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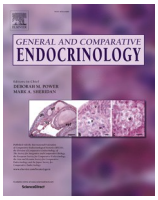

Food deprivation reduces sensitivity of liver Igf1 synthesis pathways to growth hormone in juvenile gopher rockfish (*Sebastes carnatus*)

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ABSTRACT

Growth hormone (Gh) regulates growth in part by stimulating the liver to synthesize and release insulin-like growth factor-1 (Igf1), which then promotes somatic growth. However, for fish experiencing food limitation, elevated blood Gh can occur even with low circulating Igf1 and slow growth, suggesting that nutritional stress can alter the sensitivity of liver Igf1 synthesis pathways to Gh. Here, we examined how recent feeding experience affected Gh regulation of liver Igf1 synthesis pathways in juvenile gopher rockfish (*Sebastes carnatus*) to illuminate mechanisms underlying the nutritional modulation of Igf1 production. Juvenile gopher rockfish were maintained under conditions of feeding or complete food deprivation (fasting) for 14 d and then treated with recombinant sea bream (*Sparus aurata*) Gh or saline control. Gh upregulated hepatic *igf1* mRNA levels in fed fish but not in fasted fish. The liver of fasted rockfish also showed a lower relative abundance of gene transcripts encoding teleost Gh receptors 1 (*ghr1*) and 2 (*ghr2*), as well as reduced protein levels of phosphorylated janus tyrosine kinase 2 (pJak2) and signal transducer and activator of transcription 5 (pStat5), which function to induce *igf1* gene transcription following Gh binding to Gh receptors. Relative hepatic mRNA levels for suppressors of cytokine signaling (Socs) genes *socs2*, *socs3a*, and *socs3b* were also lower in fasted rockfish. Socs2 can suppress Gh activation of Jak2/Stat5, and fasting-related variation in *socs* expression may reflect modulated inhibitory control of *igf1* gene transcription. Fasted rockfish also had elevated liver mRNA abundances for lipolytic hormone-sensitive lipase 1 (*hsl1*) and Igf binding proteins *igfbp1a, -1b* and *–3a*, reduced liver mRNAs encoding *igfbp2b* and an Igfbp acid labile subunit-like (*igfals*) gene, and higher transcript abundances for Igf1 receptors *igf1ra* and *igf1rb* in skeletal muscle. Together, these findings suggest that food deprivation impacts liver Igf1 responsiveness to Gh via multiple mechanisms that include a downregulation of hepatic Gh receptors, modulation of the intracellular Jak2/Stat5 transduction pathway, and possible shifts in Socs-inhibitory control of *igf1* gene transcription, while also demonstrating that these changes occur in concert with shifts in liver Igfbp expression and muscle Gh/Igf1 signaling pathway components.

1. Introduction

Nutritional modulation of growth in teleost fishes is regulated by the growth hormone (Gh)/insulin-like growth factor-1 (Igf1) endocrine axis (Duan, 1997; Picha et al., 2008a; Won and Borski, 2013; Sadoul and Vijayan, 2016; Bergan-Roller and Sheridan, 2018). Gh secreted into

Abbreviations: Gh, growth hormone; Hnf, hepatic nuclear factor; Hsl, hormone-sensitive lipase; Igf1, insulin-like growth factor 1; Igfals, Igfbp acid labile subunit; Igfbp, insulin-like growth factor binding protein; Jak2, janus tyrosine kinase 2; Socs, suppressors of cytokine signaling; Stat5, signal transducer and activator of transcription 5.

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blood circulation from the pituitary gland mediates metabolic processes that support somatic growth, including hepatic triglyceride uptake and adipose tissue lipolysis, and also directly stimulates growth of some tissues including skeletal muscle (Fuentes et al., 2013; Vélez et al., 2017). Gh-transgenic fish, for instance, exhibit more rapid growth via both muscle hyperplasia and hypertrophy (Hill et al., 2000; Johnston et al., 2014), and treatment of fish with exogenous Gh can stimulate both hyperplasic and hypertrophic skeletal muscle growth (Weatherley and Gill, 1987; Fauconneau and Padboef, 2000). Effects of Gh on growth, however, also emerge via Gh's influence on liver production of Igfs (Duan et al., 1993; Cao et al., 1989; Schmid et al., 2000; Leung et al., 2008). Igfs released into blood circulation by the liver in response to Gh stimulation regulate cell proliferation and growth in peripheral tissues such as skeletal muscle (Chen et al., 2000; Reinecke et al., 2005; Wood et al., 2005; Laviola et al., 2007; Fuentes et al., 2013). Accordingly, Igf1 has been shown to upregulate several genes involved in myogenesis (Garikipati and Rodgers, 2012; Jiménez-Amilburu et al., 2013; Bersin et al., 2023a). While the hepatic production of Igf1 is considered the primary pathway mediating Gh's actions on growth, Gh can also modulate synthesis of Igf1 locally within peripheral tissues including skeletal muscle, where locally-produced Igf1 may have paracrine effects on growth (Firth and Baxter, 2002; Eppler et al., 2010; Franz et al., 2016).

Circulating concentrations of Igf1 have been shown to exhibit a strong positive association with growth rate in several teleost fishes, and that relationship emerges in part from individual variation in food consumption (Andrews et al., 2011; Beckman, 2011; Journey et al., 2018). Individuals that eat more and grow quicker often show higher circulating concentrations of Igf1 (Pérez-Sánchez et al., 1995; Beckman et al., 2004a,b; Pierce et al., 2005; Wilkinson et al., 2006; Cameron et al., 2007; Breves et al., 2014, 2016; Hack et al., 2019; Strobel et al., 2020; Bersin et al., 2023a). Correspondingly, fish completely deprived of food (fasted) or given reduced feeding amounts usually slow their rate of growth and exhibit lower liver *igf1* mRNA abundance and declines in plasma Igf1 (Uchida et al., 2003; Beckman et al., 2004a; Norbeck et al., 2007; Bower et al., 2008; Hack et al., 2018, 2019; Strobel et al., 2020; Bersin et al., 2023b). Such changes in circulating Igf1 translate into shifts in growth rate under both short and long term variation in food availability and consumption (Fuentes et al., 2012; Reindl and Sheridan, 2012; Hack et al., 2019). Those influences of Igf1 on growth can also be impacted by the actions of Igf binding proteins (Igfbps), which modify the ratio of unbound:bound Igf1 (Butler and LeRoith, 2001; Duan and Xu, 2005; Shimizu and Dickhoff, 2017; Bach, 2018). Teleost fishes have several Igfbps, with whole- and partial-genome duplications during the evolutionary history of teleosts resulting in duplications of Igfbp genes (Daza et al., 2011; Shimizu and Dickhoff, 2017; Allard and Duan, 2018).

Igf1 synthesis and release from the liver is regulated by Gh binding to hepatic Gh receptors (Ghrs). There is abundant evidence that fish treated with exogenous Gh over both short- (hours, days) and long-term (weeks) periods increase circulating Igf1, liver *igf1* mRNA abundance, and rates of somatic growth (Kajimura et al., 2001; Biga et al., 2004; Peterson et al., 2005; Gahr et al., 2008; Raven et al., 2012; Vélez et al., 2017). And yet, despite the well-established role of Gh as a stimulator of liver Igf1 production, fish experiencing conditions of food limitation can show elevated circulating Gh even with low Igf1 and diminished growth (Barrett and McKeown, 1988; Gabillard et al., 2005; Gómez-Requeni et al., 2005; Norbeck et al., 2007; Reinecke, 2010; Beckman, 2011; Reindl and Sheridan, 2012; Bertucci et al., 2019). This change in the relationship of circulating Gh and Igf1 levels in fish experiencing nutritional stress appears to emerge from shifts in the sensitivity of liver Igf1 synthesis pathways to Gh (Bergan-Roller and Sheridan, 2018). Teleost fishes have two Ghrs (Saera-Vila et al., 2005; Very et al., 2005; Jiao et al., 2006), and both Ghrs bind Gh to induce liver Igf1 production via the activation of intracellular signaling cascades, including the Janus tyrosine kinase 2 (Jak2) and signal transducer and activator of transcription 5 (Stat5) pathway (Herrington and Carter-Su, 2001; Kittilson et al., 2011; Bergan-Roller and Sheridan, 2018; Dehkhoda et al., 2018). Gh binding to a Ghr results in phosphorylation of Jak2, which is already bound in an inactive state to a Gh receptor dimer (Dehkhoda et al., 2018). Activation of Jak2 subsequently enables phosphorylation and activation of Stat5 proteins, which then can be translocated to the nucleus where they function as transcription factors (Smit et al., 1996; Bergan-Roller and Sheridan, 2018; Dehkhoda et al., 2018). Stat5b in particular has been identified as a key signal transducer underlying Gh's stimulation of liver Igf1 production by upregulating hepatic expression of *igf1* as well as several hepatic nuclear factor (Hnf) genes including *hnf1α* that act as transcription factors (Han et al., 1996; Eleswarapu and Jiang, 2005; Waxman and O'Connor, 2006; Xiong et al., 2017; Dehkhoda et al., 2018). In mammals, concentrations of phosphorylated STAT5B (pSTAT5B) show Gh pulse-dependent patterns of abundance in the liver (Waxman et al., 1995; Waxman and O'Connor, 2006), and disruptions to STAT5B signaling have been found to impair growth as well as alter circulating concentrations of both IGF1 and GH (Udy et al., 1997; Hwa, 2021). While less well studied in teleost fishes, Stat5b appears to have a similarly important role, as zebrafish (*Danio rerio*) loss-of-function Stat5b mutants were found to have reduced growth and altered patterns of metabolic gene expression (Xiong et al., 2017).

Gh was previously observed to increase phosphorylated Jak2 (pJak2) and pStat5b protein abundance in hepatocytes isolated from rainbow trout (*Oncorhynchus mykiss*) that were continuously fed, but not in cells from trout that had been fasted for 4 weeks (Bergan et al., 2015), suggesting that recent feeding/fasting experience caused a shift in sensitivity of the anabolic Jak2/Stat5b transduction pathway to Gh. This change in Gh effects on the liver has been hypothesized to be an adaptive shift away from Gh as an activator of anabolic pathways including Jak2/Stat5b and toward Gh stimulation of catabolic pathways that would mobilize lipid stores during periods of energetic stress (Bergan et al., 2012; Bergan-Roller and Sheridan, 2018). Supporting this idea, fasted fish can show increased liver lipase activity (Sheridan and Mommsen, 1991; Bergan et al., 2012). Gh has been shown to induce liver expression of hormone-sensitive lipase enzymes in fasted – but not fed – rainbow trout (Bergan et al., 2012, 2015); those same fasted fish also exhibit diminished Jak2/Stat5b pathway activation (Bergan et al., 2012). Similar findings have been observed in human adipose and skeletal muscle tissues, where fasting enhanced GH stimulation of lipolysis while concurrently diminishing JAK2/STAT5B pathway activation (Moller et al., 2009). Such nutritional modulation of Gh-mediated Jak2/Stat5b activation and, ultimately, Igf1 synthesis may emerge in fasted fish and other vertebrates via several mechanisms either alone or in concert. Studies have observed declines in hepatic Gh receptor binding or *ghr* mRNA expression in fish experiencing food deprivation, suggesting a downregulation of liver Gh binding capacity (Small et al., 2006; Norbeck et al., 2007; Botta et al., 2016; Strobel et al., 2020). Alternatively, suppressors of cytokine signaling (Socs) proteins – which negatively modulate Gh stimulation of Jak2/Stat5b pathways in mammals and fish (LeRoith and Nissley, 2005; Studzinski et al., 2009; Wang et al., 2011) – may be elevated in liver by conditions of nutritional stress. For example, Arctic charr (*Salvelinus alpinus*) were observed to upregulate liver *socs1*, *socs2*, and *socs3* mRNA abundance under conditions of fasting, supporting a possible role for Socs proteins in modulating Gh stimulation of Igf1 production (Jorgensen et al., 2013). Even so, the mechanisms underlying *igf1* gene regulation by Gh – and how those mechanisms mediate changes in *igf1* regulation under conditions of nutritional stress – are not fully known (Herrington and Carter-Su, 2001; Rotwein, 2020).

In the present study, we examined the mechanisms underlying shifts in the efficacy of Gh as an activator of liver Igf1 synthesis pathways under differing nutritional status in juvenile gopher rockfish (*Sebastes carnatus*). Gopher rockfish are part of a broader group of *Sebastes* rockfishes commonly fished recreationally and commercially in the North Pacific Ocean. Some of these fishes are also being developed for aquaculture (Nakagawa et al., 2007; Guo et al., 2017). Juvenile rockfish were maintained under conditions of feeding or complete food deprivation

(fasting), and then administered recombinant sea bream Gh or vehicle solution (control). Patterns of mRNA and protein expression changes were then evaluated for evidence of the following non-exclusive mechanisms that might underlie diminished Gh stimulation of Igf1 production during fasting: 1) reduced hepatic Gh receptor expression, 2) altered expression of pJak2/pStat5 proteins and *hnf* genes involved in the intracellular signaling pathways following Gh receptor activation, and 3) changes in the expression of inhibitory *socs* genes. In addition, the influences of fasting on Gh regulation of transcript encoding lipolytic hormone-sensitive lipase 1 (*hsl1*) in liver was examined as a gauge for catabolic pathway regulation, and genes encoding Igfbps in liver and Gh receptors, Igf1 receptors, and Igfbps in skeletal muscle were quantified to evaluate how fasting altered Gh/Igf1 signaling pathways more broadly.

2. Materials and methods

2.1. Animal collection

Juvenile gopher rockfish (*S. carnatus*) were collected in the young-of-the-year life stage using a Standard Monitoring Unit for the Recruitment of Fishes (SMURF) fish trap (Ammann, 2004; Wilson et al., 2008). The SMURF trap was deployed between May and September 2016 at a depth of ~1–3 m below the ocean's surface at the California Polytechnic State University's Center for Coastal Marine Sciences (CCMS) Pier facility in Avila Beach, California, USA (35°10′12.3″N 120°44′27.2″W). Rockfish recruits captured in the SMURF were maintained in outdoor 340 L flow-through, filtered seawater tanks under conditions of ambient temperature, salinity, and photoperiod at the CCMS Pier facility. All procedures were approved by the Animal Use and Care Committee of California Polytechnic State University (Protocol #1504).

Fish were fed daily *ad libitum* a diet of commercial aquaculture pellet feed (BioPro2 pellets, BioOregon, Longview, WA, USA) for approximately 3 mo., then transferred to Moss Landing Marine Laboratories in Moss Landing, California, where fish were fed frozen squid and maintained until February 2018. In March 2018, fish were returned to the CCMS facility in Avila Beach and switched back to pellet feed until experiments began on 5 April 2018.

2.2. Experimental feeding and GH injection treatments

On 5 April 2018, eighty ($N = 80$) juvenile gopher rockfish (approx. 2 years old) were implanted intraperitoneally with passive integrated transponder tags (PIT tags, 7 mm; Loligo Systems, Inc. Viborg, Denmark) to enable identification of individual fish. Tagged fish were measured and weighed, and then placed into 340 L flow-through filtered seawater tanks (1 m diameter and 0.5 m depth). Fish ($n = 10$ per tank) were systematically assigned to each of eight replicate tanks such that mean biomass (body mass) and length (total length, TL) of fish in each tank was similar and thus balanced among tanks (mass: 5.49 ± 0.67 g [mean ± SD], one-factor ANOVA, $F_{7,72} = 0.145$, $p = 0.994$; TL: 6.79 ± 0.38 cm, $F_{7,72} = 0.705$, $p = 0.667$). All rockfish were then fed *ad libitum* for 2 weeks on a ration of BioPro2 pellet feed (Bio-Oregon, Longview, WA, USA) at an amount of 9 % feed mass · total fish mass per tank^{-1} · d^{-1}.

Following that 2 week 'baseline' feeding period, fish were again weighed and measured for the start (day 0) of the experimental feeding treatments. For the subsequent 14 d, rockfish in four of the tanks continued to receive the feed ration of 9 % feed mass · total fish mass per tank^{-1} · d^{-1} ('fed' treatment), while fish in the other four tanks were completely deprived of food ('fasted' treatment). After that 14 d period of feeding or fasting, two tanks from each food treatment were selected randomly for fish in those tanks to receive either a single injection of Gh or vehicle 'control.' The resulting experimental design thus corresponded to a 2 × 2 factorial arrangement of all combinations of feeding treatment (fed/fasted) and hormone injection (Gh injection / control), with $n = 20$ fish per each combined treatment group.

Prior to injection, fish were anesthetized (buffered MS222) and weighed. Fish were then injected intraperitoneally with either recombinant Gh (somatotropin) from gilthead sea bream (*Sparus aurata*) (2 μg per g fish mass; > 98 % purity, ProSpec TechnoGene Ltd., Ness-Ziona, Israel) or saline vehicle 'control' (0.9 % saline solution with 0.1 % bovine serum albumin; 1 μl per g body mass). This recombinant sea bream Gh is a non-glycosylated peptide of 188 amino acids with an additional Ala residue on the N-terminus, and has 89 % sequence identify to the deduced Gh polypeptide from Schlegeli's black rockfish, *Sebastes schlegelii* (GenBank accession no. AY542484), as well as 90 % identify to deduced Gh proteins translated from *gh* genes identified in the genome assemblies of tiger rockfish (*S. nigrocinctus,* AUPR02090709) and flag rockfish (*S. rubrivinctus*, KI467438). Gh injection dose was selected based on previous studies in other fishes (Duguay et al., 1996; Breves et al., 2014; Botta et al., 2016). All injections occurred between 15:00 and 18:00. Immediately following injection, fish were returned into their same experimental tanks. After being maintained for 20–24 h without further feeding, fish were recaptured, euthanized using MS222, and weighed and measured. The liver and skeletal muscle (fast-twitch 'white' muscle from the caudal region) tissues were dissected, and the liver was weighed for calculation of hepatosomatic index (HSI). The liver and skeletal muscle tissues were each subdivided into triplicate subsamples, which were then flash frozen in liquid N_2 and stored at −80 °C. One mortality occurred during the experiment in a 'fed' treatment tank during the 2 week fed/fasting period, resulting in an overall experimental sample size of N = 79 fish. Fish were maintained on ambient photoperiod and salinity (~33 ppt) conditions throughout the experiment, and water temperature recorded every 10 min averaged 11.57 ± 0.84 °C (mean ± SD) (HOBO Pendant Temp data loggers; Onset Computer Corp., Bourne, MA, USA) with no difference between treatment tanks.

2.3. Determinations of liver phosphorylated Jak2 and Stat5A/B

Liver tissue was homogenized (Fisherbrand™ Pellet Pestle™ Cordless Motor, ThermoScientific) for quantification of [pY1007/pY1008] phosphorylated Jak2 (pJak2) in hepatic cell lysates using the JAK2 (Phospho) [pY1007/pY1008] ELISA Kit (Invitrogen). Weighed liver tissue was homogenized on ice in 0.5 ml of Cell Extraction Buffer (Invitrogen) containing 1 mM phenylmethanesulfonyl fluoride (Millipore Sigma, St. Louis, MO, USA) and 5 % Protease Inhibitor Cocktail (Millipore Sigma). Following 30 min of lysis on ice with vortexing every 10 min, the lysate was centrifuged at 12,000×*g* for 10 min at 4 °C, and the resulting supernatant was collected. A 20 μl sample of the supernatant was diluted with 80 μl of Standard Dilution Buffer from the JAK2 (Phospho) [pY1007/pY1008] ELISA Kit, and 50 μl of that diluted supernatant was assayed according to protocol of the ELISA kit. Absorbance at 450 nm was quantified using a Victor X4 Multimode Plate Reader. JAK2 (Phospho) standards for the assay ranged from 50 to 1.6 units / ml, and the intra-assay % CV was 2.48 % for the assay. Concentrations of pJak2 were normalized for each fish to total protein content from the liver (Pierce™ BCA Protein Assay Kit, ThermoFisher Scientific), which did not vary with either feed ($p = 0.094$) or injection treatment ($p = 0.325$).

Concentrations of phosphorylated signal transducer and activator of transcription 5 types A and B (pStat5A/B) were quantified in hepatocyte lysates using the InstantOne ELISA™ phospho-STAT5 A/B (Tyr694/699) Kit (Invitrogen). A sample of liver tissue from each fish was weighed, homogenized as described above for JAK2, and then lysed on ice in 100 ul of 1X Cell Lysis Buffer Mix from the InstantOne ELISA™ phospho-STAT5 A/B (Tyr694/699) Kit. Lysate was subsequently assayed in 50 μl volumes along with kit-supplied negative and positive controls. Briefly, samples were incubated with 50 μl anti-pSTAT5 A/B antiserum for 1 h under shaking (~300 rpm), then washed, and incubated again for 15 min with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate according to kit instructions. Plates were subsequently read at

450 nm on a Victor X4 Multimode Plate Reader (Perkin Elmer). A serial dilution of positive control was used to generate a standard curve. Concentrations of pStat5A/B were then normalized to total protein content from the liver for each fish (Pierce™ BCA Protein Assay Kit, ThermoFisher Scientific). The mean intra-assay % CV of the assay was 4.14 %.

2.4. Quantification of RNA:DNA ratio

RNA:DNA ratios in liver and muscle tissue were quantified using a spectrophotometric method by Grémare and Vétion (1994) and adapted by Kawaguchi et al. (2013). Specific details of this method for application to rockfishes are also described in Hack et al. (2019). Frozen tissue samples from liver and skeletal muscle were weighed and then digested on ice with Protease K (20 mg/mL, Millipore Sigma) in a 20 mM phosphate buffered saline (PBS) solution with 0.15 M NaCl (pH 7.5), followed by the addition of 56 ul of 0.1 % sodium dodecyl sulfate (SDS) after digestion. Samples were then incubated on ice for 15 min and centrifuged at 4500×*g* for 15 min at 4 °C.

DNA concentrations were quantified spectrophotometrically with Hoechst 33285 (Millipore Sigma), while total nucleic acid concentrations were measured with Thiazole orange (Millipore Sigma). For DNA quantification, 100 µl of tissue supernatant was combined with 50 µl of PBS buffer and 5 µl of 0.02 mg/ml Hoechst 33258 solution, and then incubated at 37 °C for 30 min. Fluorescence was then measured at an excitation of 355 nm and emission of 460 nm (VICTOR X4 Multilabel Plate Reader, PerkinElmer, Waltham, MA, USA). For total nucleic acid quantification, 100 µl of tissue supernatant was combined with 50 µl of PBS buffer and 5 µl of 4 µg/ml Thiazole orange, followed by measurement of fluorescence at 490 nm excitation and 545 nm emission. Standard curves for both assays were generated using purified DNA from the testes of chum salmon, *Oncorhynchus keta* (Millipore Sigma). Intra-assay % CV values were 8.45 % for the Hoechst 33258 assay, and 5.59 % for the Thiazole orange assay.

2.5. Quantification of relative gene transcript abundances

2.5.1. Identification of rockfish gene sequences

Quantitative real-time reverse transcription PCR (qRT-PCR) using SYBR™ Green was used to examine how prior feeding or fasting experience affected Gh stimulation of previously identified Gh-mediated gene pathways in liver and skeletal muscle. Degenerate primers described in Hack et al., 2018, 2019 were again used to amplify and sequence partial cDNAs encoding homologous gopher rockfish Gh/Igf1 signaling genes (GenBank accession nos. MW940098 to MW940107), as described in Bersin et al. (2023a). Partial cDNAs encoding elongation factor-1α (*ef1α*) (MW940108) and 60S ribosomal protein L17 (*rpl17*) (MW940109) amplified and sequenced from gopher rockfish using degenerate primers as described by Bersin et al. (2023a) were used as internal reference genes for qRT-PCR. Additionally, a putative Igf acid labile subunit-encoding cDNA (*igfals*) was identified and sequenced from gopher rockfish as described by Bersin et al. (2023a). Each of these cDNAs were subsequently used to design gene-specific primers for use in qRT-PCR (Appendix A, Supplemental Table S1).

The availability of genome assemblies from several *Sebastes* rockfishes including the rougheye rockfish (*S. aleutianus,* GCA_001910805), Schlegel's black rockfish (*S. schlegelii*, GCA_004335315) – as well as a genome assembly for gopher rockfish (GCA_916700855) that became available following the start of this study – supplemented assemblies already available for the genomes of tiger rockfish (*S. nigrocinctus,* GCA_000475235) and flag rockfish (*S. rubrivinctus*, GCA_000475215), and allowed for identification of additional genes encoding proteins of interest for Gh/Igf1 signaling. BLAST searches followed by multiple sequence alignment was used to identify conserved nucleotide regions of the following Gh/Igf1 signaling genes in *Sebastes* rockfishes: Gh receptors 1 (*ghr1*) and 2 (*ghr2*), janus kinase 2 (*jak2*), signal transducer and activator of transcription 5B (*stat5b*), hepatic nuclear-factor 1α (*hnf1α*), suppressor of cytokine signaling (Socs) types 1 (*socs1*), 2 (*socs2*), 3a (*socs3a*) and 3b (*socs3b*), and hormone-sensitive lipase 1 (*hsl1*), as well as tubulin α-1A (*tuba*) as an additional reference gene. Gene-specific oligos were designed to conserved regions of each of those genes for use in SYBR™ Green qRT-PCR (Appendix A, Supplemental Table S1). Specificity of these qPCR primer sets was further confirmed by Sanger sequencing PCR products amplified using each set of primers.

2.5.2. qRT-PCR of relative gene transcript abundance

Total RNA was extracted from liver and fast-twitch skeletal muscle tissues of each rockfish in the experimental feed/fasting and Gh injection treatments using TRI Reagent® (Molecular Research Center, Inc.) with bromochloropropane as the phase separation agent. Resultant total RNA (P300 NanoPhotometer, Implen, Inc., Westlake Village, CA, USA) was quantified and DNase I treated (TURBO DNA-free kit, Ambion). Total RNA was then diluted to equivalent concentrations (72.5 ng/µl in liver and 23.5 ng/µl in muscle) and reverse transcribed in 50 µl reactions (liver) and 30 µl reactions (muscle) following the same thermal profile described above.

Relative mRNA levels were quantified by SYBR™ Green qPCR using a CFX96 Real-Time PCR Detection System (BioRad Laboratories, Inc.), according to the guidelines outlined by Bustin et al. (2009). PCRs were run in 10 µl reactions containing 5 µl PowerUp™ SYBR Green Master Mix (Thermo Fisher Scientific), 1.5 µl cDNA template, 2.2 µl nuclease free H_2O and 0.65 µl each of forward and reverse primer (10 µM). Reactions followed a thermal profile of 50 °C for 2 min, 95 °C for 2 min, and then 42 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a melt curve analysis. For each gene, a standard curve was made using a pool of RNA combined from fish of both feed and injection treatments. This RNA pool was serially diluted, and each standard assayed in triplicate. Correlation coefficients (r^2) for the standard curves were $r^2 > 0.96$. For each run, melt curve analyses were performed to confirm a single product. PCR efficiencies for each gene were calculated by the equation: $\%\ efficiency = [10^{(-1/\text{slope})} - 1]\times 100$. Mean reaction efficiencies for each gene assay are provided in Appendix A, Supplemental Table S1.

Relative mRNA level values were calculated using the standard curve and normalized to the geometric mean of mRNA expression for *rpl17* and *tuba* for liver tissue, and *rpl17*, *tuba* and *ef1α* for skeletal muscle, neither of which varied between treatment groups for the respective tissues. Abundance values for each gene were then further normalized to the RNA:DNA ratio for each tissue to correct for changes in liver status induced by fasting (Metzger et al., 2012), and then expressed as a relative level by dividing the resulting mRNA expression values by the mean value of the fed 'control' injection treatment group.

2.6. Statistical analyses

Data were examined for adherence to parametric assumptions of normality and equivalence of variances (Levene's test and Bartlett test) prior to all analyses. In cases when data failed to conform to equal variances, square root or $\log(x + 1)$ transformations were performed.

Initial body mass and total length (TL) values were compared between combinations of fed/fasted and injected/control treatments using one-factor ANOVA models to confirm the absence of any body size differences at the beginning of the experiment. Final body mass and TL values were compared using two-factor ANOVA models with food condition treatment ('fed' or 'fasted'), injection type ('Gh' or 'control'), and the interaction between food treatment and injection type as factors. Change (%) in body mass and length from the start of the experiment (day 0) to final sampling (day 14) was also compared between feeding treatment groups using t tests. Specific growth rate (SGR) only was calculated as the change in wet body mass (g) from initial mass (day 0) to final mass (day 14) using the following formula (Lugert et al., 2016;

Shoup and Michaletz, 2017):

$$SGR = \frac{\log(mass_{final}) - \log(mass_{initial})}{total\ days} \times 100$$

Body mass and length were also used to quantify body condition factor (K), which was calculated as: (mass/TL^3) * 100 (Lugert et al., 2016; Ricker, 1975). SGR and body condition values were each compared among treatments using two-factor ANOVA models.

The effects of recent feeding experience and Gh injection on concentrations of pStat5A/B and pJak2 in the liver were analyzed using two-factor ANOVA models with feeding treatment, injection treatment, and the feeding treatment * injection treatment interaction as factors. Two-factor ANOVA models were likewise used to test for influences of food deprivation and Gh injection on relative mRNA levels in both the liver and skeletal muscle. When a significant effect or interaction was observed in the ANOVA model, post hoc pairwise comparisons were made using Tukey's HSD tests. Detailed descriptions of these two-factor ANOVA model results are provided in Appendix A, Supplemental Table S2. All data are shown as mean ± SEM values, and all statistical tests were two-tailed and performed using JMP Pro 15 software (SAS Institute, Inc.).

3. Results

3.1. Fasting altered rockfish body mass and growth rate

Rockfish assigned to the fed and fasted treatments did not differ in body size prior to fasting. However, the 14 d fasting period resulted in fasted fish being smaller in mass at the same body length (Fig. 1a) ($F_{1,74}$ = 8.863, p = 0.0039). That difference in body size resulted from fasted rockfish losing body mass over the 14 d period of food deprivation while fed fish gained mass over that same period (Fig. 1b) (t = 17.350, p < 0.0001). That loss of body mass was also reflected in a negative mass-specific growth rate for fasted fish, while fed fish increased in mass (Fig. 1c) ($F_{1,74}$ = 192.390, p < 0.0001). Body length, however, was not significantly different after the 14 d fasting period indicating that the duration of feeding/fasting was not sufficient to result in differences in growth by length between the feeding groups (Fig. 1b) (p = 0.193). In line with that result, there was no difference in length-specific growth rate between fasted and fed treatments. The reduction in body mass did, however, generate a lower body condition factor (k) for fasted fish (Fig. 1d) (feeding effect: $F_{1,74}$ = 8.726, p = 0.0042), which was consistent with the higher mass for fed rockfish at the same length (Fig. 1a). Fasted fish also showed lower hepatosomatic index (HSI) values than fed fish (Fig. 1e) ($F_{1,74}$ = 69.056, p < 0.0001). Injection of Gh did not affect body condition or HSI in either fed or fasted rockfish.

3.2. Fasting alters the effects of Gh on liver Igf1 synthesis pathways

Fed juvenile rockfish treated with recombinant sea bream Gh had higher liver *igf1* mRNA levels compared to fed fish controls (Fig. 2). That upregulation of *igf1* transcript abundance by Gh was only observed in fed fish (feed*injection interaction: $F_{1,72}$ = 9.259, p = 0.0033), as Gh treatment did not affect liver *igf1* mRNA levels in fasted fish.

Gh action on the liver is mediated via binding to Gh receptors, and the abundance of gene transcripts encoding Gh receptor *ghr1* was lower in fasted fish than in fed fish (Fig. 3a). Messenger RNA levels of *ghr1* also showed opposing patterns of up- and down-regulation in fasted and fed

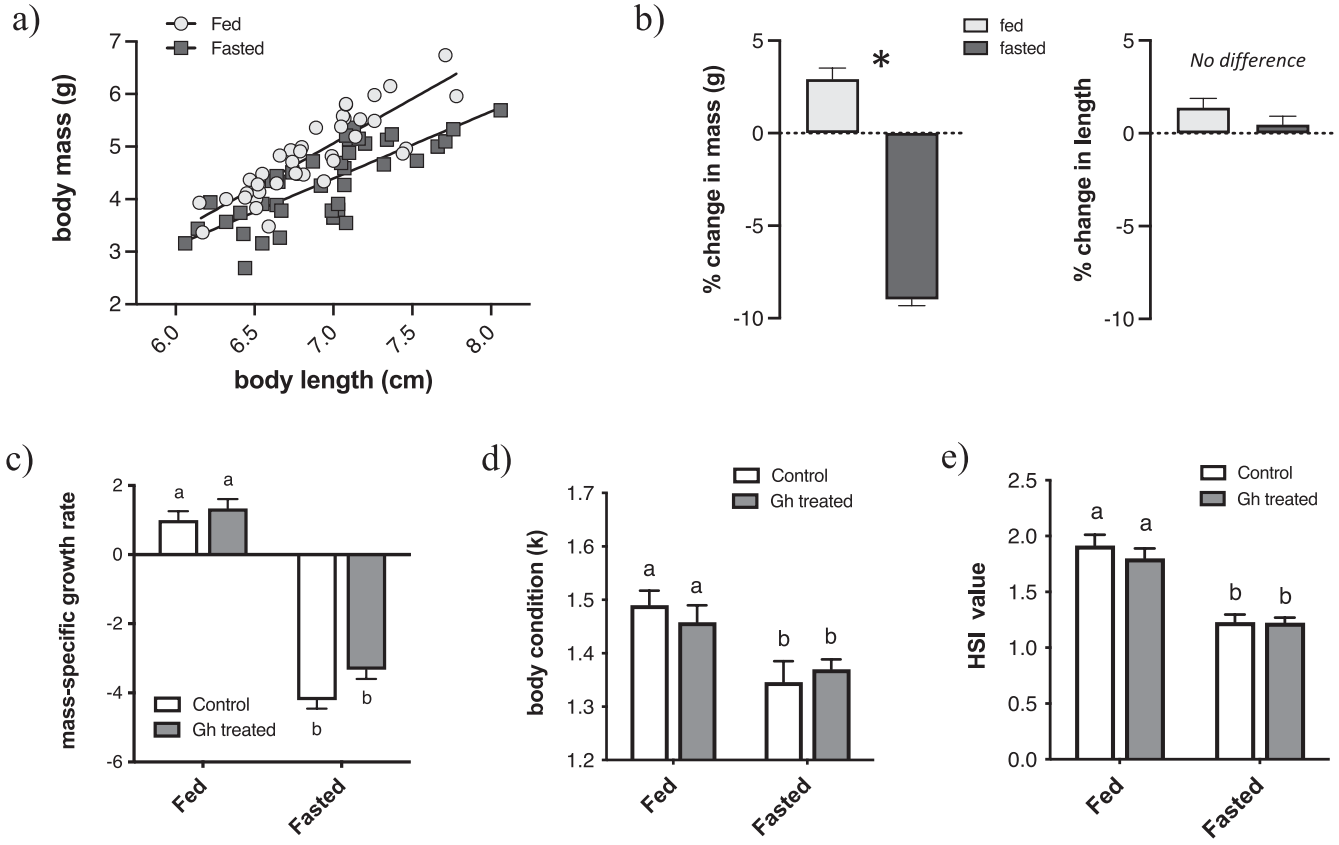

Fig. 1. (a) Food deprivation for 14 d resulted in fasting gopher rockfish having a lower body mass than continuously fed fish at the same body length (total length). (b) Fasted fish showed a decline in body mass while fed fish increased in body mass, even though no difference in change in body length was detected. (c) The change in mass resulted in a negative mass-specific growth rate for fasted rockfish. (d) Body condition factor (k) was lower in fasted rockfish. (e) Fasting also led to lower hepatosomatic index (HSI) values. Letters reflect post-hoc Tukey's HSD comparisons.

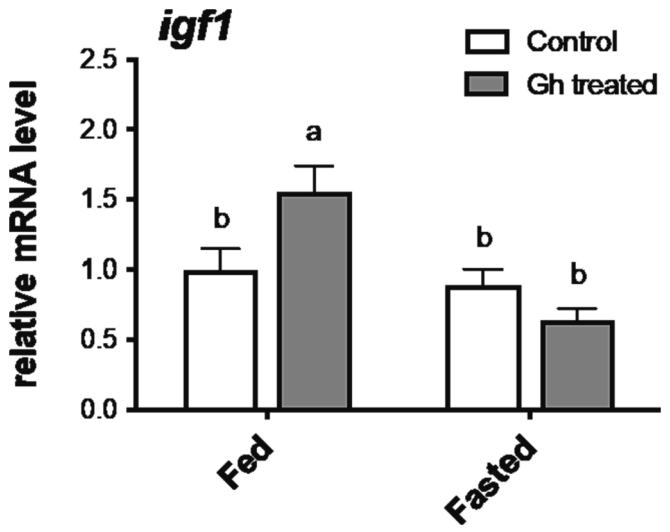

Fig. 2. Effects of fasting and Gh on liver *igf1* mRNA values. Fed rockfish treated with Gh showed elevated liver *igf1* mRNA levels compared to fed fish treated with vehicle control. Fasted fish showed no difference in hepatic *igf1* mRNA level following Gh or control injection (feed*injection interaction: $F_{1,72}$ = 9.259, p = 0.0033). Letters show post-hoc Tukey's HSD comparisons.

rockfish, respectively, following treatment with Gh (feed*injection interaction: $F_{1,74} = 4.396$, $p = 0.0394$). Fasting downregulated liver *ghr2* mRNA levels ($F_{1,68}$ = 5.266, p = 0.0248), and Gh treatment led to increased liver *ghr2* mRNA levels in both fed and fasted fish ($F_{1,68}$ = 4.122, $p = 0.0462$) (Fig. 3b), although these effects were not sufficiently large to be detected in multiple pairwise comparisons (Tukey HSD tests).

Looking downstream in the Gh transduction pathway, liver pJak2 and pStat5(A/B) concentrations differed among fish depending on fasting experience and Gh administration. Fasted rockfish showed lower concentrations of pJak2 in the liver (Fig. 4a) ($F_{1,74} = 9.118$, $p = 0.0035$). Treatment of fish with Gh also resulting in lower liver pJak2 concentrations in both fed and fasted rockfish ($F_{1,74} = 5.249$, $p = 0.0248$). Food deprivation also resulted in a reduced abundance of pStat5(A/B) protein in the liver ($F_{1,75} = 26.130$, $p < 0.001$) (Fig. 4b). Unlike pJak2, rockfish treated with Gh showed higher concentrations of pStat5(A/B) in the liver under both feeding and fasting conditions (injection effect: $F_{1,75}$ = 4.311, p = 0.0413).

We also detected fasting and Gh effects on hepatic gene transcript abundances of *jak2* and *stat5b*, although those effects were less pronounced than what was observed for the phosphorylated proteins of Jak2 and Stat5. Fasted fish had lower liver *jak2* mRNA abundance (feed effect: $F_{1,74} = 5.217$, $p = 0.0252$), with that reduction most apparent in fasted fish treated with Gh (Appendix A, Supplemental Fig. S1a). In contrast, liver *stat5b* gene transcript abundance was unaffected by the different feeding regimes; fish given exogenous Gh, however, had generally lower liver *stat5b* mRNA levels (injection effect: $F_{1,74} = 5.123$, p = 0.0265) (Appendix A, Supplemental Fig. S1b). The relative abundance of liver gene transcripts encoding the transcription factor *hnf1α* was also compared among treatment groups. Neither feeding nor injection treatment altered mRNA levels of *hnf1α* (Appendix A, Supplemental Fig. S2).

Transcripts encoding Socs genes in the liver varied in relative abundance depending on feeding treatment and Gh administration. Transcript levels of *socs1*, which inhibits anabolic pathways resulting in Igf1 synthesis (Studzinski et al., 2009), were not affected by either feeding treatment or Gh injection (Fig. 5a). However, expression levels for gene transcripts encoding three other Socs involved in the catabolic regulation of *igf1*- synthesis – *socs2, socs3a* and *socs3b* – all were lower in abundance in the liver of fasted rockfish (*socs2:* $F_{1,74}$ = 8.150, p = 0.0056; *socs3a*: $F_{1,74}$ = 4.188, p = 0.0443; *socs3b*: $F_{1,74}$ = 4.859, p =

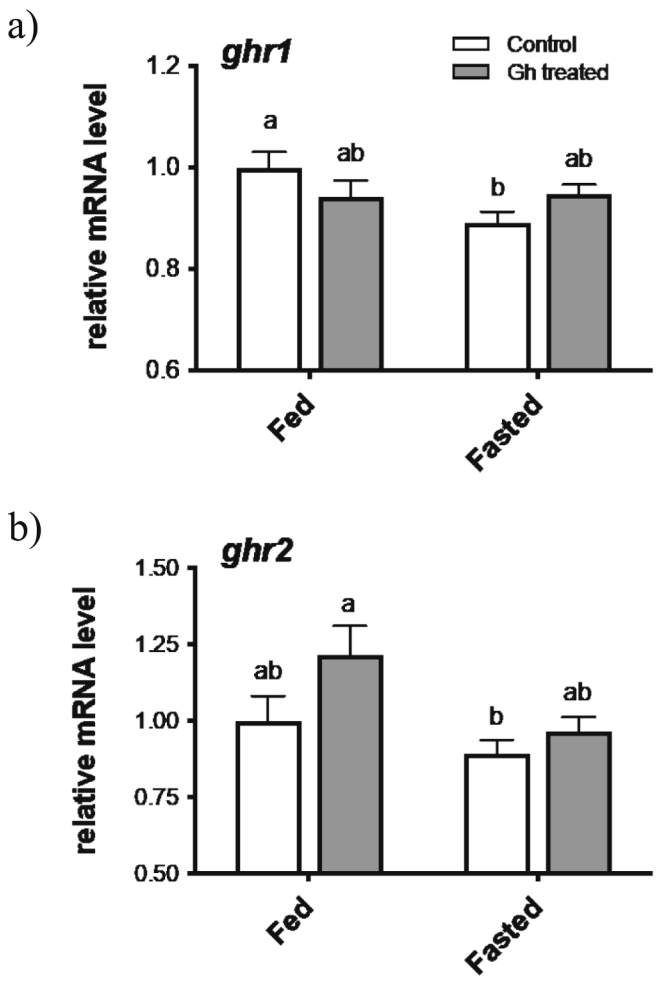

Fig. 3. Abundance of mRNAs encoding *ghr1* and *ghr2* following fasting and Gh treatment. (a) Transcripts encoding receptor *ghr1* were less abundant in the liver of fasted fish than in fed fish, and showed opposing patterns of up- and down-regulation in fasted and fed rockfish, respectively, following treatment with Gh. (b) Fasting decreased abundance of transcripts encoding *ghr2*, while Gh treatment increased transcript abundance in both fed and fasted fish. Letters show post-hoc Tukey's HSD comparisons.

0.0306) (Fig. 5b-d). Gh increased hepatic *socs2* mRNAs in fed fish ($F_{1,74}$ = 15.669, p = 0.0002), but did not affect liver *socs3a* or *socs3b* mRNA levels in either fed or fasted rockfish.

Gene transcripts for hormone sensitive lipase type 1 (*hsl1*), an enzyme that promotes lipolysis, were upregulated with fasting and showed a clear pattern of downregulated expression in fasted fish after Gh treatment, but not in fed fish given exogenous Gh (feed*fasting interaction: $F_{1,72} = 6.273$, p = 0.0145) (Fig. 6).

3.3. Fasting and Gh regulate liver Igf binding protein gene expression

Both fasting and Gh injection also influenced expression levels of gene transcripts encoding Igfbps in the liver, with specific effects varying depending on Igfbp type (Fig. 7). Fasted fish exhibited higher liver mRNA abundance for both *igfbp1a* ($F_{1,74}$ = 13.256, p = 0.0005) and *igfbp1b* ($F_{1,74} = 4.820$, $p = 0.0313$). Gh injection significantly decreased liver expression levels of transcripts encoding *igfbp1a* ($F_{1,74} = 12.012$, p = 0.0009) and *igfbp1b* ($F_{1,74}$ = 16.763, p = 0.0001). Rockfish experiencing food deprivation also showed elevated levels of *igfbp3a* mRNAs in the liver ($F_{1,73} = 12.352$, p = 0.0008). However, Gh had no effect on *igfbp3a* gene expression in either fasted or fed fish.

Other Igfbps exhibited an opposing pattern of lower relative

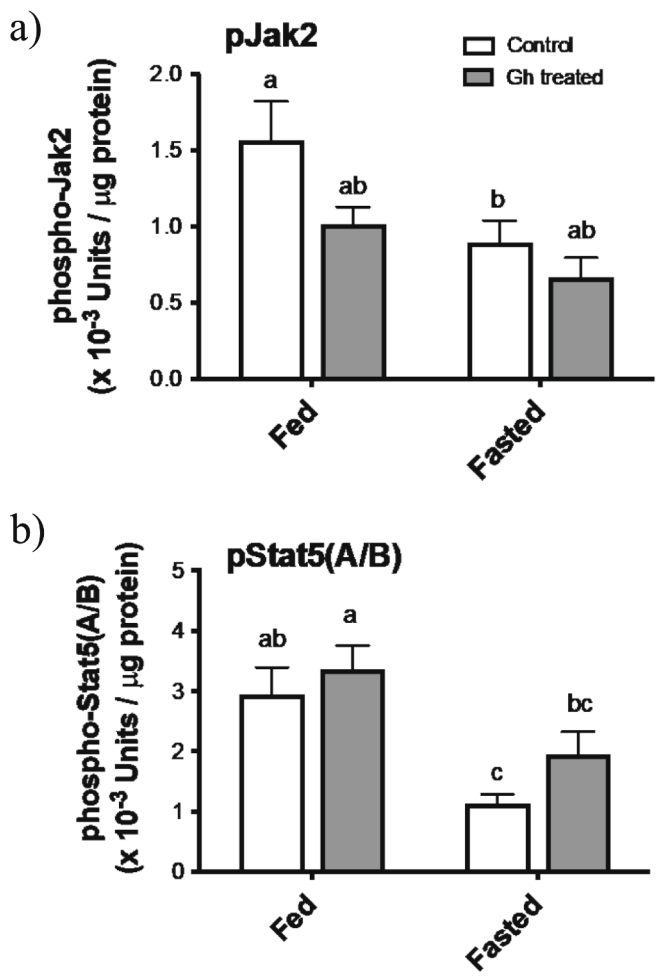

Fig. 4. Effects of fasting and Gh injection on levels of phosphorylated Jak2 and Stat5(A/B) proteins. (a) Fasted rockfish had a lower liver abundance of pJak2, and Gh decreased pJak2 concentrations in both fed and fasted fish. (b) Food deprivation for 14 d likewise reduced the liver concentration of pStat5(A/B) protein. However, unlike pJak2, exogenous Gh increased pStat5(A/B) in the liver of both fed and fasted rockfish. Letters denote pairwise differences (Tukey's HSD comparisons).

expression in the liver of gopher rockfish experiencing food deprivation. Transcript abundances for liver *igfbp2a* and *igfbp2b* (Fig. 7) were each lower in the liver of fasted rockfish (*igfbp2a*: $F_{1,74} = 7.069$, $p = 0.0096$; *igfbp2b*: $F_{1,74} = 12.049$, $p = 0.0009$), even though treatment with exogenous Gh had no effect on liver *igfbp2a* and *igfbp2b* mRNA levels in either feeding group. And, while gene transcripts encoding *igfbp3b* were at similar relative levels across fed and food-deprived rockfish ($F_{1,74} = 2.023$, $p = 0.1591$), Gh injection marginally decreased hepatic *igfbp3b* mRNA abundance ($F_{1,74} = 4.010$, $p = 0.0489$), although that effect appeared very minor in fed fish.

We also observed that fasted rockfish expressed lower relative levels of mRNAs encoding an *igfals*-like gene in the liver (Fig. 8, $F1_{,73} = 10.185$, $p = 0.0021$). Increased hepatic *igfals* mRNA abundance was also observed in both fed and fasted rockfish treated with Gh ($F_{1,73} = 5.196$, $p = 0.0256$).

3.4. Effects of fasting and Gh on skeletal muscle Gh/Igf signaling gene pathways

In skeletal muscle, mRNAs encoding Gh receptor 1 (*ghr1*) showed disparate responses to Gh depending on rockfish nutritional status, with *ghr1* mRNAs being elevated with Gh injection in fed rockfish but decreasing following Gh treatment of fasted fish (Fig. 9a) (feeding*injection interaction, $F_{1,71} = 4.797$, $p = 0.0318$), although the effects were minor enough to not be detected statistically in *post hoc* pairwise comparisons. Fasted rockfish also showed a nearly 4-fold increase in skeletal muscle Gh receptor 2 (*ghr2*) mRNA levels compared to fed fish (Fig. 9b) ($F_{1,70} = 18.661$, $p < 0.0001$). Gh increased muscle *ghr2* mRNA levels significantly in both fed and fasted rockfish ($F_{1,70} = 14.809$, $p = 0.0003$). Fasted rockfish also exhibited greater relative expression of mRNAs encoding the Igf1 receptor genes *Igf1ra* ($F_{1,70} = 5.131$, $p = 0.0266$) and *Igf1rb* ($F_{1,70} = 4.190$, $p = 0.0444$) in skeletal muscle relative to fed that continued to be fed (Fig. 9c,d). Gh had no effect on muscle mRNA levels of either *igf1ra* or *igf1rb* in either feeding group.

Food deprivation and exogenous Gh interacted to alter relative *igf1* mRNA levels in fast-twitch skeletal muscle (Fig. 9e) (feeding*injection interaction, $F_{1,69} = 4.642$, $p = 0.0347$). Fasted, control-injected rockfish showing a nearly 70 % reduction in muscle *igf1* mRNA levels relative to fed, control-injected fish. Gh administration led to an increase in muscle *igf1* transcripts in fasted rockfish, but not in fed fish. Neither feeding status nor Gh administration had any effect on muscle *igf2* mRNA abundance (Fig. 9f). As observed in the liver, both fasting and Gh injection regulated mRNA expression of genes encoding Igfbps in muscle, with direction and degree of regulation differing with type of Igfbp (Fig. 9g). Fasting led to higher relative mRNA levels of both *igfbp1a* ($F_{1,71} = 5.115$, $p = 0.0268$) and *igfbp1b* ($F_{1,71} = 5.131$, $p = 0.0272$). Gh had no effect on muscle mRNA levels for *igfbp1a* or *igfbp1b* in either feeding treatment. Neither feeding nor injection treatment affected expression of *igfbp2a* or *igfbp3b*. While fasting had no effect on muscle *igfbp2b* mRNA levels, rockfish treated with Gh showed a lower relative mRNA abundance for *igfbp2b* in skeletal muscle ($F_{1,68} = 6.260$, $p = 0.0148$), although the degree of that Gh-induced decline was minor enough to not be statistically significant in *post hoc* pairwise comparisons (Fig. 9g). In contrast, fasting increased muscle mRNA expression of transcripts encoding *igfbp3a* ($F_{1,69} = 9.912$, $p = 0.0024$), while Gh injection had no effect.

4. Discussion

When fish experience conditions of food deprivation and nutritional stress, Gh secretion can be elevated without any corresponding increase in liver Igf1 production (Fox et al., 2006, 2009). Here, we tested several non-exclusive mechanisms that might underlie changes in liver sensitivity to Gh in fish experiencing fasting using juvenile gopher rockfish as a model. We found evidence that the liver of fasted rockfish experiences several changes that shift *igf1* induction by Gh during food deprivation.

The 14 d fasting period for gopher rockfish caused significant reductions in body mass leading to a negative mass-SGR. Fasted gopher rockfish also showed changes in liver status reflected as lower HSI values compared to fed fish. The liver is a primary source of glycogen storage in teleost fishes. Studies specifically in *Sebastes* rockfishes have found that, in addition to glycogen, the liver stores high concentrations of triacylglycerols and other lipids (Oliveira et al., 2009). The lower HSI values observed here in the food-deprived gopher rockfish thus likely reflect the depletion of these energy stores from hepatocytes (Power et al., 2000; De Pedro et al., 2003; Barcellos et al., 2010), suggesting the 14 d fasting period was likely sufficient to activate catabolic pathways.

Even though fasted rockfish controls showed no difference in liver *igf1* mRNA abundance relative to fed control fish, liver *igf1* mRNAs were upregulated by Gh in fed rockfish but not in fasted rockfish. That difference in liver *igf1* mRNA responses to Gh likely reflects differences in Igf1 production. The abundance of *igf1* mRNAs in the liver has been shown to relate positively to circulating Igf1 hormone concentrations in *Sebastes* rockfishes (Hack et al., 2019; Bersin et al., 2023b), as well as in cabezon (Strobel et al., 2020), another fish belonging to order Scorpaeniformes. Furthermore, in many fish species, liver *igf1* mRNAs and plasma Igf1 concentrations both decline under conditions of food

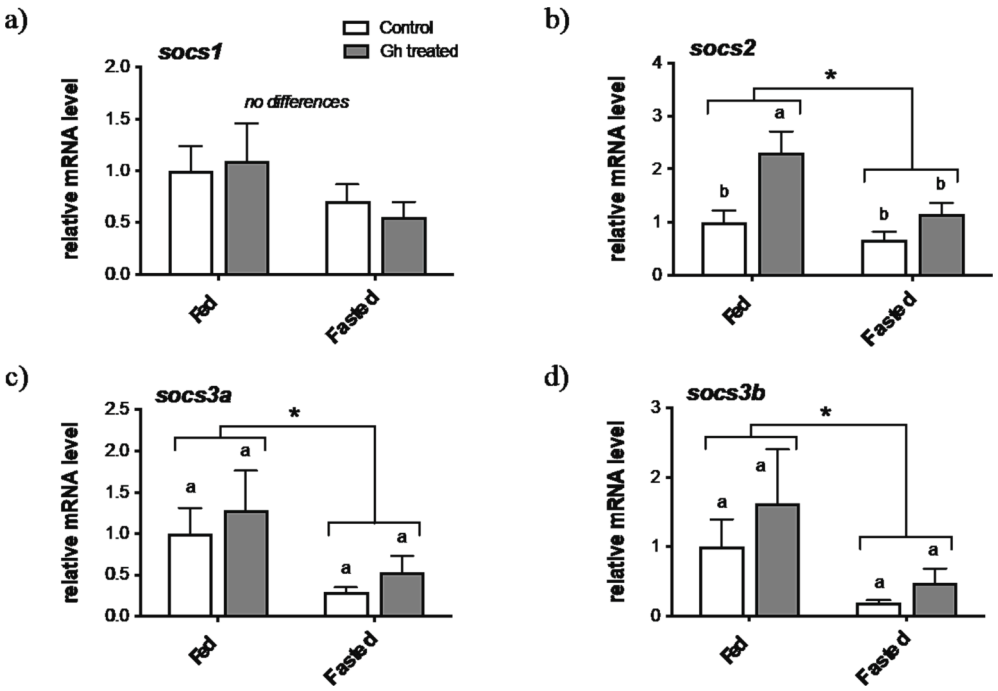

Fig. 5. Effects of nutritional experience and Gh administration on transcripts encoding suppressors of cytokine signaling (*socs*) genes in the liver. (a) Neither fasting nor Gh affected hepatic mRNA levels of *socs1*. However, fasting decreased the relative abundance of gene transcripts encoding hepatic *socs2* (b), *socs3a* (c), and *socs3b* (d). Gh injection increased hepatic mRNA levels of *socs2* mRNAs in fed fish (b), but did not affect liver *socs3a* or *socs3b* mRNA levels in either fed or fasted rockfish (c, d). Asterisks indicate $p < 0.05$ for feeding treatment main effects from ANOVA models.

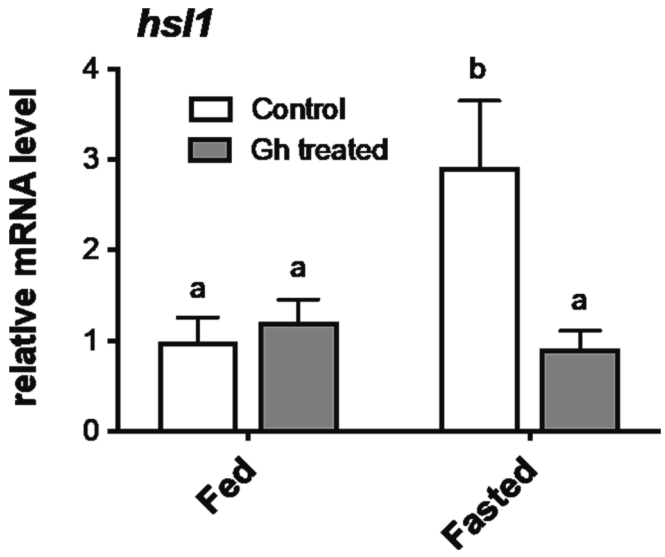

Fig. 6. Liver mRNA levels of the hormone sensitive lipase-1 (*hsl1*) gene were higher in fasted rockfish than fed rockfish given saline control injections, and also showed a reduction in fasted fish administered Gh, but not fed fish given Gh. Letters indicate *post hoc* pairwise differences (Tukey HSD test).

deprivation (Peterson et al., 2005; Pierce et al., 2005; Pedroso et al., 2006; Vera Cruz et al., 2006; Picha et al., 2008b; Fox et al., 2010; Beckman, 2011; Kawaguchi et al., 2013; Hack et al., 2019; Bersin et al., 2023a). While lower liver *igf1* mRNAs were not observed in gopher rockfish administered only 'control' vehicle injections, the observed lack of Gh stimulation of liver *igf1* mRNA levels in fasted gopher rockfish implies diminished liver Igf1 synthesis pathway sensitivity to Gh in fasted fish.

4.1. Fasting-induced changes in hepatic Gh receptor transcript expression

Fasted juvenile rockfish showed reduced hepatic expression of both Gh receptor genes *ghr1* and *ghr2*. Those reductions in liver Gh receptor mRNA abundance imply that the capacity of the liver to respond to Gh may have been altered by fasting. Both teleost Gh receptor 1 and 2 are heavily expressed in the liver (Jiao et al., 2006; Botta et al., 2016; Liang et al., 2019), and both receptors are involved in Gh induction of *igf1* gene expression (Reindl and Sheridan, 2012; Bergan-Roller and Sheridan, 2018). Several studies in fishes have documented patterns of downregulation in the expression of one or both hepatic Gh receptors by food deprivation, implying that hepatic sensitivity to Gh can be reduced under nutritional stress (Small et al., 2006; Norbeck et al., 2007; Botta et al., 2016; Strobel et al., 2020). Supporting that idea, the liver of nutritionally stressed fish can exhibit diminished Gh binding (Gray et al., 1992; Pérez-Sánchez et al., 1994; Deng et al., 2004; Norbeck et al., 2007; Liang et al., 2019). For example, black seabream (*Acanthopagrus schlegeli*) placed under conditions of complete food deprivation showed reduced hepatic Gh binding after just 2 d, and expressed liver Gh receptor mRNAs at a level only ~30 % of that of fed control fish after 30 d of fasting (Deng et al., 2004). Similarly, rainbow trout fasted for 2 or 6 weeks had lower liver ^{125}I-Gh binding capacity as well as decreased relative mRNA levels for both *ghr1* and *ghr2* (Norbeck et al., 2007).

Of the two Gh receptors in teleost fishes, studies have sometimes found that Ghr1 changes expression more under conditions of nutritional stress, with downregulation of transcripts encoding *ghr1* documented in both rainbow trout (Walock et al., 2014) and cabezon (Strobel et al., 2020). In the present study, fasting downregulated expression of both *ghr1* and *ghr2*. However, treatment of gopher rockfish with Gh upregulated mRNA levels of *ghr1* under fasted conditions but resulted in lower relative *ghr1* mRNA levels in fish under fed conditions. In contrast, Gh increased mRNA levels of *ghr2* in both fasted and fed fish. In another study with juvenile gopher rockfish, a single 2 h period of refeeding after 30 d of fasting upregulated liver gene transcript abundance for *ghr1*, but not for *ghr2* (Bersin et al., 2023b). Taken together, those observations

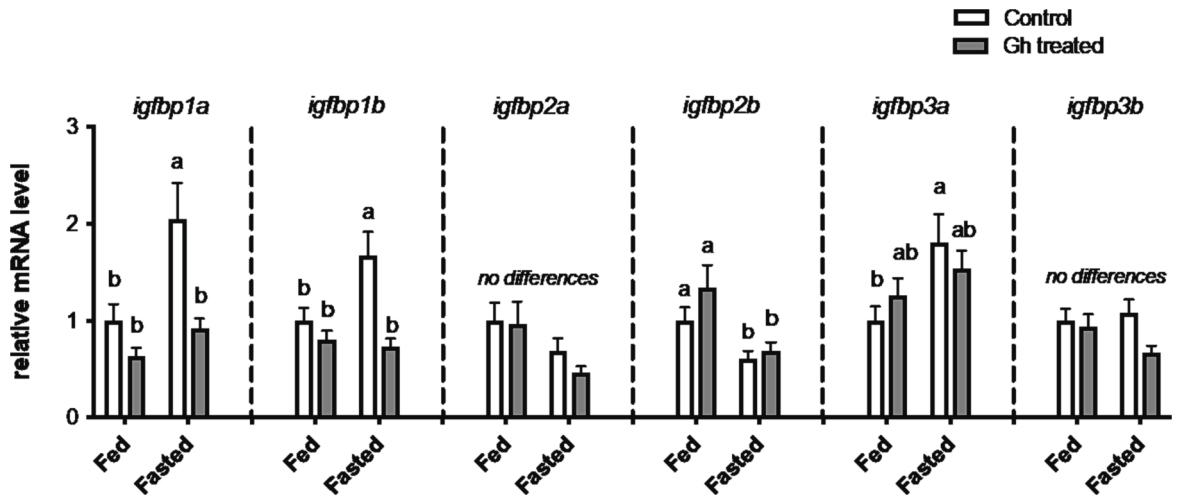

Fig. 7. Effects of fasting and Gh on relative mRNA levels of genes encoding hepatic Igf binding proteins (Igfbp) 1a, 1b, 2a, 2b, 3a, and 3b. Fasting increased relative abundance of mRNA transcripts encoding *igfbp1a*, *igfbp1b*, and *igfbp3a*, and decreased abundance of transcripts encoding *igfbp2b*. Gh injection decreased expression of transcripts encoding *igfbp1a* and *igfbp1b* in both fed and fasted fish. Letters reflect post-hoc Tukey's HSD groupings for each binding protein evaluated separately.

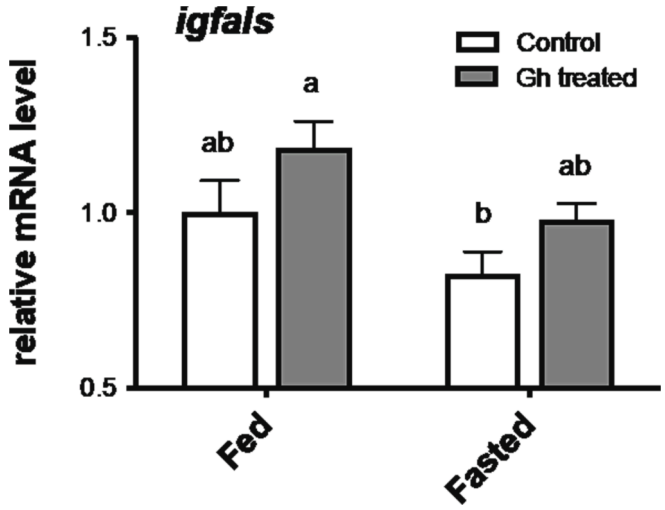

Fig. 8. Fasting and Gh effects on hepatic mRNAs encoding an acid labile subunit-like protein (*igfals)*. Fasted rockfish expressed greater relative levels of *igfals* mRNAs in the liver, and Gh administration increased hepatic *igfals* mRNAs in both feeding groups. Letters indicate Tukey's HSD pairwise comparisons.

point to differences in *ghr1* and *ghr2* regulation by Gh that are dependent in part on recent feeding experience. While such differences in Gh receptor-type expressional regulation have not been seen in all fishes (Norbeck et al., 2007), greater sensitivity of *ghr1* gene regulation to fasting/feeding was observed in gilthead sea bream (*Sparus aurata*) (Saera-Vila et al., 2005). And, in the pejerrey *Odontesthes bonariensis*, a silverside fish of southeastern and southern central South America, liver *ghr1* gene transcripts increased 4.5 fold, while *ghr2* transcripts increased only 2.1 fold, in fish administered Gh once a week over a 4 week period (Botta et al., 2016). While more work is needed to understand these differential responses of liver *ghr1* and *ghr2* expression to Gh stimulation – as well as how recent feeding experience might modulate those responses – the downregulation of Gh receptors and decreased capacity for liver Gh action under nutritional stress likely contributed to the lack of *igf1* gene expression response in faster gopher rockfish treated with Gh.

4.2. Fasting modulation of Gh regulation of liver Jak2/Stat5 signaling pathways

The Jak2/Stat5b intracellular signaling pathway acts downstream of Gh receptor activation to increase production of Igf1 by hepatocytes (Herrington and Carter-Su, 2001; Kittilson et al., 2011; Reindl et al., 2011; Chia, 2014; Bergan-Roller and Sheridan, 2018; Dehkhoda et al., 2018). Two Jak2 kinases bind to the intracellular domain of Gh receptor homodimers (Brown et al., 2005; Brooks et al., 2014). Those two Jak2s phosphorylate each other, autophosphorylate themselves, and phosphorylate tyrosine residues of the Gh receptor (Herrington and Carter-Su, 2001). That phosphorylation allows Stat5b binding to the intracellular region of the receptor (Derr et al., 2011). Receptor-bound Stat5b proteins are then themselves phosphorylated by pJak2, which results in pStat5b releasing from the receptor and translocating to the nucleus of the cell where the pStat5b homodimer acts as a transcription factor to promote *igf1* gene transcription (Mohr et al., 2012; Smit et al., 1996; Bergan-Roller and Sheridan, 2018; Dehkhoda et al., 2018).

As Jak2 kinase is activated by phosphorylation, and in turn activates Stat5b though phosphorylation as well, we quantified concentrations of both phosphorylated proteins in the rockfish liver as a measure of Jak2/Stat5b pathway activation. Juvenile gopher rockfish that were fasted for 14 d showed significantly reduced levels of both pJak2 and pStat5 in the liver. Those lower pJak2 and pStat5 levels suggest lower liver Jak2/Stat5b pathway stimulation in rockfish experiencing food deprivation. Such reduced Jak2/Stat5b pathway activation in fasted rockfish might have resulted from either changes in circulating Gh or – as suggested by the lower hepatic *ghr1* and *ghr2* mRNAs in these rockfish – down-regulated liver Gh binding capacity. Prior work in rainbow trout observed levels of pJak2 and pStat5 in the liver were also altered by food limitation (Bergan et al., 2012). In that work, trout experiencing 2 or 4 weeks of fasting showed lower pJak2 abundance in liver, slow- and fast-twitch skeletal muscle, and adipose tissues, while the refeeding of trout that experienced the 4 week period of fasting recovered those reduced pJak2 levels back to concentrations of trout that were fed continuously over that same 4 week period (Bergan et al., 2012).

Since nutrition-related changes in liver status have the potential to alter the response of the Jak2/Stat5b pathway to Gh stimulation, we also measured levels of hepatic pJak2 and pStat5 after *in vivo* Gh administration. While Gh increased hepatic levels of pStat5(A/B) in gopher rockfish from both the fed and fasting treatments, pJak2 levels were lower after Gh treatment. Prior work using isolated hepatocytes from rainbow trout found that Gh upregulated both pJak2 and pStat5 (Reindl

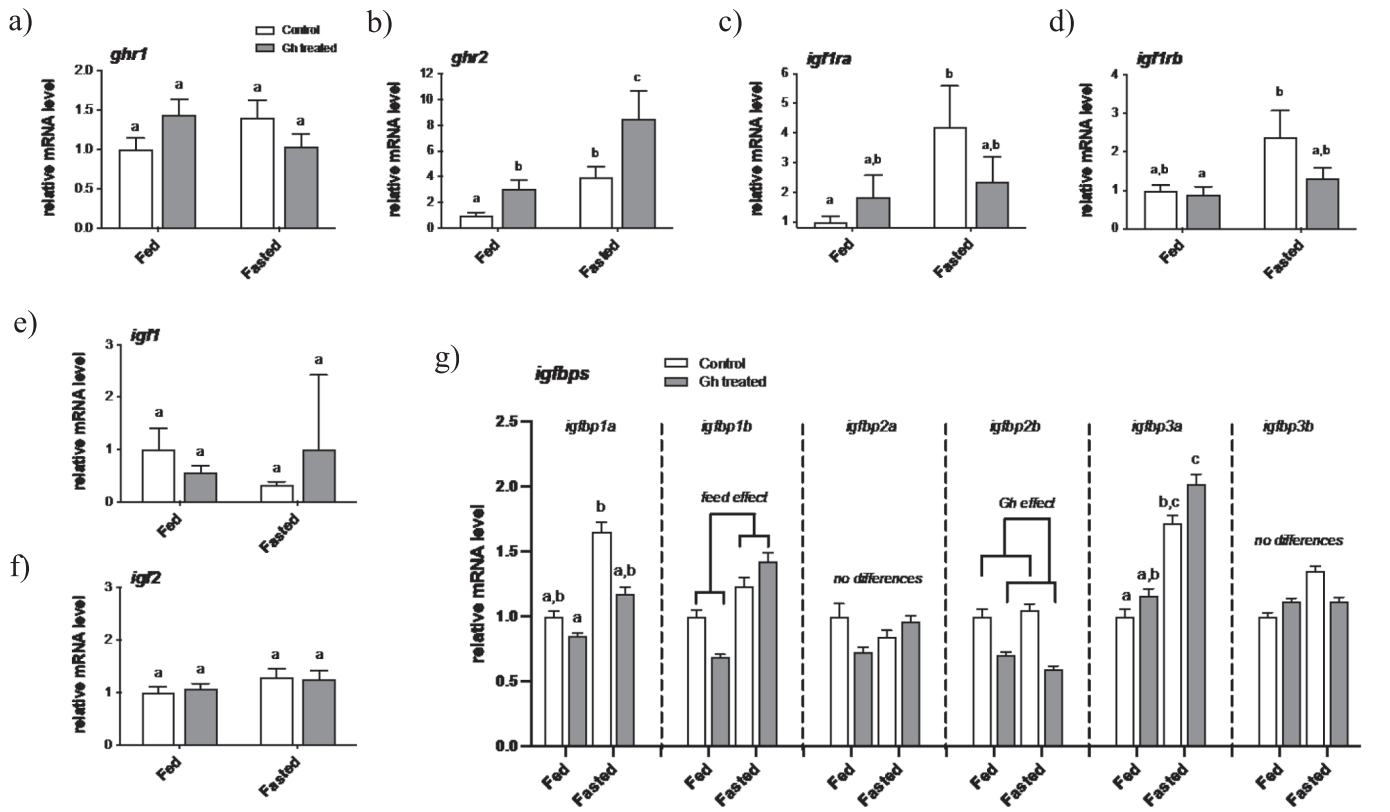

Fig. 9. Effects of nutritional experience on Gh/Igf1 signaling components in skeletal muscle. Fasting increased muscle abundance of gene transcripts encoding Gh receptors *ghr1* and *ghr2* (a,b), and Gh increased muscle *ghr2* mRNA abundance in both fed and fasted rockfish. Fasting increased relative mRNA levels for Igf1 receptors *igf1ra* and *igf1rb* (c,d). Gh had no effect on muscle mRNA levels of either *igf1ra* or *igf1rb*. (e) Even though fasting and Gh had significant interactive effects on muscle *igf1* mRNA levels (feeding*injection interaction, $F_{1,69} = 4.642$, $p = 0.0347$), those effects were not apparent in pairwise *post hoc* comparisons. (f) Neither fasting nor Gh had any effect on muscle *igf2* mRNA abundance. (g) Effects of fasting and Gh on mRNA levels of Igfbps depended on binding protein type. Bars for *igfbp1b* and *igfbp2b* indicate significant ANOVA effect without any significant pairwise differences. Letters reflect *post hoc* pairwise differences (Tukey's HSD tests).

et al., 2011). Those Gh-induced changes in pJak2 and pStat5 were rapid, with peak levels of both phosphorylated proteins occurring 5 min after Gh treatment and then remaining elevated, but slowly declining, through 1 h after Gh administration (Reindl et al., 2011). While time courses in cell culture versus *in vivo* are likely to vary, the speed by which pJak and pStat5 in trout hepatocytes responded to Gh may help explain why pJak2 abundance was actually lower in Gh-treated rockfish – and not higher as expected – while pStat5 increased. The 24 h time period between injection with recombinant Gh and tissue sampling may have missed any peak in Gh-mediated Jak2 phosphorylation. However, controlled studies using cell culture have found nucleocytoplasmic shuttling of the phosphorylated Stat5b dimer, as well as other Stat proteins, into the nucleus occurs over tens of min to h (Zeng et al., 2002; Mohr et al., 2012). Given elevated liver pStat, but not pJak2, was observed in Gh-treated gopher rockfish, it is possible that pStat5 was retained longer than pJak2 as the phosphorylated and dimerized pStat5 was shuttled to the nucleus. In any case, the fasting-associated declines in the concentrations of both pJak2 and pStat5 suggests that a down-regulation of the Jak2/Stat5b pathway may be contributing to the changing sensitivity of liver Igf1 synthesis in fish experiencing food deprivation.

Stat5 activates *igf1* gene transcript via synergistic interactions with other gene transcription factors including hepatic nuclear factors (Hnfs) (Kulik et al., 1995; Metón et al., 1999; Lin et al., 2014), so we also explored whether any variation in relative mRNA levels of *hnf1α* might be detectable in patterns linked to Gh treatment or feeding status. We did not, however, detect any changes in relative *hnf1α* mRNA levels linked either to feeding/fasting or to Gh administration (Appendix A, Supplemental Fig. S2).

4.3. Fasting effects on Igf1-inhibiting and catabolic pathways

Several other proteins have been identified that play key roles in regulating Gh intracellular signaling pathways. Socs proteins are best understood as regulators of innate and adaptive immune systems (Tamiya et al., 2011; Strebovsky et al., 2012), but have also been linked to Gh/Igf1 signaling regulation via effects on the Jak2/Stat5 pathway (Greenhalgh et al., 2002, 2005; Croker et al., 2008). In mammals, GH induces expression of SOCS1, -2 and -3 (Adams et al., 1998; Tollet-Egnell et al., 1999). Several SOCS forms interact with the mammalian GH receptor (Favre et al., 1999; Hansen et al., 1999; Greenhalgh et al., 2002), although SOCS2 appears to be the primary SOCS with inhibitory influences on GH signaling (Greenhalgh et al., 2002). SOCS2-knockout mice exhibit larger size and body mass, longer bone lengths, and increased IGF1, providing a picture of SOCS2 as a negative regulator of GH mediated IGF1 production and growth (Metcalf et al., 2000).

There also evidence in fish for Socs having a regulatory role in Gh/Igf1 signaling. Homozygous Gh-transgenic zebrafish (*Danio rerio*) expressing high concentrations of Gh have slower growth and lower liver *igf1* mRNA abundance than hemizygous individuals, with those effects linked to elevated expression of *socs1* and *socs3* (Studzinski et al., 2009). Overexpression of *socs2* in grass carp (*Ctenopharyngodon idellus*) cultured hepatocytes transfected with Gh receptor suppressed Jak2/Stat5 signaling as well as diminished *igf1* gene expression (Jiang et al., 2016). Combined, such evidence points to Socs2 acting as negative control on Igf1 production in fish by disrupting Gh stimulation of Jak2/Stat5b signaling (Bergan-Roller and Sheridan, 2018).

Here, we examined how mRNA levels of transcripts encoding *socs1*, *socs2*, *socs3a* and *socs3b* varied in the gopher rockfish liver with feeding/

fasting and Gh stimulation. While levels of transcript encoding *socs1* did not vary either fasting or Gh stimulation, mRNAs encoding *socs2*, *socs3a*, and *socs3b* were each at lower relative abundance in the liver of fasted rockfish. Similar to what was observed previously in carp hepatocytes (Jiang et al., 2016), we also found *socs2* mRNA levels to be elevated in fed rockfish after Gh treatment. Messenger RNAs encoding *socs2* – but not *socs1* or *socs3* – were similarly found to increase in the liver of juvenile male Nile tilapia (*Oreochromis niloticus*) following exogenous Igf1 treatment (Liu et al., 2018). Using recombinant Igf1 in a dual-luciferase reporter assay, Liu et al. (2018) observed that Igf1 induced *socs2* transcription but reduced *socs1* and *socs3* mRNA levels in cultured tilapia hepatocytes. Our observations here *in vivo* that mRNA abundances for *socs2* and *socs3a* and *-b* were affected by fasting – and that Gh induced an increase in liver *socs2* mRNAs – supports the idea that nutritional-related changes in liver Socs2 and -3 expression may contribute to the nutritional modulation of hepatic Igf1 synthesis.

Here, we also observed that fasted gopher rockfish showed elevated *hsl1* mRNA levels in the liver compared to their fed counterparts, and found that Gh reduced liver *hsl1* mRNA levels in fasted rockfish only. Gh can play both a stimulatory and an inhibitory role in lipolytic regulation in the liver via actions on gene expression for lipolytic pathways (Bergan-Roller and Sheridan, 2018). Bergan et al. (2015) observed Gh to stimulate increases in *hsl1* and *hsl2* gene expression in hepatocytes from fasted rainbow trout, but not in cells isolated from fed trout. Such *hsl* gene regulation has likewise been observed in other studies with fish (Bergan et al., 2013; Vélez et al., 2019), pointing to Gh stimulation of lipolytic actions under some conditions. Our observations here with rockfish support Gh having some regulatory control over liver Hsl expression – and thus modulating lipolysis mechanisms in rockfish experiencing food deprivation. However, the disparate pattern of change observed in *hsl1* mRNA levels in the rockfish liver compared to isolated trout hepatocytes (Bergan et al., 2013, 2015) is harder to interpret. While the difference of *in vivo* administration to rockfish here and *in vitro* hepatocyte or liver section culture in studies with trout and sea bream (Bergan et al., 2013, 2015; Vélez et al., 2019) might contribute to dissimilar regulation for *hsl*, it is also possible that species variation, the duration and dose of Gh exposure, or one of several other factors that generated variation in the physiological state of the liver cells when exposed to Gh in these studies might have impacted Gh's lipolytic actions (Sheridan, 1986, 1994).

4.4. Fasting- and Gh-induced changes in other components of Gh/Igf signaling in liver and skeletal muscle

Igfbps regulate Igf1 availability in blood circulation and target tissues, with some forms facilitating Igf1 action and other having inhibitory influences (Shimizu and Dickhoff, 2017; Allard and Duan, 2018). Here, we observed that fasted gopher rockfish had higher hepatic mRNA abundances for *igfbp1a, -1b*, and *igfbp-3a*, as well as reduced liver mRNA levels for *igfbp2b*. Prior studies observed hepatic mRNA and blood protein levels of Igfbp types 1a and 1b to increase in fish experiencing food limitation (Shimizu et al., 1999; Kelley et al., 2002; Peterson and Small, 2004; Picha et al., 2008b; Hevrøy et al., 2011; Kawaguchi et al., 2013; Breves et al., 2014, 2016; Kaneko et al., 2020). That pattern corresponds with another recent study in juvenile gopher rockfish that observed increases in *igfbp1a* and *igfbp1b* gene transcripts in the liver of fish fasted for 14 d (Bersin et al., 2023a). Higher liver transcript abundances for *igfbp1a* and *igfbp1b* were similarly observed in copper rockfish fasted for 12 d (Hack et al., 2019), and olive rockfish maintained under a lower feed amount for 98 d (Hack et al., 2018). Teleost Igfbp1a and -1b are thought to be negative regulators of Igf1 action (Shimizu and Dickhoff, 2017; Garcia de la Serrana and Macqueen, 2018), and elevated liver mRNA levels for *igfbp1a* and *igfbp1b* in gopher rockfish experiencing food deprivation are interpreted as a response to reduce Igf1 availability and growth promoting actions on target tissues.

Igfbp3 likely also has a regulatory role for Igf1 action in fishes, although its functions are not clear. In mammals, IGFBP3 serves as the main carrier of circulating IGF1 (Boisclair et al., 2001). Human IGFBP3 facilitates IGF1 transport via interactions as a complex with IGFALS, and low expression of IGFALS leads to lower circulating IGF1 and IGFBP3 concentrations as well as impaired growth (Domené and Domené, 2020). And yet, the role of Igfbp3 in teleost fishes – and whether that binding protein interacts with a putative Igfals protein expressed from the *igfals* gene examined here – remains unknown. Despite hepatic *igfbp1a, -1b*, and *-3a* each being elevated in mRNA abundance in fasted gopher rockfish here and in a prior study, liver *igfbp3a* is regulated by Igf1 in a different pattern than *igfbp1a* and *-1b* in rockfish (Bersin et al., 2023a). Treatment of gopher rockfish with recombinant sea bream Igf1 induced hepatic *igfbp3a* mRNA levels in fish experiencing fasting, but not in fish that were continuously fed; messenger RNAs for *igfbp1a* and *-1b*, in contrast, were both elevated by Igf1 in both fed and fasted gopher rockfish (Bersin et al., 2023a). Studies in yellowtail (*Seriola quinqueradiata*) and tilapia (*Oreochromis mossambicus*) each observed elevations to hepatic *igfbp3* mRNA levels caused by Gh (Cheng et al., 2002; Pedroso et al., 2009), although specific paralogs of *igfbp3* were not demarcated in those studies. It is also unclear whether teleost type 3 Igfbps interact with an Igfals protein, as Igfbp3 does in mammals. Other recent studies in zebrafish (*Danio rerio*) and the catfish (*Clarias magur*) also identified *igfals* genes in those species (Landi et al., 2020; Haldar et al., 2022); however, a putative Igfals protein has not yet been isolated from fish. Even so, fasting-induced declines in liver *igfals* mRNAs were observed both in the current study and in a prior study also with juvenile gopher rockfish (Bersin et al., 2023a), and the present study provides evidence that Gh can upregulate *igfals* mRNA levels in rockfish under both fed and fasting conditions. Bersin et al. (2023a), however, did not see any changes in liver *igfals* mRNAs in gopher rockfish following Igf1 treatment, indicating that the Gh-induced changes in liver *igfals* observed here were not due to Gh-mediated increases in Igf1. Still, it remains unclear if this fish *igfals* gene is translated and whether any such Igfals protein interacts with fish Igfbp3 or any other Igfbp. Future studies should focus on investigating this teleost *igfals* gene and its potential roles for regulating Igf1 signaling.

Igfbp2b is thought to be the main circulating carrier of Igf1 in teleost fishes (Shimizu and Dickhoff, 2017; Allard and Duan, 2018). Along with Igf1, Igfbp2b protein levels in blood are reduced in fasting salmonids (Beckman et al., 2004b, 2004a; Pierce et al., 2005; Gabillard et al., 2006; Shimizu et al., 2009; Cleveland and Weber, 2013), and recent work using CRISPR/Cas9 to mutate the *igfbp2b1* and *-2b2* genes of rainbow trout observed declines in circulating Igf1 as well as diminished growth (Cleveland et al., 2018). Here, we observed that fasted gopher rockfish exhibited lower mRNA levels for *igfbp2b* – but not *igfbp2a* – in the liver, a finding that aligns with what was observed previously in copper rockfish (Hack et al., 2019) and in a separate study with gopher rockfish (Bersin et al., 2023a). While we did not find any effects of Gh on liver *igfbp2b* gene transcript abundance in juvenile gopher rockfish in the current study, Igf1 was found to reduce hepatic *igfbp2b* mRNA levels in both continuously fed and fasted gopher rockfish (Bersin et al., 2023a). Studies in other fish species have similarly found liver mRNAs for *igfbp2b* to decline under conditions of food deprivation (Duan et al., 1999; Kelley et al., 2002; Gabillard et al., 2006; Safian et al., 2012; Chen et al., 2014). Such declines in *igfbp2b* mRNA levels in the liver might relate to reduced Igf1 transport to peripheral tissues, and thus reduced growth, in fish experiencing fasting.

In addition to changes in Igfbps, we also observed that fasting and Gh administration had effects on several components of Gh/Igf1 signaling in the fast-twitch skeletal muscle of juvenile gopher rockfish, which likely functioned in conjunction with changes in hepatic Gh/Igf1 pathways to generate the loss of body mass and reduced body condition of rockfish under food deprivation. Specifically, fasted rockfish exhibited higher skeletal mRNA abundances encoding the *ghr2* Gh receptor and Igf1 receptors *igf1ra* and *igf1rb*. Feeding-associated changes in skeletal muscle Gh/Igf1 pathways have previously been observed in other fishes.

Messenger RNA levels of both *ghr1* and *ghr2* were elevated in the skeletal muscle of fasted rainbow trout, but declined back to baseline pre-fasting expression levels upon refeeding (Gabillard et al., 2006). Similarly, elevated skeletal muscle mRNAs encoding *igf1ra* were observed in fasted rainbow trout (Chauvigné et al., 2003). And, in a study with juvenile copper rockfish, fish fed either higher (daily 9 % rations per body mass) or lower (3 % rations) food amounts for 140 d both showed increased skeletal muscle mRNA levels for Igf1 receptors *igf1ra* and *igf1rb* after being shifted to complete food deprivation for 12 d (Hack et al., 2019).

In gopher rockfish studied here, skeletal muscle regulation of *igf1ra* and *igf1rb* mRNA levels by Gh was dependent on recent feeding, with Gh downregulating *igf1ra* and *igf1rb* mRNA levels in fasted rockfish, but not in continuously fed fish. In teleost fishes, Gh receptor and Igf1 receptor gene expression can be modulated by several factors including recent feeding experience as well as changes in circulating Igf1 (Azizi et al., 2016), and such changes appear to mediate shifts in muscle Gh/Igf1 sensitivity as fish undergo changes in feeding conditions (Bower et al., 2008). While it is not entirely clear how such changes in muscle Gh receptor and Igf1 receptor expression in fish link to alterations in growth, a recent parallel study also in juvenile gopher rockfish observed that an increase in skeletal muscle Igf1 receptor mRNA levels occurred during fasting in conjunction with declines in the expression of myogenesis genes including myoblast determination protein 2 (*myoD2*) and myogenic factors 5 (*myf5*) and 6 (*myf6* encoding Mrf4), as well as increased expression of muscle atrophy genes ubiquitin ligase F-box only protein 32 (*fbxo32*) and muscle RING-finger protein-1 (*muRF1*) (Bersin et al., 2023a). That overall pattern of gene expression implies increased muscle Igf1 receptor expression occurred in rockfish experiencing lower circulating Igf1, diminished myogenesis, and upregulated muscle atrophy pathways caused by fasting. Those findings combined with our observations here suggest that the skeletal muscle of gopher rockfish undergoes changes in Igf1 receptor expression based on recent food intake and levels of Gh in blood circulation.

5. Conclusions

Juvenile gopher rockfish experiencing conditions of complete food deprivation showed changes in several steps of the pathways from liver Igf1 synthesis to skeletal muscle Gh/Igf-related mechanisms that, combined, help explain how Gh/Igf1 regulation of growth is altered by food limitation (Fig. 10). An absence of Gh stimulation of liver *igf1* mRNA levels observed in fasted rockfish is in line with a decrease in the sensitivity of *igf1* gene expression to Gh in fish experiencing food deprivation. That lack of upregulation of liver *igf1* mRNAs was accompanied by reduced liver Gh receptor mRNA expression – implying changes in liver Gh binding capacity – and reduced liver pJak2 and pStat5 levels, suggestive of reduced activation of the primary intracellular signaling pathway downstream of Gh receptor binding. Fasted rockfish also exhibited significant changes in Gh regulation of hepatic gene expression for several Socs genes including *socs2*, which has been

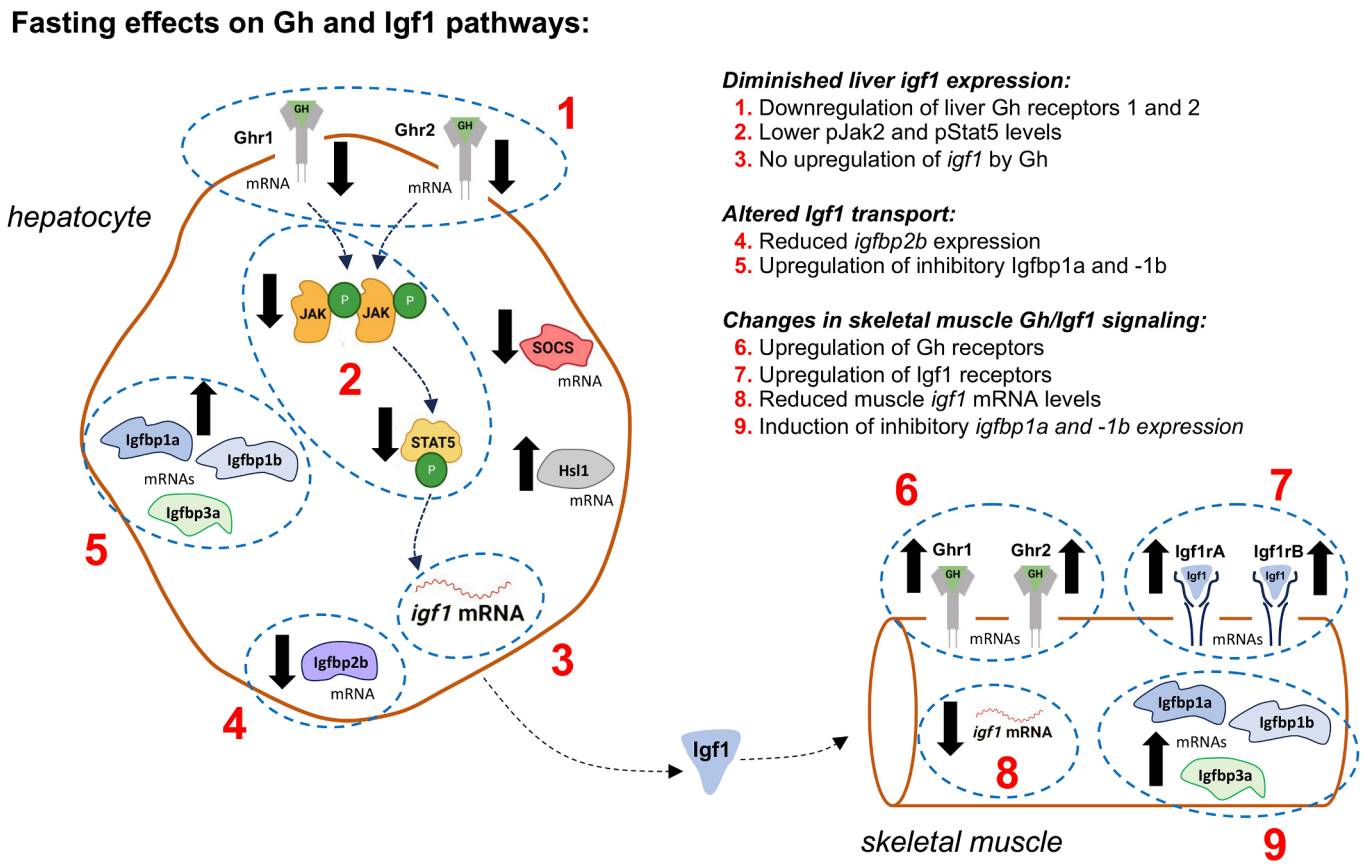

Fig. 10. Summary of changes in Gh/Igf1 signaling in rockfish under fasting. Specific pathway components are indicted with dotted ovals. Numbered points 1 to 5 (*red numbers*) describe identified changes in liver hepatocyte pathways. Points 6 to 9 outline changes in skeletal muscle Gh/Igf1 signaling components. (1) Fasted fish showed reduced liver Gh receptor mRNAs suggesting reduced liver Gh binding capacity. (2) Fasting led to reduced concentrations of pJak2 and pStat5, as well as (3) the absence of liver *igf1* gene upregulation following treatment with Gh. Hepatic mRNAs for (4) Igfbp2b – the purported main carried of Igf1 in circulation – were reduced, but (5) increased for inhibitory Igfbp1a and Igfbp1b, therein likely altering blood availability of Igf1. At the level of fast-twitch skeletal muscle, (6) mRNAs encoding Gh receptors 1 and 2 were more abundant and transcripts for (7) Igf1 receptors A and B were also upregulated, suggesting changes in muscle sensitivity to Gh and Igf1. Muscle (8) Igf1 and (9) Igfbp1a, -1b, and -3b mRNA levels were also altered by fasting, implying changes in local Igf1 production and paracrine actions. Created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previously shown to have inhibitory influences on Gh activation of the liver Jak2/Stat5 pathway (Jiang et al., 2016). In addition, observed increases in liver type 1 Igfbps, which have inhibitory influences on Igf1 action (Shimizu and Dickhoff, 2017) – as well as changes in skeletal muscle Gh and Igf1 receptor gene expression – together imply shifts in circulating Igf1 availability and action on skeletal muscle (Fig. 10). These collective findings point to changes at multiple levels of Gh/Igf1 signaling from liver Igf1 production to skeletal muscle Gh and Igf1 binding capacity and local production as together contributing to body mass loss in gopher rockfish experiencing conditions of food scarcity.

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CRediT authorship contribution statement

Theresa V. Bersin: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Formal analysis, Visualization, Funding acquisition. **Kasey L. Cordova:** Conceptualization, Methodology, Funding acquisition. **Meredith L. Journey:** Methodology. **Brian R. Beckman:** Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Funding acquisition. **Sean C. Lema:** Conceptualization, Methodology, Resources, Writing – review & editing, Formal analysis, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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