Insulin-like growth factor-I (IGF-I) is considered a critical regulator of growth in fish and other vertebrates. Little is known about its regulation under different states of growth in southern flounder (*Paralichthys lethostigma*). In this study, we cloned IGF-I cDNA in southern flounder and investigated whether alterations in circulating IGF-I and hepatic IGF-I mRNA might accompany changes in growth under different nutritional planes. Southern flounder IGF-I coding sequence has 99% homology compared to a closely related species, Japanese flounder (*P. olivaceus*). The mature IGF-I amino acid sequence of southern flounder is identical to that of Japanese flounder and composed of 68 amino acid residues compared with 70 residues in other teleost, amphibian, avian, and mammalian IGF-Is. In the 5´-untranslated region, four different cDNA sequences were detected by 5´RACE, possibly reflecting alternate transcription initiation sites and tissue-specific usage.

To establish whether IGF-I correlates with or mediates growth in southern flounder, we conducted an 8-week trial in which growth rates were manipulated through changes in feeding regimen. Fish were subjected to either four weeks of starvation (treatment group) or four weeks of *ad libitum* feeding (control group). After the initial four weeks, all fish were fed *ad libitum* for an additional four weeks. Liver and blood were collected at 4-week intervals throughout the study for measures of IGF-I mRNA
expression and plasma IGF-I levels, respectively. For the first four weeks, food-deprived fish showed little growth compared with fed fish. Upon refeeding, the treatment group showed a substantial increase in body weight (132 %) compared to control group (107 %) over the last four week period. However, the growth rate of the treatment group following realimentation was no different than that of the size-matched control group fed over the first four weeks, suggesting southern flounder were unable to compensate for the lost growth. By contrast, the hepatosomatic index (HSI) was completely restored in the treatment group following realimentation and exceeded that of the control group by the end of eight weeks. Overall, it appears flounder do not undergo significant growth compensation, despite a complete restoration of hepatic energy stores, at least under this experimental paradigm. Circulating IGF-I levels of the control group increased over the first four weeks, and then subsided slightly by the end of eight weeks where concentrations remained above that observed in animals at the start of the experiment. The food-deprived fish showed a significant decline in circulating IGF-I levels during the starvation period, and levels were completely restored to that of control fish by the end of the refeeding period. Circulating IGF-I concentrations were positively correlated to changes in specific growth rate in southern flounder ($r^2 = 0.67, P = 0.0012$). By contrast, hepatic IGF-I mRNA levels did not differ between control and the starved treatment group, although a rise was observed in fish upon refeeding ($P = 0.08$). There was no significant correlation between hepatic IGF-I mRNA and circulating IGF-I levels ($r^2 = 0.01; P = 0.75$) or specific growth rate ($r^2 = 0.16; P = 0.2$).

Although the growth of teleosts is regulated by various environmental parameters, little is known of the effects of ambient calcium concentration on this process. Therefore,
we also examined a range of ambient calcium concentrations on growth of southern flounder raised in fresh water. Growth did not significantly differ in animals exposed to 50, 100, and 150 ppm calcium (as CaCO₃) over a 10-week period. We conclude that ambient calcium concentrations as low as 50 ppm do not adversely affect southern flounder growth. However, prolonged exposures to waters with lower than 50 ppm calcium may adversely affect disease resistance and survival.
ASSESSMENT OF GROWTH AND ITS REGULATION THROUGH INSULIN-LIKE GROWTH FACTOR-I IN SOUTHERN FLOUNDER, *Paralichthys lethostigma*

by

AIKO UEDA

A thesis submitted to the Graduate Faculty of North Carolina State University at Raleigh in partial fulfillment of the requirements for the Degree of Master of Science

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DEDICATION

To my parents, Yoshiko and Shunzo Ueda.
BIOGRAPHY

The author was born January 8, 1979, in Yamatokoriyama, Nara, Japan. She graduated from Fujishiro High School, Fujishiro, Ibaraki. She attended Nihon University from 1997 to 2001, and graduated with a Bachelor of Science degree in Marine Science and Resources. In 2002 she entered Graduate School at North Carolina State University.
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INTRODUCTION

Due to its high market value, wide distribution along the Atlantic and Gulf coasts of the United States and decline in wild stocks, research has focused on developing aquaculture methods for southern flounder (*Paralichthys lethostigma*). Despite its wide market appeal and potential for aquaculture, little information is available on the environmental and nutritional control of growth in southern flounder. Likewise, virtually nothing is known of the endocrine mechanisms that mediate growth in this species.

Fish growth is regulated by various environmental factors, such as temperature, salinity, photoperiod, nutritional status, as well as internal factors such as sex and genotype. The hypothalamo-pituitary somatotropic axis, which includes growth hormone (GH), insulin-like growth factor-I (IGF-I), and their receptors and binding proteins, are core neuroendocrine factors that integrate these external and internal inputs to mediate growth in vertebrates. Insulin-like growth factor-I is a single-chain polypeptide considered central to controlling somatic and skeletal growth, as it mediates many of the actions of GH and itself may correlate better to changes in growth status relative to GH, at least under certain conditions, including poor nutrition (Uchida et al., 2003; reviewed by Duan 1998). IGF-I is also a potent negative feedback inhibitor of GH secretion and synthesis (reviewed by Fruchtman et al., 2000). Although evidence in knockout mice suggests that paracrine, rather than systemic sources of IGF, which are derived mainly from the liver, may be most critical to postnatal growth (Yakar et al., 1999), the relative contribution of circulating versus local IGF-I in controlling growth in teleosts remains uncertain (Gabillard et al., 2003 a and b).
Nutritional status has a profound effect on the GH/IGF-I growth axis in fish (Duan, 1998) as well as in mammals (Straus, 1994). In tilapia, fasting limits somatic growth and reduces both hepatic IGF mRNA and circulating IGF-I and exerts little effect on GH secretion (Uchida et al., 2003). Moriyama et al. (1994) reported that fasting of coho salmon smolts for 25 days reduces plasma IGF-I. A decrease of plasma IGFs was also observed after fasting for six weeks in rainbow trout (Foucher et al., 1992) and in sea bream (Pérez-Sánchez et al., 1994). With regard to effects on IGF-I gene expression, 21 days of fasting reduces hepatic IGF-I mRNA in barramundi (Matthews et al., 1997). A decrease in IGF-I mRNA was also observed after starvation in eel (Duan and Hirano, 1992). Duan and Plisetskaya (1993) reported that hepatic IGF-I mRNA levels decreased after four weeks of fasting and were restored by two weeks of refeeding in coho salmon. This regulation of IGF-I mRNA is restricted to the liver, and was not observed in other tissues examined. These results in salmonids are significant since the major production site of circulating IGF-I in adult fish is the liver, while all other tissues produce IGF-I locally (Duan and Plisetskaya, 1993; Duguay et al., 1992; Shamblott and Chen, 1993). Collectively, results to date suggest that poor nutrition down-regulates IGF-I production at the mRNA level (Duan, 1998) and reduces circulating IGF-I. By contrast, circulating GH may increase during fasting (Straus, 1994; McMurtry et al., 1997; Duan, 1998), which likely reflects a reduction in hepatic GH receptors (Gray et al., 1992), and a subsequent decline in circulating IGF-I and its negative feedback inhibition of pituitary GH production and secretion (Pérez-Sánchez et al., 1992; Blaise et al., 1995; Fruchtman et al. 2000). Reduced ration and dietary protein deficit have also been reported to down-regulate hepatic GH receptor number and plasma IGF-I concentration while stimulating circulating GH levels (Pérez-Sánchez et al., 1994, 1995;
Company et al., 1999, Beckman et al., 2004). There are no reports on the nutritional control of IGF gene expression and circulating hormone levels with respect to growth regulation of flounder or other flatfish that exhibit a more sedentary life history relative to many teleost fishes.

In addition to nutrition, growth in fish may also be under the influence of abiotic factors such as temperature and salinity. Although salinity may regulate growth in a species specific manner, previous studies in our laboratory show that salinities ranging from 0-30 % ppt are similarly effective in promoting southern flounder growth (Daniels and Borski, 1998). However, the optimal balance of ions that control the growth of teleosts generally, and southern flounder specifically has not been determined. It is known that environmental calcium is required for proper development and hatching of the larvae of certain euryhaline or marine teleosts (Brown and Lynam, 1981; Lee and Hu, 1983; Lee and Krishnan, 1985; Tucker, 1991). Wurts and Stickney (1989) reported that environmental calcium significantly affected red drum survival in fresh and sea water. Red drum require water with a calcium content greater than 100 ppm and chloride content greater than 150 ppm for good survival. For optimum growth the calcium content should be more than 150 ppm and chloride content greater than 1,000 ppm (Davis, 1990). Regardless, little is known about the interaction between ambient calcium and fish growth and its potential control by IGF-I. Practical information about flounder growth in low calcium waters would be beneficial to determine optimum water quality conditions for flounder production.

The objective of this study was to investigate the growth performance of southern flounder under different ambient calcium levels and to food deprivation and realimentation. We also examined whether any observed changes in growth are accompanied by similar
alterations in IGF-I protein and gene expression. The nucleotide sequences of IGF-I cDNAs have been determined in a number of teleost species including coho salmon, rainbow trout, Atlantic salmon, chum salmon, catfish, carp, tilapia, and Japanese flounder (Cao et al., 1989; Shamblott and Chen, 1992; Duguay et al., 1992; Wallis and Devlin, 1993; Kavsan et al., 1993; McRory and Sherwood, 1994; Liang et al., 1996; Reinecke et al., 1997; Tanaka et al., 1998, respectively; for review see Chan and Steiner, 1994). Depending on species, teleosts share 70-80 % homology with *Xenopus*, chicken, rat, and human mature IGF-I amino acid sequence (Le Bail et al., 1998). The sequence of IGF-I in southern flounder has not yet been reported. Therefore, we also cloned and sequenced the IGF-I gene for southern flounder and developed a real-time PCR method for detecting potential changes in IGF-I gene expression in animals induced to grow at different rates with nutritional state.
MATERIALS AND METHODS

Cloning and Sequencing of IGF-I

The first strand of cDNA was synthesized from 1 µg of total RNA extracted from southern flounder liver (Chomczynski and Sacchi, 1987) using 1 µM primer 2 (see below) in a 20 µl reaction (Omniscript™ Reverse Transcriptase, Qiagen Inc., Valencia, California, USA). The cDNA was subjected to 30 cycles of PCR consisting of 1 min of denaturing at 94 C, 1 min of annealing at 67 C, and 2 min of extension at 72 C by Hot Star Taq polymerase, using primer 1 (sense): GTGCGATGTGCTGTACCTCCT and primer 2 (antisense): TCTGTGCCCTTGTCCACTT corresponding to positions 273-293 and 626-645 of the Japanese flounder IGF-I cDNA (Tanaka et al., 1998), respectively. The locations of the primers for these studies are shown in Fig. 1.

The 3´ regions of the IGF-I cDNA were synthesized by the RACE method (Ambion, Austin, Texas, USA) using 4.8 µg total RNA extracted from southern flounder liver tissue by Trizol method (Invitrogen, Carlsbad, California, USA). Two microliters of cDNA was subjected to a first round PCR consisting of 25 cycles of 1 min of denaturing at 94 C, 1 min of annealing at 58 C, 2 min of extension at 72 C using primer 3 (sense): AAAGTGGACAAGGGCACAGA and primer 4 (antisense): CACTTGCTGCGCTGTATGTAAT corresponding to positions 627-645 and 2289-2309 of the Japanese flounder IGF-I cDNA. The second round PCR was performed from 2 µl of this PCR reaction at 30 cycles, using primer 3 (sense) and primer 5 (antisense): ACAAAAGTTCAAGCGACAGGAT corresponding to positions 1164-1184 of the Japanese flounder IGF-I cDNA.
The 5´ regions of the IGF-I cDNA were synthesized by the RACE method (Ambion, Austin, Texas, USA), using gene outer specific primer 2 (antisense):
TCTGTGCCCTTGTCACCTT and gene specific inner primer 6 (antisense):
GGGGCCATAACCTGTTGGTTTAC in the coding region of southern flounder IGF-I cDNA.

These PCR products were separated in a 1-2 % agarose gel, and the target band was excised from the gel and extracted using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, California, USA). Extracted PCR products were ligated and cloned into pCR®II vector (Invitrogen, Carlsbad, California, USA) and sequenced at the University of Chicago Cancer Research Center. The sequence was compared with known IGF-I sequences using BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information). Sequences were aligned using the ClustalW algorithm (Thompson et al., 1994) and phylogenetic analysis performed using the neighbor-joining clustering method (Saitou and Nei, 1987) in the computer software program Mac Vector 7.0 (Genetics Computer Group, Oxford Molecular, Madison, Wisconsin, USA).

Animals and Systems

Southern flounder (Paralichthys lethostigma) were obtained in July 2002 from a commercial fingerling producer (Southland Fisheries, Hopkins, South Carolina, USA) and transported to production facilities at the North Carolina State University. Fish were maintained in a 4.3 m diameter high-density polyethylene tank at 23 C and fed twice daily to satiation with a commercial pelleted feed (45 % protein, Melick Aquafeeds, Catawissa, Pennsylvania, USA) until the start of the experiment.
Growth experiments were conducted in recirculating systems consisting of bubble bead biofilters (Water Garden Gems model BBF-1, Marion, Texas, USA), heat pumps (Aquanetics model AHP-4A, San Diego, California, USA), water pumps (Little Giant Co. model 2MDQX-SC, Oklahoma City, Oklahoma, USA), UV sterilizers (Aqua Ultraviolet Co., Temecula, California, USA), and four 250-liter culture tanks (Rowland Fiberglass Inc, Ingleside, Texas, USA). Culture systems were maintained in a temperature-controlled room (21 C) under fluorescent light (12L: 12D; 1.2 m above the water level).

**Growth Experiment 1: Ambient calcium levels**

The experiment was conducted to determine the effects of ambient calcium level on southern flounder growth for 10 weeks beginning in May 2003. Fish were randomly divided into three different groups where ambient calcium levels were maintained at 50, 100, and 150 ppm as CaCO₃ in fresh water (< 0.5 ppt) (four replicate tanks/group). Fish (25 per replicate) were preacclimated for two days to the appropriate calcium levels before starting the experiment. Calcium level was measured daily as CaCO₃ by the murexide indicator method (LaMotte Chemical Co. model PHT-CM-DR-LT, Chestertown, Maryland, USA) and maintained at the same level throughout the experiment by addition of gypsum (CaSO₄·5H₂O, Garden Gypsum, Calcium A.H. Hoffman Inc. Lancaster, New York, USA) via three separate water reuse systems as described above. Experimental water was prepared from well water containing 20 ppm calcium as CaCO₃. Water temperature and salinity were maintained at 22 C, and 0.4 ± 0.3 ppt, respectively through the experiment. All fish were fed to satiation twice a day by hand and weight of feed given to fish was recorded as total feed consumption. Fish were anesthetized in tricaine methanesulfonate (MS-222) and body weights and total lengths
were measured at day 0 and every two weeks thereafter. Ammonia (NH3-N) and nitrite (NO2-N) were measured in each of the treatments throughout the course of the experiment by Nessler method and ferrous sulfate method, respectively (Hach Co, Loveland, Colorado, USA).

**Growth Experiment 2: Fed vs. Starved**

Beginning in September 2002, fish were randomly divided into two groups (control and treatment) and stocked in quadruplicate 250-liter tanks (28 fish/replicate). Animals were allowed to acclimate for four days and were maintained on feed prior to the start of the experiment. Water temperature and salinity were kept at 21.6 ± 1.2 C and 30.8 ± 3.7 ppt, respectively throughout the experiment. Control fish were fed twice daily to satiation (approximately 1 % body weight/day for 5-10 minutes per feeding) throughout the 8-week growth trial. Treatment fish were fasted for the first four weeks and then fed twice daily to satiation for the remaining four weeks. Fish were fed a 3-5 mm slow sinking flounder feed (50 % protein, Melick Aquafeed, Catawissa, Pennsylvania, USA). Feed consumption was recorded and expressed as total feed consumed and that consumed as a percentage of body weight. Feed efficiency was calculated as weight gain divided by total feed consumption X 100.

Fish were anesthetized in tricaine methanesulfonate (MS-222) and body weight and total length were recorded at the beginning of the experiment and every two weeks thereafter. Specific growth rate in body weight (SGRW; %/day) was calculated as (\(\ln BW2- \ln BW1\))/(t2 – t1) X 100, where ‘BW2’ and ‘BW1’ were fish body weight at time 2 and time 1 in days respectively. Liver and blood samples were collected from three fish from each
replicate tank at the same time intervals. Liver weight was recorded to determine the hepatosomatic index (HSI, liver weight/body weight X 100). Blood was collected from caudal vessel by a heparinized capillary tube and the plasma was separated by centrifugation for 20 min at 2660 x g. Plasma was frozen at -80 C for later radioimmunoassay (RIA). Liver samples were frozen immediately in liquid nitrogen, and stored at -80 C.

*Circulating IGF-I*

Circulating levels of IGF-I were measured from the plasma of fish in the Fed vs. Starved experiment by a “Universal Fish” IGF-I RIA according to the manufacture’s protocol (Gropep, Adelaide, South Australia, Australia). To verify that this assay reliably detects plasma IGF-I in flounder, a dilution/displacement curve of southern flounder plasma was compared to that of the fish IGF-I standard provided by the manufacturer (Fig. 15). Prior to RIA, plasma samples were first extracted by acid-ethanol (87.5 \% ethanol and 12.5 \% 2N HCl, v/v) according to Shimizu et al. (2000) to remove IGF-I binding proteins, which may interfere with IGF-I measurement. The extracted samples were diluted by the RIA buffer supplied in the kit. All samples were analyzed in triplicate and plasma samples from individuals at day 0, week four, and week eight of Fed vs. Starved experiment were measured. Intra-assay coefficient of variation was 13 \% (N=15).

*IGF-I mRNA determinations by quantitative RT-PCR*

IGF-I mRNA expression in liver tissues from the Fed vs. Starved experiment was quantified by real-time PCR using fluorogenic probes (TaqMan®, Applied Biosystems, Foster City, California, USA). Total RNA was isolated from liver using the guanidium
thiocyanate procedure as described by Chomczynski and Sacchi (1987). Liver total RNA was further extracted with lithium chloride to remove excess glycogen and other carbohydrates (Puissant and Houdebine, 1990). Any possible genomic DNA contamination within the RNA preparations was removed by treatment with a triple dose (6U of DNase-I per 40 µl reaction) and extended treatment (1 hr at 37 C) using the DNA-free™ method (Ambion, Austin, Texas, USA). Total RNA concentration was quantified and purity was assessed by spectrophotometry (NanoDrop Technologies Inc., Rockland, Delaware, USA) before the RT-PCR reaction. A260/A280 values for all samples ranged from 1.9-2.0.

The primers and TaqMan probe for southern flounder IGF-I was designed from southern flounder mature IGF-I sequence (Fig. 2) using the ABI’s Primer Express program and are shown as follows: (Forward) 5´-CAACAGGTATGGCCCCAAT-3´; (Reverse) 5´-CGCAGCTCACAGCTTTGGA-3´; and (Probe) 5´-FAM-CACGACGGTCACGCGGCATT-TAMRA-3´. EF1alpha (elongation factor 1 alpha) was used as a reference gene and the primers and probe sequences for EF1alpha are as follows: (Forward) 5´-CGAGAAAGAAGCTGCCGAGAT-3´; (Reverse) 5´-CGCTCGGCCTTCAGTTTGT-3´; and (Probe) 5´-FAM-CAAGGGCTCCTTCAAGTACGCCTGG-TAMRA-3´. Probes were labeled with the reporter dye FAM at the 5´end and the quencher dye TAMRA at the 3´end and purchased from Biosource Int. (Camarillo, California, USA). Custom primers were purchased from Invitrogen (Carlsbad, California, USA). The IGF-I primers and probes were designed to avoid overlaps in the sequences of other insulin-like polypeptides, IGF-II and insulin.

One µg of total RNA was reverse transcribed in a 20-µl reaction for 60 min at 37 C by Omniscript™ Reverse transcriptase (RT) kit (Qiagen Inc., Valencia, California, USA).
The RT reactions were set up on ice and consisted of 1 µM of Oligo-dT primer, 4U of omniscript reverse transcriptase, 1X RT buffer, 0.5 mM each of dNTP, 10U of RNase inhibitor. TaqMan PCR was performed on an ABI 7700 Sequence detector, using the standard cycling conditions (50 C for 2 min, 95 C for 10 min, followed by 40 cycles of 95 C for 15 s and 65 C for 1 min). Wells contained 25 µl of PCR mixture, made from ABI Universal PCR Master Mix (0.01 U/µl AmpErase uracil N-glycosylase, 0.025 U/µl Amplitaq Gold DNA polymerase, passive reference dye I, 200 µM dNTPs including dUTP, 3.5 mM MgCl2), with 250 nM probe and 900 nM of each of the forward and reverse primers, and 10 ng cDNA template.

Threshold was set as 0.1 for each run. Standard curves for IGF-I and EF1 alpha gene detection were run in each assay and were generated from 4-fold serial dilution of pooled cDNA (0.1-100 ng) derived from liver tissue of 13 month old southern flounder (N=3). The standard curve (log input cDNA template levels versus cycle threshold [C_T]) was linear (r^2 >0.98) at cDNA template levels ranging from 0.1-100 ng. Since the efficiency plot test derived from standard curves for IGF-I and EF1 alpha measurements passed the required validation experiment (slope < 0.01), data for IGF-I were expressed by ∆∆C_T method (ABI User Bulletin 2; Livak and Schmittgen, 2001). Data were first calculated as ∆C_T = C_T_{IGF-I}-C_T_{EF1alpha} for each respective sample. The ΔC_T for each sample was then expressed as a change from those values derived from the respective control and treatment fish at the initiation of the study or at time zero (ΔΔC_T = ΔC_T_{sample}-ΔC_T_{time zero}). A no amplification control (NAC) which lacked reverse transcriptase and a no template control (NTC) which lacked cDNA template were evaluated in real-time PCR analyses of IGF-I and EF1 alpha gene expression to
confirm the absence of genomic DNA in RNA preparations as well as the absence of any nonspecific nucleic hybridization.

**Statistical Analysis**

Differences among treatments groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer HSD test using the JMP 5.0.1 software (SAS Institute, Cary, North Carolina, USA). Differences between the time points in SGRW, HSI, circulating IGF-I, and IGF-ImRNA levels within a treatment were assessed by two-way ANOVA followed by Tukey-Kramer HSD using SAS GLM procedure (SAS version 8, SAS Institute, Cary, North Carolina, USA). Repeated measurements ANOVA assuming at first order auto regressive or AR(1) covariance for errors over time (Littell et al., 1996) were performed for the difference between time points in body weight and total length from Fed vs. Starved experiment. The differences at the $P<0.05$ level were considered to be significant. The relationship between growth rate, circulating IGF-I, hepatic mRNA IGF-I levels, and HSI were analyzed by linear regression.
RESULTS

IGF-I Sequence

A total of 1206 bp of southern flounder IGF-I cDNA including 251 bp of 5’-untranslated region and 394 bp of 3’-untranslated region was sequenced (Fig. 2). The IGF-I coding sequence (561 bp) shows 99 % homology to its congener, Japanese flounder (*P. olivaceus*) (Fig. 3). In a partial cloning of the 3’-untranslated region of IGF-I cDNA we discovered that southern flounder shows 11 nucleotide differences, 8 nucleotide deletions, and 2 nucleotide insertions compared to a similar region of the Japanese flounder IGF-I cDNA (Fig. 4). In the 5´-untranslated region, 4 different sequences were detected by the 5´RACE method (Fig. 2). The deduced amino acid sequences of southern flounder mature IGF-I were compared with those of Japanese flounder (Tanaka et al., 1998), salmon (Cao et al., 1989; Duguay et al., 1992), tilapia (Chen et al., 1997), *Xenopus* (Kajimoto and Rotwein, 1990), rat (Shimatsu and Rotwein, 1987) and human (Steenberg et al., 1991) (Fig. 5). The mature IGF-I amino acid sequence (consisting of B, C, A, and D domains) of southern flounder is identical to that of Japanese flounder and composed of 68 amino acids. The mature peptide was 77, 77, 84, 92, 92, 100 % identical to human, rat, *Xenopus*, tilapia, salmon, and Japanese flounder, respectively, further indicating that this peptide is highly conserved among vertebrates (Table 1). A phylogram was generated showing that southern flounder IGF-I falls into the same grouping as other fishes (Fig. 6).

Growth Experiment 1: Effect of ambient calcium

The growth of fish raised at different calcium levels was not significantly different at concentrations ranging from 50 ppm to 150 ppm (Fig. 7 and 8) over the 10-week period. In
the 50 ppm calcium treatment, the survival rate of fish declined at around 5-weeks and remained steady thereafter (Fig. 9). Survival rates at 50 ppm CaCO₃ (48 %) was significantly lower than fish raised at the 100 (75 % survival) and 150 ppm (80 % survival) CaCO₃ by the end of the growth trial ($P<0.05$; 10-week period). Water ammonia and nitrite levels in the 50 ppm CaCO₃ group were elevated relative that of the other treatment groups during the initial 0-5 week period (Fig. 10). Following this period, which coincided with the prominent reduction in survival rates between 4-5 weeks of fish in the 50 ppm CaCO₃ treatment, ammonia and nitrite levels subsided to background concentrations for the remainder of the experiment.

*Growth Experiment 2: Fed vs. Starved*

Somatic growth of southern flounder subjected to 4-week cycles of fasting and then daily satiation feeding is shown in Fig. 11. Body weight decreased by 10 % in fish during four weeks of fasting, but significantly increased by around 32 % upon re-feeding ($P<0.05$). Body weight of control fish, which were fed throughout the study, increased for the first six weeks and subsequently leveled off by eight weeks. By the end of the experiment control fish grew by 47 % relative to their initial body weight ($P<0.05$). In treatment fish total length did not change during the starvation period and showed a more substantial gain during the realimentation period ($P<0.05$, Fig. 12). The total length of control fish increased throughout the experiment and was significantly higher (9 %) than fish exposed to the fasting and refeeding cycle at the end of the experiment ($P<0.05$).

SGRW of control group increased over the first four weeks and then subsided over the last four weeks as body size increased (Fig. 13). By contrast, fasted fish showed a decline
in SGRW by the first two weeks of fasting and slightly rebounded between week 2 and 4 of the starvation period although the rate of growth remained close to zero. Upon refeeding, fish showed a consistent increase in SGRW through the refeeding period. However, SGRW of treatment fish following realimentation was no different than that of control animals fed over the first four weeks.

The HSI of control fish was maintained at 1.5-2.0 throughout the experiment (Fig. 14). By contrast, the HSI of the food-deprived treatment significantly decreased to 0.5 following starvation \( (P<0.05) \), but was completely restored upon refeeding and exceeded the HSI of control fish by the end of the experiment.

There was no mortality during the four weeks of starvation period in the treatment group (Table 2). The survival rates at the end of the experiment for control and treatment group were 81.0 % and 63.0, respectively and not significantly different between groups \( (P<0.05) \). Total feed consumption in control fish declined as body weight increased (Table 3). During the refeed period, treatment fish showed overall lower total feed consumption than the control group over the first 4 weeks. As a percentage of body weight, realimentated treatment fish exhibited similar feed consumption as control fish over 0-4 weeks, but higher consumption than control fish at 4-8 weeks of the study. Overall feed efficiency was significantly lower in fish on the cyclic diet than control animals on a continuous normal feed regimen \( (P<0.05) \).

*Circulating IGF-I levels in Fed vs. Starved southern Flounder*

The dilution/displacement curve of southern flounder plasma showed parallelism to the standard curve of the Gropep fish (Tuna) standard (Fig. 15) indicating this “universal”
The assay is valid for measures of circulating IGF-I in southern flounder plasma. The volume of flounder plasma that showed linearity and produced a strong parallelism to the fish IGF-I standard ranged from 2.5 - 50 µl. Our subsequent analyses utilized 20 µl of plasma for IGF-I measurements. In control animals fed a normal diet, circulating IGF-I increased during the first four weeks and subsequently leveled off over the remaining four weeks of the study (Fig. 16), similar to that seen with body weight changes. On the other hand, IGF-I declined significantly in 4-week fasted fish ($P<0.05$), but was completely restored to the level of control group upon four weeks of refeeding. A strong positive correlation was found between circulating IGF-I and SGRW ($r^2 = 0.665, P = 0.0012$, Fig. 17) and HSI ($r^2 = 0.62, P = 0.0023$, Fig. 18) in this study.

**Hepatic IGF-I mRNA expression in Fed vs. Starved southern flounder**

Serial dilutions of samples gave linear log input cDNA vs $C_T$ plots with $r^2>0.98$. PCR efficiencies ranged from 88-101 % and were consistent between IGF-I and EF1alpha measurements. IGF-I mRNA levels typically resulted in $C_T$s of 25-27, with EF1alpha $C_T$s of 20-22.

Hepatic IGF-I mRNA levels remained similar in the control group throughout the course of the feeding experiment. IGF-I mRNA levels were not altered by four weeks of fasting. Upon refeeding for four weeks (week 8 of the experiment) IGF-I mRNA levels increased by 61 %, albeit this effect was not statistically significant ($P = 0.084$; Fig. 19). There were no correlations between IGF-I mRNA level and circulating IGF-I ($r^2 = 0.01, P = 0.75$, Fig. 20) or IGF-I mRNA levels and SGRW ($r^2 = 0.16, P = 0.19$, Fig. 21).
DISCUSSION

The present investigations on IGF-I cDNA structure show the deduced amino acid sequence of southern flounder mature IGF-I (domains B, C, A, and D) is composed of 68 amino acids, which is two residues shorter than that reported for other teleosts, amphibian, bird and mammalian species (Tanaka et al., 1998). We found that the amino acid sequence of the preproIGF-I of *P. lethostigma* differs from *P. olivaceus* by three residues, one in the signal peptide region, and two in the E-domain region. Since the differences reflect conserved, nonpolar amino acid substitutions, there is likely to be minimal impact on the primary structure, and hence function or processing of the preproIGF-I between the two species. Overall, the structure of southern flounder mature IGF-I is > 90 % and 77-84 % homologous to other teleosts as well as amphibians and mammals, respectively, confirming the highly conserved nature of this peptide among all vertebrates examined to date.

A 1206 bp region of the southern flounder IGF-I cDNA was sequenced, which includes 251 bp of the 5´-untranslated and 394 bp of the 3´-untranslated regions (Fig. 2). We attempted to obtain the entire 3´ region of the IGF-I cDNA by the 3´RACE method. However, these trials were unsuccessful, probably due to the long length of the 3´-untranslated region. Nagamtsu et al., (1991) also used the RACE method for 3´ regions of hagfish IGF-I cDNA and reported similar results. Constructing a cDNA library or alternatively using an inverse PCR method (Ochman, 1990) may be required to obtain the complete 3´ region of the IGF-I cDNA. Nevertheless, we found by Northern blot analysis that the liver of southern flounder, like that of its Japanese congener, expresses two major transcripts of around 4.6 and 2.8 kb (data not shown). These two transcripts may reflect the
alternative usage of polyadenylation sites in the 3’ noncoding sequence (Steenbergh et al., 1991; Hoyt et al., 1992; Tanaka et al., 1998), which is rich in (T)nA sequences and may play a role in mRNA stability and the post-transcriptional regulation of IGF-I synthesis (Hepler et al., 1990).

We found four different sequence lengths in the 5´-untranslated region by 5´RACE method. These are likely to reflect at least four transcription initiation sites, although as many as four minor and one major 5´-flanking sites were reported in Japanese flounder by ribonuclease protection assay (Tanaka et al., 1998). Three of the southern flounder 5´-flanking sites are similar to those observed in Japanese flounder, with the transcription initiation site 225 bp upstream from the start codon in southern flounder corresponding to the major site detected in Japanese flounder. The most distal 5´-flanking initiation site (251 bp upstream from the start codon) detected in southern flounder is absent in Japanese flounder and is similar to one of three observed in chum salmon (Koval et al., 1994). Multiple transcription initiation sites have been identified in the rat and human IGF-I genes (Adamo et al., 1991a; Jansen et al., 1991; Simmons et al., 1993), and tissue-and development-specific usage of the start sites has been observed (Lowe et al., 1987; Hoyt et al., 1988; Adamo et al., 1989, 1991b; Shemer et al., 1992). Although, multiple transcription start sites in the IGF-I gene(s) have been identified in avian and fish (Kajimoto and Rotwein, 1991; Koval et al., 1994) their physiological functions are not well understood and require further investigations.

In addition to the 5´-sequence heterogeneity, four E-domain variants of IGF-I mRNA designated as Ea-1, Ea-2, Ea-3, and Ea-4 have been identified in salmonid species (Duguay et al., 1992; Wallis and Delvin, 1993; Shamblott and Chen, 1992). The IGF-I transcripts differ only in the size of the E-domain and are generated by alternative splicing of pre-
mRNA. The differential expression of these E-domain variants of salmon IGF-I mRNA is under hormonal regulation (Duguay et al., 1994). In salmon, all four transcripts are detectable in liver, and hepatic Ea-1 and Ea-3 levels increased following GH treatment while Ea-2 and Ea-4 were unchanged (Duguay et al., 1994). Most non-hepatic tissues, including heart, fat, brain, kidney, spleen, and ovary, expressed only the Ea-4 transcript, and expression was not influenced by GH (Duguay et al., 1994). Ea-1 and Ea-3 transcripts were detectable in gill samples from fish treated with GH, and juvenile salmon expressed Ea-1, Ea-2, and Ea-4 in ovary tissue (Biga et al., 2004). In the PCR of IGF-I cDNA, we found only one E domain transcript that is equivalent to Ea-4 in southern flounder. It includes the entire E-domain sequence. However, an additional splice form, Ea-2, which lacks the E-2 region altogether, is expressed in tissues from Japanese flounder (Tanaka et al., 1998). RT-PCR analysis of E-domain variants in Japanese flounder indicate that the Ea-4 is the dominant transcript expressed in most tissues, including liver, with a more limited tissue distribution (e.g. liver, spleen, brain, gill, testis, kidney only) and lower abundance of the Ea-2 IGF-I mRNA transcript. In preliminary studies, we designed primers and TaqMan probes specific to the E1-E2 region and compared them with the effectiveness of primers designed against the mature IGF-I sequence (B to D domain specific) and found that the E1-E2 domain primers were less effective at detecting muscle IGF-I mRNA than primers for the mature IGF-I sequence. However, the level of detection of liver mRNA expression was similar when using either E1-E2 or B-D domain primers. Albeit indirect, these results suggest a possible tissue specific expression of E-domain variants in southern flounder. Further analyses are required to confirm this, as has been demonstrated with Japanese flounder. Our subsequent research
on the nutritional control of IGF-I gene expression focused on the primers against the mature
IGF-I so as to detect all possible tissue IGF-I mRNA variants.

Although IGF-I is a critical regulator of somatic and skeletal growth in those
vertebrates examined to date, its role in mediating these processes in flounder or other
flatfish is unknown. We evaluated the nutritional effects on southern flounder growth and
found that feed manipulations significantly altered both skeletal and somatic growth.
Compared with control fish fed daily, fasted flounder showed a decline in body weight, total
length and specific growth rate in body weight (Fig. 11, 12, and 13). Upon refeeding, fasted
fish exhibited increases in somatic, skeletal and specific growth rate, but body mass in
treatment fish never fully caught up to control fish following the 4-week realimentation
period. Indeed, SGRW of fish following realimentation was no different than that of size-
matched control fish fed over the first four weeks. These results indicate southern flounder
did not undergo compensatory growth, a physiological response characterized by an
accelerated growth rate that typically exceeds normal rates following periods of restricted
growth associated with states of stress, overwintering and malnutrition (for reviews see

The inability of southern flounder to exhibit a compensatory growth response is not
likely due to ‘developmental’ stunting potentially linked to prolonged starvation, since Holst
(2003) found juvenile fish lack the response despite short-term periods (2-8 days) of fasting.
Rather, it appears the absence of growth compensation observed in flounder may be due to
limited hyperphagia, feed efficiency or both. We found that refeeding did not improve
overall feed efficiency or enhance hyperphagia in southern flounder (see Table 3) relative to
size-matched control fish fed at normal rates throughout the experiment (0-4 week control vs.
4-8 week treatment fish). These features typically accompany accelerated growth in fish during partial or complete growth compensation (Ali et al., 2003).

Despite the lack of catch-up or growth acceleration beyond that of normal rates, we found that southern flounder show the pattern of impaired growth and subsequent recovery reflective of the nutritional state of the animal. Fasted and refed fish showed the typical HSI dynamics and growth responses associated with the depletion and subsequent restoration, respectively, of hepatic glycogen and/or lipid stores.

Changes in somatic growth observed with feed manipulations were accompanied by similar alterations in circulating IGF-I levels as measured by RIA. Four weeks of fasting significantly reduced plasma IGF-I concentrations, and levels were restored completely to control levels by the end of the refeeding period. Interestingly, even in control fish fed daily throughout the experiment, circulating IGF-I levels corresponded well with growth, being highest at four weeks and then subsiding slightly by the end of eight weeks, similar to that observed with changes in body weight. The strong concordance between SGRW and circulating IGF-I ($r^2 = 0.67$, $P = 0.0012$) in animals under both the normal and cyclic feed regimen indicates that this IGF-I pool is strongly linked to changes in southern flounder growth. Similar findings of a positive correlation between circulating IGF-I level and growth rate have been demonstrated for coho salmon (Moriyama et al., 1994), rainbow trout (Foucher et al., 1992), and sea bream (Pérez-Sánchez et al., 1994) and more recently in tilapia (Uchida et al., 2003). We also found that circulating IGF-I levels correlate well with the HSI in flounder, suggesting nutritional metabolites are a critical facet to the regulation of systemic IGF-I, and presumably, somatic growth. The contribution of local IGF production
(i.e. chondrocytes and muscle; Chauvigné et al., 2003) to the changes in growth reported here cannot be ruled out.

It is well documented in mammals and to a lesser degree in fish that the primary source of circulating IGF-I is derived from hepatic production (Yakar et al. 1999; reviewed by Duan 1997). Consistent with this, fasting has been shown to reduce both plasma IGF-I and hepatic IGF-I mRNA levels in mammals and various fish, including tilapia (Uchida et al., 2003), barramundi (Matthews et al., 1997), coho salmon (Duan and Plisetskaya 1993) and gilthead seabream (Pérez-Sánchez et al., 1994). These findings suggest that nutritional status regulates IGF-I production at the mRNA level. Despite the extended fasting period of four weeks, we found little effect of fasting on IGF-I mRNA levels in southern flounder using the sensitive real-time quantitative PCR method for measures of gene expression. However, upon refeeding IGF-I mRNA levels increased, although the effect was only significant at the P = 0.08 level. No correlation was found between hepatic IGF-I mRNA expression and circulating IGF-I level or SGRW.

The discordant regulation in IGF-I mRNA level and circulating IGF-I in fasted flounder might be explained by several factors such as the differential regulation of IGF binding proteins (IGFBPs), mRNA stability, translation rate, or other variables. IGFBPs bind to IGF-I in circulation, prolong its half-life and are under the influence of nutrition (Jones and Clemmons 1995; Kelley et al., 2001). It is possible that a decline in the most prominent 40-50 kDa IGFBP (mammalian IGFBP-3-like), which tends to correlate with the anabolic state of the animal and is reduced under catabolic states (Kelley et al., 2001), could increase the clearance rate of IGF-I, leading to reduced circulating levels with little change in hepatic IGF-I production or expression levels during periods of starvation.
Based on the results presented here it is also possible that IGF-I mRNA levels may not change during starvation due to altered mRNA stability or changes in posttranscriptional regulation. Thissen et al. (1991) reported that translational as well as pre-translational defects could be responsible for decreased serum IGF-I concentrations in dietary protein restricted rats. Straus and Takemoto (1990) investigated the regulation of hepatic IGF-I mRNA in rat using a nuclear transcription elongation assay. They reported that there was considerable individual variability in IGF-I gene transcription within each group and the magnitude of the decrease in IGF-I gene transcription did not account for the pronounced decrease in total IGF-I mRNA, and suggested that IGF-I mRNA is regulated at least partly by at the posttranscriptional level. Whether steady-state mRNA levels are regulated at the transcriptional level or the posttranscriptional level or both in fish is presently unknown (Duan 1998).

At the beginning of this study, we hypothesized that ambient calcium levels would affect growth of southern flounder raised in fresh water. However, the growth rate was not significantly different among treatments for calcium concentrations ranging from 50 ppm to 150 ppm. On the other hand the survival rate was significantly lower at 50 ppm CaCO₃. The mortalities that account for the lower survival occurred around the fifth week of the growth trial, which coincided with spikes in nitrite, preceded by an elevation in ammonia levels in the recirculating system. It is uncertain that higher nitrite level in the system with 50 ppm calcium may have led to a lower level of biofiltration and a subsequent decline in survival. However, there are reports that environmental calcium may affect the survival of euryhaline fish independent of changes in water quality parameters *per se*. Wurts and Stickney (1989) reported that red drum stocked in fresh water (0.56-1.9 g/l TDS) with calcium concentration
of 1.7 mg/l or less had 0-33% survival after 96 h, but growth and survival were not significantly affected when calcium was between 9 and 107 mg/l or ppm. Grizzle et al., (1985) also reported that without additional calcium, juvenile striped bass (Morone saxatilis) and hybrid bass (M. chrysops × M. saxatilis) had 16% survival, compared to the groups with 80-99% survival when ambient calcium concentrations were increased from 20 to 100 mg/l as CaCO₃. Collectively, our results suggest that calcium concentrations as low as 50 ppm does not adversely affect growth, but may reduce survival of southern flounder in fresh water. Whether the effects of suboptimal ambient calcium levels on survival is a result of elevated nitrite level or not remains to be determined.

In summary, we have cloned and sequenced the southern flounder IGF-I gene, established a RIA for reliable measures of circulating IGF-I from plasma volumes as low as 5-10 µl, and developed and validated a sensitive real-time quantitative RT-PCR assay for measures of tissue IGF-I mRNA expression. Our results show that fasted and refed southern flounder exhibit typical growth responses associated with the depletion and restoration of energy stores, but exhibit little compensatory growth or growth acceleration beyond that of normal growing individuals when alleviated of growth stunting conditions associated with malnutrition. Our findings also suggest that circulating IGF-I may serve as a useful biomarker of the growth status of southern flounder under different nutritional states, including in animals that exhibit subtle changes in growth under normal nutritional conditions. Finally, it is possible to raise high-value southern flounder in a fresh water environment with no loss in growth at calcium levels as low as 100 ppm. However, further analysis is required to ascertain the relationship between the survival rates and the long time exposure to ambient calcium levels of 50 ppm or less.
Fig. 1. Schematic representation of southern flounder IGF-I cDNA. The boxes indicate the coding region, and side lines mark the 5’ and 3’-untranslated regions. The solid lines indicate the cDNA clones isolated and the dashed lines indicate the region not cloned in this study. The arrowheads show the position of the primers used for cloning southern flounder IGF-I cDNA.
Fig. 2. Nucleotide sequence (above) and deduced amino acid sequence (below) of southern flounder IGF-I cDNA. Asterisk indicates the stop codon and arrows above sequence indicate transcription initiation sites. The mature IGF-I amino acid sequence is underlined.
Fig. 3. Nucleotide sequence of preproIGF-I cDNA of southern flounder and Japanese flounder (GeneBank Accession No. AF061278). Identical nucleotides are shaded and homology between the sequences is 99%.
Fig. 4. A portion of 3’-untranslated region of southern flounder IGF-I cDNA compared with the same region in Japanese flounder (AF061278). Identical nucleotides (nc) are shaded and homology between the sequences is 95%. Eight nc deletions, 2 nc insertions, 11 nc differences are found between them.
Fig. 5. Comparison of amino acid sequence of preproIGF-I (Japanese flounder and southern flounder only) and mature IGF-I from other vertebrates. The southern flounder sequence is based on the PCR fragments from Fig. 2. References are follows: human (GeneBank Accession No. AY260957), rat (AH002176), Xenopus (M29857), tilapia (AF033796), salmon (M32792), Japanese flounder (AF061278).
Table 1. Percent identity between mature IGF-I peptide of southern flounder and other known species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Percent Identity</th>
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</thead>
<tbody>
<tr>
<td>Japanese flounder</td>
<td>100</td>
</tr>
<tr>
<td>Salmon</td>
<td>92</td>
</tr>
<tr>
<td>Tilapia</td>
<td>92</td>
</tr>
<tr>
<td>Xenopus</td>
<td>84</td>
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<tr>
<td>Rat</td>
<td>77</td>
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<tr>
<td>Human</td>
<td>77</td>
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</table>
Fig. 6. Sequence identities among IGF-I protein sequences from various vertebrates using the neighbor-joining clustering method (Saitou and Nei, 1987). Branch lengths are proportional to sequence divergence and numbers along branches reflect relative differences in number of amino acid residues compared with the southern flounder sequence.
Fig. 7. Somatic growth of southern flounder subjected to different ambient calcium levels. Each value represents the mean ± SEM of four replicates per treatment group with 25 fish/replicate at initial stocking. There was no significant difference in somatic growth between groups.
Fig. 8. Skeletal growth of southern flounder subjected to different ambient calcium levels. Each value represents the mean ± SEM of four replicates per treatment group with 25 fish/replicate at initial stocking. There was no significant difference between groups.
Fig. 9. Survival rate of southern flounder subjected to different ambient calcium levels over a 10-week rearing period. Each value represents the mean ± SEM of four replicates per treatment group with 25 fish/replicate at initial stocking. Asterisks denote significant differences from other treatments: *$P<0.05$. 
Fig. 10. Ammonia and nitrite levels in the ambient water of the 10-week growth trial of southern flounder reared at 50, 100, and 150 ppm CaCO₃.
Fig. 11. Somatic growth of southern flounder subjected to a 4-week cycle of fasting and normal feeding. Arrow indicates the beginning of the refeed (*ad libitum* twice daily) period, which follows four weeks of fasting in the treatment group. Control fish were fed *ad libitum* twice daily throughout the experiment. Each value represents the mean ± SEM of four replicates per treatment group with 28 fish/replicate at initial stocking. Asterisks denote significant differences from the control group and letters denote significant differences cross time within each group using Tukey-Kramer HSD test: \( P<0.05 \).
Fig. 12. Skeletal growth of southern flounder subjected to a 4-week cycle of fasting and normal feeding. Arrow indicates the beginning of the refeed (*ad libitum* twice daily) period, which follows four weeks of fasting in the treatment group. Control fish were fed *ad libitum* twice daily throughout the experiment. Each value represents the mean ± SEM of four replicates per treatment group with 28 fish/replicate at initial stocking. Asterisks denote significant differences from the control group and letters denote significant differences cross time within each group using Tukey-Kramer HSD test: $P<0.05$. 
Fig. 13. Specific growth rate in body weight (SGRW) of southern flounder subjected to a 4-week cycle of fasting and normal feeding. Treatment groups were fasted for the first four weeks and then subsequently fed *ad libitum* daily for the remaining four weeks. Control fish were fed *ad libitum* twice daily throughout the experiment. Each value represents the mean ± SEM of four replicates per treatment group with 28 fish/replicate at initial stocking. Asterisks denote significant differences from the control group and letters denote significant differences across time within each group using Tukey-Kramer HSD test: *P*<0.05.
Fig. 14. Hepatosomatic index (HSI) of southern flounder subjected to a 4-week cycle of fasting and normal feeding. Arrow indicates the beginning of the refeed (*ad libitum* twice daily) period, which follows four weeks of fasting in the treatment group. Control fish were fed *ad libitum* twice daily throughout the experiment. Each value represents the mean ± SEM of four replicates per treatment group with 28 fish/replicate at initial stocking. Asterisks denote significant differences from the control group and letters denote significant differences across time within each group using Tukey-Kramer HSD test: *P*<0.05.
Table 2. Survival rate of southern flounder subjected to a 4-week cycle of fasting and normal feeding. Treatment groups were fasted for the first four weeks and then subsequently fed *ad libitum* daily for the remaining four weeks. Control fish were fed *ad libitum* twice daily throughout the experiment. Each value represents the mean ± SEM of four replicates per treatment group with 28 fish/replicate at initial stocking. There was no significant difference between each group.

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Week 2</th>
<th>Week 4</th>
<th>Week 6</th>
<th>Week 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
<td>98.68 ± 1.0</td>
<td>92.19 ± 3.0</td>
<td>80.77 ± 5.0</td>
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<tr>
<td>Treatment</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
<td>98.44 ± 2.0</td>
<td>63.46 ± 8.0</td>
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Table 3. Feed consumption and feed efficiency of southern flounder subjected to a 4-week cycle of fasting and normal feeding. Treatment groups were fasted for the first four weeks and then subsequently fed ad libitum daily for the remaining four weeks. Control groups were fed ad libitum twice daily throughout the experiment. Each value represents the mean ± SEM of four replicates/group with 28 fish/replicate. For each category, asterisks denote significant differences from the control group at same time intervals: *P<0.05.

<table>
<thead>
<tr>
<th>Interval (week)</th>
<th>Total feed consumption (g)</th>
<th>Feed consumption (% body weight/day)</th>
<th>Feed efficiency (%)</th>
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</thead>
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<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
</tr>
<tr>
<td>0-2</td>
<td>76.00 ± 0.00</td>
<td>-</td>
<td>1.10 ± 0.03</td>
</tr>
<tr>
<td>2-4</td>
<td>86.00 ± 0.00</td>
<td>-</td>
<td>1.30 ± 0.08</td>
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<tr>
<td>4-6</td>
<td>58.63 ± 1.57</td>
<td>49.70 ± 2.86*</td>
<td>0.83 ± 0.04</td>
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<tr>
<td>6-8</td>
<td>33.20 ± 5.61</td>
<td>49.63 ± 5.54</td>
<td>0.54 ± 0.09</td>
</tr>
<tr>
<td>0-4</td>
<td>162.00 ± 0.00</td>
<td>-</td>
<td>1.20 ± 0.04</td>
</tr>
<tr>
<td>4-8</td>
<td>91.83 ± 6.38</td>
<td>99.33 ± 7.21</td>
<td>0.68 ± 0.06</td>
</tr>
<tr>
<td>0-8</td>
<td>253.83 ± 6.38</td>
<td>99.33 ± 7.21*</td>
<td>0.94 ± 0.04</td>
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</table>
Fig. 15. Binding inhibition curve for Universal Fish IGF-I standard and serial dilutions of flounder plasma (2.5-50 µl) in the Universal Fish IGF-I radioimmunoassay (Gropep LTD, Adelaide, Australia). A dilution/displacement curve of southern flounder plasma paralleled the universal fish standard curve.
Fig. 16. Circulating IGF-I level of southern flounder subjected to a 4-week cycle of fasting and normal feeding. Arrow indicates the beginning of the refeed (*ad libitum* twice daily) period, which follows four weeks of fasting in the treatment group. Control fish were fed *ad libitum* twice daily throughout the experiment. Each value represents the mean ± SEM of three replicates/group with 3 fish/replicate. Asterisks denote significant differences from the control group and letters denote significant differences cross time within each group using Tukey-Kramer HSD test: *P*<0.05.
Fig. 17. Correlation between circulating IGF-I level and specific growth rate in body weight (SGRW) of southern flounder. Data are derived from control animals fed *ad libitum* twice daily as well as treatment animals exposed to a 4-week cycle of fasting and *ad libitum* feeding (treatment). Each point represents the mean circulating IGF-I concentrations and SGRW of each replicate for both control and treatment groups at four and eight weeks of the feed study (3 replicates/group/time point). $f(x) = 0.0196X - 0.1609$, $r^2 = 0.665$ ($P = 0.0012$)
Fig. 18. Correlation between circulating IGF-I level and hepatosomatic index (HSI) of southern flounder. Data are derived from control animals fed *ad libitum* twice daily as well as treatment animals exposed to a 4-week cycle of fasting and *ad libitum* feeding (treatment). Each point represents the mean circulating IGF-I concentrations and HSI of each replicate for both control and treatment groups at four and eight weeks of the feed study (3 replicates/group/time point). \( f(x) = 0.0277X - 1.24, \quad r^2 = 0.62 \quad (P = 0.0023) \)
Fig. 19. Hepatic IGF-I mRNA level of southern flounder subjected to a 4-week cycle of fasting and normal feeding. Treatment groups were fasted for the first four weeks and then subsequently fed *ad libitum* daily for the remaining four weeks. Control groups were fed *ad libitum* daily throughout the experiment. Each value represents the mean ± SEM of three replicates/group with 3 fish/replicate. Asterisks denote differences from control: \( *P = 0.084. \)
Fig. 20. Correlation between hepatic IGF-I mRNA and circulating IGF-I levels in southern flounder. Data are derived from control animals fed *ad libitum* twice daily as well as treatment animals exposed to a 4-week cycle of fasting and *ad libitum* feeding (treatment). Each point represents the mean IGF-I concentrations and IGF-I mRNA levels of each replicate for both control and treatment groups at four and eight weeks of the feed study (3 replicates/group/time point). \( f(x) = -0.00186x + 1.418, r^2 = 0.0108 \) \( (P = 0.748) \)
Fig. 21. Correlation between hepatic IGF-I mRNA level and specific growth rate in body weight (SGRW) of southern flounder. Data are derived from control animals fed ad libitum twice daily as well as treatment animals exposed to a 4-week cycle of fasting and ad libitum feeding (treatment). Each point represents the mean IGF-I mRNA levels and SGRW of each replicate for both control and treatment groups at four and eight weeks of the feed study (3 replicates/group/time point). $f(x) = -0.298X + 1.062$, $r^2 = 0.1604$ ($P = 0.197$)
LIST OF REFERENCES


Davis, J.T., 1990. Red drum; site selection and pond construction. Southern Regional Aquaculture Center No. 321


