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Growth-Related Dynamics of Muscle Protein Degradation in Reared *Oncorhynchus Mykiss* during Growing Season

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Abstract

Although temperature influence on the physiology of rainbow trout have been widely studied, there is little information about the responses of muscle growth and overall protein turnover to temperatures. Therefore, body growth rate and muscle protein turnover of cultivated rainbow trout (*Oncorhynchus mykiss*) of 0+ and 1+ year-classes were studied within a growing season since June to October. Fish grown on cages on a natural lake does not face with food shortage or other growth-retarding factors, and the environmental water temperature affects fish growth primarily. There were detected that water temperature increase in-season results in maximal growth increments and maximal protein turnover rate in trout muscles. Since protein synthesis recognized to be similar between individuals, high overall protein turnover rate depended on increased protein-degrading activities mostly relying on calpain system. Trout of different year-classes significantly differs in growth rate and calpain activity coordinately decreasing with age while their temperature responses are quite similar. New data expands on aquaculture practice since based on our observations on coincidence of peak water temperatures (mid-summer) and increased muscle protease activities in trout we can recommend excluding this period for fish slaughter to avoid postmortem flesh deterioration and excessive fillet softening.

Keywords: calpain, proteolysis, skeletal muscle, growth, fish, *O. mykiss*

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1. Introduction

In aquaculture, fish growth and fillet quality are important traits that impact profitability. Fish growth rate directly depends on the accumulation of skeletal muscle mass constituting more than a half of the total body weight and containing mainly proteins [1-4]. In teleost fish, the rates of muscle protein synthesis and degradation are apparently counterbalanced to support indeterminate growth. A most studies on the mechanisms of fish muscle growth focuses on protein synthesis regulation [4, 5] but not degradation though up- or down-regulation of proteolytic enzymes significantly perturb muscle protein accumulation. Protein synthesis rates in fish appear relatively similar between

individuals when protein consumption is accounted for, but the protein retention is variable and is believed to result from differing rates of protein degradation [6]. Total protein degradation relies on three main proteolytic systems such as ubiquitin-proteasome, lysosomal-autophagy and calpain-calpastatin. A role of individual proteases as well as their relative contribution to the total protein degradation in the skeletal muscles has been partially characterized in salmonid fish [6-10]. It was shown that in the muscle of fish, in contrast to terrestrial vertebrates, ATP-independent proteolytic pathways, such as calpain and autophagy, dominate while proteasome plays a minor role [9]. Nowadays the members of calpain proteolytic system including calcium-dependent proteases and their proteinaceous inhibitor, calpastatin, have been characterized in several teleost fish including salmonids [7, 10, 11]. Calpains exert a crucial role in fish muscle physiology due to their high proteolytic capacity against large myofibrillar proteins [12, 13] and calpain-mediated mechanisms in myogenesis, including myoblast fusion [14, 15]. In addition, the expression of muscle calpains was shown to be inversely correlated with fish muscle texture and postmortem deterioration of fish fillet, indicating they may serve as potential markers of flesh quality [7, 16]. However, little is known about their contribution to proteolytic strategies of protein degradation in fish.

In contrast to salmonids of wild populations, artificially growing fish does not faced with food shortage or life stages of accelerated protein breakdown, such as gonad maturation or spawning migration, when protein degradation far outweighs protein synthesis [16-18], and their growth and protein deposition rates primarily depend on water temperature and photoperiod varying during year cycle. Temperature influences physiological variables in fishes including food consumption, growth, and metabolic rates [19]. Much research into temperature effects has been conducted on salmonid fishes, especially rainbow trout (*Oncorhynchus mykiss*) [19, 20] though little attention has been directed at temperature-related effects on protein turnover and controlling mechanisms. Maximal weight increments are achieved at thermal optimum for a species (14-16 °C for *O. mykiss*) resulting from complete digestion and assimilation of consumed nutrients due to optimal conditions for enzymatic reactions in poikilothermic organisms like fish. Besides, physiological effects of elevated temperatures approaching the incipient lethal limit for rainbow trout have received little attention though in North-Western Karelia water temperatures above the optimal range are commonplace.

The intent of this work was to assess the dynamics of body growth and calpain activity in the skeletal muscles of reared rainbow trout of 0+ and 1+ year-classes during a summer-autumn season in order to evaluate temperature dependence of the muscle growth and underlying mechanisms of muscle physiology. The results on muscle calpain

activity on intact healthy individuals provide us considered background values to further investigations on protease responses to multiple physiological and exogenous effectors such as hormones, nutrition, infectious diseases, hypoxia, temperature, pollutants, etc. Since myofibrillar-degrading proteases contribute to both muscle protein accretion in growing fish and postmortem fillet softening, the detailed knowledge of protein degradation mechanisms in fish muscle will be of benefit to the aquaculture industry.

2. Material and Methods

2.1. Material

2.1.1. Experimental fish

Rainbow trout (*O. mykiss*, Walbaum 1792) juveniles, age 0+ and 1+, grown on a commercial rainbow trout farm on Ladoga lake (Karelia Republic, Russia) were used in a study. Trout were maintained in cages at nature environmental variables; means of temperature are in Table. Fish were fed to apparent satiation twice daily with commercial trout pellets (manufacturer's analysis: 46% crude protein, 23% crude fat, 16% carbohydrates, 1.4% crude fiber, 7.3% ash, 1.2% phosphorus, and 19.9 MJ/kg digestible energy; AS BioMar, Aarhus, Denmark).

TABLE 1: Biometric parameters of rainbow trout of two year-classes (0+ and 1+) in different sampling dates. Data are shown as means ± SEM (n = 8). Alphabet letters indicate significant differences: ``a'' in comparison with 26 Jun, ``b'' in comparison with 06 Jul, ``c'' in comparison with 16 Jul, ``d'' in comparison with 27 Jul, ``e'' in comparison with 23 Aug ($p < 0.05$).

Date	Water temperature,˚C	Length, cm	Weight, g	Length, cm	Weight, g
		age 0+		age 1+	
26 Jun	11.3	9.22±0.38	10.56±0.74	29.50±2.81	404.12±124.25
06 Jul	18.4	10.14±0.59^{a}	15.44±1.07^{a}	29.70±2.31	459.38±114.65
16 Jul	18.6	13.14±0.47^{ab}	32.92±1.67^{ab}	32.67±3.04	572.95±74.66
27 Jul	18.1	14.32±1.60^{bc}	49.82±8.19^{abc}	33.90±3.31	653.24±100.22
23 Aug	16.3	18.65±2.41^{abcd}	108.73±18.39^{abcd}	36.41±4.94	916.12±170.48
11 Oct	11.2	24.12±3.26^{abcde}	244.95±44.89^{abcde}	40.87±3.83^{abcd}	1379.80±227.20^{abcd}

2.1.2. Sampling

Sampling was carried out at the dates June 26, July 6, 16, 27, August 23, October 11. Prior to sampling, fish were killed by a blow to the head and medullar section, a procedure approved by the local animal ethics committee. Fish were weighed to the

nearest 0.1 g on a calibrated electronic balance and fork length measured to the nearest mm. White (fast) skeletal muscle near dorsal fin was extracted, immediately frozen in liquid nitrogen and stored at --80°C until assayed. Biochemical indices were measured in individual fish.

2.2. Reagents and equipment

Chemical reagents, protease inhibitors and 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); spectrophotometer SP-2000 (OKB Spectrum, Russia), and microplate reader CLARIOstar (BMG LABTECH, Germany).

2.3. Methods

2.3.1. Extraction of calpain proteases

Samples (0.1 g each) were homogenized in 1:10 w/v 20 mM *Tris*-HCl (pH 7.5) with 150 mM NaCl, 20 mM dithiothreitol, 0.1% Triton X-100, and protease inhibitors (0.5 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL aprotinin, and 1 mM PMSF). Homogenates were centrifuged at 20,600 g for 30 min to obtain a pooled fraction of soluble cytoplasmic and organelle proteins referred as enzyme-containing fraction.

2.3.2. Calpain activity assay

Calcium-dependent proteolytic activity was quantified using a microplate assay and casein as a substrate [21]. 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 Ca2+ (as CaCl2) or 5.0 mM EDTA (negative control), and the enzyme-containing fraction. Following incubation at 28 °C for 30 min (heating/cooling dry block CH-100; BioSan, Latvia), remaining protein was quantified by Bradford assay [22]. 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 for 1.0 mg protein in a sample.

2.3.3. Total protein content determination

Protein levels were measured according to the method of Bradford [22] and the protein concentrations (mg of protein per g of tissue wet weight) in enzyme-containing fractions were determined by comparison with bovine serum albumin standards.

2.3.4. Statistical analyses

Raw data were initially checked for normality of distribution and homogeneity of variances by Kolmogorov-Smirnov and Levene's tests, respectively. As variances were unequal and not distributed normally, multiple groups were compared with non-parametric Kruskal-Wallis test and two groups with Mann-Whitney *U* test. Values are throughout presented as mean ± SD. A p value < 0.05 was considered statistically significant in all analyses.

3. Results

3.1. Fish growth dynamics

Linear and weight growth performance of rainbow trout is shown in Table. In both year-classes (under-yearlings and yearlings), weight and length of fish steadily increased with the growing season. Within under-yearling group, significant differences were detected between all the sampling dates. Due to high individual variability in size among yearlings, their body weight and length difference achieve statistical significance only by the end of observation date if compare with initial time point. Although insignificant differences in 1+ group, rainbow trout growth rate tended to increase with water temperature over the 16 to 18 °C range. Maximal growth rate was achieved since 6 to 16 July (2.13 and 1.25-fold in 0+ and 1+ groups, respectively) right after water temperature rise from 11.3 up to 18.4 °C.

3.2. Skeletal muscle calpain activity

Muscle Ca^{2+}-dependent proteolytic, or calpain, activity was considerably higher (though insignificant due to high individual dispersion of the parameter) in the younger individuals (age group 0+) if compare with older age group (1+) throughout a season (Figure 1). Water temperature curve and fish growth rate mostly coincide with calpain activity

dynamics in both trout groups with peak values at July sampling dates. The only exception is proteolytic maximum in 26 June samples of both age groups.

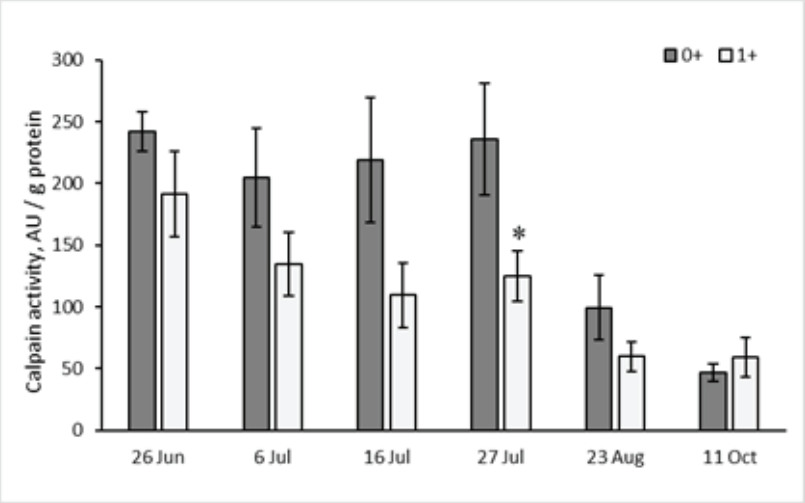

Figure 1: Calpain activity in skeletal muscle of rainbow trout (*O. mykiss*) of different year-classes. *indicates significant differences between year-classes ($p < 0.05$).

4. Discussion

4.1. Season-related changes in growth and protease activities

Seasonal patterns of physiological processes like growth in cultivated rainbow trout are determined primarily by environmental variables such as water temperature modifying in turn other parameters, such as oxygen content or organic contamination. Interpreting the mechanisms of temperature responses of rainbow trout juveniles of different year-classes, our data on growth rate and muscle protease activities show their high similarity (with a few exceptions) indicating common mechanisms governing fish growth and muscle physiology. Maximal coordinated increase in growth increments and proteolytic activity coincide with temperature rise thus indicating signal effect of thermal factor on overall protein metabolism. This metabolic effect is apparently age-independent despite confounding effects of size which tended to mask the temperature effect. Our results together probably demonstrate a shift in energy allocation with increasing temperature, with metabolism receiving a greater portion of the ingested energy than at lower temperatures. Since maximal weight and length gains in both age groups, this temperature range recognized to be optimal for rainbow trout growth, besides, it does not exceed thermal tolerance of the species quite similar among salmonids.

4.2. Age-related changes in growth and protease activities

Since the skeletal muscles comprise a substantial proportion of fish body weight, the growth of the organism to larger extent relies upon increased muscle mass [1, 2]. In fish, skeletal muscle protein pool is a reserve of amino acids that can be mobilized during fasting or protein deficit to provide substrates for hepatic gluconeogenesis and energy production [17] as well as protein synthesis. According to estimations [6, 9], rates of protein degradation in salmonid muscle are 5--35% greater than what has been calculated in mammals and nonenergy-dependent proteolysis, such as autophagy and calpains, dominate in total protein degradation due to their energetically efficiency for protein turnover. Muscle growth in juvenile fish is determined by high rates of muscle protein synthesis, accumulation, and structural integration onto new muscle fibers associated with high but not exceeding rate of protein degradation. Muscle growth is supported by persistent protein degradation involving routine turnover of endogenous proteins, recycling of amino acids, protein quality controlling machinery eliminating abnormal proteins and organelles, and protease-dependent tissue remodeling throughout hyperplastic mode of muscle growth inherent to young fish [3]. Calpains play both degradative and signaling roles contributing to turnover of myofibril and sarcomere proteins, reorganization of cytoskeleton and supramolecular complexes, and specific regulation of myocyte fusion and myocyte maturation [13-15]. The highest growth rate in fish of the youngest ages (0+) is associated with maximal muscle protease activities including calpain and proteasome [10]. As fish grow larger their rate of growth and overall metabolism decrease. Gradual age-dependent decrease in total body mass accumulation rate in salmonids is tightly connected with the decrease in both protein synthesis and degradation. In juvenile salmon of 0+ and 1+ but not in other year-classes, a positive correlation between the fish weight and protein synthesis measured by RNA/DNA ratio was described [23]. Up to 2+ year age, the mRNA expression of myosin heavy chain (MyHC), a main myofibril component susceptible to calpain-dependent hydrolysis in fish [12], positively correlates with fish body weight [23]. The age-dependent decrease in protein-degrading capacity in fish muscles particularly concerns calpain and proteasome systems. Similar thermal tolerances and muscle-growth protein turnover regulation emphasis similarity between the different age groups' physiological performances.

4.3. Practical values of the findings for trout aquaculture

In post-mortem muscles, remaining protease activities alter fillet texture. Despite partial inactivation due to chilling of fish fillet, some proteases like cathepsins become even more active being released from intracellular compartments in postmortem muscles. High proteolytic capacity of calpains and other proteases against structural myofibrillar proteins results in postmortem deterioration of fish products during storage and shelf-life [7, 24]. Calpain-dependent proteolysis of large molecules supporting muscle architecture promotes myofibril disintegration and increases susceptibility of protein substrate to cathepsins or proteasome digestion. Since that, remaining calpain activity is undesirable in the final commercial fish product in order to safe its quality. Our results support the researches indicated that calpain mRNA and activity are up regulated in both intense growth and fasting-induced muscle degradation [11] and that softening of muscle can be accelerated by activation of calpains with exogenous calcium [7]. For rational aquaculture, the results presented here suggest that in order to produce high-quality fish fillet the period of peak water temperatures (mid-summer) coinciding the period of faster growth rate and increased calpain activity should not be used for fish slaughtering. These data suggest potential use of the calpains as a biogenetic tool to monitor fish muscle growth and texture quality as well as to select fish strains with desired fillet tenderness.

5. Conclusion

Our results considerably expand the knowledge on the growth patterns and muscle physiology of reared rainbow trout of different age. Muscle growth in trout juveniles depends on overall protein turnover in their muscles mostly relying on calpain and, to a lesser extent, other protease activities. Since linear growth and body mass accumulation decrease with fish age, muscle protein turnover varies too to maintain indeterminate fish growth. High water temperatures (16-18 °C) accelerate linear and weight growth increments of trout promoting muscle protein turnover by both protein synthesis and degradation particularly by calpain-dependent pathway. Based on our observations on calpain activity in the skeletal muscles of trout juveniles, we suggest that there is similar correlation of the level of protein degradation and fish growth rate. Our results on temperature-dependent calpain response could be used in commercial production of widely cultivated fish species like rainbow trout. According to our data the period of

high temperatures coinciding with maximal proteolytic activities in fish muscle should be excluded for fish slaughtering to avoid postmortem fillet softening and deterioration.

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Conflict of Interest

The authors have no conflict of interest to declare.

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