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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.

Green PCR master mix (Applied Biosystems) in a final volume of 25 μL. Melting dissociation curves and agarose gel electrophoresis were performed to confirm that only a single product was amplified. Control reactions were run lacking cDNA template to check for reagent contamination. Relative gene expression was calculated using the Comparative CT Method (Livak and Schmittgen, 2001). Different samples were normalized to 18S gene expression.

2.4. Statistical analysis

Body mass and relative gene expression data were expressed as minimum, 1st quartile, median, 3rd quartile and maximum values using non-parametric ANOVA followed by Dunn's multiple comparison test (Zar, 2009).

Muscle fiber diameter data were analyzed by Goodman test between and within the multinomial population (Goodman, 1965). The statistical significance level was set at $P<0.05$ for all analyses.

3. Results

3.1. Body mass response to fasting and refeeding

At the end of the experimental time period (D42), F5 recovered the body mass, which was similar to that observed in FC group. However, in F10 the body mass was lower than that observed in FC and F5 at D42 ($P<0.05$) (Fig. 1).

3.2. Morphological and morphometric analyses

In FC group, from D5 to D42, as well as in F5 and F10, from D20 to D42 showed muscle morphology with round and polygonal fibers separated by a fine septum of connective tissue, called endomysium. Thicker septa of connective tissue separate muscle fibers into fascicles comprising the perimysium. Muscle fibers were distributed in a mosaic pattern characterized by fibers of different diameters. However, in F5 and F10 at D5 and D10 round muscle fibers were predominant (Fig. 2).

At the beginning of the experiment, the muscle fiber frequency distribution in diameter classes was similar in all groups: class 20: $33.67 \pm 0.24\%$; class 30: $44.8 \pm 0.14\%$; class 40: $18.23 \pm 0.17\%$; and class 50: $3.3 \pm 0.22\%$. The majority of muscle fibers (~70%) showed ≤30 μm diameters (data not shown).

After 5 and 10 days of fasting, we observed a significant increase in the smaller muscle fiber frequency (class 20) in F5 and F10 compared to FC. In class 40, muscle fiber frequency was lower in F5 and F10 compared to FC. At D10, there was an increase in muscle fiber frequency in classes 50 and 60 in all groups. At the end of the experimental period (D42), muscle fiber frequency in class 20 was higher in F5 (Fig. 3).

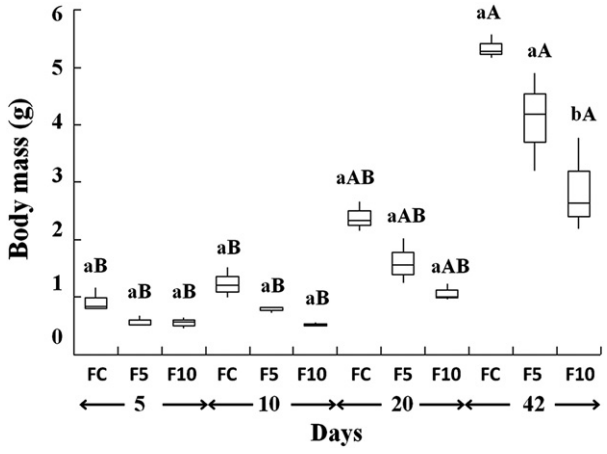

Fig. 1. Juvenile Nile tilapia body mass (g) of FC, F5 and F10 groups throughout the experiment. Data are reported median values (minimum, 1st quartile, median, 3rd quartile and maximum). Capital letters compare the body mass in the same group. Small letters compare groups in the same analysis period ($P<0.05$, ANOVA).

3.3. MyoD, myogenin and myostatin mRNA expressions

Gene expression was analyzed at D5, D10, D20, and D42. RT-qPCR results showed changes in gene expression in response to fasting and refeeding. At D5, the *MyoD* mRNA levels were higher in FC than in F5 and F10. However, at D20, the *MyoD* gene expression was higher in F5 and F10 compared to FC, indicating a possible attempt at recovery of muscle growth (Fig. 4A). At D10 and D42, the expression levels were similar between the groups. *Myogenin* gene expression was lower in F5 and F10 in relation to FC at D5. At D10, the *myogenin* mRNA level increased in F5 and F10, but only F5 showed a significant difference in relation to FC. At D20, the expression levels were similar among the groups, and at D42, the *myogenin* gene expression was higher in FC compared with F5 (Fig. 4B). At D5, the *myostatin* mRNA level was higher in F5 and F10 compared to FC, and from D10 to D42, *myostatin* gene expression was similar among all groups (Fig. 4C).

4. Discussion

The major finding of this study is that short periods of fasting (5 and 10 days) followed by refeeding in juvenile Nile tilapia (*O. niloticus*) changed the expression of muscle growth-related genes (*MyoD*, *myogenin* and *myostatin*) and muscle growth characteristics.

Except in fasting groups at D5 and D10, the other time periods analyzed in the groups showed white muscle fibers in a mosaic distribution pattern that was characterized by fibers with different diameters; this pattern was also previously reported by Almeida et al. (2010) and Leitão et al. (2011) in pacu and by Rowlerson and Veggetti (2001) in other fish species such as trout, salmon, sea bream, sea bass, and carp, among others. Muscle morphometric analysis showed significant changes in the white fiber diameter during the refeeding. At D5 and D10, in F5 and F10 groups led to a high frequency of fibers in class 20 in comparison to FC group. These findings could indicate muscle catabolism in these groups reflecting the muscle atrophy characterized by small and round fibers. In fact, Seiliez et al. (2008) observed muscle proteolysis in juvenile trout subjected to 14 days of fasting, these authors observed an activation of ubiquitin–proteasome pathway proteins during this time period; and 24-hour of refeeding induced a significant decrease in the expression of these proteins. Although we did not evaluate the ubiquitin–proteasome pathway in our experiment, it is possible that this system has contributed to protein degradation and muscle fiber atrophy during the fasting periods. Additional experiments are required to better investigate this possibility.

We observed an increase in the frequency of fibers with a diameter of ≤30 and ≥40 μm in F10 and F5, respectively, at D20 (data not shown). This result indicates a more active hyperplastic growth in F10 and the beginning of differentiation and hypertrophy in F5 (Rowlerson and Veggetti, 2001) during this time period. Based on these findings, we can infer that F10 followed by 10 days of refeeding promotes muscle fiber recruitment. On the other hand, muscle fiber hypertrophy was more evident after 5 days of fasting followed by 15 days of refeeding. At D42, a balance in muscle growth mechanisms in all groups was observed. However, the frequency of fibers in class 20 was higher in F5, which indicates that muscle fiber recruitment was still occurring during at this time period.

In this study, juvenile Nile tilapia showed a differential *MyoD* gene expression during short fasting periods and refeeding. At D5, *MyoD* mRNA levels were lower in F5 and F10 in relation to FC, and refeeding caused an increase in *MyoD* gene expression that peaked at D20 in the F5 and F10 groups. During skeletal muscle growth, *MyoD* controls satellite cell proliferation (Megeney and Rudnicki, 1995; Watabe, 2001; Kuang and Rudnicki, 2008). These cells provide nuclei for new muscle

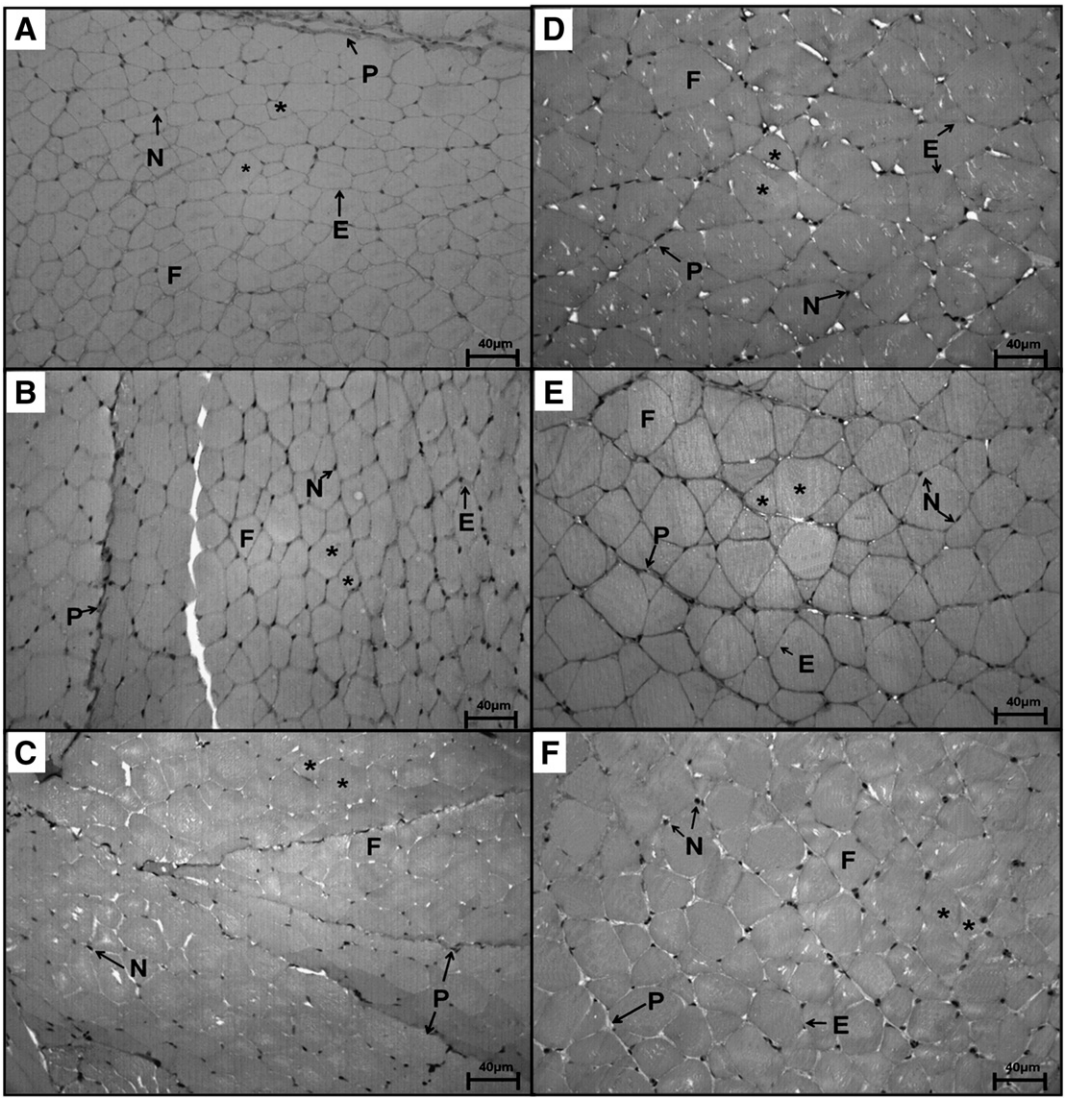

Fig. 2. Transverse sections of juvenile Nile tilapia (*Oreochromis niloticus*) white muscle of FC, F5 and F10 groups. Muscle fibers (F), nucleus (N), endomysium (E), perimysium (P). Note a mosaic pattern of muscle fibers with different diameters (*). A: FC at D5; B: F5 at D5; C: F10 at D10. D, E and F: FC, F5 and F10, respectively at D42. Hematoxylin–eosin stain. Scale bars: 40 µm.

fiber formation (hyperplasia) and hypertrophy (McCarthy et al., 2011). The mechanism underlying increased *MyoD* mRNA expression at D20 in the F5 and F10 may be related to an intense satellite cell proliferation thus, demonstrating an attempt at recovery of muscle growth by hypertrophy and hyperplasia in F5 and hyperplasia in F10, as observed by morphometric analysis. On the contrary, the lower level of MyoD (mRNA) in F5 and F10 at D5 may be related to a low muscle growth rate and increased muscle catabolism; this fact could explain the muscle fiber atrophy demonstrated by the high frequency of fibers in class 20 in these groups. For rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792), *MyoD* mRNA expression did not change in response to 30 days of starvation and 14 days of refeeding (Johansen and Overturf, 2006); similar results were also observed for Atlantic salmon (*Salmo salar* Linnaeus, 1758) when the fish were submitted to 32 days of starvation followed by 14 days of refeeding (Bower et al., 2009). To our knowledge, this is the first study that reports, in a warm water species such as the Nile tilapia, an increase in *MyoD* mRNA levels during short periods of fasting (5 and 10 days) following by refeeding. In the FC group at D5, the *MyoD* mRNA levels could reflect satellite cell proliferation, which in fact could contribute to the muscle fiber hyperplasia and hypertrophy. These processes remained active at 10 and 20 days and decreased at 42 days, but the *MyoD* expression, similar to that in the F5 and F10 groups, was enough to allow muscle growth in all groups, as shown by morphological and morphometric analyses.

The shorter fasting period studied (F5) promoted a decrease in the *myogenin* gene expression pattern in Nile tilapia. In rainbow trout, the expression of *myogenin* decreased after 30 days of fasting, suggesting a major reduction in muscle hypertrophy during this period (Johansen and Overturf, 2006). In our study, after 5 days of refeeding, the F5 group displayed high *myogenin* gene expression compared to the FC group. The high *myogenin* mRNA levels observed in the F5 group indicate that the refeeding was able to induce a high rate of satellite cell differentiation, thus contributing to hyperplasia and hypertrophy muscle growth (Levesque et al., 2007; Sandri, 2008). Although the *myogenin* expression declined until day 42, the mRNA levels detected in all groups could have been enough to promote satellite cell differentiation for hyperplasia and hypertrophy, as demonstrated by distribution of the muscle fiber diameter classes.

Myostatin mRNA levels in the F5 and F10 were higher in D5 comparing to FC groups. At D10, D20 and D42, mRNA levels decreased similarly in all groups. The same increase was observed in larvae tilapia during

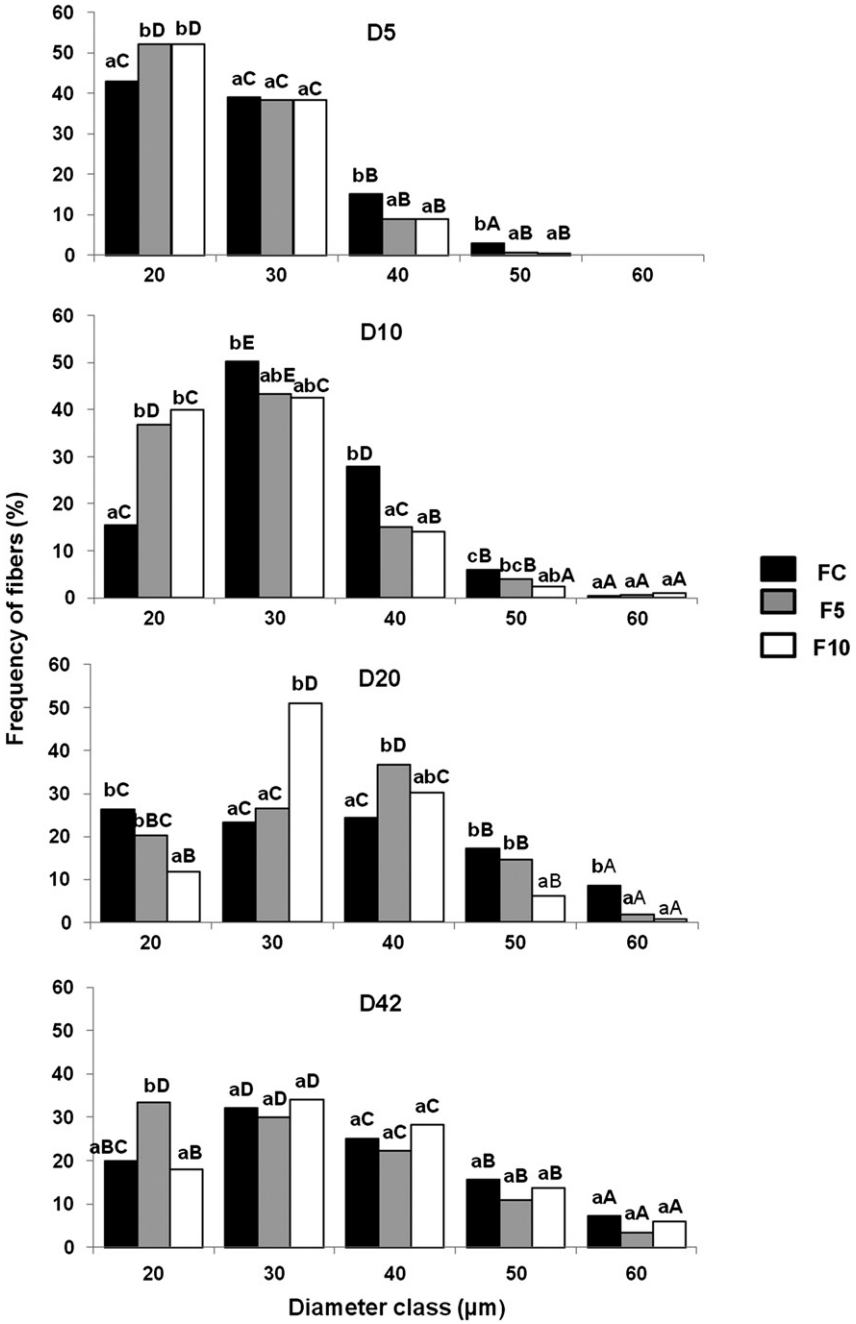

Fig. 3. Frequency distribution of white muscle fibers of juvenile Nile tilapia (*Oreochromis niloticus*) in diameter classes (d, µm): class 20 = d ≤ 20; class 30 = 20 > d ≤ 30; class 40 = 30 > d ≤ 40; class 50 = 40 > d ≤ 50 and class 60 = d > 50 at D5, D10, D20 and D42 of the experiment. Capital letters compare the frequency of fibers in the same group. Small letters compare the frequency of fibers in the same diameter class ($P<0.05$, Goodman test).

3 days of fasting, but in adult's tilapia, after 28 days of fasting, *myostatin* expression was not affected, indicating that *myostatin* levels increase during a short-term fasting but are reduced with prolonged fasting (Rodgers et al., 2003). Many studies have investigated the actions of *myostatin* in skeletal muscle development and growth and have shown that *myostatin* inhibits satellite cell proliferation by activating the cyclin-dependent kinase inhibitor p21, which forces withdrawal from the cell cycle (Thomas et al., 2000). In our study, a high *myostatin* gene expression pattern in F5 and F10 groups indicated lower satellite cell proliferation activity, which was also confirmed by low *MyoD* mRNA levels. Based on these results and the observation that quiescent satellite cells express *myostatin* (McCroskery et al., 2003), it has been suggested that one of the normal functions of *myostatin* in postnatal muscle is to maintain satellite cells in a quiescent and undifferentiated state (Manceau et al., 2008). The fasting conditions used in the present study promoted an increase in the *myostatin* gene expression. As this behavior occurred simultaneously with lower *MyoD* gene expression, we can infer that the fasting time period used may have prevented satellite cell activity. The refeeding was able to induce a decrease in *myostatin* gene expression and an increase in the *MyoD* mRNA expression, as observed at D10 in F5 and at D20 in F10. This condition was maintained until the end of the experiment and could explain the muscle fiber hyperplasia and hypertrophy phenomenon observed during this time period.

In parallel, the activation of *myostatin* has also been associated with the inhibition of myoblasts and satellite cell differentiation

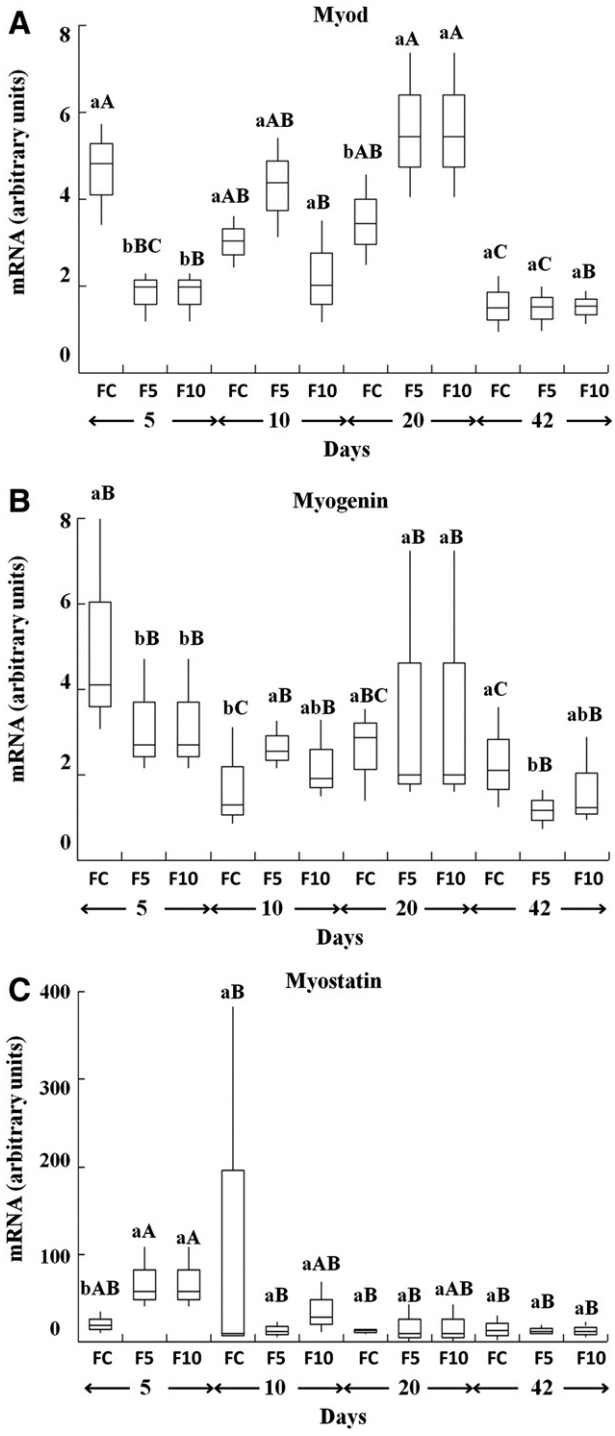

Fig. 4. Real-time RT-PCR quantification of *MyoD* (A), *myogenin* (B) and *myostatin* (C) mRNA expressions in the white muscle of juvenile Nile tilapia (*Oreochromis niloticus*). Data were expressed as minimum, 1st quartile, median, 3rd quartile and maximum values. Capital letters compare the gene expression in the same group. Small letters compare groups in the same analysis period ($P<0.05$, ANOVA).

(Fauconneau and Paboeuf, 2000; Langley et al., 2002; Rios et al., 2002; Joulia et al., 2003), which is a process controlled by *myogenin* expression (Megeney and Rudnicki, 1995; Grobet et al., 1997). Studies have shown that *myostatin* regulates the differentiation process by inhibiting *myogenin* action and that this *MRF* is probably a major target of endogenous *myostatin* (Joulia et al., 2003). However, this correlation between *myogenin* and *myostatin* expressions was not observed in the present experiment, except at 5 days in both starvation groups. Johansen and Overturf (2006) showed that for rainbow trout (*O. mykiss*, Walbaum 1792), the *myogenin* and *myostatin* mRNA levels were lower after 30 days of fasting and increased after 14 days of refeeding, indicating that *myostatin* may not control *myogenin* expression. In fact, the role of *myostatin* in the regulation of muscle growth mechanisms in fish is not yet well understood. Studies have shown that *myostatin* regulation of muscle growth mechanisms is dependent on the fish species, growth phase, muscle type and nutritional conditions (Østbye et al., 2001; Roberts and Goetz, 2001; Patruno et al., 2008).

5. Conclusion

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.

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