

Alteration in expression of atrogenes and IGF-1 induced by fasting in Nile tilapia *Oreochromis niloticus* juveniles

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Abstract The growth rate of farmed fish is an important factor regarding aquaculture success. An understanding of the cellular events that occur in skeletal muscle when fish undergo periods of fasting and refeeding provides information useful in developing alternative feeding strategies for improving muscle growth in commercially cultivated species. To evaluate the effect of 1–3 weeks of fasting and 10 weeks of refeeding in Nile tilapia juveniles, we analyzed the growth performance and changes in muscle cellularity and the expression of the following growth and muscle related genes: MyoD, myogenin, IGF-1, IGF-1 receptor, MuRF-1, atrogen-1 and myostatin. Reduced body mass was observed in all three groups of fasted fish during their time off feed, and 10 weeks of refeeding resulted in partial compensatory growth of body mass. No differences in the frequency of white muscle fiber diameters were observed between fasted and fed control fish treatments. However, changes in gene expression induced by fasting and refeeding were found. IGF-1 receptor, ubiquitin ligases MuRF1 and atrogen-1 expression increased during the 1–3 weeks of fasting, while IGF-1 levels dropped significantly ($P < 0.001$) compared to the control treatment. Furthermore, myogenin mRNA level in fish submitted to 3 weeks of fasting was higher in comparison to the control treatment ($P < 0.05$). Overall, our results showed that 1–3 weeks of fasting can induce muscle atrophy activation in Nile tilapia juveniles, and 10 weeks of refeeding is enough to induce only partial compensatory growth.

Keywords Atrogen-1 · Fasting · IGF-1 · MuRF-1 · Muscle atrophy · Muscle regulatory factors

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Introduction

Fasting is a phenomenon that can occur naturally in the environment due to low food supply, during fish migration, overwintering or during reproduction, throughout which fish demonstrate the ability to survive without food for lengthy periods (Love 1970; Bower et al. 2009). After a period of growth depression and when favorable conditions are restored, there occurs a phase of accelerated growth, called compensatory growth (Ali et al. 2003). Partial, full and over-compensation growth have all been evoked in fish. However, over-compensation has only been observed when cycles of deprivation and satiation feedings have occurred. Compensatory growth has been partially linked to hyperphagia when rates of food consumption are significantly higher than those in fish that have not experienced a previous growth depression episode. The severity of the growth depression increases the duration of the hyperphagic phase rather than maximum daily feeding rate. In some studies, growth efficiencies were notably greater during the compensatory growth stage (Ali et al. 2003; Dobson and Holmes 1984). In teleosts, the maintenance of skeletal muscle development and growth is a complex process, and may be influenced by environmental factors and the nutritional and physiological states of the animal (Johnston et al. 2008, 2011). A significant amount of research effort has been expended in understanding the regulatory factors surrounding muscle accretion, specifically evaluation of the genes involved. Myogenic regulatory factors (MRFs) are a family of four transcription factors (MyoD, myogenin, myf5 and MRF4/myf6) that are highly conserved in vertebrates and are responsible for the control of myogenesis and muscle growth. Each gene has evolved to regulate a specific area of myogenesis. For instance, MyoD and myf5 are essential regulators of muscle cell proliferation determination, whereas myogenin and myf4 act as regulators of muscle cell differentiation (Rudnicki et al. 1993; Steinbacher et al. 2007; Johnston et al. 2008).

Muscle growth is also controlled by the regulator myostatin. Myostatin is a member of the transforming growth factor- β (TGF- β) superfamily that functions as a negative regulator of skeletal muscle development and growth in mammals; however, in fish, myostatin is also present in other tissues and in other forms (Maccatrozzo et al. 2001; Rios et al. 2002; Acosta et al. 2005; De Santis et al. 2012). Another regulator of muscle growth in vertebrates is the insulin-like growth factor-1 (IGF-1) which represents the main autocrine and paracrine regulatory mechanisms of skeletal muscle by activation of the PI3 k/Akt pathway which consequently increases protein synthesis, associated with muscle hypertrophy (Glass 2003; Sacheck et al. 2004; Kandarian and Jackman 2006; Fuentes et al. 2013).

Muscle protein degradation is regulated by several processes, one of which is the ubiquitin–proteasome pathway. Two members of these muscle-specific ubiquitin ligases are the muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx/atrogen-1). Studies show that an increase in MuRF1 and atrogen-1 expression causes fiber reduction due to catabolic conditions (Glass 2005).

The Nile tilapia *Oreochromis niloticus* is globally one of the most important farmed finfish species and has been found to effectively adapt to a wide range of rearing conditions (Abdel-Hakim et al. 2009; Fox et al. 2009). However, tilapia culture is affected by water shortage, low food supply in floating cages, prolonged winter, fish reproductive period and other conditions. Understanding the molecular mechanisms that control muscle growth in tilapia would be beneficial for improving production under different conditions that occur such as short-term fasting.

Previously we showed that short periods of fasting promoted muscle cell fiber atrophy in small juvenile tilapia (0.6 g) and that refeeding caused compensatory mass gain correlated with increased expression of muscle growth related genes (MyoD, myogenin and myostatin) (Nebo et al. 2013). Thus, the aim of this study was to evaluate the effects in larger juveniles (25–30 g) under longer fasting and refeeding time intervals to test the hypothesis that the ubiquitin–proteasome pathway is activated in tilapia juveniles during periods of fasting, and that IGF-1 is overexpressed during the refeeding to induce compensatory growth.

Materials and methods

Ethical procedures

The study was approved by the Comitê de Ética no Uso de Animais (CEUA) of the Faculdade de Ciências Agrárias e Veterinárias, UNESP Univ Estadual Paulista (CEUA/FCAV/UNESP, 009436/11).

Experimental design

Fish were obtained from a commercial farm (Royal Fish, Itupeva—São Paulo, Brazil) and acclimated (15 days) to the experimental conditions at the Laboratório de Nutrição de Organismos Aquáticos, of the Centro de Aquicultura. A total of 1120 fish were stocked in thirty-two 150-L tanks (35 fish/tank) with constant water flow and aeration. Nile tilapia *Oreochromis niloticus* juveniles, Chitralada–Thai strain were weighed (initial weight 30.2 ± 0.9 g) and randomly distributed into four experimental treatments with eight replicates: (FC) control, fish were fed continuously during the 13 weeks; (F1) 1 week of fasting (1WF) and 10 weeks of refeeding (10WR), (F2) 2 weeks of fasting (2WF) and 10WR; (F3) 3 weeks of fasting (3WF) and 10WR. The experiment lasted 13 weeks, and a fixed period of 10 weeks of refeeding was maintained for all fasting treatments. When not fasted, fish were fed to apparent satiation with an extruded commercial diet (32% crude protein and 6.5% lipid) three times per day (9 am, 2 pm, and 5 pm).

White muscle samples ($n = 8$ per treatment) were collected to analyze morphology, morphometry and the expression of growth related and muscle atrophy genes, at the end of each period of fasting (1WF, 2WF and 3WF) and after 10WR. Water variables were monitored weekly and maintained at: temperature 30.1 ± 0.32 °C, pH 7.89 ± 0.14 and dissolved oxygen 4.74 ± 0.86 mg/L. For sampling, fish were anesthetized (0.1 g/L of benzocaine) and individually weighed and measured. The body mass (g) was measured at the beginning of the experiment (0 day) and after each period of fasting: one (1WF), two (2WF) and three (3WF) weeks, and after one (1WR), two (2WR), six (6WR) and 10 weeks of refeeding (10WR). A graphical representation of the experimental design is provided in Supplementary file 1.

Morphology and morphometry of muscle fibers

Fish were euthanized with benzocaine (0.4 g/L) and white muscle samples ($n = 8$ fish/treatment) were collected from the dorsal region, fixed in Karnovsky solution (8% paraformaldehyde and 2.5% glutaraldehyde in PBS—phosphate buffer saline) and embedded in Histo-resin[®] (Leica, Germany). Histological transverse sections (4 μ m) were obtained and stained with hematoxylin-phloxine B to analyze muscle fiber diameter. To estimate the degree of muscle hypertrophy, hyperplasia or atrophy the diameter of 700 white muscle fibers for each treatment ($n = 8$ per treatment) were measured from between three to four fields per sample. The fibers were binned into classes according to their diameter (d , μ m): class 10 = $d < 10$, class 30 = $10 > d < 30$, class 50 = $30 > d < 50$ and class 60 = $d \geq 50$, and white muscle fibers frequency in different diameter classes was determined. Images were captured with a Zeiss Axioplan 2 microscope and analyzed with Zeiss Axiovision 4.7 software (Carl Zeiss Microscope, LLC, USA). Morphology of muscle cells at different periods is provided in Fig. 1 and in color in Supplementary file 2.

Gene expression

White muscle samples ($n = 8$) were collected from the dorsal region of fish at the beginning of the experiment and at 1WF, 2WF, 3WF and after 10WR during the experiment and frozen in liquid nitrogen. Total RNA was extracted using TRIzol[®] Reagent according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Extracted RNA integrity was confirmed by electrophoresis on a 1% agarose gel stained with GelRed (Biotium, Hayward, CA, USA). Quantity of extracted RNA 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 were solubilized in RNase-free water and incubated with Dnase I (Life Technologies, Carlsbad, CA, USA) to remove any DNA present in the samples. For expression analysis using SYBR, total RNA (2 μ g) was reverse transcribed using the High Capacity cDNA archive kit with RNase inhibitor (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. At the end of the reaction, the samples were stored at -20 °C.

Gene expression analysis was evaluated by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). For each gene, specific primers were tested at different concentration levels to determine optimal amplification. Samples of cDNA were amplified with specific primers for *O. niloticus* *MyoD*, *myogenin*, *myostatin* (Nebo et al., 2013), IGF-1, IGF-1 receptor, and the ubiquitin regulating gene, MAFbx/atrogen-1 designed from cDNA nucleotide sequences, available in GenBank (<http://www.ncbi.nlm.nih.gov/>



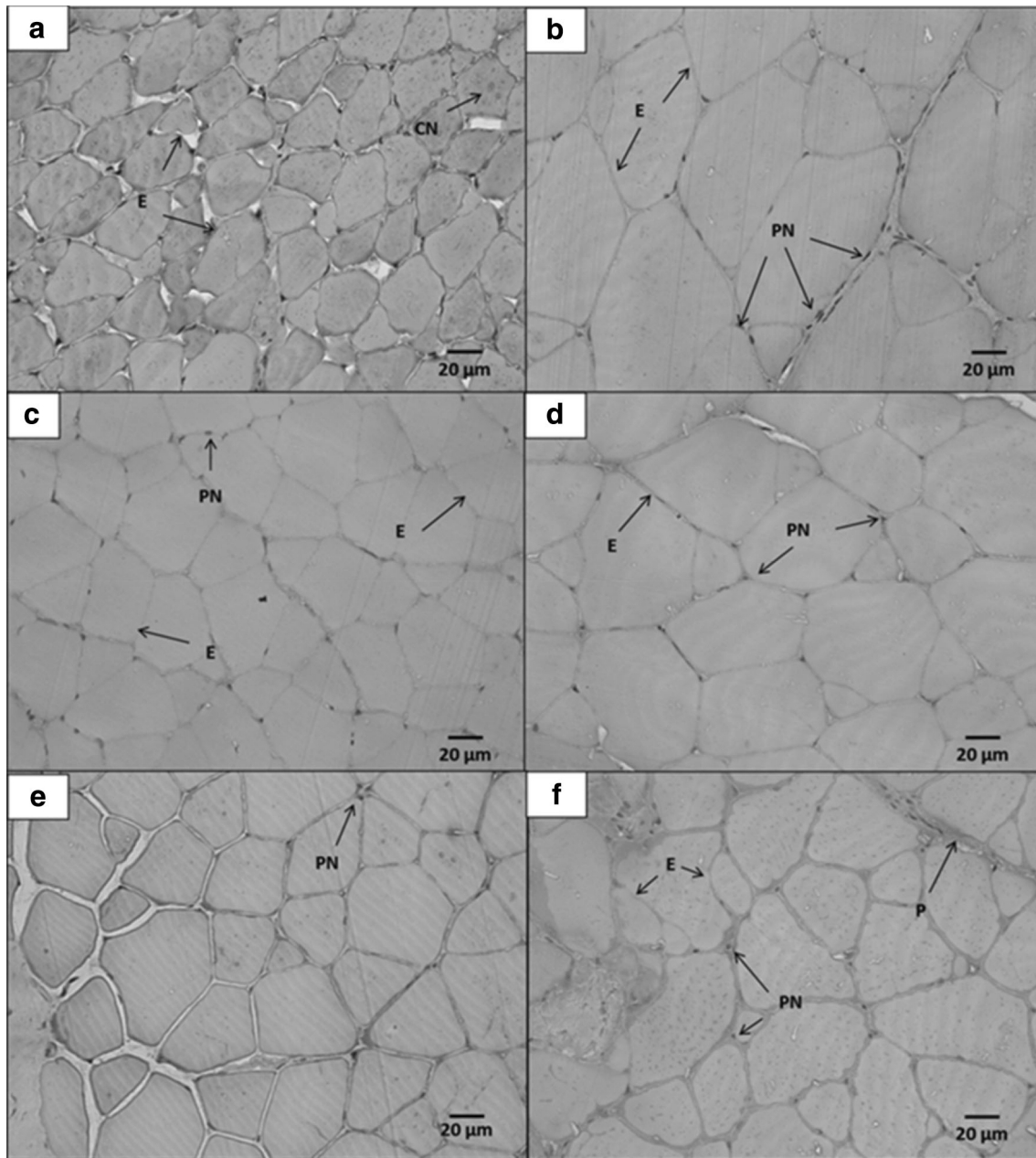


Fig. 1 Morphology of muscle cells after different periods of fasting and refeeding. Transverse section of white muscle fiber of Nile tilapia *Oreochromis niloticus* juveniles after 1 (1WF), 2 (2WF) and 3 (3WF) weeks of fasting and after 10 weeks of refeeding (10WR). **a** F1 at 1WF; **b** F1 at 10WR; **c** F2 at 2WF; **d** F2 at 10WR; **e** F3 at 3WF; **f** F3 at 10WR. Small fibers around big fibers, forming a growth mosaic pattern. Central nucleus (CN), peripheral nucleus (PN), perimysium (P) and endomysium (E). (FC) control, fed continuously during the 13 weeks; (F1) 1 week of fasting and 10WR, (F2) 2 weeks of fasting and 10WR; (F3) 3 weeks of fasting and 10WR. Hematoxylin–phloxine stain. Scale bars: 20 µm, 40 X

[pubmed/nucleotide](#)). The gene expression analyses were performed with an ABI 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The reactions were run in duplicate using Fast SYBR Green Master Mix (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Target gene expression was normalized to ubiquitin-like protein 3 (ubl3) gene expression and melting dissociation curves were performed to confirm that only a single product was amplified. Control reactions lacking cDNA template were also included to check for reagent contamination. The normalization genes were previously tested and the gene with the least variation between samples was chosen.

For the analysis of MuRF1, atrogin-1 and beta-actin genes expression primers and probes were utilized. Reactions were run on an ABI Prism 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA, USA) using Verso 1-Step RT-qPCR ROX Mix (Thermo Fisher Scientific, Waltham, MA, USA). The reactions were run in duplicate using a TaqMan Assay kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Samples were normalized to beta-actin gene expression. Relative gene expression was calculated using the ($2^{-\Delta\Delta CT}$) method using the FC group as the untreated sample (Livak and Schmittgen 2001). For IGF-1, IGF-1 receptor, beta-actin, MuRF1, and atrogin-1 genes primers and probes were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA) (Table 1).

Statistical analysis

Morphometric and gene expression data were analyzed by one-way ANOVA for differences between means, and where significant ($P < 0.05$) post hoc analyses tested with Tukey's, using the Statistical Analysis System SAS V9 software (SAS Institute Inc., Cary, NC, USA). Two comparisons were made: fed control (FC) between fasted treatments (F1, F2, and F3); and also fasted treatments were compared between groups after fasting (1WF, 2WF, and 3WF) and 10 weeks of refeeding. All data were tested for homogeneity of variance using Brown–Forsythe test ($P < 0.05$) and normality using Cramer–von Mises test ($P < 0.05$). Logarithmic

Table 1 Oligonucleotide primers and probes used for RT-qPCR amplification

Primer–Probe sequence (5'–3')	GenBank accession no.
<i>Atrogin-1</i>	
Forward: GTGGGCTCGTTCAGGACAT	XM0054505921
Reverse: CGGTGTACCCAGATGTTGATGTTT	
Probe: CAGCACAGACTTGCCC	
<i>B-actin</i>	
Forward: GCCCCACGCCATCCT	XM0034431272
Reverse: GAGTAGCCACGCTCTGTCA	
Probe: CTGGCCGTGACCTCAC	
<i>MuRF1</i>	
Forward: AGGGTGAAGTGAATGCTATCGA	XM005456465
Reverse: GCAGGCATCTTCCATCTG	
Probe: CAGGCGACCATTGCTG	
<i>IGF1</i>	
Forward: CAAGAGCACCCAAGGTTAGTAG	EU2721491
Reverse: CAGGTGAAGGTCTCTTCTTGTT	
<i>IGF1 receptor</i>	
Forward: GTGGTCAAAGACGAACCAGA	KC5067771
Reverse: CTCATGACGGAGGCTTCATT	
<i>Myogenin</i>	
Forward: GCAGCCCACTGAGGGAGAA	Nebo et al. 2013GU246717
Reverse: AAGCATCGAAGGCCTCGTT	
<i>Myostatin</i>	
Forward: TGTGGACTTCGAGGACTTTGG	Nebo et al. 2013KT987208
Reverse: TGGCCTTGATCGTTTTGGT	
<i>MyoD</i>	
Forward: TCAGACAACCAGAAGAGGAAGCT	Nebo et al. 2013FJ907953
Reverse: CCGTTTGGAGTCTCGGAGAA	
<i>Ubl3</i>	
Forward: CCTGCGGCTGATTTTGGT	XM005463259
Reverse: CAGAGTCGTTGGGAGAGAAGA	



transformations (natural logarithm) were used, in analysis of body mass, IGF-1, IGF-1 receptor, myogenin, atrogen-1 and MuRF1, as relevant correction of original data.

Results

Body mass response to fasting and refeeding

Survival rate of Nile tilapia juveniles was greater than 83% in the FC (83.9%) F1 (86.1%), and F2 (87.5%) treatments, whereas in the F3 it was much lower (79%). However, these differences were not significant.

As expected, no body mass (BM) increase was observed during the fasting period (week 1–week 3); On the other hand, fasted fish lost significant weight during fasting in relation to FC ($P < 0.001$) (Table 2). BM was increased during the refeeding periods (1WR, 2WR, 6WR and 10WR), but it was lower in all fasted fish compared to FC group ($P < 0.001$). Regarding the 1WR treatment, the BM in fish from F1 and F2 was significantly higher compared to the F3 treatment. Similar results were observed for the treatment group 2WR, the BM was higher in fish from F1 and F2 treatments compared to F3. However, at 6WR, the BM increased in fish from F3 compared to F1 and F2 treatments. After 10WR, the BM in fish from F1 was significantly higher compared to the F2 and F3 treatments (Table 2).

Morphological and morphometric analyses

Muscle morphology analysis revealed that at 1WF the F1 treatments showed a high number of muscle fibers with a central nucleus, exhibiting a pattern of mosaic hyperplasia (Table 3). Nonetheless, the muscle fiber morphology at 2WF or 3WF in groups of fish from F2 and F3 treatments was different from what was observed the week before, showing an increased number of mature muscle fibers with multinucleated cells

Table 2 Body mass in response to fasting in Nile tilapia (*Oreochromis niloticus*) juveniles

	Body mass (g)				P value
	FC	F1	F2	F3	
Fasting					
1WF	40.20 ± 0.34 ^a	30.20 ± 0.36A			< 0.001
2WF	48.75 ± 0.22 ^a		29.44 ± 0.24AB		< 0.001
3WF	52.89 ± 0.35 ^a			28.50 ± 0.31B	< 0.001
Refeeding					
1WR	48.68 ± 0.14 ^a	36.47 ± 0.38A			< 0.001
	52.89 ± 0.35 ^a		35.72 ± 0.24A		< 0.001
	64.52 ± 0.37 ^a			32.50 ± 0.12B	< 0.001
2WR	52.89 ± 0.35 ^a	40.21 ± 0.35A			< 0.001
	64.86 ± 0.37 ^a		41.12 ± 0.35A		< 0.001
	74.76 ± 0.36 ^a			36.86 ± 0.69B	< 0.001
6WR	88.45 ± 0.38 ^a	73.38 ± 0.49B			< 0.001
	104.56 ± 1.08 ^a		69.25 ± 0.83C		< 0.001
	133.42 ± 1.61 ^a			79.12 ± 0.76A	< 0.001
10WR	152.31 ± 2.02 ^a	119.03 ± 0.69A			< 0.001
	177.6 ± 3.08 ^a		98.97 ± 1.15C		< 0.001
	182.67 ± 2.12 ^a			106.8 ± 0.80B	< 0.001

Fish body mass (g, BM) after 1 (1WF), 2 (2WF) and 3 (3WF) weeks of fasting and after 1 (1WR), 2 (2WR), 6 (6WR) and 10 (10WR) weeks of refeeding. FC: control, continuously fed during the 13 weeks of treatment; F1: 1 week of fasting and 10WR; F2: 2 weeks of fasting and 10WR; F3: 3 weeks of fasting and 10WR. Uppercase letters compare between fasting treatments after fasting and refeeding

^a Significant difference ($P < 0.05$) between FC and fasting treatments. Data are mean ± SD ($n = 8$)

with peripheral nuclei (Table 3). After 10WR, in the fasted treatments (F1, F2 and F3), muscle fiber morphology exhibited a typical mosaic pattern with polygonal shaped fibers, small fibers adjacent to big fibers (Table 3).

After 1WF, 2WF and 3WF, morphometric analyses showed a higher quantity (> 95%) of smaller muscle fibers in between classes 10 and 30, and a lower quantity (< 0.6%) in class 50. The frequency of muscle fibers in diameter classes did not differ significantly between fasted fish and the respective FC group (Table 3). However, after 10WR the distribution of white muscle fiber frequency in diameter classes changed in fish from F1, F2, and F3 treatments, showing higher numbers of big fibers in classes 50 and 60 compared to classes 10 and 30. Similar to the fasting period, no differences were found among FC and the fasted fish, after 10WR (Table 3).

Increased expression of atrogenes and IGF-1 reduction during fasting

The IGF-1 gene expression, at 1WF, 2WF and 3WF, was downregulated, exhibiting significant differences ($P < 0.028$) in comparison to the FC group; and after 10WR, the IGF-1 levels were increased, being similar to the IGF-1 mRNA level in the FC group, showing a substantial response to refeeding. Furthermore, after 10WR, the F1 group was found to express IGF-1 significantly higher compared to the F2 group ($P < 0.017$) (Fig. 2a). Conversely, the IGF-1 receptor was upregulated in the fish from the F1, F2 and F3 groups during the fasting periods, but after 10WR, only the expression of IGF-1 receptor in the F3 group was found to be significantly reduced ($P < 0.03$) in comparison to the FC group (Fig. 2b).

Table 3 Muscle fibers frequency in Nile tilapia (*Oreochromis niloticus*) juveniles

Frequency of fibers (%)				
Diameter class	10	30	50	60
1WF				
FC	32.1 ± 11.6	67.6 ± 11.4	0.4 ± 0.2	
F1	42.5 ± 10.8	57.5 ± 10.7		
<i>P</i> value	0.522	1.000		
2WF				
FC	24.8 ± 4.6	74.8 ± 4.5	0.5 ± 0.4	
F2	29.2 ± 7.3	70.6 ± 7.3	0.2 ± 0.1	
<i>P</i> value	0.960	1.000	0.763	
3WF				
FC	29.5 ± 8.9	70.5 ± 9.5		
F3	36.9 ± 7.7	63.1 ± 7.8		
<i>P</i> value	0.732	1.000		
10WR-F1				
FC	0.3 ± 0.2	8.5 ± 4.3	19.5 ± 7.5	71.8 ± 11.0
F1	0.4 ± 0.1	13.0 ± 6.6	22.2 ± 12.1	65.5 ± 15.3
<i>P</i> value	0.943	0.954	1.000	0.853
10WR-F2				
FC	0.3 ± 0.1	8.3 ± 5.2	16.6 ± 10.5	74.9 ± 13.3
F2	–	14.0 ± 6.4	26.7 ± 9.1	59.4 ± 14.0
<i>P</i> value		0.552	0.413	0.390
10WR-F3				
FC	–	8.0 ± 6.9	20.8 ± 11.2	71.2 ± 15.3
F3	0.4 ± 0.4	18.3 ± 11.3	26.0 ± 10.5	55.4 ± 20.0
<i>P</i> value		0.444	0.970	0.265

Frequency of white muscle fibers in certain diameter class groups after 1 (1WF), 2 (2WF) and 3 (3WF) weeks of fasting and 10 weeks of refeeding (10WR). Muscle diameters are grouped into four diameter classes (d , μm): class 10 = $d < 10$, class 30 = $10 \leq d < 30$, class 50 = $30 \leq d < 50$ and class 60 = $d \geq 50$. (FC) control, fed continuously during the 13 weeks; (F1) 1 week of fasting and 10WR; (F2) 2 weeks of fasting and 10WR; (F3) 3 weeks of fasting and 10WR. Data are mean ± SD ($n = 8$)



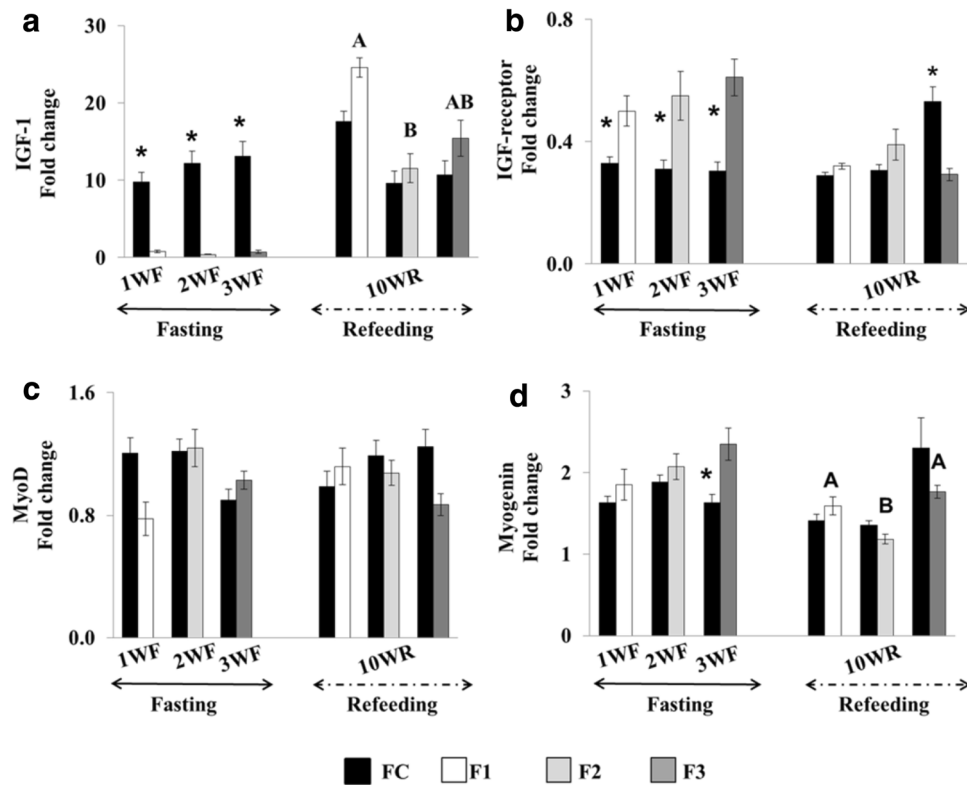


Fig. 2 Relative gene expression of IGF-1, IGF-1 receptor, MyoD and myogenin in white skeletal muscle of Nile tilapia *Oreochromis niloticus* juveniles subjected to fasting and refeeding. Gene expression was evaluated after 1 (1WF), 2 (2WF) and 3 (3WF) weeks of fasting and after 10 weeks of refeeding (10WR). **a** Relative expression of IGF-1; **b** relative expression of IGF-1 receptor; **c** relative expression of MyoD; **d** relative expression of myogenin. (FC) control, feeding continuously during the 13 weeks; (F1) 1 week of fasting and 10WR; (F2) 2 weeks of fasting and 10WR; (F3) 3 weeks of fasting and 10WR. Uppercase letters denote significant differences between F1, F2, and F3 treatments after fasting and refeeding, and *significant difference ($P < 0.05$) between control and fasted treatments. Data are mean \pm SEM ($n = 8$)

MyoD gene expression was found to not differ among all the groups, either in the fasting or the refeeding group (Fig. 2c). Regarding myogenin expression, its mRNA levels were not found to differ significantly except at 3WF where they were higher compared to the FC group (Fig. 2d).

The myostatin mRNA levels, after fasting and refeeding, did not differ between control and fasted groups (Fig. 3a). The atrogen-1 and MuRF-1 gene expression levels, at 1WF, 2WF and 3WF, increased significantly ($P < 0.004$) compared to the FC group. However, after 10WR, the atrogen-1 and MuRF-1 levels in all fasted groups were similar to the FC (Fig. 3b, c). Atrogen-1 expression was serially higher after 10WR and significantly different between the F1, F2 and F3 groups, but not significantly different compared to the control (Fig. 3b).

Discussion

In the present work, we investigated if fasting for a certain interval followed by 10 weeks of refeeding would change muscle gene expression. Concurrently, we also wanted to evaluate if this starve/refeed regime would cause alterations in white muscle fiber plasticity and morphology in Nile tilapia *Oreochromis niloticus* juveniles.

The periods of fasting evaluated, 1, 2 and 3 weeks (1WF, 2WF, and 3WF, respectively) were not sufficient to cause any significant decrease in weight in fasted tilapia juveniles during the fasting period. However, significant differences were detected between the groups during the refeeding periods. The inability of the fasted groups to achieve body mass as the FC fish after refeeding, as previously reported, could be that the fasting periods were not of sufficient to induce a strong compensatory growth response in these animals at this phase of development (Wang et al. 2000).



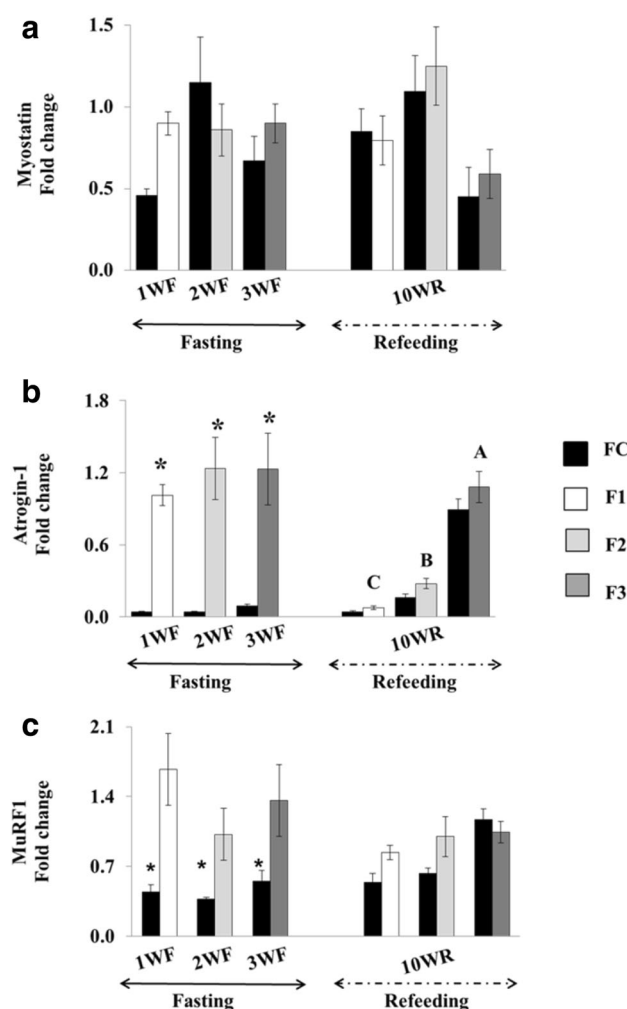


Fig. 3 Relative gene expression of myostatin, atrogin-1 and MuRF-1 in white skeletal muscle of Nile tilapia *Oreochromis niloticus* juveniles subjected to fasting and refeeding. The gene expression were calculated after 1 (WF), 2 (2WF) and 3 (3WF) weeks of fasting and after 10 weeks of refeeding (10WR). **a** Relative expression of myostatin; **b** relative expression of atrogin-1; **c** relative expression of MuRF1. (FC) control, fed continuously during the 13 weeks; (F1) 1 week of fasting and 10 weeks of refeeding; (F2) 2 weeks of fasting and 10WR; (F3) 3 weeks of fasting and 10WR. Uppercase letters denote significant differences between F1, F2, and F3 treatments after fasting and refeeding, and *significant difference ($P < 0.05$) between control and fasted treatments. Data are mean \pm SEM ($n = 8$)

To understand if fasting periods were detrimental to fish skeletal muscle growth and to determine the response of fish refeeding after 10 weeks, muscle tissue was analyzed by evaluating the morphology and the diameters of muscle fibers to determine the relative degree of hypertrophy, hyperplasia or atrophy of fibers. High frequency (over 90%) of small white muscle fibers in classes 10 and 30 during one 1WF, two 2WF and three 3WF weeks, in all treatments, showed that muscle hyperplasia was occurring in the juvenile tilapia within this study. Hyperplasia was evident within the first week of the experiment by the observation of several small fibers with central nuclei. This morphological feature characterizes skeletal muscle development in mosaic hyperplasia, with small fibers containing central nuclei surrounding big fibers throughout the myotome (Johnston et al. 2003; Johnston and Hall 2004; Johnston et al. 2008; Steinbacher et al. 2007; Valente et al. 2013). However, after 10 weeks of refeeding the growth pattern changed, with increasing the frequencies of muscle fibers in the $> 50 \mu\text{m}$ diameter classes and decreasing the frequencies in the 10 and 30 diameter classes. This change in fiber distribution was demonstrated by the fish in all the fasted and fed groups and can be considered as evidence of hypertrophic muscle growth (Veggetti et al. 1993; Valente et al. 1999; Zimmerman and Lowery 1999; Johnston 2006).

IGF-1 mRNA levels dropped significantly throughout periods of fasting (1WF, 2WF, and 3WF), but after subsequent refeeding, the IGF-1 muscle expression in previously fasted fish was identical to the FC group.

Among fasting treatments, the mRNA levels were higher in the F1 group. These findings are in agreement with other studies in which reductions in muscle IGF-1 levels as a result of fasting, and subsequent recovery following 2–4 weeks of refeeding have also been seen in studies with tilapia juveniles *Oreochromis mossambicus* (Fox et al. 2009), rainbow trout *Oncorhynchus mykiss* fry (Montserrat et al. 2007), and rainbow trout adults submitted to 10 weeks of food deprivation and 4–34 days of refeeding (Chauvigne et al. 2003).

In channel catfish, *Ictalurus punctatus* the IGF-1 expression decreased after 30 days of fasting, although after refeeding, its expression level was found to be similar to the fed control group (Peterson et al. 2009). In rainbow trout treated with IGF-1 during feed deprivation, inhibition of atrogen-1 expression was observed, along with a reduction of proteolytic activity (Cleveland et al. 2009). In the present study, IGF-1 receptor expression was upregulated significantly during 1–3 weeks of fasting in F1, F2, and F3, compared to FC. The high level of IGF-1 receptor in juvenile tilapias, during fasting periods, appears to correlate with a down-regulation of IGF-1 expression.

Other studies reported diverse results with different fish species and experimental conditions, and in general, fasting causes significant changes in MRF gene expression (Chauvigne et al. 2003; Valente et al. 2012). However, our results showed that there was no change in the expression of MyoD between FC and the fasted groups F1, F2 and F3 during the entire experimental period. Similar results were found in rainbow trout, where no significant change in MyoD expression levels was found in fish in response to starvation and refeeding (Johansen and Overturf 2006). The same results were also noted in another study with rainbow trout fasted for 1, 2 and 4 weeks, where MyoD levels in white muscle were not significantly affected by fasting (Montserrat et al. 2007). Furthermore, myogenin levels in Nile tilapia juveniles measured at the first 2 weeks of fasting (2WF) and after 10 weeks of refeeding (10WR) did not differ in relation to FC. In gilthead sea bream, myogenin gene expression was also found to not be affected after 30 days of food deprivation (Garcia et al. 2014). MyoD expression can be expected to be reduced in fasting fish as the lack of available nutrient energy would preclude muscle development and growth.

If the expression of myostatin was high, it would explain the relatively low expression of MyoD in fasted treatments. However, in our study, skeletal muscle myostatin levels were not different in Nile tilapia juveniles between fasted and control fish throughout the experiment. A long-term study of fasting in adult tilapia also showed that myostatin expression was unaffected by fasting (Rodgers et al. 2003). Our findings suggest that the level of starvation in these juveniles was enough to activate an alternative mechanism for regulating muscle growth rather than through myostatin. In rainbow trout deprived of food for 10 weeks and refeed for 34 days, the myostatin expression, similar to our findings, was not significantly changed during the experiment (Chauvigne et al. 2003). However, in tilapia fingerlings (0.6 g), myostatin expression was upregulated after 5 days of fasting in comparison to the fed control; nonetheless, after refeeding no differences were detected (Nebo et al. 2013). Likewise in another study performed with tilapia in larval phase, myostatin mRNA levels were upregulated by fasting and then reduced during refeeding (Rodgers et al. 2003). Taken into consideration the above, these results suggest that the degree of catabolism induced by fasting in tilapia is greater in larvae and fingerlings, likely due to lower body reserves, than in juvenile and adult fish that have more energy storage, and thus a higher capacity to tolerate fasting conditions.

The levels of atrogen-1 and MuRF1 expression in white skeletal muscle after 1–3 weeks of fasting increased rapidly, suggesting an increase in muscle turnover. However, the expression of atrogen-1 and MuRF1 after 10 weeks refeeding dropped to basal levels, and no significant differences were found among all groups ($P > 0.05$). Our results corroborate other reported findings with different fish species submitted to different fasting protocols. For instance, in rainbow trout juveniles, after 14 days of food deprivation the atrogen-1 levels increased and 12 h after refeeding decreased significantly (Seiliez et al. 2008); in salmon white muscle, the expression of MuRF1 increased in fasted fish compared to fed control (Tacchi and Bicherdike 2012); and in Atlantic salmon and zebrafish, atrogen-1 and MuRF1 were also found to be upregulated during fasting and downregulated after refeeding (Bower et al. 2009; Bower and Johnston 2010; Amaral and Johnston 2011).

It is well established in vertebrates that insulin-like growth factor-1 stimulates activation of the PI3 K/Akt/mTOR cascade, increasing protein synthesis and hypertrophic muscle growth (Engert et al. 1996; Zhang et al. 2008; Duan et al. 2010), and inactivates FOXO transcription factors, decreasing the expression of atrogen-1/MuRF1 and MAFbx (Sacheck et al. 2004; Edstron et al. 2006; Cleveland et al. 2009; Fuentes et al. 2013). In this context, the lower IGF-1 expression observed in fasted tilapia juveniles likely plays a role in the higher expression of MuRF1 and atrogen-1, consequently inducing muscle atrophy, over extended periods of time,



and blocking somatic growth. On the other hand, after refeeding, the expression of IGF-1 increased to the basal level, equal to the control, resulting in growth recovery.

In conclusion, the results from the present study demonstrated that the corresponding upregulation of atrogin-1 and MuRF1 and the drop in IGF-1 expression during fasting periods correlate with an initial stage of muscle atrophy. However, no signs of atrophy were observed in muscle morphology, but the expression of atrophy genes changed during fasting. Nevertheless, the increased IGF-1 levels after 10 weeks of refeeding presumably activated the Akt pathway, which correspondingly decreased the expression of genes involved in the ubiquitin–proteasome pathway, thus reducing rates of protein degradation and contributing to growth recovery. Furthermore, we observed partial compensatory growth (fish grew, but did not reach the same body mass than the control fish), after refeeding in Nile tilapia juveniles. These results contribute to a better understanding of molecular control related to skeletal muscle growth during fasting and after refeeding. This information will be beneficial in developing best management plans for feeding optimized to economically maximize growth during production specifically if phase feeding or fasting can be used.

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Author contribution Caroline Nebo was involved in planning and carrying out the experiment, data analysis and writing the manuscript. Ken Overturf was involved in planning, data analysis and writing the manuscript. Andrezas Brezas was involved with data analysis and writing. Maeli Dal-Pai-Silva was involved with experimental design, data analysis and writing of the manuscript. Maria Celia Portell was involved with experimental design, data analysis and manuscript writing.

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