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Research paper

A comparative examination of cortisol effects on muscle myostatin and HSP90 gene expression in salmonids

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ABSTRACT

Cortisol, the primary corticosteroid in teleost fishes, is released in response to stressors to elicit local functions, however little is understood regarding muscle-specific responses to cortisol in these fishes. In mammals, glucocorticoids strongly regulate the muscle growth inhibitor, myostatin, via glucocorticoid response elements (GREs) leading to muscle atrophy. Bioinformatics methods suggest that this regulatory mechanism is conserved among vertebrates, however recent evidence suggests some fishes exhibit divergent regulation. Therefore, the aim of this study was to evaluate the conserved actions of cortisol on myostatin and hsp90 expression to determine if variations in cortisol interactions have emerged in salmonid species. Representative salmonids; Chinook salmon (*Oncorhynchus tshawytscha*), cutthroat trout (*Oncorhynchus clarki*), brook trout (*Salvelinus fontinalis*), and Atlantic salmon (*Salmo salar*); were injected intraperitoneally with a cortisol implant (50 μg/g body weight) and muscle gene expression was quantified after 48 h. Plasma glucose and cortisol levels were significantly elevated by cortisol in all species, demonstrating physiological effectiveness of the treatment. *HSP90* mRNA levels were elevated by cortisol in brook trout, Chinook salmon, and Atlantic salmon, but were decreased in cutthroat trout. *Myostatin* mRNA levels were affected in a species, tissue (muscle type), and paralog specific manner. Cortisol treatment increased *myostatin* expression in brook trout (Salvelinus) and Atlantic salmon (Salmo), but not in Chinook salmon (Oncorhynchus) or cutthroat trout (Oncorhynchus). Interestingly, the VC alone increased *myostatin* mRNA expression in Chinook and Atlantic salmon, while the addition of cortisol blocked the response. Taken together, these results suggest that cortisol affects muscle-specific gene expression in species-specific manners, with unique Oncorhynchus-specific divergence observed, that are not predictive solely based upon mammalian stress responses.

1. Introduction

Stress can be defined as a condition where homeostasis is disrupted in response to a stressor (intrinsic or extrinsic) and a number of neuroendocrine, physiological, and behavioral responses occur in response to stressors in order to reestablish homeostatic equilibrium (Wendelaar Bonga, 1997; Prunet et al., 2008). Overall, the stress response is viewed as an adaptive mechanism that allows the fish to cope; however if homeostasis is not attained in the long run the stress response can be detrimental to the organism and therefore becomes maladaptive.

Elevated levels of circulating cortisol are detectable within minutes following stress induced activation of the hypothalamic-pituitary-interrenal (HPI) axis and are responsible for nervous system activation, maintaining or increasing circulating glucose levels, and restoring hepatic glycogen levels through regulating metabolism (Bamberger et al., 1996; Mommsen et al., 1999). Cortisol acts at the transcript level by regulating the expression of metabolically related genes such as glycolytic enzymes, gluconeogenic enzymes, amino acid metabolizing enzymes, and lipolytic enzymes (Mommsen et al., 1999; Schoneveld et al., 2004; Wiseman et al., 2007). The inhibitory effects of cortisol on growth and reproduction are largely a result of the metabolic shift and reallocation of energy to processes necessary for survival (Van Weerd and Komen, 1998; Mommsen et al., 1999).

The effects of stress on growth have been studied extensively in fish and elevated cortisol is directly linked to the inhibition of muscle growth (Wendelaar Bonga, 1997; Van Weerd and Komen, 1998; Mommsen et al., 1999). The inhibitory effects of cortisol involve inhibiting protein synthesis and the catabolic effects on muscle

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include the induction of protein catabolism and increased amino acid utilization (Mommsen et al., 1999). Further, cortisol inhibits muscle growth via modulating the GH-IGF axis by altering protein and mRNA levels of GH, IGF-I, and IGFBPs (Kajimura et al., 2003; Peterson and Small, 2005; Davis and Peterson, 2006; Wilkinson et al., 2006; Leung et al., 2008; Beckman, 2011; Nakano et al., 2013). Philip and Vijayan recently demonstrated that pretreatment of rainbow trout hepatocytes with cortisol can suppress GH-mediated IGF-1 expression (Philip and Vijayan, 2015). Additionally, the growth-suppressing effects of cortisol in trout results from reduced food intake likely mediated by increased hepatic leptin expression and increased hepatic IGFBP1 expression thereby reducing the growth-promoting actions on the GH/IGF axis and subsequent mobilization of energy reserves (Madison et al., 2015).

Another possible target of the glucocorticoids is myostatin, a negative regulator of muscle growth. In mammals corticosteroids have been linked to greater myostatin levels and muscle atrophy (Gilson et al., 2007; Schakman et al., 2008), and are hypothesized to function via glucocorticoid response elements (GREs) within the myostatin gene promoter. These GREs have been identified and functionally validated in the myostatin promoter of mammals (Ma et al., 2001, 2003). However, GREs have only been identified putatively in the *myostatin-1b* promoters of brook trout (*Salvelinus fontinalis*; Garikipati et al., 2006) and Atlantic salmon (*Salmo salar*; Ostbye et al., 2007). Interestingly, analyses of the rainbow trout *myostatin-1a*, *-1b*, and *-2a* promoters failed to identify putative GREs (Garikipati et al., 2006, 2007). However, the *myostatin* promoters in that species and others have not been empirically tested to verify the absence of true GREs or the presence on noncanonical GRE motifs. As a first step, this study aimed to evaluate differential regulation of myostatin homologs by cortisol within salmonids.

In addition, as cortisol is thought to be beneficial to the capacity of individuals to cope with environmental change (Wendelaar Bonga, 1997), this study aimed to demonstrate conserved cortisol action on muscle tissue by evaluating heat shock protein regulation. Cortisol is known to upregulate heat shock protein genes, such as heat shock protein 90 (*HSP90*) (Deane and Woo, 2011). Additionally, HSP90 has been recently shown to be important in protein folding in muscle tissue and being studied as a key target in ameliorating skeletal muscle abnormalities (Erekat et al., 2014). Recent evidence demonstrated elevated *HSP90* mRNA in response to cortisol in rainbow trout muscle *in vitro*, suggesting a conserved regulation across taxa (Galt et al., 2014b). However, the role of cortisol in the *in vivo* regulation of HSP90 among salmonids has not been established.

The aim of this study was therefore to evaluate the effects of exogenous cortisol treatment on the expression of *myostatin* and *HSP90* mRNAs in cutthroat trout (*Oncorhynchus clarki*), Chinook salmon (*Oncorhynchus tshawytscha*), brook trout (*Salvelinus fontinalis*), and Atlantic salmon (*Salmo salar*) in order to investigate an interaction between cortisol and muscle gene expression across salmonid taxa. Herein, we report the differential regulation of *myostatin-1a*, *-1b*, and *-2a* and *HSP90* among closely related salmonids and provide evidence supporting the *in silico* studies reporting the divergence of corticosteroid regulation of myostatin in fishes.

2. Materials and methods

2.1. Fish

Juvenile Chinook salmon (*Oncorhynchus tshawytscha*) and cutthroat trout (*Oncorhynchus clarki*) of both sexes were transferred from the United States Fish and Wildlife Service, Garrison National Fish Hatchery, Riverdale, North Dakota, and housed at North Dakota State University, Fargo. Fish were maintained in 800 L flow-through (100% daily turnover) stock tanks on a 12L:12D photoperiod at 12 °C. Juvenile Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*) of both sexes were obtained from the Kensington State Hatchery, Kensington, Connecticut, and Sandwich Hatchery, Sandwich, Massachusetts, respectively. These species were housed at the Conte Anadromous Fish Research Center, Turners Falls, Massachusetts, and maintained in 1000 L flow-through stock tanks on a natural photoperiod (10L:14D) at 13 °C. Experiments were approved in advance of experimentation by the Institutional Animal Care and Use Committees at both North Dakota State University, Fargo, and the Conte Anadromous Fish Research Center.

2.2. Experimental design

Fish were randomly assigned to three treatment groups: no injection control (n = 10), vehicle control (n = 10), or cortisol treatment (n = 10). For each treatment, fish were lightly anaesthetized in MS-222, mass and fork length were recorded, and caudal and anal fins were tagged with a non-toxic fluorescent marker to identify their specific treatment. Starting fish mass for each species was measured: cutthroat trout, 24.71 ± 1.87 g; Chinook salmon, 3.23 ± 0.23 g; Atlantic salmon, 23.97 ± 1.48 g; brook trout, 37.98 ± 2.55 g. Appropriate doses were calculated so each fish received an intraperitoneal injection of 10 μL/g BW vegetable oil: shortening (1:1) alone (vehicle control, VC) or 50 μg cortisol/g BW dissolved in the vehicle The dose of cortisol for this study was chosen based upon previous work (Specker et al., 1994) that detailed the examination of methods to achieve high physiological concentrations of cortisol in Atlantic salmon and their medium dose (50 μg/g BW) was shown to elevate plasma cortisol within 24 h and maintain elevated levels for as long as a month. The authors chose this dose to ensure that plasma levels were physiologically high and the sampling period at 48 h was chosen to ensure that changes observed in muscle mRNA levels were due to sustained cortisol levels and not immediate fluctuations. No injection controls were handled in a similar manner to injected fish but without an actual injection. After recovering, all fish were transferred to a 60 L flow-through tank. Fish were euthanized using MS-222 (>300 mg/mL) after 48 h, and blood was collected using 1 mL heparinized syringes with 25G hypodermic needles via caudal venipuncture. Red-oxidative and white-glycolytic muscle samples were removed and stored at −80 °C until further processing. Blood samples were centrifuged at 3000*g* for 15 min, plasma was extracted, and glucose levels were analyzed using an Accu-Chek Advantage glucose meter (Roche) as previously described (Galt et al., 2013). Plasma cortisol levels were determined using a competitive enzyme immunoassay as described by Carey and McCormick (1998). Rabbit anti-cortisol antibody (cat.# 20-CR50, Fitzgerald Ind. Int'l, MA) was used at a 1:25,000 dilution in coating buffer and the cortisol-HRP conjugate (cat.# 65-IC08, Fitzgerald Ind. Int'l, MA) at a 1:4000 dilution EIA Buffer.

2.3. RNA extraction and quantitative real-time PCR

Total RNA was isolated from red and white muscle samples using RNAzol (Molecular Research Center, Inc.) according to the manufacturer's instructions, and total RNA concentrations were determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). DNase (RQ1, Promega) treated RNA (1 μg) was reverse transcribed using Oligo dT primers and the ImProm-II Reverse Transcription System (Promega). Primers used for qPCR were validated via product cloning and sequencing (sequences are presented in Table 1). Standard curves were generated by serial dilution of plasmids (pGEM-T Easy Vector, Promega) containing amplicon of interest as previously described (Galt et al., 2013)

Table 1
Primer sequences uesd for qPCR.

Gene	Forward	Reverse	References
O.t., O.c. Myostatin-1a	cttcacatatgccaatacatatta	gcaaccatgaaactgagataaa	Garikipati et al. (2006)
O.t., O.c. Myostatin-1b	ttcacgcaaatacgtattcac	gataaattagaacctgcatcagattc	Garikipati et al. (2006)
S.s. Myostatin-1a	gggagtggacgtagtagcct	ctggtgtatgtgtgtccatcc	
S.s. Myostatin-1b	gcctgttggacttgacatggac	gctgcacttgattgagcttgcc	
S.f. Myostatin-1a	tatcagtgtagagcctgatt	agaacctgcgtgagatgcatt	
S.f. Myostatin-1b	cctgctgcacccccaccaag	ctctcgctcacgagcagccg	
Universal Myostatin-2a	aatctccgcataaaagcaaccac	caccagaagccacatcgatctt	Garikipati et al. (2007)
O.t., O.c., S.f. HSP90	tccagcagctgaaggagttt	tgagcttgcagaggttctca	Ings et al. (2011)
S.s. HSP90	ttgcgtggaactaaggtga	ccaatgaactgagagtgct	Gadan et al. (2012)

Briefly, 1:10 serial dilutions of stock constructs were performed, resulting in final concentrations of 1.0×10^1 copies/μL to 1.0×10^8 copies/μL. Quantitative real-time PCR (qPCR) was performed using PerfeCTa SYBR Green SuperMix (Quanta Biosciences) according to the manufacturer's recommendations and run using the Bio-Rad CFX platform. PCR cycling parameters were as follows: 94 °C (2 min) followed by 40 cycles at 94 °C for 20 s, primer specific annealing temperature for 30 s, and 68 °C for 30 s. All assays utilized a comparative baseline strategy using the ΔC_q method that standardized raw data to starting input cDNA quantity (Bustin et al., 2009; Meyer et al., 2013; Galt et al., 2014a).

2.4. Statistical analysis

Differences between plasma glucose, plasma cortisol and gene expression were evaluated by one-way ANOVA and Tukey's Multiple Comparison Test using GraphPad Prism version 6, GraphPad Software, San Diego California USA.

Differences were considered significant at $P < 0.05$. Gene expression data are reported relative to control as percent-mean ± S.E.M.

3. Results

After 48 h, plasma glucose levels were significantly elevated in response to cortisol treatment in all four species. In Chinook salmon, glucose levels were 134% and 146% higher in the cortisol treatment group relative to the no injection control (control) and vehicle control (VC), respectively (Fig. 1a). Similar results were observed for cutthroat trout (152% relative to control, 150% relative to VC; Fig. 1b), brook trout (148% relative to control, 161% relative to VC; Fig. 1c), and Atlantic salmon (174% relative to control, 188% relative to VC; Fig. 1d). No differences were detected between the control and VC in any species.

Plasma cortisol levels were significantly higher in the cortisol treatment groups relative to both the no injection control and vehicle control in cutthroat trout (187% relative to control, 196% relative to VC; Fig. 2), Atlantic salmon (858% relative to control, 851% relative to VC; Fig. 2), and brook trout (403% relative to control, 339% relative to VC; Fig. 2). Due to the small size of the Chinook salmon, the plasma quantity obtained from each individual was only sufficient for plasma glucose analysis. As such, no cortisol values are reported for Chinook salmon.

The expression patterns of *HSP90* mRNA varied among species. In Chinook salmon, no significant differences in *HSP90* mRNA levels were detected in white muscle, but cortisol increased *HSP90* in red muscle 159% and 154% compared to control and VC, respectively (Fig. 3a). Cortisol treatment reduced *HSP90* mRNA levels by 37% compared to control in cutthroat trout white muscle; however, both the VC and cortisol treatments reduced expression in red muscle (81% and 71% of control, respectively, Fig. 3b). In brook trout, cortisol treatment increased *HSP90* expression in white and red muscle (268% and 217% of control, respectively; Fig. 3c). In Atlantic salmon, white and red muscle *HSP90* expression increased in response to cortisol treatment (173% and 214% of control, respectively, Fig. 3d), however, no difference was detected between the VC and cortisol treatment in white muscle.

Expression of *myostatin-1a*, *-1b*, and *-2a* varied among species and tissues. Cortisol treatment increased red muscle *myostatin-1b expression 212%* compared to control in brook trout (Fig. 5c). In Atlantic salmon, cortisol treatment increased white muscle *mystatin-2a expression 182%* compared to control; however, no significant difference was detected between the VC and cortisol treatment (Fig. 4d). Expression of *myostatin-1a* and *-1b* decreased in cutthroat trout white muscle in response to cortisol treatment (40% and 68% of control, respectively; Fig. 4b), but no differences were detected between the VC and cortisol treatment for either paralog. Interestingly, a 432% increase in *myostatin-1a* was detected for the VC compared to the control in Chinook salmon white muscle, but no difference was detected between the cortisol treatment and control (Fig. 4a). A similar pattern was observed for *myostatin-1b* in Chinook salmon red muscle (Fig. 5a) and for *myostatin-1a* in Atlantic salmon red muscle (Fig. 5d). In addition, expression of Chinook salmon *myostatin-1b* was significantly less than the VC (40% of VC) in white muscle, but no difference was detected between the cortisol treatment and control (Fig. 4a). Finally, no differences were detected for any *myostatin* paralog in brook trout white muscle (Fig. 4c) and cutthroat trout red muscle (Fig. 5b).

4. Discussion

In this study, we analyzed the effects of cortisol on muscle gene expression utilizing similar experimental regimes in four salmonid species representing three different genera. The objectives of this study were to determine whether variation is present within closely related fish species in the effects of cortisol on *myostatin* and *HSP90* expression. A recent study conducted by our lab demonstrated that a lower dose of cortisol in lipid vehicle elevated cortisol levels at 12 and 24 h post injection and no differences in myostatin expression effect were observed between the 12 and 24 h time points in rainbow trout (Galt et al., 2014b). The results of this study indicate that cortisol regulates *HSP90* in muscle and that *myostatin* genes are not consistently regulated by cortisol across the salmonid species analyzed.

Consistent with previous work in Atlantic salmon and rainbow trout (Specker et al., 1994; Vijayan et al., 2003), intraperitoneal injections of cortisol stimulated an increase in circulating glucose in all four species following cortisol treatment (Fig. 1). Further, the cortisol treatment significantly increased circulating cortisol levels in cutthroat trout, Atlantic salmon, and brook trout (Fig. 2). Unfortunately, plasma volumes obtained from Chinook salmon were low and not sufficient to evaluate cortisol levels. These data

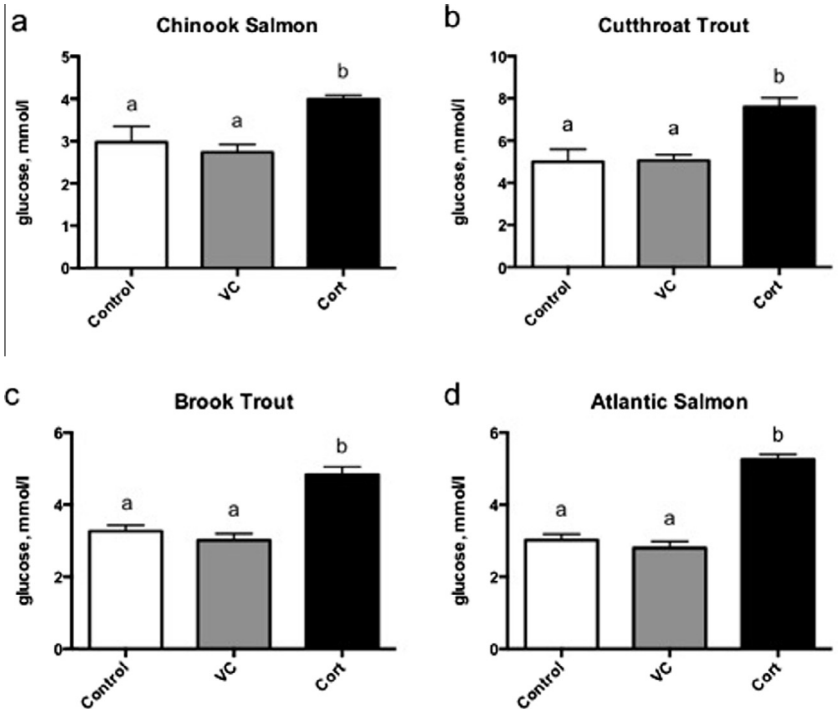

Fig. 1. Plasma glucose levels 48 h after cortisol injection. Treatment groups include: no injection control (Control), vehicle control (VC), and cortisol (Cort). Results are mean glucose (mmol/l) ± SEM. Different letters are significantly ($P < 0.05$) different.

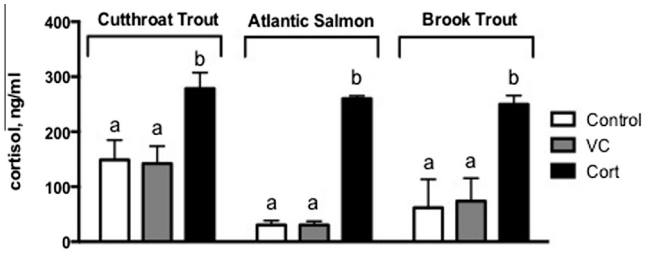

Fig. 2. Plasma cortisol levels 48 h after cortisol injection. Treatment groups include: no injection control (Control), vehicle control (VC), and cortisol (Cort). Results are mean cortisol (ng/ml) ± SEM. Different letters are significantly ($P < 0.05$) different.

verify that the cortisol treatments elicited an expected physiological response in each species.

Altered expression of the HSP family of proteins is a common biomarker of stress in fish and expression level changes in liver tissue have been associated with elevated cortisol levels (reviewed in Deane and Woo, 2011). In addition, *HSP90* has been linked to muscle development in fish (reviewed in Johnston et al., 2011) and is responsive to cortisol treatment in rainbow trout skeletal muscle myoblasts (Galt et al., 2014b). In this study, we analyzed muscle *HSP90* mRNA expression to further assess the effects of cortisol on muscle gene expression. Except for Chinook salmon white muscle, our analysis detected altered expression in all species and muscle types (Fig. 3). The lack of a detectable response in Chinook salmon white muscle is consistent with previous observations, however, red muscle expression was not characterized (Palmisano et al., 2000). In addition, we detected increased expression of *HSP90* in Chinook salmon (red but not white muscle), brook trout, and Atlantic salmon consistent with previous work. To our knowledge this is the first report of cortisol induction of *HSP90* in fish muscle, but is consistent with the upregulation of *HSP90* by cortisol in other tissue (Vijayan et al., 2003; Celi et al., 2012).

Heat shock proteins represent some of the most abundant proteins in many cell types, making up almost 2% of the soluble proteins during periods of no stress (Lai et al., 1984). The heat shock response, induction of HSPs in response to thermal stress, is thought to play a protective role against a number of factors, including oxidation, inflammation, hypoxia, and temperature changes, by repairing or breaking down denatured proteins, maintaining protein folding, and protecting cellular functions (Latchman, 2001). Increased HSP expression in muscle tissue in response to heat stress is hypothesized to protect muscle proteins from degradation and could promote muscle protein synthesis depending on the physiological load (Naito et al., 2000; Goto et al., 2003). Physical exercise also induces the expression of HSPs (primarily HSP70) in muscle tissue (Puntschart et al., 1996; Yang et al., 1997; Gonzalez et al., 2000; Liu et al., 2000; Khassaf et al., 2001), likely to protect against cellular stress related to oxygen radicals and other metabolic products that accumulate during exercise. HSP90 appears to be more sensitive to mechanical stress in muscle cells (Goto et al., 2003), compared to heat stress, and is responsive to amino acids during feeding (Garcia de la Serrana and Johnston, 2013) suggesting differential pathway regulation in relation to myogenesis.

In this study, Cutthroat trout exhibited decreased muscle *HSP90* expression in response to cortisol treatment, opposite of a predicted increase expression that was also observed in the three other salmonid species. Additionally, *myostatin-1b* expression was decreased by cortisol treatment in white muscle. Compared to Atlantic salmon and brook trout, cortisol levels were elevated in control and VC cutthroat trout. These elevated cortisol levels are likely responsible for these results which are opposite of previously observed increases in expression in other fish species and vertebrates. Interestingly, the cortisol levels observed in cutthroat trout in this study are consistent with levels reported in sexually immature kokonee salmon, migrating and hatchery-raised (*Oncorhynchus nerka kennerlyi*) (Carruth et al., 2000, 2002), and these elevated levels are thought to be in response to previous stress. In this

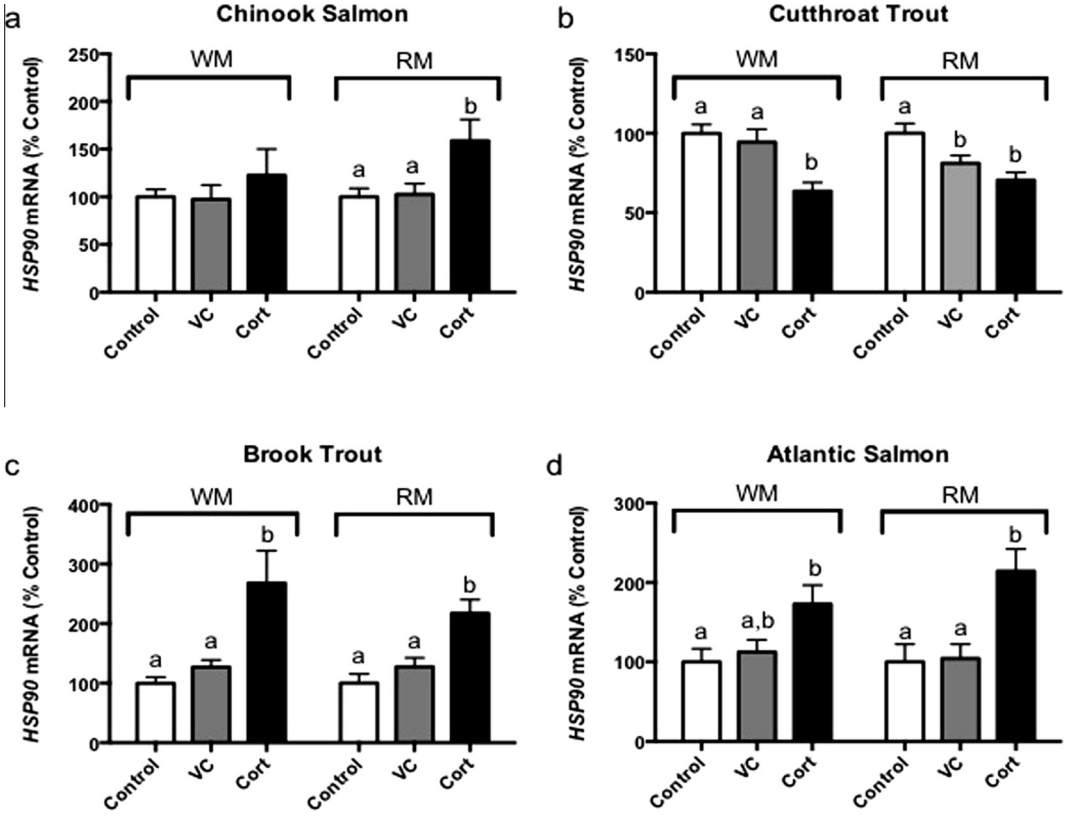

Fig. 3. White muscle (WM) and red muscle (RM) *HSP90* mRNA expression 48 h post-cortisol injection. Treatment groups include: no injection control (Control), vehicle control (VC), and cortisol (Cort). Results are percent-mean relative to control ± SEM. Different letters are significantly ($P < 0.05$) different.

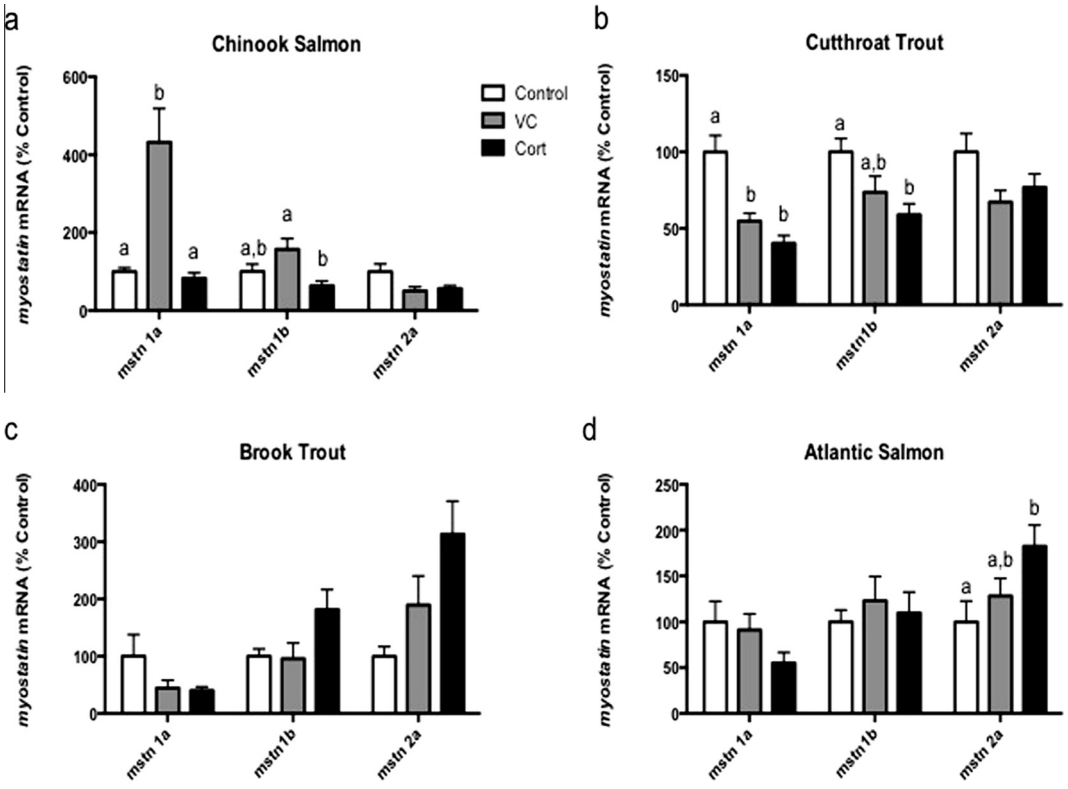

Fig. 4. White muscle (WM) expression of *myostatin-1a* (mstn 1a), *myostatin-1b* (mstn 1b), and *myostatin-2a* (mstn 2a) mRNA 48 h post-cortisol injection. Treatment groups include: no injection control (Control; white bars), vehicle control (VC; gray bars), and cortisol (Cort; black bars). Results are percent-mean relative to control ± SEM. Different letters are significantly ($P < 0.05$) different.

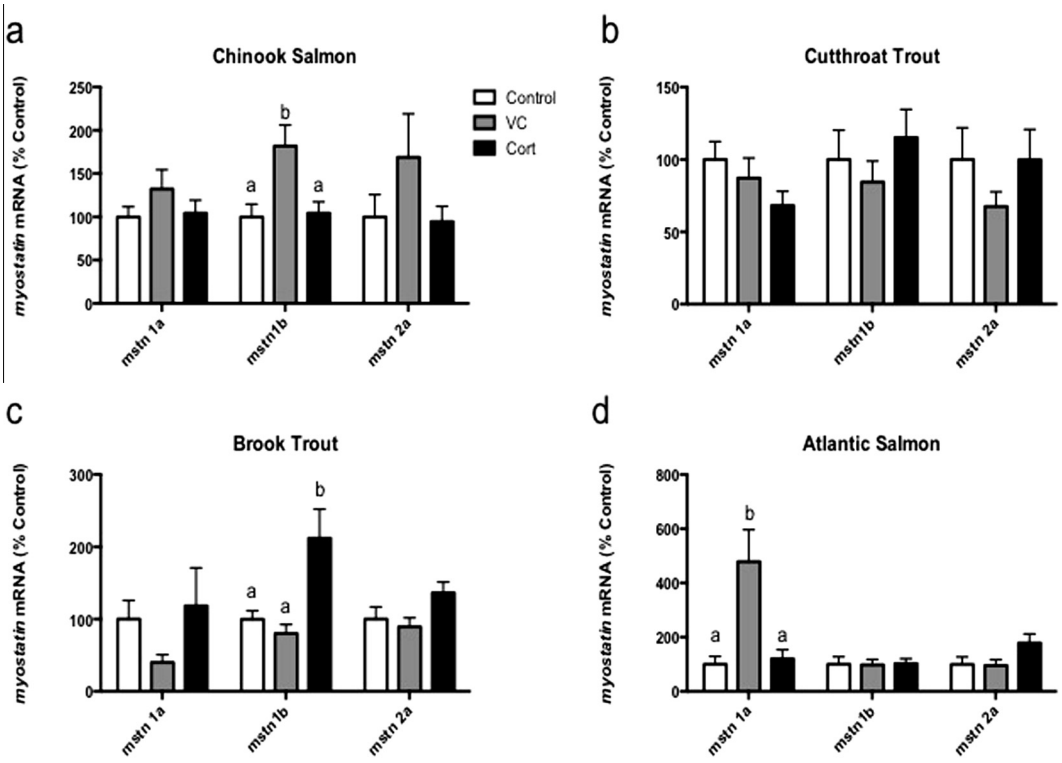

Fig. 5. Red muscle (RM) expression of *myostatin-1a* (mstn 1a), *myostatin-1b* (mstn 1b), and *myostatin-2a* (mstn 2a) mRNA 48 h post-cortisol injection. Treatment groups include: no injection control (Control), vehicle control (VC), and cortisol (Cort). Results are percent-mean relative to control ± SEM. Different letters are significantly ($P < 0.05$) different.

study, the elevated cortisol levels observed in control cutthroat trout are indicative of a prior stress, and the downregulation of *HSP90* and *myostatin* expression is likely a response to chronic stress.

In mammals, *myostatin* is upregulated by glucocorticoids through the binding of the activated glucocorticoid receptor to specific glucocorticoid response elements in the promoter of this gene (Ma et al., 2001, 2003). To date, no study has empirically tested the presence of functional GREs in the *myostatin* promoters of salmonids, but putative GREs have been identified in the *myostatin-1b* promoters in both brook trout and Atlantic salmon by *in silico* analyses (Garikipati et al., 2006; Ostbye et al., 2007). In this study, a significant increase in *myostatin-1b* was detected in red muscle of brook trout in response to cortisol treatment and a similar response (albeit not statistically significant), was observed in white muscle (Fig. 4). Interestingly, no change in Atlantic salmon *myostatin-1b* transcription was observed, suggesting the previously identified GRE may not be functional or that canonical GRE sequences may not be useful for describing GREs in salmonids (Ostbye et al., 2007). The lack of a clear cortisol response in cutthroat trout and Chinook salmon in this study (as VC treatment elicited the same responses as cortisol treatment) is consistent with previous *myostatin* promoter analyses in rainbow trout (all three being *Oncorhynchus* species), as two previous studies failed to detect putative GREs in all three paralogs (Garikipati et al., 2007; Helterline et al., 2007). Consistent with these data, a previous study in rainbow trout failed to show a clear effect of cortisol on myostatin expression *in vivo* (Galt et al., 2014b). Considering the importance of promoter evolution in gene function, the discrepancies between the *in silico* promoter analyses and the results of exogenous cortisol treatment in this and other studies demonstrates the need for functional characterization of the *myostatin* promoters in fishes by empirical means.

A number of unexpected changes in *myostatin* expression were observed in response to the lipid-based VC (vegetable oil and shortening, 1:1; Specker et al., 1994). In cutthroat trout, white muscle expression of *myostatin-1a* was significantly reduced by VC and cortisol treatment compared to control (Fig. 4b). It is possible that the VC may increase plasma cortisol levels during the first 24 h after injection and could explain the observed effects. Additionally, as the VC is a lipid injection, a slight increase in circulating free fatty acids (FFAs) could be possible and subsequent activation of cellular lipid sensors, such as the peroxisome proliferator-activated receptors (PPARs), could modulate *myostatin* expression. In rainbow trout, a sister species to cutthroat trout, *in silico* analysis of the *myostatin-1a* and *-1b* promoters identified numerous putative binding sites of transcription factors involved in lipid metabolism, including PPARs, and a previous study demonstrated that myostatin expression is lipid-sensitive (Galt et al., 2013). A functional PPAR response element (PPARRE) has been characterized in the porcine *myostatin* promoter, and putative PPARREs have been identified in the human and gilthead sea bream (*Sparus aurata*) *myostatin* promoters (Ma et al., 2001; Funkenstein et al., 2009; Deng et al., 2012).

In addition, a potential interaction between the VC and cortisol was identified. A significant upregulation of Chinook salmon *myostatin-1a* and *-1b* in white and red muscle, respectively, was detected following VC, but concurrent cortisol treatment inhibited the VC response (Figs. 4a, 5a). In Atlantic salmon, the same response was observed in red muscle *myostatin-1a* transcript levels (Fig. 5d). Very little is known about the trans-repressive behavior of the GR in fishes, but one study has described such an interaction, where PPARα activation attenuated the effects of dexamethasone-induced gene expression (Bougarne et al., 2009). Further analysis revealed that PPARα blocks recruitment of the GR to GREs, at least in a GRE-driven reporter assay. This study did not measure GR-GRE

binding due to the lack of salmonid-validated assay, nor did the study evaluate changes in PPAR expression. However, these results are the first to report potential interactions between lipid-based VC and cortisol treatments in any salmonid fishes. Further studies are needed to empirically test the hypothesis regarding lipid-sensing nuclear factors and the GR, and considering that fish muscle is highly dependent on lipid metabolism, the salmonid family of fishes may be a useful system for studying the cross talk between these factors.

The salmonid *myostatin* gene family has been recently proposed as a novel model for studying the evolutionary fate of duplicated genes, and it was concluded that this gene family is actively diverging following a comprehensive analysis of *myostatin* sequences and the myostatin literature (Lawson et al., 2012). Changes in promoter composition between the *myostatin* paralogs, and differences in expression patterns, both temporally and spatially, are strong evidence supporting functional divergence. Identifying the factors regulating expression and the functional consequences is key to determining the functional evolution of *myostatin* in salmonids and other fishes. Further study is needed to determine the mechanisms underlying the expression patterns of *myostatin* and their functional significance, both physiologically and evolutionarily.

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