# EFFECTS OF NUTRITIONAL STATE ON GHRELIN, SOMATOLACTIN, AND THE GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR AXIS IN THE TILAPIA, *OREOCHROMIS MOSSAMBICUS*

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We certify that we have read this dissertation and that, in our opinion, it is satisfactory in scope and quality as a dissertation for the degree of Doctor of Philosophy in Molecular Biosciences and Bioengineering.

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## ABSTRACT

Mozambique tilapia (*Oreochromis mossambicus*) is an important aquaculture species that is cultured in tropical climates worldwide. Similar to terrestrial vertebrates, a functional growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis has recently been identified in the tilapia which responds to nutritional status. Tilapia also possess two forms of the stomach-derived hormone ghrelin, which stimulates GH release, appetite, and fat deposition. Somatolactin (SL) is a fish-specific pituitary-derived member of the GH/prolactin (PRL) family of peptide hormones which has been shown to be involved in energy metabolism in some fish species. The primary objective of this dissertation was to investigate the physiological effects of tilapia ghrelin, and characterize modulations in ghrelin levels and the GH/IGF-I axis in response to nutrient restriction. In the present study, ghrelin treatment stimulated GH release from primary tilapia pituitary cells, and a ghrelin receptor antagonist blocked this stimulatory effect. Similarly, peripheral injection of homologous tilapia ghrelin increased plasma GH and IGF-I levels. These results suggest that a functional ghrelin-GHS-R system exists, and that ghrelin's actions in the pituitary are conserved between the tilapia and terrestrial species. Furthermore, prolonged fasting (2 and 4 weeks) brought about a significant elevation in plasma ghrelin levels, suggesting a possible role for ghrelin in long-term energy partitioning and homeostasis in the tilapia. Fasting for 4 weeks in both seawater and fresh water resulted in elevated or unchanged plasma GH levels, with consistently reduced plasma IGF-I and liver IGF-I mRNA levels suggesting GH resistance. Changes in hepatic expression of IGF-I, GH-R, and SL-R mRNA levels were minor compared

with those detected in muscle tissue suggesting a significant contribution by muscle to metabolic recovery during both the catabolic state of fasting and during the nutrient surplus that follows re-feeding. Additionally, pituitary expression of SL mRNA levels were reduced during re-feeding suggesting a role for this hormone in the partitioning and deposition of substrates during energy surplus. The findings resulting from the studies described herein suggest that ghrelin, along with the GH/IGF-I axis play important roles in coordinating metabolism according to the nutritional state of tilapia.

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## LIST OF ABBREVIATIONS

ARP.....Acidic ribosomal phosphoprotein P0

cDNA.....Complementary DNA

GH.....Growth hormone

GH-R (GH-R2).....Growth hormone receptor

GHRH.....Growth hormone –releasing hormone

GHS.....Growth hormone secretagogue

GHS-R.....Growth hormone secretagogue receptor

IGF-I.....Insulin-like growth factor-I

IGF-II.....Insulin-like growth factor-II

mRNA.....Messenger ribonucleic acid

PRL.....Prolactin

RIA.....Radioimmunoassay

rtqRT-PCR.....Real-time quantitative RT-PCR

SL.....Somatolactin

SL-R (GH-R1).....Somatolactin receptor

# CHAPTER I

## INTRODUCTION

Growth in vertebrates is governed by the integration of genetic, hormonal, and nutritional components. The most significant endocrine influence in body growth is the complex regulation of the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis, and this mechanism appears to be highly conserved among vertebrates (Duan 1997; Moriyama et al. 2000; Wood et al. 2005). Growth hormone is involved in regulating numerous physiological processes besides somatic growth in fish including immune function, lipid and protein metabolism, osmoregulation, and feeding behavior (Duan 1997; McCormick 2001; Bjornsson et al. 2004; Albalat et al. 2005). In teleost fish, secretion of GH from the pituitary is regulated by sex steroids along with several hypothalamic factors, which act in concert under the influence of the physiological and nutritional state of the animal (Peng et al. 1997; Sherwood et al. 2000; Jensen 2001; Volkoff et al. 2006).

Growth hormone acts directly on target tissue by stimulating mitosis, and indirectly by initiating the production and release of IGF-I, a mitogenic factor produced primarily in the liver (Le Roith et al. 2001; Wood et al. 2005). The physiological actions of GH are mediated through its binding to the growth hormone receptor (GH-R), located on the surface of cells in target tissue (Argetsinger et al. 1996; Pérez-Sánchez et al. 2002). We have recently identified two GH-R subtypes (GH-R1 and -R2) in the Mozambique tilapia, one of which (GH-R1) we believe to be the putative receptor for somatolactin (SL-R) and the other (GH-R2), the growth hormone-receptor (GH-R)

(Pierce et al. 2007). Somatolactin (SL) is a member of the GH/prolactin family of pituitary peptide hormones, which is present in a variety of teleost species as well as the sturgeon and lungfish, but not in tetrapods (Fukamachi et al. 2007). The signal transduction by the GH-R leads to the biological actions evoked by GH. Protein restriction during fasting has been shown to reduce circulating IGF-I and liver IGF-I mRNA levels in several teleost species including the tilapia (Uchida et al. 2003; Picha et al. 2006; Small et al. 2006). Alterations in circulating GH and IGF-I due to disruptions in metabolic rhythms in turn alter the number and post-receptor functions of GH-R through changes in the transcription and translation of the GH-R (Thissen et al. 1999; Beauloye et al. 2002; Deng et al. 2004). Insulin-like growth factor-II (IGF-II) shares a high structural homology with IGF-I, and gene expression of both mitogens appears to be regulated in fish in several tissue types by GH (Vong et al. 2003). In contrast with mammals which express IGF-II chiefly during embryonic development, IGF-II is expressed widely in both juvenile and adult fish (Reinecke et al. 2005). On the other hand, the metabolic actions of IGF-II in fish are virtually unknown. Both IGFs also activate cell proliferation suggesting that they share several overlapping physiological roles in fish (Pozios et al. 2001; Reinecke et al. 2005).

***Effects of fasting and re-feeding on GH/IGF-I axis in fish***

There have been numerous studies of the effects of partial or total nutrient restriction on the GH/IGF-I axis in teleost fish. One of the first comprehensive studies on the subject was designed to address the mechanisms that underlay growth hormone resistance (Perez-Sanchez et al. 1994). This laid the groundwork for future experiments

that sought to understand the seemingly contradictory findings that elevated plasma GH in fish is not necessarily correlated with increased growth rate. Instead, increased binding of GH to its hepatic receptor is strongly correlated with seasonal increases in growth, regardless of changes in circulating GH (Perez-Sanchez et al. 1994). Furthermore, a GH-induced catabolic state during fasting is associated with many factors including lowered circulating and hepatic expression of IGF-I, along with decreased hepatic GH-R binding in the presence of elevated plasma GH. A dose-related decrease in plasma GH, accompanied by concurrent increases in plasma IGF-I and hepatic GH binding sites was shown to occur in sea bream fed increasing dietary protein levels (Perez-Sanchez et al. 1994). A subsequent fasting study was conducted to characterize the effects of varying ration levels on plasma GH, specific growth rate, and various other growth-related endocrine factors (Toguyeni et al. 1996). The authors observed an increase in plasma GH associated with decreased ration size, but failed to see a correlation between plasma GH levels and specific growth rate.

Interestingly, Menton et al. (2000) observed a decline in IGF-I mRNA levels following a period of fasting in the sea bream that recovered upon re-feeding. On the other hand, they saw no effect of dietary protein levels on IGF-I mRNA transcript levels. The authors also observed no correlation between body growth and hepatic IGF-I mRNA levels. Similarly, food deprivation was shown to increase plasma GH in the goldfish, while subsequent re-feeding reversed the effects (Narnaware et al. 2001). Replacement of fishmeal with plant-derived protein sources in feed produced a significant increase in plasma GH and a decrease in plasma IGF-I following an overnight fast in sea bream

(Gomez-Requeni et al. 2004). Likewise, fasting resulted in an elevation in plasma GH and a decrease in plasma and hepatic expression of IGF-I in Chinook salmon (Pierce et al. 2005). Plasma GH increased, while plasma IGF-I and hepatic expression of IGF-I and GH-R decreased in fasted coho salmon (Fukada et al. 2004). Fasted rainbow trout exhibited also elevated plasma GH and suppressed plasma IGF-I with reduced IGF-I mRNA in liver and muscle (Gabillard et al. 2006a). Fasting also resulted in lowered GH-R1 (SL-R), but not GH-R2 (GH-R) mRNA levels in liver and muscle tissue. Re-feeding resulted in a normalization of plasma GH and IGF-I to fed control levels (Gabillard et al. 2006a). Hepatic and muscle expression of IGF-I and GH-R1mRNA also returned to levels of fed control fish, while expression of GH-R2 (GH-R) increased following re-feeding in liver and muscle. In the grouper, fasting brought about an elevation of pituitary expression of GH mRNA, while at the same time reduced hepatic IGF-I mRNA (Pedroso et al. 2006). Subsequent re-feeding resulted in a restoration of the expression of both genes to fed control levels. Interestingly, pituitary expression of GH mRNA increased in the rabbitfish during fasting along with a transient increase in hepatic expression of IGF-I (Ayson et al. 2007). Following re-feeding, pituitary GH mRNA levels returned to those of fed controls, and expression of hepatic IGF-I mRNA fell transiently below control values.

These results indicate that the up-regulation of plasma GH in parallel with decreased plasma IGF-I and liver expression of IGF-I mRNA represent a conserved mechanism in teleost and vertebrate evolution in general. This physiological state of

"GH-resistance" shifts metabolism in favor of mobilizing stored substrates to maintain energetic homeostasis instead of promoting tissue growth.

Mozambique tilapia are known to grow faster in seawater than in fresh water (Kuwaye, et al 1993; Ron et al. 1995; Sparks et al. 2003). Nevertheless, previous studies of the effects of fasting have been restricted to the study of fish acclimated to fresh water (Rogers et al. 1992; Weber et al. 1999; Uchida et al. 2003). In view of the fact that GH plays an important role in seawater acclimation in several euryhaline fishes including the tilapia (for review, see McCormick 2001), fasting in seawater may affect the GH/IGF-I axis of the tilapia more profoundly than in the fish in fresh water. Chapter II of this thesis describes the effects of fasting on the GH/IGF-I axis in seawater-acclimated tilapia.

***Orexigenic action of ghrelin***

The neuroendocrine control of GH release in fish is considered to be multifactorial including such stimulatory and inhibitory factors as sex steroids and neuropeptides; the sum of which regulates secretion (Peng et al. 1997; Sherwood et al. 2000). A peptide hormone called ghrelin, which originates in gastric mucosal cells of the stomach and is then released into circulation, has been identified recently and shown to have a strong stimulatory effect on GH release. Ghrelin was first characterized in the rat as the endogenous ligand for the "orphaned" growth hormone secretagogue receptor (Kojima et al. 1999). Since then, ghrelin has been identified in numerous mammalian and non-mammalian vertebrates (van der Lely et al. 2004; Kaiya et al. 2008). Two forms of ghrelin, ghrelin-C8 and -C10, were isolated from the stomach of Mozambique tilapia (*Oreochromis mossambicus*), and both forms stimulate GH release from organ-cultured

tilapia pituitaries (Kaiya et al. 2003c). Initially, research into the biological function of ghrelin focused on its potent GH-releasing activity (Cummings et al. 2003). Subsequent studies have revealed an increasing body of evidence that ghrelin's role in animal physiology is wide-ranging (Cummings et al. 2005; Kaiya et al. 2008). Ghrelin has been shown to have effects on the release of several pituitary-derived hormones including prolactin and adrenocorticotropin hormone. Ghrelin also acts on gastric motility and acid secretion and has antiproliferative effects on varying types of carcinomas, as well as on cardiovascular function and glucose metabolism, among others (Muccioli et al. 2002). Among the most interesting effects of ghrelin is its potent orexigenic, or appetite-stimulatory, activity. Ghrelin has been shown to stimulate food intake in both mammals and fish (Wren et al. 2001; Unniappan et al. 2004a; Riley et al. 2005). Chronic administration of ghrelin leads to increased body weight and fat storage in many mammalian species (Cummings 2006) as well as in tilapia (Riley et al. 2005), suggesting a role for ghrelin in feeding and energy metabolism. In humans, serum ghrelin typically exhibits a pre-prandial rise and then a return to basal levels directly after a meal. Ghrelin has also been shown to initiate sniffing, foraging, and hoarding behaviors in a variety of animals (Cummings 2006). Furthermore, studies of mammals suggest that ghrelin also regulates IGF-I release, and that IGF-I may directly regulate the expression of the GHS-Rs (Hickey et al. 1997; Sun et al. 2004). We have also identified two putative GHS-R subtypes (1a and 1b), and tissue expression patterns indicate the highest expression of these receptors in the brain of the tilapia (Fox et al. 2007). Recently, the effects of fasting on ghrelin levels have been examined in several fish species, and the combined results

suggest strong species differences exist (Kaiya et al. 2008). No attempt has been made, however, to examine time-course of changes in circulating ghrelin or expression of ghrelin genes during fasting and re-feeding in tilapia. Chapter III describes the short-term effects of two homologous tilapia ghrelins on the GH/IGF-I axis, while Chapter IV characterizes the effects of short- and long-term fasting on the GH/IGF-I axis concomitantly with changes in plasma ghrelin levels.

***Justification***

Fish exhibit intermittent periods of growth throughout their life cycles, which make them ideal model systems for studying growth during development (MacKenzie et al. 1998; Mommsen 2001; Ali et al. 2003). They also represent one of the most species-diverse and ancient vertebrate groups, living in virtually every aquatic environment on Earth. Comparative studies on the regulation of growth in teleosts can be very useful in understanding similar mechanisms in other vertebrates, including humans. Fish also have clear economic value both in terms of aquaculture and natural fisheries. Worldwide tilapia aquaculture harvests account for more than 2 million metric tons of fish annually (American Tilapia Association, 2006). Global production of aquatic animals has exceeded 45 million tons, and aquaculture accounts for nearly 50% of the food fish supply (The World Bank, 2006). The development of techniques and assays that successfully monitor fish growth, and its regulation, are of high value to both nutritional scientists who design formulated diets for aquaculture as well as fish culturists. Thus, a better understanding of growth in fish has many practical as well as scientific applications.

Fish, in contradistinction with tetrapods, exhibit two isoforms of GH-Rs (GH-R and SL-R) that are coded by separate genes (Fukamachi et al. 2007). Fish also express a hormone, primarily synthesized in the pituitary gland and structurally similar to GH and prolactin, called somatolactin that is likely involved in feeding and lipid metabolism (Forsyth et al. 2002; Kawauchi et al. 2006). Liver and muscle expression of the two GH-R subtypes has not been well characterized in fish. Hence, observed changes in these genes during fasting and re-feeding will yield important information. Measurement of the expression of tilapia GH-R and SL-R as well as IGF-I and -II in liver and muscle during fasting will offer new insight into the regulation of metabolism during a catabolic state in this economically important species. Furthermore, a better understanding of the regulation of this complex endocrine system in fish will have direct positive implications for aquaculture as well as for human biology and medicine, including the understanding of human metabolic regulation, obesity, and diabetes. This thesis was aimed at refining our understanding of the effects of nutritional status on tilapia physiology.

***Goals and Objectives***

The primary goal of this dissertation was to clarify the effects of nutrient restriction and re-feeding on GH/IGF-I axis in tilapia with special reference to the involvement of ghrelin. The following chapters describe: 1) The effects of prolonged fasting on the GH/IGF-I axis in seawater-adapted tilapia (Chapter II; published in *General and Comparative Endocrinology* 148: 340-347, 2006); 2) The physiological effects, both *in vivo* and *in vitro*, of two homologous forms of tilapia ghrelin on plasma hormones, GH receptors, and the GH/IGF-I axis in various tissues (Chapter III; published in *Zoological*

*Science* 24:391-400, 2007); 3) The effects of short, intermediate, and long-term fasting on ghrelin and the GH/IGF-I axis in the tilapia (Chapter IV; to be submitted to *General and Comparative Endocrinology*); 4) The effects of fasting and re-feeding on GH receptors, pituitary expression of GH and SL, and the GH/IGF-I axis in muscle and liver tissue (Chapter V; to be submitted to *General and Comparative Endocrinology*); and 5) Chapter VI provides general conclusions and future perspectives on the work described in this dissertation.

## CHAPTER II

## Effects of fasting on growth hormone, growth hormone receptor and insulin-like growth factor-I axis in seawater-acclimated tilapia, *Oreochromis mossambicus*


### ABSTRACT

Effects of fasting on the growth hormone (GH)-growth hormone receptor (GH-R)-insulin-like growth factor I (IGF-I) axis were characterized in seawater-acclimated tilapia (*Oreochromis mossambicus*). Fasting for 4 weeks resulted in significant reductions in body weight and specific growth rate. Plasma GH and pituitary GH mRNA levels were elevated significantly in fasted fish, whereas significant reductions were observed in plasma IGF-I and hepatic IGF-I mRNA levels. There was a significant negative correlation between plasma levels of GH and IGF-I in the fasted fish. No effect of fasting was observed on hepatic GH-R mRNA or somatolactin-receptor (SL-R) mRNA levels. Plasma glucose levels were reduced significantly in fasted fish. The fact that fasting elicited increases in GH and decreases in IGF-I production without affecting GH-R expression indicates a possible development of GH resistance.


### INTRODUCTION

Growth in vertebrates is governed by the integration of genetic, hormonal, and nutritional components whose effects are integrated to a major extent through the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis. Growth hormone exhibits many physiological roles in fish including the regulation of somatic growth, seawater adaptation, immunity and reproductive maturation (Duan 1997; Yada et al. 1999; McCormick 2001).

Growth hormone acts directly on target tissue by stimulating mitosis and affecting various aspects of energy metabolism, and indirectly by initiating the production and release of IGF-I in the liver and in the majority of peripheral tissues. As in higher vertebrates, many of the growth enhancing effects of GH in fish are mediated by IGF-I (Duan 1997; Wood et al. 2005).

It is well established in mammals that the physiological actions of GH are mediated through its binding to the Class I cytokine growth hormone receptor (GH-R) located on the surface of cells in target tissue (Argetsinger et al. 1996). Following binding of GH, a dimerization of GH-R occurs, which activates an intracellular phosphorylation cascade involving Janus kinase 2 (JAK2) and members of the signal transducer and activator of transcription (STAT) family of proteins. This signal transduction pathway elicits the biological actions of GH. Recently, GH-R have been cloned from the liver of several teleost species including turbot, goldfish, sea bream, masu and coho salmon, and channel catfish (Calduch-Giner et al. 2001; Lee et al. 2001; Tse et al. 2003; Deng et al. 2004; Fukada et al. 2004; Small et al. 2006). We have recently identified two GH-R subtypes (GH-R1 and -R2) in the Mozambique tilapia, one of which (GH-R1) we believe to be the putative receptor for somatolactin (SL-R) and the other (GH-R2), the growth hormone-receptor (GH-R) (Pierce et al. 2007). In all species examined to date, GH-R mRNA is expressed in almost all the tissues examined with the highest expression in the liver. Deng et al. (2004) and Fukada et al. (2004) reported reductions in hepatic GH-R mRNA in fasted black sea bream and coho salmon. Beyond this, little is known at present about the regulation of GH-R expression in fish.

The GH/IGF-I axis is governed in part by the nutritional state of the animal (Duan 1997; Thissen et al. 1999). Protein restriction or fasting has been shown to reduce circulating IGF-I and liver IGF-I levels in several teleost species that include the coho salmon (Moriyama et al. 1994; Duan 1997), Chinook salmon (Pierce et al. 2005), sea bream (Perez-Sanchez et al. 1994), barramundi (Matthews et al. 1997), and the tilapia (Uchida et al. 2003). By contrast, normal or elevated levels of GH have been observed in fasted salmonids (Duan et al. 1993; Kakizawa et al. 1995; Pierce et al. 2005) and tilapia (Weber et al. 1999). The generally held view is that fasting produces an increase in circulating GH levels that is consequent to a reduced negative feedback by hepatic IGF-I (Wood et al. 2005). Indeed, studies on fasted rats have shown that IGF-I in hepatocytes is reduced in the presence of elevated GH levels, indicating a decrease in the sensitivity of the liver to GH stimulation (Thissen et al. 1999). These observations suggest nutritional deficit contributes to a state of GH resistance. In mammals, primary GH resistance, comprising a failure to grow despite the presence of normal, or even elevated, GH secretion, has been linked to defects in GH-R (Rosenfeld et al. 2004). In black sea bream, fasting induced a significant increase in plasma GH accompanied by a decreased GH-R (Deng et al. 2004). Similarly, both plasma IGF-I and hepatic GH-R mRNA were reduced significantly in fasted catfish (Small et al. 2006). These results suggest that a reduction in hepatic GH-R gene expression might serve as a mechanism for the reduction of circulating IGF-I and growth during the period of food deprivation.

We have shown previously that 2 weeks of fasting caused significant reductions in circulating IGF-I and hepatic IGF-I mRNA in freshwater-acclimated tilapia without

affecting circulating GH levels (Uchida et al. 2003). By contrast, elevated levels of GH have been observed after 3 weeks of fasting in this species (Weber et al. 1999). A delayed GH response relative to IGF-I was also observed during fasting in channel catfish (Small 2005b). According to Vijayan et al. (Vijayan et al. 1996), food-deprivation seems to affect the initial stages of seawater acclimation in the tilapia. In view of the fact that GH plays important roles in seawater acclimation in some euryhaline fishes including the tilapia (Sakamoto et al. 1997; McCormick 2001), fasting in seawater may affect GH/IGF-I axis of the tilapia more profoundly than in the fish in fresh water. The present study was conducted to examine the effects of 4 weeks of fasting on the GH/GH-R/IGF-I axis in the seawater-acclimated tilapia.

## MATERIALS AND METHODS

*Animals*

Mozambique tilapia (*Oreochromis mossambicus*) were reared outdoors in 5000 L seawater flow-through tanks under natural photoperiod at the Hawaii Institute of Marine Biology. Male tilapia were used in this study, since Vijayan et al. (2001) reported that estradiol impairs hyposmoregulatory capacity in the tilapia, although nothing is known on sex difference in saltwater adaption with respect to GH/IGF-I axis. We observed no difference in specific growth rate between sexes during a 4 week experiment (2 weeks of fasting) in the tilapia in fresh water (Uchida et al. 2003). Twenty-eight fish, weighing approximately 40 g, were divided into 4 groups (2 fed and 2 fasted) and maintained in outdoors in oval 60 L fiberglass aquaria (7 fish in each tank). Water temperature was 25-

28 °C. They were fed approximately 2% of the body weight per day twice daily with Silver Cup Trout Chow (Nelson and Sons, Murray, UT). Individual animals were identified by clipping the pelvic, anal, or dorsal fin. Once positive growth and appetite in all fish was established for 2 weeks, the fish in two tanks were fasted for 4 weeks, while those in the other two tanks were continued at the same feeding rate. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

*Sampling*

Body weight was measured weekly from the start of the experiment (week 2) to the end of the experiment (week 4) under anesthesia in tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, 0.5 g/L). Standard length was also measured at the termination of the experiment. Condition factor, (body wt, g)/(standard length, cm)$^3$ x 100, and specific growth rate, (ln $W_f$-ln $W_i$)/t x 100, where $W_f$ is the final weight (g), $W_i$ is the initial weight (g) at each time interval and t is growth time (days), were calculated. Blood was withdrawn by caudal puncture using 1 ml heparinized syringes. Plasma was separated by centrifugation at 10,000 x g for 5 min, and stored at -20° for later analyses of GH and IGF-I by radioimmunoassays. At the end of the experiment (week 4), fish were weighed rapidly and decapitated following blood collection. Livers and pituitaries were excised, snap-frozen in liquid nitrogen, and stored at -80°C until dot blot hybridization and real-time PCR analysis for GH, IGF-I, and GHR gene expression.

*Analyses*

Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5100C, Logan, UT). Plasma levels of GH were measured by homologous radioimmunoassay (Ayson et al. 1993). For measurement of total plasma IGF-I, 25 µl of plasma was extracted with 100 µl of acid-ethanol (87.5% ethanol and 12.5% 2 N HCl, v/v) as described by Moriyama et al. (1994) and Shimizu et al. (1999). Total IGF-I levels were then measured by radioimmunoassay following Moriyama et al. (1994) with some modification (Kajimura et al. 2002). Recombinant salmon IGF-I and anti-barramundi IGF-I were purchased from GroPep (Adelaide, Australia). Plasma glucose concentrations were estimated using a commercially available kit (Glucose Assay Kit, GAHK-20, Sigma, St. Louis, MO).

Dot blot hybridization for pituitary GH mRNA was carried out as described by Riley et al. (2002b). Homologous tilapia GH and β-actin cDNA probes were generated with the use of $^{32}$P-ATP and Prime-it II random primer labeling kit (Stratagene, La Jolla, CA). Quantification of GH mRNA was carried out by Northern dot blot hybridization. Relative density was determined by exposing the membranes to Phospho-Imager screens (Amersham, Piscataway, NJ). To ensure equal loading of total RNA, GH levels were normalized to the relative amount of β-actin mRNA in each sample.

*Real-time quantitative RT-PCR*

Total RNA was extracted from liver tissue using a RNA extraction solution (TRI-Reagent, MRC, Cincinnati, OH) according to the manufacturer's instructions. Liver IGF-I, SL-R, and GH-R mRNA transcripts were analyzed using real-time quantitative RT-PCR (rtqRT- PCR). Equal amounts of RNA were reverse transcribed using BioRad

(Hercules, CA) iScript cDNA synthesis kit. Liver RNA from several fish was pooled, reverse transcribed and used as an inter-assay standard sample. In addition, samples were added to the cDNA synthesis reaction mix without the addition of reverse transcriptase enzyme for a no-reverse transcriptase control.

IGF-I, GH-R, and SL-R rtqRT-PCR was performed as described previously (Kajimura et al. 2004; Pierce et al. 2007) using BioRad iCycler iQ Real-Time PCR Detection System. Each plate well contained 15 µl of standard primer and probe concentrations in iQ supermix (BioRad). Standards were made from plasmids and diluted to known copy numbers. Inter-plate variation was normalized using the positive control and data expressed as copy number per µg RNA. The cycling parameter was as follows: 2 min at 95°C, 2 min at 50°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Data were analyzed by iCycler software. Primer pairs and probe sequences for all assays are shown in Table 1.

*Statistical analysis*

Group comparisons were performed using a Student's t test or two-way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Levels of correlation were determined by the Pearson correlation test. Significant level was set at $P < 0.05$. Calculations were performed using a computer program, Statistica (StatSoft, Tulsa, OK). Data are expressed as means ± S.E.M.

## RESULTS

No significant difference was found between replicate tanks in any measured parameters, and thus individual fish were used as replicates in the statistical analyses. At

the beginning of the experiment (2 weeks prior to the start of fasting), there was no significant difference in body weight between the control and fasted groups. All fish gained weight at similar rates during the following 2 weeks (control period), and there was no significant difference in specific growth rate (Figs. 1A and B). Fasting resulted in a gradual reduction in body weight ($r = -0.305$, $P < 0.001$). Specific growth rate decreased significantly ($P < 0.001$) to a negative value in the fasted group after 1 week of fasting, and remained at the same level until the end of the experiment (week 4). Specific growth rate of the control group declined significantly during the last 3 weeks of the experiments ($r = -0.471$, $P < 0.001$). At the end of the experiment, there was a significant reduction ($P < 0.001$) in condition factor of the fasted fish ($2.78 \pm 0.044$, $n = 14$) compared with the fed fish ($3.20 \pm 0.078$, $n = 14$).

Plasma GH and pituitary GH mRNA levels were elevated significantly ($P < 0.01$) in fish fasted for 4 weeks (Figs. 2A and B). On the other hand, 4 weeks of fasting produced highly significant ($P < 0.001$) reductions in plasma IGF-I and hepatic IGF-I mRNA (Figs. 2C and D). Significant correlations were observed between plasma IGF-I and hepatic IGF-I mRNA levels ($r = 0.47$, $P < 0.05$), when all the data were combined. No effect of fasting was observed on hepatic GH-R mRNA or SL-R mRNA (Fig. 3A and 3B). There was a significant negative correlation between plasma levels of GH and IGF-I in the fasted fish ($r = -0.64$, $P < 0.05$), whereas a positive correlation was observed in the fed fish ($r = 0.64$, $P < 0.05$) (Fig. 3C).

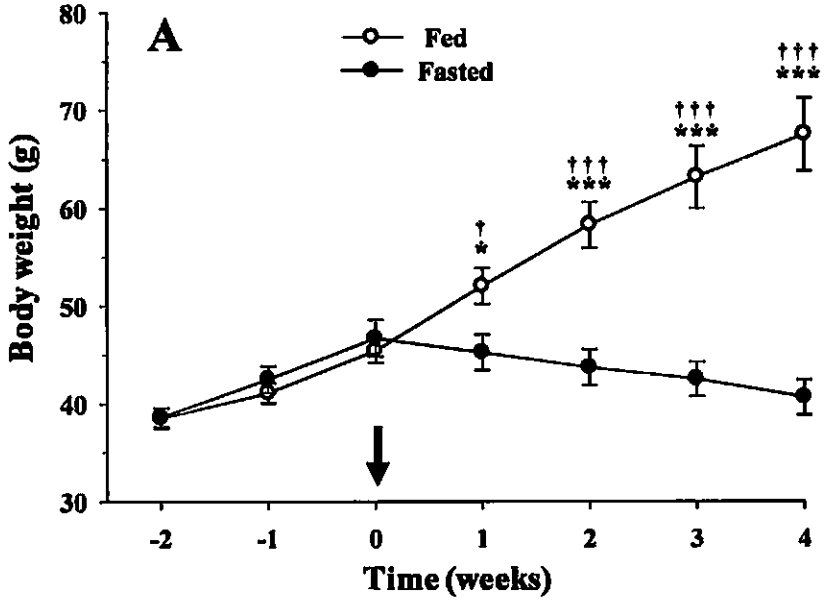
A significant reduction ($P < 0.001$) was also observed in plasma glucose levels in the fasted fish ($61.4 \pm 2.65$ mg/dl, $n = 14$) compared with those in the control ($46.9 \pm$

1.07, n = 14 (Fig. 4A). When both fed and fasted groups were combined, plasma glucose was correlated significantly and inversely with plasma GH ($r = -0.70$, $P < 0.005$). There was no difference in plasma osmolality between the fed control (305 ± 6.5 mOsm, n = 14) and the fasted fish (299 ± 1.9 mOsm, n = 14) at the end of the experiment (Fig. 4B).

**Table 1.** Nucleotide sequences for primers and probes used in rtqRT-PCR assays

| Gene | | Sequence |
|---|---|---|
| SL-R | Forward | 5'-CACAGACTTCTACGCTCAGGTCA-3' |
| | Reverse | 5'-TGAGTTGCTGTCCAGGAGACA-3' |
| | Probe | 5'-CAATGTTATGCCAACTGGTGGTGTGGTG-3' |
| GH-R | Forward | 5'-CACACCTCGATCTGGACATATTACA-3' |
| | Reverse | 5'-CGGTTGGACAATGTCATTAACAA-3' |
| | Probe | 5'-CGTCCAGCTCCGCTCCAGGGA-3' |
| IGF-I | Forward | 5'-CTGCTTCCAAAGCTGTGAGCT-3' |
| | Reverse | 5'-GATCGAGAAATCTTGGGAGTCTTG-3' |
| | Probe | 5'-CAGCGCCTTGAGATGTACTGTGCACCT-3' |
| IGF-II | Forward | 5'-TATAAGAGGCACGCGGAGGAG-3' |
| | Reverse | 5'-GCTTGCTAGGAAGGCTGATCA-3' |
| | Probe | 5'-AAGGGAGCAGGCTATCTTTCCACAGGCC-3' |
| GH | Forward | 5'-TTACATCATCAGCCCGATCG-3' |
| | Reverse | 5'-AGATCGACAGCAGCTTCAGGA-3' |
| | Probe | 5'-CAAACACGAGACCCAGCGCAGCTCG-3' |
| SL | Forward | 5'- GGCTGGCTTTGCATGTATCA-3' |
| | Reverse | 5'-AGTGGAGCAACCATTTATCAGATATCT-3' |
| | Probe | 5'-TTACCGATCCCCAGTCTTAAAAGTGAA-3' |
| ARP | Forward | 5'-TTTGAAAATCATCCAACTTTTGGAT-3' |
| | Reverse | 5'-GCAGGGACAGACGGATGGT-3' |
| | Probe | 5'-ACTATCCAAAATGCTTCATCGTGGGCG-3' |
| Ghrelin | Forward | 5'- GGGTTGGTTCAGCTGTCATT-3' |
| | Reverse | 5'- ACGTTTGGGTGTTTGGTAGAC-3' |

Fig. 1. Changes in body weight (A) and specific growth rate (B) during 4 weeks of fasting in seawater-acclimated tilapia. Twenty-eight fish, weighing approximately 40 g, were divided into 4 groups (2 fed and 2 fasted) and maintained in outdoors in oval 60 L fiberglass aquaria (7 fish in each tank). They were fed approximately 2% of the body weight per day twice daily. The experiment was started once it was determined all of the animals exhibited similar appetite and growth. Once positive growth and appetite in all fish was established for 2 weeks (week 0, shown by arrow), two tanks of fish were fasted for 4 weeks, while the other two were fed at the same rate. Vertical bars indicate mean ± SEM (n = 14). *, *** Significantly different from the fed control at each time point at $P < 0.05$ and 0.001, respectively (two-way ANOVA). †, †††Significantly different from week 0 of each treatment at $P < 0.05$ and 0.001, respectively (two-way ANOVA).


A
Fed
Fasted
Body weight (g)
80
70
60
50
40
30
-2
-1
0
1
2
3
4
Time (weeks)
†
*
†††
***
†††
***
†††
***
†††
***

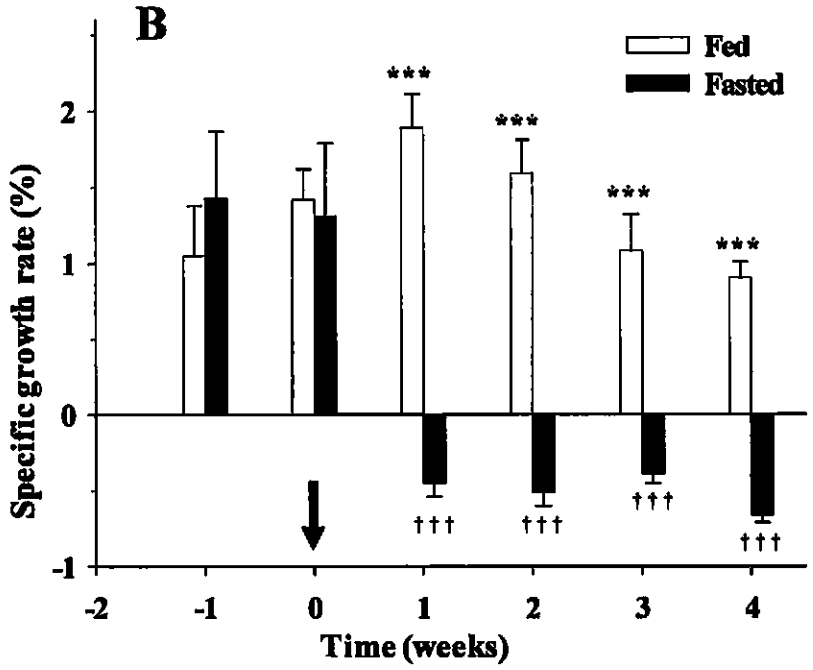

B
Fed
Fasted
Specific growth rate (%)
2
1
0
-1
-2
-1
0
1
2
3
4
Time (weeks)
***
***
***
***
†††
†††
†††
†††

Fig. 2. Effects of 4 weeks of fasting in seawater-acclimated tilapia on plasma GH (A), pituitary GH mRNA (B), plasma IGF-I (C), and hepatic IGF-I mRNA (D). The pituitary GH mRNA levels were estimated by dot blot hybridization and normalized to relative amounts of $\beta$-actin mRNA, while hepatic IGF-I mRNA levels were measured by real-time PCR and normalized to copy number per ng total RNA. Vertical bars indicate mean ± SEM (n = 14). **, *** Significantly different from the fed control at $P < 0.01$ and 0.001, respectively, by Student's *t* test.

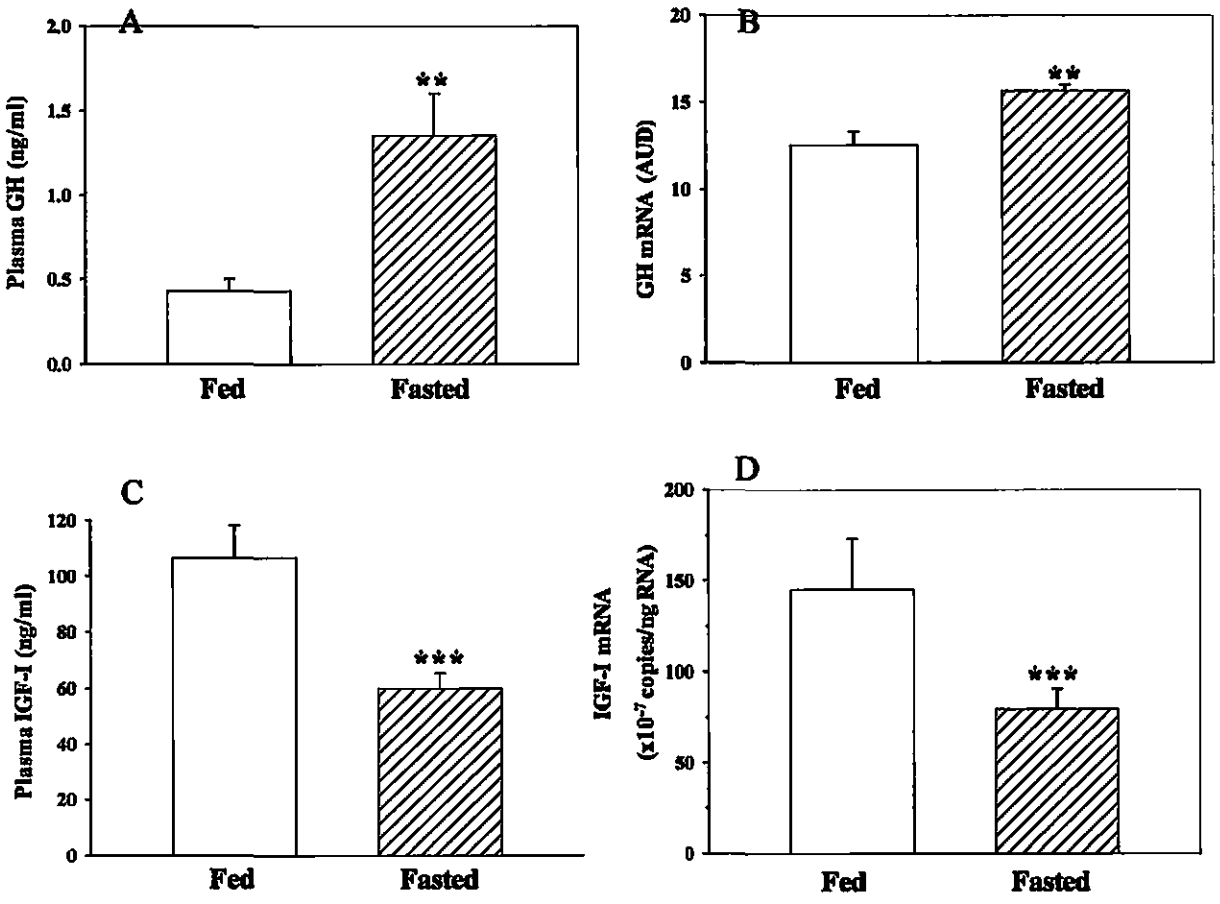

A
Plasma GH (ng/ml)
2.0
1.5
1.0
0.5
0.0
**
Fed
Fasted
B
GH mRNA (AUD)
20
15
10
5
0
**
Fed
Fasted
C
Plasma IGF-I (ng/ml)
120
100
80
60
40
20
0
***
Fed
Fasted
D
IGF-I mRNA
(x10⁻⁷ copies/ng RNA)
200
150
100
50
0
***
Fed
Fasted

Fig. 3. Effects of 4 weeks of fasting in seawater-acclimated tilapia on hepatic SL-R (A) and GH-R mRNA (B), and correlation between plasma GH and IGF-I (C). Open and closed circles indicate values in fed and fasted fish, respectively. Hepatic mRNA levels were measured by real-time PCR, normalized to copy number per ng total RNA, and expressed as percent of fed controls. Vertical bars indicate mean ± SEM (n = 14). *** Significantly different from the fed control at $P < 0.01$ and 0.001, respectively, by Student's *t* test.

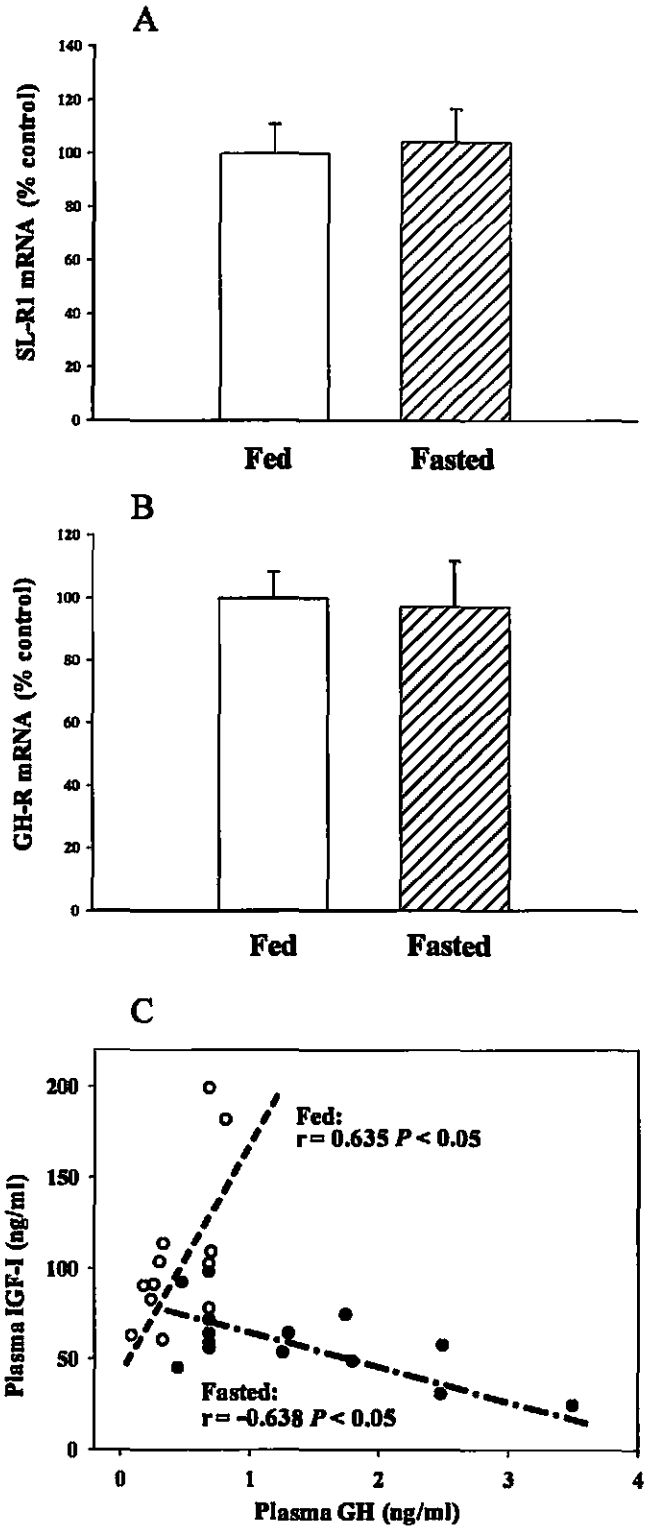
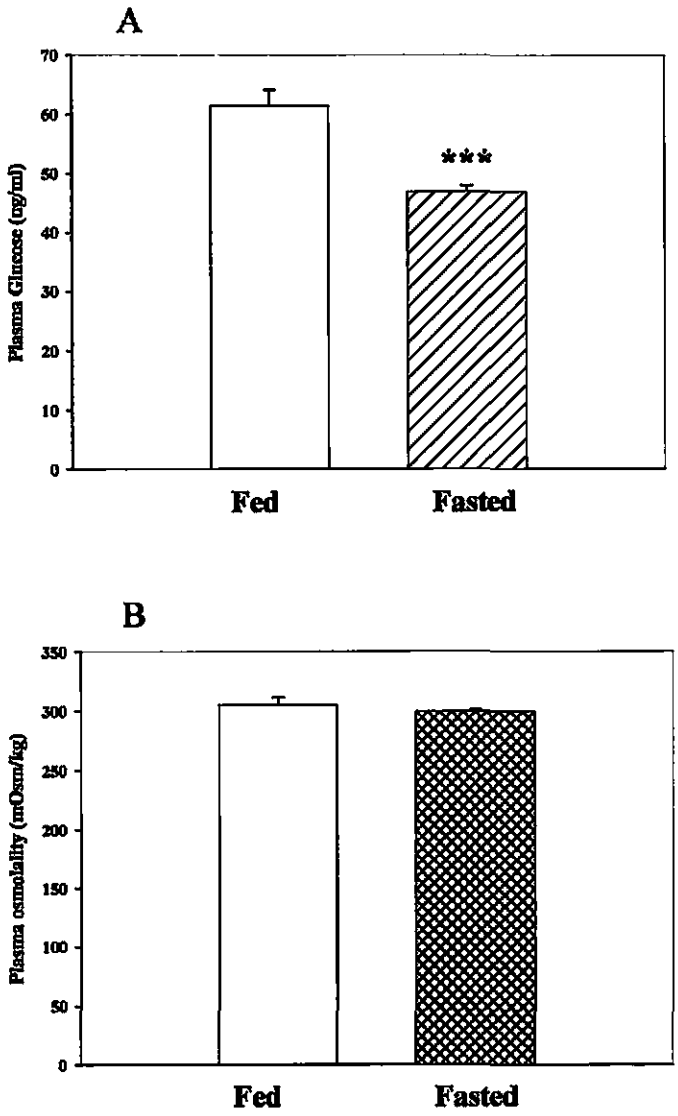

A
SL-R1 mRNA (% control)
140
120
100
80
60
40
20
0
Fed
Fasted
B
GH-R mRNA (% control)
120
100
80
60
40
20
0
Fed
Fasted
C
Plasma IGF-I (ng/ml)
200
150
100
50
0
Fed:
r= 0.635 P < 0.05
Fasted:
r= -0.638 P < 0.05
0
1
2
3
4
Plasma GH (ng/ml)

Fig. 4. Effects of 4 weeks of fasting in seawater-acclimated tilapia on plasma glucose (A) and plasma osmolality (B). Vertical bars indicate mean ± SEM (n = 14). *** Significantly different from the fed control at $P < 0.01$ and 0.001, respectively, by Student's *t* test.


A
Plasma Glucose (mg/ml)
70
60
50
40
30
20
10
0
***
Fed
Fasted
B
Plasma osmolality (mOsm/kg)
350
300
250
200
150
100
50
0
Fed
Fasted

## DISCUSSION

Fasting for 4 weeks produced significant reductions in both body weight and specific growth rate in seawater-acclimated tilapia. Reduction in specific growth rate in the fed fish during the last 4 weeks of experiment is in accord with our previous finding that growth rate is reduced once tilapia reach 50-60 g (Kuwaye et al. 1993; Sparks et al. 2003). Fasting also resulted in significant reductions in plasma IGF-I and liver expression of IGF-I mRNA, while concurrently causing an elevation in plasma GH and pituitary GH mRNA. Hepatic GH-R mRNA remained unchanged. There was a significant negative correlation between plasma levels of GH and IGF-I in the fasted fish. These results indicate that fasting may inhibit the stimulation of IGF-I by GH at a point that is downstream from the GH-R. It is also possible that IGF-I deficiency may be due to defects of GH-R or post-GHR signaling or primary defects of IGF-I synthesis. Similarly, there were no effects on hepatic SL-R mRNA levels. Without plasma SL levels to correlate these data with, it is difficult to make conclusions about the physiological relevance of these findings. Nevertheless, it appears SL-R mRNA expression levels may not be important during fasting in seawater-acclimated tilapia, or perhaps similar post-SL-R defects to the GH-R exists during nutrient restriction in fish.

The increases in plasma GH and pituitary GH mRNA levels after 4 weeks of fasting in seawater-acclimated tilapia are consistent with previous findings in our laboratory that: (1) plasma GH and pituitary GH content increased in freshwater tilapia

fasted for 21-31 days in fresh water (Weber et al. 1999), (2) spontaneous release of GH from the organ-cultures pituitary increased when tilapia were fasted for 2 weeks (Rogers et al. 1992), and (3) there was a significant increase in plasma GH 3 days after transfer to seawater in tilapia that were fasted for 2 weeks (Vijayan et al. 1996). Increases in plasma GH have also been observed in fasted sea bream in seawater (Deng et al. 2004), masu and Chinook salmon in fresh water (Fukada et al. 2004; Pierce et al. 2005), and also in Nile tilapia (*O. niloticus*) in fresh water (Toguyeni et al. 1996) after 2-3 weeks of fasting. Taken together, these findings indicate that a rise in plasma GH is a general feature that accompanies fasting whether in seawater or in fresh water. Although Uchida et al. (2003) failed to see changes in plasma GH in fasted tilapia in fresh water, 2 weeks of fasting may not have been sufficient to induce an increase in plasma GH.

We have shown earlier that the release of GH from the tilapia pituitary *in vitro* is inhibited by human IGF-I, indicating the presence of negative feedback (Kajimura et al. 2002). This interpretation is supported by the present finding that increased circulating GH was accompanied by a fall in plasma IGF-I, liver IGF-I expression and specific growth rate. More importantly, present study revealed a significantly negative correlation between circulating levels of GH and IGF-I specifically in fasted fish. We have observed similar reductions in plasma IGF-I and hepatic IGF-I expression in freshwater-acclimated tilapia fasted for 2 weeks, although there was no change in plasma GH levels (Uchida et al. 2003). Similar increases in plasma GH accompanied by decreased plasma IGF-I and IGF-I gene expression have been observed in fasted

salmonid species, indicating an apparent GH resistance (Duan et al. 1993; Moriyama et al. 1994; Fukada et al. 2004; Pierce et al. 2005).

In this study, we found no change in the hepatic expression of GH-R mRNA after 4 weeks of fasting. By contrast, Deng et al. (2004) reported a progressive decrease in hepatic GH-R mRNA from day 2 to day 30 of fasting in the black sea bream. In the coho salmon, there was no change in the expression of GH-R gene during the first 2 weeks of fasting, but a significant reduction was observed after 3 weeks (Fukada et al. 2004). Fasting or malnutrition is generally associated with low plasma levels of IGF-I regardless of unchanged or elevated plasma GH also in higher vertebrates, indicating that a state of GH resistance generally develops during food restriction (Rosenfeld et al. 2004). It is likely that the elevated plasma levels of GH and suppressed plasma levels of IGF-I observed in fasted animals may be a result from either primary IGF deficiency or IGF resistance. Primary IGF deficiency may be due to defects of GH-R or post-GH-R signaling or primary defects of IGF-I synthesis. IGF resistance may result from defects of IGF receptor, post-IGF signaling, or IGF binding proteins (Rosenfeld et al. 2004). It is well established in mammals that a dimerization of GH-R occurs following binding of GH, resulting in an intracellular phosphorylation cascade involving JAK2 and members of STAT family of proteins (Argetsinger et al. 1996). Beauloye et al. (2002) have found that IGF-I gene expression is reduced significantly in the hepatocytes isolated from fasted rats, whereas there was no difference in GH-R protein contents, suggesting that impairment of the GH-stimulated JAK-STAT signaling pathway may be responsible for the decreased IGF-I production. Fukada et al. (2004) suggested similar mechanisms may

mediate the apparent GH resistance observed in fasted coho salmon, and a similar mechanism may be operating in fasted tilapia. Recently, two GH-R subtypes (GH-R1 and GH-R2) have been identified in the black sea bream, each with different signal transduction and tissue distribution as well as different regulation of the expression by steroid hormones (Jiao et al. 2006). We also observed no difference in hepatic expression of SL-R mRNA levels in the current study. It has been suggested, as is the case in closely-related mammalian GH-R and prolactin-receptor (PRL-R), that heterodimerization may occur between fish GH-R/SL-R/PRL-R as well (Pérez-Sánchez et al. 2002). Further studies are needed to clarify the functional significance to the two receptors and their signal transduction pathways during development of GH resistance in response to food deprivation.

In nature, the Mozambique tilapia occurs in a wide range of habitats ranging from fresh water to hypersaline seawater (Pullin 1991). They are known to grow faster in seawater than in fresh water (Kuwaye et al. 1993; Ron et al. 1995; Riley et al. 2002b). The increased growth rate in seawater-raised tilapia has been correlated with a decrease in resting metabolic rate, suggesting that seawater-acclimated tilapia have more energy available for growth than in freshwater tilapia (Ron et al. 1995; Sparks et al. 2003). There was no difference in plasma osmolality between fed and fasted fish in this study. Besides growth, GH appears to play an integral role in seawater acclimation in euryhaline fishes including the tilapia (Sakamoto et al. 1997; McCormick 2001). Plasma GH levels are increased during the first 3 days of seawater acclimation in the tilapia (Yada et al. 1994; Vijayan et al. 1996). Osmoregulation appears to use a high proportion of the

available energy, ranging from 20 to more than 50% of the total energy expenditure in some teleost species (Boef et al. 2001). In the present study, we observed no difference in plasma osmolality between fed and fasted tilapia. The increased plasma GH levels in fasted fish suggest that GH may be involved in the reallocation of metabolic energy for supporting growth toward the maintenance of homeostasis.

The response of fish to fasting is characterized by a sequential utilization of glycogen, lipid and protein reserves (Collins et al. 1995). Stress-induced elevation of plasma cortisol concentration is commonly used as an indicator of the primary stress response, while increases in plasma glucose levels are used as indicators of the secondary stress response in fish (Mommsen et al. 1999; Kajimura et al. 2003). Plasma glucose was reduced significantly in fasted tilapia in our study. Rogers et al. (1992) also observed a reduction in plasma glucose in fasted tilapia after 3 weeks. On the other hand, Vijayan et al. (1996) observed an elevation in plasma glucose in food-deprived tilapia 3 days following transfer to seawater. They ascribed the increased plasma glucose to elevated plasma cortisol and up-regulation of hepatic glycogenolytic enzymes. Decreases in plasma glucose concentrations after fasting have been observed also in yellow perch, sea raven and rainbow trout (Foster et al. 1991; Vijayan et al. 1994; Reddy et al. 1995). Reduction in plasma glucose observed in the present study may reflect the exhaustion of liver glycogen stores. To the degree that this is true, reduced plasma glucose may be tolerated because of the increased utilization of lipid in fasted fish (Pottinger et al. 2003). Recently, Albalat et al. (2005) have shown clear lipolytic effect of GH in adipocytes isolated from gilthead sea bream. It is interesting to note that a significantly negative

correlation was observed between plasma levels of GH and glucose in the present study, suggesting again possible involvement of GH in reallocation of metabolic energy during fasting.

In summary, hepatic expression of GH-R and SL-R in response to fasting was characterized in tilapia for the first time. Fasting for 4 weeks resulted in a significant reduction in body weight and specific growth rate. Plasma GH and pituitary GH mRNA levels were elevated significantly in fasted fish. Fasting also produced significant reductions in plasma IGF-I and hepatic IGF-I mRNA levels. A significant negative correlation was observed between plasma levels of GH and IGF-I in the fasted fish. No effect was observed on hepatic GH-R or SL-R mRNA levels. The increased GH and decreased IGF-I production indicate the development of GH resistance in fasted tilapia; however, the lack of effect on GH-R mRNA expression suggests mechanisms post-GH-R transcription are involved.

## CHAPTER III

## Effects of Homologous Ghrelins on the Growth Hormone/Insulin-like Growth Factor-I Axis in the Tilapia, *Oreochromis mossambicus*


### ABSTRACT

Ghrelin is a gut-brain peptide synthesized mainly in the oxyntic mucosal cells of the stomach with potent growth hormone (GH)-releasing and orexigenic activities. Recently, two forms of ghrelin, ghrelin-C8 and -C10, were identified in the Mozambique tilapia (*Oreochromis mossambicus*). The present study describes *in vitro* and *in vivo* effects of the endogenous ghrelins on the GH/insulin-like growth factor-I (IGF-I) axis. Ghrelin-C8 (100 nM) stimulated GH release from primary culture of pituitary cells after 4 and 8 h of incubation, whereas no effect was seen on prolactin (PRL) release. Stimulatory effects of ghrelin-C8 and -C10 (100 nM) on GH release during 6 h of incubation were blocked by pre-incubation with GHS receptor antagonist, [D-Lys3]-GHRP-6 (10 M). Intraperitoneal injection of ghrelin-C8 (1 ng/g body weight) and -C10 (0.1 and 1 ng/g) increased plasma GH levels significantly after 5 h. Significant increases were observed also in hepatic expression of IGF-I, somatolactin-receptor (SL-R), and GH-receptor (GH-R) genes following injections of both forms of ghrelin (0.1 and 1 ng/g), although there was no effect on plasma levels of IGF-I. In the next experiment, both forms of ghrelin (1 ng/g) increased plasma IGF-I levels significantly at 10 h after the

injection. No significant effect of either ghrelin was observed on plasma PRL levels. Both forms of GHS receptor (GHSR-1a and -1b) were found in the pituitary, clearly indicating that tilapia ghrelins stimulate GH release primarily through GHS receptor. Stimulation of hepatic expression of IGF-I, SL-R, and GH-R suggests metabolic roles of ghrelin in tilapia.

## INTRODUCTION

In teleosts, secretion of growth hormone (GH) is regulated by several hypothalamic factors that are influenced by the physiological state of the animal. In fish, growth hormone-releasing hormone (GHRH), gonadotropin-releasing hormone (GnRH), neuropeptide Y, and thyrotropin-releasing hormone (TRH) stimulate the release of GH, effects that have been shown to be influenced by gonadal sex steroids and the nutritional state of the animal (Nishioka et al. 1988; Peng et al. 1997; Sherwood et al. 2000). The actions of somatostatin, the primary inhibitory factor of GH release, are also influenced by gonadal sex steroids in fish (Peng et al. 1997).

Both growth hormone and prolactin (PRL) play important roles in osmoregulation, growth, development and reproduction in teleosts (McLean et al. 1993; Blazquez et al. 1998; Manzon 2002). The regulation of PRL release in fish is equally as complex. Release of PRL is stimulated by TRH, estradiol-17β and PRL-releasing peptide (PrRP), and inhibited by somatostatin, dopamine, and urotensin II (Nishioka et al. 1988; Seale et al. 2002). Priming of PRL-secreting cells by pre-incubation with estradiol enhances the ability of TRH and GnRH to stimulate PRL release from organ-cultured tilapia pituitary (Barry et al. 1986; Weber et al. 1997). Thus, the multifactoral function

and regulation of both GH and PRL make it difficult to clarify the mechanisms that regulate their release from the pituitary.

In 1999, Kojima and co-workers identified ghrelin, an endogenous ligand for a previously characterized orphan receptor in rat stomach, known as the growth hormone secretagogue receptor (GHSR) in rat stomach (Kojima et al. 1999). Ghrelin potently stimulates GH release both *in vivo* and *in vitro* in rat (Kojima et al. 1999), bullfrog (Kaiya et al. 2001), rainbow trout (Kaiya et al. 2003a), and goldfish (Unniappan et al. 2004b), and also *in vivo* GH release in humans (Hataya et al. 2001) and the channel catfish (Kaiya et al. 2005). Ghrelin also stimulates PRL release *in vivo* in humans (Peino et al. 2000; Takaya et al. 2000; Nagaya et al. 2001) and the bullfrog (Kaiya et al. 2001). We have shown earlier that rat ghrelin stimulates *in vitro* release of GH and PRL in tilapia (Riley et al. 2002a). Amidated ghrelin isolated from eel stomach was also effective in stimulating *in vitro* release of GH and PRL in tilapia (Kaiya et al. 2003b). Recently, two forms of ghrelin, ghrelin-C8 and -C10, were isolated from tilapia stomach (Kaiya et al. 2003c). Similar to all ghrelins isolated thus far, tilapia ghrelins possess an acylated modification on the $Ser_3$ residue. The major form of tilapia ghrelin (ghrelin-C10) possesses an n- decanoic modification, which is similar to that found in eel, chicken, and bullfrog (Kaiya et al. 2001; Kaiya et al. 2002; Kaiya et al. 2003b), whereas in rats, the major form of ghrelin possesses an n-octanoic modification (Kojima et al. 1999). The acylated modification, which occurs post-translationally, is essential for receptor binding (Muccioli et al. 2001) and ghrelin transport across the blood-brain barrier (Banks et al. 2002). Octanoylated ghrelin in the tilapia (ghrelin-C8) exists in the stomach in lower

abundance than ghrelin-C10 (Kaiya et al. 2003c). To date, tilapia is the only animal to possess both C8 and C10 forms of ghrelin. Both forms of tilapia ghrelin stimulated GH and PRL release from the organ-cultured tilapia pituitary.

In vertebrates, many of the actions of GH are mediated by insulin-like growth factor-I (IGF-I), a mitogenic factor produced primarily in the liver (Le Roith et al. 2001; Duan 1997; Wood et al. 2005). Repeated oral administration of the GHS, MK-0677, produced sustained increases in plasma levels of both GH and IGF-I in the beagle (Hickey et al. 1997). According to Sun *et al.* (2004), GHSR-null mice exhibited modestly reduced serum levels of IGF-I compared with wild-type littermates, suggesting that ghrelin is a regulatory factor for IGF-I release. Conversely, IGF-I has been shown recently to down-regulate GHSR mRNA levels in rat pituitary directly, suggesting that IGF-I may modulate GH secretion through modulation of expression of the GHSR (Kamegai et al. 2005). However, little is known about the effect of ghrelin on IGF-I production or on the involvement of GHSR in stimulating GH (and PRL) release in lower vertebrates (Kaiya et al. 2008). The present study was undertaken to examine possible *in vitro* and *in vivo* effects of ghrelin-C8 and -C10 on GH and PRL release from the tilapia pituitary.

Effects of homologous ghrelins on GH/IGF-I axis were also examined by following changes in plasma IGF-I and hepatic expression of IGF-I, SL-R, and GH-R genes after intraperitoneal injections.

## MATERIALS AND METHODS

*Animals*

Mozambique tilapia (*Oreochromis mossambicus*) were reared outdoors in 5000-L freshwater flow-through tanks under natural photoperiod at the Hawaii Institute of Marine Biology. Animals were fed approximately 2% of body weight per day twice daily with Silver Cup Trout Chow (Nelson and Sons, Murray, UT). Water temperature was 25-28 °C. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

*Primary culture of pituitary cells*

*In vitro* effects of ghrelin were examined using cells dispersed from whole pituitary. Mature tilapia of both sexes, weighing 300-500 g, were used. After anesthesia in tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, 0.5 g/L, buffered with 0.5 g/L $NaHCO_3$), pituitaries were collected aseptically in isotonic medium (Krebs bicarbonate-Ringer solution, 330 mOsmolal, pH 7.4) as described by Wigham et al. (Wigham et al. 1977), supplemented with penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and nystatin (250 IU/ml, all from Sigma, St. Louis, MO). The pituitaries were diced with a sterile razor blade, and trypsinized for 1 h at room temperature in 2.5 ml of trypsin-EDTA solution (0.25% trypsin + 0.02% EDTA in phosphate buffered saline, pH 7.4). Tissues were aspirated repeatedly through a pipette during trypsinization to promote dissociation of cells. The process was terminated by the addition of 0.5 ml (20%) fetal bovine serum (Sigma). Cells were counted on a hemocytometer under a light microscope,

and viability determined by trypan blue exclusion. Viability of the cells was always greater than 95%. Cells were then plated at a density of 4.0 x $10^5$ cells/well into a 24-well plate (Falcon, Primaria 24, Becton Dickinson, Franklin Lakes, NJ) at a volume of 300 µl per well of isotonic medium supplemented with 10% fetal bovine serum. The cells were pre-incubated for 4 days at 26-28°C under a humidified atmosphere of 95% $O_2$ and 5% $CO_2$, with one change of culture medium at 48 h post-plating. Prior to each experiment, cells were washed once with serum-free medium. A final 300 µl serum-free medium was added containing tilapia ghrelin-C8, -C10, human $GHRH_{1-29}$ amide (Sigma) or control medium without hormones. Medium was replaced at 4 h. Incubations were terminated at 8 h, and hormone release was quantified for the 0-4 and 4-8 h intervals. GH and PRL release was expressed as secretion per unit volume of medium (ng/ml). Medium samples were stored at -20 °C for further analyses of GH and PRL concentrations by homologous radioimmunoassays. To examine whether ghrelin acts through the GHSR, dispersed pituitary cells were exposed to the GHSR antagonist, [D-$Lys_3$]-GHRP-6 (Sigma). On the day of treatment, cells were pre-incubated for 1 h with either control medium or medium containing [D-$Lys_3$]-GHRP-6. Pre-incubation medium was then aspirated and replaced with fresh medium containing either tilapia ghrelin-C8 or -C10 without the antagonist. Incubation was terminated after 6 h, and the medium was stored at -20 °C for later analyses of GH and PRL concentrations.

*Injection and blood sampling*

Male tilapia, weighing 30–50 g, were used in this study. Prior to the experiment, fish were transferred to oval 60-L flow-through freshwater tanks. The animals were allowed

to acclimate for 14 days, during which time they were fed to satiety twice daily, and food was withheld 24 h before the experiment. In the first experiment, 48 fish (n=8/tank) were given a single intraperitoneal injection of vehicle (saline) or vehicle-containing tilapia ghrelin-C8 or -C10 at two doses (0.1 or 1 ng/g body weight or 0.043 or 0.43 pM/g) or human GHRH (Sigma, 10 ng/g or 2.97 pM/g), under anesthesia in MS-222. In the second experiment, 24 fish (n=6/tank) were given a single intraperitoneal injection of vehicle (saline) or vehicle containing tilapia ghrelin-C8 or -C10 (1 ng/g body weight) or GHRH (10 ng/g). The injection volume was 1 µl/g body weight for all treatments.

Blood was collected by caudal puncture using a heparinized syringe (200 U/ml, ammonium heparin, Sigma) at 5 h post-injection for the first experiment, and sampled serially from the same fish at 5 and 10 h post-injection for the second experiment. Plasma was separated by centrifugation at 10,000 rpm for 15 min, and stored at –20°C for later analyses of GH, PRL, IGF-I, and glucose. Following blood collection, livers and pituitaries were excised, snap-frozen in liquid nitrogen, and stored at –80°C until real-time PCR analysis for GH, IGF-I, SL-R, and GH-R gene expression.

*Radioimmunoassays*

Plasma levels of GH and PRL188 were estimated by homologous radioimmunoassays (Yada et al. 1994). The tilapia pituitary secretes two distinct PRL molecules, PRL177 and PRL188, which are encoded by separate genes (Yamaguchi et al. 1988). Since we found no significant difference in response to various treatments between the two forms of PRL (Seale et al. 2003), only PRL188 release was examined. For measurement of total plasma IGF-I, 25 µl of plasma were extracted with 100 µl of

acid-ethanol (87.5% ethanol and 12.5% 2 N HCl, v/v) and neutralized with Tris. Total IGF-I levels were then measured by radioimmunoassay following Moriyama *et al.* (1994). Recombinant salmon IGF-I (as a standard and for labeling) and anti-barramundi IGF-I were purchased from GroPep (Adelaide, Australia). Plasma glucose concentrations were estimated using a commercially available kit (Glucose Assay Kit, GAHK-20, Sigma), which was modified for a microplate reader (SpectraCount, Packard, Meriden, CT).

*Real-time quantitative RT-PCR*

Total RNA was extracted from pituitary and liver tissues by guanidinium thiocyanate-phenol-chloroform extraction, using a commercial extraction solution (TRI-Reagent, MRC, Cincinnati, OH) according to the manufacturer's instructions. Liver IGF-I, SL-R, GH-R, and pituitary GH mRNA transcripts were analyzed using real-time quantitative RT-PCR (rtqRT- PCR). Equal amounts of RNA (100 ng/µl) were reverse transcribed using a BioRad (Hercules, CA) iScript cDNA Synthesis Kit. Separately, liver and pituitary RNA from several fish was pooled, reverse transcribed, and used as inter-assay standard samples. In addition, samples were added to the cDNA synthesis reaction mix without the addition of reverse transcriptase enzyme for a no-reverse transcriptase control. IGF-I, SL-R, GH-R, and GH rtqRT-PCR followed previously described methods (Kajimura et al. 2004; Pierce et al. 2007) using a BioRad iCycler iQ Real-Time PCR Detection System. Each plate well contained 15 µl of iQ Sybrgreen supermix (BioRad). Inter-plate variation was normalized using the positive control and data expressed as percent of control. The cycling parameters were 2 min at 50°C and 2 min at 95°C,

followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Data were analyzed by iCycler software. Primer-pair sequences for IGF-I, SL-R, and GH-R analyses were the same as those described in Table 1.

*Tissue distribution of GHSR subtypes*

RNA was extracted from stomach, liver, gill, kidney, muscle, rostral pars distalis (RPD), posterior pars distalis (PPD), and 3 brain sections (diencephalon, metacephalon, and telechephalon) as described above. Total RNA was treated with DNase (DNA-*free* kit, Ambion, Austin, TX). After centrifugation at 10,000×g for 1.5 min, the supernatant containing the RNA was transferred to a fresh tube. First-strand cDNA was reverse transcribed (Improm-II Reverse Transcription System, Promega, Madison, WI) using 500 ng of total RNA and random hexamer primers. PCR was performed using gene-specific primers for GHSR-1a, 5'-AAG ATG CTG GTG GTT GTT GTG-3' (forward) and 5'-TGG CGG CAG GCT GTC GGT CAG-3' (reverse), and GHSR-1b, 5'-AGT GCT CTA CAG CCT GAT AGG-3' (forward) and 5'-TTG CGC GTT CTG GAA ACT TAC-3' (reverse) to amplify a 250 bp and 134 bp product, respectively. Thermal cycling conditions for GHSR-1a were 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Thermal cycling conditions for GHSR-1b were 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min. Both PCRs had a final extension of 72°C for 5 min. Each PCR reaction (50 µl) contained 3 µl of cDNA corresponding to the tissue of interest, 200 µM dNTPs, 0.3 mM each primer, and 2 mM or 1.5 mM MgCl2 for GHSR-1a or GHSR-1b, respectively. Each PCR was analyzed on a 1.6% agarose gel with 20 µl of sample stained with ethidium bromide.

*Statistics*

Data were analyzed by one-way ANOVA and means were compared using Fischer's least significant difference test for predetermined comparisons at each time point. Calculations were performed using the computer program STATISTICA (StatSoft, Tulsa, OK).

## RESULTS

*In vitro effects of tilapia ghrelins on GH and PRL release from dispersed pituitary cells*

In the first experiment, the effects of ghrelin-C8 and human GHRH on GH and PRL release were examined using dispersed cells from the whole pituitary. Both ghrelin-C8 (100 nM)and GHRH (10 nM) stimulated GH release significantly after 4 and 8 h of incubation. There was no effect of ghrelin-C8 or GHRH on PRL release (Fig. 5). Fig. 6 depicts the effects of GHSR specific antagonist, [D-Lys3]-GHRP-6, on GH and PRL release. Pre-incubation of dispersed cells with [D-Lys3]-GHRP-6 (10 ⌈M) for 1 h produced a significant suppression of the stimulatory effects of tilapia ghrelin-C8 and -C10 (100 nM) on the release of GH following 6 h of incubation. There was no effect of tilapia ghrelins on PRL release with or without [D-Lys3]-GHRP-6 pretreatment.

*In vivo effects of tilapia ghrelins on plasma levels GH, PRL and IGF-I and on hepatic expression of IGF-I, SL-R, and GH-R genes*

In the first experiment, intraperitoneal injection of ghrelin-C8 (1 ng/g body weight) and ghrelin-C10 (0.1 and 1 ng/g) produced elevated plasma GH levels significantly at 5 h post-injection (Fig. 7). The low dose of ghrelin-C8 (0.1 ng/g) and

human GHRH (10 ng/g), however, failed to affect plasma GH levels. There was no effect of two doses of ghrelin-C8 or -C10 on pituitary GH mRNA or on plasma levels of PRL or IGF-I. In contrast, significant increases were observed in hepatic expression of IGF-I, SL-R, and GH-R genes following injections of low and high doses of both forms of ghrelin and also of human GHRH (10 ng/g) (Fig 7D and 8). In the second experiment, the effects of the two forms of ghrelin (1 ng/g) and human GHRH (10 ng/g) were examined at 5 and 10 h post injection. Significant increases in plasma GH were observed after the injection of ghrelin-C10; ghrelin-C8 failed to elicit a significant effect in this experiment. On the other hand, both forms of ghrelin as well as GHRH increased plasma IGF-I significantly at 10 h after the injection; there was no effect after 5 h. No significant effect of either ghrelin or GHRH was observed on plasma levels of PRL or glucose (Fig. 9).

*Tissue distribution of GHSR-1a and GHSR-1b*

Fig. 10 illustrates expression of GHSR mRNA (GHSR-1a and -1b) by RT-PCR in various tissues. All tissues assayed expressed both subtypes of GHSR-1; these tissues included the liver, the RPD, which contains PRL cells almost exclusively, and the PPD which contains GH cells. The highest expression was observed in the diencephalon.

Fig. 5. Effect of tilapia ghrelin-C8 and human GHRH on GH (A) and PRL (B) release from dispersed pituitary cells. Cells were exposed to 100 nM ghrelin-C8 or 10 nM human GHRH for 8 h. Values are mean □ SEM, *, **, *** Significantly different from the control (0 nM) at each time point at $P < 0.05$, 0.01, and 0.001, respectively (n = 8).

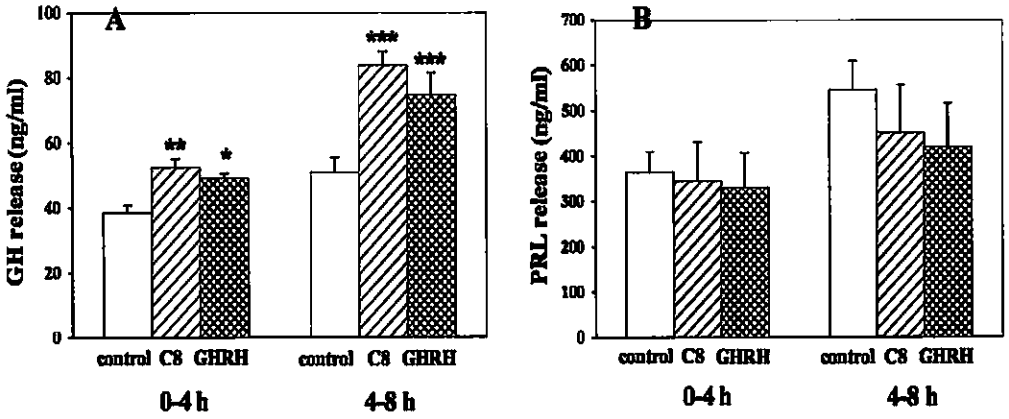

A
GH release (ng/ml)
100
80
60
40
20
0
**
*
***
***
control C8 GHRH
control C8 GHRH
0-4 h
4-8 h
B
PRL release (ng/ml)
700
600
500
400
300
200
100
0
control C8 GHRH
control C8 GHRH
0-4 h
4-8 h

Fig. 6. Effects of the growth hormone secretagogue-receptor-specific antagonist, [D-Lys3]- GHRP-6 on stimulatory effects of tilapia ghrelin-C8 and -C10 on GH (A) and PRL (B) release from dispersed pituitary cells. Cells were pre-treated with [D-Lys3]-GHRP-6 (10 μM) for 1 h, and then incubated with tilapia ghrelin-C8, -C10 (100 nM), or medium control for 6 h. Values are mean □ SEM, **, *** Significantly different from the control (0 nM) at each time point at $P < 0.01$, and 0.001, respectively (n=8)

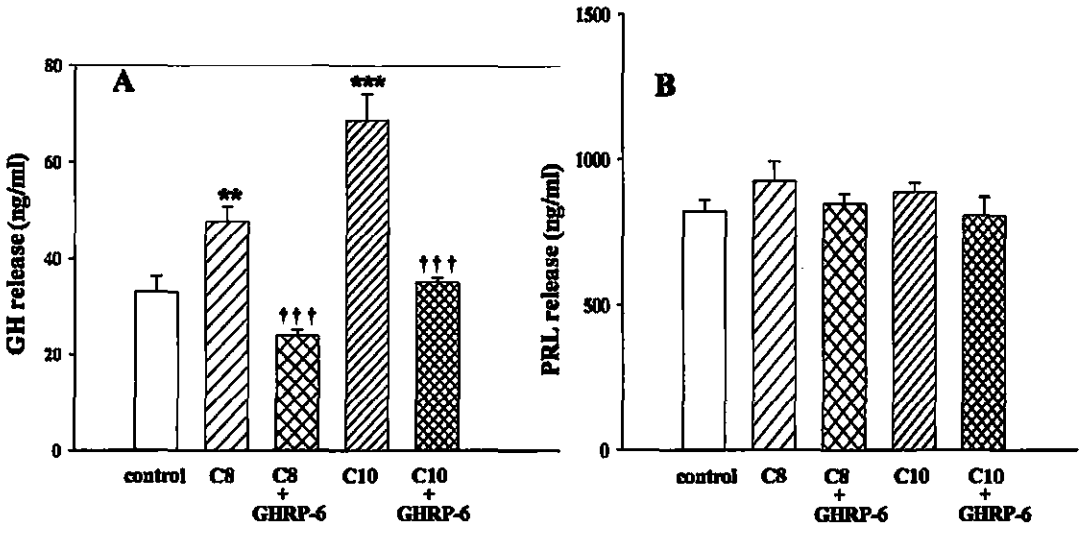

A
B
GH release (ng/ml)
PRL release (ng/ml)
80
60
40
20
0
1500
1000
500
0
**
***
†††
†††
control
C8
C8 + GHRP-6
C10
C10 + GHRP-6

Fig. 7. Effects of intraperitoneal injection of tilapia ghrelin-C8 (0.1 or 1 ng/g), -C10 (0.1 or 1 ng/g), GHRH (10 ng/g), or vehicle (saline) on plasma GH (A), pituitary GH mRNA (B), plasma IGF-I (C), hepatic IGF-I mRNA (D), and plasma PRL (E). Samples were taken at 5 h post injection. Values are mean ± SEM, *, **, ***Significantly different from the control (saline) at $P < 0.05$, 0.01, and 0.001, respectively (n = 8).

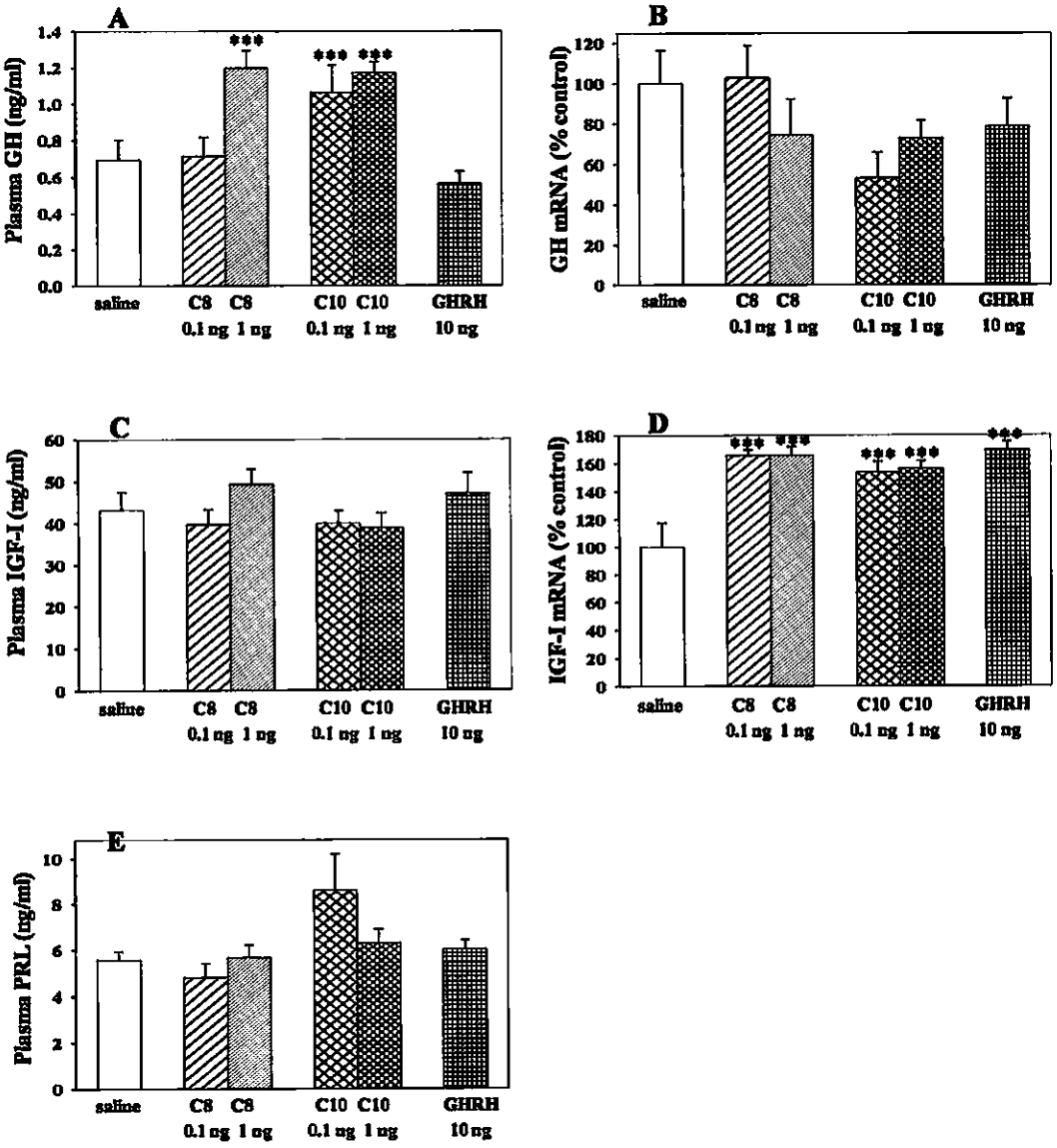
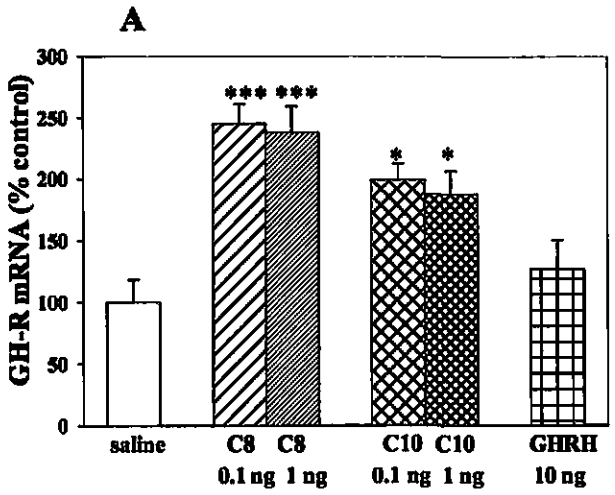

A
Plasma GH (ng/ml)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
saline
C8 C8
0.1 ng 1 ng
C10 C10
0.1 ng 1 ng
GHRH
10 ng
B
GH mRNA (% control)
120
100
80
60
40
20
0
saline
C8 C8
0.1 ng 1 ng
C10 C10
0.1 ng 1 ng
GHRH
10 ng
C
Plasma IGF-I (ng/ml)
60
50
40
30
20
10
0
saline
C8 C8
0.1 ng 1 ng
C10 C10
0.1 ng 1 ng
GHRH
10 ng
D
IGF-I mRNA (% control)
180
160
140
120
100
80
60
40
20
0
***
***
***
***
***
saline
C8 C8
0.1 ng 1 ng
C10 C10
0.1 ng 1 ng
GHRH
10 ng
E
Plasma PRL (ng/ml)
10
8
6
4
2
0
saline
C8 C8
0.1 ng 1 ng
C10 C10
0.1 ng 1 ng
GHRH
10 ng

Fig. 8. Effects of intraperitoneal injection of tilapia ghrelin-C8 (0.1 or 1 ng/g), -C10 (0.1 or 1 ng/g), GHRH (10 ng/g), or vehicle (saline) on, hepatic GH-R mRNA (A) and hepatic SL-R mRNA (B). Samples were taken at 5 h post injection. Values are mean ± SEM, *, **, *** Significantly different from the control (saline) at $P < 0.05$, 0.01, and 0.001, respectively (n = 8).


A
GH-R mRNA (% control)
300
250
200
150
100
50
0
*** ***
* *
saline
C8 C8
0.1 ng 1 ng
C10 C10
0.1 ng 1 ng
GHRH
10 ng

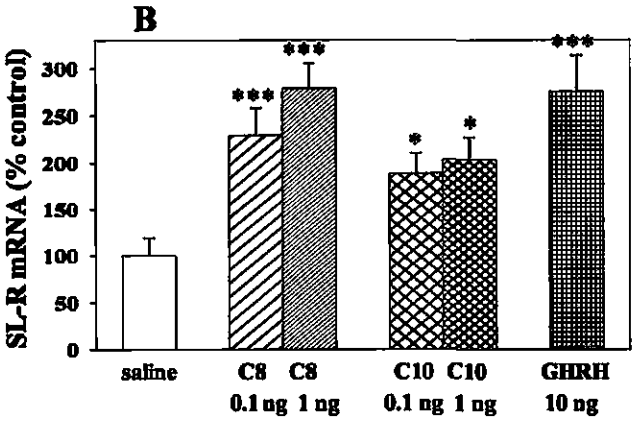

B
SL-R mRNA (% control)
300
250
200
150
100
50
0
*** ***
* *
*** ***
saline
C8 C8
0.1 ng 1 ng
C10 C10
0.1 ng 1 ng
GHRH
10 ng

Fig. 9. Effects of intraperitoneal injection of tilapia ghrelin-C8 (1 ng/g), -C10 (1 ng/g), GHRH (10 ng/g BW), or vehicle (saline) on plasma levels of GH (A), IGF-I (B), PRL (C), and glucose (D) 5 and 10 h after injection. Values are mean ± SEM, *, **, *** Significantly different from the control (saline) at each time point at $P < 0.05$, 0.01, and 0.001, respectively (n = 6).

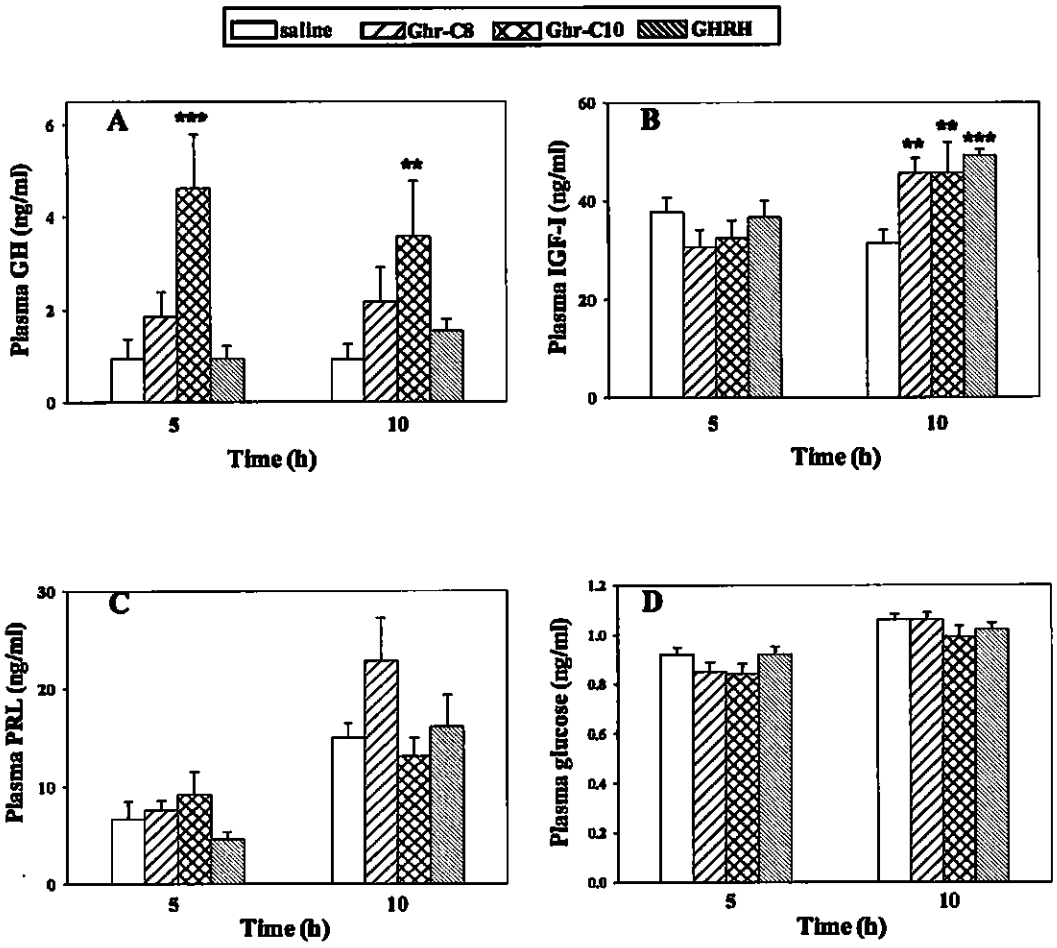
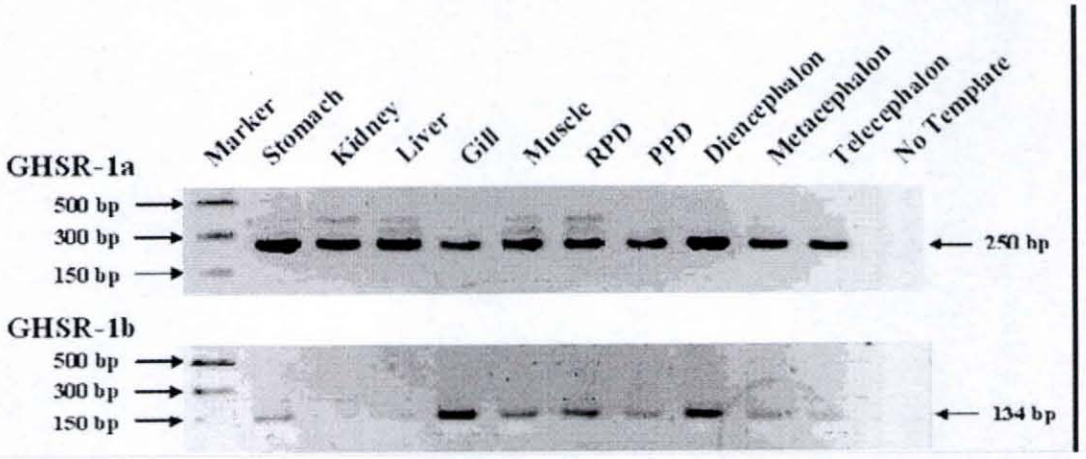

saline
Ghr-C8
Ghr-C10
GHRH
A
Plasma GH (ng/ml)
Time (h)
B
Plasma IGF-I (ng/ml)
Time (h)
C
Plasma PRL (ng/ml)
Time (h)
D
Plasma glucose (ng/ml)
Time (h)

Fig. 10. Expression of GHSR mRNA (GHSR-1a and -1b) detected by RT-PCR.


Marker
Stomach
Kidney
Liver
Gill
Muscle
RPD
PPD
Diencephalon
Metacephalon
Telecephalon
No Template
GHSR-1a
500 bp
300 bp
150 bp
250 bp
GHSR-1b
500 bp
300 bp
150 bp
134 bp

## DISCUSSION

The present study focused on *in vitro* and *in vivo* effects of the two native forms of tilapia ghrelin on GH/IGF-I axis in tilapia. To our knowledge, this is the first report of the effect of peripheral administration of homologous ghrelins on hepatic expression of GH-R or SL-R in fish. Ghrelin-C8 (100 nM) stimulated GH release from primary culture of pituitary cells after 4 and 8 h of incubation, whereas no effect was seen on PRL release. Stimulatory effects of ghrelin-C8 and -C10 (100 nM) on GH release during 6 h of incubation were blocked by pre-incubation with GHSR antagonist, [D-Lys3]-GHRP-6 (10 nM), clearly indicating that endogenous ghrelins stimulate GH release through GHSR in the pituitary. Intraperitoneal injection of ghrelin-C8 and -C10 (0.1 and 1 ng/g body weight) increased plasma GH levels after 5 h. Significant increases were observed also in hepatic expression of IGF-I, SL-R, GH-R genes following injections of both forms of ghrelin, although there was no effect on plasma levels of IGF-I. In the next experiment, both forms of ghrelin (1 ng/g) increased plasma IGF-I levels significantly at 10 h after the injection, whereas there was no effect after 5 h. No significant effect of either of the two tilapia ghrelins was observed on plasma PRL levels. Our current observations using primary culture of pituitary cells differ with our previous findings, which showed that both forms of tilapia ghrelin stimulated GH and PRL release from the organ-cultured tilapia pituitary (Kaiya et al. 2003c). We have also shown that rat and eel ghrelins stimulate *in vitro* release of GH and PRL from the organ-cultured tilapia pituitary (Riley et al. 2002a; Kaiya et al. 2003b). Ghrelin has been shown to stimulate PRL release *in vivo*

in humans (Peino et al. 2000; Takaya et al. 2000; Nagaya et al. 2001). According to Kaiya *et al.* (2001), bullfrog ghrelin stimulated PRL release from dissociated bullfrog pituitary cells. In the present study using dispersed pituitary cells, however, tilapia ghrelins stimulated GH release specifically without affecting PRL release. Absence of ghrelins' effect on PRL release was also confirmed in the second experiments using GHRP-6 (Fig. 6). The lack of a PRL response to ghrelin has also been reported in rats (Kojima et al. 1999). Kaiya et al. (2003a) also failed to observe a stimulation of PRL release *in vitro* and *in vivo* in rainbow trout. It is not clear why the effects of different ghrelins on PRL release were different between incubations of intact pituitaries and those using dispersed pituitary cells in the tilapia. According to Parhar et al. (2003), ghrelin expression in the tilapia (*O. niloticus*) stomach was greater in females than in males. There may be a sexual difference in GH and PRL release in response to ghrelin. Further studies using positive controls such as angiotensin II (see Eckert et al. 2003) will also be necessary. In the current study, significant increases in plasma GH levels were observed after intraperitoneal injection of homologous ghrelins, whereas there was no effect on plasma PRL. In the tilapia, homologous ghrelins may be less potent in stimulating PRL release compared with their GH-releasing activity.

The actions of ghrelin are modulated by the GHSR in mammals (Kojima et al. 1999; Sun et al. 2004). We have reported earlier that plasma GH levels in the tilapia increases at 6 and 12 h following an intraperitoneal injection of a GHS (i.e. ghrelin agonist), KP-102 (Shepherd et al. 2000). In the current study, the GHSR antagonist, [D-Lys$_3$]-GHRP-6, completely abolished the stimulation of GH release by both forms of

tilapia ghrelin from dispersed pituitary cells, indicating that ghrelins' effects are mediated through GHSR. Our observation that both GHSR-1a and GHSR-1b gene transcripts are present in the RPD and PPD support the hypothesis that ghrelin's action is mediated by GHSR. At this point it is not clear which of the two GHSR subtypes mediates ghrelin's action. Two GHSR subtypes, GHSR-1a and GHSR-1b, have been identified in mammals. Both types are found in a variety of endocrine and non-endocrine tissues including the hypothalamus and a variety of other brain regions, as well as the pituitary, liver, lung, heart, muscle, kidney and gonads (van der Lely et al. 2004). Three GHSR were also identified in pufferfish (Palyha et al. 2000). Recently, two cDNA transcripts for GHSR, sbGHSR-1a and sbGHSR-1b, were identified from the sea bream pituitary (Chan et al. 2004). The sbGHSR-1a transcript had the highest expression level in the pituitary, whereas the sbGHSR-1b transcript showed its highest expression level in the telencephalon. In the current study, it appears that the highest expression of GHSR-1 is in the diencephalon. Functional expression of these two receptor constructs in human embryonic kidney 293 (HEK293) cells indicated that stimulation of sbGHSR-1a by GHS could evoke increases in intracellular $Ca^{2+}$ concentration, whereas sbGHSR-1b appeared to play an inhibitory role on the signal transduction activity of sbGHSR-1a. In tilapia, cDNA for GHSR-1a and -1b have been identified (Kaiya, H., unpublished). In addition to the RPD and PPD, both GHSR subtypes were found in the stomach, kidney, liver, gill, muscle and brain. Further studies are called for to clarify signal transduction cascades to exert physiological functions of tilapia ghrelins resulting in increased GH secretion.

In this study, intraperitoneal injection of tilapia ghrelin-C8 (1 ng/g body weight) and ghrelin-C10 (0.1 and 1 ng/g) elevated plasma GH significantly after 5 h (Fig. 7). A lower dose of ghrelin-C8 (0.1 ng/g) was without effect. Plasma GH also increased at 5 and 10 h after administration of ghrelin-C10 (1 ng/g) in the second experiment, whereas there was no significant effect of ghrelin-C8 (Fig. 9). These results suggest that ghrelin-C10, a major form of tilapia ghrelin (Kaiya et al. 2003c), is more potent than ghrelin-C8 in stimulating GH release from the tilapia pituitary. In the current study, human GHRH (10 ng/g) did not alter plasma GH levels in either experiment at any time point. In our previous study, an intraperitoneal injection of bovine GHRH (10 ng/g) produced a significant increase in plasma GH after 6 h in tilapia, whereas no effect was seen after 12 h (Shepherd et al. 2000). In rainbow trout, plasma GH levels increased at 0.5, 1, and 3 h after the injection of ghrelin, and returned to initial levels after 6 h (Kaiya et al. 2003a). An increase in plasma GH was observed only 1 h after ghrelin injection in catfish (Kaiya et al. 2005). Administration of ghrelin in goldfish brought about an increase in serum GH levels 15 and 30 min post-injection, after which levels did not differ from those in saline injected fish (Unniappan et al. 2004b). Thus more frequent samplings may be necessary to establish stimulatory effects of ghrelin-C8 and GHRH on plasma GH.

Ghrelin's effect on pituitary GH synthesis in mammals is somewhat contradictory. A single intra-cerebraventricular (icv) injection of ghrelin was found to elevate plasma GH in the rat after 15 min, but failed to alter GH mRNA levels (Date et al. 2000). Conversely, GHRP-6 caused increases in GH and PRL mRNA in dissociated anterior pituitary cells from infant rats through an increase in the Pit-1 transcription factor

starting after 1 h and lasting at least 12 h (Garcia et al. 2001). The difference in responsiveness of rat pituitary cells between these two studies may be attributed to differences in methods (*in vivo* versus *in vitro*) or in the developmental status of the animals tested (adult versus infant) (Date et al. 2000; Garcia et al. 2001). In the current study, no effect was observed on pituitary GH mRNA levels 5 h after intraperitoneal administration of ghrelin-C8 or -C10. In contrast, Riley et al. (2005) reported a significant increase in GH mRNA after long-term treatment by C-8 ghrelin for 21 days.

Although no measurement was done on GH content in the pituitary in this study, incubation of organ-cultured tilapia pituitaries with rat ghrelin for 24 h failed to alter the GH content in the pituitary (Riley et al. 2002a). The absence of ghrelin's effects on GH mRNA in this study may be due to relatively small amounts of GH released during 5 h post injection. In the goldfish, however, 10 nM of goldfish ghrelin-19, but not ghrelin-12 (a different ghrelin isoform), elevated GH mRNA levels significantly after 2 h in a static incubation of primary pituitary cells (Unniappan et al. 2004b). Additionally, intraperitoneal injection of ghrelin in the channel catfish produced a significant elevation in pituitary GH mRNA after 3 h (Kaiya et al. 2005). In contrast, GH mRNA levels in primary pituitary cells isolated from black sea bream were unchanged by incubation with various GHSs, including human ghrelin during 45 min to 48 h of exposure, suggesting that the GHS-stimulated GH secretion in sea bream is independent of any change in GH gene transcription (Chan et al. 2004). Taken together, these results suggest that the action of ghrelin on GH synthesis and release may vary with species among vertebrates.

In mammals, the physiological actions of GH are mediated through its binding to growth hormone receptor (GH-R) located on the surface of cells in target tissue (Argetsinger et al. 1996). Recently, the GH-R has been cloned from the liver of several teleost species (Saera-Vila et al. 2005; Small et al. 2006). In all species examined to date, GH-R mRNA is expressed in almost all the tissues examined with the highest expression in the liver. We have recently identified two GH-R subtypes (GH-R1 and -R2) in the Mozambique tilapia, one of which (GH-R1) we believe to be the putative receptor for somatolactin (SL-R) and the other (GH-R2), the growth hormone-receptor (GH-R) (Pierce et al. 2007). In the present study, both forms of tilapia ghrelin significantly elevated hepatic expression of both SL-R and GH-R mRNA. The increased expression of these GH-Rs may be due to increased SL and GH in the circulation. To our knowledge, this is the first report of peripherally administered ghrelin on SL-R or GH-R expression. Furthermore, it is interesting to note that a peripheral injection of human GHRH in the current study increased hepatic expression of SL-R but not GH-R mRNA (Fig. 8). This differential regulation may be due to sampling time, and further injection studies examining the time-course of relative changes between these two GH-Rs in other tissues like muscle are highly warranted to confirm these findings. Also, homologous tilapia ghrelins may stimulate SL and GH release differentially, and thus plasma levels of both hormones should also be examined in parallel with changes in tissue receptor expression in future studies.

Growth hormone is considered to be an anabolic hormone, increasing protein synthesis and also inducing lipolysis and fatty acid oxidation (Mauras et al. 2005). Many

of the actions of GH are mediated by IGF-I, produced primarily in the liver (Le Roith et al. 2001; Wood et al. 2005). In the present study, both forms of ghrelin as well as human GHRH produced a significant elevation in both plasma IGF-I levels and hepatic expression of IGF-I 10 h after injection; no effect was seen after 5 h. The increased IGF-I levels after ghrelin injection would appear to be due to an increased release in circulating GH, although a direct effect of ghrelin on hepatic IGF-I synthesis and release may also be possible. Continuous jugular vein infusion of ghrelin in weanling pigs had no effect on plasma IGF-I levels for 8 days, although plasma GH was elevated significantly at 15, 30, and 60 min (Salfen et al. 2004). Similarly, ghrelin injection elevated plasma GH, but had no effect on plasma IGF-I after 15 min in humans (Akamizu et al. 2004; Zizzari et al. 2005). In a previous study using osmotic mini-pump for a continuous administration of ghrelin-C10, we observed a significant reduction in circulating levels of IGF-I after 21 days of treatment (Riley et al. 2005). Taken together, these findings suggest that there may be a difference in responsiveness of the animal to chronic verses short-term exposure to ghrelin. Recently, GHSR isoforms have been identified in the liver of the human, rat, chicken, and tilapia (Gnanapavan et al. 2002; Geelissen et al. 2003; Kageyama et al. 2005, this study). The presence of the GHSR in hepatocytes suggests a possible role for ghrelin in the regulation of circulating IGF-I. This points to the importance of investigating the possible effects of ghrelin on the expression of GHSR in the tilapia liver, in conjunction with additional possible effects on IGF-I production and on GHR expression.

We have reported earlier that the administration of ghrelin-C10 with osmotic mini-pump produced decreases in plasma IGF-I and increases total fat content in tilapia, suggesting that chronic exposure to ghrelin may generate a positive energy balance in fish. In the present study, the injection of tilapia ghrelin-C8 or -C10 had no effect on plasma glucose levels. Ghrelin has been shown to have variable effects on glucose release in mammals (Ukkola et al., 2003). The infusion of acylated human ghrelin had no effect on plasma glucose in weanling pigs (Salfen et al. 2004). In a separate study, acylated human ghrelin stimulated, while non-acylated ghrelin inhibited glucose release from porcine primary hepatocytes after 20 min (Gauna et al. 2005). In humans, acylated ghrelin increases plasma glucose (Arosio et al. 2003; Akamizu et al. 2004), whereas non-acylated ghrelin had no effect (Broglio et al. 2004). Non-acylated ghrelin, which is unable to bind to GHSR-1a, circulates at concentrations of more than twice that of acylated ghrelin (Muccioli et al. 2002). Thus, different isoforms of ghrelin appear to have disparate effects on glucose metabolism in humans. Further studies are warranted to clarify the effects of ghrelins on lipid and glucose metabolism in tilapia.

In summary, tilapia ghrelin-C8 and -C10 stimulated the release of GH both *in vitro* and *in vivo*. These stimulatory effects of both forms of ghrelin were blocked by the GHSR antagonist, [D-Lys3]-GHRP-6. There was no effect on PRL release. Intraperitoneal administration of both forms of ghrelins increased hepatic expression of IGF-I and GHR 5 h after injection. Both forms of the GHSR were found in the pituitary, indicating clearly that endogenous ghrelins stimulate GH release through the GHSR. Stimulation of hepatic expression of IGF-I, SL-R, and GH-R suggests metabolic roles of

ghrelin. Further studies into the regulation of the dynamic expression of GHSR may explain some of the metabolic effects described here and further elucidate the role of ghrelin in the GH/IGF-I axis.

## CHAPTER IV

## Effects of short- and long-term fasting on plasma and stomach ghrelin, and the growth hormone/insulin-like growth factor I axis in the tilapia, *Oreochromis mossambicus*


## ABSTRACT

Ghrelin is a highly conserved peptide hormone secreted by the stomach, which is involved in regulation of food intake and energy expenditure. Ghrelin stimulates growth hormone (GH) release and increases appetite in a variety of mammalian and non-mammalian vertebrates, including several fish species. Studies were conducted to investigate the effect of feeding and fasting on stomach ghrelin mRNA expression, the content of ghrelin protein, as well as plasma levels of ghrelin, GH, insulin-like growth factor-I (IGF-I), and glucose in the tilapia. There were no postprandial changes in ghrelin levels. Postprandial plasma GH, IGF-I and glucose all increased in line with the anabolic roles for these factors. Fasting for 4 and 8 days likewise did not affect ghrelin levels. Plasma GH was elevated after 4 and 8 days of fasting, while plasma IGF-I levels were reduced. Plasma ghrelin levels were elevated significantly after 2 and 4 weeks of fasting, but no change was detected in stomach ghrelin mRNA levels. Similarly, 4 weeks of fasting did not affect plasma GH levels, although plasma IGF-I and glucose were reduced significantly during this period, indicating that GH resistance exists during a prolonged nutrient deficit (catabolic state). These results indicate that ghrelin is not acting as a

meal-initiating signal in daily-fed tilapia, although it may be acting as a long-term indicator of negative energy balance.

## INTRODUCTION

The most significant endocrine influence in the control of nutrient intake, assimilation, and somatic growth in fish is the complex interaction of the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis (Duan 1997; Mauras et al. 2005; Wood et al. 2005). Nutritional status is intimately linked to a variety of peripheral and central signals, which maintain energetic homeostasis (Thissen et al. 1994; MacKenzie et al. 1998; Jensen 2001; Volkoff et al. 2006). During the catabolic state associated with fasting, energy is diverted from growth to support vital processes. Elevated plasma GH shifts metabolism towards mobilizing energy substrates (lipolysis) to maintain basal metabolism, while decreased plasma IGF-I ensures less energy is spent on muscle cell growth and proliferation. In several fish species prolonged fasting causes a reduction in plasma IGF-I levels and liver expression of IGF-I mRNA, while plasma GH levels rise or remain unchanged (Moriyama et al. 2000; Uchida et al. 2003; Pierce et al. 2005; Small 2005b; Fox et al. 2006; Pierce et al. 2007). This apparent paradox of GH resistance has also been observed in mammals (Thissen et al. 1999).

Regulation of GH release in fish is multifactorial, and is under the influence of several stimulatory as well as inhibitory factors from both central and peripheral origins (Canosa et al. 2007). Ghrelin is a peptide hormone originally isolated from the stomach of rats, and identified as the endogenous ligand for the previously orphaned GH

secretagogue receptor (GHS-R) (Kojima et al. 1999). Over time, it has become increasingly clear that ghrelin plays an integral role not only in the neuroendocrine control of GH release, but also in the regulation of feeding, energy metabolism, cardiovascular performance, and immune responses in a variety of animals (Kojima et al. 2005; Kaiya et al. 2008). Thus, ghrelin represents a key link for nutritional status in the stomach and the neuroendocrine response in the brain (Cummings et al. 2005; Kaiya et al. 2008).

Both the structure and functions of ghrelin are highly conserved among both mammalian and non-mammalian vertebrates (Kaiya et al. 2008). We have previously identified two forms of tilapia ghrelin (ghrelin-C8 and -C10), both of which stimulate *in vivo* and *in vitro* GH release (Kaiya et al. 2003c; Fox et al. 2007). Ghrelin is the only known gut-derived orexigenic hormone, and its actions are mediated centrally by neuropeptide Y (NPY), as well as peripherally by stimulation of vagus nerve in mammals (Nakazato et al. 2001; Date et al. 2002; Chen et al. 2004) and the goldfish (Unniappan et al. 2002; Miura et al. 2006). Ghrelin levels have been shown to rise preprandially and fall directly after a meal in mammals (Bagnasco et al. 2002; Kim et al. 2003; Tups et al. 2004), Japanese quail (Shousha et al. 2005), and goldfish (Unniappan et al. 2004a), but no effect of feeding was observed in the rainbow trout (Jönsson et al. 2007). Plasma ghrelin or stomach expression of ghrelin mRNA has also been shown to increase in response to fasting in mammals (Kim et al. 2003; Tups et al. 2004), Japanese quail (Shousha et al. 2005), bullfrog (Kaiya et al. 2006), and goldfish and sea bass (Unniappan et al. 2004a; Terova et al. 2008). Collectively, these studies have led the authors to

suggest a major role for ghrelin in the initiation of feeding, and perhaps in the regulation of energy homeostasis during a state of negative energy balance in these species. Interestingly, a significant reduction in plasma ghrelin levels has been observed in fasted burbot (Nieminen et al. 2003) and rainbow trout (Jönsson et al. 2007). Furthermore, no effect of fasting on ghrelin levels has been observed in either the Nile or Mozambique tilapia (Parhar et al. 2003; Riley et al. 2008).

The aim of the present study was to investigate the effects of feeding and fasting on ghrelin and the GH/IGF-I axis, to clarify whether nutritional status influences these important metabolic endocrine factors in the tilapia (*Oreochromis mossambicus*). Three experiments were conducted. Stomach ghrelin mRNA and protein levels as well as circulating ghrelin, GH, IGF-I and glucose were measured to assess: 1) pre- and postprandial levels within 24 h; 2) the effects of an intermediate fast (4 and 8 days); and 3) the effect of prolonged nutrient restriction (4 weeks) on plasma and stomach ghrelin and the GH/IGF-I axis.

## MATERIALS AND METHODS

### *Animals*

Male tilapia (*Oreochromis mossambicus*) were reared outdoors in 700-L, freshwater, flow-through tanks under natural photoperiod at the Hawaii Institute of Marine Biology. All Experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

*Gastric emptying experiment*

Sixty-four fish, weighing 60-80 g, were separated and distributed randomly into 8 tanks (60 L, 8 fish per tank). Water temperature was maintained between 27-28 °C, using submersible aquarium heaters (Ebo-Jager Inc., El Segundo, CA). Adult male tilapia appear to be"social" feeders under the experimental conditions, requiring a minimum of 6 fish per tank for optimal and consistent food consumption (unpublished observations). The fish were allowed to acclimate to the experimental tanks for 4 weeks, during which time they were fed floating trout pellets (Silver Cup, Nelson & Sons Inc., Murray, UT) *ad libitum* for 0.5 h (from 0730-0800 h) once daily. Following each feeding interval, uneaten pellets were removed from each of the tanks with a hand net. All fish exhibited positive growth throughout the acclimation period. On the day of the experiment, all tanks were fed at the usual time. Then, one tank was sampled at each of the following time points: 0, 2, 4, 6, 8, 10, 12, and 14 h following the feeding interval (where 0 h represents sampling immediately after feeding).

During sampling, all eight fish per tank were killed in excess 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO) and immediately placed on ice. The fish were then rapidly dissected, and their whole stomachs including the pyloric sphincter were removed for analysis of stomach content. The entire stomach content was dried overnight in an oven at 100 °C, and then weighed the next day. Because the fish were held in groups (8 fish per tank), we were unable to observe the exact number of pellets consumed per individual. Nevertheless, we have shown previously that the number of pellets eaten is

directly proportional to body weight ($R=0.901$, $P<0.05$, unpublished observations). The amount of dried stomach content remaining from each fish was normalized to the body weight of that individual (g dry content/100 g body weight).

*Short-term fasting (pre- and post-prandial changes)*

Sixty-four fish, weighing 80-100 g, were randomly distributed into 8 tanks (100 L, 8 fish per tank). Water temperature was maintained between 27-28 °C using submersible aquarium heaters. The fish were allowed to acclimate to the experimental tanks for 8 weeks, during which time they were fed floating trout pellets to apparent satiation at 0800 h to entrain a daily feeding behavior. All fish exhibited positive growth throughout the acclimation period. To minimize the effect of sampling stress on entrained feeding behavior and digestion, fish were removed only once per tank per day (two replicate tanks were sampled per day per treatment) over the course of three days. On the first day of the experiment, 8 fish ($n=4$ from two replicate tanks) were samples pre-prandially at 0730 h, or 0.5 h before the scheduled feeding time. A further 2 tanks were fed at the usual time of 0800 h, and the fish were sampled post-prandially ($n=8$ total or 4 fish per tank) 10 h following the scheduled feeding time. On the second day of the experiment, 8 more fish were sampled pre-prandially (at 0730 h), and post-prandial samples were taken from replicate tanks fed at the usual time of 0800 h; one at 1000 h and one at 0730 h the following day (or 2 and 24 h post-prandial). On the third day of the experiment, 8 fish were sampled pre-prandially (0730 h), and 8 fish were sampled 0.5 h post-prandially (0830 h).

At the time of sampling, all fish were netted, anesthetized in water containing 2-phenoxyethanol (0.2 mL/L). Blood was collected from the caudal vasculature by a needle and syringe treated with ammonium heparin (200 U/mL, Sigma-Aldrich). Plasma was separated by centrifugation at 10,000 x g for 10 min at 4°C, and stored at -20°C until analyses for ghrelin, GH, and IGF-I by radioimmunoassay as well as for plasma glucose. Fish were rapidly decapitated and whole stomachs were collected, snap frozen and stored at -80°C until analysis for protein content and mRNA expression of ghrelin.

*Intermediate fasting*

Thirty two fish, weighing 80-100 g, were distributed randomly into 8 tanks (50 L, 4 fish per tank). Fish were acclimatized as described in the previous section. At the beginning of the experiment, food was withheld from 8 fish for 4 days; while a further 8 fish were deprived of food for 8 days. The remaining fish were fed once daily at 0800 h throughout the experiment; 8 fish were sampled after 4 days and the remaining 8 fish were sampled after 8 days. All sampling occurred pre-prandially at 0730 h or 24 h after the last meal. Fish were sampled for blood and tissue as described above.

*Long-term fasting*

One hundred and twenty fish, weighing 30-45 g, were tagged individually with passive integrated transponder tags (PIT tags; Destron IDI, Boulder, CO), and divided randomly into 4 tanks representing two treatment groups (2 fed and 2 fasted). The animals were then maintained in outdoors in oval 700 L fiberglass aquaria (30 fish per tank). Water temperature was maintained between 27-28 °C using submersible aquarium heaters. The fish were allowed to acclimate to the tanks for 2 weeks prior to the

beginning of the experiment. Following the acclimation period, food was withheld from 2 tanks for a period of 4 weeks, while the remaining two tanks were fed continuously throughout the experiment by automatic belt feeder. Twelve fish, 6 fed and 6 fasted (3 fish from each of the 4 tanks), were killed in excess anesthesia and sampled at 4 time points; 0, 1, 2, and 4 weeks. To eliminate the effect of crowding stress on growth rate, stocking densities were maintained at approximately 5 g/L by adjusting the height of the vertical standpipe as fish were removed for sampling. Fish were sampled for blood and tissue as described above.

*Plasma measurements*

Plasma GH levels were measured by homologous radioimmunoassay (RIA) according to Yada et al. (1994). Total plasma IGF-I levels were measured from a 25 µl of plasma that was extracted with 100 µl of acid-ethanol (87.5% ethanol and 12.5% 2 N HCl, v/v) as described by Shimizu *et al.* (1999). Total IGF-I levels were measured using recombinant salmon IGF-I as a standard and anti-barramundi IGF-I (GroPep, Adelaide, Australia) following Shimizu et al. (1999) and Kajimura et al. (2002). Plasma ghrelin levels were measured following the protocol described by Riley et al. (2008). Briefly, anti-rat ghrelin [1-11] was incubated with standards (tilapia ghrelin-C8) and 200 µl of plasma or 100 µl extracted stomach samples for 24 h at 4°C. One hundred µl of $^{125}$I-human ghrelin (Millipore, St. Charles, MO) was added and incubated for an additional 24 h at 4°C. One hundred µl of Pansorbin cells (CalBiochem, La Jolla, CA, 1:35 in RIA buffer) were added and the tubes were incubated overnight at 4°C. Free and bound tracers were separated by centrifugation at 3000 x g for 1 h at 4°C. The supernatant was

aspirated and the samples were counted using the Cobra II gamma counter (Packard, Meriden, CT). Plasma glucose concentrations were estimated using a commercially available kit (Glucose Assay Kit, GAHK-20, Sigma), which was modified for a microplate reader (SpectraCount, Packard, Meriden, CT).

*Preparation of stomach tissue*

Ghrelin protein was extracted from stomach tissue in a manner similar to that described by Hosoda et al. (2000). Approximately 50 mg of stomach tissue was removed, diced into < 1 mm pieces, boiled for 7 min in a 5-fold volume of water, and then immediately immersed in an ice bath. Glacial acetic acid was then added to bring the resulting solution to a final concentration of 1 M. The mixture was then homogenized using a Polytron mixer (Brinkmann Instruments, Westbury, NY). The mixture was centrifuged at 10,000 rpm for 0.5 h and the supernatant collected and lyophilized. The resulting lyophilized extract was then reconstituted in 500 µl ghrelin RIA buffer. Recovery of ghrelin peptides from stomach tissues was greater than 80% using this protocol. The displacement curves of serial dilutions of tilapia stomach extracts were parallel with ghrelin-C8 standards (data not shown).

*RNA extraction, cDNA Synthesis, and qPCR*

Stomach ghrelin mRNA levels were determined by quantitative PCR (qPCR). Total RNA was extracted from stomach tissues using Tri-Reagent (MRC, Cincinnati, OH) and then reversed transcribed in a manner similar to that described by Pierce et al. (2007). The middle or "fundic" portion of the stomach was dissected based on the gross anatomy of the Nile tilapia described by Caceci et al. (1997). Sakata et al. (2004) showed

a higher density of ghrelin-producing cells in both the fundic and pyloric regions of rainbow trout stomachs, than in the cardiac region. Briefly, first strand cDNA was synthesized with an iScript kit (BioRad Laboratories, Hercules, CA). For ghrelin mRNA, a specific primer pair was used (Table 1). Serial dilutions of the plasmid DNAs ($10^2$ – $10^7$ copies) containing the amplified fragments of target mRNAs were prepared as standards. All qPCR assays were performed in a manner that were similar to those described by Pierce et al. (2007). All qPCR assays were run using iQ Sybergreen Supermix (BioRad), in a 15 μl reaction volume, using primers at 200nM concentration. Assays were run on an iCycler iQ Real-Time PCR detection system (BioRad), using the manufacturer's recommended cycling conditions (2 min at 95 °C, 2 min at 50 °C followed by 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min). All qPCR data were normalized to ARP (acidic ribosomal phosphoprotein; reference gene) mRNA transcript levels after it was determined ARP levels did not change with treatment.

*Statistics*

Group comparisons were performed using two-way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significant level was set at $P<0.05$. Calculations were performed using computer programs, Statistica (StatSoft, Tulsa, OK). Data are expressed as means ± S.E.M.

## RESULTS

Based on analysis of dry stomach content collected immediately after the feeding, fish consumed an average of approximately 2.5% of their body weight per day (data not

shown). Under the experimental conditions described above, gastric emptying proceeded in a linear manner with relation to time. Approximately 12-14 h following a single meal, no food remained in the stomachs examined (Fig. 11).

In the first experiment, there was a significant ($P<0.05$) main effect of sampling time after feeding on plasma ghrelin by two-way ANOVA, but no main effect of treatment was observed (Fig. 12A). There was no effect of feeding (within 24 h) on either stomach ghrelin content or mRNA levels (Figs. 12B and C).

Feeding resulted in significant main effects on plasma GH levels in relation to time ($P<0.01$) and treatment ($P<0.001$). Postprandial plasma GH levels were significantly higher than pre-prandial levels 0.5 h after the feeding; nevertheless, postprandial levels were not otherwise different from pre-prandial controls (Fig. 13A). Feeding also resulted in significant main effects over both time and treatment ($P<0.01$) on plasma IGF-I. Postprandial levels of plasma IGF-I were significantly higher than pre-prandial controls at 2 h after feeding (Fig. 13B). Postprandial plasma IGF-I levels were not otherwise different from preprandial controls. Similarly, feeding resulted in significant main effects on plasma glucose levels over time ($P<0.04$) and treatment ($P<0.001$). Plasma glucose levels were reduced significantly at 0.5 h postprandially (Fig. 13C). Levels then increased postprandially at 2, 10, and 24 h, compared with preprandial controls (Fig. 13C).

Fasting for either 4 or 8 days had no effect on plasma ghrelin, stomach ghrelin content, or on stomach ghrelin mRNA levels (Figs. 14A, B, and C). Plasma GH levels were elevated significantly in fasted animals compared with continuously-fed controls

after both 4 and 8 days (Fig. 15A). Plasma IGF-I was reduced significantly after both 4 and 8 days of fasting (Fig. 15B). Plasma glucose levels in the fish fasted for 4 days were significantly lower than those in fed fish, whereas no difference was observed on day 8.

In the long-term experiment, plasma ghrelin was elevated significantly compared with fed control after 2 and 4 weeks of fasting (Fig. 16A). Expression of stomach ghrelin mRNA levels remained unchanged throughout 4 weeks of fasting (Fig. 16B). Ghrelin contents in the stomach were not examined in this experiment. Similarly, plasma GH levels were not affected by 4 weeks of fasting (Fig. 17A). Long-term fasting resulted in significant time and treatment effects ($P<0.001$) on plasma IGF-I. Plasma IGF-I was reduced significantly after 1, 2, and 4 weeks of fasting compared with continuously-fed controls (Fig. 17B). There was also a significant ($P<0.001$) main effect of fasting on plasma glucose, which was reduced after 1, 2, and 4 weeks (Fig. 17C).

Fig 11. Gastric emptying in the tilapia. Remaining feed was removed from the stomach, and dried stomach content was normalized to body weight. Fish were sacrificed immediately (0 h) and 2, 4, 6, 8, 10, 12, and 14 h after feeding. Stomachs were essentially empty 14 h following feeding.

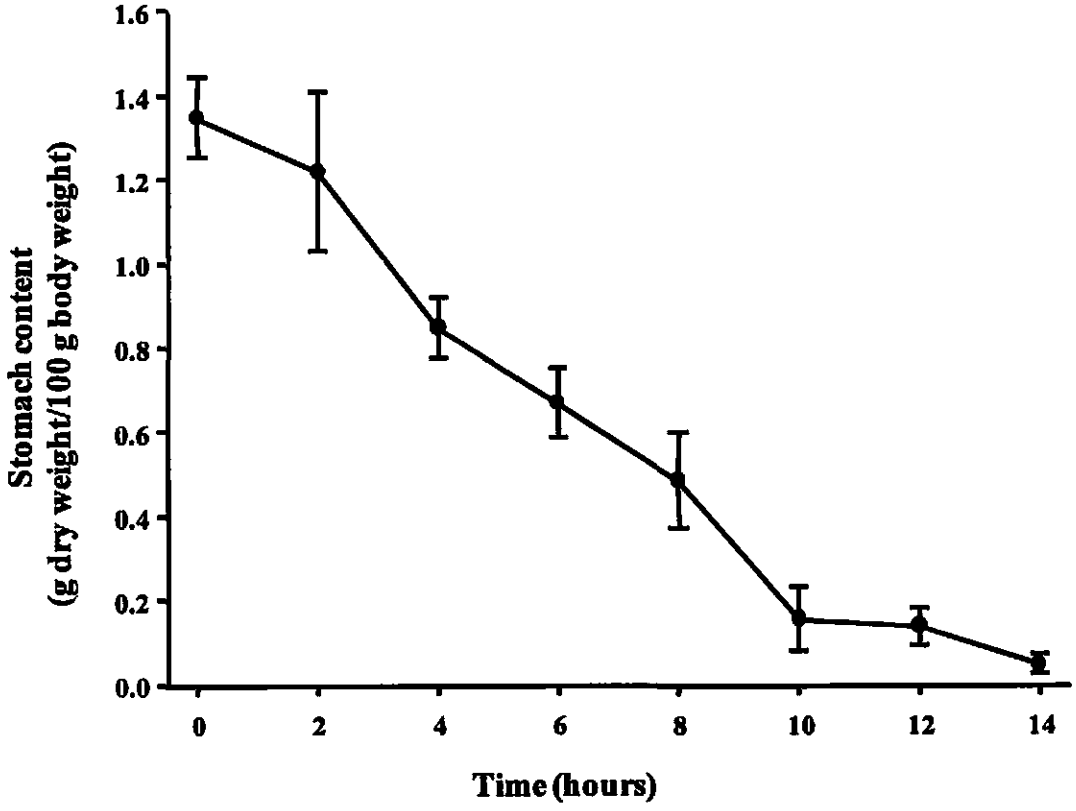

Stomach content
(g dry weight/100 g body weight)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
Time (hours)

Fig 12. Periprandial changes in plasma ghrelin (A), stomach ghrelin content (B), and stomach ghrelin mRNA levels (C). Fish were sacrificed preprandially at 0730 h, then postprandially at 0.5, 2, 10 and 24 h. There were significant ($P<0.05$) main effects of sampling time on plasma levels of ghrelin by two-way ANOVA.

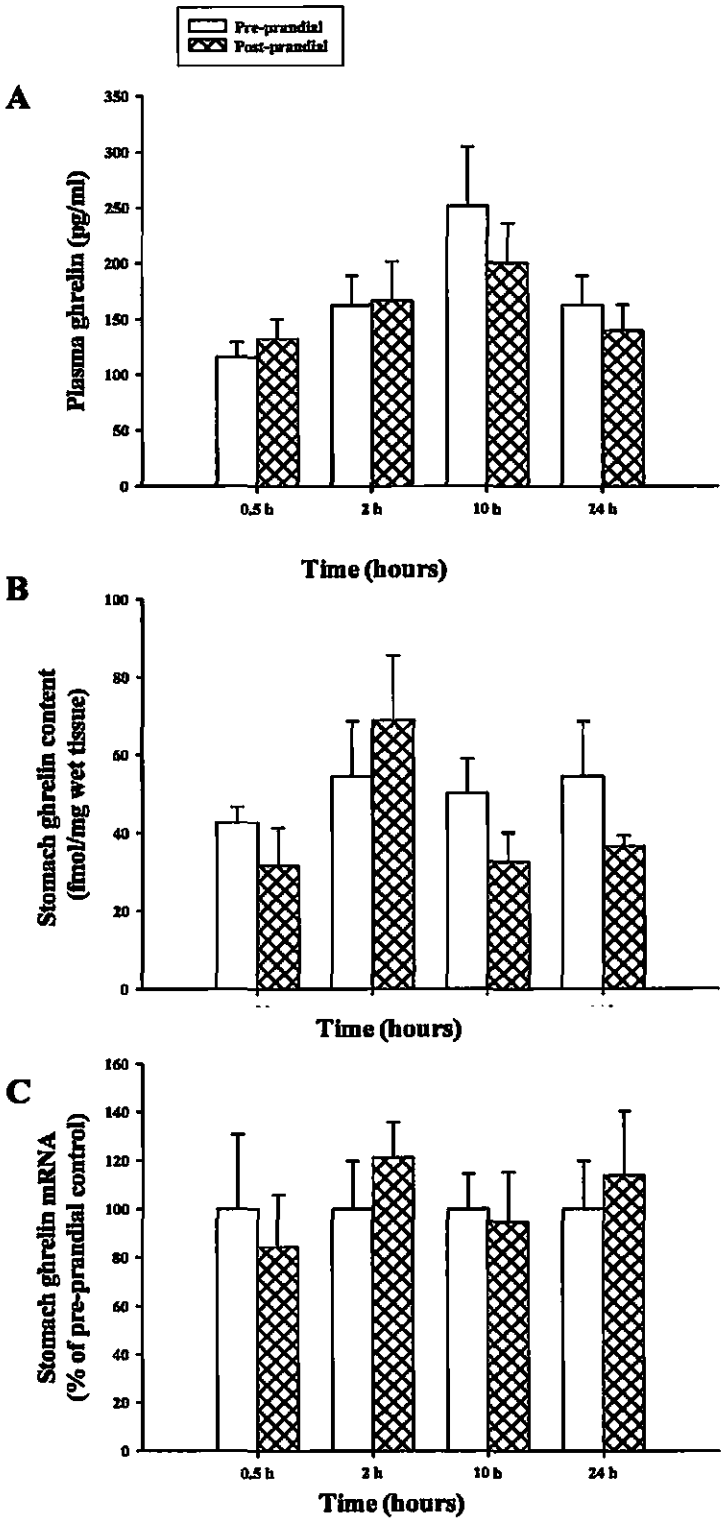

Pre-prandial
Post-prandial
A
Plasma ghrelin (pg/ml)
350
300
250
200
150
100
50
0
0.5 h
2 h
10 h
24 h
Time (hours)
B
Stomach ghrelin content (fmol/mg wet tissue)
100
80
60
40
20
0
Time (hours)
C
Stomach ghrelin mRNA (% of pre-prandial control)
160
140
120
100
80
60
40
20
0
0.5 h
2 h
10 h
24 h
Time (hours)

Fig 13. Periprandial changes in plasma GH (A), IGF-I (B), and glucose (C). Fish were sacrificed preprandially at 0730 h, then postprandially at 0.5, 2, 10 and 24 h. There were significant ($P<0.01$) main effects of treatment and sampling time on plasma levels of GH and IGF-I by two-way ANOVA. There were also significant ($P<0.001$) main effects of treatment and sampling time ($P<0.05$) on plasma levels of glucose by two-way ANOVA. *, *** Significantly different from the corresponding preprandial control at $P < 0.05$ and 0.001, respectively, by LSD.

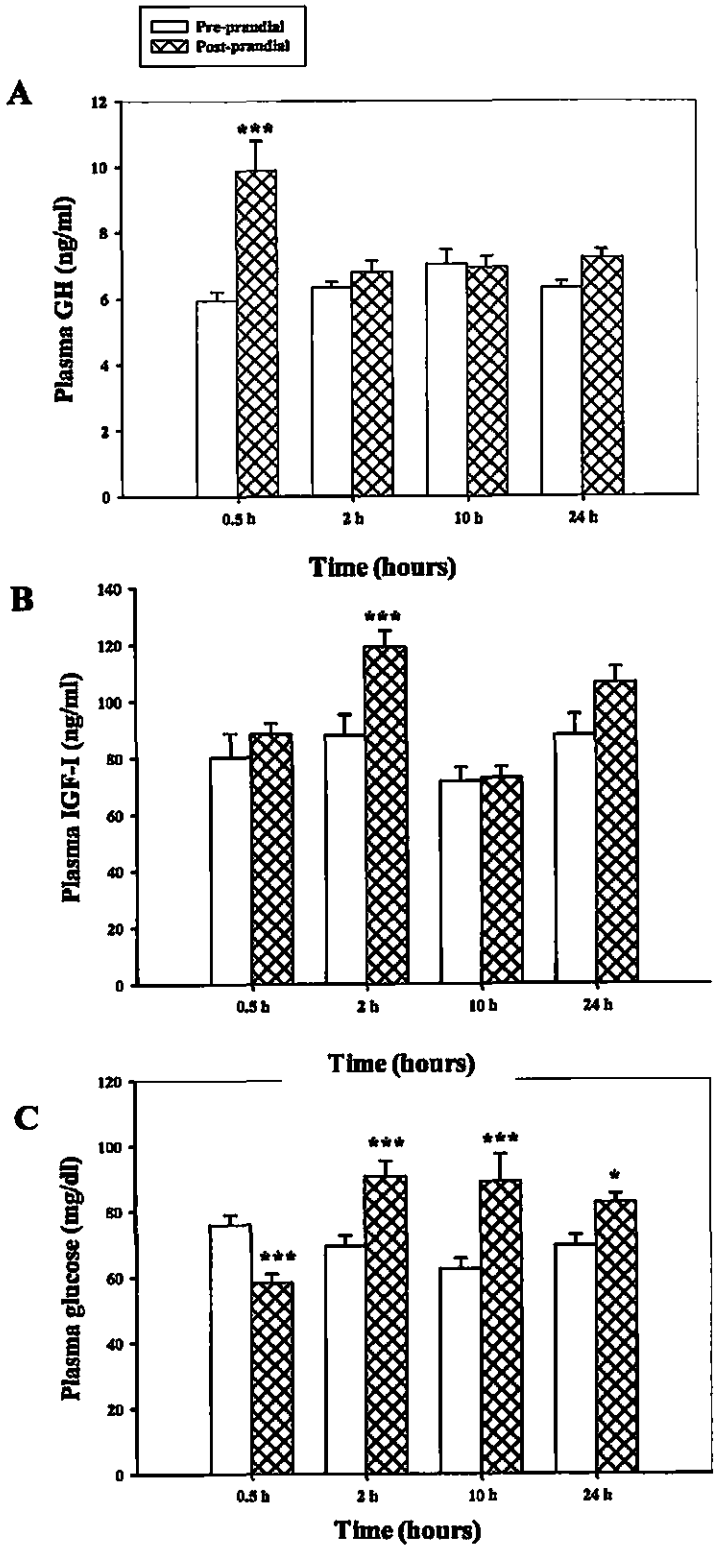

Pre-prandial
Post-prandial
A
Plasma GH (ng/ml)
0
2
4
6
8
10
12
***
0.5 h
2 h
10 h
24 h
Time (hours)
B
Plasma IGF-I (ng/ml)
0
20
40
60
80
100
120
140
***
0.5 h
2 h
10 h
24 h
Time (hours)
C
Plasma glucose (mg/dl)
0
20
40
60
80
100
120
***
***
***
*
0.5 h
2 h
10 h
24 h
Time (hours)

Fig 14. Effects of intermediate fasting on plasma ghrelin (A), stomach ghrelin content (B), and stomach ghrelin mRNA levels (C). Fish were sacrificed following 4 and 8 days of fasting. No significant effects were observed.

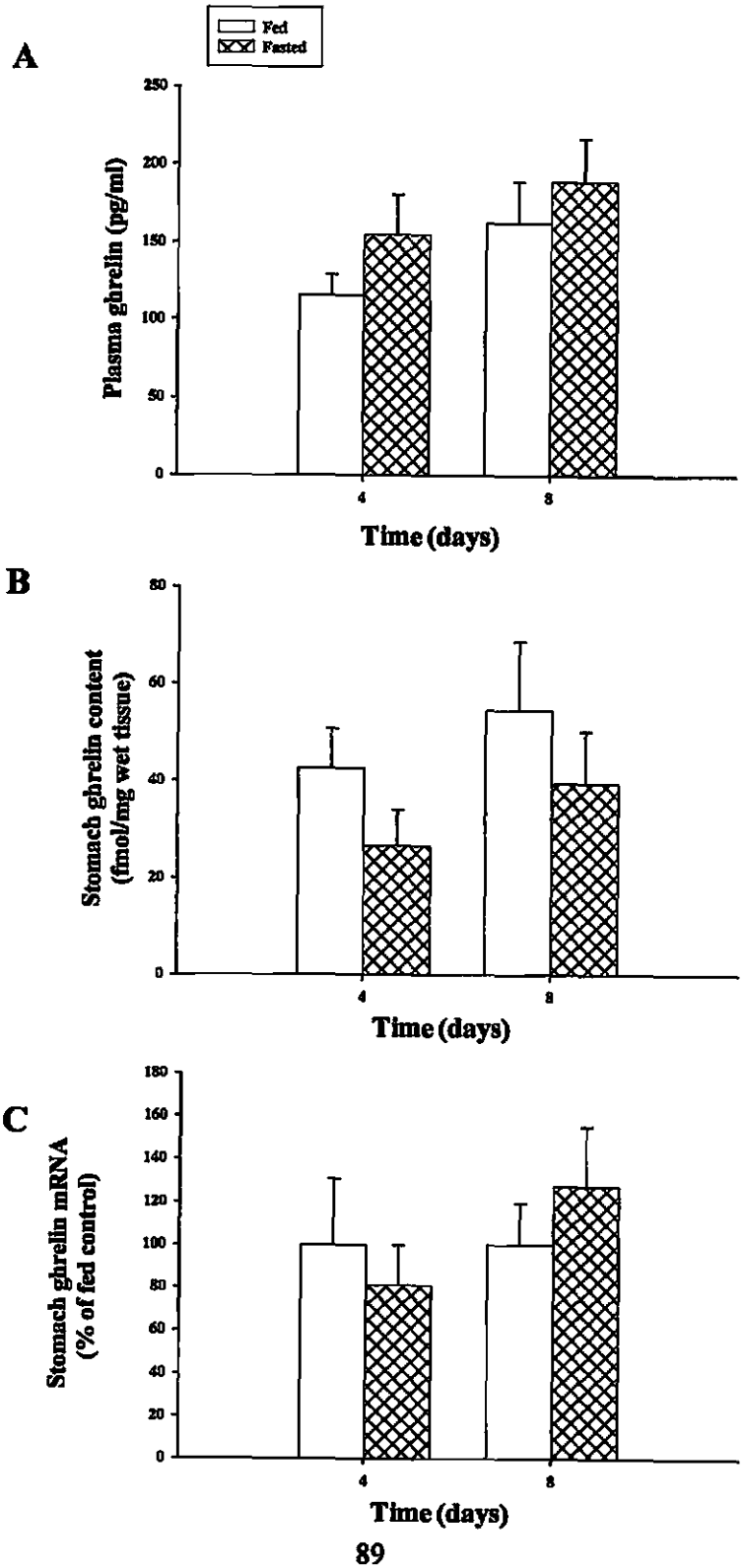

A
Fed
Fasted
Plasma ghrelin (pg/ml)
250
200
150
100
50
0
4
8
Time (days)
B
Stomach ghrelin content
(fmol/mg wet tissue)
80
60
40
20
0
4
8
Time (days)
C
Stomach ghrelin mRNA
(% of fed control)
180
160
140
120
100
80
60
40
20
0
4
8
Time (days)

Fig 15. Effects of intermediate fasting on plasma GH (A), IGF-I (B), and glucose (C). Fish were sacrificed following 4 and 8 days of fasting. There were significant ($P<0.001$) main effects of treatment on plasma levels of GH and IGF-I by two-way ANOVA. Plasma glucose was reduced after 4 but not 8 days of fasting. **, *** Significantly different from the corresponding fed control at corresponding day at $P < 0.01$ or $P < 0.001$ by LSD.

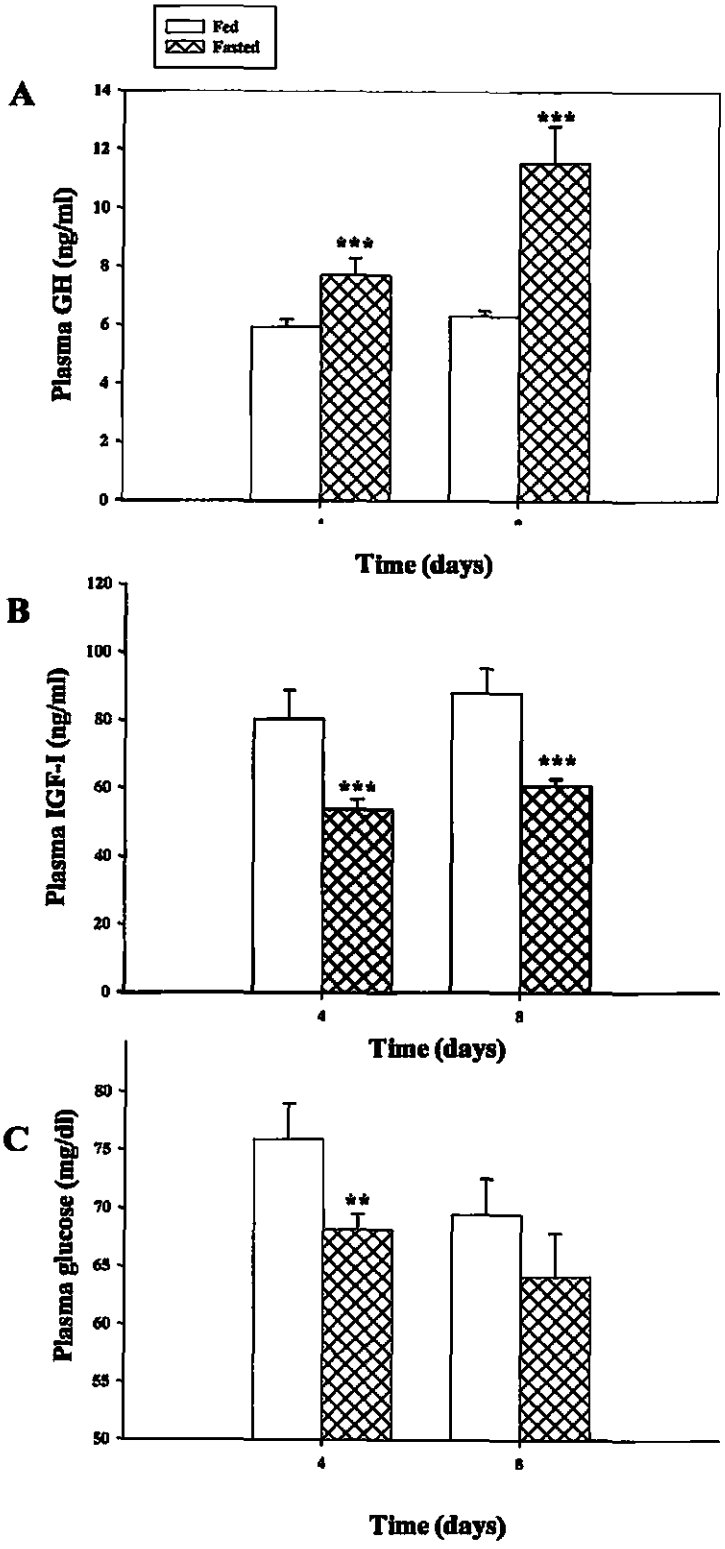
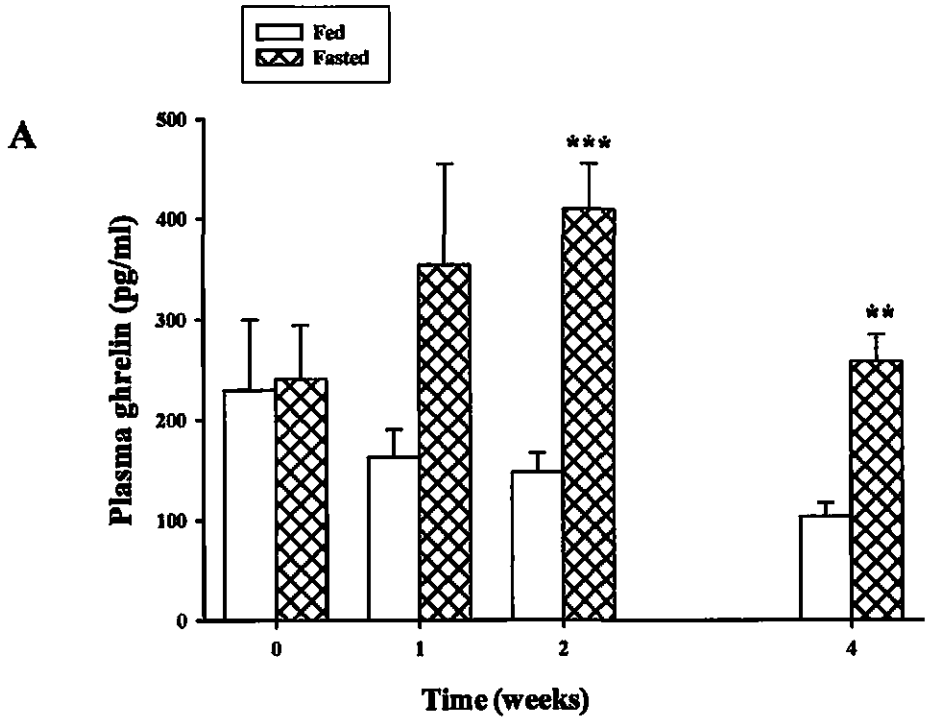

Fed
Fasted
A
Plasma GH (ng/ml)
Time (days)
B
Plasma IGF-I (ng/ml)
Time (days)
C
Plasma glucose (mg/dl)
Time (days)

Fig 16. Effects of long-term fasting on plasma ghrelin (A) and stomach ghrelin mRNA levels (B). Fish were sacrificed at the beginning of the experiment (week 0), and after 1, 2, and 4 weeks of fasting. There were significant ($P<0.001$) main effects of treatment on plasma levels of ghrelin. No effects were observed on stomach ghrelin mRNA levels. *** Significantly different from the corresponding fed control at corresponding day at $P < 0.001$ by LSD.


A
Fed
Fasted
Plasma ghrelin (pg/ml)
500
400
300
200
100
0
***
**
0
1
2
4
Time (weeks)

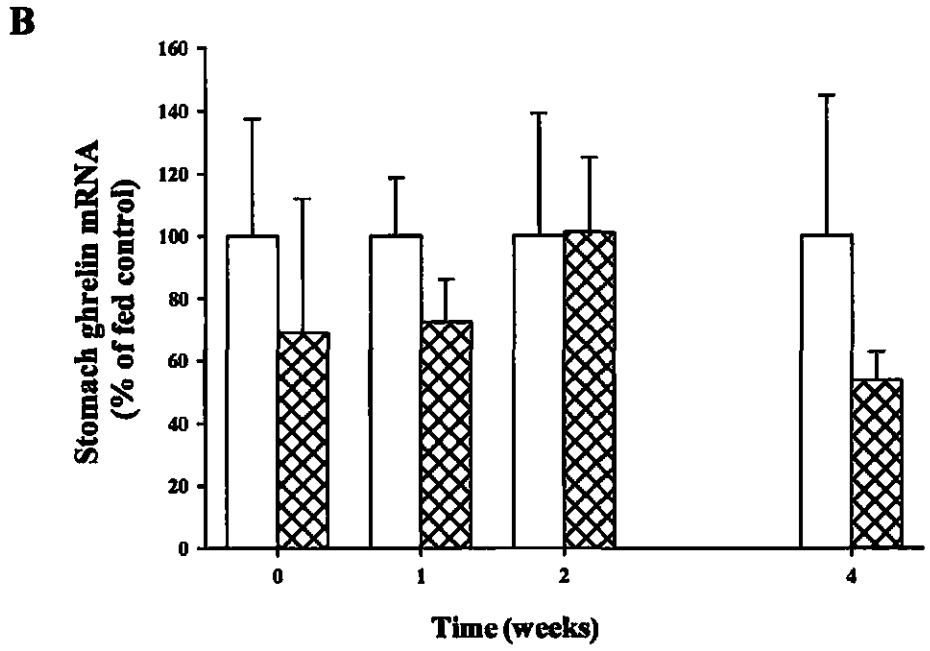
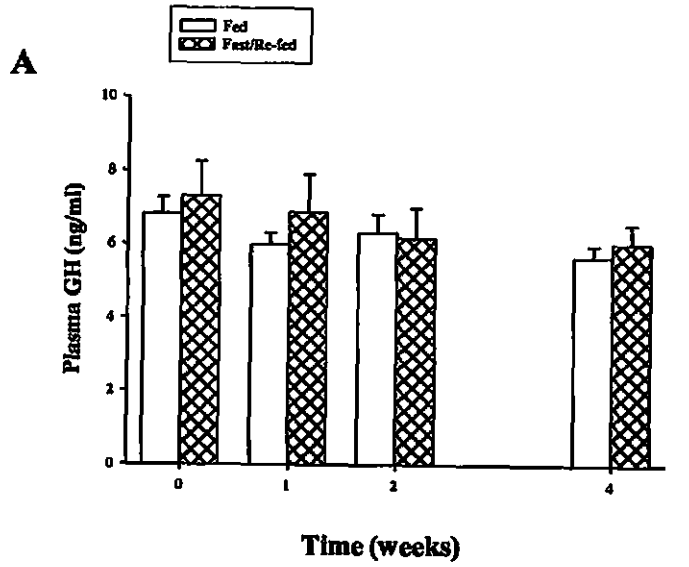
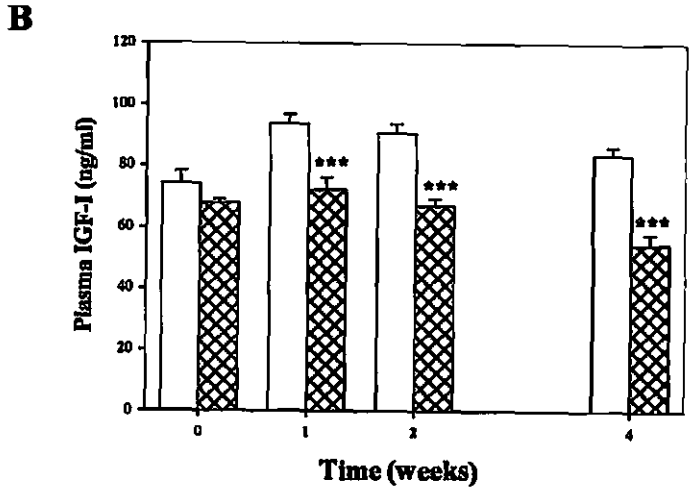
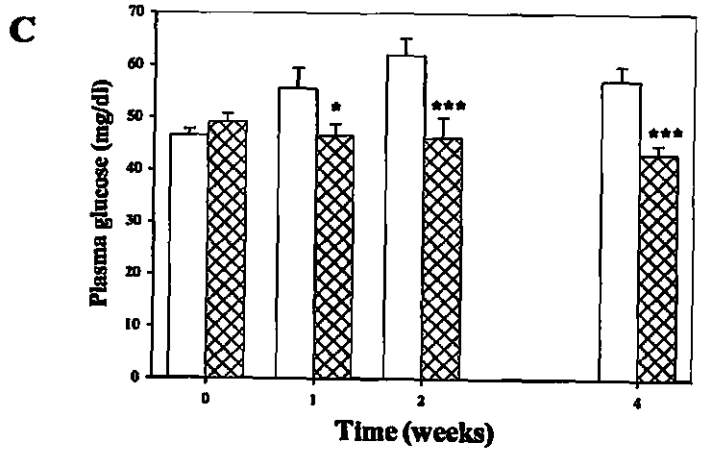

B
Stomach ghrelin mRNA
(% of fed control)
160
140
120
100
80
60
40
20
0
0
1
2
4
Time (weeks)

Fig 17. Effects of long-term fasting on plasma GH (A), IGF-I (B), and glucose (C). Fish were sacrificed at the beginning of the experiment (week 0), and after 1, 2, and 4 weeks of fasting. No effects of fasting on plasma GH were observed. There were also significant ($P<0.001$) main effects of treatment and sampling time on plasma levels of IGF-I by two-way ANOVA. There were also significant ($P<0.001$) main effects of treatment on plasma glucose by two-way ANOVA. *, *** Significantly different from the corresponding fed control at corresponding day at $P < 0.05$ and 0.001, respectively, by LSD.


A
Fed
Fast/Re-fed
Plasma GH (ng/ml)
0
2
4
6
8
10
0
1
2
4
Time (weeks)


B
Plasma IGF-I (ng/ml)
0
20
40
60
80
100
120
***
***
***
0
1
2
4
Time (weeks)


C
Plasma glucose (mg/dl)
0
10
20
30
40
50
60
70
*
***
***
0
1
2
4
Time (weeks)

## DISCUSSION

In the present study, we established a system, in which tilapia were entrained to a feeding schedule under tightly controlled conditions. This allowed us to characterize the effects of one daily feeding on the endocrine rhythm of several metabolically important factors. Food intake in fish is influenced by many factors including the concentration of dissolved oxygen, animal density, temperature, and photoperiod, while gastric emptying rate can also be influenced by temperature, feed composition, frequency of feeding, and fullness of stomach (Olsson et al. 2001; (Alanara et al. 2001; Volkoff et al. 2006). We have demonstrated that gastric emptying is essentially complete within 14 h after eating in the Mozambique tilapia. Previously, Heng et al. (2007) used radiography to determine that gastric emptying in hybrid tilapia (*O. niloticus* x *O. mossambicus*) occurred from 4-15 h following a forced meal containing barium sulfate. Differences between these results and those in the current study are likely due to differences in feed composition, temperature, and experimental procedure; the fish may have been stressed when handled multiple times postprandially during the radiographic measurements.

The highest expression of ghrelin mRNA has been shown to be in the stomach of both the Mozambique and Nile tilapias (Kaiya et al. 2003c; Parhar et al. 2003). The Mozambique tilapia produces two forms of ghrelin with Ser-3 modified by octanoic (ghrelin-C8) or decanoic (ghrelin-C10) acid (Kaiya et al. 2003c). Tilapia ghrelin stimulates both *in vitro* and *in vivo* GH release (Kaiya et al. 2003c; Fox et al. 2007). We have previously reported that chronic administration of the major circulating form tilapia

ghrelin (ghrelin-C10) for 21 days by mini-osmotic pump modestly increased food intake and weight gain in the tilapia, suggesting an orexigenic role for ghrelin (Riley et al. 2005).

In the current study, however, we observed no changes in postprandial plasma ghrelin, stomach ghrelin protein content, or stomach mRNA expression. These results suggest that feeding does not affect ghrelin levels in the tilapia, and that plasma ghrelin may not act as a signal of hunger or satiety. These results are in agreement with a recent study conducted by Jonsson et al. (2007) in rainbow trout, in which no postprandial changes in plasma ghrelin were observed. In mammals, however, a pre-prandial increase in circulating ghrelin is typically followed by rapid postprandial decrease in humans (Muller et al. 2002), rats (Bagnasco et al. 2002), and dogs (Yokoyama et al. 2005). Interestingly, the presence of nutrients, but not distension of the stomach, seems to be the key regulator of the reduction in post-meal plasma ghrelin in rats (Williams et al. 2003). Based on our gastric emptying experiment, we sampled fish postprandially with and without the presence of gastric nutrients. Thus, it appears that the ghrelin "nutrient sensing" mechanism may not be present in the tilapia. In the goldfish, however, Unniappan et al. (2004a) observed a clear postprandial decrease in serum ghrelin, together with a simultaneous decrease in intestinal ghrelin mRNA levels. The goldfish does not have a well-defined stomach and the majority of goldfish ghrelin expression occurs in the intestine (Unniappan et al. 2002). It is also to be noted that no postprandial change in plasma ghrelin was observed in the rainbow trout, which has a distinct stomach (Jönsson et al. 2007). Further studies are called for using other fish species with or

without stomach and also with various feeding behaviors and food preferences such as herbivorous, omnivorous and carnivorous.

In the intermediate fasting experiment, fasting tilapia for 4 and 8 days did not affect plasma ghrelin, stomach ghrelin content, or stomach ghrelin expression. These results are in agreement with our previous finding that fasting for 1, 3, 5, or 7 days did not affect plasma ghrelin levels in the tilapia (Riley et al. 2008). Also in agreement with these findings, there was no change in stomach ghrelin mRNA levels in the Nile tilapia (*O. niloticus*) fasted for 7 days (Parhar et al. 2003). In mammals, expression of stomach ghrelin mRNA increased within 48 h of fasting in the rat (Kim et al. 2003) and Siberian hamster (Tups et al. 2004). Similarly, stomach ghrelin mRNA increased in the Japanese quail after only 24 h of fasting (Shousha et al. 2005). Gut expression of ghrelin mRNA in the goldfish increased after 7 days, while plasma ghrelin and stomach ghrelin content increased in the bullfrog following 10 and 20 days of food restriction (Kaiya et al. 2006). Recently, Terova et al. (2008) observed a significant increase in stomach ghrelin mRNA levels after 35 days of fasting in the sea bass. In the long-term fasting experiment, we observed a significant increase in plasma ghrelin following 2 and 4 weeks of fasting in the tilapia in the current study, while no change was detected in stomach ghrelin mRNA levels. Kaiya et al. (2006) observed a similar increase in plasma ghrelin without a parallel increase in stomach ghrelin mRNA after 20 days of fasting in the bullfrog. In contrast with these results, plasma levels of ghrelin were suppressed after 1, 2, and 3 weeks of fasting in the rainbow trout (Jönsson et al. 2007), and after 2 weeks in the burbot (Nieminen et al. 2003). As suggested by Kaiya et al. (2006) and Terova et al.

(2008), the temporal differences in the effects of starvation on ghrelin (long-term vs. short-term) that were observed between homeotherms (mammals and birds) and poikilotherms (fish and amphibians) may be due to differences in the energy metabolism between the two classes of animals.

In the current study, postprandial plasma GH levels increased after 0.5 h, but levels were not otherwise different from preprandial values. Very little is known about the effects of feeding on plasma GH levels. Infusion of the amino acid L-arginine into healthy human subjects stimulated GH release within 40 min (Fisker et al. 1999). Postprandial increases in plasma GH were observed within 0.5 h in both weanling calves (Katoh et al. 2004) and weanling goats (Kobayashi et al. 2006). In goldfish, Canosa et al. (2005) observed a postprandial rise in plasma GH also within 0.5 h. The lack of correlation between the postprandial GH surge and plasma ghrelin levels in the current study suggests that circulating ghrelin may not be responsible for the elevated levels of plasma GH in the tilapia. After at meal GH may act to stimulate protein synthesis, digestive capacity, nutrient uptake, and/or initiate the production of hepatic IGF-I following the influx of ingested nutrients into the blood compartment (Mommsen 2001).

Following the rise in GH, we observed a significant rise in plasma IGF-I at 2 h after a meal. This rise in plasma IGF-I may be a response to the rise in plasma GH. In the current study, plasma glucose was reduced initially at 0.5 h postprandially, but then increased at 2, 10, and 24 h. Small (2005b) did not observe such a postprandial change in either plasma GH or IGF-I in channel catfish which were fed twice daily. Plasma glucose increased in common carp at 1 h after eating (*Cyprinus carpio*), but levels returned to

preprandial levels after 6 h (Huising et al. 2006). Plasma glucose also increased at 6 h postprandially in juvenile rainbow trout fed a high carbohydrate diet (Kirchner et al. 2008). We were unable to find any reports of postprandial hypoglycemia in fish. The initial decrease in postprandial plasma glucose levels may derive from a rapid elevation in metabolism and utilization of mobilized energy substrates related to specific dynamic action (Mommsen 1998; McCue 2006).

Fasting resulted in a significant increase in plasma GH after 4 and 8 days in the intermediate fasting experiment. Nevertheless, there was no effect of fasting on plasma GH during long-term fasting in the current study. The short- or intermediate-term increases in plasma GH are in agreement with our previous study, in which plasma levels were elevated after 3, 5, and 7 days of fasting in the tilapia (Riley et al. 2008). Pierce et al. (2005) observed an increase in plasma GH in Chinook salmon fasted for 4 days. Long-term fasting for a few to several weeks resulted in an elevation or no change of plasma GH levels in salmonids (Duan et al. 1993; Pierce et al. 2005; Jönsson et al. 2007), gilthead sea bream (Company et al. 1999), channel catfish (Small et al. 2005), and tilapia (Weber et al. 1999; Uchida et al. 2003; Fox et al. 2006; Pierce et al. 2007). In contrast with the observed increases in plasma GH, plasma IGF-I levels were reduced after 4 days of fasting in the intermediate fasting experiment, and remained below control levels throughout the length of the experiment (4 weeks). These short-term reductions in plasma IGF-I are in agreement with our previous study, in which plasma IGF-I was reduced after 1, 3, and 5 days of fasting (Riley et al. 2008). Similarly, reductions in plasma IGF-I due to fasting have also been observed in salmonids (Moriyama et al. 1994;

Fukada et al. 2004; Pierce et al. 2005), channel catfish (Small et al. 2005), and tilapia (Uchida et al. 2003; Fox et al. 2006; Pierce et al. 2007). We have recently observed reduced plasma IGF-I and liver expression of IGF-I mRNA concurrently with unchanged plasma GH levels after 4 weeks of fasting in the tilapia (Pierce et al. 2007). In this study, GH-receptor mRNA expression remained unchanged in liver, indicating an apparent state of GH resistance. A reduction in GH-signaling in the liver resulting in a decrease in plasma IGF-I has also been observed in mammals (Jenkins et al. 1996; Thissen et al. 1999), and may be occurring also in the tilapia. This disconnection of GH from IGF-I likely represents a conserved mechanism in animals that suppresses body growth and muscle proliferation during fasting, in favor of mobilizing energy stores for the maintenance of homeostasis.

In the present study, there was a significant reduction in plasma glucose following 4 days of fasting, although no effect was seen after 8 days. In previous studies, we observed a significant decrease in plasma glucose after 3 and 7 days of fasting (Riley et al. 2008), and a sustained reduction in plasma glucose throughout 1 to 30 days of fasting in the tilapia (Rodgers et al. 1992). Plasma glucose decreased after 1 week, and remained reduced throughout the 4 weeks of fasting in the current study. We observed a similar decrease in plasma glucose after 4 weeks of fasting in SW-acclimated tilapia (Fox et al. 2006). Together, these findings suggest reductions in plasma glucose appear to be a good indicator of poor nutritional status in the tilapia.

In summary, we have shown that the GH/IGF-I axis is regulated in association with daily patterns of feeding as well as under conditions of intermediate and prolonged

negative energy balance. Unlike the case of mammals and goldfish, ghrelin does not appear to play an important role in feeding or short-term energy homeostasis in the tilapia. Prolonged nutrient restriction increased plasma ghrelin, suggesting a possible role for ghrelin as a long-term metabolic endocrine signal. These new data on ghrelin and the GH/IGF-I axis contribute to a better understanding of the nutritional regulation of metabolic hormones in fish. The up-regulation of ghrelin during pronounced negative energy balance along with our previous observations that ghrelin stimulates food intake and fat deposition in this species, indicates ghrelin could potentially be used to promote growth and weight gain in this important aquaculture species

## CHAPTER V

## Tissue-specific regulation of the growth hormone/insulin-like growth factor-I axis during fasting and re-feeding: Importance of muscle expression of IGF-I mRNA in the tilapia


### ABSTRACT

The effects of prolonged nutrient restriction (fasting) and subsequent restoration (re-feeding) on the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis were investigated in the tilapia (*Oreochromis mossambicus*). Using real-time quantitative PCR, we examined changes in plasma levels of GH, IGF-I and glucose along with pituitary mRNA levels for GH and somatolactin (SL), and muscle and liver expression of IGF-I, IGF-II, and the GH receptor (GH-R), and the SL receptor (SL-R). Mean weight and specific growth rate declined within 1 week, a trend continued throughout 4 weeks of fasting. Similarly, plasma levels of IGF-I and glucose were reduced after 1, 2, and 4 weeks of fasting suggesting that the fasted fish were in a profound catabolic state. Following re-feeding, fasted fish gained weight continuously, but did not attain the weight of fed controls 8 weeks after re-feeding. Specific growth rate also increased above that of the fed controls during 6 weeks of re-feeding, but declined to control levels after 8 weeks. Plasma IGF-I levels increased by 1 week of re-feeding and levels were not thereafter different from those in fed controls. Plasma glucose remained reduced during the first week of re-feeding, but levels also recovered to those of control fish after 2 weeks. Plasma GH levels were unaffected by either fasting or re-feeding. Pituitary expression of GH mRNA was reduced only after 2 weeks of fasting, but did not

otherwise differ from control levels. Pituitary SL mRNA, however, was reduced after 4 weeks of fasting and remained reduced throughout the next 4 weeks of re-feeding. Fasting had little effect in the liver except for significant increases in the expression of SL-R and GH-R after 1 and 4 weeks, respectively, and a significant reduction in IGF-I expression after 4 weeks. A significant reduction in IGF-II expression was observed following 2 weeks of re-feeding. By contrast, muscle expression of GH-R increased markedly during 1, 2, and 4 weeks of fasting and reduced below the control levels 1 and 2 weeks of re-feeding. In contrast, muscle expression of IGF-I was reduced throughout the fasting period, and levels recovered 2 weeks after re-feeding. Muscle expression of SL-R and IGF-II were reduced after 1 and 2 weeks of re-feeding. These results indicate that GH/IGF-I axis, particularly muscle expression of GH-R and IGFs are sensitive to nutritional status in the tilapia. Findings also suggest that SL may be involved during the rebuilding of energy stores following starvation.

## INTRODUCTION

Growth in fish, as in higher vertebrates, is controlled in large part through the complex regulation of the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis in relationship to available nutrients (Duan 1997; Wood et al. 2005). Growth hormone is a member of the GH/ prolactin (PRL)/somatolactin (SL) family of pituitary hormones which are involved in regulating numerous physiological processes besides somatic growth including behavior, immune function, lipid and protein metabolism, osmoregulation, and feeding behavior (Duan 1997; McCormick 2001; Bjornsson et al.

2004; Albalat et al. 2005). Growth hormone acts directly on target tissues by stimulating mitosis and other aspects of energy metabolism, and indirectly by initiating the production and release of IGF-I in the liver and the majority of peripheral tissues. In fish, as in higher vertebrates, many of the growth promoting effects of GH are mediated by circulating, liver-derived IGF-I (Duan 1997; Le Roith et al. 2001; Wood et al. 2005). These physiological actions of GH result from its binding to a transmembrane-spanning Class I cytokine growth hormone receptor (GH-R) located on the surface of cells in target tissues (Argetsinger et al. 1996; Pérez-Sánchez et al. 2002). Growth hormone receptors have been cloned recently from numerous teleost fish species, and have been shown to be widely-distributed throughout the body (Pérez-Sánchez et al. 2002; Wood et al. 2005; Saera-Vila et al. 2007). As sequences have accumulated, the presence of two distinct phylogenetic clades in fish GHR, GH-R1 and GH-R2 sequences, have been recognized (Jiao et al. 2006; Saera-Vila et al. 2005). Binding studies with recombinant salmon receptors identified a GHR that binds specifically to salmon GH but not SL, and an SLR that binds salmon SL strongly and salmon GH more weakly (Fukada et al. 2004; Fukada et al. 2005). In addition, a phylogenetic study showed that the fish GH-R1 clade contains salmon SL-R (Fukamachi et al. 2007). For the Mozambique tilapia, we have identified a GH-R1 as a putative SL-R and a GH-R2 as a putative GH-R (Kajimura et al. 2004; Pierce et al. 2007). Recently, it was suggested that SL-R is a teleost-specific paralogue of the GH-R which was likely acquired through the duplication of GH-R due to a fish-specific genome duplication event (Fukamachi et al. 2007). Fish GH-Rs have been shown to be differentially-regulated by fasting (Saera-Vila et al. 2005), temperature (Gabillard et al.

2006b), as well as by cortisol and testosterone (Jiao et al. 2006), suggesting different physiological roles for these receptors.

Many fish have been shown to be able to compensate for long periods of nutrient restriction that occur as a normal part of their lives (MacKenzie et al. 1998). Changes in nutrient availability modulate the activity and expression of a variety of genes associated with the GH/IGF-I system (Wood et al. 2005; Gabillard et al. 2006). When food supplies are low, energy is diverted from growth and mobilized from energy stores to support essential physiological processes. Fasting increases plasma GH levels, which shifts metabolism in favor of mobilizing energy substrates (lipolysis) to maintain basal metabolism. At the same time, decreases in plasma IGF-I, hepatic expression of IGF-I and GH-R mRNA levels are induced during fasting to suppress the actions of GH and IGF-I (Thissen et al. 1994; Deng et al. 2004; Fukada et al. 2004). These endocrine influences on the control of growth and metabolism are vital to survival, and are reinforced by local autocrine/paracrine reductions in the production and influence of IGF-I in non-hepatic tissues (Le Roith et al. 2001; Reinecke et al. 2005). In several fish species, prolonged fasting causes a reduction in plasma IGF-I and liver expression of IGF-I mRNA, while plasma GH levels rise or remain unchanged (Moriyama et al. 2000; Pierce et al. 2005; Small et al. 2005; Fox et al. 2006). This apparent paradox of GH resistance has also been observed in mammals (Thissen et al. 1994).

On the other hand, the metabolic actions of IGF-II in fish remain to be characterized Insulin-like growth factor-II shares a high structural homology to IGF-I, and gene expression of both mitogens appear to be regulated in several tissue types by

GH in fish (Vong et al. 2003). Both IGFs also activate cell proliferation as well, suggesting that they share some overlapping physiological roles in fish (Pozios et al. 2001; Reinecke et al. 2005). Nevertheless, fish appear to differ from mammals which express IGF-II chiefly during embryonic development. By contrast, IGF-II is expressed widely in both juvenile and adult fish (Reinecke et al. 2005).

While the relationship between GH and IGF-I during changes in nutritional state has been well-characterized in fish, very little is known about the metabolic roles or regulation of SL. Fukada et al. (2005) observed highest levels of SL-R mRNA in the liver and fat tissue of salmon, which supports earlier studies suggesting SL's roles in lipid metabolism in sea bream and sea bass (Mingarro et al. 2002; Vega-Rubin de Celis et al. 2003). Nevertheless, observations to date have failed to show a consistent pattern of change in plasma SL levels during fasting in salmonids (Company et al. 1999; Pottinger et al. 2003). No change in pituitary SL mRNA levels was observed in fasted rabbitfish (Ayson et al. 2007). We have recently cloned SL in the tilapia and observed significant reductions in pituitary SL mRNA levels after fasting in both fresh water and seawater, suggesting a possible role for this hormone in the regulation of energy metabolism (Uchida et al. 2008).

When fasted vertebrates are re-fed animals have been often observed to gain weight at an accelerated rate. This phenomenon, termed compensatory growth, has been found to occur in numerous vertebrate species including fish (Ali et al. 2003). While previous experiments have examined compensatory growth in the tilapia (Wang et al. 2000; Wang et al. 2005), the coordinated regulation of endocrine function during re-

feeding has not yet been addressed. Recently, attempts have been made to study the time-course of events that lead to the restoration of the GH/IGF-I axis during re-feeding in salmonid fish (Chauvigné et al. 2003; Gabillard et al. 2006; Montserrat et al. 2007). In the current study, we examined the time-course of effects of long-term fasting and subsequent re-feeding on the GH/IGF-I axis in the tilapia. To this end, we followed changes in plasma levels of GH, IGF-I, and glucose concomitantly with variations in mRNA expression of pituitary GH, SL, and muscle and hepatic expression of IGF-I, IGF-II, GH-R, and SL-R.

## MATERIALS AND METHODS

### *Animals*

Male tilapia (*Oreochromis mossambicus*), were reared outdoors in fresh water, flow-through tanks under natural photoperiod at the Hawaii Institute of Marine Biology. Animals were fed approximately 5% of their body weight per day by belt feeder (12-hour belt feeder, Aquatic Ecosystems Inc., Apopka, FL) with Silver Cup Trout Chow (Nelson and Sons, Murray, UT). All Experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

### *Fasting and re-feeding*

One hundred and twenty sexually mature fish, weighing approximately 38 g, were tagged individually with passive integrated transponder tags (PIT tags; Destron IDI, Boulder, CO) and divided randomly into 4 tanks representing two treatment groups (two fed and two fasted/re-fed). The animals were then maintained outdoors in oval 700 L fiberglass aquaria (30 fish in each tank). Water temperature was maintained at between 27 °C and 28 °C using submersible aquarium heaters (Ebo-Jager Inc., El Segundo, CA). The fish were allowed to acclimate to the tanks for 2 weeks prior to the beginning of the experiment. Following the acclimation period, food was withheld from the fish in two tanks for a period of 4 weeks, while the fish in the remaining two tanks were fed continuously throughout the experiment by automatic belt feeder. After the 4 weeks, fasted animals were fed continuously for the remaining 8 weeks. Twelve fish, 6 fed and 6 fasted/re-fed (3 fish from each of the 4 tanks), were killed in excess anesthesia and sampled at 8 time points; weeks –4, -3, -2, 0, 1, 2, 4, and 8 respectively. Week -4 represents the beginning of the fasting period, while week 0 represents the beginning of the re-feeding period, and week 8 represents the termination of the experiment. To eliminate the effect of crowding stress on growth rate, stocking densities were maintained at approximately 5 g/L by adjusting the height of the vertical standpipe as fish were removed for sampling.

*Sampling*

Body weight and standard length were measured at each sampling time point from the beginning of the experiment. Condition factor, (body weight, g)/(standard length,

cm)$^3$ x 100, and specific growth rate (ln $W_f$-ln $W_i$)/t x100, where $W_f$ is the final weight (g), $W_i$ is the initial weight (g) at each time interval and t is growth time (days), were calculated also at each time point. At the time of sampling, all fish were netted, anesthetized in buckets containing 2-phenoxyethanol (0.2 mL/L), and blood was collected from the caudal vasculature by a needle and syringe treated with ammonium heparin (200 U/mL, Sigma-Aldrich, St. Louis MO). Plasma was separated by centrifugation at 10,000 x g for 10 min at 4°C, and stored at -20°C until analyses for GH, and IGF-I by radioimmunoassay as well as for plasma glucose. Fish were decapitated rapidly and whole pituitaries were collected along with approximately 100 mg of liver and muscle tissues, snap frozen and stored at -80°C until analyses for mRNA expression of GH, SL, IGF-I, IGF-II, GH-R, or SL-R.

*Plasma measurements*

Plasma GH levels were measured by homologous radioimmunoassay (RIA) according to Yada et al. (1994). Total plasma IGF-I levels were measured from a 25 µl of plasma that was extracted with 100 µl of acid-ethanol (87.5% ethanol and 12.5% 2 N HCl, v/v) as described by Shimizu et al. (1999). Total IGF-I levels were measured using recombinant salmon IGF-I as the standard and anti-barramundi IGF-I (GroPep, Adelaide, Australia) following Shimizu et al. (1999) and Kajimura et al. (2002). Plasma glucose concentrations were estimated using a commercially available kit (Glucose Assay Kit, Sigma), which was modified for a microplate reader (SpectraCount, Packard, Meriden, CT).

*RNA extraction, cDNA Synthesis, and qPCR*

Pituitary, liver, and muscle mRNA levels were determined by quantitative PCR (qPCR). Total RNA was extracted from individual tissues using Tri-Reagent (MRC, Cincinnati, OH) and then reversed transcribed in a manner similar to that described by Pierce et al. (2007). Briefly, first strand cDNA was synthesized with an iScript kit (BioRad Laboratories, Hercules, CA). For GH, SL, IGF-I, IGF-II, GH-R and SL-R mRNA, a specific primer pair was used (Table 1). Serial dilutions of PCR products containing the qPCR amplicon were used as standards. Gene specific fluorogenic probes were purchased from Biosearch Technologies (Novato, CA). All qPCR assays were performed in a similar manner to those described by Pierce et al. (2007). Briefly, all qPCR assays were run using iQ Supermix (BioRad), in a 15 μl reaction volume, using primers and probes at 200 nM concentration. Assays were run on an iCycler iQ Real-Time PCR detection system (BioRad), using the manufacturer's recommended cycling conditions (2 min at 95 °C, 2 min at 50 °C followed by 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 min). All qPCR data were normalized to ARP (acidic ribosomal phosphoprotein; reference gene) mRNA transcript levels after it was determined ARP levels did not change with treatment. Values are expressed as percent of the fed control at each corresponding time point.

*Statistics*

Group comparisons were performed using a Student's *t* test or two-way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Levels of correlation were determined by the Pearson correlation test. Significant level was set at

$P<0.05$. Calculations were performed using computer programs, Statistica (StatSoft, Tulsa, OK). Data are expressed as means ± S.E.M.

## RESULTS

No significant difference was found between replicate tanks in any measured parameters, and thus individual fish of the two tanks were combined and used for statistical analyses. At the beginning of the experiment (2 weeks prior to the start of fasting or week -6), there was no difference in body weight or condition factor between the fed and fasted groups (Table 2, Fig. 18A). All fish gained weight at similar rates during the following two weeks (acclimation period), and no difference in specific growth rate was observed during this time (Figs. 18A and B). Fasting for 2 and 4 weeks resulted in a significant reduction in body weight compared with fed controls (Fig. 18A). Following re-feeding, previously fasted fish gained weight continuously for the remaining 8 weeks of the experiment, but were unable to attain the final weight of continuously-fed control animals (Fig. 18A). Specific growth rate decreased significantly to a negative value in the fasted group following 2 and 4 weeks of fasting (Fig. 18B). There was also a significant reduction in condition factor after 2 and 4 weeks of fasting (Table 2). Following 2, 4, and 6 weeks of re-feeding, the specific growth rate of fasted fish increased significantly above fed controls, but was not different at the end of the experiment (Fig. 18B). Specific growth rate of fed animals decreased gradually from week -2 to week 4 ($r=0.98$, $P<0.001$), and stayed at the same level throughout the remaining experimental period.

Plasma GH levels were not affected by 4 weeks of fasting or by subsequent re-feeding (Fig. 19A). Plasma IGF-I levels were reduced significantly after 1, 2, and 4 weeks of fasting compared with fed controls at each time point (Fig. 19B). Plasma IGF-I levels were then recovered to control levels after 1 week of re-feeding and during the rest of the experiment. Plasma glucose was reduced significantly after 1, 2, 4 weeks of fasting and remained lower than the control level following 1 week of re-feeding (Fig. 19C). Plasma glucose then returned to fed control levels following 2 weeks of re-feeding, and values were not otherwise different from those in fed controls (Fig. 19C).

Pituitary expression of GH mRNA was reduced significantly after 2 weeks of fasting, but levels did not otherwise differ from those in controls (Fig. 20A). Pituitary SL mRNA levels were reduced significantly after 4 weeks of fasting, and this trend continued through the next 4 weeks attaining significance after 1 and 4 weeks of re-feeding; however, levels did not differ at the end of the experiment (Fig. 20B).

Liver GH-R mRNA was elevated significantly after 4 weeks of fasting, but levels were not otherwise different from those in fed controls (Fig. 21A). Similarly, liver expression of SL-R mRNA was elevated significantly after 1 week of fasting, but no other difference was detected (Fig. 21B). Expression of liver IGF-I mRNA was reduced significantly after 4 weeks of fasting, but did not otherwise differ from that observed in fed controls (Fig. 21C). Liver expression of IGF-II mRNA was reduced significantly during the re-feeding period (week 2), but transcript levels did not otherwise differ from those in controls (Fig. 21D).

Muscle GH-R mRNA was elevated significantly after 1, 2, and 4 weeks of fasting. Muscle GH-R mRNA levels were then reduced during 1 and 2 weeks of re-feeding, but levels remained the same as fed controls during the rest of the experiment (Fig. 22A). Muscle expression of SL-R mRNA was elevated significantly compared with that in controls after 4 weeks of fasting, and then fell significantly during 1 and 2 weeks of re-feeding; no difference was observed during the rest of the experiment (Fig. 22B). Levels of muscle IGF-I mRNA were reduced after 1, 2, and 4 weeks of fasting. Muscle IGF-I mRNA abundance remained lower than in fed controls after 1 week of re-feeding, returned to control levels for the next 2 weeks of re-feeding, however levels were elevated significantly at the end of the experiment (Fig. 22C). Muscle expression of IGF-II mRNA was reduced significantly during 1 and 2 weeks of re-feeding, but levels were not otherwise different from those in fed controls (Fig. 22D).

Table 2

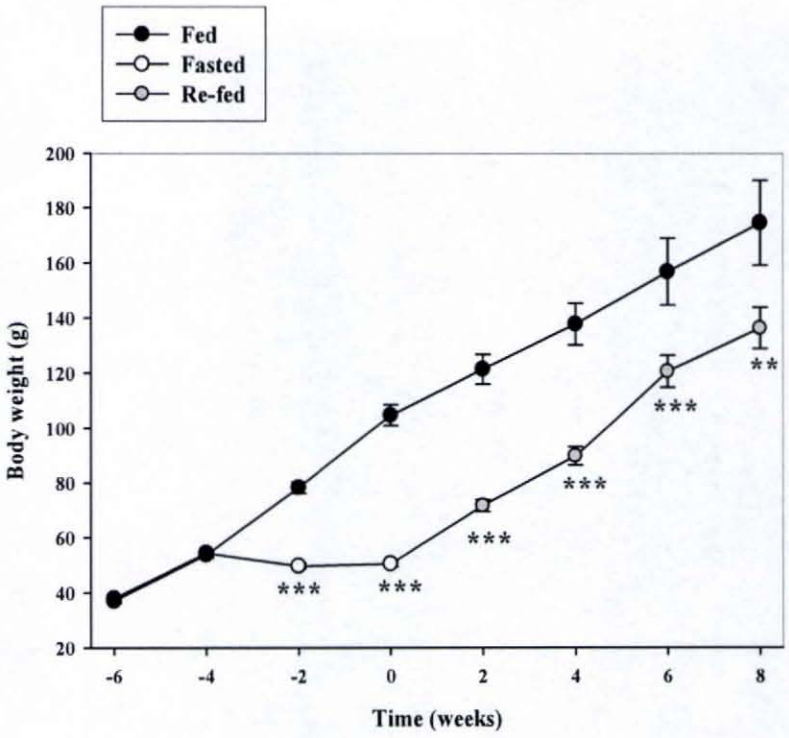
Effect of fasting and re-feeding on condition factor

| Week | Fed | Fasted/Re-fed |
|---|---|---|
| -6 | 3.64 ± 0.04 | 3.66 ± 0.04 |
| -4 | 3.66 ± 0.05 | 3.67 ± 0.04 |
| -2 | 3.89 ± 0.04 | 3.42 ± 0.04*** |
| 0 | 4.11 ± 0.06 | 3.46 ± 0.05*** |
| 2 | 3.78 ± 0.05 | 3.66 ± 0.05 |
| 4 | 3.78 ± 0.06 | 3.69 ± 0.06 |
| 6 | 3.79 ± 0.09 | 3.79 ± 0.05 |
| 8 | 3.78 ± 0.09 | 3.72 ± 0.06 |

*** Significantly different from fed controls at $P < 0.001$.

Fig. 18. Changes in body weight (A) and specific growth rate (B) during 4 weeks of fasting and 8 weeks of re-feeding in tilapia. One hundred and twenty fish, weighing approximately 40 g, were divided into 4 groups (two fed and two fasted) and maintained in outdoor 700-L fiberglass aquaria (thirty fish per tank). They were fed approximately 5% of their body weight per day continuously by automatic belt feeder. The experiment was started once it was determined all animals exhibited similar appetite and growth. Once positive growth and appetite in all fish were established for 2 weeks (week -4), two tanks of fish were fasted for 4 weeks, while the other two were fed at the same rate. Following the 4 weeks (week 0), fasted animals were re-fed for further 8 weeks. Vertical bars indicate ± S.E.M. (n=8). *** Significantly different from the corresponding fed control at each time point at $P < 0.001$.

A


Fed
Fasted
Re-fed
Body weight (g)
200
180
160
140
120
100
80
60
40
20
-6
-4
-2
0
2
4
6
8
Time (weeks)
***
***
***
***
***
**

B

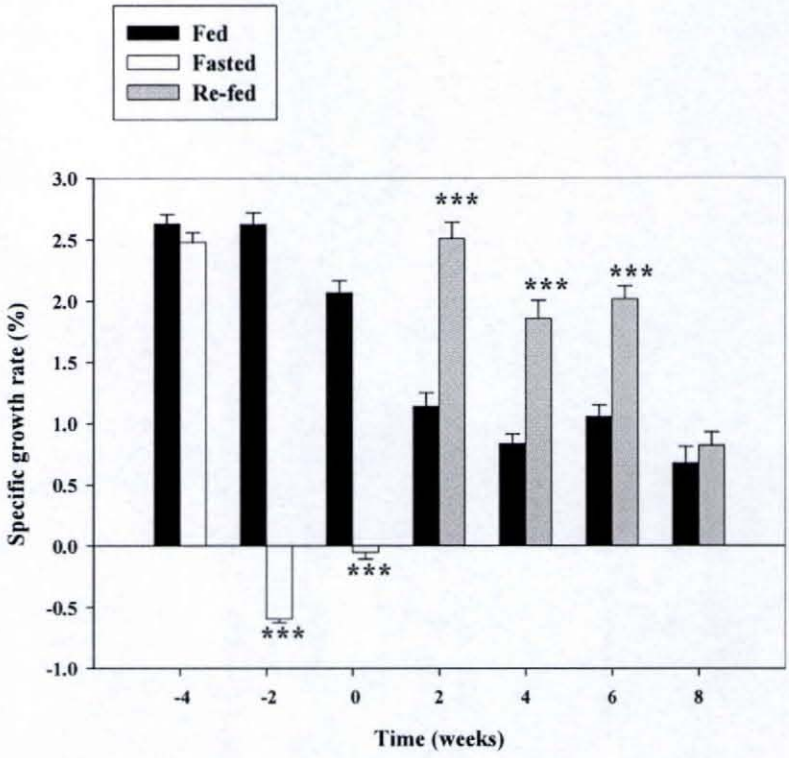

Fed
Fasted
Re-fed
Specific growth rate (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-4
-2
0
2
4
6
8
Time (weeks)
***
***
***
***
***

Fig. 19. Effects of fasting and re-feeding in tilapia on plasma levels of GH (A), IGF-I (B), and glucose (C). No effect of fasting was observed on plasma GH. There were significant ($P<0.001$) main effects of treatment and sampling time on plasma levels of IGF-I and glucose by two-way ANOVA. Vertical bars indicate ± S.E.M. (n=8). *, *** Significantly different from the corresponding fed control at each time point at $P < 0.05$, and $P < 0.001$, respectively.

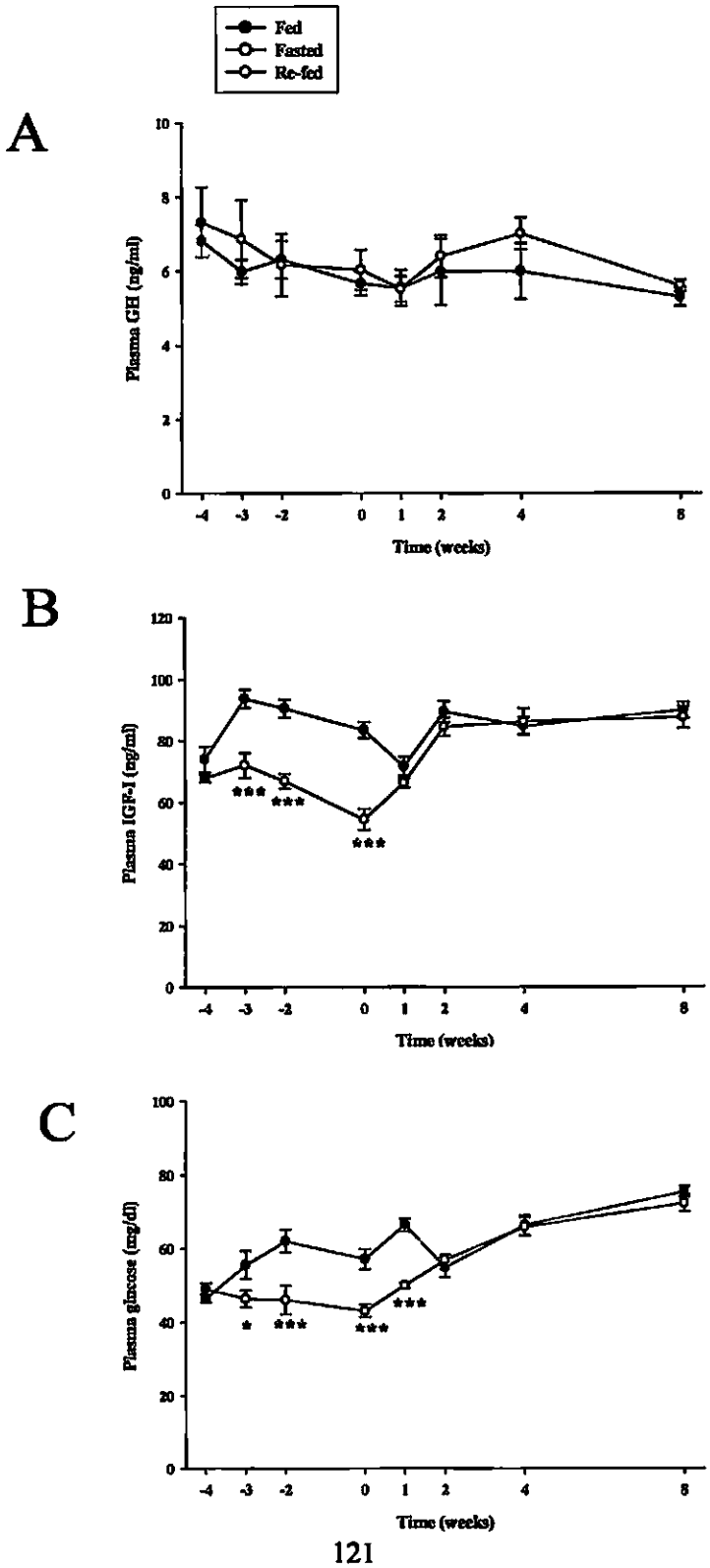
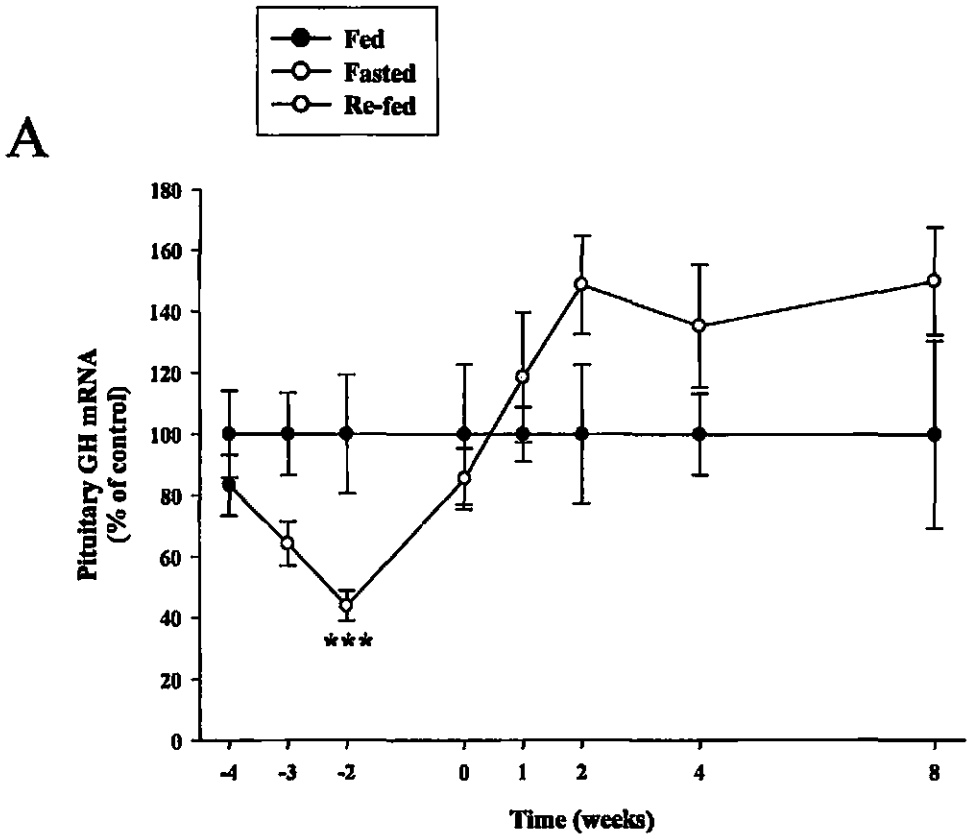

A
Fed
Fasted
Re-fed
Plasma GH (ng/ml)
Time (weeks)
B
Plasma IGF-I (ng/ml)
Time (weeks)
C
Plasma glucose (mg/dl)
Time (weeks)

Fig. 20. Effects of fasting and re-feeding in tilapia on pituitary expression of GH (A) and SL (B). *** Significantly different from the corresponding fed control at each time point at $P < 0.001$.


A
Fed
Fasted
Re-fed
Pituitary GH mRNA
(% of control)
180
160
140
120
100
80
60
40
20
0
***
-4
-3
-2
0
1
2
4
8
Time (weeks)

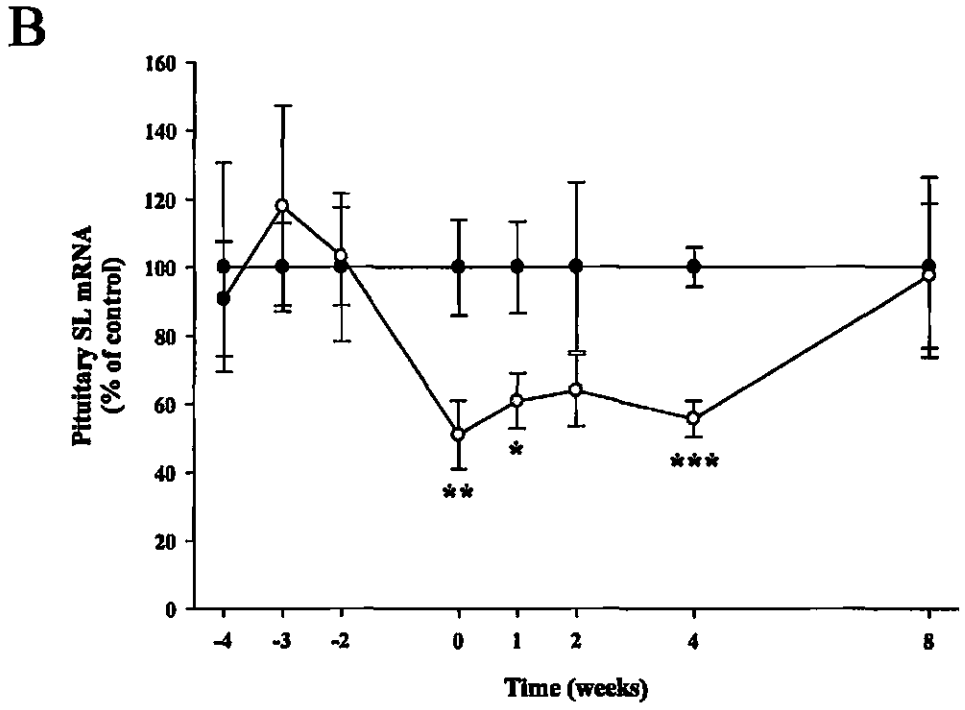
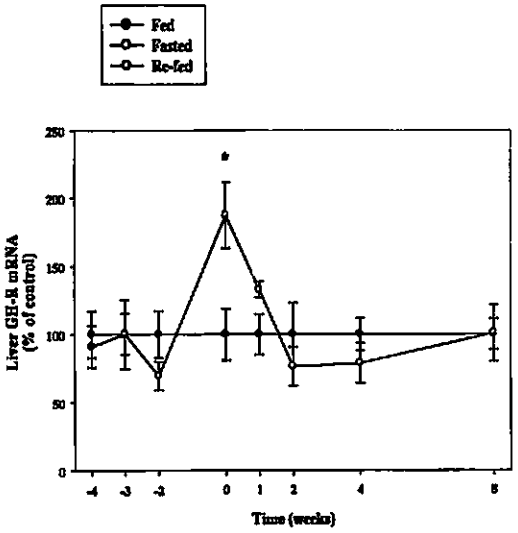
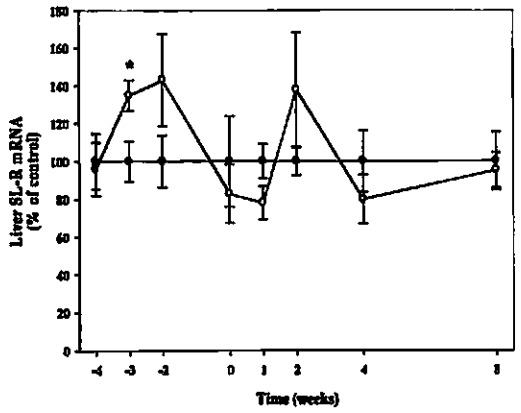

B
Pituitary SL mRNA
(% of control)
160
140
120
100
80
60
40
20
0
**
*
***
-4
-3
-2
0
1
2
4
8
Time (weeks)

Fig. 21. Effects of fasting and re-feeding in tilapia on liver expression of GH-R (A), SL-R (B), IGF-I (C), and IGF-II (D). *, *** Significantly different from the corresponding fed control at each time point at $P < 0.05$ and $P < 0.001$, respectively.

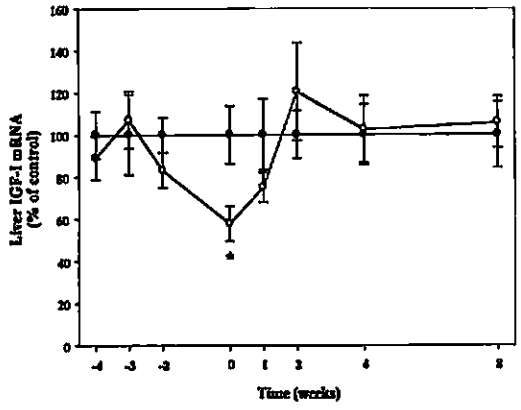
A


Fed
Fasted
Re-fed
Liver GH-R mRNA (% of control)
Time (weeks)

B


Liver SL-R mRNA (% of control)
Time (weeks)

C


Liver IGF-I mRNA (% of control)
Time (weeks)

D

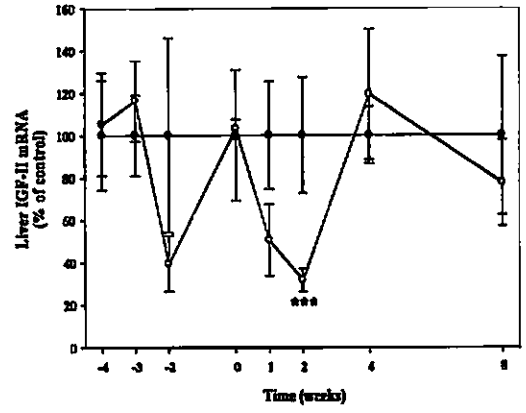
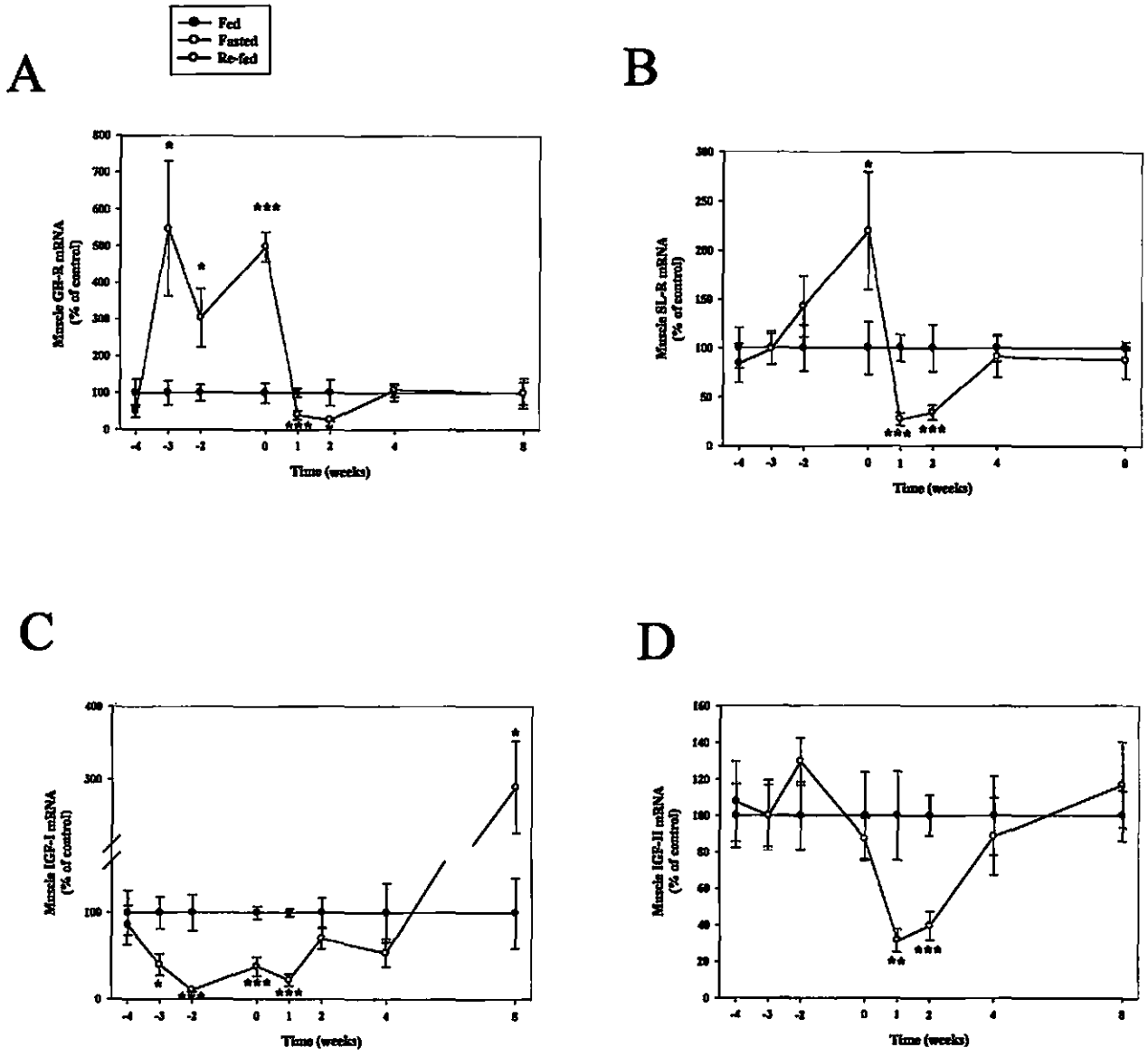

Liver IGF-II mRNA (% of control)
Time (weeks)

Fig. 22. Effects of fasting and re-feeding in tilapia on muscle expression of GH-R (A), SL-R (B), IGF-I (C), and IGF-II (D). *, **, *** Significantly different from the corresponding fed control at corresponding day at $P < 0.05$, $P<0.01$, and $P < 0.001$, respectively.


A
Fed
Fasted
Re-fed
Muscle GH-R mRNA (% of control)
Time (weeks)
B
Muscle SL-R mRNA (% of control)
Time (weeks)
C
Muscle IGF-I mRNA (% of control)
Time (weeks)
D
Muscle IGF-II mRNA (% of control)
Time (weeks)

## DISCUSSION

Compensatory or "catch-up" growth following a period of growth depression has been described to some degree in most teleost species studied (see Ali et al. 2003). The degree of compensatory growth appears to vary by species and environmental conditions, ranging from partial, full and even over-compensation for weight loss during nutrient restriction. Nevertheless, the endocrine control of this accelerated weight gain following periods of food restriction has not been well studied. In the present study, the body weights of tilapia which were fasted for 4 weeks were still significantly lower than the continuously-fed controls after 8 weeks of re-feeding. In agreement with these findings, body weights of juvenile hybrid tilapia (*O. mossambicus* x *O. niloticus*) fasted for 4 weeks in both seawater and fresh water failed to catch up with continuously-fed control fish (Wang et al. 2000; Wang et al. 2005), suggesting that tilapia are capable of only partial compensatory growth following prolonged nutrient restriction. Specific growth rate also decreased to a negative value during fasting and increased during re-feeding in the current study. We have previously observed a similar pattern of growth depression, characterized by a decrease in specific growth rate with minimal weight loss during a period of food restriction in the tilapia (Uchida et al. 2003; Fox et al. 2006).

To compensate for a lack of nutrients during fasting, many animals decrease their basal metabolic rate to shift the use of stored energy from growth to support essential physiological processes (Fuglei et al. 2000; Fu et al. 2005). The endocrine control

mechanisms involved in this metabolic shift are complex and poorly understood, and many factors are thought to be involved including the GH/IGF-I axis (Mommsen 2001). In the current study, fasting for 4 weeks, and subsequent re-feeding for 8 weeks caused no change in plasma GH levels. In contrast, plasma IGF-I levels were reduced significantly after 1, 2, and 4 weeks of fasting, and levels were recovered following 1 week of re-feeding. These findings are in agreement with other studies, in which fasting for a few to several weeks resulted in either the elevation or no change of plasma GH with coordinate reduction in plasma IGF-I levels in salmonids (Duan et al. 1993; Fukada et al. 2004; Pierce et al. 2005; Gabillard et al. 2006a; Norbeck et al. 2007; Jönsson et al. 2007), gilthead and black sea bream (Company et al. 1999; Deng et al. 2004), channel catfish (Small et al. 2005b), and tilapia (Weber et al. 1999; Uchida et al. 2003; Fox et al. 2006; Pierce et al. 2007). We have shown previously that pituitary GH content and mRNA expression increase with fasting in the tilapia (Weber et al. 1999; Fox et al. 2006). The increase in plasma GH during fasting may be necessary for the sparing of structural proteins and mobilization of fatty acids from adipose and muscle tissue during prolonged nutrient restriction in the tilapia. Indeed, GH has been shown to have potent lipolytic effect in both mammals and fish (Richelsen 1997; Albalat et al. 2005). Interestingly, pituitary expression of GH mRNA was reduced significantly after 2 weeks of fasting, but levels did not otherwise differ from those in controls. The observed reduction in pituitary GH mRNA levels may not be physiologically important.

Somatolactin is a member of the GH/PRL family of pituitary peptide hormones. It is present in a variety of teleost species as well as the sturgeon and lungfish, but not in

tetrapods (Fukamachi et al. 2007). We have recently cloned tilapia SL (Uchida et al. 2008). Studies on the physiological regulation of SL are limited, and the majority of these studies have been conducted in salmonids (Fukada et al. 2005). Injection of SL inhibited food intake in gilthead sea bream (Vega-Rubín de Celis et al. 2004) and stimulated lipid catabolism in European sea bass (Vega-Rubin de Celis et al. 2003). In the current study, we observed a significant decline in pituitary expression of SL mRNA after 4 weeks of fasting. These results are in agreement with our recent finding that SL mRNA is reduced after 4 weeks of fasting in both fresh water and seawater-acclimated tilapia (Uchida et al. 2008). This reduction in pituitary SL mRNA continued following 4 weeks of re-feeding with a return to control levels after 8 weeks. To our knowledge, this is the first description of pituitary SL mRNA expression following a long-term fasting and re-feeding. In gilthead sea bream, plasma SL has been shown to increase following 8 days of fasting, but levels returned to control values after 18 days (Company et al. 2001). Nevertheless, other studies in salmonids have failed to produce consistent effects from fasting and re-feeding on plasma SL (Company et al. 1999; Pottinger et al. 2003). For example, long-term starvation also resulted in reduction in size and number of SL cells in the pituitary of the European eel (Olivereau et al. 1997). On the other hand, no change was detected in pituitary expression of rabbitfish fasted for 15 days (Ayson et al. 2007). The current observation of reduced pituitary SL mRNA after 4 weeks of fasting and during 4 weeks of re-feeding suggests that SL may play a role in starvation and/or the re-building of energy reserves during re-feeding in the tilapia. Certainly, the measurements

of plasma levels of tilapia SL as well as more detailed investigations into the physiological actions of SL are warranted before any conclusions can be drawn.

We have recently cloned two GH-Rs in the Mozambique tilapia; GH-R1 and –R2 (Pierce et al. 2007). Based on phylogenetic analyses, we believe the GH-R1 to be the somatolactin receptor (SL-R) and GH-R2 the GH-R. Recent evidence indicates that the SL-R is a fish-specific paralogue of the vertebrate GH-R, and it was likely acquired through duplication of the GH-R gene that resulted from fish specific genome duplication events (Fukamachi et al. 2007). Previously, we identified high levels of expression of these two receptors in both liver and muscle tissue (Pierce et al. 2007). In the current study, we observed a significant increase in hepatic expression of SL-R mRNA after 1 week and in the expression of GH-R mRNA after 4 weeks of fasting, but levels did not otherwise differ from those in controls. In agreement with these findings, we observed no effect of fasting for 4 weeks on liver expression of SL-R mRNA in the tilapia (Fox et al. 2006; Pierce et al. 2007; Uchida et al. 2008). Together, these studies suggest that changes in liver expression of GH-R or SL-R may not play critical roles during fasting or re-feeding in the tilapia. Reduced hepatic binding of GH in the presence of elevated plasma GH levels has been observed in the gilthead sea bream (Perez-Sanchez et al. 1994) and rainbow trout (Norbeck et al. 2007). Likewise, Deng et al. (2004) observed a comparable decrease in hepatic binding of GH, as well as reductions in SL-R mRNA levels in the black sea bream following starvation. Furthermore, fasting has resulted in reductions in hepatic GH-R mRNA levels in the masu salmon (Fukada et al. 2004), gilthead sea bream (Saera-Vila et al. 2005), and channel catfish (Small et al. 2006).

Interestingly, fasting resulted in both increases and decreases in hepatic SL-R and GH-R mRNA levels in rainbow trout (Gabillard et al. 2006a; Norbeck et al. 2007). To further complicate interpretation of these data, it appears that both salmonid GH-R1 and -R2 are in the same phylogenetic clade being GH-Rs and not SL-Rs (Fukamachi et al. 2007). These variations in teleost GH-R evolution, coupled with the fact that some fish have undergone duplications of the SL and/or GH genes as well, make generalizations about the functional regulation of this hormone-receptor family difficult, if not impossible (Fukamachi 2007). Nonetheless, these peptide hormones and their associated receptors undoubtedly play integral roles in the metabolic regulation of somatic growth in fish.

In contrast with the liver, we observed significant increases in muscle mRNA levels of both GH-R and SL-R in response to food deprivation. We observed a rapid and marked increase in muscle GH-R mRNA after 1 week of fasting, and this trend continued throughout the fasting period after which the levels were reduced for 2 weeks following re-feeding. Likewise, there was a significant elevation in muscle SL-R mRNA level after 4 weeks of fasting, after which levels decreased for 2 weeks following re-feeding. These results are in agreement with our previous findings that both GH-R and SL-R mRNA levels increased in muscle after 4 weeks of fasting in the tilapia (Pierce et al. 2007). Similar increases in muscle GH-R mRNA levels due to starvation have been described recently in the gilthead seabream and rainbow trout (Saera-Vila, 2005; Gabillard et al. 2006a). The increase in GH-R mRNA may reflect a mechanism through which energy reserves are mobilized preferentially from skeletal muscle during long-term fasting. The transient decrease in muscle GH-R and SL-R mRNA levels following re-feeding may

indicate a down-regulation of GH's lypolytic effects on muscle tissue as reserves are deposited during a period of nutrient excess. To clarify these proposed effects in tilapia, further studies examining the influence of GH and SL on lipolysis and/or lipogenesis in muscle tissue are highly warranted.

In the current study, plasma IGF-I levels were reduced significantly after 1 week, a trend continued throughout 4 weeks of fasting. Re-feeding for 1 week restored plasma IGF-I to control levels. The reduced plasma IGF-I during long-term fasting in the current study, without a change in plasma GH, suggests a state of GH resistance. We have observed similar declines in plasma IGF-I due to fasting in the tilapia (Uchida et al. 2003; Fox et al. 2006; Pierce et al. 2007). We have also observed a comparable reduction in liver IGF-I mRNA levels after 2 and 4 weeks of fasting (Uchida et al. 2003; Fox et al. 2006), but not consistently (Pierce et al. 2007). By contrast, we observed a significant reduction in hepatic IGF-I mRNA levels only after 4 weeks of fasting in the current study. Fish in the current study were fed continuously by belt feeder to maximize growth rates and typically consumed up to 5% of their body weight per day or more, while fish in previous studies were essentially fed maintenance rations (2% body weight per day) and grew modestly, if at all (Pierce et al. 2007). Recently, Gomez-Requini et al. (2004) demonstrated a significant decrease in hepatic IGF-I mRNA levels in the sea bream that was tied to a reduction in the fish meal content of the diet. These data suggest a minimum intake (i.e., ration size and dietary protein/energy ratio) of essential nutrients is required to maintain basal hepatic IGF-I mRNA expression, and may explain the

differences we observed between tissue IGF-I mRNA expression in fish fed at maintenance vs. at maximal levels.

In contrast with hepatic IGF-I mRNA levels, we observed a significant reduction in muscle IGF-I mRNA levels after 1, 2, and 4 weeks of fasting, with levels remaining below those of control fish after 1 week of re-feeding. Muscle IGF-I mRNA levels then returned to fed controls after 2 and 4 weeks of re-feeding. This reduction in muscle IGF-I mRNA levels mirrored the reduction in plasma levels of IGF-I, except during the re-feeding period. Thus, fasting and re-feeding appear to reduce IGF-I production both systemically and locally in the tilapia. Most investigators have argued that the bulk of circulating IGF-I is produced in the liver, and that plasma IGF-I is the most important promoter of linear growth in vertebrates (Ohlsson et al. 2000). Nevertheless, this paradigm has been challenged lately in studies of post-natal mice in which liver-specific inactivation of the IGF-I gene did not completely abolish plasma IGF-I nor alter growth and development (Yakar et al. 1999). It is possible that muscle, as well as liver tissue contribute significantly to circulating levels of IGF-I in the tilapia. Reductions in muscle IGF-I mRNA due to fasting and subsequent recovery of levels following re-feeding have also been observed in the rainbow trout (Chauvigné et al. 2003; Gabillard et al. 2006a; Montserrat et al. 2007). Collectively, these studies suggest the reduction and recovery of muscle IGF-I mRNA following fasting and re-feeding is a conserved mechanism, and indicates an importance of autocrine and/or paracrine IGF-I in extrahepatic tissue in the regulation of growth in fish.

No effect of fasting was observed on expression of IGF-II in either liver or muscle tissue. In contrast, expression of IGF-II mRNA was reduced during 1 and 2 weeks of re-feeding in muscle and after 2 weeks after re-feeding in the liver. Plasma IGF-II and expression of IGF-II mRNA in the liver and muscle were reduced after 4 weeks of fasting in the rainbow trout, and levels remained reduced following re-feeding (Gabillard et al. 2006a). More recently however, no change in muscle or liver expression of IGF-II mRNA was detected in the rainbow trout after 4 weeks of fasting or after subsequent re-feeding (Montserrat et al. 2007). Interestingly, a transient rise in hepatic expression of IGF-II mRNA levels was observed in the rabbitfish after 3 and 6 days of fasting, but no other changes were detected (Ayson et al. 2007). In all of the studies described above, it appeared that IGF-I and IGF-II are regulated differentially during altered nutritional status, and that this may also be a conserved mechanism in fish. More studies are needed to clarify the metabolic roles of IGF-II and its contribution to skeletal growth in adult fish.

Finally, plasma glucose was reduced significantly after 1, 2, and 4 weeks of fasting in the current study. This hypoglycemia persisted after 1 week of re-feeding with levels returning to controls 2 weeks after re-feeding. We have observed similar reductions in plasma glucose in response to fasting in the tilapia (Rodgers et al. 1992; Fox et al. 2006; Riley et al. 2008). These results are also consistent with those reported for the rainbow trout and catfish that were re-fed after various periods of fasting (Pottinger et al. 2003; Peterson et al. 2004; Montserrat et al. 2007). Thus, reductions in plasma glucose appear to be a reliable indicator of impaired nutritional status in fish.

In summary, we observed partial significant resumption in growth in re-fed tilapia following 4 weeks of fasting with concomitant changes in the GH/IGF-I axis. Plasma IGF-I levels were reduced with fasting, while plasma GH levels remained unchanged, suggesting fasting-induced GH resistance. Levels of pituitary SL mRNA were suppressed during 4 weeks of re-feeding suggesting a possible role for SL in metabolism during energy surplus. Modest and inconsistent changes were detected in liver expression of GH-R, SL-R, and IGF-I mRNA during fasting and re-feeding. By contrast, my findings argue that muscle GH-R, SL-R, and IGF-I mRNA levels are regulated acutely and decisively by nutrient availability during the same period, suggesting a complimentary role of these genes in muscle growth and metabolism. Furthermore, IGF-I and IGF-II mRNA appeared to be regulated differentially by nutritional status in the tilapia suggesting different roles for these closely-related hormones during varied nutritional states.

# CHAPER VI

# CONCLUDING REMARKS

A full understanding of metabolic and neuroendocrine control of growth in vertebrates, and in fish in particular, requires further study. The pattern and regulation of growth in many fish differ from the situation observed in other vertebrates. By contrast with mammals, somatic growth in fish continues throughout life and the maximum size that most species attain is unknown (Mommsen 2001). Indeed, fish also differ from mammals in that post-natal muscle recruitment in fish continues during much of life, with both hypertrophy and hyperplasia contributing to increases in muscle mass (Johnston 1999). The majority of somatic growth in fish is devoted to the accretion of anaerobic white fiber muscle tissue, which typically accounts for more than half of the total body mass (Mommsen 2001).

Growth in fish is influenced by a variety of environmental factors including temperature, salinity, and photoperiod (Ron et al. 1995; Sparks, 2003; Johnston 1999). Diet composition and ration size are also crucial determinants of growth in fish as well (Perez-Sanchez et al. 1994; Jensen 2001; Volkoff et al. 2006). Overlaying these external influences, fish growth is controlled to a great extent by the internal state of the animal, with age and sex of fish affecting growth in particular (Kuwaye et al. 1993; Taylor et al. 2008). Similarly, the digestion, absorption and utilization of ingested nutrients, along with the excretion of nitrogenous waste, require considerable energy that must be diverted from growth to support these vital processes (Nelson et al. 2006). Thus, the regulatory mechanisms by which energy is partitioned and allocated are extremely

important to the regulation of growth in fish and vertebrates generally. In this regard, emerging research suggests that metabolism in fish is governed through the integrated control that is exercised by the neuroendocrine and gastroenteropancreatic systems (Volkoff et al. 2005; Nelson et al. 2006). In this dissertation, studies were undertaken with the goal of understanding how altered nutritional status modulates the hormonal regulation of growth in the tilapia.

Tilapia has emerged as an important food fish in recent years and worldwide production of farmed tilapia has exceeded 2 million metric tons, making it second only to carp as the most widely-cultured freshwater fish species (American Tilapia Association, 2006). Fish are among the most recently domesticated animals, and the improvement of production traits in aquatic livestock will become increasingly crucial (De-Santis et al. 2007). Several species of commercially important fish, including tilapia, have been modified genetically and stable transgenic lines have been established in recent years (Zbikowska 2003). The ability to manipulate a process, by which a fish can be made to grow faster and larger with less feed, is extremely valuable to farmers. Characterization of these mechanisms on a molecular level is invaluable to fish breeding programs that are driven by the selection of genes involved in growth.

Growth hormone and somatolactin are important metabolic hormones and members of the same pituitary protein family in fish. Moreover, their receptors (referred to collectively as growth hormone-receptors, or GH-Rs) appear to share a high degree of structural similarity (Perez-Sanchez 2002; Fukamachi 2007). Additionally, many of the metabolic actions of GH are mediated by the mitogenic endocrine factor insulin-like

growth factor-I (IGF-I) (Le Roith et al. 2001; Wood et al. 2005). Furthermore, a related mitogen called insulin-like growth factor-II (IGF-II) appears to be regulated in adult fish and have some overlapping physiological roles with IGF-I (Reinecke et al. 2005).

The need for a better understanding of how growth is regulated is underlined by the fact that strategies that have used the over-expression of GH genes in fish to promote growth have met with mixed success. The further understanding of how the expression GH receptors changes during nutrient restriction will undoubtedly assist researchers in that field (Zbikowska 2003). While by no means exhaustive, the present studies provide insight into the regulation of somatic growth by modulations in ghrelin levels and the GH/IGF-I axis due to fasting and re-feeding.

This dissertation focused on the effects of nutrient restriction on GH, SL, IGFs, GH-Rs, and ghrelin; however, questions as to the effects that fasting and re-feeding may have on other growth-related parameters are raised. The anabolic nature of GH and IGF-I are well established, and both of these hormones work independently as well as synergistically with one another to promote growth in fish and other vertebrates (Thissen et al. 1999; Wood et al. 2005). In this dissertation, it was shown that plasma GH increases, or remains unchanged, during a prolonged fast, while plasma IGF-I and liver and muscle expression of IGF-I mRNA levels are reduced. These data suggest a shift in the actions of the GH/IGF-I axis from anabolism and the stimulation of the growth of tissues, to catabolism of stored substrates to preserve energy balance. Furthermore, plasma ghrelin levels are upregulated during this catabolic state, while pituitary SL mRNA and liver and muscle IGF-II mRNA levels are reduced suggesting roles for these

hormones in energy partitioning. Nevertheless, future studies investigating how manipulating the levels and expression of anabolic sex steroid hormones such as androgens, peptide hormones, including leptin and insulin, or tissue specific myogenic regulatory factors (MRFs), and members of the Transforming Growth Factor-β family such as myostatin, may enhance growth rate and increase metabolism should be conducted in parallel with the studies described in this dissertation (Mommsen 2001; Riley et al. 2002; De-Santis et al. 2007). Furthermore, the role of IGF-binding proteins (IGFBPs), which modulate the physiological actions of circulating and locally produced IGF-I, in the regulation of fasting metabolism and recovery growth is clearly lacking in these studies.

Our laboratory has shown previously that Mozambique tilapia grow faster in seawater than in fresh water (Ron et al. 1995; Sparks et al. 2003). Similarly, we have observed disruptions in the GH/IGF-I axis in fresh water tilapia due to fasting (Weber et al. 1999; Uchida et al. 2003). These findings, coupled with the observations that GH is integral to the ability of the tilapia to acclimate to seawater (McCormick 2001), suggest alterations in nutritional status may affect the GH/IGF-I axis in seawater-adapted tilapia more significantly than those raised in freshwater.

Chapter II of this dissertation described the effects of prolonged fasting on the GH/IGF-I axis in seawater-acclimated tilapia. Fasting of tilapia in seawater resulted in a state of GH resistance. This condition was characterized by elevated plasma GH with reduced levels of plasma IGF-I and liver IGF-I mRNA, indicating that tilapia adapt to periods of starvation by up-regulating catabolic endocrine pathways and reallocating

energy stores. Growth hormone has lipolytic actions independent of IGF-I, and elevated levels of GH have been shown to be a good indicator of a catabolic and growth-impaired state in vertebrate animals. Along with this rise in plasma GH, a coordinate reduction of IGF-I with no changes in hepatic expression of GH-R mRNA or SL-R mRNA further indicates an overall metabolic shift from anabolic to catabolic conditions in nutrient-deprived fish. Interestingly, the only differences between the effect of fasting on the GH/IGF-I axis in seawater or fresh water-acclimated tilapia (Chapter II vs. Chapter V) was in plasma GH levels and liver expression of GH-R mRNA. Plasma GH increased after 4 weeks of fasting in seawater with no change in GH-R or SL-R mRNA, while GH levels remained unchanged in fresh water with no change in SL-R mRNA, but an increase in GH-R mRNA levels. The higher plasma GH levels in seawater-acclimated tilapia may have caused a down-regulation in hepatic GH-R mRNA to control levels. Additionally, the increase in plasma GH in fasted seawater-acclimated fish supports an important role for GH in maintenance of seawater acclimation during a catabolic state in the Mozambique tilapia.

Ghrelin has been identified as a potent stimulator of GH release in numerous mammalian and non-mammalian vertebrates, and has been shown to act to link signals from the gastroenteropancreatic system in relaying metabolic information to the neuroendocrine system (Kaiya 2008). Ghrelin was first identified as the endogenous ligand for the "orphaned" growth hormone secretagogue-receptor (GHS-R) (Kojima 1999), and has since been isolated in a variety of mammalian and non-mammalian vertebrates (Kaiya 2008). Recently, two forms of homologous ghrelins were isolated

from the tilapia stomach (Kaiya et al. 2003). The aim of the work described in Chapter III was to characterize the physiological effects of these ghrelins both *in vivo* and *in vitro*. In agreement with observations in mammals and other fish, tilapia ghrelin stimulated GH release from primary cultures of pituitary cells. This stimulatory effect was blocked by pre-incubation with a ghrelin receptor antagonist, suggesting the stimulatory actions of ghrelin occurred through its receptor.

To further confirm a functional ghrelin-GHS-R system in the tilapia, we identified two subtypes of the GHS-R in the pituitary. Peripheral injections of tilapia ghrelin stimulated both plasma GH and IGF-I. This stimulatory effect of ghrelin on the GH/IGF-I axis appears to be conserved across vertebrates (Hickey et al., 1997). For the first time, we also observed the concurrent regulation of both the hepatic GH-R mRNA and SL-R mRNA levels by ghrelin injection in fish. Taken together, these *in vivo* findings suggest a possible role for ghrelin, SL, and the hepatic SL-R in short-term metabolism and energy balance in the tilapia.

Plasma ghrelin levels have been shown in mammals to rise preprandially and fall directly after a meal (Bagnasco et al. 2002; Kim et al. 2003; Tups et al. 2004). Similar changes have been observed in some, but not all, non-mammalian vertebrates (Jönsson et al. 2007; Kaiya et al. 2008). Recently, we failed to observe any change in plasma ghrelin after 1, 3, 5, or 7 days of fasting in the tilapia (Kaiya et al. 2008).

The studies in Chapter IV were designed to clarify the role of ghrelin levels in feeding and fasting. In contrast with observations in mammals and the goldfish (Kojima et al. 1999; Unniiappan et al. 2004a), no post-prandial change in plasma ghrelin was

observed in the tilapia. Similarly and in agreement with our previous studies, plasma ghrelin levels were unaltered by an "intermediate" fasting period (4 and 8 days). As shown in Chapter V, however, we observed significant increases in plasma ghrelin levels after prolonged fasting (2 and 4 weeks).

There appears to be considerable variation in the effects of fasting on ghrelin among species. There are also temporal differences (long-term vs. short-term) among homeotherms (mammals and birds), and poikilotherms (fish and amphibians) in the effects of fasting on ghrelin. Homeotherms, which regulate the temperature of their internal environment, typically have a higher metabolic rate than poikilotherms whose internal temperature varies with changes in the temperature of the external environment. Ghrelin levels increase rapidly (within 12-14 hours) in response to fasting in most homeotherms studied. By contrast, ghrelin levels do not increase until after several days or weeks of fasting in most poikilotherms (Kaiya 2008). Thus, these differences may derive from differences in the energy metabolism between the two classes of animals (Kaiya et al. 2008). Overall, the studies described in Chapters IV and V of this dissertation suggest a possible role for ghrelin as a long-term metabolic endocrine signal in the tilapia.

Alterations in diet have profound effects on the physiology of fish and vertebrate in general (Perez-Sanchez et al. 1994; Gomez-Requeni et al. 2004). Understanding the baseline endocrine parameters that occur in fish (farmed or wild) allows for the design of nutrient and hormone supplements, which will maximize growth. The existence of the compensatory growth response in many vertebrate species suggests that, much of the

time, animals maintain growth rates which are sub-maximal (Ali et al. 2003). Understanding the underlying control mechanisms, which direct these varying rates of growth, has obvious implications in aquaculture. The use of varied patterns of feeding to optimize the compensatory growth response in farmed fish is a widely used strategy (Ali et al. 2003). Liver and muscle expression of the two GH-R subtypes has not been well characterized in fish (Jiao et al. 2006; Gabillard et al. 2006; Fukamachi et al. 2007). Hence, observed changes in these genes during fasting and re-feeding will yield important information.

Chapter V of this dissertation described the effects of prolonged fasting and subsequent re-feeding on plasma levels of GH and IGF-I, concomitantly with changes in the expression of GH, SL, GH-R, SL-R, IGF-I, and IGF-II mRNA levels in pituitary, muscle, and liver tissue. Plasma IGF-I levels were reduced during fasting, and levels recovered with re-feeding, while plasma GH remained unchanged throughout the same period suggesting GH resistance. These changes in the GH/IGF-I axis appear to be conserved in vertebrates. What has been little studied, however, is the coordinate regulation of GH, IGF-I, and IGF-II in adult vertebrates. The metabolic actions of IGF-II are virtually unknown in fish (Reinecke et al. 2005). Both IGFs (IGF-I, and II) activate cell proliferation, suggesting that they share some overlapping physiological roles in fish (Pozios et al. 2001; Reinecke et al. 2005). Tissue expression of IGF-I and IGF-II mRNA appeared to be regulated differentially by nutritional status in the tilapia suggesting possible divergent roles for these mitogens during fasting.

We have recently cloned SL in the tilapia and observed significant reductions in pituitary SL mRNA levels after fasting, suggesting a possible role for this hormone in energy metabolism (Uchida et al. 2008). Generalization of this conclusion requires caution, however, as the effects of fasting on SL levels in salmonids, for example, have yielded inconsistent results (Company et al. 1999; Pottinger et al. 2003). Levels of pituitary SL mRNA were suppressed during 4 weeks of re-feeding in the tilapia, suggesting a possible role for SL in metabolism during energy surplus.

Modest and inconsistent changes were also detected in liver expression of GH-R, SL-R, and IGF-I mRNA during fasting and re-feeding. In contrast, muscle GH-R, SL-R, and IGF-I mRNA levels appeared to be regulated acutely and decisively by nutrient availability during the same period, suggesting a complimentary role of these genes in muscle growth and metabolism. It is difficult to make generalizations about teleost GH-R or SL-R regulation based on these results because they appear to be functionally and genetically diverse among fish (Fukamachi et al. 2007). Future studies which estimate plasma SL levels in the tilapia under similar metabolic conditions, as well as tissue-specific receptor binding studies are required to truly determine the relationships between GH, SL, GH-R, and SL-R will help to clarify the roles of these important endocrine factors.

Fasting results in GH resistance in the both seawater and fresh water-acclimated tilapia which characterized by elevated or unchanged plasma levels of GH in combination with reduced plasma IGF-I and liver expression of IGF-I mRNA. These findings indicate that GH resistance is a conserved mechanism among vertebrates. In tilapia, GH

resistance is likely to allow the mobilization of energy reserves along with the suppression of growth. Furthermore, plasma IGF-I and muscle expression of IGF-I mRNA and GH-R mRNA levels change within week of fasting, while levels are recovered rapidly during re-feeding. These findings suggest that these physiological parameters may be useful and sensitive indicators of nutritional status in tilapia. Pituitary expression of SL mRNA, as well as liver and muscle expression of IGF-II, are reduced during re-feeding suggesting that these hormones may play a role in energy substrate deposition, and that they may also be useful indicators of energy surplus.

This dissertation provides novel information about the neuroendocrine modulation of somatic growth by fasting and re-feeding in a major aquaculture species, the tilapia. Results herein suggest ghrelin modulates GH release from the pituitary via a functional ghrelin-GHS-R system. Ghrelin also influences plasma IGF-I as well as hepatic GH-R mRNA and SL-R mRNA levels, suggesting roles for all of these parameters in energy metabolism. Importantly, my findings suggest that ghrelin, SL, and IGF-II may be a long-term indicator of negative energy balance in the tilapia, while muscle IGF-I and GH-R levels are reliable short-term indicators of energy homeostasis. The results presented herein prompt further questions regarding additional aspects of regulatory physiology and metabolic control in vertebrates, including changes in signaling mechanisms, physiological relevance of changes in closely related hormones and their cognate receptors, and the identification of candidate growth genes to improve somatic growth in finfish.

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