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Lipopolysaccharide regulates myostatin and MyoD independently of an increase in plasma cortisol in channel catfish (*Ictalurus punctatus*)

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Abstract

The effects of lipopolysaccharide (LPS) on plasma cortisol and the expression of MyoD and myostatin (MSTN) mRNAs were evaluated in channel catfish. In addition, the effect of dexamethasone (Dex) on MyoD and MSTN mRNAs was examined. For the LPS injection experiments, juvenile channel catfish were injected intraperitoneally with 1.5 mg/kg LPS or sterile PBS. Blood was collected at 1, 3, 12, and 24 h post-injection for cortisol determination, and muscle samples were collected at 3, 12, and 24 h for mRNA analysis. For the Dex injection experiment, fish were injected with 1.0 mg/kg Dex or saline and muscle samples were collected at 12 and 24 h. There was no effect of LPS on plasma cortisol at any of the time points measured. Injection with LPS increased the abundance of MyoD mRNA at 3 and 12 h, and decreased the abundance of MSTN mRNA at 24 h. There was no effect of Dex injection on the abundance of MyoD mRNA. However, Dex injection decreased the abundance of MSTN mRNA at 12 h post-injection. These results suggest that LPS regulates the expression of MyoD and MSTN independently of an increase in plasma cortisol, and that the regulation of MyoD in the channel catfish differs from mammals in response to inflammatory stimuli. These results also confirm that exogenous glucocorticoids decrease the expression of MSTN as shown in other fish species.
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1. Introduction

The regulation of genes involved in muscle growth may play a role in maintaining homeostasis in response to stressors such as pathogen exposure. Indeed, proinflammatory

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cytokines and glucocorticoids regulate the expression of genes involved in mammalian muscle growth and development. The expression of myostatin (MSTN), a negative regulator of skeletal muscle mass [1], is upregulated in response to exogenous glucocorticoids in mammalian models [2]. Furthermore, a subsequent increase in endogenous cortisol in response to LPS would most likely lead to an increase in MSTN expression because glucocorticoids increase the abundance of MSTN mRNA in muscle cells [2,3]. In mammals, proinflammatory cytokines directly regulate the expression of the muscle regulatory factor MyoD. The proinflammatory cytokine, tumor necrosis factor-α (TNFα), decreases the abundance of MyoD mRNA [4] and protein [5]. MyoD plays a role in skeletal muscle regeneration [6], and decreased expression of MyoD in response to pathogen exposure would likely lead to decreased ability to synthesize new muscle protein.

Proinflammatory cytokines, which are synthesized and secreted by numerous cell types in response to lipopolysaccharide (LPS), alter metabolic processes and growth in mammalian species [7,8]. The proinflammatory cytokines, interleukin-1β (IL-1β) [9] and TNFα [10], have been cloned and characterized in various fish species. Indeed, as found in mammals, LPS upregulates the expression of IL-1β [11] and TNFα [10] in warm-water fish species. It has also been demonstrated that either LPS or recombinant IL-1β increases circulating cortisol concentrations in trout [12]. An increase in cortisol elicited by proinflammatory cytokines is one mechanism via which pathogen exposure can modulate metabolism and ultimately growth in fish.

In fish it has been demonstrated that either chronic stress [13] or exogenous cortisol [14] decreases the abundance of MSTN mRNA. This indicates that the regulation of MSTN mRNA expression in fish differs from that of mammals. Furthermore, the regulation of MyoD in response to inflammatory stimuli and glucocorticoids in aquatic species has not been determined. Additionally, the regulation of MSTN in response to inflammatory stimuli in fish species remains unknown. The objectives of the current experiments were to determine whether LPS alters the abundance of the mRNAs for MSTN and MyoD, and determine whether any alterations of these mRNA transcripts were associated with an increase in circulating cortisol concentrations. Another objective was to confirm that exogenous glucocortoicoids regulate MSTN in the channel catfish.

2. Materials and methods

2.1. Research animals and sampling

The fish used in these experiments were a channel catfish strain (NWAC103) maintained by the National Warmwater Aquaculture Center, and reared at the USDA-ARS Catfish Genetics Research Unit aquaculture facility located at Stoneville, MS. For the first LPS injection study, 36 fish (body weight 46.1 ± 3.9 g) were randomly assigned to six 76-1 tanks (6 fish/tank), and for the dexamethasone (Dex) injection study, 36 fish (body weight 78.1 ± 1.3 g) were randomly assigned to four 76-1 tanks (9 fish/tank). In the second LPS injection study, 12 fish (body weight 82.0 ± 2.7 g) were randomly assigned to two 76-1 tanks (6 fish/tank). In each study, the fish were allowed to acclimate for 7 days prior to experimental treatments. For all experiments, fish were reared in 26.0 °C flow-through well

water under a 14:10 h light/dark photoperiod, and were fed a commercial floating catfish feed (36% crude protein; Land O'Lakes Farmland Feed LLC, Fort Dodge, IA, USA) once daily to apparent satiation. Water quality (pH ∼ 8.5; dissolved oxygen levels > 5.0 mg/L) and flow rates (3.75 L/min) were similar between all tanks.

For the LPS injection experiments, fish were injected intraperitoneally (i.p.) with LPS (1.5 mg/kg; *Escherichia coli* O127:B8; Sigma Chemical Co., St. Louis, MO, USA) or sterile PBS (vehicle for LPS). Muscle and blood samples were collected at 3, 12, and 24 h post-injection from six fish/treatment group at each time point. For the second LPS injection experiment, blood samples were collected at 1h after LPS or PBS injection from all fish. For the Dex injection experiment, fish were injected i.p. with Dex (1.0 mg/kg; Sigma) or sterile PBS, and muscle samples were collected at 12 and 24 h post-injection from nine fish/treatment group. At sampling times, the fish were euthanized with an overdose (0.3 g/L) of tricainemethane sulfonate (Finquil; Argent Chemical Laboratories, Redmond, WA, USA). The fish were bled from the caudal vasculature into heparin-coated syringes, and the resulting plasma was separated and frozen at −80 °C and later analyzed for cortisol. Muscle samples from the dorsal white musculature were collected, snap frozen in liquid nitrogen, and stored at −80 °C until RNA isolation.

2.2. Plasma cortisol determination

Plasma concentrations of cortisol were measured using a DELFIA® time-resolved fluroimmoassay kit (Perkin-Elmer Life Sciences, Boston, MA, USA). This assay has been previously validated for the quantification of plasma cortisol in channel catfish [15] and is used routinely in our laboratory [16,17].

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated using TRI-reagent® (Molecular Research Center, Cincinatti, OH, USA) according to the manufacturer's protocol, and the RNA pellets were resuspended in TE-8 (Tris–EDTA, pH 8.0). To eliminate possible genomic DNA contamination, the RNA samples were treated with a DNase I kit (DNA-free, Ambion, Inc., Austin, TX, USA) per the manufacturer's instructions. The total RNA was quantified by measuring the absorbance at 260 nm using a NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA), and the purity was assessed by determining the ratio of the absorbance at 260 and 280 nm (NanoDrop). All samples had 260/280 nm ratios above 1.8. Additionally, the integrity of the RNA preparations was verified by visualization of the 18S and 28S ribosomal bands stained with ethidium bromide after electrophoresis on 1.0% agarose gels. Total RNA (1 μg) was reverse transcribed using a commercially available cDNA synthesis kit (iScript, BioRad Laboratories, Hercules, CA, USA).

2.4. Quantification of mRNA expression by quantitative RT-PCR

Real-time PCR detection of MSTN and MyoD mRNAs was conducted utilizing the SYBR Green assay. Amplification was carried out in a total volume of 25 μL containing 1× iQ™ SYBR® Green Supermix (Bio-Rad), forward and reverse primers (0.1 μg/μL; Table 1) and

Table 1
Primer sequences used for quantitative PCR

Gene	Primer sequences[a]	Accession number
Myostatin	(S) CTTGCAGATATCGCGACTCA (AS) CCTGATTCTCTCCTGGTTCG	AF396747
MyoD	(S) TCCAACTGCTCAGATGGC (AS) TCCACGATGCTGGACAGA	AY534328

[a] S: sense primer, AS: antisense primer.

1 μL of the cDNA reaction. The reactions were cycled 45 times under the following parameters: 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min and 85 °C for 5 s. Optical detection was carried out at 85 °C. At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. Thermal cycling conditions and real-time detection were conducted using a Bio-Rad iCycler iQ system (Bio-Rad). A nontemplate control was run with every assay, and all determinations were performed in duplicates. External cDNA standards for MSTN and MyoD were constructed by cloning the corresponding RT-PCR product into a pCR® 4-TOPO® vector (Invitrogen, Carlsbad, CA, USA). The identities of the cloned inserts were confirmed by sequencing at the USDA Mid-South-Area Genomics Laboratory. The concentration (μg/μL) of the standards was determined by measuring the absorbance of the plasmid preparation at 260 nm. The copy number of plasmids per μL (CN) was calculated with the following formula [18]: $CN = 9.1 \times 10^{11}$ (concentration of the plasmid/size of the plasmid in kb). The number of templates present in the experimental reactions is calculated by the Bio-Rad iCycler iQ system software (Bio-Rad) based on the standard curve data. Using the amounts of cDNA reverse-transcribed from a known amount of total RNA, the number of specific molecules of mRNA/μg total RNA can be calculated.

2.5. Statistical analysis

The statistical analyses were conducted as one-way ANOVAs using the Statistical Analysis System Version 8.0 software (SAS Institute, Inc., Cary, NC, USA). Separate ANOVAs were conducted for each sampling time point. For each variable measured, fish served as the experimental unit. Data are presented as means ± S.E. A significance level of $P < 0.05$ was used.

3. Results

Plasma cortisol concentrations were not altered ($P > 0.30$) by LPS at any of the time points measured (3, 8, and 24 h) in the first LPS injection study (Fig. 1). It is possible that plasma cortisol may have peaked and returned to basal levels by 3 h post-injection. Therefore, we injected a second group of fish with LPS and collected blood at 1 h post-injection. Similar to the first LPS injection study there was no effect ($P > 0.90$) of LPS on plasma cortisol concentrations (Fig. 1).

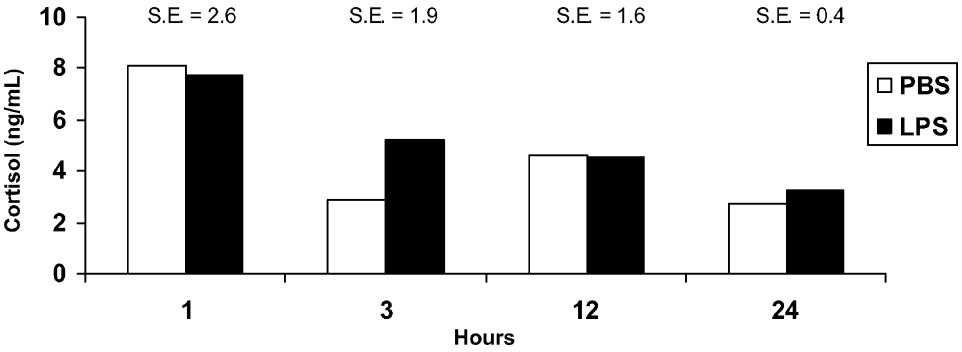

Fig. 1. Effect of *E. coli* lipopolysaccharide (LPS) on plasma cortisol concentrations in channel catfish. Fish were injected i.p. with either sterile PBS or LPS (1.5 mg/kg body weight). Blood was collected from the fish at 3, 12, and 24 h post-injection. The values presented are means for 6 fish per treatment at each time point. Significance: 1 h, $P < 0.91$; 3 h, $P < 0.42$; 12 h, $P < 0.98$; 24 h, $P < 0.30$.

The abundance of MSTN mRNA in muscle was not altered ($P > 0.86$) by LPS injection at 3 and 12 h post-injection (Fig. 2). However, at 24 h post-injection there was a decrease ($P < 0.05$) in MSTN mRNA abundance in the muscle of fish injected with LPS as compared to fish injected with sterile PBS. Injection of fish with LPS led to an increase ($P < 0.05$) in the abundance of MyoD mRNA at 3 h and 12 h post-injection (Fig. 3). At 24 h post-injection, levels of MyoD mRNA in muscle were similar ($P > 0.20$) between LPS and sterile PBS injected fish.

The injection of channel catfish with Dex decreased ($P < 0.05$) the abundance of MSTN mRNA in muscle at 12 h post-injection (Fig. 4). The abundance of MSTN mRNA was not different ($P > 0.56$) between Dex and PBS injected fish at 24 h post-injection. Levels of MyoD mRNA in muscle tissue were not affected ($P > 0.36$) by Dex injection at 12 or 24 h post-injection (Fig. 5).

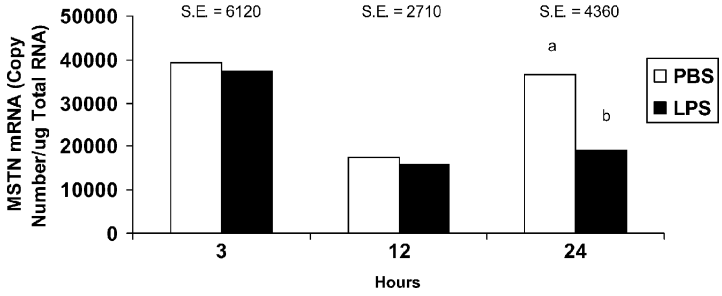

Fig. 2. Effect of *E. coli* lipopolysaccharide (LPS) on the abundance of myostatin (MSTN) mRNA in the muscle of channel catfish. Fish were injected i.p. with either sterile PBS or LPS (1.5 mg/kg body weight). Muscle samples were collected from the fish at 3, 12, and 24 h post-injection. The values presented are means for 6 fish per treatment at each time point. Within a time point, treatment means with different letters are significantly different ($P < 0.05$).

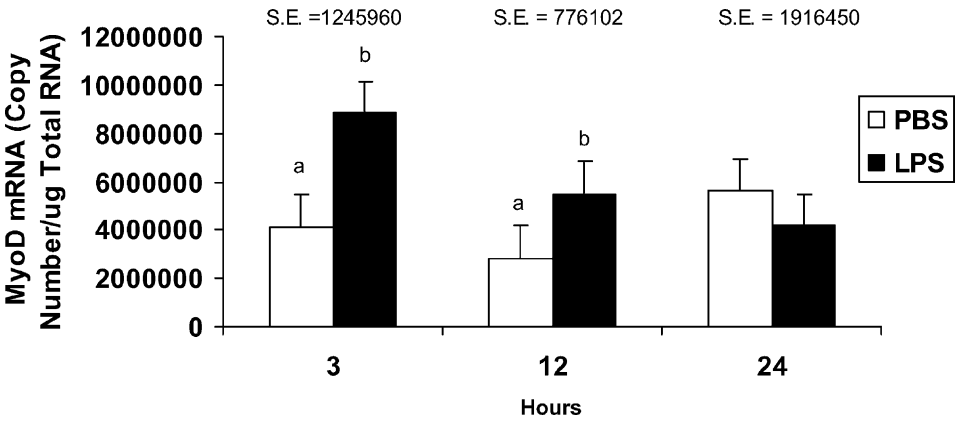

Fig. 3. Effect of *E. coli* lipopolysaccharide (LPS) on the abundance of MyoD mRNA in the muscle of channel catfish. Fish were injected i.p. with either sterile PBS or LPS (1.5 mg/kg body weight). Muscle samples were collected from the fish at 3, 12, and 24 h post-injection. The values presented are means for 6 fish per treatment at each time point. Within a time point, treatment means with different letters are significantly different ($P < 0.05$).

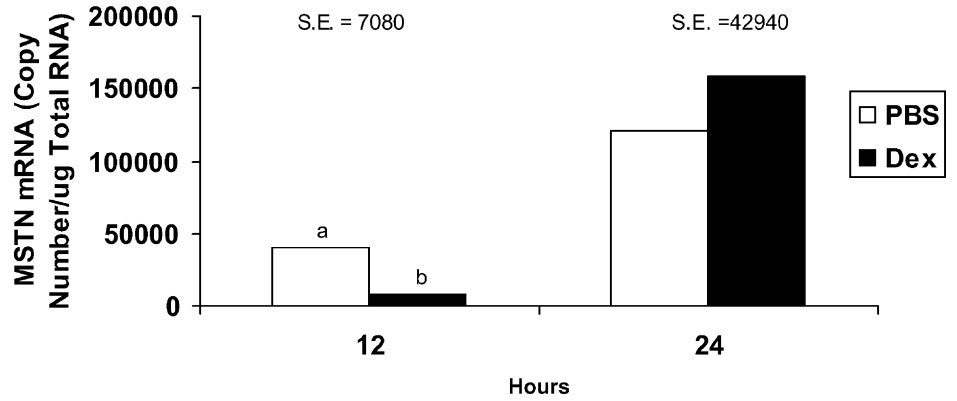

Fig. 4. Effect of dexamethasone (Dex) on the abundance of myostatin (MSTN) mRNA in the muscle of channel catfish. Fish were injected i.p. with either sterile PBS or Dex (1.0 mg/kg body weight). Muscle samples were collected from the fish at 12 and 24 h post-injection. The values presented are means for 9 fish per treatment at each time point. Within a time point, treatment means with different letters are significantly different ($P < 0.05$).

4. Discussion

Injection of channel catfish with a dose of LPS at 1.5 mg/kg bodyweight failed to alter plasma cortisol concentrations. Cortisol levels of the fish injected with LPS measured below the levels found in channel catfish which were fasted for 30 days (22.8 ng/mL) [17] or subjected to 2 h confinement stress (45.3 ng/mL) [19]. In fact, cortisol levels of LPS injected fish were similar to rested levels reported for channel catfish [17,20]. The lack of a cortisol response to LPS injection is in contrast to what is observed in rainbow trout injected with

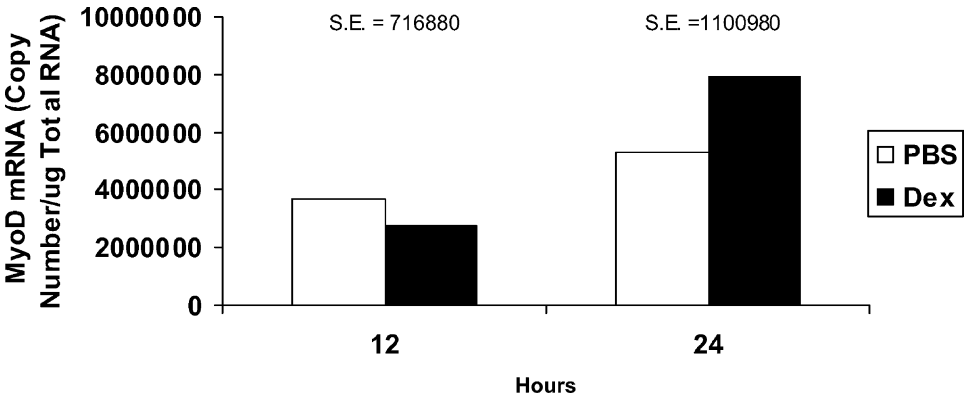

Fig. 5. Effect of dexamethasone (Dex) on the abundance of MyoD mRNA in the muscle of channel catfish. Fish were injected i.p. with either sterile PBS or Dex (1.0 mg/kg body weight). Muscle samples were collected from the fish at 12 and 24 h post-injection. The values presented are means for 9 fish per treatment at each time point.

LPS or recombinant IL-1β [12]. Injection with LPS or IL-1β in rainbow trout leads to increases in cortisol at 3 and 8 h post-injection. It is unlikely that we missed an increase in cortisol in response to LPS as cortisol was measured at 1, 3, 12 and 24 h post-injection. The dosage of LPS used in the current study (1.5 mg/kg body weight) may not have been sufficient to alter plasma cortisol concentrations, but it was greater than that used to elicit cortisol increases in trout (1.3 mg/kg body weight). Furthermore, the lack of a cortisol response indicates that channel catfish may respond different physiologically than trout to injection with LPS. Likewise, it is also possible that channel catfish are less sensitive than rainbow trout to LPS and thus require larger doses to elicit a cortisol response.

Injection of channel catfish with LPS decreased the abundance of the mRNA encoding MSTN and increased the abundance of MyoD mRNA. This is the first report of the regulation of MSTN and MyoD by inflammatory stimuli in any aquatic species. Our finding that Dex injection decreases the abundance of MSTN agrees with the finding that immersion of Tilapia larvae in cortisol decreases MSTN mRNA levels [14]. The decrease in MSTN mRNA abundance in response to LPS, Dex, chronic stress [13], and exogenous cortisol [14] indicates a general stress mechanism in fish which culminates in a decrease in the expression of the gene encoding MSTN. The increase in the abundance of MyoD mRNA is opposite of what is found in mammalian models where inflammatory stimuli decrease the expression of MyoD [4]. These findings indicate MSTN and MyoD expression are differentially regulated in fish as compared to mammalian species.

We concede that the observed changes in mRNA may not have resulted in changes in protein levels under these circumstances. However, we were primarily interested in the mechanisms at the genetic level in order to compare with what has been noted in mammalian models [4] in terms of mRNA regulation. Nonetheless, these data indicate regulation of myostatin and MyoD at the genetic level by glucocorticoids and inflammatory stimuli in channel catfish. Other studies which have investigated the regulation of myostatin in fish by glucocorticoids [14] also fail to address the question of protein abundance, likely due to the

lack of available antibodies. Our laboratory is currently attempting to acquire and screen antibodies that might be useful for these proteins in catfish, but have not had success with them at this point.

It is interesting that both proinflammatory (LPS) and anti-inflammatory (Dex) agents decrease the abundance of MSTN mRNA in the channel catfish. The lack of an increase in cortisol in response to LPS indicates that LPS regulates MSTN expression independently of glucocorticoids. It is possible that LPS acts directly upon skeletal muscle cells to alter muscle gene expression. Indeed, mouse muscle cells have the ability to recognize bacterial LPS through the toll-like receptor 4 (TLR-4) [21]. Futhermore, C2C12 myocytes produce inflammatory cytokines (both mRNA and protein) when treated with LPS [21]. Activation of TLR-4 by LPS or activation of cytokine receptors by cytokines (i.e. TNFα; IL-1) leads to the activation of nuclear factor kappa B (NFkB) [22]. Therefore, it may be possible that LPS acts directly upon myocytes to regulate MSTN and MyoD. It is also possible that proinflammatory cytokines induced by LPS act in an endocrine or autocrine fashion to activate the NFkB pathway. The promoter region of both the human [3] and brook trout [23] MSTN genes contain NFkB regulatory elements. This suggests that MSTN gene expression is under the control of NFkB in both mammalian and aquatic species. However, it remains to be determined whether channel catfish muscle cells express a TLR-4 homologue, as has been found in the zebrafish [24], which would allow a direct response to LPS and thus regulation of MSTN expression via NFkB activation.

In the channel catfish, it is unknown whether Dex decreases the in vivo expression of MSTN in a direct or indirect fashion. It is possible that Dex injection indirectly regulates the expression of MSTN through the alteration of other factors that regulate MSTN. Furthermore, preliminary data collected in our laboratory indicates that Dex, in vitro, does not alter the abundance of MSTN mRNA in channel catfish primary muscle cell culture. However, the promoter region of the brook trout MSTN does contain a glucocortioid regulatory element [23]. This suggests that the MSTN gene of brook trout is under the control of glucocorticoids. More research is necessary to determine whether MSTN expression in fish muscle cells is directly or indirectly altered by glucocorticoids.

The biological implications of the regulation of MSTN and MyoD expression in channel catfish by LPS and glucocorticoids remains to be determined. It is believed that in stressful situations, such as pathogen exposure, muscle protein breakdown is necessary to liberate amino acids for gluconeogenic substrates and for the synthesis of acute phase proteins [25]. Intuitively, a decrease in MyoD and increase in MSTN expression would be hypothesized in response to LPS or Dex. However, we found exactly the opposite in the current study. In order to compensate for muscle degradation, it is possible in channel catfish that muscle cells are being reprogrammed for muscle regeneration or repair after LPS injection. Indeed, in primary mouse muscle cells it has been found that the proinflammatory cytokine, TNFα, has mitogenic activity [26]. This is in contrast to what was observed in L8 myoblasts in which proliferation is inhibited by TNFα [27]. This suggests that primary and continuous cell-lines respond differently to inflammatory cytokines, and further suggests the need to characterize the muscle's response to inflammatory stimuli in both mammalian and aquatic species, in vivo. Nonetheless, given the known function of MSTN and MyoD in mammaliam models, a reduction in MSTN and an increase in MyoD expression indicates that LPS may stimulate muscle regeneration in channel catfish.

In conclusion, an increase in MyoD mRNA abundance and a decrease in MSTN mRNA abundance was observed with LPS injection. Injection with LPS failed to elicit a cortisol response, indicating that the regulation of MyoD and MSTN by LPS is independent of cortisol. Nonetheless, consistent to what was observed in other fish species, exogenous glucocorticoid administration decreased MSTN mRNA in the channel catfish. These results provide further evidence that the regulation of muscle regulatory factors in fish species differ from that of mammals.

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