min to obtain the enzyme-containing fraction.

Calpain activity assay

Calcium-dependent proteolytic activity was quantified using a microplate assay and casein as a substrate (Enns and Belcastro 2006). 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, remaining protein was quantified by Bradford assay (1976). 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.

Proteasome activity assay

The chymotrypsin-like activity of the proteasome was determined in the enzyme-containing fraction using a fluorescence assay (Rodgers and Dean 2003). 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-

Table 2 Length-weight parameters and condition factor of brown trout from the Indera River

Group	Number	Length, cm	Weight, g	CF
3+ (parr)	6	13.4 ± 1.3	24.4 ± 7.0	1.01 ± 0.06
4+ (parr)	4	16.5 ± 0.4a	46.4 ± 4.9a	1.04 ± 0.07
3+ (smolt, female)	5	15.3 ± 1.3	29.4 ± 8.8b	0.82 ± 0.07a, b
3+ (smolt, male)	5	15.1 ± 0.4b	32.7 ± 7.4b	0.95 ± 0.13
4+ (smolt, female)	5	18.5 ± 1.2a	55.9 ± 9.1c	0.88 ± 0.04a, b
4+ (smolt, male)	4	17.5 ± 0.3a, b, d	50.8 ± 6.0d	0.94 ± 0.08c

Letters indicate significant differences: "a" in comparison with parr 3+, "b" in comparison with parr 4+, "c" in comparison with smolt (3+, female), "d" in comparison with smolt (3+, male)

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 and 440 nm, respectively. The change in proteasome activity was normalized to sample protein concentration and expressed as relative fluorescence fold change (FU).

Statistical analysis

Data are expressed as the mean ± SD. Raw data were initially checked for normality of distribution and homogeneity of variances (Kolmogorov-Smirnov and Levene's tests, respectively) and then analyzed with a Kruskal-Wallis test. Differences between groups were evaluated by Mann-Witney *U* test. The significance threshold was set at 0.05.

Results

Fish length-weight parameters and condition factor (CF)

Length and weight parameters of Atlantic salmon parr and smolts at the same age were significantly different (Table 1). Atlantic salmon smolt CF (excepting male 2+ CF) was significantly lower than that of parr. Significant differences in growth between brown trout parr and smolts at ages 3+ and 4+ (excepting length data between male smolts and parr) were not shown. In brown trout, significant difference in CF was found between parr and female smolts only (Table 2).

Atlantic salmon protease activities

Calpain activity was lower in smolts than in parr of the same age group (2+). Significant differences in calpain activity between smolts 3+ and parr 2+ were also shown (Fig. 1). Proteasome activity was lower in smolts 3+ (both sexes) as well as in male smolts 2+ compared to the parr 2+ (Fig. 2).

Brown trout protease activities

There were no significant differences in calpain and proteasome activities between parrs and smolts at the age 3+. Calpain activity in smolts 4+ was higher than that of parr 4+ (Fig. 3). Proteasome activity in smolts 4+ showed a significant drop compared with those of parr 3+ and parr 4+. Both female and male smolt proteasome activities significantly differed between studied age groups (3+ and 4+, Fig. 4).

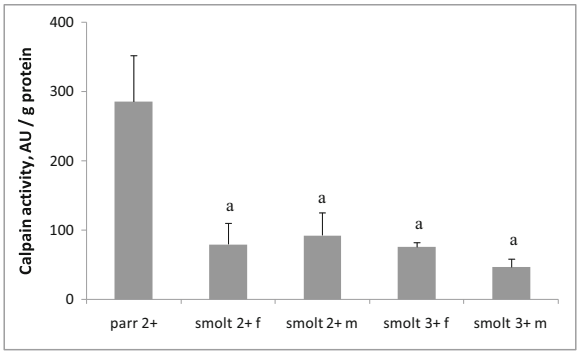

Fig. 1 Calpain activity in *S. salar* of different age (2+, 3+), stage (parr, smolt), and sex (m = male, f = female). Letter *a* indicates a significant difference in calpain activity in comparison with parr 2+

Discussion

Results indicate different muscle protein degradation rates throughout cytosolic degradation system (calpain and proteasome) between Atlantic salmon parr and smolts. Overall, calpain and proteasome activity decreased during parr-smolt transformation while fish size increased. As stated by Overturf and Gaylord (2009), muscle protein degradation management acts as the checkpoint in directing the regulation of protein turnover, muscle deposition, and growth.

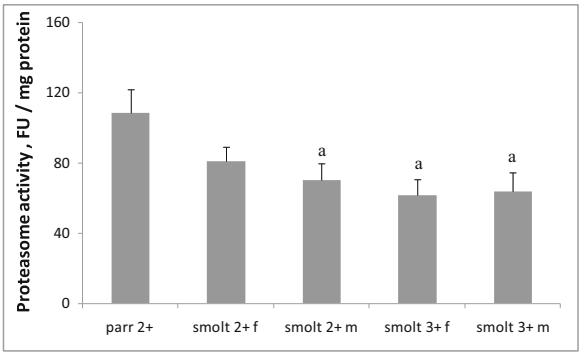

Fig. 2 Proteasome activity in *S. salar* of different age (2+, 3+), stage (parr, smolt), and sex (m = male, f = female). Letter *a* indicates a significant difference in proteasome activity in comparison with parr 2+

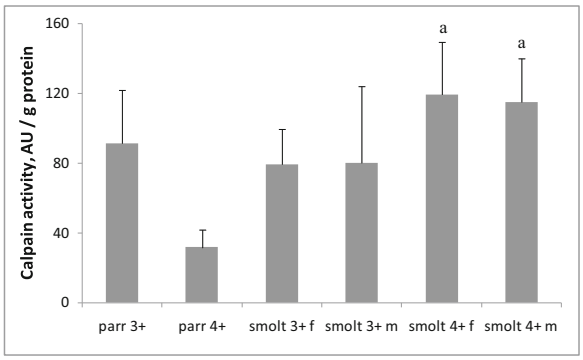

Fig. 3 Calpain activity in *S. trutta* of different age (3+, 4+), stage (parr, smolt), and sex (m = male, f = female). Letter *a* indicates a significant difference in proteasome activity in comparison with parr 4+

Atlantic salmon smoltification is a size-related process: fish that have achieved a minimum threshold size will become smolts (Stefansson et al. 2008). The difference in size of future salmon smolts and individuals which delay smoltification for at least one more year is so distinct that histograms of fish size have a bimodal distribution (Thorpe 1977; Thorpe et al. 1982; Kristinsson et al. 1985; Nicieza et al. 1994). Size-related development of salmon is regulated by hormone status; plasma levels of GH, IGF-I, cortisol, and thyroid hormones differ between upper and lower mode fish as well as between parr and smolts (Stefansson et al. 2008). Although direct or through IGF-I, anabolic effects of GH leading to protein accretion have been described in Atlantic salmon (Björnsson et al. 2002), little information on hormonal regulation of protein catabolism is available for teleosts (Johnston et al. 2011).

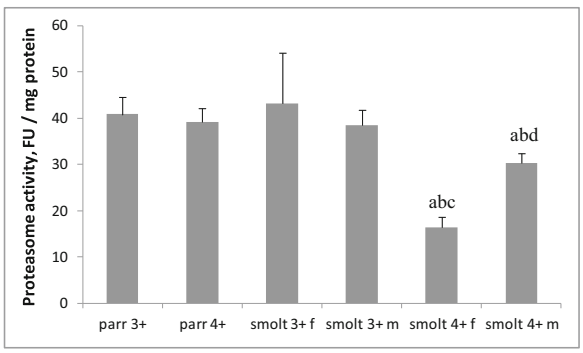

Fig. 4 Proteasome activity in *S. trutta* of different age (3+, 4+), stage (parr, smolt), and sex (m = male, f = female). Letters indicate a significant difference: *a* in proteasome activity in comparison with parr 3+, *b* in comparison with parr 4+, *c* in comparison with smolt 3+ (female), *d* in comparison with smolt 3+ (male)

Results indicate that reduced muscle protein degradation provides intense muscle growth in smoltifing Atlantic salmon parr and confirm that fish growth depends not only on protein synthesis but also equally on protein degradation.

The present study showed no significant differences in length and mass between brown trout parr and smolts at the same age (excepting length data between male smolts 4+ and parr 4+). Results are consistent with data describing size differentiation between brown trout parr and smolts (Leonko and Chernitskiy 1986). Moreover, in most studies on brown trout parr, bimodality of size distribution is not shown (Tanguy et al. 1994; Dêbowski et al. 2010). Size variation between future smolts and parr that delay smoltification for at least 1 year cannot be used for predicting the number of brown trout smolts (Dêbowski et al. 2010), unlike what is observed for Atlantic salmon smolts (Bagliniere and Champigneulle 1986). Thus, it is unlikely that brown trout smoltification is a size-dependent process. It should be noted that trout smoltification and the decision to migrate are affected by spring food availability regardless of conditions in the previous autumn or winter (Jones et al. 2015). Unlike the decision of Atlantic salmon to smoltify depending on size and energetic threshold, which is reached in the previous year (Stefansson et al. 2008), brown trout decide in the current spring whether to smoltify; for brown trout, reduced food increases smoltification and seaward migration (Jones et al. 2015). Apparently, brown trout have no need for intense muscle growth for parr-smolt transformation. Protease assay indicates no significant differences in both calpain and proteasome activities between parr and smolts 3+ that corresponds with smoltification features of this salmonid species previously discussed.

Atlantic salmon smolts CF reduction observed in the present study supports the observations that salmon smolts grow more in length than weight (McCormick et al. 1998). Apparently, it indicates both an adaptive change in morphology during smolting (e.g., increase of swimming performance in ocean) and high energetic demands of smolt transformation. It is known that condition factor reduction is associated with a non-proportional growth of the caudal peduncle of smolts (Winans and Nishioka 1987) as well as with a decrease in total lipid content (Sheridan 1989). The CF and muscle protein degradation reduction by a similar manner during salmon smoltification indicates specific features of smolting salmon metabolism. In contrast, in

brown trout smolts, CF either decreased (in females) or did not change (in males) during smoltification. We did not describe clear relationship between changes of condition factor and muscle protein degradation rates in smolting brown trout. Similar observations on negligible changes or even increases of CF in smolting brown trout have been previously described (Tanguy et al. 1994; Quigley et al. 2006). It is known that smoltification-induced non-proportional caudal peduncle shape change is more expressed in Atlantic salmon than in brown trout (Quigley et al. 2006). It is an additional evidence that brown trout smoltification is not as clear or complete as that of Atlantic salmon (McCormik 1994; Tanguy et al. 1994).

Interestingly, brown trout 4+ smoltification is associated with increased calpain activity. Due to spontaneous parr-smolt transitioning, brown trout need to develop hypoosmoregulatory mechanisms in a short time. Along with Na^+/K^+-ATPase upregulation—a key mechanism of osmoregulation in fish (Marshall 2002)—a contribution of accumulated free amino acids resulting from increased calpain-mediated protein degradation can be attributed to trout salinity tolerance. The osmolyte role of free amino acids has been demonstrated for several euryhaline species, including salmonid species such as rainbow trout (Kaushik and Luquet 1979). It should be noted that proteasome activity decreased in brown trout 4+ following smoltification. It is known that protein degradation in fish muscle relies more on calcium-dependent proteolysis (Salem et al. 2004, 2005a, b; Overturf and Gaylord 2009), while proteasomal digestion does not function as the primary method of muscle degradation in teleosts (Kolditz et al. 2008; Seiliez et al. 2008; Overturf and Gaylord 2009). Future research will need to study specific features of proteasomal digestion in muscle fish.

Discovered differences in calpain and proteasome activities between brown trout pre-smolts (parr 4+) and smolts, as well as between smolts 3+ and 4+, reflect specific features of brown trout smoltification, which is considered more facultative and plastic in comparison with the process in Atlantic salmon (McCormik 1994; Klemetsen et al. 2003). Seawater tolerance development occurs in a shorter period for brown trout than in Atlantic salmon (Tanguy et al. 1994; Quigley et al. 2006). Atlantic salmon smolts migrate to the open seas for feeding, whereas brown trout remain feeding in coastal waters with variable salinity levels (Klemetsen et al. 2003; Thorstad et al. 2007).

In conclusion, these findings indicate that muscle protein degradation systems use different mechanisms to contribute to smoltification in Atlantic salmon and brown trout. Results indicate underlying mechanisms of Atlantic salmon parr-smolt transformation depend on size threshold achievement as well as on flexibility of brown trout smoltification. Obtained results enhance our knowledge of such mechanisms and the regulation of salmonid growth and development.

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