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A comparative evaluation of crowding stress on muscle HSP90 and myostatin expression in salmonids

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ABSTRACT

Stress is a major factor that contributes to poor production and animal welfare concerns in aquaculture. As such, a thorough understanding of mechanisms involved in the stress response is imperative to developing strategies to mitigate the negative side effects of stressors, including the impact of high stocking densities on growth. The purpose of this study was to determine how the muscle growth inhibitor, *myostatin*, and the stress-responsive gene *HSP90* are regulated in response to crowding stress in rainbow trout (*Oncorhynchus mykiss*), cutthroat trout (*Oncorhynchus clarki*), brook trout (*Salvelinus fontinalis*), and Atlantic salmon (*Salmo salar*). All species exhibited higher cortisol and glucose levels following the handling stress, indicating physiological response to the treatment. Additionally, all species, except rainbow trout, exhibited higher *HSP90* levels in muscle after a 48 h crowding stress. Crowding stress resulted in a decrease of *myostatin-1a* in brook trout white muscle but not red muscle, while, *myostatin-1a* and *-2a* levels increased in white muscle and *myostatin-1b* levels increased in red muscle in Atlantic salmon. In rainbow trout, no significant changes were detected in either muscle type, but *myostatin-1a* was upregulated in both white and red skeletal muscle in the closely related cutthroat trout. The variation in response to crowding suggests a complex and species-specific interaction between stress and the muscle gene regulation in these salmonids. Only Atlantic salmon and cutthroat trout exhibited increased muscle myostatin transcription, and also exhibited the largest increase in circulating glucose in response to crowding. These results suggest that species-specific farming practices should be carefully examined in order to optimize low stress culture conditions.

1. Introduction

1.1. Stress in aquaculture

Stress is one of the major factors negatively affecting production efficiency and animal welfare in aquaculture. Stressors such as handling, transport, stocking density, aggression, and disease are inevitable and, if not managed properly, can negatively impact growth, reproduction, immune function, and survival (Ashley, 2007). Extensive research has been conducted to develop the best practices possible for avoiding stressful conditions and to further our understanding of the stress response in fishes (Pickering, 1992; Pickering, 1993; Wendelaar Bonga, 1997; Barton, 2002; Conte, 2004; Davis, 2006; Ashley, 2007). The effects of stress on growth have been well studied in fish and elevated levels of the primary stress hormone in fish, cortisol, have been linked to protein degradation and the inhibition of muscle growth (Wendelaar Bonga, 1997; Van Weerd and Komen, 1998; Mommsen et al., 1999). In teleosts, cortisol is a pleiotropic hormone that serves as both the main glucocorticoid and mineralocorticoid. The inhibitory effects of cortisol involve inhibiting protein synthesis and the catabolic effects on muscle include the induction of protein catabolism and increased amino acid utilization (Mommsen et al., 1999). Elevated cortisol levels can suppress growth hormone (GH) secretion and responsiveness (Hotta et al., 1988; Magiakou et al., 1994; Pinto et al., 1999) while increasing negative regulators or growth, like somatostatin and myostatin (Takahashi et al., 1992; Leal-Cerro et al., 1993; Dieguez et al., 1996; Rodgers et al., 2003).

1.2. Stress and muscle growth

Myostatin is well known for the “double muscled” phenotype observed when it is mutated or deleted in mice, cattle, domestic dogs, and humans (Grobet et al., 1997; Kambadur et al., 1997; McPherron and Lee, 1997; Schuelke et al., 2004; Mosher et al., 2007; Shelton and Engvall, 2007), and is generally thought to be inhibitory to muscle growth. The teleost-specific genome duplication resulted in two distinct

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myostatin genes in salmonids (*myostatin-1* and *-2*), and an additional salmonid-specific duplication event resulted in additional paralogs (*myostatin-1a*, *-1b*, *-2a*, and *-2b*) (Phillips et al., 1992; Garikipati et al., 2006; Garikipati et al., 2007; Ostbye et al., 2007). Salmonid *myostatin-1a*, *-1b*, and *-2a* are putatively functional and ubiquitously and differentially expressed; however, relaxed selection pressures have presumably resulted in the loss of *myostatin-2b* function in these fishes (Ostbye et al., 2007; Gabillard et al., 2013). The coding sequences for vertebrate *myostatin* genes are highly conserved, and studies in fish have found the function of myostatin to be at least partially conserved. However, the divergent expression patterns (relative to mammals) suggests the function of myostatin may be more diverse in fishes (reviewed by Gabillard et al., 2013), and it is unclear how stress regulates *myostatin* in teleosts.

Cortisol is known to negatively affect muscle growth, likely by affecting upstream regulators of protein synthesis, like myostatin and heat shock proteins (HSPs) (Kawada and Ishi, 2005). In addition, cortisol affects the expression of HSPs during stress as a mechanism of proteostasis (Whitesell et al., 2003). One HSP, HSP90, is a key molecular chaperone, crucial for cortisol mediated cellular action, including glucocorticoid receptor (GR) signal transduction (Pratt and Toft, 1997). Through interactions with various transcription factors, protein kinases, steroid receptors, structural proteins (actin and tubulin), and proteasomes, HSP90 functions to mediate intracellular signaling (Csermely et al., 1998). Even though muscle protein maintenance and metabolism is regulated by HSPs, it is unclear how stress and cortisol regulate muscle HSP expression in teleosts.

From an aquaculture perspective, understanding the regulation of *myostatin* and *HSP90* in muscle by stress, and potentially glucocorticoids (GCs) like cortisol, is paramount. In mammals, *myostatin* is strongly regulated by GCs via direct binding of the glucocorticoid receptor (GR) to glucocorticoid response elements (GREs) in the *myostatin* promoter (Ma et al., 2001; Ma et al., 2003). These regulatory elements have been identified in some, but not all, of *myostatin* promoters sequenced in fishes, although these GREs have not been experimentally validated (Kerr et al., 2005; Garikipati et al., 2006; Garikipati et al., 2007; Funkenstein et al., 2009; De Santis and Jerry, 2011; Nadjar-Boger et al., 2012). Interestingly, two separate studies have failed to identify putative GREs in the promoters of fish *myostatin* paralogs (Garikipati et al., 2006; Garikipati et al., 2007), a finding that is consistent with a lack of impact of in vivo cortisol treatment on *myostatin* transcription (Galt et al., 2014). In addition, few studies have directly tested the relationship between the stress response through stimuli (i.e., crowding and others named above) and myostatin and/or HSP90 in fish, particularly salmonids (Rodgers et al., 2003; Vianello et al., 2003; Weber et al., 2005; Helterline et al., 2007).

The hypothesized role for myostatin in muscle development and growth makes it an excellent candidate for selection, but further work is needed to determine how myostatin is regulated in response to various conditions, particularly those involving stressors. Additionally, it is not clear how elevated cortisol levels affect the heat shock proteins in muscle, which could have downstream effects on muscle metabolism and growth as well. The purpose of this study was to test the hypothesis that *myostatin* paralogs will be differentially regulated within and among salmonid species related to life history, while *HSP-90* expression will be elevated in all species following a canonical stress response. To accomplish this, the study determines the effects of a 48 h crowding stress on *myostatin* and *HSP90* mRNA expression in four salmonid species: rainbow trout (*Oncorhynchus mykiss*), cutthroat trout (*Oncorhynchus clarki*), brook trout (*Salvelinus fontinalis*), and Atlantic salmon (*Salmo salar*). To the authors' knowledge, this is the first study to directly compare the effects of a standardized stressor on *myostatin* and *HSP90* mRNA expression among closely related salmonids.

2. Materials and methods

2.1. Fish

Juvenile rainbow trout and cutthroat trout of mixed sex 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. These fishes were maintained in 800 L flow-through (100% daily turnover) stock tanks on a 12 L:12D photoperiod at 12 °C. Rainbow trout and cutthroat trout were fed ad libitum daily commercially available trout grower diet (45% crude protein, 16% fat, 3% crude fiber; PMI Nutrition International, Brentwood, MO, USA). Juvenile Atlantic salmon parr and brook trout of mixed sex were obtained from the Kensington State Hatchery, Kensington, Connecticut, and Sandwich Hatchery, Sandwich, Massachusetts, respectively, and housed at the Conte Anadromous Fish Research Laboratory, Turners Falls, Massachusetts. Fish were maintained in 1000 L flow-through stock tanks on a natural photoperiod (10 L:14D) at 13 °C. Atlantic salmon and brook trout were fed a commercial trout pellet (42% protein, 16% fat, Finfish Gold, Ziegler Bros, Gardners PA, USA). Experiments were approved in advance of experimentation by the Institutional Animal Care and Use Committees at both North Dakota State University, Fargo, and the USGS, Conte Anadromous Fish Research Laboratory.

2.2. Experimental design

Plastic mesh cages, 30 cm in diameter, were constructed and attached to the side of the holding tanks. Ten fish were transferred from the stock tank into the cage, and the water level was adjusted inside the cage to yield a density of ~75 kg/m^3 (similar density used in transporting fish and known to elicit a stress response (Carey and McCormick, 1998)). Starting fish mass for each species was measured: cutthroat trout, 19.14 ± 1.13 g; rainbow trout, 36.36 ± 1.53 g; Atlantic salmon, 23.24 ± 1.35 g; brook trout, 43.55 ± 1.76 g. After 48 h, the cage was removed and the fish were immediately euthanized using MS-222 (> 300 mg/mL, Leary, 2013). Blood was collected using 1 mL heparinized syringes with 25G hypodermic needles via caudal venipuncture. Ten control fish were then removed from the stock tank (stocking density of ~1 kg/m^3), and blood was collected after euthanasia using the protocol above. Experiments all began at ~1500 h and sampling began 2 days later at ~1500 h. Stocking densities (control and crowded) were consistent across all four species and feed was restricted for the 48-h duration of all experiments (control and crowded). All muscle tissue samples were stored at − 80 °C until further analysis.

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. 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) was used to produce cDNA. Rainbow trout *myostatin-1a*, *-1b*, *-2a*, and *HSP90* primers, and Atlantic salmon *HSP90* primers were used as previously described (Table 1) (Garikipati et al., 2006; Garikipati et al., 2007; Ings et al., 2011; Gadan et al., 2012; Galt et al., 2013). Further, Atlantic salmon *myostatin-1a* and *-1b*, brook trout *myostatin-1a* and *-1b*, a universal *Salmo/Salvelinus* primer pair for *myostatin-2a*, and cutthroat trout and brook trout *HSP90* primers were validated via product cloning and sequencing, with primer efficiencies determined and standard curves generated as previously described (Galt et al., 2013). Standard curves were generated by serial dilution of plasmids (pGEM-T Easy Vector, Promega) containing amplicon of interest as previously

Table 1
Primer sequences used for qPCR.

Gene	Forward	Reverse	Reference
O.m., O.c. Myostatin-1a	cttcacatatgccaatacatatta	gcaaccatgaaactgagataaa	Garikipati et al., 2006
O.m., 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	aatctcccgcataaaagcaaccac	caccagaagccacatcgatctt	Garikipati et al., 2006
O.m., O.c., S.f. HSP90	tccagcagctgaaggagttt	tgagcttgcagaggttctca	Ings et al., 2011
S.s. HSP90	ttgcgtggaactaaggtga	ccaatgaactgagagtgct	Gadan et al., 2012

described (Galt et al., 2013). 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 anneal temperature for 30 s, and 68 °C for 30 s. A dissociation curve was then performed for each assay to ensure primer specificity by running a single cycle as follows: 95 °C for 1 min followed by a rising temperature ramp of 55–95 °C over 5 min. All data were analyzed using Bio-Rad software (Hercules, CA). 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).

2.4. Plasma glucose and cortisol determination

Immediately after euthanasia, blood samples were collected via caudal venipuncture. Blood samples were centrifuged at 3000*g* for 15 min before plasma was isolated from packed cells and stored at − 80 °C. Plasma glucose levels were analyzed using an Accu-Chek Advantage glucose meter (Roche) as previously described (Galt et al., 2013; Meyer et al., 2013). A competitive binding immunoassay, previously described by Carey and McCormick (1998), was used to evaluate plasma cortisol levels. The rabbit anti-cortisol antibody and cortisol-HRP conjugate (Fitzgerald Industries International, Concord MA) were used at a final dilution of 1:25,000 and 1:4000 in coating buffer and EIA Buffer, respectively.

2.5. Statistical analysis

Differences between plasma glucose levels and plasma cortisol levels were determined by Students's *t*-test, and transcript expression levels were compared using ANOVA and pairwise comparison analyses (Sidak's) using GraphPad Prism version 6, GraphPad Software, San Diego California USA. Differences were considered significant at $p < 0.05$. All gene expression data are reported relative to control as percent-mean ± S.E.M.

3. Results

Both plasma cortisol and glucose levels were significantly elevated following a 48 h crowding stress in all four salmonids (Figs. 1 and 2). Plasma glucose levels increased 1.3-fold in rainbow trout, 1.6-fold in cutthroat trout, 1.5-fold in brook trout, and 3.8-fold in Atlantic salmon relative to the control. Plasma cortisol levels increased 1.7-fold in rainbow trout, 3.9-fold in cutthroat trout, 17.0-fold in brook trout, and 11.3-fold in Atlantic salmon relative to the control.

Expression of *HSP90* mRNA following a 48-h crowding stress was significantly affected in muscle tissue in all species analyzed, however the directionality of effects is not consistent (Fig. 3). In rainbow trout, crowding reduced *HSP90* transcription 40% compared to control in red muscle with no detectable response in white muscle (Fig. 3a). Conversely, crowding increased the transcription of *HSP90* in white muscle of cutthroat trout, brook trout, and Atlantic salmon relative to the controls (128%, 144%, and 177%, respectively; Fig. 3b–d), but no significant differences were detected in red muscle.

Similarly, differential transcription was detected across species, between muscle type, and within *myostatin* paralogs in response to crowding (Fig. 4). No statistically significant differences were detected in the transcription of *myostatin-1a*, *-1b*, and *-2a* in response to a 48 h crowding stress in rainbow trout (Fig. 4a). However, *myostatin-1a* increased in both white and red muscle from cutthroat trout (179% and 170% relative to controls, respectively; Fig. 4b) and white muscle from Atlantic salmon (131% relative to the controls; Fig. 4d). Additionally, crowding increased *myostatin-2a* in white muscle and *myostatin-1b* in red muscle in Atlantic salmon relative to the controls (135% and 207%; respectively; Fig. 4d). In brook trout, crowding decreased *myostatin-1a* transcription in white muscle, but not in red muscle (46% of control; Fig. 4c).

4. Discussion

4.1. Stress as a factor for fish production

Farmed fish are routinely exposed to various stressors and, if not managed properly, these conditions can negatively impact growth rates and animal welfare (Ashley, 2007). A common issue faced by producers is high stocking density or “crowding” that can be associated with intense production protocols, transportation, and/or harvesting. As these conditions have been shown to hinder fish growth, we aimed to evaluate the effects of a standardized crowding stress on the expression of the multiple *myostatin* genes in four closely related salmonids. As a common measure of metabolic change associated with the stress response, plasma glucose and cortisol levels were quantified (Benfey and Biron, 2000; Trenzado et al., 2003; Cairns et al., 2008; Gatica et al., 2010). In this study, the 48-h crowding stress resulted in significantly elevated cortisol and glucose levels, thereby eliciting significant elevation of physiological stress responses.

4.2. Local muscle gene expression affected by crowding stress

Primary (catecholamines and glucocorticoids) and secondary (e.g. glucose and lactate) stress responses can lead to tertiary changes in performance, growth, and immunity by affecting local tissue gene expression. Altered expression of HSPs in liver tissue is a common biomarker of stress, as elevated levels are correlated to elevated cortisol levels (reviewed in (Deane and Woo, 2011)). In addition, *HSP90* functions in muscle development and myotube differentiation (Johnston et al., 2011), and cortisol treatment elevates *HSP90* expression during myoblast differentiation in Atlantic salmon and in rainbow trout myoblasts in vitro (Bower and Johnston, 2010; Galt et al., 2014). Increased *HSP90* expression in muscle tissue in response to stress is hypothesized to protect muscle proteins from degradation and possibly

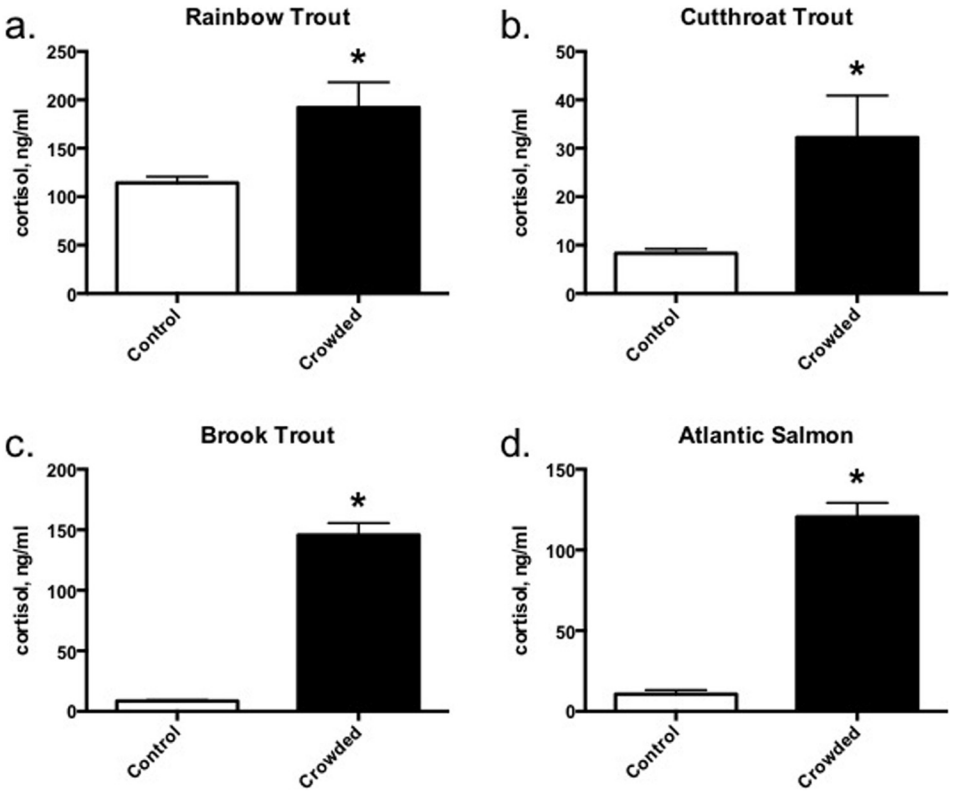

Fig. 1. Circulating plasma cortisol levels after 48 h of crowding. Results are mean plasma cortisol ng/ml ± SEM (*$p < 0.05$).

promote protein synthesis or recycling (Naito et al., 2000; Goto et al., 2003). Herein, crowding increased *HSP90* in white muscle in cutthroat trout, brook trout, and Atlantic salmon consistent with cortisol-induced *HSP90* elevation observed in other teleost tissues (Vijayan et al., 2003; Celi et al., 2012). These data suggest that crowding stress activates protein homeostatic pathways in white muscle tissue fairly consistently across species, with the exception of rainbow trout. The lack of response detected in red muscle (except in rainbow trout) is consistent with the phenotype of red muscle as a tissue involved more in locomotion as compared to contributing to overall growth (Glancy and Balaban,

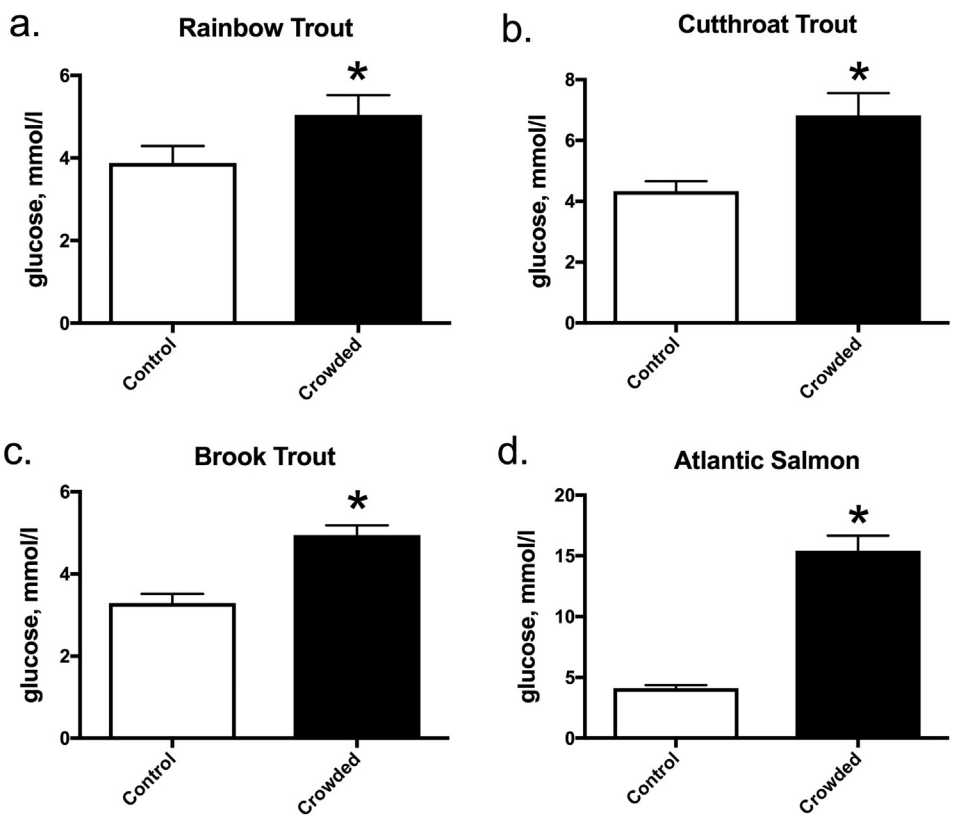

Fig. 2. Circulating plasma glucose levels after 48 h of crowding. Results are mean plasma glucose mmol/l ± SEM (*$p < 0.05$).

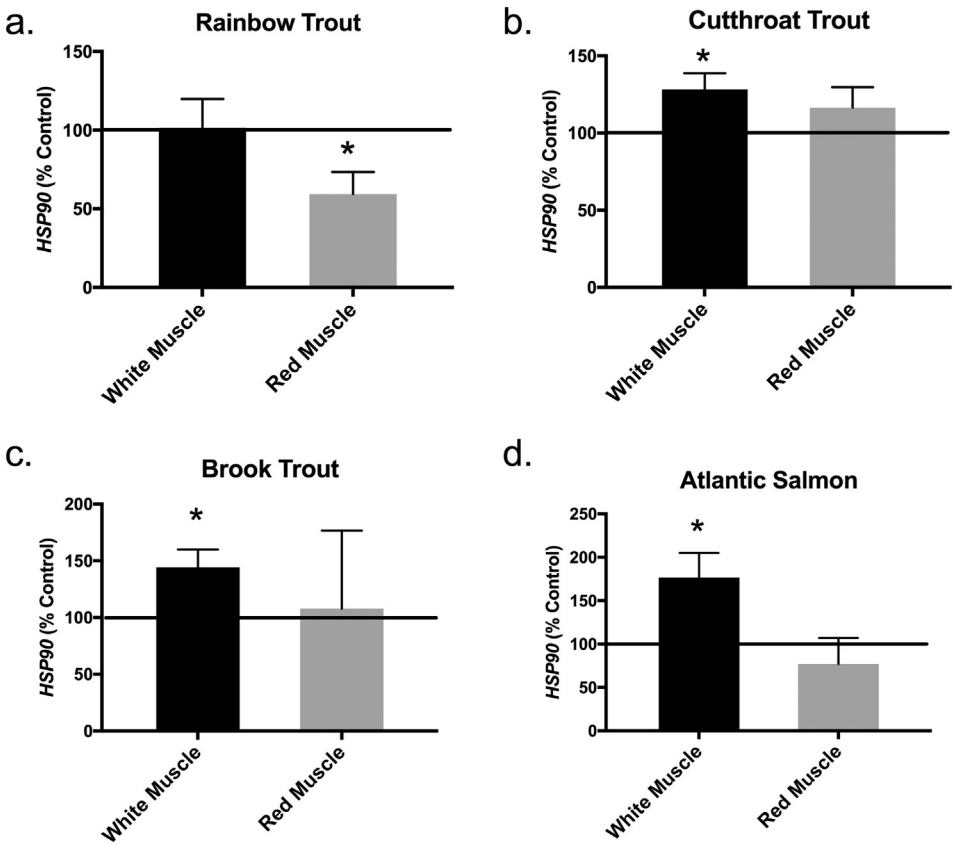

Fig. 3. White muscle (WM) and red muscle (RM) *HSP90* mRNA expression. Results are percent-mean relative to control ± SEM (*$p < 0.05$).

2011). The lack of detectable changes in white muscle *HSP90* transcription in rainbow trout suggest that rainbow trout muscle is less sensitive to elevated cortisol than some other salmonid species. This reduced sensitivity to elevated cortisol might be due to the high level of domestication that hatchery raised rainbow trout have endured (Teletchea and Fontaine, 2014), compared to cutthroat trout and brook trout who have undergone closed captive breeding for fewer generations than rainbow trout. Although (hatchery raised) Atlantic salmon are considered as highly domesticated as rainbow trout (Teletchea and Fontaine, 2014), the Atlantic salmon used in this study were the progeny of wild fish that had returned to the Connecticut River.

4.3. Stress and muscle growth regulation in fishes

In fish, very little evidence is available regarding the regulation of *myostatin* by components of the stress axis and other metabolic mediators. In contrast to stress- and glucocorticoid-induced positive regulation of *myostatin* transcription in mammals, previous reports demonstrate reduced muscle *myostatin* expression in response to crowding or elevated cortisol levels in zebrafish, tilapia larvae, and channel catfish (Rodgers et al., 2003; Vianello et al., 2003; Weber et al., 2005). Interestingly in this study, no significant changes in *myostatin* transcription were detected in rainbow trout muscle, while the *myostatin* paralogs of Atlantic salmon appear to be most sensitive to crowding stress as all three paralogs were upregulated: *myostatin-1a* and *-2a* transcription in white muscle and *myostatin-1b* in red muscle. *Myostatin-1a* was also upregulated in white and red muscle of cutthroat trout in response to crowding. These data are consistent with previous reports of differential regulation of *myostatin* paralogs in salmonids (Ostbye et al., 2001; Roberts and Goetz, 2001; Roberts and Goetz, 2003; Biga et al., 2004; Roberts et al., 2004; Garikipati et al., 2006; Garikipati et al., 2007; Ostbye et al., 2007); however, this study is the first to report differential regulation following the activation of the stress response. In addition, variation across muscle fiber types was observed in *HSP90* and *myostatin* expression. The white-glycolytic muscle and red-oxidative muscle are metabolically distinct, and white muscle is known to be more susceptible to chronic stress, which may explain the observed responses in *HSP90* and *myostatin*; however, the functional significance of these transcription patterns is not known, and the complexity is confounded by interspecies variation (Mommsen et al., 1999). The intra- and interspecies variation in *myostatin* transcription in response to stress and metabolic perturbations could be the result of differential selection on the *myostatin* paralogs within and among species, specifically resulting in sequence divergence of the *myostatin* promoters. The protein coding sequences of the salmonid homologs are highly conserved, but sequence analysis of their promoters has revealed considerable variation in the type, number, and location of *cis*-regulatory elements (reviewed by Gabillard et al., 2013).

Myostatin polymorphisms associated with growth performance have been identified in numerous fishes: gilthead sea bream (*S. aurata*; Sanchez-Ramos et al., 2012), bighead carp (*Aristichthys nobilis*; Liu et al., 2012), common carp (*Cyprinus carpio*; Sun et al., 2012), yellow catfish (*Pelteobagrus fulvidraco*; Zhu et al., 2012), spotted halibut (*Verasper variegatus*; Li et al., 2012), pufferfish (*Takifugu rubripes*; Wang et al., 2014), and Atlantic salmon (Penaloza et al., 2013). In the present study, we found evidence that links the stress response to the regulation of *myostatin*, and given the allelic variation recently described, this gene may present an opportunity for both scientists and aquaculturists to select favorable traits associated with *myostatin* polymorphisms and their regulation by stress. Despite this, further work is needed to identify differences in the regulation of *myostatin* by stress within species, particularly the outcomes of increased or decreased *myostatin* transcription (depending on species). Additionally, these data suggest that salmonids likely exhibit tissue-specific stress sensitivity and further work is needed to better understand the effects of stress on muscle proteostasis.

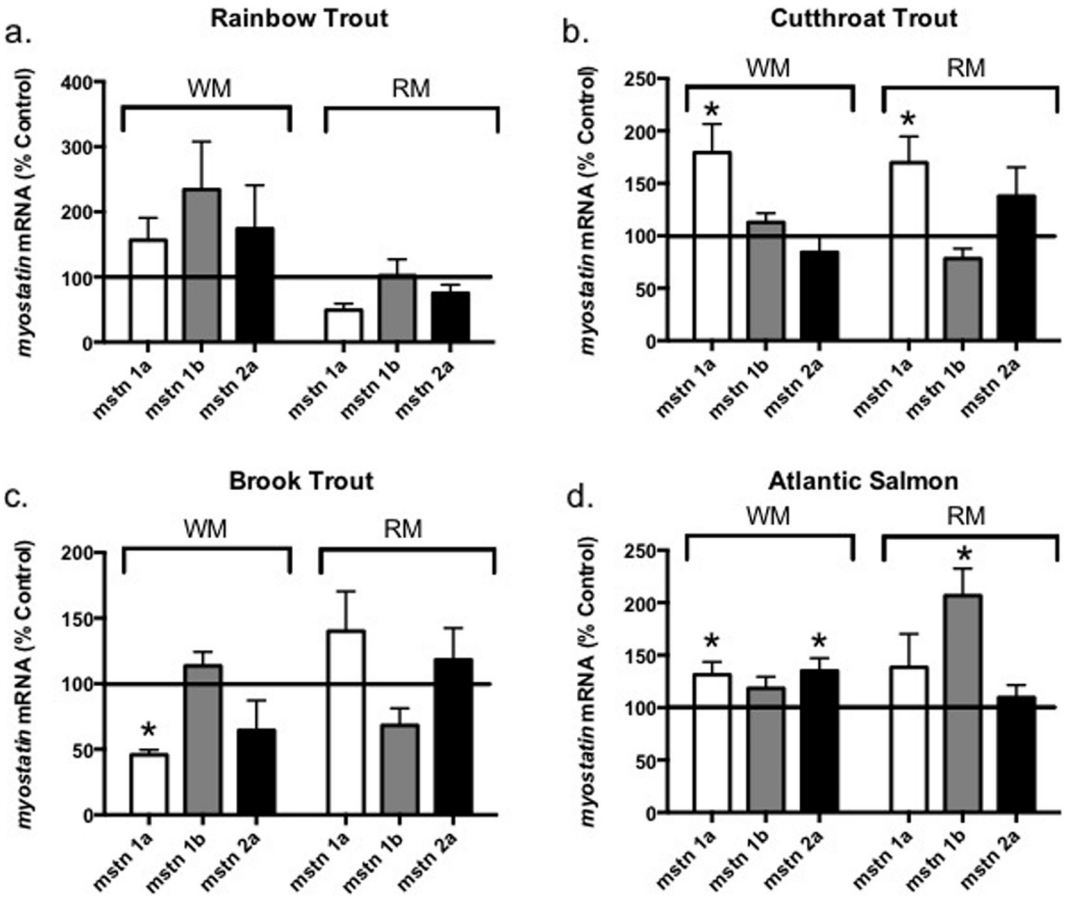

Fig. 4. Expression of *myostatin-1a* (mstn-1a), *-1b* (mstn-1b), and *-2a* (mstn-2a) mRNA in white muscle (WM) and red muscle (RM). Bar represents control level at 100%. Results are percent-mean relative to control ± SEM (*$p < 0.05$).

4.4. Stress, metabolic response, and growth

This study provides valuable evidence supporting a divergence in myostatin regulation among the fishes, particularly in those of *Salmonidae*:*Salmoninae*. Only two of the species tested, cutthroat trout and Atlantic salmon, exhibited increased muscle *myostatin* transcription in response to crowding, and interestingly, these two species exhibited the largest glucose responses after crowding. In addition, Atlantic salmon was the only wild species analyzed, and the only species exhibiting upregulation of all three *myostatin* paralogues, as well as *HSP90*, in muscle. It is likely that generations of domestication result in relaxed stress responsiveness, resulting in reduced local tissue sensitivity to elevated cortisol levels leading to muted changes in stress-induced gene expression. Rainbow trout exhibited a much lower response to crowding stress than the other species analyzed, with only a 45% increase in cortisol levels, suggesting that rainbow trout might acclimate to increased density more rapidly. It is also important to note that rainbow trout resting cortisol levels were higher than resting cortisol levels in the other species, indicating a possible variation in response to the experimental conditions among these species. This elevated resting cortisol and reduced cortisol response crowding are likely a result of domestication and are relevant to hatchery operations.

These data are the first to link the stress response to *myostatin* transcription in four salmonids subjected to a standardized stressor. While we did not directly test for the presence or absence of GREs in the *myostatin* promoters of these species, future studies should investigate the mechanisms involved in the up- or down-regulation of *myostatin* paralogs in salmonids and other fishes Collectively, these data highlight the need for the development of species-specific farming practices in order to optimize culture conditions, improve fish welfare, and increase production efficiency.

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