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Age- and stage-dependent variations of muscle-specific gene expression in brown trout *Salmo trutta* L.

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ABSTRACT

This study was conducted to characterize the features of muscle-specific genes expression during development of brown trout *Salmo trutta* inhabiting the river Krivoy ruchey (Kola Peninsula, Russia). Gene expression levels of myogenic regulatory factors (MRFs – *MyoD1* paralogs (*MyoD1a*, *MyoD1b*, *MyoD1c)*, *Myf5*, *myogenin*), myostatin paralogs (*MSTN-1a*, *MSTN-1b*, *MSTN-2a*), fast skeletal myosin heavy chain (*MyHC*) were measured in the white muscles of brown trout parr of ages 0 + (under-yearling), 1 + (yearling) and 2 + (two year old) and smolts of age 2 +. Multidirectional changes in *MyoD1* and *MSTN* paralogs expression along with *myogenin, Myf 5* and *MyHC* expression levels in white muscles in parr of trout with age were revealed. The expression of *MyoD1c*, *myogenin*, *MSTN-2a* was the highest in 0 + parr and then decreased. *MyoD1a/b* expression levels didn't differ between age groups. The simultaneous elevation of *MyHC*, *Myf5*, *MSTN-1a*, and *MSTN-1b* was found in trout yearlings. In smolts, expression levels of *MSTN* paralogs, *MyHC*, *Myf5*, *MyoD1a* was lower than in parr. But in contrast, the *MyoD1c* and *myogenin* mRNA levels was higher in smolts. The study revealed that there are definite patterns in simultaneous muscle-specific genes expression in age groups of parr and smolts. As *MyoD* and *MSTN* paralogs expression changed differently in dependence on age and stage, it was suggested that paralogs of the same gene complementarily control myogenesis during development.

1. Introduction

In most fish, skeletal muscles comprise the larger part of the body (about 60% of weight) and therefore play a great role in the metabolism of the entire organism and determine total growth rate (Houlihan et al., 1993). Postnatal muscle growth in fish occurs by both mechanism hyperplasia and hypertrophy and is a result of the activation of adult skeletal muscle-specific stem cells, myogenic precursor cells (MPCs). During hypertrophy, MPCs activate, proliferate and fuse with existing myofibers to become new myonuclei. In hyperplastic growth, MPCs proliferate to generate fields of myoblasts that fuse to form multi-nucleated myotubes (Johnston et al., 2011).

The main role in myogenesis regulation belongs to specific myogenic regulatory factors (MRF), the transcription factors of a bHLH family: *MyoD*, *Myf5*, *myogenin* and MRF4 (Watabe, 2001). These factors have highly conserved basic helix-loop-helix (bHLH) domain which is linked to the DNA sequence *E*-box, which is found in the promoting region of many skeletal muscle-specific genes. Transcription factors *MyoD* and *Myf5* play a key role in specification and proliferation of myoblasts and *myogenin* and MRF4 control the differentiation and fusion of myoblasts to form myofibers (Watabe, 2001). The quantitative expression of MRFs in reference to hypertrophic and hyperplastic muscle growth mechanisms was studied in rainbow trout *Oncorhynchus mykiss* (Johansen and Overturf, 2005) and pacu *Piaractus mesopotamicus* (Almeida et al., 2008, 2010) at different developmental stages. It was shown on wild and farmed salmonids, that MRF expression depends on various factors, such as temperature (Macqueen et al., 2007), feed composition and feeding regime (Johansen and Overturf, 2006; Bower et al., 2009, Overturf et al., 2010).

The mechanism of myogenesis regulation by transcription factor *MyoD* is complicated by presence of its paralogs. In result of tetraploidization salmonids expressed three *MyoD1* paralogs – *MyoD1a*, *MyoD1b*, and *MyoD1c* (Macqueen and Johnston, 2008). The *MyoD* paralogues have sub-functionalized and they exhibit distinct expression patterns during development and in different fiber types (Macqueen and Johnston, 2006). *MyoD* paralogs are differentially expressed during myotube maturation, and that suggests that *Myod1b* and *Myod1c* are primarily expressed in proliferating cells and *MyoD1a* in differentiating cells. This provides evidence for their subfunctionalization following whole genome duplication in Atlantic salmon. Based on this it is possible, that *Myod1b* and *Myod1c* regulate cell cycle and *myoD1a* is involved in terminal differentiation (Bower and Johnston, 2010).

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A key regulator of myogenesis is myostatin (*MSTN*), a member of transforming growth factors-β (TGF-β). *MSTN* is a negative regulator of muscle growth and inhibits proliferation and differentiation of muscle cells in mammals (Gabillard et al., 2013). However, according to recent studies, the mechanism of *MSTN* action on growth in fish probably differs from mammals. *MSTN* is conserved as two genes in most teleost and four genes in salmonids and is found to be expressed in muscles and other tissue. Consequently, it may possess different functions (Østbye et al., 2001; Gabillard et al., 2013). In salmonids, *MSTN* is presented by genes *MSTN-1a*, *MSTN-1b*, *MSTN-2a* and *MSTN-2b* (which is a pseudogene). Studies in salmonids have shown that *MSTN* regulation of muscle growth mechanisms are dependent on the muscle type, developmental stage and nutritional conditions (Østbye et al., 2001; Johansen and Overturf, 2005, 2006).

Myosin, the primary protein of muscles, amounts to 25% of total protein in the whole organism and 50% of the muscle proteins (Watabe and Ikeda, 2006). Thus it is advantageous protein for growth studies. It was revealed that *MyHC* mRNA level can be correlated with the growth rate of rainbow trout *Oncorhynchus mykiss* (Overturf and Hardy, 2001), wolffish *Anarhichas minor* (Imsland et al., 2006), walleye Sanders vitreous (Dhillon et al., 2009) and weight of Atlantic salmon parr within different age groups (Churova et al., 2015).

The Salmonidae, including brown trout *Salmo trutta* L., are the most important and interesting objects to study myogenesis. The life cycle of Salmonidae includes various developmental stages with a complex system of adaptations. One of the primary processes is parr-smolt transformation (smoltification), the preparing for life in the sea (Quigley et al., 2006). That is why the growth of salmonid fishes is influenced by numerous factors and differs among the age groups and as a result of smoltification. As it was mentioned, due to genome tetraploidization of salmonids there are several paralogs of genes that regulate the process of muscle formation and growth, which function and features of expression is needed to investigate. In spite of numerous studies devoted to myogenesis of Salmonidae in ontogenesis, there is not enough information about age-related differences and alterations during smoltification in muscle-specific genes expression. Moreover, the study on *MyoD* and *MSTN* paralogs expression in correspondence to other MRFs could elucidate their age- and stage- dependent features in expression and functional differentiation. Based on this, gene expression levels of myogenic regulatory factors (*MyoD1* paralogs: *MyoD1a*, *MyoD1b*, *MyoD1; Myf5*, *myogenin*), myostatin paralogs (*MSTN-1a*, *MSTN-1b*, *MSTN-2a*) and fast skeletal myosin heavy chain (*MyHC*) were studied in white muscles in wild brown trout parrs and smolts of different ages.

2. Materials and methods

2.1. Fish samples

Parr of brown trout (*Salmo trutta* L.) at ages 0 + (under-yearling), 1 + (yearling) and 2 + (two year old) and smolts at age 2 + was inhabited the Krivoj Ruchey River (the Kola Peninsula, Russia) were studied. Salmonid fish catch and study were conducted in the accordance with a resolution no. 51 2015 03 0119 by Barentsevo-Belomorskoye territorial department of the Federal Agency for Fisheries.

Samples were collected in June 2015 at a water temperature of 11,5 °C. The parr were caught by electric fishing gear (model Fa_2, Norway). To avoid the effect of electrofishing, fingerlings were held for 24 h in cages located in the mainstream portion of the river. Smolts were caught by smolt catches, located in the River 300 m from an estuary. Fish were individually measured and weighed. The age of trout was determined from their scales. Tissue pieces were frozen in liquid nitrogen and kept at − 80 °C prior to analysis. The mean body weight, total length, and some fish are presented in Table 1.

This study was carried out at the Scientific Center of Collective Usage Platform of the Institute of Biology of the Karelian Research Centre of the Russian Academy of Sciences.

Table 1
Mean (M ± SE) weight and length of parr and smolts of different ages.

Age	n	Weight, g	Fork length, cm
Parr			
0 +	8	0.10 ± 0.05	2.44 ± 0.33
1 +	8	1.34 ± 0.75	5.27 ± 0.65
2 +	7	9.19 ± 0.95	9.95 ± 0.83
Smolts			
2 +	7	27.20 ± 3.21	14.47 ± 0.95

2.2. Myf5, MyoD1, myogenin, MSTN, MyHC mRNA gene expression

Total RNA was isolated from the dorsal white muscles samples using a kit “RNA-extra,” analog of TRIzol (Evrogen, Russia) according to the manufacturer protocol. Then total RNA was treated with DNase (Sileks, Russia). RNA integrity and quality was assessed by 1% agarose gel electrophoresis and spectrophotometrically at 260/280 nm absorbance ratio (SmartSpec Plus, BioRad, USA). RNA was reverse transcribed using MMLV-reverse transcriptase and random hexamer primers (Evrogen, Russia).

Real-time-PCR assay was conducted using the iQ5 real-time PCR detection systems (BioRad, USA). The primers for the fast skeletal *MyHC*, *myogenin*, *MyoD1a*, *MyoD1b*, *MyoD1c*, *Myf5*, *MSTN-1a*, *MSTN-1b*, *MSTN-2a* and elongation factor-1 (*Ef-1α*) were selected using the Beacon Designer 5.0 software (Premier Biosoft, USA). The primer to genes *MyHC*, *myogenin*, *MyoD1a*, *MyoD1b*, *Myf5*, *MSTN-1a*, *MSTN-1b*, *MSTN-2a* were designed with reference to the nucleotide sequences from Atlantic salmon *Salmo salar* L and rainbow trout *Oncorhynchus mykiss* Walb. The specificity of real-time reaction product was tested by agarose gel electrophoresis. The primer sequences are given in Table 2.

Amplification of 2 μl cDNA (1:5 dilution of RT reaction) occurred using 5 μl qPCRmix-HS SYBR Green 5 × (Evrogen, Russia) and 500 nM primers to yield a final volume of 25 μl. The real-time conditions were as follows: DNA denaturation for 5 min at 95 °C; repeat cycles (40):

Table 2
Oligonucleotide primers used for RT-qPCR amplification.

Gene	Sequence 5′-3′	Size of amplified fragment, bp	GenBank accession no.
EF-1a	F: GCAAGAACGACCCTCCAATG R: CAGGCGATGTGAGCAGTATG	124	EF406271.1
Myogenin	F: GTGGAGATCCTGAGGAGTGC R: CTCACTCGACGACGAGACC	147	DQ294029
MyoD1a	F: CATTTCCAGTTGGCAAGGCG R: CACTTCCTGTGTGTTGTTCGT	128	AJ557148
MyoD1b	F: ATTTCGTTCCCTGTCACCTCTG R: TCGTCTTCGTTGTAATGG	146	AJ557150
MyoD1c	F: ACGGCGAAAACTACTACCCTTC R: TAGCTGCTTCGTCTTGCGGA	133	DQ366710
Myf5	F: ACGCCATCCAGTACATCGAG R: AGTCAACCATGCTGTCGGAG	132	DQ452070
MyHC	F: TGAGAATGTTCGCCAGGTCAA R: TCCTCAATCGCCCTCTTCAG	171	Z48794.1
MSTN-1a	F: CGGAAACCCAAGTGTTGCTTATTC R: GGCATCAGGCGGGAGATTTG	137	NM_001123549.1
MSTN-1b	F: TCTGAGTTTTATGGTTGCTTTCGG R: TTGTGACTTGATGGCGTGTAATC	151	NM_001123634.1
MSTN-2a	F: TTGTTGTTTCTTCAATCTCAGTCC R: ATCCGTATGCGTGAGTTTCC	156	EF392863

Forward and reverse primer sequences (5′-3′), bp- base pairs; *EF1a* - elongation factor 1a; *Myf5* - myogenic factor 5; *MyoD* – myoblast determination protein; *MyHC* – myosin heavy chain; *MSTN* – myostatin.