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Short periods of fasting followed by refeeding change the expression of muscle growth-related genes in juvenile Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Muscle growth mechanisms are controlled by molecular pathways that can be affected by fasting and refeeding. In this study, we hypothesized that short period of fasting followed by refeeding would change the expression of muscle growth-related genes in juvenile Nile tilapia (*Oreochromis niloticus*). The aim of this study was to analyze the expression of *MyoD*, *myogenin* and *myostatin* and the muscle growth characteristics in the white muscle of juvenile Nile tilapia during short period of fasting followed by refeeding. Juvenile fish were divided into three groups: (FC) control, feeding continuously for 42 days, (F5) 5 days of fasting and 37 days of refeeding, and (F10) 10 days of fasting and 32 days of refeeding. At days 5 (D5), 10 (D10), 20 (D20) and 42 (D42), fish (n = 14 per group) were anesthetized and euthanized for morphological, morphometric and gene expression analyses. During the refeeding, fasted fish gained weight continuously and, at the end of the experiment (D42), F5 showed total compensatory mass gain. After 5 and 10 days of fasting, a significant increase in the muscle fiber frequency (class 20) occurred in F5 and F10 compared to FC that showed a high muscle fiber frequency in class 40. At D42, the muscle fiber frequency in class 20 was higher in F5. After 5 days of fasting, *MyoD* and *myogenin* gene expressions were lower and *myostatin* expression levels were higher in F5 and F10 compared to FC; at D42, *MyoD*, *myogenin* and *myostatin* gene expression was similar among all groups. In conclusion, this study showed that short periods of fasting promoted muscle fiber atrophy in the juvenile Nile tilapia and the refeeding caused compensatory mass gain and changed the expression of muscle growth-related genes that promote muscle growth. These fasting and refeeding protocols have proven useful for understanding the effects of alternative warm fish feeding strategies on muscle growth-related genes.

1. Introduction

Methods for maximizing growth have been tested for many years in aquaculture, i.e. the use of fasting conditions that result in low growth rate followed by refeeding, when many organisms attempt to accelerate the growth rate (Hornick et al., 2000). This accelerated growth is identified by being significantly faster than the growth rate of those individuals that have not experienced growth depression and have been kept under the same conditions (Nikki et al., 2004). There are several hypotheses that attempt to explain the increased growth following a fasting period such as an increase of feed intake (hyperphagia) (Jobling and Johansen, 1999; Hayward et al., 2000), protein synthesis (Bower et al., 2009) and hormonal responses (Gaylord and Gatlin, 2001). Muscle is one of the most important tissues that are considerably affected by fasting and refeeding. Gene expression induced by starvation and refeeding also changes muscle metabolism, growth rate and sometimes can impair muscle growth (Hornick et al., 2000; Hagen et al., 2009).

In most fish, skeletal muscle comprises 40–60% of total body mass (Weatherley and Gill, 1985) and predominantly consists of white muscle, the edible part of the fish (Zhang et al., 1996; Sänger and Stoiber, 2001). Researchers have shown that fasting protocols lead to a substantial decrease in white muscle fiber size, thus implying that this muscle is the main target in this condition (Fauconneau et al., 1995; Martínez et al., 2002). Fish muscle growth involves a population of adult myoblasts, also called satellite cells (Johnston, 1999), that provide the essential nuclei for hyperplasic and hypertrophic muscle growth mechanisms (Zammit et al., 2006; McCarthy et al., 2011).

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These muscle growth processes are controlled by several molecules such as myogenic regulatory factors (MRFs) and myostatin. The MRFs, including *MyoD*, *Myf5*, *myogenin*, and *MRF4* (Watabe, 2001; Pownall et al., 2002), are transcription factors that have a highly conserved basic helix–loop–helix (bHLH) region (Funkenstein et al., 2007), which is linked to the DNA sequence E-box found in the promoting region of many muscle specific genes (Lassar et al., 1989; Murre et al., 1989; Blackwell and Weintraub, 1990). During fish muscle growth, *MyoD* and *Myf5* regulate the activation and proliferation of satellite cells, whereas *myogenin* and *MRF4* act on cell differentiation (Watabe, 2001).

Muscle growth is also controlled by the expression of *myostatin*, known as growth and differentiation factor-8 (GDF-8), member of the transforming growth factor-β (TGF-β) superfamily of proteins (McPherron et al., 1997). *Myostatin* functions as a negative regulator of skeletal muscle growth, and in fish, their role may not be restricted to muscle growth regulation but may have other possible functions both in muscle and other tissues (Østbye et al., 2001; Rodgers et al., 2001; Acosta et al., 2005; Patruno et al., 2008; Lee et al., 2009).

Because muscle growth mechanisms are dependent on *MyoD*, *myogenin* and *myostatin* expressions and these growth factors can be influenced by extrinsic factors, we hypothesized that short periods of food restriction followed by refeeding would change the expression of muscle growth-related genes in juvenile Nile tilapia (*Oreochromis niloticus*). The aim of this study was to analyze the expression of *MyoD*, *myogenin* and *myostatin* and the muscle growth characteristics in white muscle of juvenile Nile tilapia during short fasting followed by refeeding.

2. Materials and methods

2.1. Fish rearing conditions and experimental design

The experiment was conducted at the Laboratory of Aquatic Organisms Nutrition from the Aquaculture Center, UNESP, SP. We used the juvenile Nile tilapia (*O. niloticus*) chitralada Thai strain. Juvenile fish with body mass of 0.6 ± 0.19 g and total length of 35.6 ± 29.4 mm were stored (100 juvenile/tank) in 150 L polyethylene tanks with continuously flowing water and constant aeration. The experiment lasted 42 days. Fish were randomly distributed into three groups with three replicates per group: (FC) control, feeding continuously to apparent satiation with a commercial diet for 42 days, (F5) 5 days of fasting and 37 days of refeeding, and (F10) 10 days of fasting and 32 days of refeeding. After 5 or 10 days of food restriction, fish from F5 and F10 were fed to apparent satiation with a commercial diet. During the experimental period, the following values of tank water quality were observed: temperature 26.7 ± 0.6 °C, pH 8.3 ± 0.55 and dissolved oxygen 6.5 ± 0.17 mg/L. At the beginning of the experiment (day 0) and at 5 (D5), 10 (D10), 20 (D20), and 42 (D42) days, fish from all groups (n = 14) were anesthetized using benzocaine (0.1 g L^{-1}), individually weighted (g), and measured (mm), and muscle samples were collected. This experiment was approved by the Ethics Committee of the Biosciences Institute, UNESP, Botucatu, SP, Brazil (Protocol 106/2009).

2.2. Morphological and morphometric analyses

White muscle samples (n = 7 for each group) were collected from the dorsal region, near the head, fixed in Karnovsky solution (8% paraformaldehyde and 2.5% glutaraldehyde in PBS) and embedded in Historesin® (Leica, Germany). Histological transverse sections (4 μm) were obtained and stained with hematoxylin–eosin to analyze muscle fiber diameter and morphology (Dubowitz and Brooke, 1973). To estimate the degree of muscle hypertrophy and hyperplasia, the smallest diameter of 200 white muscle fibers from each animal per group was measured using an image analysis system (Leica Qwin, Germany). Based on the methodology used by Valente et al. (1999), the fibers were distributed into classes according to their diameter (d, μm): class 20 = d ≤ 20; class 30 = 20 > d ≤ 30; class 40 = 30 > d ≤ 40; class 50 = 40 > d ≤ 50; and class 60 = d > 50. Muscle fiber frequency was expressed as the number of fibers from each diameter class relative to the total number of fibers measured.

2.3. MyoD, myogenin and myostatin mRNA expressions

2.3.1. RNA isolation and cDNA synthesis

Muscle samples (n = 7 for each group) were collected from the dorsal region in groups FC, F5 and F10. Total RNA was extracted using TRIzol® Reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Extracted RNA integrity was confirmed by electrophoresis on a 1% agarose gel stained with GelRed (Biotium, Hayward, CA, USA) and visualized under ultraviolet light (not shown). The amount of RNA extracted was determined using a NanoVue™ Plus Spectrophotometer (GE Healthcare, Piscataway, NJ, USA). RNA purity was ensured by obtaining a 260/280 nm OD ratio ≥ 1.8.

Total RNA was treated with Dnase I Amplification Grade (Invitrogen) following the manufacturer's protocol to remove any potential genomic DNA contamination present in the samples.

Total RNA (2 μg) was reverse transcribed using the a High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) with 10 μL of reverse transcriptase buffer (10× RT buffer), 4 mL of dNTP (25×), 10 μL of random primers (10×), 2.5 μL of MultiScribe™ Reverse Transcriptase (50 U/μL), and 2.5 μL of Recombinant Ribonuclease Inhibitor RNaseOUT (40 U/μL) (Invitrogen), and the final volume was adjusted to 100 μL with RNase-free water. The samples were incubated at 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min; then, the reaction products were stored at −20 °C.

2.3.2. RT-qPCR analysis of target gene expression

Samples were amplified with specific primers to the *MyoD*, *myogenin*, *myostatin*, and 18S genes from cDNA nucleotide sequences from other teleost fishes, available in GenBank (http://www.ncbi.nlm.nih.gov/pubmed/nucleotide). All PCR products were sequenced using a BigDye Terminator v.3.1 Cycle Sequencing kit (GE Healthcare, Piscataway, NJ, USA). For the sequencing reaction, samples were subjected to the following conditions: denaturation for 1 min at 96 °C followed by 25 cycles at varying temperatures (10 s at 96 °C, 5 s at 55–57 °C and 4 min at 60 °C), after which samples were kept at 4 °C. The annealing temperature (55–57 °C) varied according to the specific annealing temperature of each primer. The partial nucleic acid sequences obtained (unpublished data) were analyzed by a BLASTN search at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/blast) and were used to design primer pairs for the RT-qPCR analysis with Primer Express® software (Applied Biosystems) (Table 1).

MyoD, *myogenin*, and *myostatin* mRNA expression analyses were performed with 2 μL of cDNA at a 1:10 dilution as template in the real-time qPCR performed in a 7300 Real-Time PCR System (Applied Biosystems). Cycling conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The reactions were run in duplicate using 0.4 μM of each primer and 2× Power SYBR

Table 1
Oligonucleotide primers used for RT-qPCR amplification.

Genes	Primers (5′→3′)	AT, °C	Size (bp)
Myostatin	Forward: TGTGGACTTCGAGGACTTTGG Reverse: TGGCCTTGTAGCGTTTTGGT	59	59
MyoD	Forward: TCAGACAACCAGAAGAGGAAGCT Reverse: CCGTTTGGAGTCTCGGAGAA	58	60
Myogenin	Forward: GCAGCCACACTGAGGGAGAA Reverse: AAGCATCGAAGGCCTCGTT	60	58
18S rRNA	Forward: GCAGCCGCGGTAATTCC Reverse: ACGAGCTTTTTAACTGCAGCAA	58	62

AT: annealing temperature; bp: base pairs.