Molecular and Cellular Endocrinology 518 (2020) 110989

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Cortisol regulates *insulin-like growth-factor binding protein* (*igfbp*) gene expression in Atlantic salmon parr

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ARTICLE INFO

Keywords:
Fish
Gill
Growth
Liver
Muscle
Stress

ABSTRACT

The growth hormone (Gh)/insulin-like growth-factor (Igf)/Igf binding protein (Igfbp) system regulates growth and osmoregulation in salmonid fishes, but how this system interacts with other endocrine systems is largely unknown. Given the well-documented consequences of mounting a glucocorticoid stress response on growth, we hypothesized that cortisol inhibits anabolic processes by modulating the expression of hepatic *igfbp* mRNAs. Atlantic salmon (*Salmo salar*) parr were implanted intraperitoneally with cortisol implants (0, 10, and 40 μg g^{-1} body weight) and sampled after 3 or 14 days. Cortisol elicited a dose-dependent reduction in specific growth rate (SGR) after 14 days. While plasma Gh and Igf1 levels were unchanged, hepatic *igf1* mRNA was diminished and hepatic *igfbp1b1* and *-1b2* were stimulated by the high cortisol dose. Plasma Igf1 was positively correlated with SGR at 14 days. Hepatic *gh receptor* (*ghr*), *igfbp1a*, *-2a*, *-2b1*, and *-2b2* levels were not impacted by cortisol. Muscle *igf2*, but not *igf1* or *ghr*, levels were stimulated at 3 days by the high cortisol dose. As both cortisol and the Gh/Igf axis promote seawater (SW) tolerance, and particular *igfbps* respond to SW exposure, we also assessed whether cortisol coordinates the expression of branchial *igfbps* and genes associated with ion transport. Cortisol stimulated branchial *igfbp5b2* levels in parallel with Na^+/K^+-ATPase (NKA) activity and *nka-α1b*, *$Na^+/K^+/2Cl^-$-cotransporter 1* (*nkcc1*), and *cystic fibrosis transmembrane regulator 1* (*cftr1*) mRNA levels. The collective results indicate that cortisol modulates the growth of juvenile salmon via the regulation of hepatic *igfbp1s* whereas no clear links between cortisol and branchial *igfbps* previously shown to be salinity-responsive could be established.

1. Introduction

Cortisol is the primary corticosteroid produced by teleost interrenal tissue and exhibits both glucocorticoid and mineralocorticoid activities (Wendelaar-Bonga, 1997; Gorissen and Flik, 2016). Activation of the hypothalamic-pituitary-interrenal axis in response to a perceived stressor results in elevated plasma cortisol levels that constitute a key facet of the 'primary' stress response (Barton, 2002). In combination with other hormones, cortisol promotes a suite of 'secondary' stress responses that include the elevation of plasma glucose levels. While catecholamines initiate the mobilization of glucose via glycogenolysis, cortisol sustains hyperglycemia via protein catabolism and gluconeogenesis (Mommsen et al., 1999; Faught and Vijayan, 2016). These metabolic responses represent the reallocation of energy away from somatic growth toward physiological and/or behavioral processes aimed at restoring the organism to homeostasis (Wendelaar-Bonga, 1997; Bernier, 2006). Sustained long-term stress may severely impact organismal fitness. These 'tertiary' aspects of the stress response include negative effects on growth, immunity, and other processes (Wendelaar-Bonga, 1997; Barton, 2002). The suite of endocrine interactions that underlies the well-documented consequences of mounting a glucocorticoid stress response on growth must be identified to gain a better understanding of how environmental stressors impact the physiology of wild and domesticated fish populations.

The allocation of acquired nutrients toward anabolic processes such as somatic and linear growth is principally controlled by the actions of the growth hormone (Gh)/insulin-like growth-factor (Igf) system (Duan et al., 2010; Pérez-Sánchez et al., 2018). Endocrine Gh directly stimulates the growth of target tissues by acting as a mitogen upon binding to transmembrane Gh receptors (Ghrs) (Butler and LeRoith, 2001;

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https://doi.org/10.1016/j.mce.2020.110989
Received 7 July 2020; Received in revised form 10 August 2020; Accepted 11 August 2020
Available online 21 August 2020

Bergan-Roller and Sheridan, 2018). Gh indirectly promotes growth through the synthesis and secretion of Igfs (LeRoith et al., 2001; Rotwein, 2020). While plasma Igf levels are primarily determined by their rate of secretion from the liver, the localized muscle production of Igfs is also important in regulating tissue and organismal growth (LeRoith et al., 2001; Wood et al., 2005; Duan et al., 2010). Igfs stimulate somatic growth by controlling cell differentiation, proliferation, migration, and survival in tissues such as skeletal muscle and bone (Wood et al., 2005; Castillo et al., 2004; Codina et al., 2008; Capilla et al., 2011). Igfs interact with a suite of Igf binding proteins (Igfbps) that affect their availability, transport, and receptor binding (Shimizu and Dickhoff, 2017; Allard and Duan, 2018). Teleosts harbor an expanded Igfbp family in comparison to tetrapods due to lineage-specific genome duplications (Allard and Duan, 2018; Garcia de la Serrana and Macqueen, 2018). Following recent insights into the evolution of teleost Igfbps (Ocampo Daza et al., 2011; Macqueen et al., 2013; Garcia de la Serrana and Macqueen, 2018; Pérez-Sánchez et al., 2018), the current challenge is to resolve how Igfbps mediate adaptive responses to various environmental circumstances.

Among the ways that mounting a stress response influences growth (Mommsen et al., 1999; Barton, 2002; Bernier et al., 2004; Bernier, 2006; Madaro et al., 2015, 2016; Madison et al., 2015), cortisol affects pathways within the Gh/Igf system. For example, cortisol diminishes hepatic sensitivity (Igf1 responsiveness) to endocrine Gh (Pierce et al., 2005, 2011; Nakano et al., 2013; Philip and Vijayan, 2015). Glucocorticoids and/or environmental stressors also influence plasma Igfbp levels and hepatic *igfbp* expression patterns (Kelley et al., 2001; Kajimura et al., 2003; Peterson and Small, 2005; Davis and Peterson, 2006; Pierce et al., 2006; Shepherd et al., 2011; Shimizu et al., 2011a; Madison et al., 2015; Garcia de la Serrana et al., 2017). Thus, a nuanced understanding of how cortisol connects with Igfs and Igfbps is critical to developing a mechanistic understanding of how cortisol impacts the growth of fishes.

In anadromous Atlantic salmon (*Salmo salar*), elevated plasma cortisol levels underlie, in part, the timing of 'parr-smolt transformation' or 'smoltification' (Shrimpton and McCormick, 1998; McCormick et al., 2002; Sundell et al., 2003). This life-stage transition entails the orchestrated development of physiological, morphological, and behavioral traits that support the survival of smolts in the ocean (Hoar, 1988). Reflecting its mineralocorticoid activities, cortisol is widely regarded as a 'seawater (SW)-adapting' hormone in teleosts because it directly, and indirectly, promotes SW tolerance. For instance, cortisol directly stimulates the activities and/or expression of Na^+/K^+-ATPase (NKA) and ion transporters/channels that mediate branchial ion secretion; cortisol acts indirectly by synergizing with the Gh/Igf1 system (Sakamoto et al., 1993; Björnsson, 1997; McCormick, 2001; Pelis and McCormick, 2001; Kiilerich et al., 2007; Tipsmark and Madsen, 2009). In turn, springtime elevations in plasma cortisol, Gh, and Igf1 coordinate the acquisition of hyposmoregulatory capacities associated with parr-smolt transformation (Hoar, 1988). Within this context of heightened corticosteroid and somatotropic axis signaling, Atlantic salmon exhibit smoltification-related changes in the expression of branchial *igfbp* transcripts (Breves et al., 2017). Moreover, particular branchial *igfbps* acutely respond to SW exposure (Breves et al., 2017). The underlying regulators of these developmentally- and/or salinity-driven *igfbp* patterns stand entirely unresolved. Cortisol thus emerges as a candidate regulator of branchial *igfbps* given its roles in mediating parr-smolt transformation and acute responses to SW exposure.

In the current study, we test the hypothesis that cortisol influences growth and ionoregulation in juvenile Atlantic salmon by modulating the Gh/Igf system, including expression of *igfbps*. Atlantic salmon express at least 22 *igfbp* genes (Garcia de la Serrana and Macqueen, 2018). Here, we focused on particular *igfbps* that exhibit robust expression in the liver and gill (Macqueen et al., 2013). We chose to investigate the effects of exogenous cortisol on juvenile parr for two primary reasons. First, mounting a cortisol stress response is associated with reduced growth and Gh/Igf1 signaling during this crucial freshwater stage (McCormick et al., 1998; Madaro et al., 2015, 2016). Since the parr-smolt transformation is dependent upon individuals reaching a requisite size, parr with compromised growth rates are faced with life-history consequences such as the delayed onset (by $\geq$ 1 year) of smoltification (McCormick, 2013). Secondly, juvenile salmon activate osmoregulatory systems supportive of SW tolerance in response to cortisol administration (Bisbal and Specker, 1991; Specker et al., 1994; Veillette et al., 1995; Pelis and McCormick, 2001), and thus, are amendable to assessing how cortisol regulates molecular and cellular mediators of hydromineral balance.

2. Materials and methods

2.1. Animals

Atlantic salmon parr were obtained in October of 2015 from the Kensington National Fish Hatchery (Kensington, CT), and held at the U. S. Geological Survey Conte Anadromous Fish Research Laboratory (Turners Falls, MA) in 1.5 m diameter fiberglass tanks receiving flow-through Connecticut River water (4 l min^{-1}), maintained under natural photoperiod and ambient river temperatures (2–15 °C). Fish were fed to satiation twice daily with commercial feed (Bio-Oregon, Longview, WA). All experiments were carried out in accordance with U.S. Geological Survey institutional guidelines and an approved IACUC protocol (LSC-9070).

2.2. Experimental design

Atlantic salmon parr (10.0–12.8 cm fork length; $n = 68$) of mixed sex were randomly distributed into a 190 l tank maintained at 10 °C (range of daily temperature measurement of 9.4–10.7 °C) with particle and charcoal filtration, continuous aeration, and supplied with dechlorinated tap water at 2 l h^{-1}. Fish were acclimated to the experimental tanks for three weeks prior to the beginning of the experiment. Following the acclimation period, fish were anesthetized with MS-222 (100 mg l^{-1}; pH 7.0; Sigma, St. Louis, MO) and randomly assigned to one of three groups (0, 10 and 40 μg cortisol g^{-1} body weight). In order to monitor individual fish for changes in growth rate, fish that were to be sampled at day 14 were implanted with a passive integrated transponder tag (12.0 mm × 2.12 mm HDX; Oregon RFID, Portland, OR). The remaining fish (to be sampled on day 3) were given a different color paint mark between the anal fin rays to identify their group. Fish were injected with 10 μl g^{-1} body weight of 1:1 vegetable oil:shortening to achieve doses of 0, 10, and 40 μg g^{-1} cortisol (hydrocortisone; Pfaltz and Bauer, Waterbury, CT). After recovery for 0.5 h all fish were returned to their original tank. Animals were fed to satiation once daily (10:00 local time). At 3 and 14 days, 8 animals were sampled from each treatment tank at 09:00. This feeding/sampling schedule was selected to account for well-characterized post-prandial responses by the Gh/Igf system in salmonids (Shimizu et al., 2009).

At the time of sampling, fish were netted and anesthetized in MS-222 (200 mg l^{-1}; pH 7.0). Blood was collected from the caudal vasculature by a needle and syringe treated with ammonium heparin. Blood samples were collected within 5 min of the initial netting. Blood was separated by centrifugation at 4 °C and plasma stored at −80 °C until subsequent analyses. Body mass (BM) and standard length (SL) were measured for calculation of condition factor (CF) and specific growth rate (SGR). CF was calculated as $CF = (BM \times SL^{-3}) \times 100$. SGR (% body mass gain day^{-1}) was calculated as $SGR = ((\ln BM_{day\ 14} - \ln BM_{day\ 0})/14) \times 100$. Liver, white muscle, pituitary, and gill were collected and immediately frozen directly on dry ice and stored at −80 °C. Four to six additional gill filaments were placed in ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and stored at −80 °C. At the time of sampling, all gut contents were removed, dried overnight, and subsequently weighed.

2.3. Plasma parameters and branchial NKA activity

Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay as described by Carey and McCormick (1998). Plasma glucose concentrations were assayed by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase (Glucose Assay Reagent, G3293, Sigma). Plasma Gh levels were measured by radioimmunoassay (RIA) validated for Atlantic salmon by Björnsson et al. (1994). Plasma Igf1 levels were measured by a RIA validated for salmonids (Moriyama et al., 1994). Ouabain-sensitive branchial NKA activity was measured as described by McCormick (1993). This assay couples the production of ADP to NADH using lactate dehydrogenase and pyruvate kinase in the presence and absence of 0.5 mmol l^{-1} ouabain. Ten microliters of samples were run in duplicate in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min on a BioTek Synergy 2 spectrophotometer (BioTek, Winooski, VT). Protein concentration of the homogenate was determined using a BCA protein assay (Thermo Fisher Scientific, Rockford, IL).

2.4. RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissue by the TRI Reagent procedure (MRC, Cincinnati, OH) according to the manufacturer's protocols. RNA concentration and purity were assessed by spectrophotometric absorbance (Nanodrop 1000, Thermo Fisher Scientific). First strand cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Relative mRNA levels were determined by qRT-PCR using the StepOnePlus real-time PCR system (Life Technologies). We employed previously described primer sets for all target and normalization genes aside from *gh* and *prolactin* (*prl*) (Kiilerich et al., 2007, 2011; Nilsen et al., 2007; Bower et al., 2008; Tipsmark and Madsen, 2009; Murashita et al., 2011; Macqueen et al., 2013; Madaro et al., 2015). Primer sequences and assay efficiencies are provided in Supplementary Table 1. Primers for *gh* and *prl* were designed using NCBI Primer-BLAST to span predicted exon-exon junctions and to amplify products of 70 and 93 base pairs, respectively. Non-specific product amplification and primer-dimer formation were assessed by melt curve analyses. We followed the nomenclature for Atlantic salmon *igfbps* presented by Macqueen et al. (2013). qRT-PCR reactions were setup in a 15 µl final reaction volume with 400 nM of each primer, 1 µl cDNA, and 7.5 µl of 2x SYBR Green PCR Master Mix (Life Technologies). The following cycling parameters were employed: 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. After verification that levels did not vary across treatments, *elongation factor 1α* (*ef1α*) levels were used to normalize target genes in liver and muscle. *beta actin* (*ba*) levels were used to normalize gill and pituitary target genes. Reference and target gene levels were calculated by the relative quantification method with PCR efficiency correction (Pfaffl, 2001). Standard curves were prepared from serial dilutions of control liver, muscle, gill, or pituitary cDNA and included on each plate to calculate the PCR efficiencies for target and normalization genes (Supplementary Table 1). Relative mRNA levels are reported as a fold-change from the day 0 group.

2.5. Statistical analyses

Group comparisons of SGR were performed by one-way ANOVA followed by Tukey's HSD test. All other data were analyzed by two-way ANOVA with treatment (cortisol dose) and time as main effects. Significant main or interaction effects are indicated in figure panels: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. When a significant effect of treatment, or an interaction between treatment and time was detected, Tukey's HSD was employed at each time point. Pearson product-moment correlation coefficients were used to assess the relationship between plasma hormones (Gh and Igf1) and SGR. All statistical analyses were performed using GraphPad Prism 6 (San Diego, CA). Significance for all tests was set at $P < 0.05$.

3. Results

3.1. Plasma cortisol, plasma glucose, biometrics, and growth

There were significant treatment, time, and interaction effects on plasma cortisol levels. On days 3 and 14, parr implanted with the high dose of cortisol had significantly elevated plasma cortisol levels compared to vehicle controls (Fig. 1A). We detected significant treatment and time effects on plasma glucose, which was elevated on day 3 in both the low and high doses of cortisol compared to controls (Fig. 1B). With respect to growth performance, there were treatment and time effects on BM and CF, with both parameters reduced on day 3 by the high cortisol dose (Fig. 1C and D). On day 14, SGR was significantly reduced in parr implanted with the high cortisol dose. Mean SGR in the low-cortisol group was intermediate to, and not significantly different from, the vehicle and high cortisol groups (Fig. 2). On day 14, there were no significant differences in gut contents (dry mass BM^{-1}) between the three treatment groups (data not shown).

3.2. Plasma Gh, Igf1, and hepatic mRNA levels

There were no significant treatment or interaction effects on plasma Gh and Igf1 levels (Fig. 3A and B). On day 14, plasma Gh levels were not significantly correlated with SGR ($r^2 = 0.01$) whereas plasma Igf1 levels were correlated with SGR ($r^2 = 0.25$) (Supplementary Fig. 1). There was a significant interaction effect on hepatic *igf1* levels. On day 3, *igf1* levels were reduced from vehicle controls by the high cortisol dose (Fig. 3C). There were significant treatment, time, and interaction effects on hepatic *igf2* levels; *igf2* levels were ~2.0-fold higher in the low-dose cortisol group compared with vehicle controls on day 3 (Fig. 3D). There were no clear effects of cortisol on hepatic *ghr* levels (Fig. 3E).

There were no significant treatment or interaction effects on hepatic *igfbp1a1*, *-2a*, *-2b1*, and *-2b2* levels (Fig. 4A, D-F). There were significant treatment, time, and interaction effects on hepatic *igfbp1b1* levels. On day 3, *igfbp1b1* levels were elevated by ~3-fold from vehicle controls by the high dose of cortisol (Fig. 4B). There was a significant treatment effect on hepatic *igfbp1b2* levels; *igfbp1b2* was stimulated ~2-fold from vehicle controls by the high dose of cortisol on day 3 (Fig. 4C). There were no significant treatment, time, or interaction effects on the expression of hepatic *glucocorticoid receptor 1* (*gr1*), *-2*, or *mineralocorticoid receptor* (*mr*) (Supplementary Figs. 2A–C).

3.3. Muscle mRNA levels

There were no significant treatment or interaction effects on muscle *igf1* and *ghr* transcript levels (Fig. 5A, C). There was a significant treatment effect on muscle *igf2* levels; *igf2* was modestly stimulated by the high dose of cortisol on day 3 (Fig. 5B).

3.4. Pituitary mRNA levels

There were no significant treatment or interaction effects on pituitary gene transcript levels. A significant main effect of time was detected for *gh* and *pomca1* levels (Supplementary Fig. 3).

3.5. Branchial NKA activity and mRNA levels

There were significant effects of treatment and time on branchial NKA activity. At 3 days, the low dose of cortisol stimulated NKA activity compared to vehicle controls (Fig. 6A). While no significant treatment or interaction effects were detected for *nka-α1a* (Fig. 6B), there were significant treatment effects on *nka-α1b*, *nkcc1*, and *cftr1* levels (with additional time and interaction effects for *nka-α1b* and *nkcc1*,

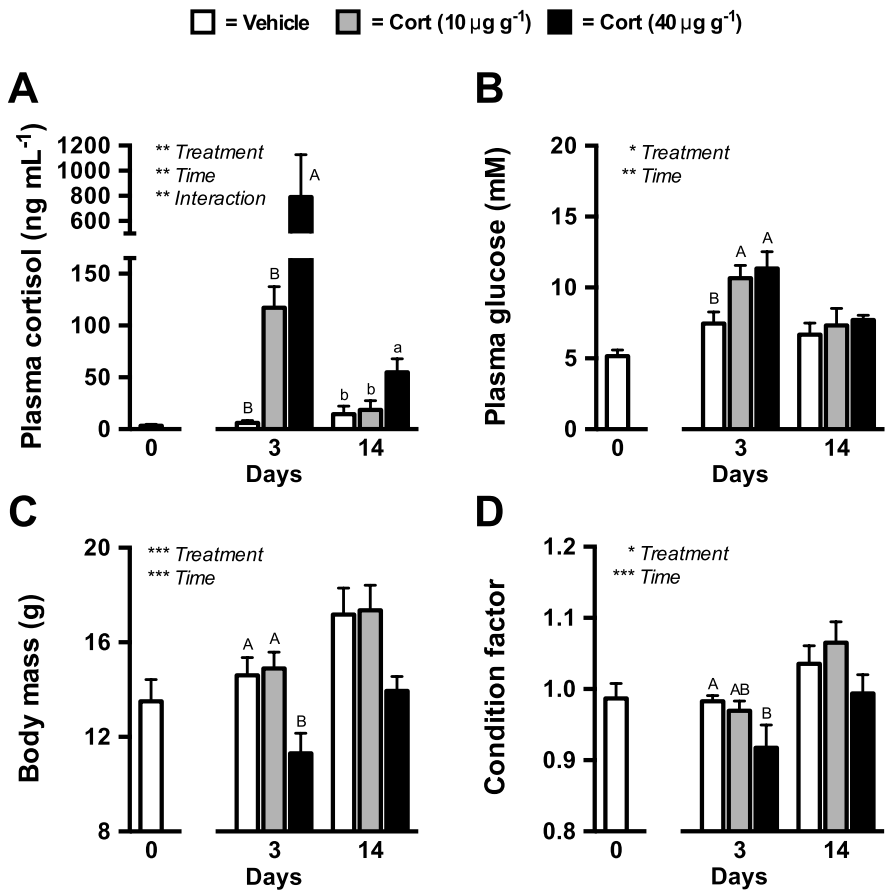

Fig. 1. Effects of cortisol implants on plasma cortisol (A), plasma glucose (B), body mass (C), and condition factor (D). Means ± SEM (n = 8–10). Parr were intraperitoneally implanted with cortisol at 0 (vehicle; open bars), 10 (shaded bars), or 40 μg g^{-1} body weight (solid bars) and sampled after 3 or 14 days. Significant effects of treatment, time, or an interaction between treatment and time are indicated in respective panels (two-way ANOVA): $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. When there was a significant effect of treatment, *post-hoc* comparisons (one-way ANOVA, Tukey's HSD test, $P < 0.05$) were made between groups at each time point. For a given time point, denoted by uppercase or lowercase letters, means not sharing the same letter are significantly different.

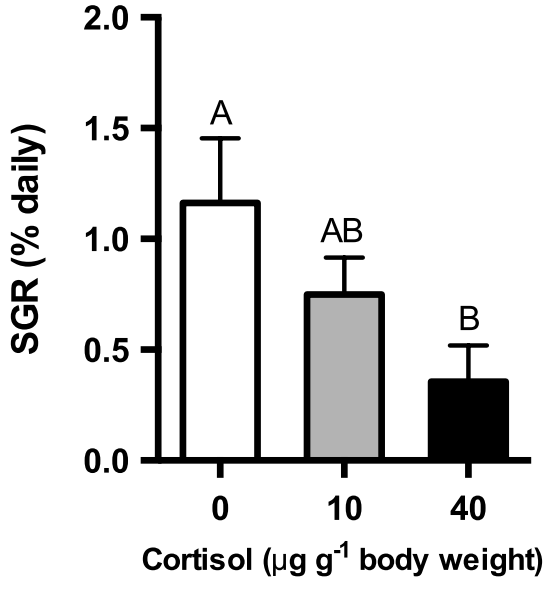

Fig. 2. Effect of cortisol implants on specific growth rate (SGR) on day 14. Means ± SEM (n = 8–10). Parr were intraperitoneally implanted with cortisol at 0 (vehicle; open bars), 10 (shaded bars), or 40 μg g^{-1} body weight (solid bars) and sampled after 14 days. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD test, $P < 0.05$).

respectively). *nka-α1b*, *nkcc1*, and *cftr1* were stimulated by ~2.0–2.5-fold by the low dose of cortisol on day 3 (Fig. 6C–E).

There were no clear effects of cortisol on branchial *igfbp4, -5a*, *-5b1*, *-6b1*, and *-6b2* mRNA levels (Fig. 7A–C, E, F). On the other hand, there were significant treatment, time, and interaction effects on branchial *igfbp5b2* mRNA levels. *igfbp5b2* was stimulated ~2-fold by both doses of cortisol on day 3 (Fig. 7D). There were significant treatment effects on branchial *gr1* and *mr* levels. On day 14, *gr1* and *mr* were diminished by the low and high cortisol doses, respectively (Supplementary Figs. 2D and F). There were no significant main or interaction effects on branchial *gr2* levels (Supplementary Fig. 2E).

4. Discussion

The primary objective of the current study was to identify cortisol-responsive factors within the Gh/Igf/Igfbp network of salmon parr and relate their dynamics to somatic growth patterns. Among the assayed hepatic *igfbp1* and *-2* gene transcripts, *igfbp1b1* and *-1b2* had elevated expression in parallel with the attenuation of growth by cortisol. Therefore, we contextualize these cortisol-*igfbp1* links in Atlantic salmon by considering what is currently known regarding the biological activities and glucocorticoid-regulation of plasma Igfbps in other teleost models. Recognizing the multiplicity of physiological processes regulated by the Gh/Igf/Igfbp system (Björnsson, 1997), we then address how branchial ionoregulatory processes and *igfbps* previously shown to respond to salinity challenges were impacted by cortisol.

The implants administered in the current study were clearly effective in elevating plasma cortisol levels, and the early increase in plasma glucose levels reflect the well-described gluconeogenic effects of cortisol (Faught and Vijayan, 2016). Any given physiological response to an environmental stressor is shaped by a variety of characteristics inherent

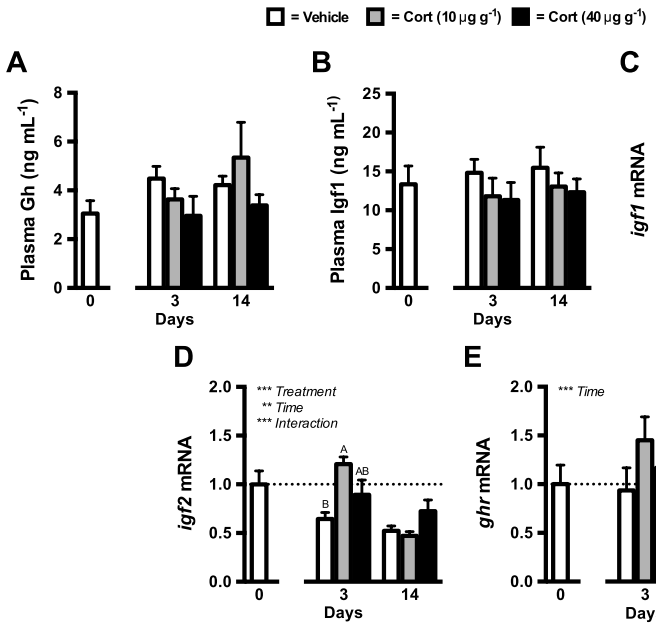

Fig. 3. Effects of cortisol implants on plasma Gh (A), Igf1 (B), and hepatic *igf1* (C), *igf2* (D), and *ghr* (E) mRNA levels. Means ± SEM (n = 8–10). mRNA levels are presented as a fold-change from the day 0 group. Parr were intraperitoneally implanted with cortisol at 0 (vehicle; open bars), 10 (shaded bars), or 40 μg g^{-1} body weight (solid bars) and sampled after 3 or 14 days. Significant effects of treatment, time, or an interaction between treatment and time are indicated in respective panels (two-way ANOVA): $^{**}P < 0.01$ and $^{***}P < 0.001$. When there were significant treatment or interaction effects, *post-hoc* comparisons (one-way ANOVA, Tukey's HSD test, $P < 0.05$) were made between groups at each time point. For a given time point, means not sharing the same letter are significantly different.

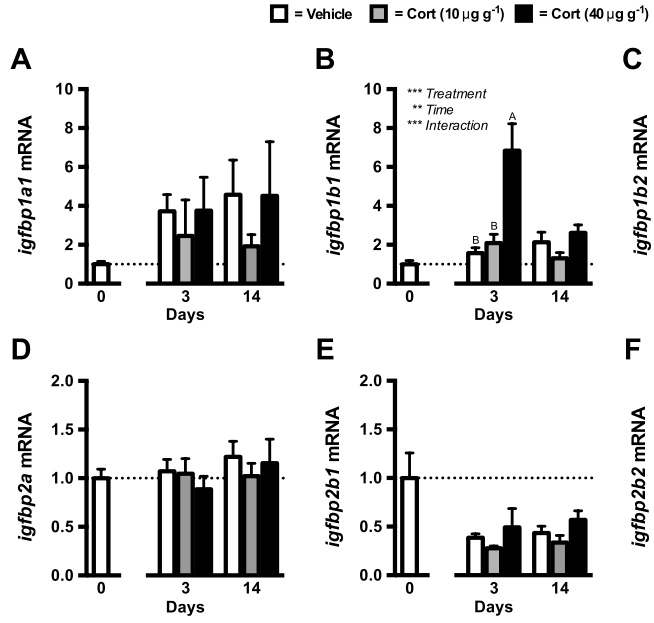

Fig. 4. Effects of cortisol implants on hepatic *igfbp1a1* (A), *-1b1* (B), *-1b2* (C), *-2a* (D), *-2b1* (E), and *-2b2* (F) mRNA levels. Means ± SEM (n = 8–10). mRNA levels are presented as a fold-change from the day 0 group. Parr were intraperitoneally implanted with cortisol at 0 (vehicle; open bars), 10 (shaded bars), or 40 μg g^{-1} body weight (solid bars) and sampled after 3 or 14 days. Significant effects of treatment, time, or an interaction between treatment and time are indicated in respective panels (two-way ANOVA): $^{**}P < 0.01$ and $^{***}P < 0.001$. When there was a significant effect of treatment, *post-hoc* comparisons (one-way ANOVA, Tukey's HSD test, $P < 0.05$) were made between groups at each time point. For a given time point, means not sharing the same letter are significantly different.

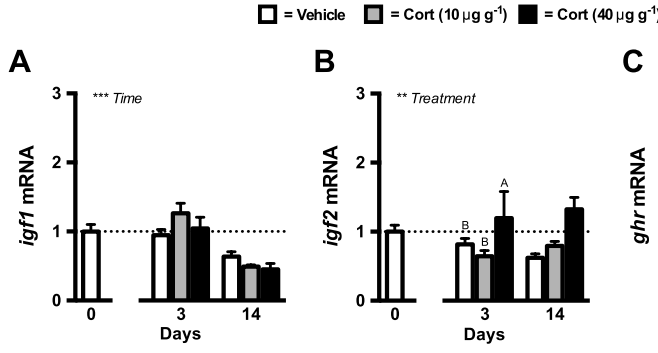

Fig. 5. Effects of cortisol implants on muscle *igf1* (A), *igf2* (B), and *ghr* (C) mRNA levels. Means ± SEM (n = 8–10). mRNA levels are presented as a fold-change from the day 0 group. Parr were intraperitoneally implanted with cortisol at 0 (vehicle; open bars), 10 (shaded bars), or 40 μg g^{-1} body weight (solid bars) and sampled after 3 or 14 days. Significant effects of treatment or time are indicated in respective panels (two-way ANOVA): $^{**}P < 0.01$ and $^{***}P < 0.001$. When there was a significant effect of treatment, *post-hoc* comparisons (one-way ANOVA, Tukey's HSD test, $P < 0.05$) were made between groups at each time point. For a given time point, means not sharing the same letter are significantly different.

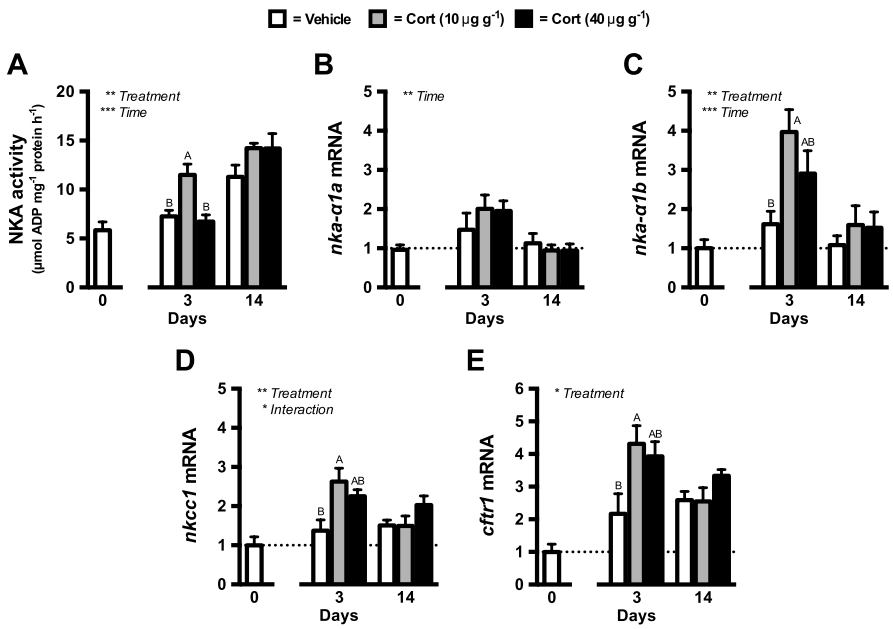

Fig. 6. Effects of cortisol implants on branchial Na^+/K^+-ATPase (NKA) activity (A), *nka-α1a* (B), *nka-α1b* (C), *nkcc1* (D), and *cftr1* (E) mRNA levels. Means ± SEM ($n = 8–10$). mRNA levels are presented as a fold-change from the day 0 group. Parr were intraperitoneally implanted with cortisol at 0 (vehicle; open bars), 10 (shaded bars), or 40 μg g^{-1} body weight (solid bars) and sampled after 3 or 14 days. Significant effects of treatment, time, or an interaction between treatment and time are indicated in respective panels (two-way ANOVA): $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. When there was a significant effect of treatment, *post-hoc* comparisons (one-way ANOVA, Tukey's HSD test, $P < 0.05$) were made between groups at each time point. For a given time point, means not sharing the same letter are significantly different.

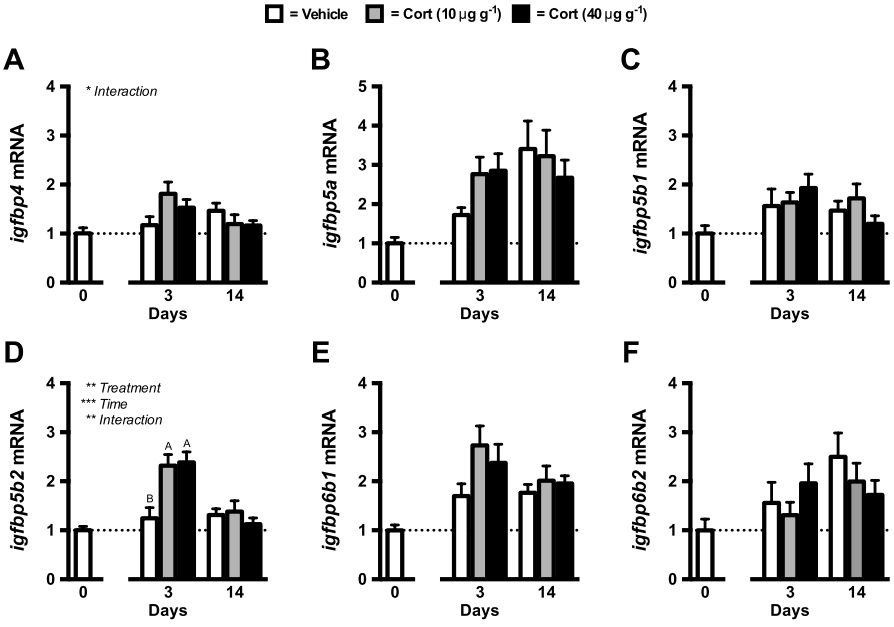

Fig. 7. Effects of cortisol implants on branchial *igfbp4* (A), *-5a* (B), *-5b1* (C), *-5b2* (D), *-6b1* (E), and *-6b2* (F) mRNA levels. Means ± SEM ($n = 8–10$). mRNA levels are presented as a fold-change from the day 0 group. Parr were intraperitoneally implanted with cortisol at 0 (vehicle; open bars), 10 (shaded bars), or 40 μg g^{-1} body weight (solid bars) and sampled after 3 or 14 days. Significant effects of treatment, time, or an interaction between treatment and time are indicated in respective panels (two-way ANOVA): $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. When there were significant treatment or interaction effects, *post-hoc* comparisons (one-way ANOVA, Tukey's HSD test, $P < 0.05$) were made between groups at each time point. For a given time point, means not sharing the same letter are significantly different.

to the responding organism (Barton, 2002). For instance, the amount of cortisol released following the perception of a stressor depends on the species/strain and life stage of a given organism. Salmonids exhibit particularly robust elevations in plasma cortisol when exposed to stressors (Pickering and Pottinger, 1989; Barton, 2002). Immature Atlantic salmon exposed to reduced water levels in rearing tanks exhibited plasma cortisol levels of ~200 ng mL^{-1} (Einarsdottir and Nilssen, 1996). Madaro et al. (2015, 2016) reported that cortisol levels ranged between ~50 and 160 ng mL^{-1} in Atlantic salmon parr exposed to various stressors; smolts subjected to confinement and handling exhibited cortisol levels >200 ng mL^{-1} (Carey and McCormick, 1998). Stressors associated with the upstream migration of kokanee salmon (*Oncorhynchus nerka kennerlyi*) resulted in plasma cortisol levels >600 ng mL^{-1} (Carruth et al., 2000) whereas spawning masu salmon (*O. masou*) exhibited levels between 200 and 400 ng mL^{-1} (Westring et al., 2008). Given the plasma cortisol concentrations induced by the implants employed in the current study, and the extended nature of their elevation, animals in the high cortisol treatment are representative of salmon exposed to a severe and prolonged stressor(s).

We characterized patterns of plasma Gh and Igf1, and hepatic *igf1* gene expression, to consider how the Gh/Igf system was affected by our cortisol implants. In the face of stable plasma Gh levels (and pituitary *gh* mRNA), hepatic *igf1* expression was diminished by the high cortisol treatment (Fig. 3A, C; Supplementary Fig. 3A) and plasma Igf1 was significantly correlated with reduced SGR on day 14 (Supplementary Fig. 1). During catabolic periods, the uncoupling of plasma Gh from plasma Igf1/hepatic *igf1* enables the attenuation of somatic growth in a fashion independent from altering systemic Gh levels (Jenkins and Ross, 1996; Björnsson, 1997). Cortisol modulates hepatic sensitivity to Gh both *in vivo* (Kajimura et al., 2003; Nakano et al., 2013) and *in vitro*

(Pierce et al., 2005, 2011; Philip and Vijayan, 2015), and by day 3, this activity likely contributed to reducing hepatic *igf1* expression. Glucocorticoids modulate Gh-sensitivity by acting directly on hepatocytes; cortisol can down-regulate Ghr/*ghr* expression (Small et al., 2006; Nakano et al., 2013) and/or activate intracellular pathways that attenuate Ghr-initiated signal transduction (Philip and Vijayan, 2015). To the extent that hepatic *ghr* gene expression reflects Gh-binding capacity (Norbeck and Sheridan, 2011; Hanson et al., 2017), we did not find evidence that cortisol modulated Gh-sensitivity via Ghr expression (Fig. 3E). Factors that attenuate Ghr-initiated signal transduction in response to glucocorticoids, such as suppressors of cytokine signaling (Socs), are activated through glucocorticoid response elements (GREs) in their promoter regions. Socs 1 and -2, for example, coordinate the expression of growth-related genes (such as *igf1*) when trout repartition energy use during a stress response (Philip and Vijayan, 2015). Given the stable *ghr* expression in the current study, future investigations should resolve whether Socs contribute to regulating *igf1* in Atlantic salmon mounting a cortisol stress response. We cannot, however, discount a direct link between glucocorticoid receptor activation and basal *igf1* gene transcription (Leung et al., 2008). While not yet described for teleost *igf1* genes, mammalian *igf1* genes possess GREs in their promoter regions (He et al., 2016).

An interesting pattern observed in this study was the disparate responses by *igf1* and *-2* to cortisol; *igf2* expression in liver and muscle was modestly stimulated by cortisol rather than inhibited, as was the case for *igf1* (Figs. 3D and 5B). The hepatic *igf2* response to cortisol is consistent with the synthetic corticosteroid dexamethasone acting independently, and synergistically with Gh, to promote *igf2* expression in cultured coho salmon (*O. kisutch*) hepatocytes (Pierce et al., 2010). Muscle *igf2* expression was also potentially elevated in direct response to circulating cortisol levels, a regulatory pattern revealed in cultured Atlantic salmon myotubes (Garcia de la Serrana et al., 2017). Thus, the current study provides important *in vivo* evidence for glucocorticoid regulation of *igf1* and *-2* expression in salmon liver and muscle. While a comprehensive understanding of how Igf2 regulates growth in fishes is not currently at hand, the fact that plasma Igf2 levels and hepatic *igf2* expression are regulated by Gh (Reindl and Sheridan, 2012) and native Igf2 promotes growth of juvenile tilapia (Chen et al., 2000), Igf2 is gaining recognition as a regulator of post-embryonic growth in fishes. From a functional perspective, the differential regulation of *igf1* and *-2* by glucocorticoids may reflect uncharacterized capacities for their encoded hormones to regulate organ and tissue growth (Pierce et al., 2011). Therefore, at present, it is difficult to conclude how *igf2* expression patterns related to the reduced growth of cortisol-treated parr.

Igfbp1a and -1b are two of the three major circulating Igfbps in salmonids (Shimizu and Dickhoff, 2017). By restricting Igfs from binding cognate receptors, Igfbp1s are negative regulators of somatic growth, development, reproduction, and glucose metabolism (Lee et al., 1997; Kajimura et al., 2005; Kamei et al., 2008; Garcia de la Serrana and Macqueen, 2018; Tanaka et al., 2018). Circulating hormones such as Gh, insulin, glucagon, thyroid hormones, 17β-estradiol, in addition to glucocorticoids, regulate hepatic *igfbp1* expression in salmonids (Pierce et al., 2006; Breves et al., 2014, 2018; Madison et al., 2015). Here, we found that among the three Atlantic salmon *igfbp1* paralogs highly expressed in the liver (Macqueen et al., 2013), *igfbp1b1* and *-1b2* were sensitive to cortisol whereas *igfbp1a1* was unresponsive (Fig. 4A–C). *igfbp1a2* was not measured in the present study because of very low levels in the liver (Macqueen et al., 2013). Interestingly, Atlantic salmon previously subjected to the catabolic conditions associated with food restriction exhibited increased *igfbp1a1* expression (Breves et al., 2016). We therefore propose that expansion of the *igfbp1* gene family in Atlantic salmon has facilitated paralog-specific responses to endogenous hormones and nutritional cues. The specific induction of *igfbp1b1* and *-1b2* gene transcripts, and presumably their translated proteins (Pierce et al., 2006), during a cortisol stress response plays a role in associating the activity of Igfs with the redirection of energy away from somatic growth and toward processes aimed at restoring homeostasis. Importantly, the stimulation of hepatic *igfbp1b1* and *-1b2* gene expression seen here in Atlantic salmon aligns with increased plasma Igfbp1b (23-kDa Igfbp) levels in cortisol-injected rainbow trout (*O. mykiss*) (Shimizu et al., 2011a). Moreover, links between cortisol and plasma Igfbps (albeit of unresolved molecular identity) extend beyond salmonids; exogenous cortisol stimulated 24- and 30-kDa Igfbps in Mozambique tilapia (*Oreochromis mossambicus*) and a 20-kDa Igfbp in channel catfish (*Ictalurus punctatus*) (Kajimura et al., 2003; Peterson and Small, 2005). At least for Atlantic salmon, the dual regulation of plasma (produced and secreted from liver) and muscle Igfbps (Garcia de la Serrana et al., 2017) by cortisol permits systemic and organ-level responses to the energetic demands associated with a stress response.

In addition to *igfbp1s*, Atlantic salmon express multiple *igfbp2* genes in liver (Macqueen et al., 2013), a pattern that is consistent across multiple teleosts (Funkenstein et al., 2002; Kamei et al., 2008; Zhou et al., 2008; Pedroso et al., 2009; Peterson and Waldbieser, 2009; Shimizu et al., 2011b; Safian et al., 2012; Yang et al., 2020). In contrast to mammals where Igfbp3 is the main Igf1-carrier, Igfbp2b serves as the primary Igf1-carrier and is the third major circulating Igfbp in salmonids (Shimizu et al., 2011a; Shimizu and Dickhoff, 2017; Allard and Duan, 2018). Varied patterns of plasma Igfbp2 levels and hepatic *igfbp2* expression in response to environmental conditions render it difficult to ascribe a universal function to Igfbp2s in teleosts (Garcia de la Serrana and Macqueen, 2018). Nonetheless, multiple studies have documented that teleost Igfbp2s (like mammalian Igfbp3) are subject to regulation by Gh (Schmid et al., 1994; Shimizu and Dickhoff, 2017). For example, Gh stimulated plasma Igfbp2b levels in coho salmon and hepatic *igfbp2b* expression in Mozambique tilapia (Shimizu et al., 1999, 2003; Breves et al., 2014). When considering these links between Gh and plasma Igfbp2b/hepatic *igfbp2b*, the unchanged expression of *igfbp2s* in cortisol-implanted parr (Fig. 4D–F) may reflect the stable plasma Gh levels throughout our experiment (Fig. 3A). In contrast, *igfbp2* mRNA levels were directly stimulated by dexamethasone in cultured Atlantic salmon myotubes (Garcia de la Serrana et al., 2017) and enhanced in the muscle of fine flounder (*Paralichthys adspersus*) subjected to chronic stress (Valenzuela et al., 2018). These results indicate that the regulation of Igfbp2s by glucocorticoids is more strongly associated with modulating Igf activities within target tissues than at the systemic level.

We previously reported that among the multiple *igfbp* transcripts expressed in the gill (Macqueen et al., 2013), *igfbp4*, *-5a*, *-5b1*, *-5b2*, *-6b1*, and *-6b2* had altered mRNA levels within 48 h of exposure to SW (Breves et al., 2017). To probe regulatory links between plasma cortisol and branchial *igfbp* expression patterns during SW acclimation, we determined whether these six *igfbps* responded to our cortisol implants. We first assessed whether our cortisol implants were sufficient to stimulate adaptive responses to SW (Pelis and McCormick, 2001; Tipsmark et al., 2002; Nilsen et al., 2007; McCormick et al., 2013) by confirming that cortisol-implanted parr exhibited elevated NKA activity and *nka-α1b*, *nkcc1*, and *cftr1* gene expression (Fig. 6A, C-E). On the other hand, *nka-α1a* expression, which decreases during SW acclimation, was not impacted by cortisol (McCormick et al., 2013) (Fig. 6B). Among the six *igfbp* genes we assayed, only *igfbp5b2,* a transcript previously diminished after SW transfer (Breves et al., 2017) was stimulated by cortisol (Fig. 7D). While Igfbp5b was shown to exert ligand-independent activity in zebrafish (*Danio rerio*) (Dai et al., 2010) it has not been linked with any ionoregulatory processes in fishes. Taken together, the discordant *igfbp5b2* responses to cortisol and SW do not suggest that a cortisol-Igfbp5b2 connection is vital to SW acclimation. Furthermore, the current study provides no indication that elevations in *igfbp4* and *-6b1* during SW acclimation (Breves et al., 2017) are linked with coincident changes in plasma cortisol. Clearly, future investigations are required to resolve the regulatory mechanisms that underlie SW-induced changes in the expression of *igfbps*/Igfbps in teleosts (Shepherd et al., 2005; Breves et al., 2017).

In summary, our principal findings include the identification of

igfbp1b1 and *-1b2* as transcripts that strongly respond to exogenous cortisol in salmon parr. The next challenge is to more precisely infer whether changes in circulating Igfbp1b1-and/or -1b2 attenuate growth. Gene editing via CRISPR/Cas9 now enables the functional characterization of specific Igfbps in salmonids (Cleveland et al., 2018). The life-history strategy, endangered status, and intense aquaculture of Atlantic salmon (Parrish et al., 1998) positions it as an important model species from which to resolve how the Igfbp system links the cortisol stress response with growth. Atlantic salmon life-history transitions are deeply interconnected with somatic growth patterns, and body size underlies overall fitness at key stages (McCormick and Saunders, 1987). From a comparative perspective, the responses observed here provide further support that hepatic *igfbp1* expression in teleosts is enhanced under catabolic conditions. Cortisol is a highly pleiotropic hormone, and its intricate modulation of the Igfbp system at both the systemic and tissue levels complements behavioral and metabolic aspects of the stress response (Pickering et al., 1982; Mommsen et al., 1999; Barton, 2002; Bernier et al., 2004; Bernier, 2006; Madaro et al., 2015, 2016; Madison et al., 2015; Conde-Siera et al., 2018). Together, these interactions underlie the long-term consequences of elevated cortisol on growth in response to external stressors.

Credit authorship statement

Jason Breves: Conceptualization, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. **Ryan Springer-Miller:** Investigation. **Damaris Chenoweth:** Investigation. **Amanda Paskavitz:** Investigation. **Annaliese Chang:** Investigation. **Amy Regish:** Investigation. **Ingibjörg Einarsdottir:** Investigation. **Thrandur Björnsson:** Formal analysis, Writing - Review & Editing, Supervision. **Stephen McCormick:** Conceptualization, Formal analysis, Writing - Review & Editing, Supervision, Project administration.

Funding

This work was supported by the National Science Foundation [IOS-1755131 to J.P.B.] and Skidmore College [Start-Up Funds to J.P.B.]. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government. The funding sources had no involvement in study design, interpretation of data, writing of the report, or in the decision to submit the article for publication.

Declaration of competing interest

The authors declare there are no competing interests that could be perceived as prejudicing the impartiality of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2020.110989.

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