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# Analysis of the effect of feeding regimen on GH/IGF axis in channel catfish (Ictalurus punctatus)

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## Contents

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## Abstract

Channel catfish (*Ictalurus punctatus*) are the most widely farmed aquatic animal in the United States. Assessing the effect of feeding regimens on their overall growth requires grow-out trials and a subsequent inefficient use of time and money. The growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis regulates growth based upon environmental factors such as feed intake in many teleost fish species. In order to increase the efficiency of aquaculture, the components of the GH/IGF-1 axis might be used as endocrine biomarkers to evaluate instantaneous growth rate. The goal of this study was to better understand the effects of feeding regimen on the regulation of growth in channel catfish. Accelerated growth during re-feeding periods after fasting is of particular interest. These periods, termed compensatory growth (CG) periods, have been observed in several teleost fishes. To study this, mRNA expression of GH/IGF-1 axis components was examined in the liver, skeletal muscle and pituitary of channel catfish during two weeks of fasting and one week of re-feeding with a control group of animals fed daily. In addition, hepatosomatic index (HSI) and condition factor (CF) were calculated for each fish. CF data suggests CG after only a week of re-feeding. Fasting induced a significant decrease ($P<0.05$) in HSI, which returned to normal after re-feeding. Fasting induced a significant increase in muscular *ghr-1* ($P<0.01$) and a significant decrease in muscular *igf-1* ($P<0.001$). Re-feeding induced a significant increase in hepatic *igf-1* ($P<0.001$), *igfbp-5a* ($P<0.01$), *igfr-1a* ($P<0.001$), and *ghr-1* ($P<0.01$) and in muscular *igf-2* ($P<0.01$), *igfbp-1* ($P<0.001$), *igfbp-2* ($P<0.01$), *igfbp-5a* ($P<0.05$), and *igfr-1a* ($P<0.05$). Results indicate that the GH/IGF-1 axis is highly sensitive to re-feeding in channel catfish and that CG can be induced.

## Introduction

Channel catfish (*Ictalurus punctatus*) are the most widely commercially cultured aquatic animal in the United States. First cultivated in Oklahoma in the early 1950's, channel catfish are now farmed all throughout the Deep South. The channel catfish aquaculture industry produces $450 million a year, with the majority of commercial production located in Mississippi, Arkansas, Alabama, and Louisiana (Food and Agriculture Organization of the United Nations. Fishery Information, Data, and Statistics Service, 1987; Chapman, 2009). Considering the scale of this industry, the ability to minimize cost and maximize product quality and quantity is paramount. Tracking growth by biomarkers, would remove the need for grow-out trials and save time and resources in research. These biomarkers could be used to indicate whether or not a certain feeding regimen or feed composition will accelerate growth, translating to production efficiency.

As in all vertebrates, somatic growth in teleost fishes has been shown to be regulated by growth hormone produced (GH) in the pituitary gland. GH directly induces the production of insulin-like growth factor-I (IGF-1) in the liver which carries out most of the interaction with growing tissues (Uchida et al., 2003). GH, IGF-I and several other proteins together make up the growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis shown in figure 1, which regulates growth.

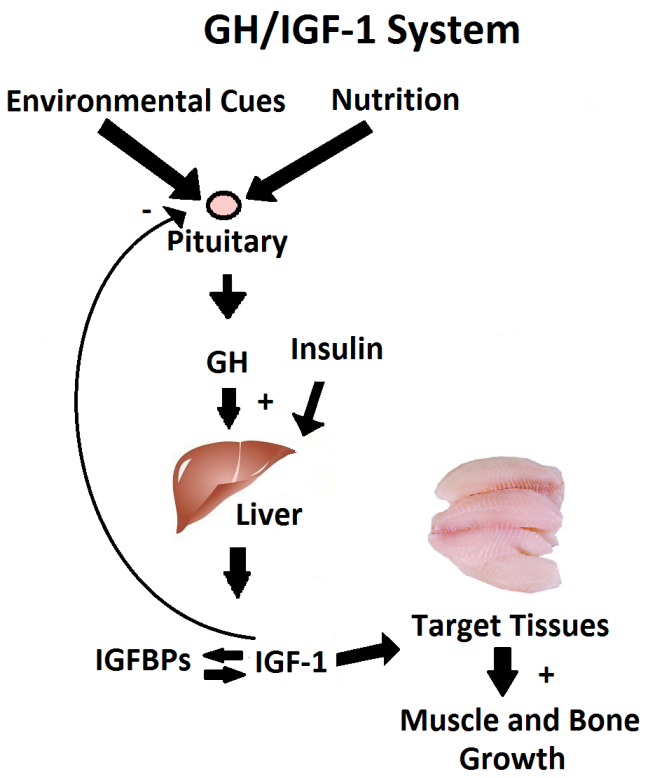


**Figure 1: Illustration of the GH/IGF-1 axis controlling growth in teleost fish.**

Multiple hormonal and nutritional factors may stimulate (+) the production and/or modify (+/-) the activity of IGF-1. Negative feedback (-) by IGF-1 inhibits GH secretion by the pituitary. The possibility is being explored of using the proteins of the GH/IGF-1 axis as endocrine biomarkers for growth in aquaculture production (Picha et al., 2008a). In order to accomplish this, the effects of feeding regimen on and the regulation of growth by the GH/IGF-1 axis must be understood in aquacultured fishes in particular.

The other proteins involved in the GH/IGF-1 axis include IGF-2, GH receptors (GHRs), IGF receptors (IGFRs) and IGF binding proteins (IGFbps). IGF-2 is mostly produced locally in the growing muscle and affects paracrine and autocrine regulation of growth. However, IGF-2 in fish has been shown to have a similar effect to IGF-1 and even binds to the same receptors. In addition, both IGF-1 and IGF-2 are regulated similarly by GH in several tissue types in fish (Fox

et al., 2010; Picha et al., 2008b). The GH and IGF receptors are obvious components of this system, as they are the sensors that activate change in cellular activities. Their presence or absence is able to regulate growth since it affects tissue sensitivity to GH and the IGFs. IGFbps perform several different tasks in fish, the bulk of which are not fully understood. Some IGFbps help IGF-1 and IGF-2 remain in circulation and avoid degradation (Shimizu et al., 2011a; Picha et al., 2008b). They may, in addition, prevent potential cross-activation of the insulin receptor and mediate the movement of IGFs from the circulation to the cell surfaces. IGFbp-1 inhibits IGF-1 activity by competing with IGFR-1a for the same binding site on IGF-1 (Kajimura and Duan, 2007). IGF-1 binds to IGFbp-1 with a higher affinity than to its receptor and so IGFbp-1 regulates the amount of IGF-1 in circulation that is available to interact with the receptors (Bower et al., 2008).

Many studies have been performed to analyze the effect of fasting on the GH/IGF-1 axis in vertebrates and in several teleost fishes. In channel catfish specifically, fasting has been shown to decrease hepatic IGF-1 mRNA expression (Small and Peterson, 2005; Peterson et al., 2009). In another study, pituitary GH mRNA was shown to increase during fasting, while hepatic GHR and plasma IGF-1 decreased (Peterson et al., 2009). During re-feeding, many teleost fishes have been shown to demonstrate a period of accelerated growth termed “compensatory growth” (Fox et al., 2010). Some studies have focused heavily on the effect of re-feeding and this compensatory growth period on the GH/IGF-1 axis. In hybrid striped bass, hepatic GHR-1, IGF-1, and IGF-2 and plasma IGF-1 have been shown to increase during re-feeding (Picha et al., 2008b). Though hormonal effects of re-feeding have been examined in many other teleost fishes, no research has been done to examine the effect of re-feeding in channel catfish.

The purpose of this study was to analyze how fasting and re-feeding periods affect the mRNA production levels for the proteins of the GH/IGF-1 axis in channel catfish. Possible feedback mechanisms were also identified in the regulation of the mRNA production for various proteins. mRNA expression for *igf-1*, *igf-2*, *igfbp-1*, *igfbp-2*, i*gfbp-5a*, *igfr-1a*, and *ghr-1* was measured in muscle and liver samples. mRNA expression of *gh* was measured in the pituitary. In addition, the body weight, length, blood glucose, and liver weight were measured for each sampled fish and the hepatosomatic index (HSI) and the condition factor (CF) were calculated.

## Materials and Methods

### *Building of the aquatic system for the experiments*

The system depicted in figure 2 was designed and built to house the catfish. It was made up of four circular-200 gallon tanks, all of which drain by means of gravity through the bottoms of the tanks into a joint pipe running the length of the four tubs. This pipe drained into a sump. A pump pulled water from the sump and through an Aquadyne© .60-B bead filter coupled to a UV sterilizer (Aquatic Ecosystem, Apopka, FL) and through a pipe above the tanks with outlet valves running into each tank.

**Figure 2: Completed aquatic system for the experiments.**

Using Microsoft Excel 2003 the flow rates and friction through each of the pipes were analyzed assuming a total flow rate through the pump of 300 gal/min. This was determined from the median flow rate from the pump curve. The drainage pipe and the pipe returning the water to the tanks were specified to be 2 in. Schedule 40 PVC. The valves and outlet pipes from the top pipe were specified to be ¾ in. Schedule 40 PVC. In order to allow the tanks to drain at an acceptable rate, they were elevated on two layers of four cinder blocks each with plywood between the cinder blocks and the tubs. Bulkhead fittings were used to deliver the water through the wall of the tanks to the drain pipe. In order to maintain a constant water level in the tubs and remove the possibility of fish getting sucked through the bottom, two foot long stem pipes were inserted to

the bulk head fittings. In order to allow for future changes in water level and cleaning away of sediments the stem pipes were not connected with adhesive. Caps were added to the tops of these stem pipes and small holes were drilled so that fish and food could not be sucked down the stem pipes. The valves controlling water into tanks were adjusted to equalize friction. A hose was put on each outlet to run the water along the side of the tub to create a current and reduce splashing. Two to three pieces of PVC pipe and/or fittings were added to the tubs to provide the fish with the appropriate habitat.

Bubble aerators were added to the tubs for oxygenation. An Aqua Logic®, Inc. Delta Star In-Line Heat Pump® (Aquatic Ecosystems) was added to the system after installation. A submersible pump was installed to draw water from the sump through the heater, and put it back into the sump.

***Animals***

All procedures and animal protocols were approved by and performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas. Approximately 60 channel catfish fry were obtained from the University of Arkansas at Pine Bluff (UAPB) aquaculture facility. These were housed in a 500 gallon circular tank and fed as much as they could eat in twenty minutes (to satiation) daily with a commercial catfish diet (Crude Protein (min) 32.00%, Crude Fate (min) 4.00%, Crude Fiber (max) 7.00%; Nutrisource, Inc., Stewart, MN) until the majority of the fish were between 15 and 45 grams in mass.

***Experimental design***

Catfish were individually weighed and their length was measured. They were sorted into 4 batches (labeled A-D) of 12 catfish each, such that each batch had a similar mean condition factor (CF; $100 \times \text{Fish weight} \div (\text{length})^3$) and a similar standard error mean for condition factor.

Each batch was placed in a separate tank from the system described above in "Building of the aquatic system for the experimentation". The residual catfish were either heavier or lighter than desired. These were placed in a separate 100 gallon tank (labeled E).

The fish in tubs A and C (control fish) were fed to satiation once daily for three weeks; the fish in tubs B and D (treated fish) were fasted for the first 2 weeks and re-fed to satiation once daily during the last week. The basic feeding regimen is displayed in table 1. At the end of week 1, 2, and 3, 4 fish from each of the 4 batches were sampled.

**Table 1: Feeding Regimen.**

| | Week | | |
|---|---|---|---|
| Batch | 1 | 2 | 3 |
| A | | | |
| B | | | |
| C | | | |
| D | | | |

| Fed | Fasted |
|---|---|

The residual fish in tank E. were fed to satiation daily and sampled for a wide range of tissues for the tissue distribution study. This was done to provide information on which target genes have particular relevance in the pituitary, liver and muscle, respectively.

For a more detailed view of the experimental design, see Appendix A.

***Sample collection***

All utensils and surfaces were sterilized with 75% ethanol. On each of the sampling days, four fish from each batch A, B, C, and D were randomly selected with a net and anesthetized in a 100

ppm concentration of tricane methane-sulfate buffered with bicarbonate. Fish were weighed and length was measured. Heparinized 1 ml syringes were used to draw blood from the caudal vein and between 0.4 and 0.8 ml of blood were removed and placed in prepared 1.5 ml microcentrifuge tubes on ice. The blood glucose was measured using a Clarity Plus Blood Monitoring System (Diagnostic Test Group, Boca Raton, FL). The spinal cord was then cut with scissors and the head was severed from the body behind the gill. The top of the skull was removed and the pituitary gland dissected. Next, the liver was removed and weighed, and two liver samples were collected. The fish was filleted and two skeletal muscle samples were collected. All were placed in microcentrifuge tubes on dry ice.

The blood samples were centrifuged with an Eppendorf Centrifuge 5804 R (Eppendorf, Hamburg, Germany) for 5 min. at 12,000 *g* and the plasma was removed into separate microcentrifuge tubes. Plasma and tissue samples were stored at -80°C until needed for further analysis. To establish the tissue distribution of our chosen target genes, the fish from tank E were sampled for brain, kidney, gill, heart muscle, and posterior intestine in addition to the skeletal muscle, liver and pituitary. All experimental procedures were approved by University of Arkansas Animal Care and Use Committee.

***Purification of total RNA***

Each tissue sample was homogenized in 500 µl of Tri-Reagent (Sigma-Aldrich, St. Louis, MO) with a VWR AHS200 PowerMax homogenizer (PRO Scientific, Inc., Oxford, CT) and 50 µl of BCP (1-bromo-3-chloropropane; Alfa Aesar, Shore Road, Heysham, Lancs.) was added. They were then shaken for 15 seconds and let stand for 2-15 minutes. The samples were then centrifuged at 12,000 *g* for 15 minutes at 4°C. Centrifugation separates the mixture into 3 phases: red organic phase (containing protein), an interphase (containing DNA), and a colorless upper

aqueous phase (containing RNA). The top phase was transferred into new microcentrifuge tubes. This is accordance with the manufacturer's recommendations (Sigma-Aldrich Tri-Reagent T9424: *Technical Bulletin*). Each mRNA sample was mixed with 250 µl of 2-propanol and let stand for 5-10 minutes. The samples were centrifuged at 12,000 *g* for 10 min. The liquid was removed and the RNA pellet was washed in 500 µl of 75% ethanol. The samples were centrifuged at 7,500 *g* for 5 min. The pellet was air dried for 5-10 minutes and dissolved in 20 µl of DEPC water (VWR, West Chester, PA). Total RNA was measured using a Thermo Scientific NanoDrop 2000 Spectrophotometer (Waltham, MA) at 260 nm and diluted to a standard concentration of 500 ng/µl.

***Reverse transcription***

A 2X RT (reverse transcription) master mix was prepared from High Capacity cDNA-RT Kit (Applied Biosystems, Foster City, CA). It contained the following per sample of RNA to be reverse transcribed: 2 µl 10X RT Buffer, 0.8 µl 25X dNTP Mix (100 mM), 2 µl 10X RT Random Primers, 1 µl MultiScribe™ Reverse Transcriptase, and 12.2 µl Nuclease-free water. This solution was mixed on ice. Each tube was filled with 18 µl of 2X RT master mix and 2 µl of an RNA sample. Reverse transcription was performed using an Eppendorf Mastercycler Gradient thermal cycler (Eppendorf). The cycling protocol was as follows: 10 min. at 25°C, 2 hr at 37°C, 5 min. at 85°C, then hold at 4°C. These cDNA samples were transferred to separate microcentrifuge tubes and stored at -20°C.

***Quantitative polymerase chain reaction (QPCR)***

For each target analyzed, the following process was followed. For each tissue, a 96-well plate was used. A solution was mixed including Sybr® Green (Sigma-Aldrich), 200nM forward and reverse primers for the desired target, and DEPC water. In each well of the 96-well plate, 14 µl

of solution was mixed with 1 µl of cDNA for each sample. All qPCR assays were run on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Cycling conditions were 95°C for 15 s and 60°C for 60 s for 40 cycles. The primers were obtained from Sigma-Aldrich and are displayed in table 2.

**Table 2. Primers for qPCR**

| Gene | Forward Primer | Reverse Primer | Gene Accession No. |
|---|---|---|---|
| *gh* | GCTCTGTGCTGAAACTGCTG | TCAGGTCAGCCAGCTTCTCT | NM_001200245 |
| *ghr-1* | CTTCAACCAGTGCCCAAGAT | TGTGCAGGAGAAACAACAGG | NM_001200214 |
| *igf-1* | CGTCTCCTGCCTGCTAAATC | AGAAGCGGAGAGGAGGAAAC | NM_001200295 |
| *igf-2* | GGCTTGCAAGAGTCAAGGAC | TTCTGCCTTGGGAGAATGTT | NM_001200199 |
| *igfbp-1* | TGGGATCGAGTGAACTACCC | TTCCTTAGGGCCTGAATGTG | NM_001200308 |
| *igfbp-2* | CCTGAGGAAGGCCAGTCAAAG | TCATCCACACGGTTCATCAT | FJ668942 |
| *igfbp-5a* | GCAGAGGCCCTCCATTTAAC | TTTGATCCGGAAAGACGAAG | UniGene Ipu.41172 |
| *igfr-1a* | GGAGCTAGGCCAAGGTTCTT | CACCCTGGTCTCAGGTTCAT | EF470421 |
| *ef-1a* | ACACTGCTCACATTGCTTGC | TTCAGGTTCTTGGGGTTGTC | DQ353797 |
| *β-actin* | GACCACCTTCAACTCCATCA | CTTCTGCATCCTGTCTGCAA | AY555575 |

For liver and skeletal muscle tissue samples qPCR assays were performed for *ghr-1, igf-1, igf-2, igfbp-1, igfbp-2, igfbp-5a, igfr-1a,* and normalizing genes, *ef-1a* and *β-actin.* For pituitary samples qPCR assays were performed for *gh,* and normalizing genes, *ef-1a* and *β-actin.*

***Data analysis***

In quantitative PCR analysis, the cycle threshold (Ct) value is defined as the cycle of the PCR protocol during which the fluorescence of the Sybr-conjugated cDNA product of the PCR reaction becomes detectable. Therefore, the more cDNA for a specific gene there is (the copy number), the sooner the product becomes detectable (the Ct value). Relative copy numbers of the target genes were calculated as $10^{-Ct/Ea}$ where Ea is the amplification efficiency for the primer

set. This transformed qPCR data for the liver was normalized using the geometric mean of elongation factor-1a (*ef1a*) transformed data and *β-actin* transformed data. Pituitary qPCR data was similarly normalized using only *ef1a*. Skeletal muscle qPCR data was normalized to total RNA, because using the normalization genes increased the standard error mean instead of decreasing it. Muscle qPCR assays will be replicated for future analysis. Treatment group comparisons were performed using a two-way ANOVA test. All statistical analysis was performed using computer programs, Prism5 for Windows (GraphPad Prism, San Diego, CA).

## Results

### *Effect of feeding regimen on growth*

Condition factor (CF) was calculated as 100 × Fish weight ÷ (length)$^3$. The results for CF are displayed in figure 3. CF was significantly higher ($P<0.05$) in the control (fed) fish than the treated (fasted/re-fed) fish during fasting (7 days and 14 days), but there is no significant difference after a week of re-feeding (21 days).

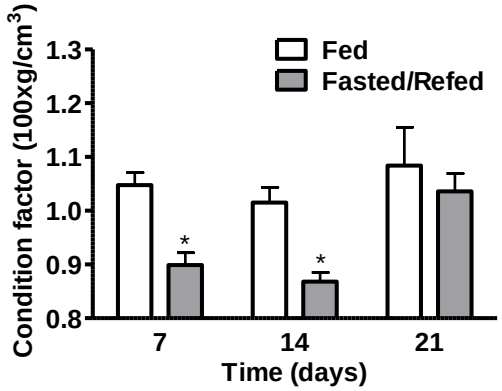


**Figure 3. Condition factor for sampled fish.**

***Hepatosomatic index***

Hepatosomatic index (HSI) is the liver weight as a percentage of the total body weight. HSI data is displayed in figure 4. Liver weight was not measured for the 7 day sample collection. HSI is significantly lower ($P<0.001$) in the treated fish relative to the control fish during fasting (14 days), but there appears to be a regaining of HSI after a week of re-feeding (21 days).

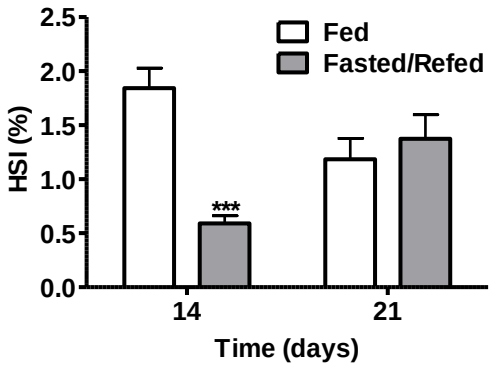


**Figure 4. Hepatosomatic index (HSI) for sampled fish.**

***Liver, muscle, and pituitary gene expression***

Normalized gene expression data for liver samples is depicted in figure 5. Because these values are only relative to one another and do not indicate any definite amount they are expressed as multiples of the fed group level at 7 days (the first bar in each graph). In the liver, *igf-1* (5A), *igfbp-5a* (5E), *igfr-1a* (5F), and *ghr-1* (5G) in treated fish show no significant difference from control values during fasting, but show a significant increase after a week of re-feeding. There is also an observable, but not significant increase in *igfbp-1* (5C) after a week of re-feeding. *Igf-2* (5B) and *igfbp-2* (5D) do not show any significant difference between expression in control and treated fish at any point in time.

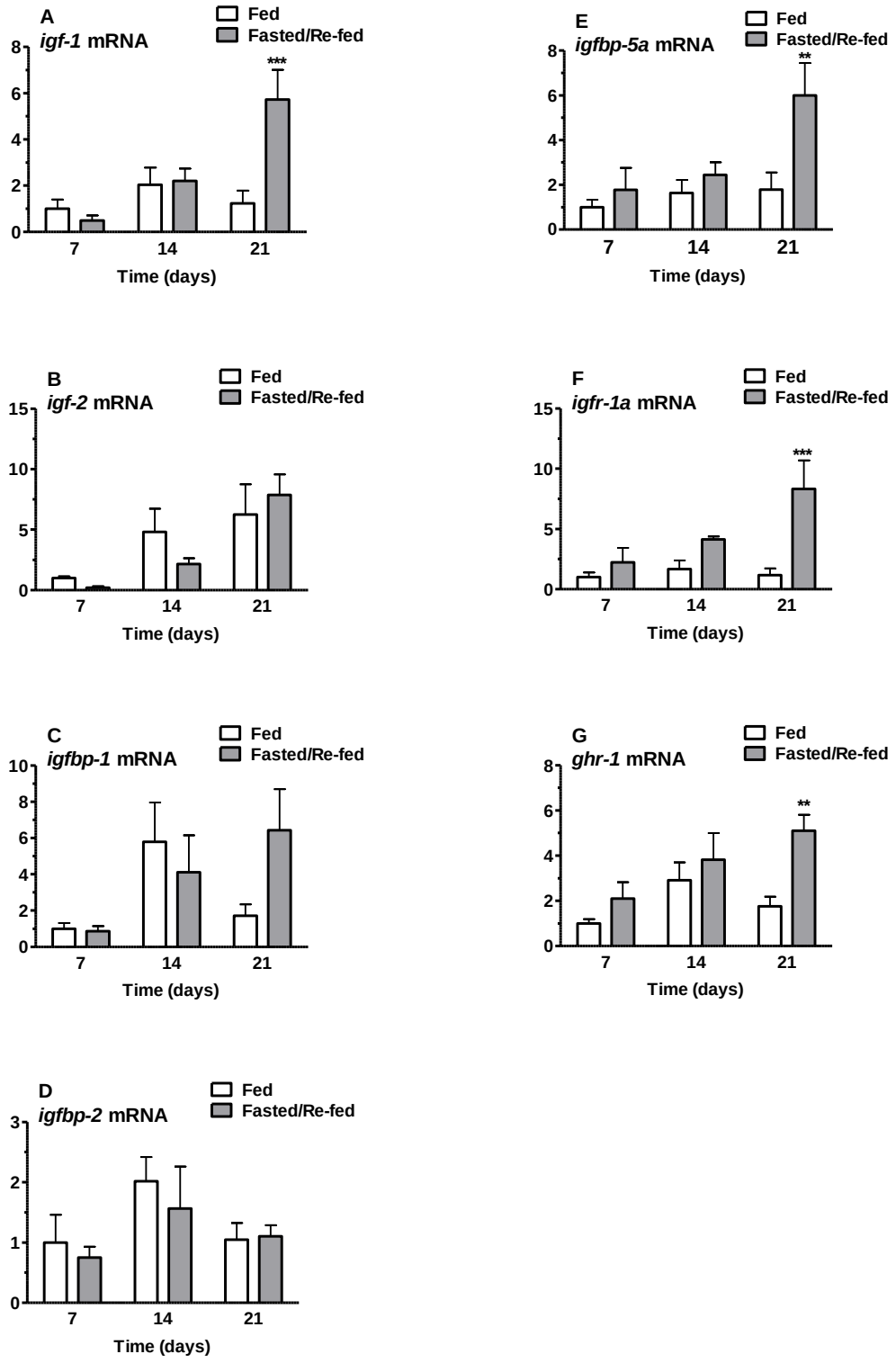


**Figure 5. Hepatic GH/IGF axis gene expression data.** Data is displayed relative to the fed group data at 7 days. Asterisks represent significant differences between groups (**P<0.01; ***P<0.001).

Normalized gene expression data for skeletal muscle samples is depicted in figure 6. In the muscle *igf-2* (6B), *igfbp-1* (6C), *igfbp-2* (6D), *igfbp-5a* (6E), and *igfr-1a* (6F) all show no significant difference between control and treated groups during fasting, but show a significant increase in treated fish after a week of re-feeding. *Igf-1* (6A) shows a significant decrease in treated fish after two weeks of fasting and a return to control levels after a week of re-feeding. There is a significant increase in *ghr-1* (6G) in the treated fish after two weeks of fasting as compared to the control fish and a subsequent return to control levels after a week of re-feeding.

The pituitary sample gene expression data (data not shown) indicated no significant difference in *gh* between control and treated fish at any of the time points.

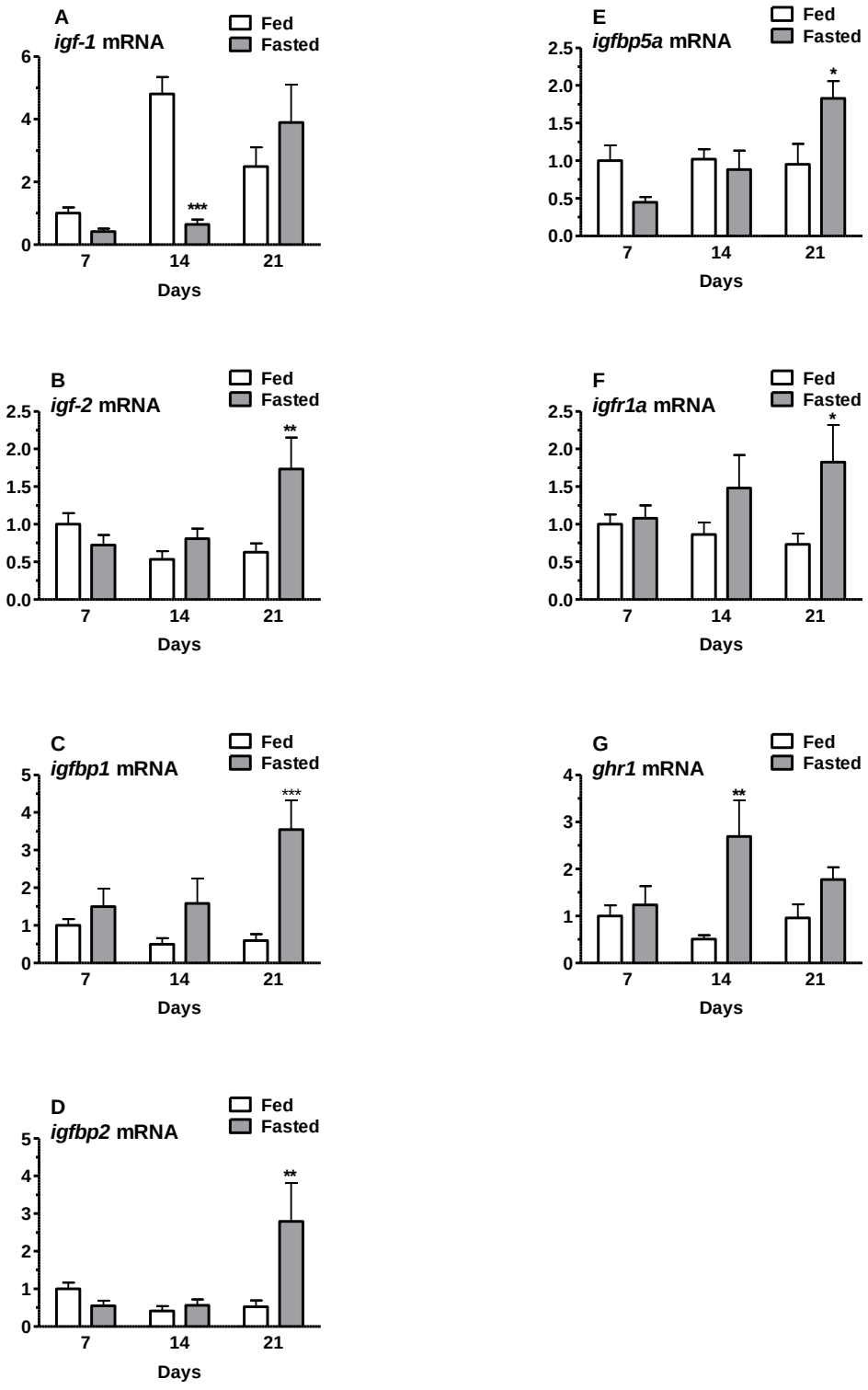


**Figure 6. Skeletal muscle GH/IGF axis gene expression.** Data is displayed relative to the fed group data at 7 days. Asterisks represent significant differences between groups (*P<0.05; **P<0.01; ***P<0.001).

## Discussion

Growth rate after re-feeding can only be inferred because there was only one re-feeding time point; however, there appears to be a significant indication of a compensatory growth period (fig. 3) due to the fact that the treated fish seem to have regained CF after only a week of re-feeding. Also, HSI demonstrates that the liver growth was extremely significant during the week of re-feeding. This compensatory growth appears to be regulated by the GH/IGF-1 axis.

Pituitary *gh* data shows no significant difference between control and treated fish. This demonstrates that GH production in channel catfish is not regulated by fasting and re-feeding at the mRNA level.

Hepatic GH/IGF-1 axis data seems to demonstrate a significant amount of regulation due to re-feeding. Contrary to what was expected in correlation with the stunted growth and the decrease in HSI, hepatic *ghr-1* remained the same throughout fasting (Norbeck et al., 2007). In addition, fasting did not produce the expected decrease in *igf-1*. In tilapia, fasting has been shown to decrease *igf-1* levels significantly within two weeks. In coho salmon, however, fasting did not significantly reduce *igf-1* levels until 4 weeks (Picha et al., 2006). It is possible that *igf-1* levels would have decreased upon longer fasting; however, the maintained *igf-1* makes sense with the maintained levels of *ghr-1* and could denote a difference of regulatory mechanism between species. The increase in *ghr-1* after re-feeding indicates that liver tissue became more sensitive to circulating GH. Since GH triggers IGF-1 production in the liver this makes sense with the increase in *igf-1*. In fact, in this study re-feeding appears to increase hepatic *igf-1* well above normal fed levels (6-fold). In teleost fishes, IGF-1 has been shown to be released from the liver with IGFbp-1 and IGFbp-2 (Shimizu et al., 2011a, Shimizu et al., 2011b). The up-regulation of

hepatic *igfbp-1* and *igfbp-5a* corresponding to the up-regulation of *igf-1* suggests that IGF-1 could also be released from the liver with IGFbp-5a.

In skeletal muscle, the raised *ghr-1* in the fasted fish would increase the tissue sensitivity to circulating GH. This makes sense because during fasting, growth hormone typically has a catabolic effect on non-vital tissues, such as skeletal muscle (Fuentes et al., 2012). Norbeck et al. (2007) demonstrates this catabolism in adipose tissue. Stimulation of lipolysis and gluconeogenesis would help direct resources to more vital tissue like the nervous system. Elevated skeletal muscle GHR during fasting appears to be a genetically conserved strategy in fishes. Thus increased expression has been reported in rainbow trout and Mozambique tilapia (Gabillard et al., 2006; Pierce et al., 2007) and the receptor appears to have a physiologically important role in muscle metabolism during fasting. This increase was not observed during re-feeding suggesting that the re-feeding down-regulates *ghr-1* production in skeletal muscle tissue to compensate for the break-down of the muscle during fasting. Because there was no significant decrease in hepatic *igf-1* expression during fasting and no significant decrease in muscular *igfr-1a* it is uncertain as to the mechanism of the slowed growth during fasting except for the decrease in autocrine/paracrine production of *igf-1*. However, the circulating IGF-1 plasma data will hopefully make this clearer. The increase in *igfbp-1*, *igfbp-2*, and *igfbp-5a* in skeletal muscle tissue during re-feeding indicate that these are involved in the regulation of compensatory growth. There is also an increase in *igf-2*, which seems to act in an autocrine/paracrine manner to promote compensatory growth in the skeletal muscle tissue.

## Conclusion

It can be concluded that compensatory growth can be induced in channel catfish. It can also be concluded that this may be driven by an up-regulation of *igf-1* production in the liver and *igfr-1a* in the skeletal muscle tissue as well as autocrine/paracrine production of *igf-2.*

This is among the first of many steps in devising a way to use the GH/IGF-1 axis as biomarkers for growth in channel catfish. However, it is the beginning of understanding the protein interaction in such a way that we can predict growth rates effectively and cheaply.

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## Appendix A: Sampling Plan and Experimental Design