

Regulation of endocrine and paracrine sources of Igfs and Gh receptor during compensatory growth in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*)

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Abstract

Compensatory growth (CG) is a period of growth acceleration that exceeds normal rates after animals are alleviated of certain growth-stunting conditions. In hybrid striped bass (HSB, *Morone chrysops* × *Morone saxatilis*), 3 weeks of complete feed restriction results in a catabolic state that, when relieved, renders a subsequent phase of CG. The catabolic state was characterized by depressed levels of hepatic Type I and II GH receptor (*ghr1*, *ghr2*) and *igf1* mRNA, along with considerable decreases in plasma Igf1. The state of catabolism also resulted in significant declines in hepatic *igf2* mRNA and in circulating 40 kDa Igf-binding protein (Igfbp). Skeletal muscle expression of *ghr2* mRNA was significantly increased. Upon realimentation, specific growth rates (SGRs) were significantly higher than sized-matched controls, indicating a period of CG. Hepatic *ghr1*,

ghr2, *igf1* and *igf2* mRNA levels along with plasma Igf1 and 40 kDa Igfbp increased rapidly during realimentation. Plasma Igf1 and total hepatic *igf2* mRNA were significantly correlated to SGR throughout the study. Skeletal muscle *igf1* mRNA also increased tenfold during CG. These data suggest that endocrine and paracrine/autocrine components of the GH–Igf axis, namely *igf1*, *igf2*, and *ghr1* and *ghr2*, may be involved in CG responses in HSB, with several of the gene expression variables exceeding normal levels during CG. We also demonstrate that normalization of hepatic mRNA as a function of total liver production, rather than as a fraction of total RNA, may be a more biologically appropriate method of quantifying hepatic gene expression when using real-time PCR.

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Introduction

Growth in teleost fishes involves a complex array of neuroendocrine processes under the direct influence of variables such as temperature, nutrition, and stress. Fish are an excellent model to study the physiological control of variable and often dramatic differences in growth rate since they exhibit indeterminate and relatively plastic rates of growth. Compensatory growth (CG) is a period of growth acceleration that exceeds normal rates after animals are alleviated of certain growth-stunting conditions (Wilson & Osbourn 1960; see Ali *et al.* 2003 for review). Experimental regimes that elicit states of CG provide the opportunity to elucidate the underlying mechanisms of stunted (negative), normal, and accelerated (compensatory) growth. While numerous studies in fishes have examined the endocrine growth axis during periods of weight loss, typically induced through feed restriction (see Picha *et al.* 2008 for review), few have examined the response of the axis during the subsequent period of CG when the stunting condition is removed.

The endocrine control of growth in fishes and other vertebrates is regulated primarily through the growth hormone (Gh)/insulin-like growth factor (Igf) axis (reviewed by Duan 1997, Pérez-Sánchez & Le Bail 1999, Reinecke 2006). Under

anabolic conditions, Gh released from the pituitary acts on its hepatic receptors to stimulate the production and subsequent release of Igf1 into the circulation. Systemic Igf1 can then act on target tissues to promote cell proliferation, differentiation, and ultimately body growth (reviewed by Wood *et al.* 2005). Accordingly, its levels have been correlated to specific growth rate (SGR) in numerous teleosts (Uchida *et al.* 2003, Beckman *et al.* 2004, Picha *et al.* 2006, 2008, Vera Cruz *et al.* 2006), and the recent evidence indicates that the presence or absence of a single IGF1 nucleotide polymorphism may determine body size in dogs (Sutter *et al.* 2007). In addition to hepatic binding, GH may also act directly on target tissues such as skeletal muscle to stimulate the production of IGF1, which in turn can act in a paracrine/autocrine fashion to stimulate tissue growth (Chauvigné *et al.* 2003). The potential contribution of locally produced Igf1 to anabolism is further underscored by studies in Gh-transgenic tilapia (*Oreochromis niloticus*), which showed elevated growth and skeletal *igf1* mRNA but reduced plasma Igf1 relative to wild-type controls (Eppler *et al.* 2007). Unlike postnatal mammals, circulating as well as locally produced Igf2 may also have mitogenic effects in adult fishes (Chen *et al.* 2000, Peterson *et al.* 2004, Gabillard *et al.* 2006, Terova *et al.* 2007). The anabolic effects of both Igf1 and Igf2 are mediated by Igf-binding

proteins (Igfbps), four of which have been identified in fishes at the protein level (Igfbp-1, -2, -3, and -5; Shimizu *et al.* 2003, Kamangar *et al.* 2006). The putative Igfbp-3 (40–50 kDa), which actually may be an Igfbp-2 paralog (Rodgers *et al.* 2008), is most commonly associated with anabolic states in fishes and may facilitate Igf actions by increasing Igf half-life in the blood and by mediating their transport from the vascular space to target tissues (reviewed by Kelley *et al.* 2006). Despite evidence supporting the role of the Gh/Igf axis in regulating growth in ectotherms generally and teleosts specifically, few studies have addressed how most components correspond to variable growth phases, including the accelerated state seen with CG.

The purpose of this experiment was to examine the growth, metabolic, and endocrine characteristics of hybrid striped bass (HSB, *Morone chrysops* × *Morone saxatilis*) during stunted, normal, and accelerated (compensatory) growth. Specifically, we were interested in how Igf1, Igf2, 40 kDa Igfbp, Igf receptor, and Type I Ghr and Type II Ghr might change during catabolism (weight loss) and more particularly during the period of rapid (compensatory) growth that follows the growth-stunting condition.

Materials and Methods

Animals and experimental design

Procedures were approved by the North Carolina State University (NCSU; Raleigh, NC, USA) Institutional Animal Care and Use Committee. Freshwater, juvenile (71.4 ± 0.8 g; mean ± S.E.M.) HSB were transported from ponds at the Tidewater Research Station (Plymouth, NC, USA) to indoor tanks at NCSU. One week following transport, fish were evenly distributed to eight 650 l tanks within two identical freshwater recirculating systems (4 tanks/system) equipped with biofiltration and u.v. sterilization. Fish were allowed to acclimate to photoperiod (12-h light:12-h darkness) and water quality parameters (hardness = 170 mg/l; alkalinity = 250 mg/l) for 1 month prior to the start of the experiment. All fish were maintained on a daily feeding regimen prior to initiation of the experiment using a commercial pelleted floating feed (Zeigler Bros., Gardners, PA, USA; 40% protein, 10% fat). Two tanks from each of the recirculating systems were randomly assigned to control and treatment groups (*N* = 4 tanks/group at 50 fish/tank). Control fish were fed twice daily to apparent satiation throughout the 63-day experiment. Treatment fish were fasted for the initial 3 weeks (days 0–21) of the experiment, after which time they were placed on the control, twice daily satiation diet for 6 weeks (days 22–63). Water temperature averaged 24.4 ± 0.04 °C (mean ± S.E.M.) throughout the experiment.

Sample procedures

Body weights (g) and total lengths (mm) were taken from both control and treatment groups at the following points: time 0, the end of 3 weeks of feed restriction (day 21), and 3 and 6 weeks

into refeeding (days 42 and 63). The blood and tissue (liver, muscle) samples were taken from a subsample of fish at these same time points, as well as 8 days into the refeeding period (day 29) to better track the time-course response of variables during CG. Group weights and lengths were taken in fish anesthetized with quinaldine sulfate (B L Mitchell Inc., Leland, MS, USA), while terminal sampling for the blood and tissue collection was conducted on fish anesthetized with buffered tricane methane-sulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA). The blood was collected using heparinized 1 cc syringes with 22-gauge needles, dispensed into heparinized 1.5 ml tubes containing aprotinin and kept on ice. Plasma was separated by centrifugation at 4 °C and stored at -70 °C until analyses. The liver and muscle samples were rapidly removed, snap frozen in liquid nitrogen, and then stored at -70 °C. Fish were deprived of feed 20 h prior to sampling or group weight and length determinations.

Growth rate and metabolic calculations

Specific growth rate (SGR) was calculated as $((\ln W_2 - \ln W_1) / (T_2 - T_1) \times 100)$, where W_2 is the weight at the end of the growth interval and W_1 is the weight at the beginning of the growth interval, while $T_2 - T_1$ represents the duration (days) of the growing interval. SGRs were calculated for control and treatment fish during the following time intervals: days 0–21, 22–42, 43–63. Hepatosomatic index (HSI) was calculated as $((\text{liver wt}/\text{body wt}) \times 100)$ and feed conversion ratio (FCR) as $(\text{feed consumed}/\text{weight gain})$. Percent body weight consumed per day (% BW/d) at specific intervals throughout the experiment was calculated as $(\text{total feed consumption per time interval})/(\text{total fish weight estimated at midpoint of the interval}) \times 100$.

Gene cloning

Striped bass (*M. saxatilis*) *igf2* (180 bp), Type I Ghr (*ghr1*; 476 bp), and Type II Ghr (*ghr2*; 670 bp) genes were partially cloned in order to design effective primers and probes for measures of mRNA by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from striped bass liver samples using TRI Reagent isolation solution (Molecular Research Center; Cincinnati, OH, USA), DNase treated (Ambion; Austin, TX, USA), and then quantified via Nanodrop spectrophotometry (NanoDrop Technologies; Wilmington, DE, USA). One microgram of total RNA was reverse transcribed using oligo d(t) primers and then 10% of the RT reaction used for PCR (Qiagen). Degenerate *igf2* primers (Forward: 5'-TGTGGRG-GAGARCTGGTGGGA-3'; Reverse: 5'-ACTTGGCRGGT-TTGGCACAG-3') were designed within the B and A/D domains of the *igf2* gene using known *Cottus scorpius* (GenBank Accession Number Y16643), *Oncorhynchus mykiss* (M95184), *Paralichthys olivaceus* (AF091454), and *Oreochromis mossambicus* (Y18691) *igf2* sequences. Degenerate *ghr1* primers (Forward: 5'-TCCTGCACCYAAAATYAAAGG-3'; Reverse: 5'-CTGGGSCCCYCCAGTTTGG-3') were designed within the

Box 1 region and downstream of the Box 2 region of the cytoplasmic domain using *ghr1* sequences from *Scophthalmus maximus* (GenBank Accession Number AF352396), *Acanthopagrus schlegelii* (AF502071), *Sparus aurata* (AF438176), *O. mossambicus* (AB115179), and *Dicentrarchus labrax* (AF438177). Degenerate *ghr2* primers (Forward: 5'-CCTGGGTGGARTT-CATCGA-3'; Reverse: 5'-CACCYTCKACCACRG-TGTAGACA-3') were designed within and downstream of the Box 2 region of the cytoplasmic domain using *ghr2* sequences from *A. schlegelii* (AY662334), *S. aurata* (AY573601), *O. niloticus* (AY973233), and *D. labrax* (AY642116). PCR cycling conditions were as follows for both genes: 1 cycle at 95 °C for 15 min; 30 cycles at 94 °C for 30 s, 50–65 °C for 30 s, and 72 °C for 1 min; and 1 cycle at 72 °C for 10 min. PCR products were ligated into pCR 2.1 vectors and transformed using INVαF' chemically competent *Escherichia coli* cells (Invitrogen). Plasmids were sequenced at the University of Chicago DNA sequencing facility and verified by BLAST search (NCBI: Bethesda, MD, USA).

RNA isolation for quantitative real-time PCR

Total RNA was extracted from liver samples using TRI Reagent isolation solution with sequential high salt (Molecular Research Center) and LiCl precipitation steps intended to remove glycogen contamination (Barlow *et al.* 1963). The mean 260:230 nm ratio for these RNA samples ranged from 1.35 to 2.28 as determined by Nanodrop spectrophotometry. This wide range and low values in 260:230 ratios suggested residual polysaccharide contamination (Nanodrop Technical Support Bulletin T009), which can artificially alter RNA quantification since glycogen can absorb at 260 nm (unpublished data). Therefore, samples were further purified with Plant RNA Isolation Aid (Ambion) to completely remove glycogen. Specifically, Plant RNA Isolation Aid was added to RNA samples dissolved in water at a 1:1 v/v, incubated at room temperature for 10 min, and then spun at 15 000 g for 15 min. The RNA-containing supernatant was removed from the glycogen pellet and then reprecipitated with LiCl (2.5 M). Subsequently, all samples had 260:230 values of 2.0–2.2, indicating that they were essentially glycogen free. This, combined with the sample 260:280 ratios of 1.9–2.0, indicates high purity of nucleic acids and minimal contamination by proteins and polysaccharides. Muscle samples were extracted using the standard Tri Reagent RNA isolation protocol (Molecular Research Center).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) primers and Taq Man probes for HSB (Order Perciformes) were designed for *igf1*, *igf2*, *igfr*, and *ghr1* and *ghr2* in regions with low identity to related genes (Table 1). The *igf1* primers and probe showed no cross-reactivity when run with *igf2* cDNA template. Likewise, no cross-reactivity was observed using *igf2* primers and probe with *igf1* cDNA template. HSB *igfr* primers and probe

Table 1 Primer and Taq Man probe sequences for various hybrid striped bass genes measured in liver and muscle tissues by quantitative RTPCR

Gene	For primer	Taq Man probe	Rev primer
<i>igf1</i>	5'-TTGTGTGTGGAG AGAGAGGCTTT-3'	5'-TTTCAGTAAACCT ACAGGCTATGGCC-3'	5'-TGACC GCC GTGCATTG-3'
<i>igfr</i>	5'-CGTCTCTCCG ACCGAAAGAG-3'	5'-TGGTCGAGCCTGT CACTCCCTCCTC-3'	5'-GACCGGCCATCT GAAGCATCTC-3'
<i>igf2</i>	5'-AAAGAACAGAC GGACCCAGAA-3'	5'-CGTGGGATCGTAGA GGAGTGTGTTCC-3'	5'-AGGAAGTTGAG GCACAGCTA-3'
<i>ghr1</i>	5'-TCAGCAACCACA TGAACATAGGA-3'	5'-TGCCAAATGTCAT CAGCTCCCGG-3'	5'-GCGACGGCC TGAGTCATC-3'
<i>ghr2</i>	5'-CCCAGG CATTAGCTT-3'	5'-CCTCTCATCCCAA TCAACCCTCAGTG-3'	5'-GCTGTCGGGA ACGATGTTTC-3'
18S	5'-TGAAACATTC TTGGCAATGC-3'	5'-TTCGCTTTCGTC CGTCTGGCC-3'	5'-GCCGTAGAGGT GAAATCTTC-3'
β-actin	5'-GCCTTCCTTC CTCGGTATGG-3'	5'-CCTGCGGAATC CACGAGACCACC-3'	5'-CCGACTTCAT GATGCTGTGTG-3'

were designed within the tyrosine kinase domain (AF402674) that exhibits only 63% nucleic acid sequence similarity to insulin receptor of another perciform, the tilapia (*O. mossambicus*, AF493794). No cross-reactivity was observed using *ghr1* primers and probe with *ghr2* cDNA template or when using *ghr2* primers and probe with *ghr1* cDNA template.

Following total RNA extraction, muscle and liver RNA from experimental samples were double DNase treated, diluted to the same concentration, and then subjected to reverse transcription (RT) using random hexamer primers (Applied Biosystems; Foster City, CA, USA). Enzymatic reactions for all samples for each tissue type were performed at the same time with identical reagents, as to reduce the variability of DNase and RT efficiency between the samples. Housekeeping genes were also run for all tissue samples to further account for these same variables. cDNAs of 10 (liver) or 100 ng (muscle) from the RT reaction were then loaded with 900 nM primers and 250 nM probes into 20 μ l qRT-PCR volumes containing Brilliant QPCR master mix (1 \times ; Stratagene; La Jolla, CA, USA). Quantitative RT-PCR assays for each gene were run on an ABI 7900 Thermal Cycler (Applied Biosystems) under the following conditions: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. No amplification (DNase-treated RNA instead of cDNA) as well as no template (no cDNA) controls were run in parallel with experimental samples to confirm the absence of genomic DNA and qRT-PCR reagent contamination respectively. The absolute quantity of specific muscle mRNAs was calculated using cDNA copy number standard curves (10^0 to 10^{-9} copy number) generated through gene-specific PCR amplification and isolation according to previously established procedures (Bustin 2000, Picha *et al.* 2006). Copy number data for all experimental muscle samples were then normalized to total RNA loaded into each qRT-PCR assay, and are therefore expressed as copy number per ng RNA. Our initial studies found that changes in skeletal muscle mRNA are similar whether normalized to total RNA or the β -actin housekeeping gene.

Liver *igf1*, *igf2*, *ghr1*, and *ghr2* mRNA data were initially expressed as copy number as a fraction of total RNA (copy number per ng RNA) as described above (Fig. 3A, Table 3). We found here and previously that hepatic mRNA data were similar whether expressed as copy number per ng total RNA or when normalized to the housekeeping gene *18S* (Picha *et al.* 2006), indicating that the processing of tissue samples into cDNA did not result in significant errors in RNA quantification or variability in RT efficiency. Our studies found that β -actin was regulated in the liver by nutritional state. Hepatic gene expression data were also expressed as total copy number for the entire liver relative to body weight ((ng total liver RNA \times copy number of gene per ng RNA) / (g body weight); Figs 3 and 4). We argue that this latter method is more physiologically relevant for quantifying liver gene expression levels when hepatosomatic indices and total liver RNA content fluctuates dramatically due to nutritional state.

RIA

Circulating levels of total Igf1 were measured from acid/ethanol extracted plasma by RIA using recombinant barramundi Igf1 as tracer and standard, rabbit anti-barramundi Igf1 primary antibody (Novozymes GroPep; Adelaide, Australia) and goat anti-rabbit secondary antibody (Sigma) according to previously described methods (Shimizu *et al.* 2000, Picha *et al.* 2006). Barramundi Igf1 was iodinated using the chloramine-T method and purified by column chromatography. Tracer (125 I-barramundi Igf1) was diluted to 20 000 c.p.m. for each assay tube. Our previous validation shows that serially diluted HSB plasma produces a displacement curve that parallels that of the barramundi standard (Picha *et al.* 2006). All samples were run in triplicate.

Western ligand blot

HSB 40 kDa Igfbp was measured in plasma by western blot according to previously published procedures used for striped bass (Siharath *et al.* 1995a,b). HSB plasma samples (4 μ l) along with rat serum (positive control) were run on discontinuous 4% stacking, 12% separating SDS-PAGE gels under non-reducing conditions. Following electrophoresis, proteins were electro-transferred overnight onto nitrocellulose membranes, stained with Coomassie blue, and then blocked with TBS + 1% BSA for 4 h. Hybridization took place overnight at room temperature using 125 I-barramundi Igf1 at 200 000 c.p.m./ml incubating buffer. Membranes were exposed to film in cassettes using intensifying screens at -70 °C. All data were analyzed using Image Quant 5.2 software (GE Healthcare, Piscataway, NJ, USA) and are expressed as arbitrary density units.

Statistical analyses

Body weight was analyzed by Repeated Measures ANOVA followed by Fisher's least significant difference (LSD) test for predetermined comparisons (Steele & Torrie 1980). Correlations were analyzed by regression analysis. All other data were analyzed with a two-way ANOVA (treatment \times time) followed by Fisher's LSD test. Statistical analyses were performed with Statistica 7.0 software (Stat Soft, Tulsa, OK, USA). The *N* value for all growth (weight, SGR) and feed (% BW/d, FCR) data was represented by mean values of tanks, while individual fish served as the *N* value for all endocrine parameters. Statistical significance was set at a level of $P \leq 0.05$. All data are presented as mean \pm S.E.M.

Results

Effects of cyclic feeding on growth and metabolic indices

Three weeks of complete feed restriction (days 0–21) in treatment fish resulted in a 7.7% decrease in body weight (Fig. 1A), indicated by a negative SGR (-0.38 ± 0.05 ; Fig. 1B).

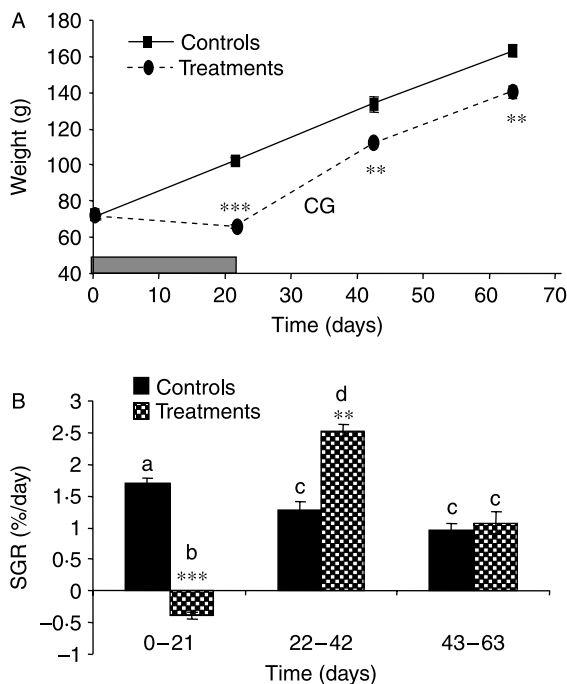


Figure 1 (A) Mean body weight (g) and (B) specific growth rates (SGR; %/day) of control HSB fed to satiation twice daily and treatment fish subjected to 3 weeks feed restriction (grey bar) followed by 6 weeks of refeeding. CG indicates a period of compensatory growth for treatment fish as defined by elevated SGRs relative to similar-sized controls (time 0 controls = 71.4 ± 1.3 g; day 21 treatments = 65.8 ± 0.7 g). Asterisks represent significant differences between groups at each time point (** $P < 0.01$; *** $P < 0.001$; N = average values of individual tanks with 4 replicate tanks/group and 30–50 fish/tank). Letters represent significant differences within and between groups across time ($P < 0.05$).

During the subsequent 3 weeks of refeeding (days 22–42), however, SGRs for treatment fish were significantly higher ($P < 0.05$) than those for controls at all growth intervals throughout the experiment (Fig. 1B). Since growth rates are dependent on size and show an allometric relationship with body mass, it is also important to compare growth of similar sized fish. We found that elevations in SGR in treatment fish during the realimentation period was still higher compared with control fish of a similar initial size (Time 0 controls = 71.4 ± 1.3 g; day 21 treatments = 65.8 ± 0.7 g). The elevated SGR that characterizes CG subsequently declined back to basal levels during weeks 4–6 of realimentation (days 43–63), where there was no significant difference in SGR in treatment fish relative to size-matched controls (control SGR days 22–42 = $1.3 \pm 0.1\%$ /day; treatment SGR days 43–63 = $1.1 \pm 0.2\%$ /day; Fig. 1).

In conjunction with the weight loss experienced during feed restriction (days 0–21), HSI values for treatment fish dropped 3.5-fold (3.85 ± 0.11 to 1.11 ± 0.11 ; $P < 0.001$) and were significantly lower than controls (Fig. 2; $P < 0.001$). Eight days into realimentation and the initial CG response (day 29), treatment HSI values increased by 168%, and

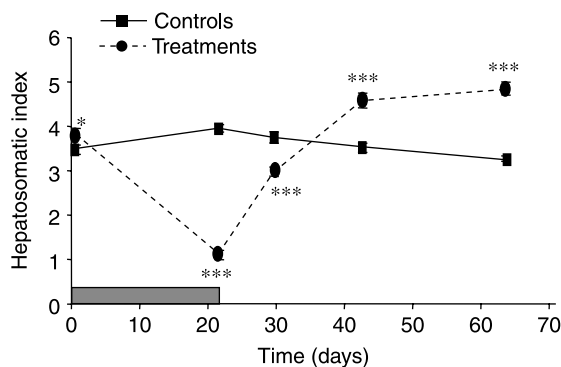


Figure 2 Hepatosomatic index of control HSB fed to satiation twice daily and treatment fish subjected to 3 weeks feed restriction (grey bar) followed by 6 weeks of refeeding twice daily to satiation. Asterisks represent significant differences between groups at each time point (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $N = 16$ fish/group).

reached higher levels than controls by 3 weeks (day 42: controls = 3.53 ± 0.09 ; treatments = 4.60 ± 0.16 ; $P < 0.001$). This overcompensation persisted through the end of the experiment (day 63: controls = 3.27 ± 0.07 ; treatments = 4.85 ± 0.14 ; $P < 0.001$).

Feed consumption and feed conversion

Control fish, which were fed twice daily to apparent satiation, had a gradual yet significant increase in FCR throughout the experiment (Table 2; $P < 0.01$), coupled with a gradual and significant decrease in feed consumption (% BW/d; Table 2; $P < 0.001$). While no data was available for either of these parameters during feed restriction for treatment HSB, the subsequent CG response (days 22–42) was marked by both hyperphagia ($2.46 \pm 0.05\%$ BW/d for treatments, $1.48 \pm 0.05\%$ BW/d for controls; $P < 0.01$) and improved feed conversion (0.87 ± 0.03 for treatments, 1.14 ± 0.07 for controls; $P < 0.01$; Table 2). Both feed consumption and FCRs returned to control values 4–6 weeks into the refeeding period in treatment fish, coinciding with the decline in CG response. Overall, FCR (days 0–63) for treatment fish was statistically similar to that of controls. A highly significant and positive correlation was obtained between feed consumption (% BW/d) and HSI values ($R^2 = 0.64$; $P < 0.001$), suggesting that HSI may be used as a biomarker for varying degrees of feeding intensity, including periods of feed deprivation.

Gene cloning

Sequencing results indicated that the striped bass (*M. saxatilis*) amplicon generated with degenerate *igf2* primers (EU419619) shared 98% nucleotide identity with the closely related European sea bass (*D. labrax*) *igf2* (Terova *et al.* 2007), confirming we cloned a partial sequence of *igf2* in striped bass. The striped bass amplicon generated with degenerate *ghr1* primers (EU419618) shared 84 and 94% nucleotide identity with gilthead sea bream (*S. aurata*; Saera-Vila *et al.* 2005; Jiao

Table 2 Feed conversion ratios and feed consumption of control fish fed to satiation twice daily and treatment fish subjected to 3 weeks feed restriction (days 0–21) followed by 6 weeks of refeeding twice daily to satiation (days 22–42, 43–63). No data was available for treatments during days 0–21 because fish were not fed during this time. Treatment HSB had improved feed conversion and increased feed consumption during the CG response (days 22–42).

Days	Feed conversion ratio		Feed consumption	
	Control	Treatment	Control	Treatment
0–21	0.97 ± 0.01	N/A	1.56 ± 0.07	N/A
22–42	1.14 ± 0.07	0.87 ± 0.03*	1.48 ± 0.05	2.46 ± 0.05*
43–63	1.20 ± 0.07	1.23 ± 0.08	1.11 ± 0.06	1.25 ± 0.12

* $P < 0.01$ represents significant differences from controls at the same time point. $N = 4$ tanks/group with 30–50 fish/group.

et al. 2006) and European sea bass *ghr1* sequences respectively, and only 54% identity with the striped bass *ghr2* sequence (EU595732). Based on these identities with closely related species, we consider the striped bass amplicon a partial clone of the putative Type I Ghr and hence refer to it here as Ghr1. The striped bass amplicon generated with degenerate *ghr2* primers (EU595732) shared 83 and 96% nucleotide identity with gilthead sea bream (*S. aurata*; Saera-Vila *et al.* 2005, Jiao *et al.* 2006) and European sea bass *ghr2* sequences respectively. Based on these identities with closely related species, we consider the striped bass amplicon a partial clone of the putative Type II Ghr and hence refer to it here as Ghr2.

It should be noted that it is not certain whether the Ghr1 in non-salmonid fishes is a true Ghr (Jiao *et al.* 2006, Li *et al.* 2007) or instead the somatolactin receptor (SLR; Fukada *et al.* 2005, Fukamachi *et al.* 2005, Pierce *et al.* 2007). Recent phylogenetic and evolutionary analysis suggests that the SLR may in fact be a duplicated Ghr that occurred during the fish-specific genome duplication (Fukamachi & Meyer 2007). The striped bass *ghr1* amplicon showed 70 and 72% nucleotide identity with medaka (*Oryzias latipes*; DQ002886; Fukamachi *et al.* 2005), and masu salmon (*Oncorhynchus masou*; AB121047; Fukada *et al.* 2005) *slr* respectively, while our striped bass *ghr2* amplicon only showed 50 and 53% nucleotide identity to these same *slr* sequences.

Hepatic gene expression

When hepatic *igf1* qRT-PCR data were normalized to copy number/ng total RNA (Fig. 3A), 3 weeks of feed restriction in treatment fish (days 0–21) resulted in a 27% increase in *igf1* copy number ($P < 0.05$), followed by a subsequent decline 8 days into realimentation and the CG response (day 29; $P < 0.001$). These same trends were observed when data were normalized to the housekeeping gene *18S*. While data normalized in either of these ways represent the most common methods for expressing hepatic mRNA levels and are an accurate reflection of gene expression trends relative to a fraction of total liver RNA, they do not take into account the total amount of mRNA produced by the liver and presumably the protein released into the circulation. Indeed, during nutritional manipulations, the liver shows dramatic fluctuations in size relative to body weight, as indicated by HSI values in this study (see Fig. 2). Similarly, total

hepatic RNA content also fluctuates with HSI ($R^2 = 0.41$; $P < 0.001$), whereby feed-deprived fish having lower HSI values have the least amount of total liver RNA, and vice versa for fish in an anabolic state. Therefore, in order to determine the actual amount of a specific mRNA and potentially the protein produced in response to nutritional status, we accounted for gene expression output for the entire liver. For this reason, we expressed the results for *igf1* and all other hepatic mRNAs as total liver mRNA as a function of body weight (total liver copy number/BW).

When hepatic *igf1* gene expression was expressed as total amount of hepatic mRNA as a function of body weight (total liver *igf1* copy number/body weight), control fish experienced no significant variations throughout the experiment (Fig. 3B). This pattern of gene expression in control fish is virtually identical to that seen when hepatic gene expression is normalized as a fraction of total RNA (Fig. 3A). This would be expected in livers where little change occurs in HSI. Following feed restriction, treatment HSB experienced a 3.5-fold decline in total hepatic *igf1* mRNA levels ($P < 0.001$; Fig. 3B) that were 64% lower than control fish at day 21 ($P < 0.01$). Upon refeeding, levels rose 8 days into the CG response, the shortest time point measured (day 29; $P < 0.05$). By 3 weeks of realimentation, a 530% increase in total *igf1* mRNA was observed that exceeded control values (day 42; $P < 0.001$). This dramatic rise and overcompensation in total hepatic *igf1* mRNA that coincides with the duration of the CG response was subsequently restored to control levels by the end of the experiment.

With regard to *igf2* gene expression, no significant changes in total hepatic mRNA levels were observed for control fish during the initial 3 weeks. After this point levels slowly declined and plateaued by the end of the experiment (Fig. 4A), possibly representing an allometric pattern of gene expression associated with increased body size in naturally growing individuals. Treatment HSB, on the other hand, experienced an over sixfold decline in total liver *igf2* mRNA following 3 weeks of complete feed restriction ($P < 0.001$). Despite this precipitous decline, levels rose by 377% ($P < 0.001$) and had completely caught up to controls 8 days into refeeding and the CG response. After this initial increase, values continued to rise for the remainder of the CG

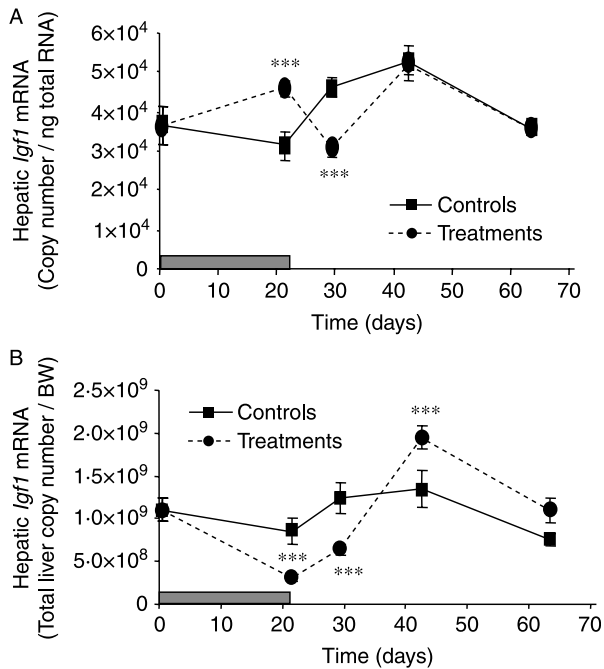


Figure 3 Hepatic mRNA levels of *igf1* in control HSB fed to satiation twice daily and treatment fish subjected to 3 weeks feed restriction (grey bar) followed by 6 weeks of refeeding twice daily to satiation. Hepatic mRNA levels were expressed as (A) *igf1* copy number/ng total RNA and (B) total hepatic *igf1* copy number/body weight. Asterisks represent significant differences between groups at each time point (***) $P < 0.001$; $N = 6-8$ fish/group).

response (day 42), exceeding that of control fish by 2.7-fold ($P < 0.001$; Fig. 4A). However, this elevation at day 42 relative to controls may be somewhat diminished when considering mRNA levels as an allometric function of body weight. Similar to trends for hepatic *igf1* gene expression, *igf2* mRNA declined back to control levels over the final 3 weeks of realimentation (days 43–63), the period over which CG was not observed. Altogether, a significant correlation was achieved between total liver *igf2* mRNA and SGR ($R^2 = 0.43$; $P < 0.001$; Table 4).

Alterations in *ghr1* and *ghr2* mRNA were similar to that observed for *igf2* mRNA. For *ghr1*, no significant changes were observed in controls during the first 29 days of the experiment, after which time they underwent a slight decrease and then remained level for the remainder of the trial (Fig. 4B). Treatment fish experienced a fivefold decline in total hepatic *ghr1* mRNA levels during 3 weeks of feed deprivation ($P < 0.001$), followed by a rapid, 3.2-fold increase upon 8 days of refeeding ($P < 0.001$). By 3 weeks of realimentation, *ghr1* levels surpassed that of control fish ($P < 0.05$) and reached similar levels by the end of the experiment. Strong correlations were achieved between total hepatic *ghr1* mRNA and both total liver *igf1* and *igf2* ($R^2 = 0.41$ and 0.67 , respectively; $P < 0.001$; Table 4). Control levels of total hepatic *ghr2* mRNA did not change throughout

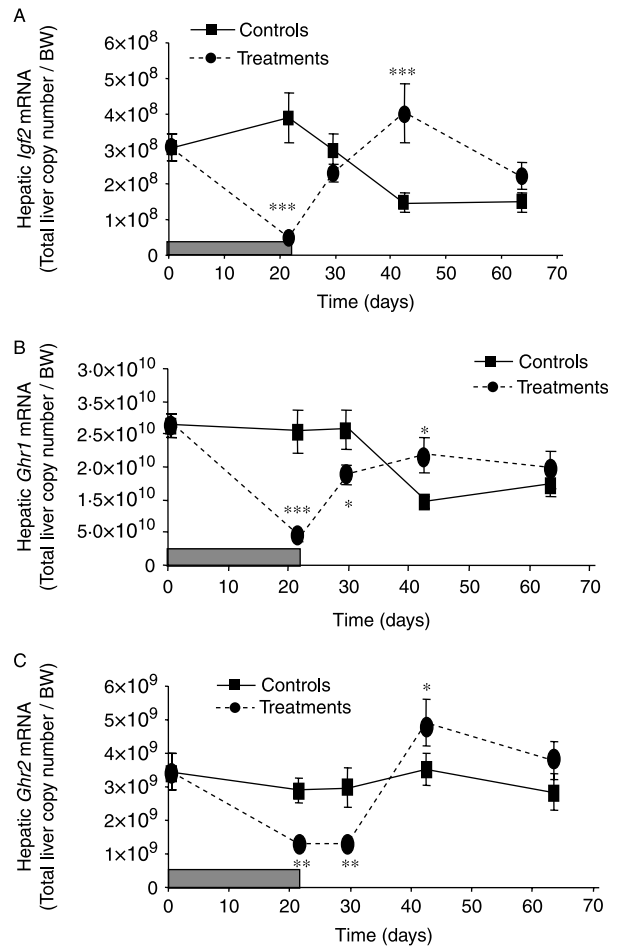


Figure 4 Total hepatic mRNA levels for (A) *igf2*, (B) the Type I Ghr (*ghr1*), and (C) Type II Ghr (*ghr2*) in control HSB fed to satiation twice daily and in treatment fish subjected to 3 weeks feed restriction (grey bar) followed by 6 weeks of refeeding twice daily to satiation. Asterisks represent significant differences between groups at each time point (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $N = 6-8$ fish/group).

the experiment (Fig. 4C). By contrast, treatment fish experienced a 63% decline following starvation ($P < 0.001$). Expression levels did not change 8 days into refeed (day 29) and the CG response; however, values increased by over 275% ($P < 0.001$) to levels above controls after 3 weeks of realimentation (day 42; $P < 0.05$). No significant differences were observed between groups by day 63. Strong correlations were achieved between total hepatic *ghr2* mRNA and both total liver *igf1* and *igf2* ($R^2 = 0.68$ and 0.31 respectively; $P < 0.001$; Table 4).

Data for hepatic *igf2*, *ghr1*, and *ghr2* mRNA expressed as copy number/ng total RNA are also provided for normalization comparison purposes (Table 3). However, we do not discuss these data in the results or discussion because we argue that the most physiologically relevant normalization is total hepatic gene expression/BW.

Table 3 Hepatic mRNA levels of *igf2*, Type I GH receptor (*Ghr*; *ghr1*), and Type II *Ghr* (*ghr2*) when normalized as copy number/ng total RNA. Data in this table are provided solely for purposes of qRT-PCR normalization comparisons, as Fig. 4 shows data normalized to total liver copy number/body weight, which we argue is more biologically relevant. Control HSB were fed to satiation twice daily and treatment fish were subjected to 3 weeks feed restriction (days 0–21) followed by 6 weeks of refeeding (days 22–63)

Day	<i>igf2</i>		<i>ghr1</i> ^a		<i>ghr2</i> ^a	
	Control	Treatment	Control	Treatment	Control	Treatment
0	9831 ± 1068	9831 ± 1068	6886 ± 318	6886 ± 318	1109 ± 164	1109 ± 164
21	14 205 ± 1731	6182 ± 497 [†]	7634 ± 543	6615 ± 298	1202 ± 134	1850 ± 131 [†]
29	13 462 ± 1654	12 147 ± 1092	7804 ± 396	7159 ± 506	1352 ± 151	717 ± 92 [†]
42	6390 ± 974	10 756 ± 1580*	4395 ± 325	4693 ± 479	1643 ± 185	1387 ± 74
63	7688 ± 979	7185 ± 616	5543 ± 517	4981 ± 315	1426 ± 153	1224 ± 66

Time 0 samples were combined between groups for each gene. Statistical analyses for each gene are between groups within time. * $P < 0.05$, [†] $P < 0.001$. $N = 8$ fish/group.

^aAll values are divided by 100.

Plasma *Igf1*

Plasma *Igf1* rose slightly in control fish over the course of the experiment (Fig. 5A). By contrast, treatment HSB experienced a 61% decline in plasma *Igf1* ($P < 0.001$) following feed restriction (days 0–21) to a value that was significantly lower than control levels at any time during the experiment ($P < 0.001$; Fig. 5A). Upon refeeding, plasma *Igf1* increased by 8 days and reached control levels by 3 weeks of realimentation, after which point levels remained similar to controls. A significant correlation was seen with plasma *Igf1* and SGR ($R^2 = 0.54$, $P < 0.001$; Fig. 6). Responses in plasma *Igf1* in treatment fish subjected to the CG feeding protocol were also similar to that observed for total hepatic *igf1* mRNA levels ($R^2 = 0.50$, $P < 0.001$).

Plasma 40 kDa *Igfbp*

The 40 kDa *Igfbp* is thought to be a putative mammalian *Igfbp-3* based on physiological responses and molecular weight (Siharath *et al.* 1995a,b, Shimizu *et al.* 2003), but according to recent phylogenetic analysis may be an *Igfbp-2* paralog (Rodgers *et al.* 2008). The 40 kDa *Igfbp* in our study displays distinct expression patterns in relation to feed restriction and CG (Fig. 5B). Specifically, levels of the 40 kDa *Igfbp* decreased by 74% ($P < 0.001$) following 3 weeks of feed restriction (days 0–21) to values well below those of controls ($P < 0.001$). Similar to that observed with circulating *Igf1*, the 40 kDa *Igfbp* was completely restored to control values 3 weeks into the CG response when fish were refed.

Skeletal muscle gene expression

The catabolic state induced through complete feed restriction in treatment fish resulted in an 89% decrease in skeletal muscle *igf1* mRNA ($P < 0.001$) to levels significantly lower than controls ($P < 0.05$; Fig. 7a). The *igf1* mRNA increased tenfold ($P < 0.001$) from their previously depressed levels and were completely restored to control values by 8 days of

realimentation. Gene expression levels were similar between control and treatment groups for the remainder of the experiment. A significant, albeit mild, correlation was derived between muscle *igf1* mRNA and SGR ($R^2 = 0.25$; $P < 0.001$) in control and treatment groups. There were no significant differences in skeletal muscle *igf2* or *igf1r* levels either within treatment across time or between treatments within time (data not shown). While there were no significant differences in muscle *ghr1* mRNA expression between or within groups

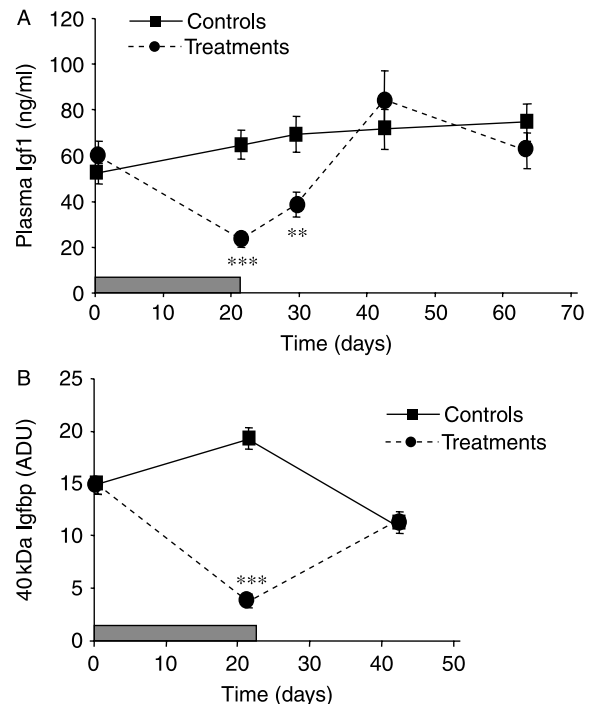


Figure 5 Plasma (A) *Igf1* (ng/ml) and (B) 40 kDa *Igf*-binding protein (arbitrary density units, ADU) in control HSB fed to satiation twice daily and in treatment fish subjected to 3 weeks feed restriction (grey bar) followed by 6 weeks of refeeding twice daily to satiation. Asterisks represent significant differences between groups at each time point (** $P < 0.01$; *** $P < 0.001$; $N = 6-8$ fish/group).

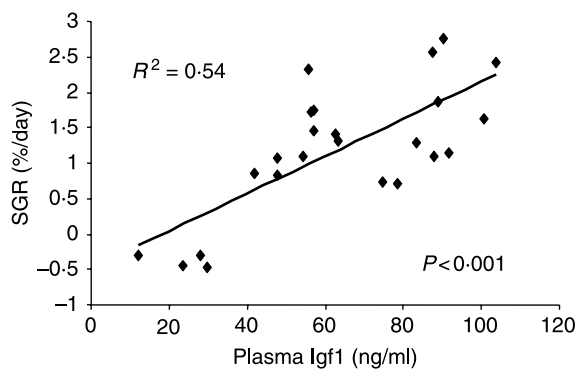


Figure 6 Correlation between plasma Igf1 (ng/ml) and specific growth rate (SGR; %/day) in control HSB fed to satiation twice daily and in treatment fish subjected to 3 weeks feed restriction followed by 6 weeks of refeeding twice daily to satiation. Specific growth rates (days 0–21, 22–42, 43–63) reflect the pairing of mean weights of fish with mean plasma Igf1 values from 8 tanks (4 tanks/group). Fish were sampled at days 21, 42, and 63.

throughout the experiment (Fig. 7b), 3 weeks of starvation in treatment fish resulted in a 340% increase in *ghr2* mRNA expression ($P < 0.001$; Fig. 7c) to levels 3.2-fold higher than controls ($P < 0.001$). Upon refeeding and the CG response, *ghr2* mRNA was restored to levels of control fish.

Discussion

This study demonstrates that a sufficient period of feed restriction and catabolism can induce subsequent periods of CG upon refeeding in HSB. The response is characterized by elevated SGRs, hyperphagia, and improved feed conversion. Furthermore, when hepatic gene expression data were normalized to total liver mRNA relative to body weight, significant increases were observed in *igf1*, *igf2*, *ghr1*, and *ghr2* mRNA during CG, and for some of the genes the increase even exceeded that of normal animals fed continuously throughout the experiment. The CG response was also accompanied by considerable increases in plasma Igf1, a 40 kDa Igfbp, and skeletal muscle gene expression of *igf1*. Fasting resulted in elevated muscle *ghr2* mRNA levels, which was restored to control values during CG. No change in skeletal muscle gene expression was detected for *igfr*, *igf2* or *ghr1* during the different metabolic states associated with CG feeding protocols.

CG is a period of growth acceleration that exceeds normal rates after animals are alleviated of certain growth-stunting conditions. In fishes, feeding manipulation remains the most common means of inducing the response, with periods of feed restriction and typically some degree of weight loss being followed by satiation feeding (see Ali *et al.* 2003 for review). In those species where CG has been demonstrated, either partial or full catch-up growth was induced in Atlantic halibut (Heide *et al.* 2006), channel catfish (Gaylord & Gatlin 2000),

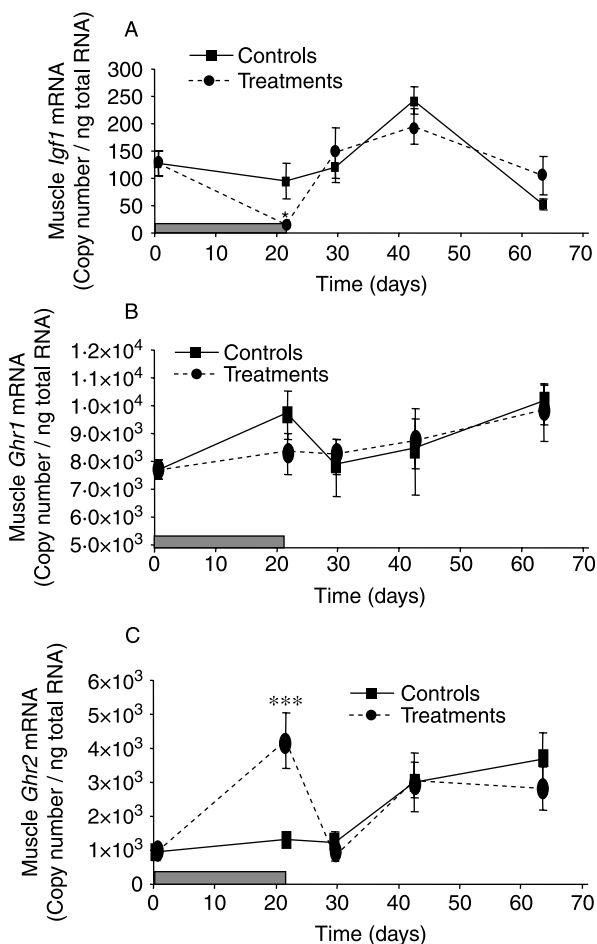


Figure 7 Skeletal muscle tissue mRNA expression of (A) insulin-like growth factor-I (*igf1*), (B) Type I Ghr (*ghr1*), and (C) Type II Ghr (*ghr2*) in response to normal (control) and cyclic (treatment) feeding. Data are expressed as copy number/ng total RNA. Grey bar denotes a period of feed restriction for treatment fish. Asterisks represent significant differences between groups at each time point ($*P < 0.05$; $N = 8–16$ fish/group).

European sea bass (Terova *et al.* 2006), hybrid sunfish (Hayward *et al.* 1997), and rainbow trout (Montserrat *et al.* 2007) through variations of this protocol. In HSB, partial feed restriction has led to full compensation in individually housed fish (Skalski *et al.* 2005), along with partial CG in group-housed HSB in both ponds (Turano *et al.* 2007) and tanks (Picha *et al.* 2006).

In this study, we demonstrate that tank-raised, group-housed HSB exhibit strong CG responses following complete feed restriction, and that the response is characterized by almost twofold elevations in SGR relative to control fish over the same time interval. An additional consideration that has received little attention in most CG studies (see Ali *et al.* 2003), including those evaluating the endocrine basis of the response, is the allometric relationship between growth rates and body mass (Jobling 1994). Indeed, smaller fish tend to

grow faster than larger fish, raising the question as to whether elevations in SGR of growth-stunted fish are the result of their smaller body size or a 'true' CG response. We found that SGR was 50% higher than control fish during the CG response when initial body size was accounted for (control fish days 0–21, 1·70% BW/day versus treatment fish days 22–42, 2·52% BW/day). This is in agreement with our earlier reports where CG was shown to occur following a period of partial feed restriction in HSB, even when accounting for potential differences in size-dependent growth rate (Skalski *et al.* 2005, Picha *et al.* 2006).

Hyperphagia and improved feed conversion were observed alongside the elevated SGRs during the CG response (days 22–42; Table 2). Hence, these behavioral and metabolic responses likely contribute to phases of accelerated growth associated with CG. Based on previous reports and results herein, it would appear that a certain catabolic threshold is necessary to drive these higher growth rates, feed consumption, and improved feed conversion. In previous studies, we found that a combination of low HSI (<1·5) and body weight loss are necessary to induce subsequent CG responses (Picha *et al.* 2006, Turano *et al.* 2007). In the current study, both of these conditions were met following feed restriction (7·7% BW decrease; HSI=1·11).

Feeding manipulation protocols that induce periods of stunted (negative), normal, and accelerated (compensatory) growth can provide methods for elucidating the endocrine control of variable growth rates and its control by the Gh–Igf axis. In cattle and pigs, levels of circulating IGF1 corresponded to periods of feed restriction and CG (see Hornick *et al.* 2000 for review; Leon *et al.* 2004, Therkildsen *et al.* 2004). Similarly, we found that circulating Igf1 paralleled altered growth states during CG feeding protocols, declining during growth depression and increasing during CG. Likewise, total hepatic *igf1* mRNA levels also changed in a fashion similar to both circulating hormone and growth, suggesting that the synthesis and secretion of hepatic Igf1 is important in mediating growth responses observed with stunted and rapid growth states. Interestingly, we found that *igf1* mRNA levels exceeded control fish during the CG response, raising the possibility that extra elevated transcript levels may contribute to the rapid rise in circulating Igf1 during CG. The significant correlation between total hepatic *igf1* mRNA and circulating Igf1 supports this notion, and suggests the liver is the primary source of circulating hormone, which has been firmly established in mammalian models using selective knockouts of the hepatic *igf1* gene (Sjogren *et al.* 1999, Yakar *et al.* 1999).

The majority of circulating Igf1 in fish is bound to Igfbps, making them prime mediators of the mitogenic effects of Igf1 (Shimizu *et al.* 1999, Duan & Xu 2005). Based on molecular weight (MW) and physiological responses, including those in striped bass (Kelley *et al.* 1992, Siharath *et al.* 1995a,b), the 40 kDa Igfbp detected in this study is likely the mammalian equivalent of Igfbp-3, or alternatively an Igfbp-2 paralog, which is reported to increase Igf1 half-life and regulate its availability to target tissues (Kelley *et al.* 2001, Shimizu *et al.*

2003, Rodgers *et al.* 2008). Levels of the 40 kDa Igfbp paralleled those of circulating Igf1, declining during catabolism and increasing to levels of control fish by termination of the CG response. These studies are the first to show circulating 40 kDa Igfbp trends during CG responses in fishes, with the strong up-regulation during realimentation possibly contributing to increasing Igf1's half-life in the plasma and the hormones elevation during CG. This also corroborates earlier studies in which similar trends in 40 kDa Igfbp were observed with fluctuations in nutritional state of the more primitive salmonid teleosts (Beckman *et al.* 2004, Pierce *et al.* 2005). In cattle, IGFBP-3 levels corresponded to fluctuations in reduced and compensatory growth, although the onset of puberty was a confounding variable (Luna-Pinto & Cronje 2000). Evidence in mammals also suggests that some IGFBPs may have ligand-independent mitogenic effects (see Duan & Xu 2005), underscoring the need to further investigate their role in fishes during CG.

During catabolic states preceding CG responses or following most periods of feed restriction in fishes, a state of Gh resistance is induced in which elevated plasma Gh is generally countered by depressed hepatic *ghr* gene expression and binding, which ultimately results in low levels of both hepatic *igf1* mRNA and plasma Igf1 (Gray *et al.* 1992, Duan *et al.* 1995, Pierce *et al.* 2005, Norbeck *et al.* 2007). In an alternative form of Gh resistance seen during feeding manipulation, plasma Gh increases and hepatic *igf1* gene expression declines with no change in hepatic *ghr* mRNA (Fox *et al.* 2006, Gabillard *et al.* 2006), raising the possibility that Ghr signaling may be impaired (Beauloye *et al.* 2002). In our investigation, hepatic *ghr1* and *ghr2* mRNA levels declined during feed deprivation along with hepatic *igf1* mRNA and circulating Igf1 peptide. This, along with evidence that pituitary *gh* mRNA, Gh content, and plasma Gh increase in these fish during the feed deprivation period (Turano 2006; unpublished results) suggests a state of Gh resistance. Furthermore, a strong correlation between both

Table 4 Correlations (R^2) between specific growth rate (SGR) and total hepatic mRNA levels of insulin-like growth factor-I (*igf1*), *igf2* and the Type I growth hormone (GH) receptor (*ghr1*) and Type II Ghr (*ghr2*). Specific growth rates were calculated from mean tank weights of control and treatment fish during days 0–21, 22–42, and 43–63 and were plotted against individual fish gene expression (mRNA) values. Correlations between gene expression (mRNA) values were derived from individual control and treatment fish on days 0, 21, 29, 42, and 63.

	SGR	<i>igf1</i>	<i>igf2</i>	<i>ghr1</i>
SGR				
<i>igf1</i>	0·48			
<i>igf2</i>	0·44	0·43		
<i>ghr1</i>	0·37	0·41	0·67	
<i>ghr2</i>	0·41	0·68	0·31	0·34

All correlations are statistically significant ($P < 0·001$). $N = 54$ –80 animals/parameter.

ghr1 and *ghr2* and *igf1* mRNA (Table 4) suggests that the *Ghr* may be responsible for mediating *Igf1* transcription, in particular during periods of Gh resistance and subsequent periods of CG. However, further research is required to confirm that *Ghr* binding declines in conjunction with mRNA levels, as has been recently shown for rainbow trout (Norbeck *et al.* 2007). Previous work in HSB has shown that *Igf1* binds with high affinity to the pituitary and acts as a powerful negative feedback inhibitor of Gh synthesis and secretion (Fruchtman *et al.* 2000, 2002). It is likely, therefore, that elevations in pituitary Gh activity and plasma Gh levels are a reflection of the prevailing low circulating *Igf1* seen during states of catabolism and vice versa during anabolism.

As previously indicated, we found that total hepatic *igf1* mRNA levels decline during catabolism and increase upon realimentation. However, when hepatic *igf1* gene expression is normalized according to standard procedures, i.e. to copy number/ng RNA or a housekeeping gene, an elevation in *igf1* mRNA is observed during the catabolic state of feed deprivation in HSB (see Fig. 3A), which is similar to that we previously reported with partial feed restriction in HSB (Picha *et al.* 2006). Studies by others using similar normalizations have reported either increases or no change in hepatic *igf1* mRNA but declines in plasma *Igf1* and/or growth following feed restriction (Ayson *et al.* 2007, Pierce *et al.* 2007). The lack of concordance in hepatic *igf1* mRNA and circulating hormone in these studies may be resolved by the use of an alternative method of mRNA normalization presented here. Since HSI fluctuates dramatically between feed restriction and CG and its values positively correlate to total liver RNA, we argue that expressing hepatic gene expression data as a function of the total amount in the organ relative to body size (total liver copy number/BW) is more physiologically appropriate than standard normalization methods. The final step of dividing by body weight puts the total hepatic mRNA production for a particular gene in an organismal context (total hepatic production per unit of body mass), which is critical when comparing gene expression trends between fish of different sizes and in variable metabolic states. Overall, the amount of transcript produced by the entire liver relative to the size of the fish provides a more accurate indication of the *Igf1* levels present in the circulation (see Figs 3B and 5A; $R^2=0.50$ for total hepatic *igf1* mRNA versus plasma *Igf1*). A similar qRT-PCR normalization scheme was utilized in gene expression analyses of spermatogenesis in rainbow trout where testes size varies considerably with reproductive state (Kusakabe *et al.* 2006). Hepatic *Ghr*-binding assays in fed and fasted rainbow trout were also argued to be more biologically relevant when expressed as total liver binding, as opposed to *Ghr* binding per unit of liver weight (Yao *et al.* 2006). We suggest that this normalization procedure be considered for all endocrine organs whose size changes significantly with physiological state.

Relative to *Igf1*, little is known about the role of *Igf2* in postnatal growth despite its high expression levels in the liver

of fish, ability to bind with equal affinity as *Igf1* to the *Igf* receptor, and potential for up-regulation by Gh (Shamblott *et al.* 1995, Fruchtman *et al.* 2002, Vong *et al.* 2003, Peterson *et al.* 2005, Moriyama *et al.* 2008). While barramundi and salmon *Igf1* were more potent in stimulating protein synthesis than *Igf2* in rat myoblasts (Degger *et al.* 2001, Wilkinson *et al.* 2004), injection of homologous *Igf2* resulted in similar elevations in weight gain in tilapia as injection with *Igf1* (Chen *et al.* 2000). Furthermore, *Igf2* has recently been shown to have significant mitogenic effects in trout myocytes (Codina *et al.* 2008). In HSB, total hepatic *igf2* mRNA levels fell dramatically during feed deprivation and rose rapidly upon realimentation, eventually exceeding those of control fish by day 21 of the experiment. In fact, a significant correlation was achieved between total hepatic *igf2* mRNA and SGR, indicating that the growth factor, assuming a similar translation pattern, could be facilitating phases of accelerated growth. This is the first study, to our knowledge, to report a correlation between SGR and any *Igf2* value in fishes, including during exposure to sequential cycles of catabolism and CG. An even stronger correlation was reported between total liver *ghr1* and *igf2* mRNA (Table 4), supporting the idea that plasma GH may work through hepatic receptors to regulate *Igf2* transcription and growth (Shamblott *et al.* 1995, Vong *et al.* 2003).

Studies in European sea bass showed that both muscle and liver *igf2* mRNA changed with growth state and feeding (Terova *et al.* 2007), while feed restriction in Atlantic salmon resulted in significant decreases in the circulating *Igf2* peptide (Wilkinson *et al.* 2006). Hepatic and muscle *igf2* mRNA is also higher in fast versus slow-growing families of catfish (Peterson *et al.* 2004), while hepatic levels of *igf2a* mRNA were higher in larger compared with smaller glass eels, *Anguilla japonica* (Moriyama *et al.* 2008). In rainbow trout, both hepatic *igf2* mRNA and plasma *Igf2* were depressed during fasting and were subsequently up-regulated during refeeding (Gabillard *et al.* 2006). This is in contrast to cattle where plasma *Igf2* did not correspond to feed restriction and CG (Hayden *et al.* 1993). Our results with hepatic *igf2* gene expression suggest that systemic or endocrine sources of *Igf2* may contribute to the rapid growth characteristic of CG and the negative growth seen with catabolism. This, along with the research of others, strengthens the hypothesis that *Igf2* might serve as an important regulator of postnatal growth in fish. It would appear based on gene expression analysis that paracrine or locally produced *Igf2* may not be contributing to differential growth in HSB, as we found no difference in muscle *igf2* mRNA expression between control and treatment fish. Whether striped bass or its hybrids possess duplicated forms of *Igf2* that might be differentially regulated with metabolic state as seen in eel (Moriyama *et al.* 2008) is unknown.

Aside from the endocrine effects of *Igf1* and possibly *Igf2*, local autocrine/paracrine dynamics of *Igf1* may also contribute to the phase of accelerated growth (Chauvigné *et al.* 2003, Luckenbach *et al.* 2007). In particular, transgenic

tilapia overexpressing Ghs were twofold heavier than wild types yet had depressed levels of plasma Igf1 and elevated skeletal muscle *igf1* gene expression (Eppler *et al.* 2007). This raises the possibility that autocrine/paracrine sources of Igf1 may be a significant contributor to somatic growth. In studies here, we found that skeletal muscle *igf1* mRNA increased tenfold from previously depressed catabolic levels and was completely restored to control values by 8 days into the CG response. These findings and those in rainbow trout and European sea bass (Montserrat *et al.* 2007, Terova *et al.* 2007) show that paracrine Igf1 may facilitate the rapid growth characteristic of CG in fish, although studies have yet to validate that locally produced *igf1* mRNA corresponds to protein. We speculated that the Igf receptor might increase to enhance tissue sensitivity to Igfs during CG. However, we found that gene expression of the receptor in skeletal muscle was not altered with either feed deprivation or the rapid growth phase. This does not preclude the possibility, however, that the receptor itself might be up-regulated, that an alternate form might be differentially regulated, or that it might show greater responsiveness to rapid fluctuations in systemic or local (muscle) Igf1 compared with relatively constant levels of ligand with fish on continuous daily feeding (Borski *et al.* 2000, Schlueter *et al.* 2006).

Although levels of *igf1* transcript were down-regulated during fasting, skeletal muscle *ghr2* expression was significantly up-regulated, while no change was observed with *ghr1*. We were unable to determine total muscle mRNA transcript levels as a function of body size during different metabolic states, as was done for the liver, because all muscle tissue cannot be reliably excised. Nevertheless, the elevations in skeletal muscle *ghr2* mRNA during catabolism are consistent with that observed in gilthead sea bream, rainbow trout, and tilapia (Saera-Vila *et al.* 2005, Gabillard *et al.* 2006, Pierce *et al.* 2007), and suggest an alternative form of GH resistance. That is, signaling by GH through the Ghrs could be impaired (Beauloye *et al.* 2002), leading to reduced Igf1 transcription as shown here. At the same time, increased expression of *ghr2* mRNA and presumably its protein during fasting, in parallel with elevations in plasma GH (data not shown) could facilitate either mobilization of energy stores (i.e. amino acids; Inui *et al.* 1985) or, conversely, protein sparing (Gamrin *et al.* 2000).

In conclusion, we have found that complete feed restriction can prime HSB for CG responses and that this catabolic state is characterized by weight loss, a drop in HSI below threshold levels and an endocrine state of Gh resistance. During the subsequent phase of refeeding and the ensuing CG response, elevations in growth rates are potentially driven by a combination of hyperphagia, improved feed conversion, and heightened levels of Igf1, 40 kDa Igfbps, and possibly Igf2. Interestingly, elevations in certain hepatic transcripts (e.g. *igf2*, *ghr1*, and *ghr2*) to levels exceeding those in normally fed fish might be a contributing factor to the accelerated growth rates characteristic of CG responses. Our results also suggest

that paracrine/autocrine Igf1 may be contributing to enhanced growth during CG. In addition, we propose that hepatic mRNA levels of specific genes be expressed as the total amount produced by the organ relative to body weight. This method provides a more physiologically relevant idea of the amount of gene, and presumed protein, produced by a tissue whose size changes dramatically with nutritional state.

Declaration of Interest

There is no conflict of interest that would prejudice this manuscript's impartiality.

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