

**GHRELIN STIMULATES GROWTH HORMONE AND PROLACTIN RELEASE  
IN THE TILAPIA,  
*OREOCHROMIS MOSSAMBICUS***

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I dedicate this thesis to my father, who remains a constant inspiration to me.

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## ABSTRACT

Ghrelin (Ghr) is a gut-brain peptide with potent growth hormone (GH)-releasing and orexigenic activities. Ghrelin is synthesized mainly in the oxintic mucosal cells of the stomach, but is expressed to varying degrees in many other tissues, thus suggesting both endocrine and paracrine functions. Ghrelin is the endogenous ligand of the growth hormone secretagogue (GHS) receptor. The characterization of ghrelin's actions has demonstrated that the control of growth hormone cell function and growth is more complex than previously thought. Recently, two forms of ghrelin, ghrelin-C8 and -C10, were identified in the tilapia, *Oreochromis mossambicus*. The present study describes *in vitro* and *in vivo* effects of the endogenous ghrelins on the release of GH, prolactin (PRL), and insulin-like growth factor-I (IGF-I) in the tilapia. Ghrelin-C8 (100 nM) stimulated GH release *in vitro* from primary culture of cells prepared from proximal pars distalis containing GH cells and also from whole pituitary after 4 and 8 h. Both forms of ghrelin (100 nM) stimulated GH release from organ-cultured pituitaries after 4 and 8 h, although effect of ghrelin-C8 was not significant at 4h. Lower doses of ghrelin-C10 (0.1 and 1 nM) stimulated PRL release from organ-cultured pituitaries after 4 and 8 h. No effect was seen at higher doses (10 and 100 nM). Ghrelin-C8 was without effect on PRL release at any dose. The GHS-receptor-specific antagonist [D-Lys<sup>3</sup>]-GHRP-6 significantly blocked the stimulatory effect of both forms of ghrelin on GH release from primary cell incubations of pituitary cells. No effect of GHRP-6 was observed on PRL release. Intraperitoneal injection of ghrelin-C10 at 1ng/g body weight elevated plasma GH levels 5 and 10 h. Plasma levels of IGF-I were also increased by both forms of

ghrelin (1 ng/g) after 10 h following injection, whereas no effect was seen on plasma PRL. These results indicate that ghrelin is involved in regulation of GH release through GHS receptor, although it is still possible that ghrelin stimulates IGF-I release acting directly on hepatocytes.

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## CHAPTER I

### INTRODUCTION

In teleosts, secretion of growth hormone (GH) is regulated by several hypothalamic factors that are influenced by the physiological state of the animal (Peng et al., 1997). For example, growth hormone-releasing hormone (GHRH), gonadotropin-releasing hormone (GnRH), neuropeptide Y, and thyrotropin-releasing hormone (TRH) stimulate the release of GH, and effects of which 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 (Peng et al. 1997). The regulation of prolactin (PRL) is equally as complex. Prolactin, for example, is inhibited by somatostatin, dopamine, and urotensin II (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1982), and stimulated by TRH and estradiol-17 $\beta$  (E<sub>2</sub>) (Wigham et al., 1984; Barry et al., 1986). Priming of PRL-secreting cells by pre-incubation with E<sub>2</sub> enhances the ability of TRH to stimulate PRL release from organ-cultured tilapia pituitaries (Barry et al. 1986). In teleosts, GH and PRL play regulatory roles in osmoregulation, growth, development and reproduction (McLean et al., 1993; Blazquez et al., 1998). In sum, the multifactoral function and regulation of both GH and PRL make it difficult to clarify the mechanisms that regulate their release from the pituitary.

Many of the actions of GH in all vertebrates, are mediated to an important extent by insulin-like growth factor-I (IGF-I), a mitogenic factor produced primarily in the liver (Jones et al., 1995; LeRoith et al., 1995; Duan 1997; Lupu et al., 2001). As with GH, the

nutritional state of the animal strongly influences circulating levels of IGF-I (Tannenbaum et al., 1983; Bermann et al., 1994). Plasma levels of IGF-I and liver and intestine mRNA levels of IGF-I are reduced in fasted barramundi (Matthews et al., 1997) and salmon (Moriyama et al., 1994), and restored by refeeding.

In 1999, Kojima and co-workers identified an endogenous ligand, ghrelin, for a previously characterized orpin receptor known as the growth hormone secretagogue receptor (GHSR) (citation), which they isolated from rat stomach (Kojima et al. 1999). Ghrelin has been shown to potently stimulate GH release *in vitro* in rat, bullfrog, chicken and tilapia (Kojima et al. 1999; Kaiya et al. 2001, Kaiya, 2003; Riley et al. 2002) as well as *in vivo* GH release in rat, human, and chicken (Kojima et al. 1999; Hataya et al. 2001; Kaiya et al. 2002). Ghrelin has also been shown to stimulate prolactin (PRL) release *in vivo* in bullfrog and human (Takaya et al. 2000; Kaiya et al. 2001) and *in vitro* release in tilapia (Kaiya et al. 2003a).

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 (Jones et al. 1995; LeRoith et al. 1995; Duan 1997; Lupu et al. 2001). However, ghrelin's role in IGF-I regulation remains unclear. 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 to wild-type littermates, suggesting that ghrelin is a regulatory factor for IGF-I release. Other evidence suggests that ghrelin may act on the GH/IGF-I axis by modulating the "set-point" of GHRH neurons. Conversely, IGF-I has recently been shown to directly down-regulate GHS-receptor

mRNA levels in rat pituitary cells, suggesting IGF-I may modulate GH secretion through modulation of expression of the GHS-receptor (Kamegai et al. 2005).

Recently, two forms of ghrelin, ghrelin-C8 and -C10, were isolated from tilapia stomach (Kaiya et al. 2003b). Similar to all ghrelins isolated thus far, tilapia ghrelins possess an acylated modification on the Ser<sup>3</sup> residue (Kojima et al. 1999; Kaiya et al. 2001, Kaiya, 2002, Kaiya, 2003). The major form of tilapia ghrelin (ghrelin-C10) possesses an n-decanoic modification which is similar to that found in the eel, chicken, and bullfrog (Kaiya et al. 2001, Kaiya, 2002, Kaiya, 2003), whereas in rats, the major form of ghrelin possesses an n-octanoic modification (Kojima et al. 1999). This 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 is also found in the tilapia (ghrelin-C8), existing in the stomach in lower abundance than ghrelin-C10 (Kaiya et al. 2003a). To date, tilapia is the only animal to possess both C8 and C10 forms of ghrelin. In the present study, we examined the *in vivo* and *in vitro* effects of both forms of native ghrelin on GH, IGF-I, and PRL release in the tilapia.

## CHAPTER II

### MATERIALS AND METHODS

#### *Animals*

Male tilapia (*Oreochromis mossambicus*), weighing 50-80 g, 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 their body weight per day twice daily with Silver Cup Trout Chow (Portco Corporation, Vancouver, WA). 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 either posterior pars distalis (PPD) or whole pituitary. Pituitaries were collected aseptically in isotonic medium (330 mosmol/kg H<sub>2</sub>O, Krebs bicarbonate-Ringer solution, pH 7.4) as described by Wigham et al. (1977), supplemented with penicillin (100 IU/ml), streptomycin (0.1 mg/ml) and nystatin (250 IU/ml, Sigma, St. Louis, MO). The PPDs were dissected as described by (Nishioka et al. 1985). Whole pituitaries and PPDs were pooled, diced with a sterile razor, 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). During trypsinization, tissues were aspirated frequently through a pipette 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 their viability assessed by trypan blue exclusion. Cells were then plated at a density of  $4.0 \times 10^5$  cells/well into a 24-well plate (Falcon, Primaria 24, Becton Dickinson, Franklin Lakes, NJ) at a volume of 300  $\mu$ l per well of isotonic medium supplemented with 10% v/v fetal bovine serum. The cells were pre-incubated for 4 days at 26-28°C under a humidified atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>, with one change of culture medium at 48 h post-plating. Prior to each experiment, cells were washed once with 300  $\mu$ l antibiotic-antimycotic-free and serum-free medium. A final 300  $\mu$ l volume was added containing either tilapia ghrelin-C8 (100 nM), GHRH (10 nM, 1-29, Sigma) or control medium without hormones. Incubated medium was removed at 4 h and replaced with fresh medium containing various peptides. The culture was terminated at 8 h, and hormone release was quantified for the 0-4 and 4-8 h intervals. Growth hormone and PRL release was expressed as secretion per volume medium (ng/ml). Medium samples were stored at -20 °C for further analysis of GH and PRL release by homologous radioimmunoassays.

To examine whether ghrelin acts through the GHS-receptor, dispersed whole pituitaries were exposed to the GHS-receptor antagonist [D-Lys<sup>3</sup>]-GHRP-6 (Sigma). On the day of the treatment, cells were pre-incubated for 1 h with either control medium or medium containing [D-Lys<sup>3</sup>]-GHRP-6 (10  $\mu$ M). Pre-incubation medium was then aspirated and replaced with fresh medium containing either tilapia ghrelin-C8 or -C10 (100 nM). Following 6-h incubation, the culture was terminated, and the medium was stored at -20° C for later analyses of GH and PRL.

### *Pituitary organ culture*

Whole pituitaries were collected aseptically in isotonic medium as described above but without nystatin and fetal bovine serum and pre-incubated individually in a 96-well plate for 20 h in 200  $\mu$ l medium under a humidified atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The pre-incubation medium was removed and replaced with fresh medium (200  $\mu$ l) containing tilapia ghrelin-C8 or -C10 (0, 0.1, 1, 10, or 100 nM) or GHRH (10 nM). Medium was removed at 4 h and replaced with fresh medium containing the various treatments. The culture was terminated at 8 h, and hormone release was quantified for the 0-4 and 4-8 h intervals. Growth hormone and PRL release was expressed secretion per unit body weight (ng/100g BW) for organ-cultured pituitaries. Medium samples were stored at -20° C until analysis of GH and PRL contents.

### *Injection experiment and blood sampling*

Prior to the experiment, fish were transferred to oval 60 l flow-through freshwater tanks. The fish were allowed to acclimate for 14 days, during which time they were fed to satiety once daily and food was withheld 24 h before the experiment. They 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  $\mu$ l/g body weight for all treatments. Blood was collected by caudal puncture using 1 ml heparinized syringes at 5 and 10 h post-injection, and plasma was separated by centrifugation at 10,000 rpm for 15 minutes, and stored at -20° C for later analyses of GH, PRL, and IGF-I.

### *Radioimmunoassays*

Plasma levels of GH and PRL<sub>188</sub> were estimated by homologous radioimmunoassays (Ayson et al. 1993) as modified by Yada et al. (1994). Total IGF-I levels in the plasma were measured by a heterologous RIA, following Moriyama et al. (1994) and Shimizu et al. (1999) with some modifications (Kajimura et al. 2002). In brief, 25 µl of plasma was extracted with 100 µl of acid-ethanol solution (87.5% ethanol and 12.5% 2 N HCl, v/v) for 30 min at room temperature. Then, the samples were neutralized with 50 µl of 0.855 M Tris base, and centrifuged at 2000 g for 30 min at 4 °C. Salmon IGF-I (GroPep, Adelaide, Australia) was used as a standard. Series of standards or extracted plasma samples were diluted in 10 mM phosphate buffer (pH 7.3) containing 0.1% Triton-X 100, 1% BSA (Sigma), and 0.01% sodium azide (Sigma) to a final volume of 60 µl. Fifty microliters of primary antibody (anti-barramundi IGF-I antibody, GroPep, Adelaide, Australia, 1:10,000 dilution) and 50 µl of iodinated salmon IGF-I (13,000-15,000 cpm) dissolved in assay buffer were added to each tube. Following overnight incubation at 4 °C, the antibody-bound hormone complexes were separated from the free tracer by adding 0.5% Pansorbin (Calbiochem, La Jolla, CA) in 100 µl of the assay buffer. The tubes were centrifuged, aspirated and the pellet was counted in a gamma counter (Cobra II, Packard, Meriden, CT).

### *Statistics*

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

## CHAPTER III

### RESULTS

#### *In vitro effects of tilapia ghrelins*

In the first experiment, effects of ghrelin-C8 (100 nM) and GHRH (10 nM) were examined using dispersed cells from the PPD or whole pituitary. Ghrelin-C8 stimulated GH release significantly from both dispersed PPD cells and those from whole pituitaries after 4 and 8 h (Figs. 1A and B). Growth hormone release was also stimulated by GHRH from dispersed PPD cells and dispersed whole pituitary cells except for release from PPD cells during 0-4 h of incubation. Absolute amounts of GH released (ng/ml) in control medium during 0-4 and 4-8 h of incubation were significantly ( $P < 0.05$ ) smaller in the dissociated cells from the whole pituitary compared with those from PPD. This is likely to be due to the fact that GH cells are segregated into the PPD and when plated and the same density ( $4.0 \times 10^5$  cells/well), PPD culture wells would be expected have higher numbers of GH cells than would wells containing cells from whole pituitaries. There was no effect of either form of ghrelin on PRL release from dispersed whole pituitary cells (Fig. 1C).

Inasmuch as incubations of cells from whole pituitaries and PPD showed similar response patterns, organ-cultured pituitaries were used in subsequent experiments to examine dose-response effects of ghrelin-C8 and -C10 on GH and PRL release. Growth hormone release was normalized to body weight of the fish; we have shown earlier a highly significant correlation between amount of the hormone released from organ-

cultured pituitary and body weight (Eckert et al. 2003; Uchida et al. 2004). As shown in Fig. 2, both ghrelin-C8 and -C10 at 100 nM as well as 10 nM GHRH significantly stimulated GH release. No effect of ghrelin was observed at doses 0.1-10 nM. On the other hand, ghrelin-C10 at doses of 0.1 and 1 nM significantly stimulated PRL release during the first 4 h of incubation (Fig. 3). PRL release declined considerably during the next 4 h, but significant stimulation was still observed at 0.1 and 1 nM. Ghrelin-C8 had no effect on PRL release at any dose tested, while GHRH significantly ( $P < 0.05$ ) stimulated PRL release at 4h (Fig. 3).

#### *Effects of GHS-receptor antagonist*

Effects of GHS-receptor-specific antagonist [D-Lys<sup>3</sup>]-GHRP-6 on GH and PRL release were examined using dissociated cells from the whole pituitary (Fig. 4). Pre-incubation of dissociated cells with [D-Lys<sup>3</sup>]-GHRP-6 (10  $\mu$ M) for 1 h significantly blocked the stimulatory effect of tilapia ghrelin-C8 and -C10 (100 nM) following 6 h of incubation. There was no effect of either [D-Lys<sup>3</sup>]-GHRP-6 or tilapia ghrelins on PRL release (Fig. 4).

#### *In vivo effects of tilapia ghrelins*

Intraperitoneal injection of ghrelin-C10 (1 ng/g body weight) significantly elevated plasma GH levels 5 and 10 h post-injection (Fig. 5). By contrast, ghrelin-C8 and GHRH had no effect on plasma GH levels. Plasma levels of PRL were unaffected by any treatment at either time point. Ghrelin-C8 and -C10 significantly elevated plasma levels

of IGF-I 10 h post-injection, whereas no effect was seen after 5 h. Plasma IGF-I levels were also elevated by GHRH (10 ng/g) significantly after 10 h.

Figure 1. Effect of tilapia ghrelin-C8 and GHRH on GH release from dispersed PPDs (A), dispersed whole pituitaries (B), and on PRL release from dispersed whole pituitaries (C). Cells were pre-incubated for 4 days in fetal bovine serum-supplemented medium, and then exposed to 100 nM ghrelin or 10 nM GHRH in serum-free medium for 8 h. Incubation medium was changed at 4 and 8 h. Values are mean  $\pm$  SEM, \*, \*\*, \*\*\* significantly different from the control (0 nM) at each time point at  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively (n=8).

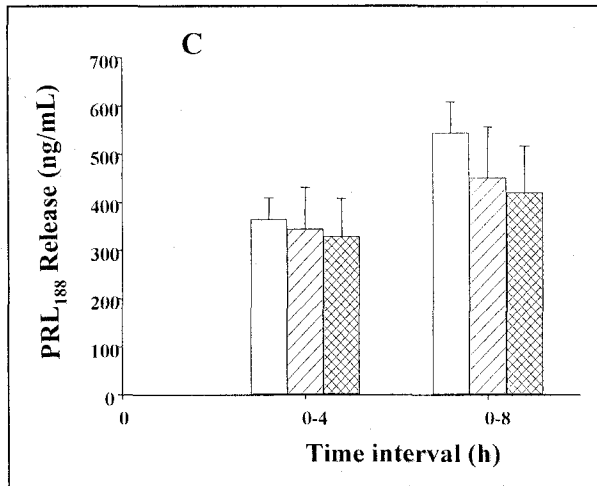
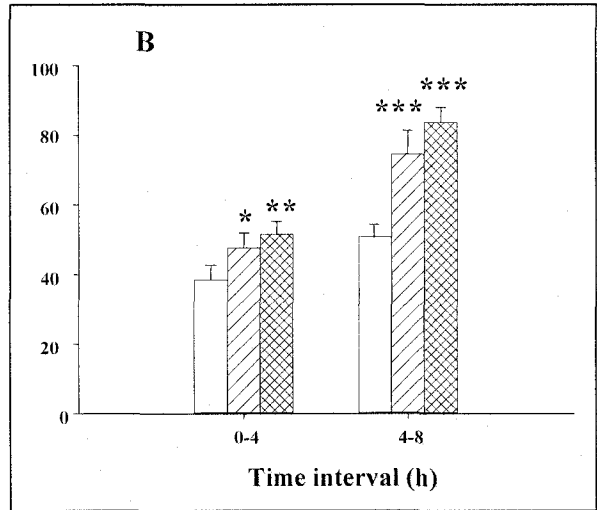
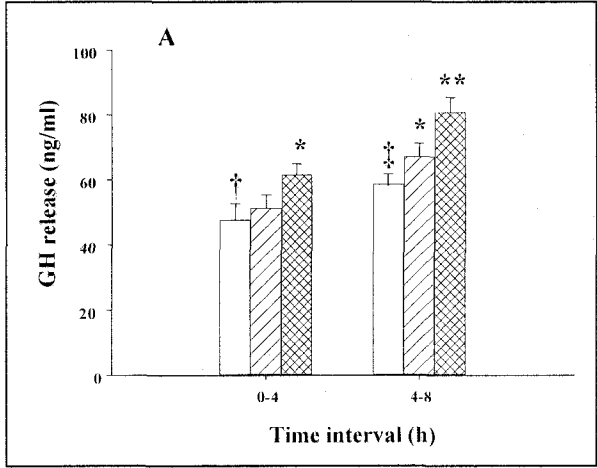


Figure 2. Effect of tilapia ghrelin-C8 (A) and ghrelin-C10 (B) on GH release from organ-cultured pituitaries. Whole pituitaries were pre-incubated for 20 h in serum-free isotonic medium (330 mOsm), and then exposed to 0.1-100 nM tilapia ghrelin-C8, -C10, or GHRH (10 nM) for 8 h. Incubation medium was change at 4 and 8 h. Values are mean  $\pm$  SEM, \*, \*\*, \*\*\* significantly different from the control (0 nM) at each time point at  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively (n=8).

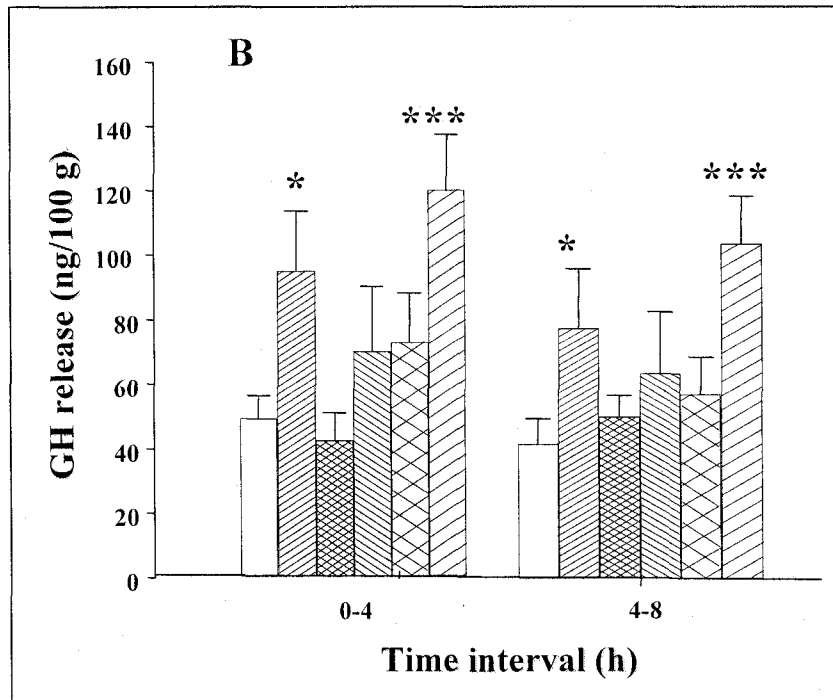
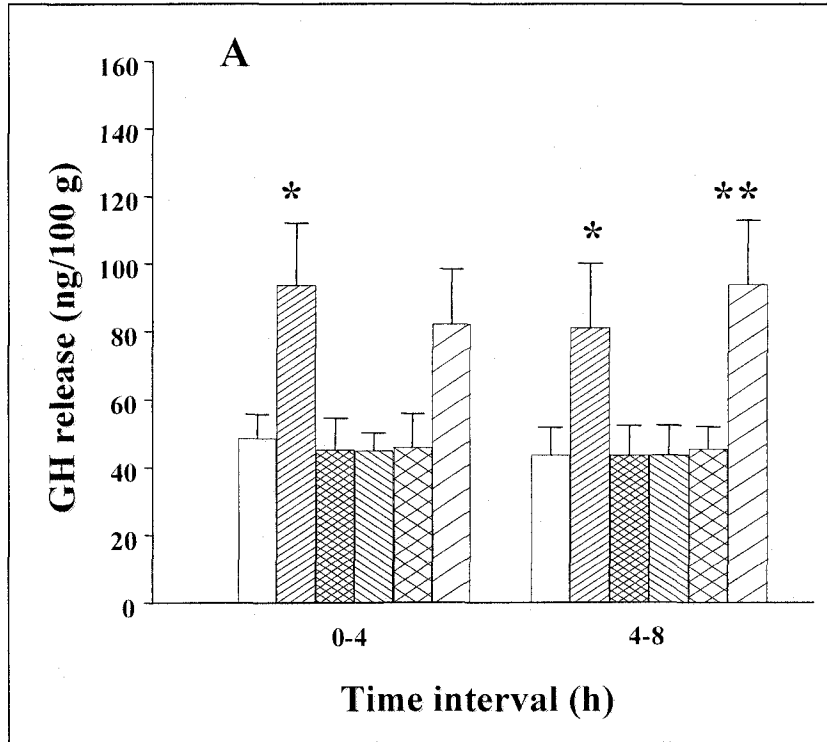
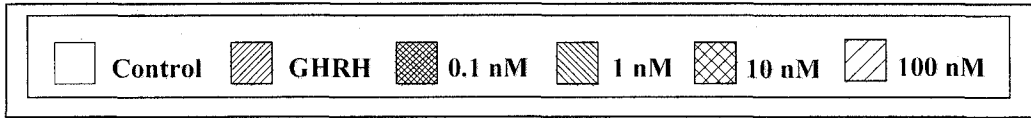


Figure 3. Effect of tilapia ghrelin-C8 (A) and ghrelin-C10 (B) on PRL<sub>188</sub> release from organ-cultured pituitaries. Whole pituitaries were pre-incubated for 20 h in serum-free isotonic medium (330 mOsm), and then exposed to 0.1-100 nM tilapia ghrelin-C8, -C10, or GHRH (10 nM) for 8 h. Incubation medium was change at 4 and 8 h. Values are mean  $\pm$  SEM, \*, \*\*\* significantly different from the control (0 nM) at each time point at P < 0.05, and P < 0.001, respectively (n=8).

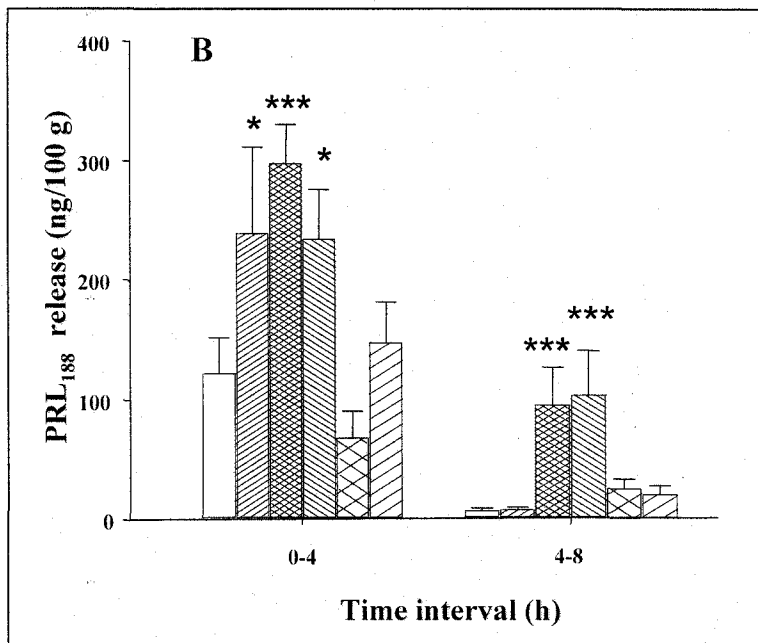
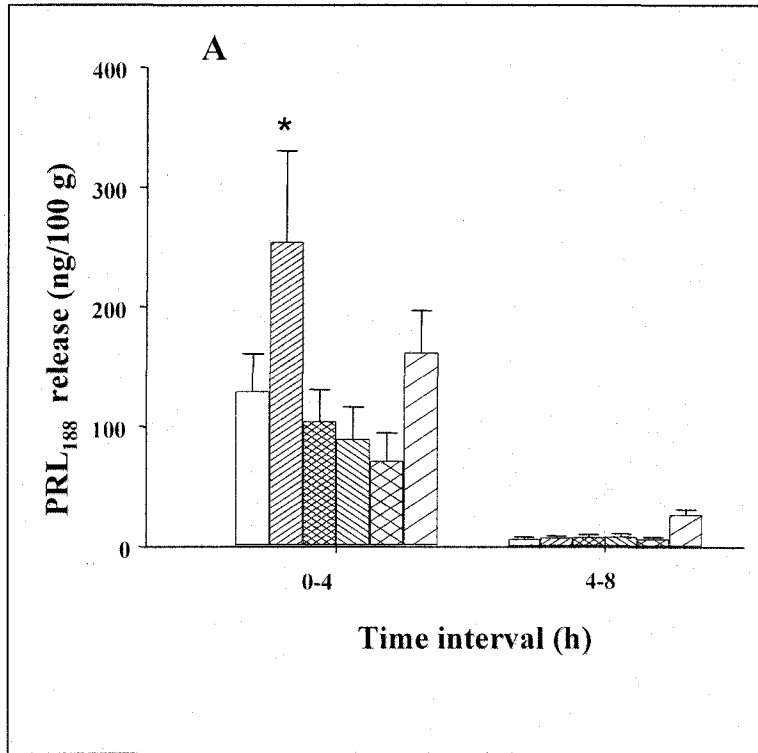
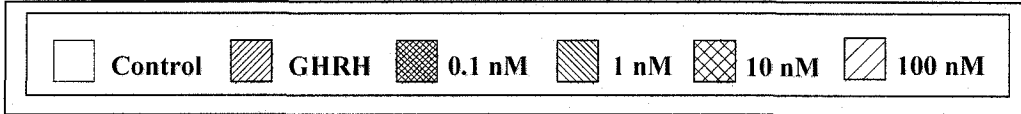


Figure 4. Effects of the growth hormone secretagogue-receptor-specific antagonist, [D-Lys<sup>3</sup>]-GHRP-6 on GH (A) and PRL (B) release from dispersed tilapia pituitary cells. Cells were pre-incubated for 4 days in fetal bovine serum-supplemented medium, pre-treated with [D-Lys<sup>3</sup>]-GHRP-6 (10  $\mu$ M) for 1 h in serum-free medium, and then incubated with tilapia ghrelin-C8, -C10, or medium control for 6 h. Values are mean  $\pm$  SEM, \*\*, \*\*\* significantly different from the control (0 nM) at each time point at  $P < 0.01$ , and  $P < 0.001$ , respectively (n=8).

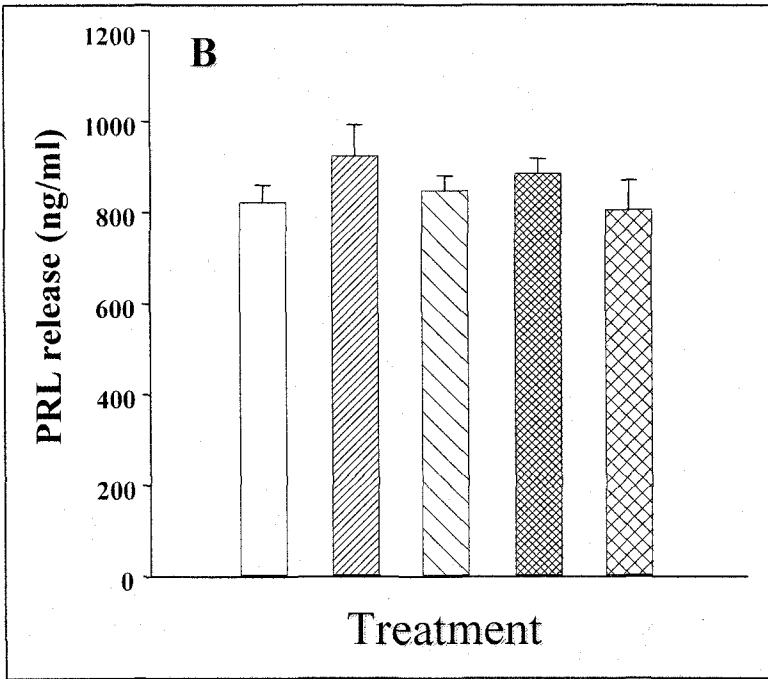
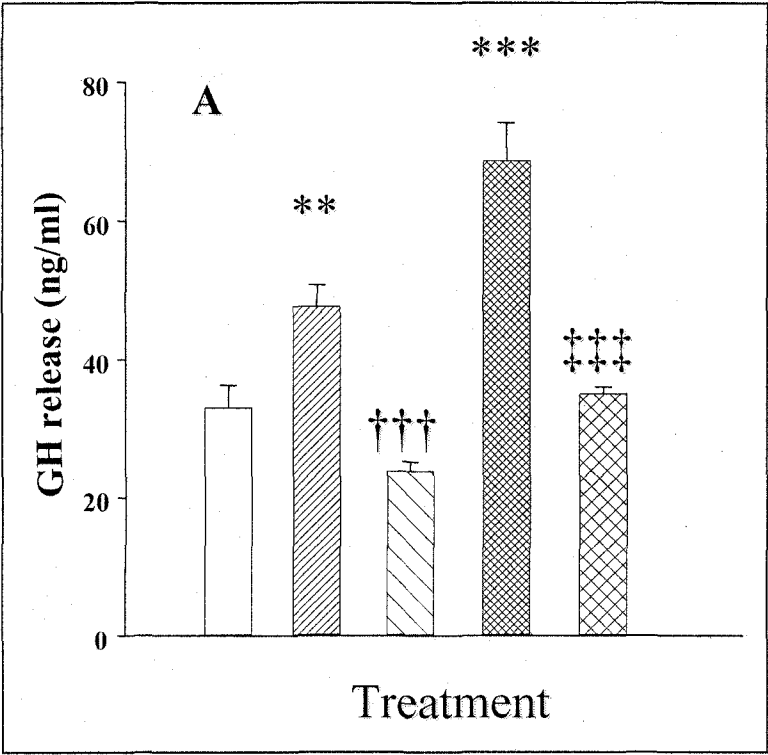
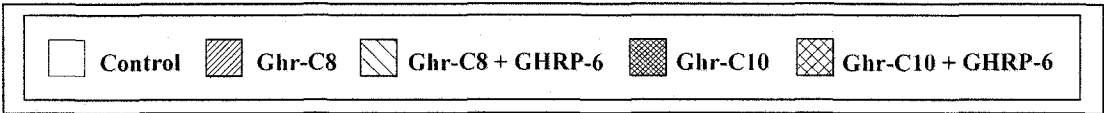
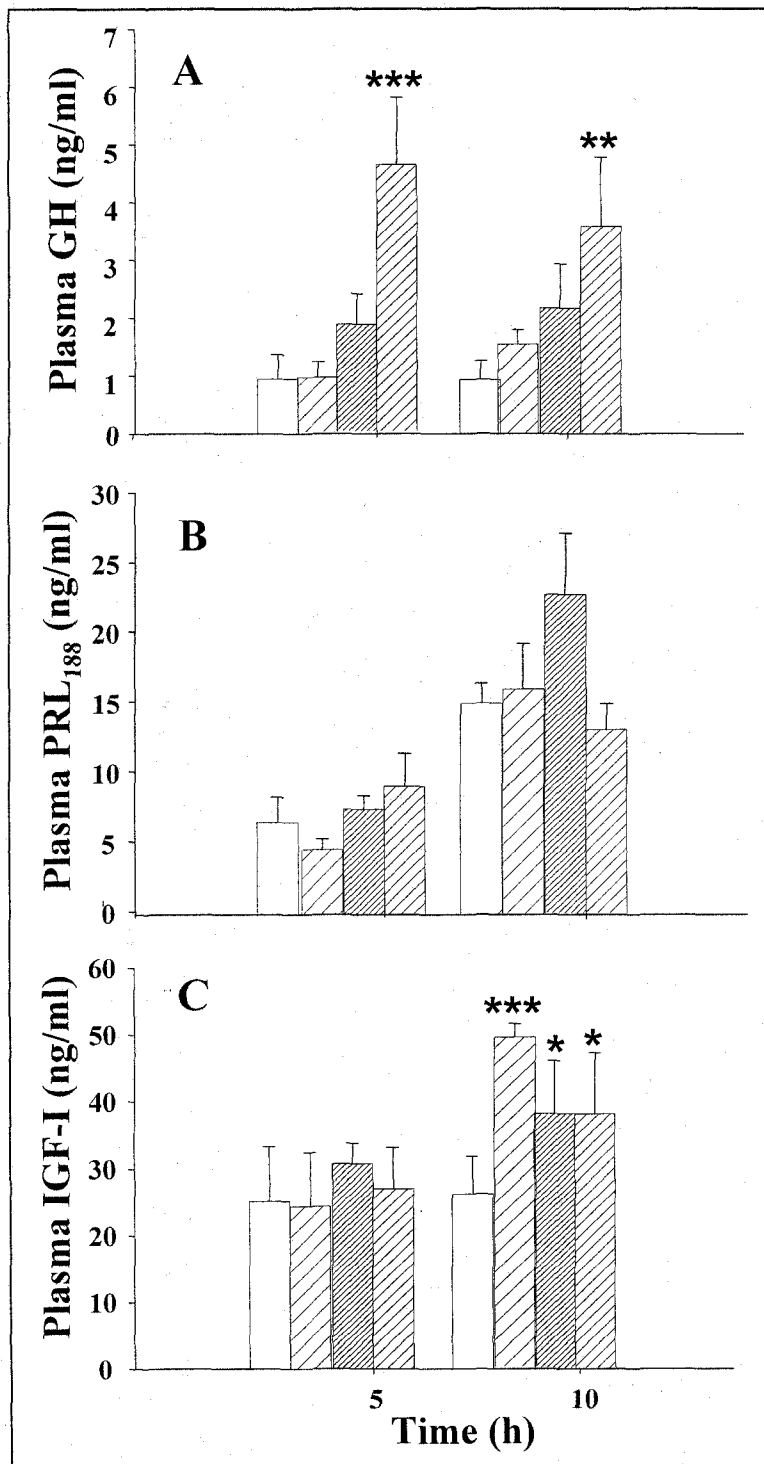


Figure 5. Effects of intra-peritoneal injection of tilapia ghrelin-C8 (1 ng/g), -C10 (1 ng/g), GHRH (10 ng/g), or vehicle (saline) on plasma levels of (a) GH, (b) PRL, and (c) IGF-I sampled at 5 and 10 h post injection. Values are mean  $\pm$  SEM, \*, \*\*, \*\*\*significantly different from the control (0 nM) at each time point at  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively (n=6).



## CHAPTER IV

### DISCUSSION

The present study focused on the effects of the two native forms of tilapia ghrelin on GH and PRL release both *in vitro* and *in vivo*. Ghrelin-C8 (100 nM) stimulated GH release from primary cultures of cells prepared from PPD containing GH cells and also from whole pituitary after 4 and 8 h. Both forms of ghrelin (100 nM) stimulated GH release from organ-cultured pituitaries after 4 and 8 h. Low doses of ghrelin-C10 (0.1 and 1 nM) stimulated PRL release from organ-cultured pituitaries after 4 and 8 h. Intraperitoneal injection of ghrelin-C10 stimulated GH release after 5 and 10 h. Plasma levels of IGF-I were also increased by both forms of ghrelin (1 ng/g) after 10 h following injection, whereas no effect was seen on PRL release. The stimulatory effect of ghrelin on GH release from primary culture of pituitary cells was abolished by the GHS-receptor antagonist [D-Lys<sup>3</sup>]-GHRP-6, indicating that ghrelin's actions are mediated through a GHS-receptor. No effect of GHRP-6 was observed on PRL release. Taken together, these results suggest that ghrelin acts on the GH cell independently of GHRP-6, mostly likely through the GHS receptor.

Organ culture allows for intimate intercellular cytoplasmic connections found *in vivo* to be retained. Paracrine and autocrine interactions are also maintained in this system, thus allowing for physiological responses to regulatory factors that are similar to those found *in vivo*. In organ culture, however, the special arrangement of cells may not allow them to be exposed to hormones in the incubation medium either simultaneously or

to the same degree. We have reported earlier that the release of GH and PRL from dispersed pituitary cells of the tilapia was similar to that from organ-cultured pituitary during 7 days of culture (Yada et al. 1995). According to Melamed et al. (1999), however, dispersed pituitary cells of tilapia hybrids (*Oreochromis aureus* × *O. niloticus*) containing a rich population of gonadotrophs showed characteristic cytoplasmic extensions not seen in the somatotrophs, which appeared small and failed to form aggregates. Many somatotrophs had undergone nuclear condensation and fragmentation typical of apoptosis. They suggested that estradiol and IGF-I, both generated from gonadotrophs or other cells may exert antiapoptotic effects on somatotrophs. For this reason, we have first compared effects of ghrelin-C8 and GHRH between dispersed cells from PPD and those from whole pituitary. Ghrelin-C8 and GHRH significantly stimulated GH release from both dispersed PPD cells and those from whole pituitaries after 4 and 8 h, absolute amounts of GH released in control medium were significantly smaller in the dissociated cells from the whole pituitary compared with those from PPD. Since the same number of the cells ( $4.0 \times 10^5$  cells/well) was plated in each well, numbers of somatotrophs are likely to be less in primary culture of whole pituitary than in that of dispersed PPD cells. The differential responsiveness between whole pituitary and PPD cell populations in the response of somatotrophs to ghrelin or GHRH would seem to suggest that paracrine or autocrine factors present in whole pituitary cell populations, but absent in PPD cell incubations, may preserve responsiveness of the somatotrope population to regulatory factors.

In this study, significant stimulation of GH release by two forms of tilapia ghrelin were observed in both organ-cultured, and dispersed pituitary cells during 4 and 8 h of

incubation. In a previous study, we have observed that rat ghrelin stimulates GH release from organ-cultured tilapia pituitaries after 4 h of incubation (Riley et al. 2002). Kaiya *et al.* (2003a) observed an increase in GH release from organ-cultured pituitaries by synthetic tilapia ghrelin-C8 after 2 h of incubation. In rats, however, ghrelin stimulated GH release within 15 min from dispersed pituitary cells (Kojima et al. 1999). The rapid (within 1 h) response to ghrelin was not observed in the tilapia (unpublished observation). Mechanisms underlying differential responsiveness of GH cells to ghrelin between rats and tilapia are completely unknown.

Ghrelin-C10 also stimulated PRL release from organ-cultured pituitaries at doses of 0.1 and 1 nM after 4 and 8 h of incubation. A similar effect of synthetic octanoylated ghrelin (1 and 10 nM) on PRL release was observed by Kaiya et al. (2003a) after 4 and 8 h. Riley et al. (2002) observed stimulation of PRL release from organ-cultured pituitaries after 8 and 24 h by rat ghrelin (0.1-10 nM). The reason why higher doses of ghrelin-C10 (10 and 100 nM) were without effect is unknown. Ghrelin has also been shown to stimulate PRL release in human (*in vivo*) and bullfrog (*in vitro*) (Kaiya, 2001; Nagaya, 2001; Peino, 2000). To our knowledge, there is only one report demonstrating the lack of a PRL response to ghrelin in rats (Kojima et al. 1999). Kaiya et al. (2003b) also failed to observe a stimulation of PRL release *in vitro* and *in vivo* in rainbow trout. The lack of PRL response by rat prolactin cells to ghrelin may be due to the limited exposure time (15 min) of the cells to ghrelin. Inasmuch as PRL and GH are members of the same peptide family, descended from a common ancestor gene and possess actions that are similar or antagonistic (Goffin et al. 1996), it is likely that regulatory peptides such as ghrelin modulate their synthesis and secretion in a similar manner.

The actions of ghrelin are modulated by the GHS-receptor in mammals (Kojima et al., 1999; Sun et al., 2004). In the current study, the GHS-receptor antagonist [D-Lys<sup>3</sup>]-GHRP-6 completely abolished the stimulation of GH release by both forms of tilapia ghrelin from dispersed pituitary cells. A similar suppression of ghrelin's actions by [D-Lys<sup>3</sup>]-GHRP-6 was observed on superoxide production in phagocytic cells from peripheral blood leucocytes and head kidney leucocytes of the rainbow trout (Yada et al. 2005). The results in both instances indicate that ghrelin's effects are mediated through GHS-receptor mediation, in accord with our previous observation for the presence of a functioning GHS-receptor in the tilapia pituitary (Shepherd et al. 2000). In the present study, the failure of ghrelin to stimulate PRL release from tilapia PRL cells may be due to the high dose used (100 nM).

A single intraperitoneal injection of ghrelin-C10 at a dose of 1 ng/g body weight increased plasma GH levels after 5 and 10 h. Shepherd et al. (2000) observed a similar increase in plasma GH levels 6 and 12 h following an intraperitoneal injection of the GHS, KP-102, in the tilapia. They also reported that KP-102 failed to elicit an effect on plasma PRL, which is consistent with the results of the present study. A rapid effect of ghrelin, increasing plasma GH levels within 30 min has been observed in the rat (Kojima et al. 1999) and rainbow trout (Yada et al. 2005). We have been unable to observe a similar rapid action in the tilapia, the earliest time point ghrelin increases plasma GH being 5 h post-injection. The differences between the experiments may be due to species and environmental differences. Rainbow trout are coldwater, carnivorous species while omnivorous tilapia inhabit more temperate environments. The significantly shorter gut system in carnivorous species may require a more rapid response of GH secretion and

gastric motility than omnivorous or herbivorous species. Interestingly, neither human GHRH and ghrelin-C8 altered plasma GH levels at either time point.

Growth hormone is considered to be an anabolic hormone, increasing protein synthesis, and lean body mass, along with inducing lipolysis and fatty acid oxidation (Mauras et al. 2005). Many of the actions of GH are mediated by IGF-I, primarily produced in the liver (Jones et al. 1995; LeRoith et al. 1995; Duan 1997; Lupu et al. 2001). In the present study, GHRH and both forms of ghrelin significantly elevated plasma IGF-I levels 10 h post-injection. It is unclear why IGF-I levels were not affected by GHRH and tilapia ghrelin-C8, which also failed to increase plasma levels of GH. It is possible plasma GH levels were elevated at a different time point and had already become suppressed by negative feedback from elevated circulating IGF-I. At any rate, it is highly probable that the increased IGF-I levels after ghrelin injection is due to increased release of GH into circulation.

Recently, GHS-receptor isoforms have been identified in the liver of the human, chicken, and black seabream (Gnanapavan et al. 2002; Geelissen et al. 2003; Kageyama et al. 2005). The presence of the GHS-receptor in hepatocytes suggests a possible role for ghrelin in the regulation of circulating IGF-I. To our knowledge, there is no evidence to indicate ghrelin's direct role in IGF-I regulation. In a previous study using micro-osmotic pumps to deliver a continuous administration of ghrelin-C10, we observed a significant reduction in circulating levels of IGF-I after 21 days of treatment, suggesting that chronic exposure to ghrelin may generate a positive energy balance since ghrelin decreased plasma IGF-I and increased total fat content (Riley et al. 2005). There appears

to be a difference in responsiveness of the animal to chronic versus short-term exposure to ghrelin.

In summary, both forms of tilapia ghrelin stimulated the release of GH both *in vivo* and *in vitro*. Only ghrelin-C10 stimulated PRL release *in vitro* and increased plasma levels of GH, whereas neither forms of ghrelin elevated plasma PRL levels. These results provide further evidence of ghrelin's role in regulating GH and PRL secretion in fish, and suggest that ghrelin's action on GH release is mediated by GHS-receptor. Further studies into the dynamic expression of GHS-receptor may explain some of the effects described here and elucidate further the role of ghrelin in GH and PRL secretion in the tilapia.

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