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High-fat diet reduces local *myostatin-1* paralog expression and alters skeletal muscle lipid content in rainbow trout, *Oncorhynchus mykiss*

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Abstract

Muscle growth is an energetically demanding process that is reliant on intramuscular fatty acid depots in most fishes. The complex mechanisms regulating this growth and lipid metabolism are of great interest for human health and aquaculture applications. It is well established that the skeletal muscle chalone, myostatin, plays a role in lipid metabolism and adipogenesis in mammals; however, this function has not been fully assessed in fishes. We therefore examined the interaction between dietary lipid levels and myostatin expression in rainbow trout (*Oncorhynchus mykiss*). Five-weeks of high-fat (HFD; 25% lipid) dietary intake increased white muscle lipid content, and decreased circulating glucose levels and hepatosomatic index when compared to low-fat diet (LFD; 10% lipid) intake. In addition HFD intake reduced *myostatin-1a* and *-1b* expression in white muscle and *myostatin-1b* expression in brain tissue. Characterization of the *myostatin-1a*, *-1b*, and *-2a* promoters revealed putative binding sites for a subset of transcription factors associated with lipid metabolism. Taken together, these data suggest that HFD may regulate *myostatin* expression through *cis*-regulatory elements sensitive to increased lipid intake. Further, these findings provide a framework for future investigations of mechanisms describing the relationships between myostatin and lipid metabolism in fish.

Keywords

myostatin; high-fat diet; lipid; muscle growth; rainbow trout

1. Introduction

Fatty acids (FAs) are metabolized as the main energy source in many fishes and are imperative for processes such as growth, reproduction, movement, and homeostasis. In some fishes, >50% of body mass is comprised of skeletal muscle, making this tissue a major energy consumer. Interestingly, slow-oxidative ‘red’ and fast-glycolytic ‘white’ skeletal

muscle are two of the major storage depots for lipids in piscine species (Sheridan 1988). Lipid utilization and the molecular mechanisms regulating lipid metabolism have been extensively reviewed among the fishes (Sheridan 1988, 1994; Leaver et al. 2008). The energetically costly process of muscle growth is particularly dependent on FA metabolism, a process that is tightly regulated to ensure efficient energy allocation to other organismal processes (e.g., reproduction). Numerous studies have evaluated the growth factors and regulatory networks associated with muscle growth in fish, and this topic has been reviewed extensively in the literature (Mommsen 2001; Johnston et al. 2011). As with all physiological processes, positive and negative regulators (i.e., genes, *cis*-regulatory elements, and signaling pathways) are required for proper control of energy flux, storage, and utilization. However, the interaction between muscle growth and dietary lipids is poorly understood, particularly among the fish lineages.

The myostatin gene is highly conserved throughout the vertebrates (Rodgers and Garikipati 2008). In mice, deletion of myostatin results in increased skeletal muscle mass (McPherron et al. 1997), a finding that has been supported in other eutherian species such as cattle, dogs, and even one human (Schuelke et al. 2004). Exogenous administration of myostatin leads to cachexia (Zimmers et al. 2002) and cancer-induced cachexia is associated with increased levels of myostatin (Costelli et al. 2008). Further, overexpression of myostatin in skeletal muscle reduces muscle mass as expected (Reisz-Porszasz et al. 2003). Thus, mammalian myostatin appears to be a potent chalone of skeletal muscle, and its function in these species raises questions about its function in other vertebrates.

In fish, several studies have confirmed the conserved myogenic actions of myostatin, although reports failing to confirm this conservation do exist. In zebrafish, medaka, and rainbow trout, disruption of myostatin caused an increase in muscle mass and myofiber number, suggesting myostatin plays a role in myogenic precursor cell (MPC) proliferation and differentiation (Xu et al. 2003; Lee et al. 2009; Sawatari et al. 2010; Lee et al. 2010). In an *in vitro* context, rainbow trout MPCs and myoblasts display similar proliferative responses to mammalian myoblasts when treated with myostatin (albeit of mammalian origin) (Garikipati and Rodgers 2012b; Seiliez et al. 2012). In a complementary article, Garikipati and Rodgers (2012c) demonstrated that myostatin signaling *enhances* myoblast differentiation, while Seiliez and colleagues (2012) found that myostatin had no effect on the differentiation markers myogenin and myosin in these cells, a finding which conflicts with mammalian data (Joulia et al. 2003; Thomas et al. 2000; McFarlane et al. 2011). Whatever the conclusion of these *in vitro* studies, *in vivo* experiments among several piscine species have produced results consistent with some degree of conservation of myostatin function, from weak (Acosta et al. 2005; Amali et al. 2004) to strong (Lee et al. 2009; Sawatari et al. 2010; Chisada et al. 2011). It is important to note that these referenced studies were executed in small fish species, one of which is known to be a determinate-like grower (Biga and Goetz 2006) and may not reflect the function of myostatin across all *Teleostei*, particularly those with a large, indeterminate body size.

The study of myostatin in teleost fishes poses a great opportunity to study the fate of duplicated genes, in that all examined species possess at least two paralogs. In rainbow trout, four paralogs exist, with *myostatin-2b* being truncated and is putatively nonfunctional

(Garikipati et al. 2007). Like in many fishes, myostatin is ubiquitously expressed in rainbow trout (Garikipati et al. 2006a). *Myostatin-1* (-a and -b) predominates in skeletal muscle, brain, testes, spleen, and eye (Garikipati et al. 2006a), while mature *myostatin-2a* is only detectable in the brain (Garikipati et al. 2007) or under IGF-I stimulation in myoblasts (Garikipati and Rodgers 2012a).

Outside of skeletal muscle, suppression or inhibition of myostatin leads to high-fat diet induced obesity (HFDIO) resistance in mice (Zhao et al. 2005; Hamrick et al. 2006; Lyons et al. 2010). Further, ablation of myostatin in skeletal muscle leads to heightened insulin sensitivity in murine models of HFDIO (Guo et al. 2009) and can even prevent type II diabetes in lipodystrophic mice (Guo et al. 2012). At the cellular level, myostatin appears to both inhibit and promote adipogenesis *in vitro* (Kim et al. 2001; Guo et al. 2008; Artaza et al. 2005; Feldman et al. 2006). Clearly, these findings suggest that myostatin may play a role in energy balance, either through increased lipid utilization, modulation of adipogenesis, and/or lipid deposition.

To our knowledge, only one study has investigated the relationship between myostatin and increased dietary lipids in fishes. In flatfish (*Solea senegalensis*), a panel of growth-related genes, including myostatin, was examined under increased dietary lipid consumption. *Myostatin-1* expression appears to be highest in the fish fed a diet with high lipid content (20%); however, no statistical difference was noted (Campos et al. 2010). In zebrafish, HFDIO has been reported to be similar to the mammalian condition (Oka et al. 2010), although this analysis did not consider myostatin expression. Importantly, Oka and colleagues did identify several dysregulated genes involved in HFDIO. Of particular note, peroxisome proliferator-activated receptor (PPAR)- α/γ expression was involved with the HFDIO phenotype. This intracellular mediated pathway operates through PPAR-response elements (PPARREs) in the promoters of genes. The human *myostatin* promoter contains two PPARREs (Ma et al. 2001), while the porcine *myostatin* promoter has been shown experimentally to contain at least one PPARRE, although a bioinformatics approach failed to identify a consensus sequence (Deng et al. 2012). Taken together, these data suggest that lipids via PPARs may regulate *myostatin* expression.

Several studies have examined the promoters of teleost *myostatin* genes, largely mining for myogenic regulatory elements (Garikipati et al. 2006a; Garikipati et al. 2007; Ostbye et al. 2007; Funkenstein et al. 2009). While these studies demonstrated some degree of myogenic element conservation, no published myostatin promoter analyses have specifically aimed to identify transcription factor binding sites involved in lipid metabolism in rainbow trout. As many fishes, including rainbow trout, rely on lipid stores for muscle growth and myostatin is likely involved with such growth, there is a clear need for this type of characterization, both *in silico* and experimentally.

In this study, we further characterized rainbow trout as a model organism for studying the interaction between lipid metabolism and myostatin. In addition to being the best studied of the salmonid fishes, its size particularly suits it for traditional studies of physiology, and its pseudotetraploid nature offers an opportunity to study changes in paralogous gene expression and/or function. To this end, we profiled *myostatin-1a*, -1b, and -2a mRNA

expression in these fish in response to high and low lipid diets. Additionally, we characterized the *myostatin* promoters in rainbow trout for the presence of putative binding sites recognized by transcription factors known to be involved with lipid metabolism in mammals.

2. Materials and Methods

2.1 Animal Care

All animals were handled in accordance with approval from the Institutional Animal Care and Use Committee at North Dakota State University in advance of any experimentation. Juvenile rainbow trout (*Oncorhynchus mykiss* Shasta strain; 64.13 ± 2.03 g) of both sexes were obtained from the United States Fish and Wildlife Service, Garrison National Fish Hatchery, Riverdale, North Dakota, and housed at North Dakota State University, Fargo. All fish were maintained in 800-liter dechlorinated city water flow-through tanks maintained at 12°C with 100% daily turnover on a 12L:12D photoperiod. Fish were fed to apparent satiation twice daily with a standard diet (AquaMax Grower 400, Purina Mills, Gray Summit, MO; 45% protein and 16% lipid) >6 weeks prior to experimentation and introduction of treatment diets (low-fat, LFD; high-fat, HFD).

2.2 Diet Formulation and Manufacture

The two experimental diets were formulated to vary primarily in total lipid content by replacing wheat flour with fish oil to produce the HFD and LFD. Both diets were produced with commercial manufacturing methods using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland). The premixed diet mash was exposed to an average of 117°C for 16 s in five barrel sections and the last section was water cooled to an average temperature of 83°C. Pressure at the die head was fluctuated between 28 to 31 bar. The pellets were then dried in a pulse bed drier (Buhler AG) supplied with 121°C air for 19 min and the air exiting the drier never exceeded 93°C. Pellets were cooled with forced air at ambient air temperatures to reach final moisture levels of <10%. Fish oil was top-dressed for the HFD using vacuum coating (A.J. Mixing, Oakville, ON, Canada) after the pellets were cooled. All fish oil was incorporated in the mash for the LFD prior to extrusion. Diets were stored in plastic-lined paper bags at room temperature until used. All diets were fed within 4 months of manufacture. Nutritional content of each diet is included in Table 1.

2.3 Experimental Protocol: Effects of high-fat diet intake on myostatin expression

Twenty-four juvenile rainbow trout (see above) were equally and randomly assigned (n=12 per group) to one of two treatments: high-fat, HFD (25%) or low-fat, LFD (10%) diet, based on menhaden oil content of the respective diet. Individual fish were initially weighed and implanted with an opercular tag under anesthesia (100 mg/L tricaine methanesulfonate in buffered fresh water). Fish were fed experimental diets for five weeks to apparent satiation twice daily. Fish were weighed weekly, and food intake was calculated daily. Upon experiment termination, fish were euthanized by overanesthetization in tricaine methanesulfonate (>300 mg/L; AVMA), 1–2 mL of whole blood was collected by caudal venipuncture, livers were weighed, and samples of slow-oxidative red muscle and fast-glycolytic white muscle and whole brains were immediately flash-frozen on dry ice for

subsequent total RNA isolation and qPCR analyses (see below). Total quantification of lipids in white skeletal muscle (due to this tissue's overwhelming contribution to the mass of this species and importance to the aquaculture industry) was carried out essentially as described in Rosauer et al. (2011) using the chloroform-methanol method. Circulating blood glucose levels were determined using plasma and an AccuCheck Advantage glucose meter (Roche). Specific growth rates were calculated as $[(\ln \text{ final fish mass} - \ln \text{ initial fish mass}) * 100 / \text{days}]$. Hepatosomatic index was calculated as $[(\text{liver mass} / \text{fish mass}) * 100]$.

2.2 Quantitative real-time PCR

Total RNA was isolated from red muscle, white muscle, and whole brain using RNeasy (Molecular Research Center, Inc.) according to the manufacturer's instructions. RNA concentrations were determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific) and 1 μg of total RNA was reverse transcribed into first-strand cDNA using ImProm-II Reverse Transcription System (Promega). Quantitative real-time PCR (qPCR) was performed using PerfeCTa SYBR Green SuperMix (Quanta Biosciences) according to the manufacturer's recommendations using the Mx3000P system (Stratagene). All reactions contained 2 μL sample cDNA (produced from 1 μg total RNA and diluted 1:10) or 1 μL vector at desired concentrations for standard curve. All primers for *myostatin-1a*, *-1b*, and *-2a* transcripts were used as previously described (Table 2) (Garikipati et al. 2007; Garikipati et al. 2006a) at a concentration of 300nM. All assays utilized a comparative baseline strategy using the C_q method that standardized to starting input cDNA quantity {Bustin, 2009 #1366; De Santis, 2011 #1113; Meyer, 2013 #747}. Primer PCR efficiencies were calculated for each primer: tissue pair using $\log_{10}$ dilution series of input cDNA as well as generated standards. Efficiency (E) was determined by the equation $[E = 10^{(-1/\text{slope})}]$ and ranged from 90.3% to 94.6%. Reproducibility was represented by the R^2 value of the standard curve and was always greater than 0.98. Quantification was conducted using standard curves generated for each *mstn* isoform. Standards were generated using pGEM-T Easy Vector system (Promega, Madison, WI). The coefficient of variation (CV, %) of C_q values was calculated ($CV < 0.45$). The PCR cycling parameters were as follows: 94°C (2 min) followed by 40 cycles at 94°C for 20 s, 60°C for 30 s, and 68°C for 1 min. A dissociation curve was performed for each assay to ensure primer specificity by running a single cycle as follows: 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s.

2.4 In silico Promoter Analysis

To identify putative transcription factor binding elements in the promoters of rainbow trout *myostatin-1a*, *-1b*, and *-2a*, promoter sequences were obtained from GenBank (NCBI; Accession numbers: DQ136028, DQ138300, and DQ138301; respectively). Sequences were then input into MatInspector software (Genomatrix, Inc., www.genomatrix.de) for detection of a subset of consensus *cis*-regulatory elements known to function in lipid metabolism by a position weight matrices model. Matrix similarity thresholds were set to satisfy similarity 0.80.

2.5 Statistical Analyses

Differences between mean circulating glucose levels, hepatosomatic indices, specific growth rates, *myostatin* isoform transcript expression, and mean skeletal muscle lipid content were determined by Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1 Physiological Indices

Plasma glucose concentrations were decreased 22% in fish fed HFD ($p = 0.02$; Figure 1a). In addition, high-fat diet intake decreased mean hepatosomatic index (HSI) 42.9% (Figure 1b). Specific growth rate (Figure 1c) and overall final fish mass were not changed by high-fat diet intake. In addition, feed intake was not different between treatment groups (data not shown). However, high-fat diet intake did increase white skeletal muscle (fast-glycolytic) lipid content 59.6% compared to LFD-fed fish (Figure 1d).

3.2 *Myostatin* mRNA Isoform Expression

Quantitative real-time PCR revealed that *myostatin-1a* and *-1b* mRNA levels in slow-oxidative red muscle were unchanged by HFD administration (Figures 2a, 2b). In fast-glycolytic white muscle, *myostatin-1a* and *myostatin-1b* were downregulated 50.3% and 53.9%, respectively, by HFD intake (Figure 2c, 2d). *Myostatin-2a* transcripts were undetectable in both muscle types (data not shown). In brain tissue, high-fat diet intake downregulated *myostatin-1b* by 63.2%, while *myostatin-1a* and *-2a* appear to be downregulated (Figure 3) although no statistical differences were noted.

3.3 *In silico* Promoter Analyses

Rainbow trout *myostatin* promoters were analyzed for a subset of putative transcription factor (TF) binding sites for TFs known to be involved in lipid metabolism in vertebrates, including activating protein-1 (AP-1), cAMP response element-binding protein (CREB), interferon regulatory factor (IFNRF), peroxisome proliferator-activated receptor (PPAR), retinoid X receptor (RXR), serum response factor (SRF), CCAAT binding factor (CCAAT), GC-box factors and SP1 (GC-box/SP1), and nuclear factor of activated T-cells (NFATC). AP-1 sites were found in all three paralogs, as were CREB, IFNRF, RXR, SRF, and GC-box/SP1 sites (Figure 4a, b, c). PPAR and CCAAT binding sites were only identified in *myostatin-1a* and *-1b*, while NFATC sites were only found in *myostatin-2a* (Figure 4c).

4. Discussion

While many rodent models have been developed for studies of lipid metabolism and metabolic changes induced by differences in dietary lipid intake (Panchal and Brown 2011), few useful teleost fish models exist to investigate the effects of high lipid (saturated or unsaturated) dietary intake and metabolic physiology. We have identified three studies, one in zebrafish (Oka et al. 2010), one in rainbow trout (Figueiredo-Silva et al. 2012), and one in sole (Campos et al. 2010) in which the effects of HFD were examined. Zebrafish are obviously advantageous for many reasons (i.e., small size, high fecundity, sequenced genome) but are difficult to work with in traditional physiological studies. At the onset of

this study, we set out to further establish the rainbow trout as a model organism for metabolism and obesity research by examining myostatin expression under high-fat dietary insult.

At first, though the demonstration of lower mean circulating glucose levels in HFD fish may seem counterintuitive, it is consistent with literature generated using other model organisms. Increased dietary fat content has been shown by our group and others (Lyons et al. 2010) to increase whole blood glucose levels in mice. Further, HFD-induced hyperglycemia has been demonstrated in humans and other mammals (Calder et al. 2011; Lichtenstein and Schwab 2000). However, lipid content in our experimental diet was increased using menhaden oil, a fish-based product rich in mono- and polyunsaturated fatty acids (MUFA and PUFA, respectively) (Joseph 1985). In mice, the addition of PUFA-enriched Menhaden oil to a lard-based HFD actually restored normal glucose levels in the C57Bl/6J line (Lamping et al. 2012). Results from rats appear to be in conflict, as improved glucose tolerance was not displayed in diabetic (streptozotocin-induced) rats (Coppey et al. 2012) but glucose intolerance was prevented in BHE/cdb rats (Berdanier et al. 1992; Bunce et al. 1992).

In this study, high levels of lipid supplied by menhaden oil (25%) decreased glucose levels compared to the 10% lipid diet, contrary to what has been previously found in this species (Figueiredo-Silva et al. 2012). Rainbow trout, as they are carnivorous teleost fish, are considered 'glucose-intolerant,' a phenomenon which is caused by weak peripheral actions of insulin and not due to low insulin secretion (Moon 2001; Plisetskaya et al. 1976; Hilton et al. 1987; Mommsen and Plisetskaya 1991). However, it does appear that the lack of feedback on the gluconeogenic pathway may play a role as well (Panserat et al. 2001). Our HFD-fed trout consumed similar amounts of protein as our LFD-fed trout, while the LFD-fed fish ingested approximately 50% more protein than did HFD-fed fish in the preceding study (Figueiredo-Silva et al. 2012). Thus, the main difference between our study's experimental diets was the concentration of menhaden oil (Figure 1b) versus the isoenergetic diets used in the Figueiredo-Silva study. This may account for the conflicting glucose data. As with glucose concentrations, lower hepatosomatic indices (HSI) were detected in HFD-fed fish. Menhaden oil has been shown to lower HSI in channel catfish (Lim et al. 2010); however, the reason for lower HSI in our or the Lim study is not clear. Lim and coworkers did not identify differences in final masses between treatment groups, a finding our data support.

While most research investigating the role(s) of myostatin have focused on its regulation of skeletal muscle mass, an accumulating amount of evidence suggests that this protein may play a pivotal role in energy partitioning, particularly by promoting adiposity (Zhang et al. 2012; Burgess et al. 2011; Lyons et al. 2010; Guo et al. 2009; Hamrick et al. 2006; Zhao et al. 2005). These studies have convincingly demonstrated that knockdown, inhibition, or deletion of myostatin promotes 'normal' body lean mass under high fat dietary insult. Because skeletal muscle is the primary energy consumer in teleost fish, we focused our analysis of *myostatin* transcript expression to this tissue, which exists as two distinct layers in these animals: 'red' slow-oxidative and 'white' fast-glycolytic. In this study, *myostatin-1a* and *-1b* transcripts were significantly downregulated in white muscle in response to HFD (Figure 2c, 2d), suggesting that, like in mammals, lipids can alter myostatin expression. It is

important to note that, in both the LFD and HFD groups, *myostatin-2a* transcripts were not detectable in either muscle type, which contradicts previous tissue distribution reports (Garikipati et al. 2007). The decreased myostatin detected in response to increased dietary lipid does support a correlation between lipid deposition, muscle mass, and energy partitioning, one that warrants further investigation.

A negative trend in *myostatin-1a*, *-1b*, and *-2a* was observed in whole brain tissue, but only *myostatin-1b* was significantly decreased (Figure 3b). This finding coincides with the observed *myostatin-1b* response in white muscle. Previous reports of *myostatin* expression have indicated that *myostatin-2a* is highly expressed in the brain (Garikipati et al. 2007). Further, *myostatin-2a* is only fully processed in the brain or upon IGF-1 stimulation in skeletal muscle (Garikipati and Rodgers 2012c; Garikipati et al. 2007). Therefore, we predicted that expression of this paralog would be altered by increased dietary lipids. Our data, however, did not confirm this prediction.

Like myostatin, PPARs, especially PPAR γ , can have profound effects on skeletal muscle. Constitutive activation of PPAR γ in mice has been shown to induce resistance to HFD-induced obesity and promote fiber switching (Wang et al. 2004). Therefore, we aimed to determine whether PPAR response elements (PPARREs) exist in the promoters of the three myostatin genes found in rainbow trout (Garikipati et al. 2006b; Garikipati et al. 2007). After confirming the conservation of three PPAR genes in *Oncorhynchus mykiss* (Jia, C., Zhang, Q., Liu, P., Zhu, H., Fu, H., Xu, S. and Yang, G., unpublished results; NCBI GenBank), our *in silico* analyses of the promoters of these genes indicates that one PPAR-response element (PPARRE) exists in the promoter of *myostatin-1a* and two PPARREs are present in the *myostatin-1b* gene: one in the promoter and one in the 5'-untranslated region (5'-UTR) (Figure 4). We speculate that these PPARREs, if functionally active, negatively regulate *myostatin* expression in these fish, but this prediction requires empirical experimentation. At the transcriptional level of *myostatin* regulation, increased PPAR activation has been shown to repress the ability of nuclear factor- κ B (NF- κ B) (Staels et al. 1998) and activating protein-1 (AP-1) (Delerive et al. 1999) to activate gene expression. Similarly, PPAR γ has been demonstrated to negatively regulate the SP1-dependent activation of follistatin transcription (Necela et al. 2008). Of these three transcription factors (NF- κ B, AP-1, and SP1), AP-1 and SP1 sites were found in all *myostatin* isoforms (Figure 4), while we failed to detect any putative NF- κ B binding sites.

In line with our *in silico* data, only *myostatin* isoforms containing PPARREs (*myostatin-1a* and *-1b*) were found to differ in expression levels upon experimentation. Consistently, *myostatin-1b* was downregulated in white skeletal muscle and brain in response to HFD administration. Additionally, *myostatin-1a* transcripts were downregulated only in white skeletal muscle, although a trend was noted in brain ($p=0.17$). Interestingly, the promoter of *myostatin-2a* does not appear to contain any PPARREs, and experimental data failed to reveal any changes in the transcription of this paralog. At the protein level, PPAR $\beta\delta$ has been shown to upregulate Gasp-1 expression, a protein that attenuates the actions of myostatin (Bonala et al. 2012). Further, crosstalk between PPAR γ and myostatin signaling has been demonstrated in human mesenchymal stem cells (hMSCs) (Guo et al. 2008). Clearly, myostatin may play a role in the interplay between energy partitioning and muscle

growth; however, further experimentation involving the perturbation of myostatin is needed to confirm such a hypothesis.

Based on this mammalian data and our *in silico* analyses of rainbow trout *myostatin* promoters, we hypothesize that myostatin and PPARs play an important role in lipid utilization and muscle metabolism in rainbow trout. As lipid content in fish fillets is of importance to the aquaculture industry, a better understanding of the regulation of lipid deposition in skeletal muscle would be of great impact to the production of high quality fish. From a biomedical perspective, understanding lipid metabolism in a basal vertebrate may be helpful in combating disorders related to increased lipid intake in more derived vertebrate lineages (i.e., humans). Indeed, the foundational data presented here provide a platform for future investigations of the actions in myostatin in teleost fishes, particularly at the interface between lipid metabolism and skeletal muscle growth.

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Fig. 1. Circulating blood glucose levels (a), hepatosomatic index (HSI) (b), specific growth rates (SRG) (c) and white muscle lipid content (d) after 5 week treatment. Treatment groups included: low-fat diet (LFD) and high-fat diet (HFD). (mean $\pm$ SEM, $n=12/\text{trt}$, $*P < 0.05$)

Fig. 2. Red muscle *myostatin-1a* (a), *-1b* (b) and white muscle *myostatin-1a* (c), *-1b* (d) mRNA levels after 5-week treatment. Results are mean relative copy number $\pm$ SEM, $n=12$ fish/trt. Treatment groups included: low-fat diet (LFD) and high-fat diet (HFD). (mean $\pm$ SEM, * $P < 0.05$)

Fig. 3. Whole brain *myostatin-1a* (a), *-1b* (b), and *-2a* (c) mRNA levels after 5 week treatment. Results are mean relative copy number $\pm$ SEM, $n=12$ fish/trt. Results are mean relative copy number $\pm$ SEM, $n=12$ fish/trt. Treatment groups included: low-fat diet (LFD) and high-fat diet (HFD). (mean $\pm$ SEM, * $P < 0.05$)

Fig. 4.

Map of *cis*-regulatory elements associated with lipid metabolism within the promoter region of *myostatin-1a* (a), *-1b* (b), and *-2a* (c). Black and gray regions represent 100 bp segments. Symbols superior to DNA strand are transcription factor binding sites identified on the positive strand; those inferior are transcription factor binding sites identified on the negative strand.

Table 1

Ingredient and nutrient composition of experimental diets.

Composition of low-fat diet (LFD) and high fat diet (HFD) formulated (a) and nutrient composition of the LFD and HFD (b).

Ingredient	g/100 g	
	High	Low
Fish meal, menhaden ^a	39.75	39.75
Fish oil, menhaden ^b	19.90	4.80
Poultry by-product meal ^c	18.12	18.12
Wheat flour ^d	15.10	30.20
Blood meal ^e	5.13	5.13
Vitamin Premix, ARS 702 ^f	1.00	1.00
Choline CL	0.60	0.60
Vitamin C ^g	0.30	0.30
Trace Mineral Premix ^h	0.10	0.10
Calculated Composition, %		
Crude protein	40.0	40.0
Crude lipid	25.1	10.1
Phosphorus, total	1.54	1.54

^aOmega Proteins Inc., Menhaden Special Select, 628 g/kg protein

^bOmega Proteins Inc., Menhaden Virginia Prime

^cIDF Inc., pet food grade, 602 g/kg protein

^dManildra Milling, 120 g/kg protein

^eIDF Inc., 832 g/kg protein

^fContributed, per kg diet: vitamin A 9650 IU; vitamin D 6600 IU; vitamin E 132 IU; vitamin K3 1.1 g; thiamine mononitrate 9.1 mg; riboflavin 9.6 mg; pyridoxine hydrochloride 13.7 mg; pantothenate DL-calcium 46.5 mg; cyanocobalamin 0.03 mg; nicotinic acid 21.8 mg; biotin 0.34 mg; folic acid 2.5 mg; inositol 600 mg.

^gRovimix Stay-C, 35%, DSM Nutritional Products

^hContributed in mg/kg of diet: zinc 40; manganese 13; iodine 5; copper 9

Table 2

Primer sequences used for quantitative real time PCR.

Primer sequences used for qPCR			
Gene isoform	Forward	Reverse	reference
Myostatin-1a	cttcacatagccaatacatatta	gcaacatgaaactgagataaa	Garikipati et al., 2006
Myostatin-1b	ttcagcaaatacgtattcac	gataaattagaacctgcatcagattc	Garikipati et al., 2006
Myostatin-2a	aatctccgcataaaagcaaccac	caccagaagccacatcgatctt	Garikipati et al., 2007