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REGULATION OF PROLACTIN AND CHANGES IN PROLACTIN AND GROWTH HORMONE IN OSMOREGULATION, METABOLISM, AND REPRODUCTION IN THE TILAPIA, OREOCHROMIS MOSSAMBICUS.

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY MAY 1995

By Gregory Martin Weber

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E. Gordon Grau, Chairman
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Fred I. Kamemoto
I dedicate this dissertation to my wife, Sue, in appreciation of her constant support, and to my daughter, Katelyn, who likes to see her name in books.

(MMS)
ACKNOWLEDGEMENTS

I wish to extend my thanks to my thesis committee, and especially to my advisor, Prof. E. Gordon Grau for his support that allowed me to pursue a diversity of projects, including some which are not encompassed in this dissertation. I would like to acknowledge the contributions of Buel Rodgers with whom I ran the first experiments of this work. This dissertation also includes studies conducted with Drs. Hal Richman and Russell Borski. I would like to thank Drs. Tatsuya Sakamoto and Takashi Yada for teaching me the RIA procedures and Prof. Tetsuya Hirano for providing the antibodies. Among the many other people who have helped me along the way and may have also contributed to this work are: Benny Ron, Craig Morrey, Brian Shepherd, Steve Shimoda, Prof. Milton Stetson, Prof. Howard Bern, Prof. Yoshitaka Nagahama, Dr. Richard Nishioka, Prof. Nancy Sherwood, Dr. Matthew Grober, and Philippa Melamed.
ABSTRACT

These studies addressed the regulation of the prolactins (PRL) and growth hormone (GH) in the tilapia, Oreochromis mossambicus. Regulation of the PRL cell was investigated in the context of its potential roles in osmoregulation, metabolism, and reproduction. The in vitro release of the tPRLs (tPRL177 and tPRL188) was measured as well as changes in serum concentrations and pituitary content of the PRLs and GH.

These studies provide evidence that it is the osmotic gradient across the cell membrane that leads to an increase in osmotic pressure, and not osmolality per se, which accounts for the osmoreceptivity of the tilapia PRL cell. Prolactin release from pituitary tissues (rostral pars distalis; RPD) was inhibited when RPD were incubated in medium made hyperosmotic by the addition of NaCl or the membrane-impermeant molecule, mannitol; but not by the addition of the permeant molecules, urea or ethanol.

Injections of a gonadotropin-releasing hormone (GnRH) analog elevated serum concentrations of the tPRLs. Furthermore, native forms of GnRH stimulated PRL release in vitro with the following order of potency: chicken-GnRH-II > salmon-GnRH > seabream-GnRH. Prolactin release was accompanied by an increase in intracellular free Ca^{2+}, suggesting Ca^{2+} operates as a second messenger in mediat-

Serum concentrations of the tPRLs were elevated in response to fasting, followed by increases in serum concentrations and pituitary content of GH. Serum concentrations and pituitary content of GH and serum concentrations of PRL were highest late in the brooding phase of the reproductive cycle. Reduced food intake during brooding may contribute to changes in PRL and GH serum concentrations and pituitary content. Nevertheless, patterns of changes in serum and pituitary levels of the tPRLs and GH observed during the reproductive cycle had characteristics that were both similar to and distinct from patterns observed during fasting, suggesting the hormones may have actions in both metabolism and reproduction. In addition, salinity altered the patterns of changes in serum and pituitary levels of the tPRLs, but not GH, observed during the reproductive cycle.
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<tr>
<td>BW</td>
<td>Body weight</td>
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<tr>
<td>cAMP</td>
<td>3'5'-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGnRH-I</td>
<td>Chicken-gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>cGnRH-II</td>
<td>Chicken-gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>CF</td>
<td>Condition factor</td>
</tr>
<tr>
<td>cfGnRH</td>
<td>Catfish-gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol-17β</td>
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<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FW</td>
<td>Fresh water</td>
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<tr>
<td>GH</td>
<td>Growth hormone</td>
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<tr>
<td>GHRH</td>
<td>Growth hormone-releasing hormone</td>
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<td>GnRH</td>
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<tr>
<td>GSI</td>
<td>Gonadosomatic index</td>
</tr>
<tr>
<td>GtH</td>
<td>Gonadotropin</td>
</tr>
<tr>
<td>HSI</td>
<td>Hepatosomatic index</td>
</tr>
<tr>
<td>mGnRHa</td>
<td>Mammal-gonadotropin-releasing hormone analog</td>
</tr>
<tr>
<td>mOsmolal</td>
<td>Milliosmolal</td>
</tr>
<tr>
<td>mOsm</td>
<td>Milliosmolal</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>RPD</td>
<td>Rostral pars distalis</td>
</tr>
<tr>
<td>sbGnRH</td>
<td>Seabream-gonadotropin-releasing hormone</td>
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<td>sGnRH</td>
<td>Salmon-gonadotropin-releasing hormone</td>
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<tr>
<td>SL</td>
<td>Standard length</td>
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<tr>
<td>SW</td>
<td>Seawater</td>
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<tr>
<td>T</td>
<td>Testosterone</td>
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Chapter I

INTRODUCTION

Prolactin (PRL) and growth hormone (GH) are members of the same polypeptide family and are thought to be derived from a common ancestral gene (Niall et al., 1971). Not surprisingly, PRL and GH have many overlapping actions and are regulated by many of the same factors, although often in opposite directions. The spectrum of actions of each of these hormones throughout the vertebrates includes effects on reproduction, growth and development, metabolism, and osmoregulation (Clarke and Bern, 1980; Loretz and Bern, 1982; Sakamoto et al., 1993; Nicoll, 1982).

Often, the hormones regulate these different functions simultaneously. The cells secreting the hormones must, therefore, receive regulatory information from a variety of sources to be used in determining the secretion rate of the hormones. In order to understand how PRL and GH accomplish the regulation of diverse functions, changes in hormones that are related to these activities must be characterized and the factors regulating PRL and GH must be identified. This has been the thrust of my research and is the subject of this thesis.
Two tilapia prolactins

The rostral pars distalis (RPD) of the adenohypophysis of the tilapia releases two distinct PRL molecules which are encoded by different genes and are not derived through the differential processing of the same translation product (Specker et al., 1985b; Yamaguchi et al., 1988). The larger PRL (tPRL_{188}) contains 188 amino acid residues and has a molecular weight of 20.8 kDa, whereas the smaller PRL (tPRL_{177}) contains 177 amino acid residues and has a molecular weight of 19.6 kDa (Yamaguchi et al., 1988).

There is limited evidence that the two tPRLs may have distinct as well as overlapping functions. The two PRL molecules show similar effects in the tilapia sodium retaining assay, a well characterized bioassay that measures the osmoregulatory activity of PRL (Specker et al., 1985b). Nevertheless, Borski and colleagues (1992) have shown that the ratio of the total amount of tPRL_{188} to tPRL_{177} is greater in the pituitary of freshwater (FW)-adapted tilapia, Oreochromis mossambicus, compared with seawater (SW)-adapted tilapia. Specker and colleagues (1985a) found that tPRL_{188} and not tPRL_{177} has growth-promoting activity in the tilapia. On the other hand, Shepherd and colleagues (1994) found that injections of tPRL_{177} and not tPRL_{188} are effective at restoring
sulfate and thymidine incorporation in vitro by branchial cartilage of hypophysectomized tilapia. The assay is a well characterized assay for skeletal growth. Shepherd and colleagues also found that tPRL_{177}, but not tPRL_{188}, ovine-PRL or salmon-PRL, can displace GH from high-affinity, low-capacity binding sites in the tilapia liver. A functional distinction between the two PRLs in regards to reproduction or metabolism within the tilapia has not been elucidated. Rubin and Specker (1992) found similar activities of the two PRLs in affecting steroid release from testicular tissues of courting and non-courting male tilapia.

Tilapia pituitary gland morphology

The anatomical arrangement of the tilapia pituitary gland imparts certain advantages as a model to study PRL and GH cell physiology. The distinctive features of the tilapia pituitary are described below in the next section, followed with a description of how these features have been exploited to study PRL and GH cell function. The morphology of the tilapia pituitary gland has been described in detail (Dharmamba and Nishioka, 1968; Bern et al., 1975; Nishioka et al., 1988). Two features of the pituitary gland common to most teleosts including the tilapia, are strikingly different from those of mammals.
They are: 1) hormone-producing cell types are segregated into discrete areas of the pituitary gland, and 2) hypothalamic fibers innervate the adenohypophysis (Dharmamba and Nishioka, 1968; Bern et al., 1975; Ball, 1981). In the tilapia, the proximal pars distalis contains GH cells in addition to gonadotropin (GtH) and thyrotropin cells. The RPD contains PRL and adrenocorticotropin cells. The PRL cells are located in the anterior region of the RPD and adrenocorticotropin cells are located close to the neurohypophysis. Hypothalamic fibers innervate the proximal pars distalis and terminate in close proximity to the hormone-secreting cells. In contrast, hypothalamic fibers do not leave the neurohypophysis in the RPD, but terminate on an adjacent basement membrane. Separating the PRL cells and the hypothalamic fibers are the basement membrane, the adrenocorticotropin cells, and a layer of stellate cell processes (see review, Nishioka et al., 1988). Nishioka and colleagues (1988) pose the possibility that the stellate cells are involved in the movement of neurohormonal factors from the neurohypophysial nerve endings to the PRL cells.

Tilapia PRL and GH cells as models for studying PRL and GH cell function

Interactions among hormones in heterogeneous cell
populations complicate the study of the regulation of individual cell types. Segregation of cell types within the teleost pituitary facilitates separation of certain cell types for study. A tissue containing a nearly homogeneous population of PRL cells can be obtained for in vitro study by a simple dissection of the anterior RPD. The RPD tissue obtained in this way is 95-99% PRL cells. An additional attribute of the PRL cell which lends itself to study is its sensitivity to small physiological changes in osmolality. This sensitivity allows for the control of baseline release to facilitate the investigation of potentially important regulators of PRL secretion (cf. Grau et al., 1982). Cells of the proximal pars distalis can also be separated from cells of the pars intermedia and RPD by simple dissection. Separation of GH, GtH and thyrotropin cells from each other is more difficult. Currently, most investigators utilize mammalian clonal cell lines derived from PRL tumors to study the regulation of PRL cells (Tashjian et al., 1970; Lamberts and MacLeod, 1990; Sato et al., 1990). A concern with the use of tumor cell lines is that one can not be sure that they are studying normal cell function as opposed to tumor cell function.

Direct innervation of the tilapia adenohypophysis by hypothalamic neurosecretory fibers enables the tracing of fibers from the hypothalamus to the region of the
pituitary where the hormone is released. Since the hor-
mone-secreting cells of the pituitary are segregated, 
neurosecretory fibers can be followed to determine at
which cells the neurosecretory factors are released, and
therefore, may regulate. Furthermore, neurosecretory
factors reaching the pituitary can be easily identified
due to the high quantity of the factors in the nerve
terminals in the pituitary. I have taken advantage of
this feature and determined with colleagues, that sea-
bream-gonadotropin-releasing hormone is the most abundant
form of gonadotropin-releasing hormone (GnRH) in the
tilapia pituitary (Weber et al., 1994).

Hormonal regulation of teleost PRL and GH cell activity

The teleost PRL cell responds to many molecules in a
way that is similar to the manner in which the molecules
have been shown to be effective in mammals. Basal PRL
secretion in teleosts and mammals is under inhibitory
control from the hypothalamus. When connections with the
hypothalamus are severed at the pituitary stalk, as in
auto-transplants, the pituitary secretes high levels of
PRL. In mammals, this inhibition appears to be controlled
predominantly by dopamine (cf. Lamberts and MacLeod, 1990)
while in the tilapia, somatostatin appears to be the
predominant suppressor molecule (Grau et al., 1982, 1985).
Thyrotropin-releasing hormone stimulates PRL release in mammals and tilapia, while vasoactive intestinal peptide is a stimulator in mammals and an inhibitor in tilapia (Barry and Grau, 1986; Kelley et al., 1988; cf. Lamberts and MacLeod, 1990). A non-hypothalamic hormone, cortisol, has been shown to be a potent inhibitor of PRL release in the tilapia (Wigham et al., 1977; Borski et al., 1991).

More related to reproduction, it has been shown that the tilapia PRL cells are stimulated to release PRL in response to estradiol-17β (E₂) and testosterone (T), (Wigham et al., 1977; Barry and Grau, 1986; Borski et al., 1991). Other steroids including progestins have been found to have no effect on PRL release (Borski et al., 1991). Furthermore, stimulation of PRL cells by thyrotropin-releasing hormone was only observable following pretreatment with E₂ (Barry and Grau, 1986). Stimulation of PRL release from PRL cells by E₂ and T suggests an increase in PRL activity during phases of the reproductive cycle when circulating E₂ and T levels are elevated.

Control of GH cell activity in teleosts is not as well characterized as control of PRL cell activity (see review, Nishioka et al, 1988). In mammals, GH secretion is primarily under the control of hypothalamic regulators. Growth hormone-releasing hormone (GHRH) stimulates GH secretion and somatostatin inhibits GH secretion. It is
clear that the inhibitory role of somatostatin on GH cell activity has been conserved in teleosts including the tilapia (Fryer et al., 1979). Studies examining the effects of GHRH on GH release have been mixed. Nevertheless, GHRH was found to be a potent stimulator of GH release in the only study to use native GHRH (Vaughan et al., 1992). Vaughan and colleagues (1992) isolated and synthesized GHRH from the common carp and found it able to elevate circulating GH levels and stimulate release of GH from pituitary cells. In this same study a native GnRH, salmon GnRH (sGnRH), was found to be equipotent with carp GHRH at the one concentration compared (100 nm). Marchant et al. (1989) have shown that GnRH is a potent stimulator of GH release in the goldfish. Injections of a human GHRH fragment, fragment 1-29, elevated plasma GH levels in a tilapia hybrid. Furthermore, GnRH fragment 1-29 and carp GHRH stimulated GH release from pituitary fragments with similar potency (Melamed et al., 1995). In the same study, injections of a sGnRH superactive analog was more effective than the human GHRH fragment in elevating GH levels. In addition, sGnRH was more potent than either GHRH form in stimulating GH release in vitro.

Cortisol is a potent stimulator of GH release, yet direct effects of sex steroids on GH secretion in the tilapia have not been demonstrated (Nishioka et al., 1985;
Helms et al., 1987). There is evidence, however, that androgens have modulatory effects on GH-releasing factors, similar to the effects of E2 on the induction of PRL release by thyrotropin-releasing hormone described earlier (Barry and Grau, 1986). Melamed (1993) has shown that GHRH and GnRH are only effective in vivo and in vitro in a tilapia hybrid when the fish are reproductively mature. Furthermore, the releasing factors can be effective in vivo with reproductively immature animals if the fish are first injected with T or the synthetic androgen, 17α-methyltestosterone. Growth hormone-releasing hormone and GnRH are also effective in vitro with tissues from reproductively immature tilapia, if the tissues are co-incubated with these steroids (Melamed, 1993). In contrast to sex steroids, the glucocorticoid cortisol has a direct and potent stimulatory effect on GH secretion (Nishioka et al., 1985; Helms et al., 1987). Finally, insulin-like growth factor-I inhibits GH release in teleosts as in mammals (Perez-Sanchez et al., 1992).

Possible role of GnRH in PRL regulation

One area of regulation of reproduction in which the teleost system may differ from the mammalian system is with regard to GnRH. First, while most eutherian mammals have only one form of GnRH, mammalian-GnRH, teleosts, like
most vertebrates examined to date, have multiple forms (see review, Sherwood et al., 1994). By convention, the GnRH molecules are named for the animals from which they were first identified. There is evidence for specificity of function for the different forms of the peptide. Chicken-GnRH II (cGnRH-II) was found to be more potent in releasing GtH from perifused fragments and dispersed cells of the goldfish pituitary than sGnRH, while sGnRH was found to be the more potent of the two in releasing growth hormone (GH) from the pituitary fragments, although equipotent with dispersed cells (Chang et al., 1989; Peter et al., 1990).

The possibility that one or more of the multiple forms of GnRH present in the tilapia may stimulate PRL release was investigated as part of the studies described in this dissertation. Marchant and colleagues (1989) have shown that GnRH stimulates GH release in the goldfish, and Melamed and colleagues (1995) have shown the same for a tilapia hybrid. However, Cook and colleagues (1991) were not able to detect GnRH binding to PRL cells of the goldfish despite binding to GtH and GH cells.

The involvement of GnRH in PRL regulation is not well understood in mammals or in fishes. In mammals, GnRH has been shown to stimulate PRL release in vivo when basal PRL release is within normal ranges, but inhibit PRL release
when basal PRL release is unusually high as in hyperprolactinemia (Debeljuk et al., 1985; Kugu et al., 1988; Sridaran et al., 1988). Injections of GnRH have been shown to increase PRL cell activity in the Atlantic salmon, based on an immunocytochemical and electron microscopic study (Ekengren et al., 1978). The authors suggest that this effect was mediated via GnRH stimulation of GtH release which in turn, stimulated E₂ release, a potent stimulator of PRL cell activity.

One example of PRL stimulation by GnRH in a mammal may not fit the teleost. Denef and Andries (1983) have shown that in the fourteen-day-old rat, GnRH stimulates gonadotroph cells to release a paracrine factor that stimulates PRL release. This factor is not one of the GtHs. They demonstrated that PRL release is stimulated when a population of dispersed pituitary cells, including both PRL and GtH cells, are treated with GnRH. However, if GtH cells are removed from the culture, GnRH has no effect. Furthermore, when PRL cells are incubated in media from GnRH stimulated GtH cells, PRL release is increased. Recent studies suggest that angiotensin II may be this paracrine factor (Becu-Villalobes et al., 1994). The anatomical arrangement of the tilapia pituitary makes this same paracrine interaction less likely. The PRL
cells of the tilapia are segregated into the RPD while the GtH cells are in the proximal pars distalis.

The role of Ca\(^{2+}\) in tilapia PRL release

Indirect evidence suggests that PRL release in response to an osmotic signal is Ca\(^{2+}\) dependent in the tilapia (see reviews, Grau and Helms, 1989; Grau et al., 1994). Recently, Borski (1993) has used the Ca\(^{2+}\)-sensitive fluorescent dye, fura-2, to show that intracellular free Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) increase within 20 sec after exposure to reduced osmolality medium, further supporting a role for Ca\(^{2+}\) in PRL release. Now that homologous radioimmunoassays are available for tilapia PRLs, the time-course for PRL release in conjunction with changes in [Ca\(^{2+}\)]\(_i\), in response to osmotic stimuli, should be investigated. Jobin and Chang (1992) have also shown that native GnRH stimulates increases in [Ca\(^{2+}\)]\(_i\) in GtH and GH cells of goldfish. As part of my studies, I examined simultaneous changes in [Ca\(^{2+}\)]\(_i\) and the release of the two tPRLs in response to reductions in medium osmolality and GnRH.

Roles and regulation of PRL and GH in osmoregulation

The tilapia, *O. mossambicus*, is a euryhaline teleost fish that reproduces and thrives in habitats ranging in
salinity from fresh water (FW) to hypersaline seawater (SW). Prolactin and GH are regulators of physiological processes in the tilapia, which confer the ability to adapt to various salinities.

Prolactin plays a central role in FW adaptation and GH is increasingly believed to play a role in SW adaptation in tilapia and other euryhaline teleost fishes. Prolactin maintains extracellular salt and water balance when the fish are in a low salinity environment by acting on virtually all osmoregulatory tissues including the gills, integument, urinary bladder, intestine and kidney to reduce water permeability and increase sodium retention (Clarke and Bern, 1980; Hirano, 1986). Growth hormone has been shown to act in SW adaptation although its mode of action is not as well known as for PRL. Studies suggest that GH affects ion transport at the gills and may be working in conjunction with cortisol and insulin-like-growth factors, or through a stimulation of cortisol and insulin-like-growth factors (see review, Sakamoto et al., 1993, Sakamoto et al., 1994).

Consistent with the roles of PRL and GH in osmoregulation, PRL cell activity is enhanced when tilapia are maintained in FW and is reduced in SW fish, while the reverse is true for GH cell activity (Dharmamba and Nishioka, 1968; Clarke et al., 1973; Nagahama et al.,
1975; Nishioka et al., 1993; Borski et al., 1994). Small changes in medium osmolality, well within the physiological range of the tilapia, alter PRL and GH release from pituitary tissues (Nagahama et al., 1975; Grau et al., 1982; Helms et al; 1987). Grau and colleagues (1982) have shown that PRL release from PRL cells is increased in response to reductions in medium osmolality within 10-20 min; time-course studies have not been conducted for GH cells. Nagahama and colleagues (1975) have shown that the PRL cells respond to changes in medium osmolality and not sodium or chloride ions per se, by demonstrating that the response of PRL cells to changes in medium osmolality are the same whether the changes in medium osmolality are due to differences in NaCl concentration or are made by changes in the membrane-impermeant molecule, mannitol. It has not been determined whether changes in medium osmolality or the osmotic gradient across the cell membrane leading to a change in osmotic pressure, evoke the osmotic response. This question was addressed as part of this dissertation.

Circulating levels of GH and PRL change during adaptation of O. mossambicus to different salinities. Circulating and pituitary levels of PRL were observed to decrease when tilapia were transferred or acclimated from FW to SW and to increase when transferred or acclimated
from SW to FW (Nicoll et al., 1981; Borski et al., 1992; Ayson et al., 1993; Yada et al., 1994).

Borski and colleagues (1994) found that the pituitary content of GH was almost twice as high in male tilapia raised in SW for 7 months compared with male tilapia raised in FW for 7 months. In addition, pituitary GH levels were reduced in male fish transferred from SW to FW and increased in fish transferred from FW to SW after 49 days. Ayson and colleagues (1993) did not see an increase in pituitary GH in *O. mossambicus* (sex not reported) 3-4 weeks after transfer from FW to SW. Yada et al. (1994) observed an increase in plasma GH concentrations shortly after transfer of male *O. mossambicus* from FW to 70% SW but no change in levels in females. Growth hormone concentrations in the plasma were reduced shortly after transfer from SW to FW in both sexes, compared to animals transferred from SW to SW, at the same time points. Circulating levels of GH were no longer significantly different from controls, in either direction of transfer and either sex, by 7 days after transfer. Sakamoto and colleagues (1994) also observed an increase in plasma GH concentrations following transfer of *O. mossambicus* from FW to SW, whereas plasma GH was not altered following transfer from SW to FW. Furthermore, the increase in plasma GH was observed in both males and females and at
the end of the study, day 14 after transfer, plasma GH was elevated in males but not in females (Sakamoto, personal communication).

The patterns of circulating GH concentrations observed in *O. mossambicus* following transfer from FW to SW or SW to FW are similar to those observed in salmonids undergoing comparable salinity changes (see review, Sakamoto et al., 1993; Yada et al., 1994; Sakamoto et al., 1994). Circulating levels of GH increase transiently or do not change following transfer from FW to SW or SW to FW in salmonids (see review, Sakamoto et al., 1993). Transfer of rainbow trout and coho salmon to SW was accompanied by an increase in metabolic clearance rate and the calculated secretion rate of GH whereas clearance kinetics did not change in coho salmon transferred from SW to FW (Sakamoto et al., 1990, 1991). Whether there are changes in metabolic clearance rate or secretion rate of GH in tilapia following adaptation to FW or SW has not been determined.

Roles and regulation of PRL and GH in metabolism

Prolactin and GH have effects on protein, carbohydrate, and lipid metabolism in mammals (see reviews, Nicoll, 1974; Fain, 1980; Davidson, 1987). These metabolic actions appear to be conserved for GH in teleosts,
while the actions of PRL in metabolism have received little attention.

Prolactin can be lipolytic or lipogenic in teleosts, depending on the influences of temperature and photoperiod, circadian rhythms, and development (Lee and Meier, 1967; de Vlaming and Pardo, 1974; de Vlaming et al., 1975; Horseman and Meier, 1979; Sheridan, 1986). There has been only one study examining circulating PRL concentrations with changes in metabolic state. In this study, circulating PRL levels were not altered in Kokanee salmon (Oncorhynchus nerka), fasted for 30 days (McKeown et al., 1975). Nevertheless, Rodgers and colleagues (1992) found both pituitary content of PRL and basal release from RPD in vitro are decreased after 2 weeks of fasting in O. mossambicus. In this same study, PRL release in vitro was negatively correlated with the concentration of essential amino acids added to the incubation medium and was unaffected by D-glucose concentration. Although PRL appears to have metabolic actions in teleosts, the roles of PRL in regulating metabolism are still unclear. I examined changes in serum and pituitary levels of PRL in response to fasting as part of this thesis.

The role of GH in metabolism has received more attention in teleosts than has the role of PRL, but is not without controversy. Growth hormone treatment has been
shown to increase free fatty acids (FFA) in the circulation of rainbow trout and goldfish and to mobilize lipid reserves from the liver of rainbow trout and coho salmon (Minick and Chavin, 1970; Leatherland and Nuti, 1981; Sheridan, 1986). In addition, GH injections increased muscle FFAs but not plasma FFAs or glucose, and increased liver glycogen in Kokanee salmon (McKeown et al., 1975). Different responses to GH treatment were observed with the eel, *Anguilla japonica*, and tilapia, *O. mossambicus*. Injections of bovine-GH in hypophysectomized eels increased serum amino acid concentration and did not alter plasma lipid concentration (Inui et al., 1985). Injections of bovine-GH stimulate the release of glycogen and not lipid from liver reserves of the tilapia, and increase serum concentrations of amino acids and glucose but not protein, lipid or cholesterol (Leung et al., 1991). Consistent with this elevation in circulating amino acids and glucose with fasting, Rodgers and colleagues (1992) found an increase in GH release from pituitary tissues in response to reductions in essential amino acids and glucose in the incubation medium, in the same species. Together the results of these studies suggest that GH cells act in the regulation of, and are directly responsive to changes in the blood concentrations of amino acids and glucose in the tilapia.
Increases in circulating and pituitary levels of GH have been observed in fasted teleosts. Increases in plasma GH levels have been observed within 3 weeks of fasting in rainbow trout by Wagner and McKeown (1986), and within 1 week by Sumpter et al. (1991). No change in plasma GH was observed after 30 days of fasting in Kokanee salmon (McKeown et al., 1975). Farbridge and Leatherland (1992b) observed a bi-modal response to fasting in rainbow trout. Plasma GH concentrations were lower in fasted fish than in fed fish at 2 weeks of fasting, followed by an elevation in circulating GH concentrations in fasted fish at 4 weeks. Plasma GH concentrations were elevated at 2 weeks of fasting in a repeat of the study. Melamed (1993) observed a rise in plasma GH within 16 days of fasting in a tilapia hybrid. Also in tilapia, O. mossambicus, Rogers and colleagues (1992) found that pituitary content of GH was elevated with 2 weeks of fasting.

Farbridge et al. (1992) found that feeding rainbow trout only once every five days resulted in reduced plasma GH and suggested that GH concentrations are depressed by low feeding rates. This is not consistent with the study on the tilapia hybrid, by Melamed (1993). The tilapia at the start of the experiment by Melamed, were described as "sub-optimally fed". When the fish were switched to an increased feeding rate, plasma GH levels fell within 8
days. The nutritional state of the animal prior to fasting and changes in nutritional state during fasting is likely to affect the hormonal response to subsequent fasting. For this reason, I examined changes in serum and pituitary levels of GH and PRL, in response to fasting, in tilapia which had been well-fed and tilapia which had been on a restricted diet. I also measured changes in body parameters including body weight, length, gonad weight, and liver weight in response to fasting as a means of assessing changes in the nutritional state of the animals.

The reproductive cycle of the female tilapia

Prolactin and GH have actions in reproduction in teleosts. Before reviewing these actions, I will first discuss what is known about the reproductive cycle of the female tilapia. Tilapias are important food fishes worldwide and therefore their reproductive habits and physiology have received much attention. The tilapia O. mosambicus, can reproduce in FW and in SW. After the female lays her eggs and they are fertilized by the male, she picks up the eggs and broods them in her buccal cavity. Brooding continues for about 3 weeks. The female tilapia O. mosambicus, can reproduce every 20-25 days if they do not brood. If the female does brood, the inter-spawn period is extended to approximately 40 days. Smith and Haley
(1987, 1988) have described sex steroid and ovarian morphology profiles of the reproductive cycle for brooding and non-brooding female O. mossambicus. Plasma steroid hormone levels and ovarian tissue responsiveness to GtH at different phases of the breeding cycle have also been examined for another tilapia species, O. aureus, by Bogomolnaya and colleagues (1984).

Smith and Haley (1987) found that postovulatory follicles remain viable over the length of the brooding period in fish that brood, but regress quickly in those females which do not. They have provided evidence that these structures produce steroid hormones, most strongly during the first 7 days after spawning. In addition, blood levels of E₂ and T are elevated during the latter phase of the brooding cycle, even though oocytes constituting the next clutch to be spawned are arrested in early vitellogenesis during this time. Both E₂ and T are also elevated during brooding in O. aureus (Bogomolnaya and colleagues, 1984). Smith and Haley (1987) found that ovarian growth is not arrested in non-brooding female tilapia that are fasted, however, the oocytes show signs of atresia at latter stages. Smith and Haley (1987, 1988) suggest that E₂ and testosterone, and postovulatory follicles, may be involved in parental care behavior, as well as the arresting of oocyte growth during brooding and the
protection of oocytes from atresia in the tilapia. Based on what is known about the actions of PRL and GH in reproduction and metabolism, PRL and GH should also be considered in these roles. Furthermore, PRL and GH may be involved in the elevation of the steroid hormone levels and in prolonging the life of the postovulatory follicles.

In addition to the reproductive roles PRL and GH may have during the reproductive cycle of the tilapia, the hormones may be called upon to regulate osmotic homeostasis and metabolism. This would depend on both the salinity in which the animals reproduce and on metabolic changes that may occur in response to reduced feeding during the brooding phase of the reproductive cycle. For this reason I characterized changes in serum and pituitary levels of the tPRLs and GH during the reproductive cycle of FW- and SW-adapted female tilapia as well as fed and fasted tilapia as part of my studies.

Prolactin and teleost parental care behaviors

The roles of PRL in vertebrate reproduction are diverse; often they are associated with the nurturing of young. These include nestbuilding and protective behaviors as in birds, rabbits and rats, and mitogenic effects associated with maternal tissue derived feeding, such as
mammary development and lactation and pigeon cropsac development (cf. Nicoll and Bern, 1971).

Similar actions have been attributed to PRL in teleosts; these include parental care behaviors such as fanning of eggs and nests by sticklebacks, bluegills and cichlids. Also in cichlids, "calling movements" to fry, a behavior exhibited by *O. mossambicus*, and reduced feeding behavior suggested to prevent the cannibalism of young during brooding, have been attributed to PRL. Prolactin has been shown to induce mucus secretion in the discus fish, a source of nutrition for developing fry (Noble et al., 1938; Blum and Fielder, 1965; Slijkhuys et al., 1984; DeRuiter et al., 1986; Kindler et al., 1991). The tilapia is a cichlid and displays parental care behaviors similar to those attributed to PRL in other cichlids. Thus, PRL may be involved in the regulation of parental care behavior in the tilapia.

Prolactin cell activity was shown to be increased in male sticklebacks displaying fanning behavior, based on ultrastructure morphometry and \[^{3}H\]-lysine incorporation rate of PRL cells (Slijkhuys et al., 1984). However, the same group (Wendelaar Bonga et al., 1984) found no differences in PRL cell activity between brooding and non-brooding female tilapia using these same techniques. While
this conclusion may be correct, further investigation is warranted.

Roles of PRL in teleost reproduction

Evidence suggests that PRL has roles in reproduction in teleosts in addition to regulating behavior. As I have discussed earlier, the sex steroids E₂ and T stimulate PRL cell activity directly and sensitize PRL cells to thyrotropin-releasing hormone stimulation. Studies examining changes in circulating or pituitary PRL levels with reproductive cycles are limited but do provide support for PRL having roles in reproduction. Prolactin has been implicated in the regulation of vitellogenesis (covered in detail in the next section) and steroidogenesis. Finally, specific binding of ovine-PRL has been observed in membrane preparations of ovary and testis of tilapia (Edery et al., 1984).

Hirano et al. (1986) and Prunet et al. (1990) both cite preliminary data suggesting plasma PRL concentrations change during the reproductive cycle in female salmonids and suggest an inverse correlation with plasma progestins. The rise in PRL in rainbow trout described by Prunet and colleagues (1990) did not occur until 4 weeks after ovulation. Changes in prolactin bioactivity in sera and pituitary of the FW catfish, Clarias batrachus, assessed
using a pigeon crop sac assay, paralleled changes in GtH concentrations (Singh and Singh, 1981). In contrast, no changes in ultrastructure of PRL cells were observed during the reproductive cycle of the sailfin molly, *Poecilia latipinna* (Young and Ball, 1983).

To date, there have been only three studies on the effects of teleost PRL on steroidogenesis. Singh and colleagues (1988) used purified salmon PRL to examine gonadal steroidogenesis in hypophysectomized *Fundulus heteroclitus*, and found salmon PRL significantly increased plasma concentrations of T in males but had no effect on steroid levels in females and no effect on steroid release in vitro by either testes or ovaries despite preventing the decline in gonadal weight usually associated with hypophysectomy. Both tPRLs, tPRL188 and tPRL177, were tested for their effects on E2 production by vitellogenic oocytes of the guppy, *Poecilia reticulata*, (Tan et al., 1988). Prolactin188 stimulated E2 production from all stages of vitellogenic oocytes of the guppy with the strongest response elicited from oocytes at the beginning of vitellogenesis. Prolactin177 was found to have no consistent effect. Neither of these studies used homologous PRL, and therefore, the specificity of the responses to the hormones is open to question.

Rubin and Specker (1992) conducted the only study to
examine the effects of homologous PRL on steroidogenesis. They examined the effects of the two tPRLs on steroid production by testicular tissues of courting and non-courting male tilapia. Rubin and Specker (1992) found similar effects with both tPRLs. The tPRLs stimulated T production in testicular tissue from courting males, but not in testicular tissue from non-courting males. The tPRLs also increased ovine-luteinizing hormone-stimulated T production in courting males but were inhibitory in non-courting males.

Blum and Weber (1968), showed that injections of ovine-PRL increased steroid-3β-ol-dehydrogenase activity in the cichlid, *Aquadus pulcher*. Young and colleagues (1983), reported that ovine-PRL increases E₂ and 17α,20β-dihydroxy-4-pregnen-3-one production by ovarian follicles. Collectively, changes in circulating PRL levels during the reproductive cycle, effects of exogenous PRL on steroidogenesis and vitellogenesis, and detection of ovarian and hepatic PRL receptors strongly indicate a role for PRL in control of fish reproduction.

**Roles of GH in teleost reproduction**

Several lines of evidence suggest GH may have roles in fish reproduction. Circulating GH levels change during the reproductive cycle of fishes; GH preparations affect
steroidogenesis and gonadal development; and GH receptors are present in the ovary and in the liver, the site of vitellogenin production.

The recent availability of homologous radioimmunoassays led to the discovery that GH is present at moderate levels in the circulation during vitellogenesis (oocyte growth) or spermatogenesis in several fish species, and then increases abruptly during or just prior to the spawning period (Stacey et al., 1984; Marchant and Peter 1986; Bjornsson et al., 1991; Swanson 1991). In pituitaries of vitellogenic striped bass (genus Morone), immunoreactive GH cells were strongly labeled with a heterologous antiserum to fish GH (Huang and Specker, 1994). The density of GH cells and their intensity of staining then decreased in spawning fish, suggesting changes in GH secretion are linked to final maturation. Changes in serum GH and GtH levels are closely correlated during the ovulatory GtH surge in goldfish (Yu et al., 1991). Sumpter and colleagues, (1991b) on the other hand, found no significant elevations in GH with reproductive cycle in female rainbow trout until after ovulation. They suggest the rise in GH after ovulation and the rise in GH observed in maturing males, were due to starvation effects and were not associated with reproduction per se.
In salmonids, mammalian GH preparations have long been known to enhance ovarian growth, increase circulating levels of sex steroids and promote in vitro ovarian steroidogenesis (Higgs et al., 1976; Fostier et al., 1983; Young et al., 1983). Similar effects of bovine-GH were recently observed in spotted seatrout (Singh and Thomas, 1993). In combined treatments, stimulation of steroidogenesis by bovine-GH and human chorionic GtH were additive, confirming that bovine-GH did not merely potentiate GtH action. Furthermore, bovine-GH increased follicular aromatase activity, an action dependent upon the synthesis of new RNA and regulatory protein(s). The gonadotropic and steroidogenic actions of mammalian GH have been confirmed using purified and recombinant fish hormones. Injections of recombinant GH stimulate gonadal growth in hypophysectomized killifish and elevate circulating sex steroids in immature rainbow trout (Singh et al., 1988; Danzmann et al., 1990). The recombinant GH stimulated steroidogenesis by follicles isolated from hypophysectomized killifish or trout, even in the absence of GtH preparations (Singh et al., 1988). Purified carp or chum salmon GH potentiated carp GtH-II stimulation of in vitro steroidogenesis by both vitellogenic and preovulatory goldfish follicles (Van Der Kraak et al., 1990). However, GH was ineffective when used alone.
Most oocyte growth in fishes can be accounted for by the uptake of a yolk precursor protein (vitellogenin) that is synthesized and secreted by the liver of maturing females (vitellogenesis) under the influence of circulating estrogens, primarily $E_2$ (Specker and Sullivan, 1994). In frogs and turtles, GH stimulates hepatic synthesis of vitellogenin *in vitro* (Carnevali *et al.*, 1992; Ho *et al.*, 1985). The effect is also seen *in vivo* in hypophysectomized turtles (Ho *et al.*, 1982), implying direct action of GH on the liver independent of circulating GtH. Recently, Kwon and Mugiya (1994) demonstrated that GH or PRL as well as $E_2$ are essential for vitellogenin synthesis in the eel. Although $E_2$ injections could induce vitellogenin synthesis in intact and sham operated immature eels, they were ineffective in hypophysectomized animals, confirming that pituitary hormones play a role in initiating vitellogenin synthesis. Growth hormone or PRL added to the incubation medium of cultured eel hepatocytes greatly potentiated weak responsiveness to $E_2$ alone. Growth hormone and insulin are known to regulate uptake of vitellogenin (growth) by cultured oocytes of various vertebrates, including fishes (reviewed by Specker and Sullivan, 1994).

Finally, specific membrane receptors for GH have recently been detected in salmon ovary and testes (Le Gac *et al.*, 1991; Mourot *et al.*, 1992), confirming that the
teleost gonad is a GH target. Hepatic receptors for GH were described earlier for a variety of teleosts (Gray and Kelley, 1991; Hirano, 1991; Sakamoto and Hirano, 1991; Yao et al., 1991). Collectively, the maturational changes in circulating GH levels, effects of exogenous GH on steroidogenesis and vitellogenesis or oocyte growth, and detection of ovarian and hepatic GH receptors strongly indicate a role for GH in control of fish reproduction.

Research objectives

The spectrum of actions of PRL and GH in teleosts include effects on osmoregulation, metabolism and reproduction. Often, the hormones are called upon to regulate these different functions simultaneously. The cells secreting the hormones must therefore receive regulatory information from a variety of sources to be used in determining the secretion rate of the hormones. In order to understand how PRL and GH may be involved in regulating diverse functions, hormone changes with these functions must be characterized and the factors regulating PRL and GH must be identified.

Changes in serum and pituitary levels of the tPRLs and GH with changes in environmental salinity have been well characterized in the tilapia O. mossambicus, but changes with metabolic or reproductive state have received
little attention. There is still much to learn about the regulation of the tPRLs and GH in osmoregulation, metabolism, and reproduction. The overall objectives of my thesis research were to characterize changes in serum concentrations and pituitary content of the tPRLs and GH with changes in metabolic and reproductive states and to characterize further the regulation of these hormones.

Prolactin and GH cells of the tilapia can detect changes in the osmotic concentration of extracellular fluids and respond by altering the release of their hormones (Helms et al., 1987; cf. Grau et al., 1994). It is not known whether osmoreceptive cells, such as the PRL and GH cells, detect changes in the osmolality of fluids or changes in the osmotic gradient across the cell membrane which lead to an increase in osmotic pressure on the cell membrane. The first objective of my thesis research was to make this determination.

Gonadotropin-releasing hormone has been shown to stimulate not only GtH release in teleosts, including the tilapia, but also GH release (Marchant et al., 1989; Melamed et al., 1995). Since PRL and GH are closely related molecules, derived from a common ancestral gene and regulated by many of the same factors, my second objective was to determine whether GnRH has effects on PRL release. As part of this study, it was also my objective
to determine whether calcium operates as a second messenger in mediating the effects of GnRH on PRL release.

Little is known about the roles of PRL and GH in the regulation of metabolism in teleosts. Treatment of teleosts including the tilapia, with heterologous PRLs and GHs indicates that these hormones have effects on the mobilization of metabolites. There have been few studies inquiring into the existence of correlations between changes in circulating and pituitary levels of GH with alterations in metabolic state in teleosts. Furthermore, there has been only 1 study describing changes in circulating PRL concentrations and 1 describing changes in pituitary PRL content with changes in metabolic state (McKweon et al., 1975; Rodgers et al., 1992). The third objective of my research was to characterize changes in serum concentrations and pituitary content of the tPRLs and GH in relation to alterations in metabolic state induced by fasting.

Prolactin and GH have reproductive actions in teleosts. There have been few studies which examine changes in these hormones with the reproductive cycle of teleosts. The fourth objective of my thesis work was to characterize changes in serum concentrations and pituitary content of the tPRLs and GH during the reproductive cycle of the female tilapia. As part of this study, my objective was
to determine whether environmental salinity alters the patterns of serum and pituitary levels of the tPRLs and GH observed during the reproductive cycle.
Chapter II

ALTERATIONS IN OSMOTIC PRESSURE AND NOT OSMOLALITY ARE COUPLED TO THE SUSTAINED RELEASE OF THE OSMOREGULATORY HORMONE, PROLACTIN, IN THE EURYHALINE TELEOST, TILAPIA (OREOCHROMIS MOSSAMICUS)

INTRODUCTION

Prolactin is the FW-adapting hormone of many euryhaline teleosts including the tilapia, *O. mossambicus*. Prolactin acts on all osmoregulatory tissues to stimulate ion transport and to reduce ion and water permeability (see reviews by Clarke and Bern, 1980; Hirano, 1986; Brown and Brown, 1987; Grau and Helms, 1990). Consistent with this osmoregulatory action, prolactin cell activity, and blood and pituitary PRL levels are higher in FW-adapted tilapia than in SW-adapted tilapia, (Dharmamba and Nishio-ka, 1968; Nicoll et al., 1981; Borski et al., 1992; Ayson et al., 1993). Clearly defined osmoregulatory actions have yet to be defined in mammals.

In addition to the osmoregulatory actions of PRL in the tilapia, the tilapia PRL cell appears to be an osmoreceptor. Support for this notion comes from 3 lines of evidence. First, studies have demonstrated changes in
sustained PRL release, PRL synthesis and PRL mRNA levels in direct response to small changes in medium osmolality that are well within the range of plasma osmolalities observed in vivo (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1981; Kelly et al., 1988; Grau et al., 1994; Yoshikawa et al., 1995). Furthermore, the effect of changes in osmolality on PRL release appears to be mediated by second messenger systems. Prolactin release in response to changes in medium osmolality is Ca^{2+} and cAMP dependent and is accompanied by an increase in intracellular Ca^{2+} (Grau et al., 1981, 1982; Richman et al., 1990, 1991; Borski, 1992; Grau et al., 1994). The increased release of PRL in response to decreases in osmolality is specific to PRL cells. The secretory response of PRL cells to changes in osmolality is not shared by the closely related GH cells of the tilapia. The release of GH from GH cells of the tilapia is either unchanged or inhibited by decreases in medium osmolality (Helms et al., 1987; c.f. Grau and Helms, 1989).

The studies demonstrating that the PRL cells of the tilapia are osmoreceptive cells were conducted using pituitary tissues of the tilapia which are a nearly homogeneous population of PRL cells (95-95%; Nishioka et al., 1988). These tissues are from the anterior most region of the rostral pars distalis (RPD). Thus, the PRL cells were
responding directly to changes in medium osmolality. The ability to isolate a nearly homogeneous population of PRL cells obviates the impediments to investigating most osmoreceptive cells.

The vasopressin cells of the mammalian hypothalamus are an example of the complexity of most osmoreceptive cells. The cell bodies of these neurosecretory cells are located mainly in the supraoptic nucleus with a smaller number in the paraventricular nucleus. In both sites, the vasopressin cells are found among a variety of cell types and synapse with many others. The axons of vasopressin neurons project a considerable distance from these locations to capillaries in the posterior pituitary, the site of vasopressin secretion. This kind of complexity in morphology and arrangement has virtually blocked attempts to clarify the mechanisms by which the osmotic signals alter vasopressin secretion. In fact, it is still unknown whether vasopressin neurons are directly osmosensitive, or are governed by adjoining osmoreceptive cells, possibly around the anteroventral border of the third ventricle (see Guyton, 1991).

The combination of PRL's actions in regulating osmotic homeostasis together with the PRL cell's ability to respond directly to changes in osmolality demonstrates that the PRL cell both monitors and regulates the osmolal-
ity of extracellular fluids. Little is known about the cellular mechanisms which monitor the osmolality of extracellular fluids and lead to changes in the release of osmoregulatory hormones. Basic to this understanding is the identification of the osmotic signal recognized by the osmoreceptive cell. Nagahama and colleagues (1975) using the tilapia PRL cell as a model, showed that osmoreceptive cells respond to changes in osmolality and not sodium. They demonstrated that changes in medium osmolality by the addition of NaCl or the membrane-impermeant molecule mannitol, result in similar reductions in PRL releases from PRL cells. Since this study, there has been little progress towards characterizing the osmotic signal recognized by osmoreceptive cells. Studies on cells which are not involved in the osmoregulation of extracellular fluids point to the possibility that osmotic pressure and not osmolality may be the osmotic signal recognized by osmoreceptive cells.

Studies have shown that many cells, including mammalian PRL cells, release hormones in response to changes in medium osmotic pressure and not medium osmolality per se (Blackard et al., 1975; Sato et al., 1991; Wang et al., 1991). These cells include adenohypophysial cells and β-cells of the pancreatic islets of the rat. Thyrotropin is released by thyrotropin cells and insulin is released from
pancreatic cells in response to reductions in medium osmolality. These studies have shown that when the NaCl concentration of isosmotic media is reduced and replaced with membrane-impermeant molecules, release is not altered, however, when the same amount of NaCl is replaced with membrane-permeant molecules release is increased. Unlike membrane-impermeant molecules, membrane-permeant molecules reach an equilibrium across the cell membrane and do not contribute appreciably to the osmotic pressure on the cell membrane. The osmolality inside and outside the cell are increased equally by membrane-permeant molecules.

Unlike the tilapia PRL cells, the release of hormones by the rat PRL, thyrotropin and β-pancreatic cells is not sustained with continued reduced osmolality (Blackard et al., 1975; Sato et al., 1991; Wang et al., 1991). There is a burst of release followed by a return to baseline levels of release within 10 min of exposure to reduced osmolality. This temporally limited response to changes in osmolality is inconsistent with osmolality or osmotic pressure being a signal which regulates the release of hormones to serve osmoregulatory functions. Unlike PRL in the tilapia, the hormones released by these cells have not been shown to posses clearly defined osmoregulatory actions in mammals. Thus, it is not likely that a sustained
response to osmotic stimuli would be required. Blackard and colleagues (1975) noted that a reduction in serum osmolality of the magnitude required to increase insulin release (20 mOsmolal) does not occur physiologically in the rat and therefore the significance of this response is questionable. Furthermore, Sato and colleagues (1991), also working with the rat, made isotonic medium containing the membrane-permeant molecule ethanol by removing NaCl to reduce the osmolality of the medium 80 mOsmolal and then replacing it with ethanol. The reduction in medium osmolality before the addition of ethanol in the studies by Sato and colleagues (1991) was an even greater change in osmolality than in the studies by Blackard and colleagues (1975).

The studies just described raise the question of whether the sustained release of PRL from PRL cells of the tilapia, in response to reductions in medium osmolality, results from differences in osmotic pressure and not osmolality per se. Do cells respond to the overall osmolality of the medium or do they respond to the osmotic pressure of the medium which results from the osmotic gradient between the cell cytoplasm and the medium? To this end, I examined the response of PRL cells to various medium osmolalities achieved by varying the concentrations of membrane-permeant or membrane-impermeant molecules.
Tilapia RPD were incubated individually in wells for 18-20 hr to examine whether the effect of reduced medium osmotic pressure on PRL release is sustained. Prolactin tissues were also incubated in perifusion to characterize the short-term response of PRL cells to reduced medium osmotic pressure and osmolality. Prolactin release in response to a reduction in osmotic pressure must be sustained for osmotic pressure to be the physiologically important signal recognized by osmosensitive cells in monitoring extracellular osmotic conditions. Sustained increases in PRL cell activity and hormone effects are necessary for PRL to maintain osmotic homeostasis in the tilapia.

**MATERIALS AND METHODS**

**Animals**

Tilapia, *O. mossambicus* were collected from brackish water streams and maintained in FW in outdoor tanks at a temperature of 22-25°C for at least 2 months before use. The fish were fed to satiation twice daily with Purina trout chow (Purina Mills, Inc., St. Louis, MO).

**Static incubations**

Mature male tilapia 30-60 g were decapitated and
their pituitaries removed. Each pituitary was dissected and the RPD was placed into a well of a Falcon 96-well culture plate with 100 µl of Kreb's bicarbonate-Ringer solution containing glucose (500 mg/l), L-glutamine (290 mg/l), and Eagles's minimal essential medium (50X MEM, 20 ml/l: GIBCO; Grand Island, NY) (Wigham et al., 1977). The osmolality of the incubation medium was adjusted by varying the concentration of NaCl (or treatment molecules) and measured using a Wescor vapor osmometer (Wescor; Logan, UT). Treatment media were hyposmotic medium, (300 mOsmolal), or hyposmotic medium made hyperosmotic (355 mOsmolal) by the addition of either NaCl, the permeant molecules; urea or ethanol, or the impermeant molecule; D-mannitol. The medium was gassed for 10 min with 95% O₂/5% CO₂ (pH = 7.3). The tissues were incubated at 28 ± 1°C under a humidified atmosphere of 95% O₂/5% CO₂ and placed on a gyratory platform (80 rpm) for 18-20 hr. All chemicals were purchased from Sigma, St. Louis, MO unless otherwise indicated.

Perifusion incubations

Mature male tilapia 200-300 g were used for the perifusion studies. The RPD were dissected and placed 8 per chamber in parallel chambers with 3 chambers per treatment. The perifusion apparatus consisted of a peris-
taltic pump (Technicon proportional pump model III, Dublin, Ireland) connected to a fraction collector (ISCO model 328, Lincoln, NE). The chambers containing the tissues consisted of a 3.5 cm length of glass tubing (cut from a 100-200 µl microdispenser tube; Drummond, Broomall, PA) sealed at both ends with 120 µl mesh Nitex screen. The chambers were placed after the pump. The dead volume between the introduction of the test media and the fraction collector was 1.1 ml. Tissues were preincubated in control medium (355 mOsmolal) overnight and continued until the introduction of treatment media. Unlike the static culture medium, the medium used in the perifusion studies contained 50 I.U./ml penicillin and 0.05 mg/ml streptomycin. Fractions (100 µl) were collected every 2 minutes into 1.5-ml polypropylene microcentrifuge tubes containing 50 µl of 2% bovine serum albumin in phosphate buffered saline.

Electrophoresis

Prolactin release during 18-20 hr incubations was quantified using a combination of gel electrophoresis and densitometry (Specker et al., 1985a; modified by Kelley et al, 1988). At the termination of the incubations, media and tissue were placed in sodium dodecyl sulfate (SDS)-2-mercaptoethanol buffer, ultrasonically disrupted (Heat
Systems W-385 sonicator, Heat Systems-Ultrasonics, Inc.; Farmingdale, NY) and boiled for 3 min. Samples were then stored at -80°C or measured immediately.

The tPRLs were separated using SDS-polyacrylamide gel electrophoresis (PAGE). A vertical slab gel electrophoresis apparatus (Bio-Rad; Richmond, CA) was used. The samples were stacked in a 4% 37.5:1 acrylamide:bis-acrylamide gel and separated in a 15% 37.5:1 acrylamide:bis-acrylamide gel (12 cm long, 0.15 cm thick). Samples were subject to electrophoresis at 30 mA constant current/gel for 4-5 hr using a voltage- and current-regulated power supply (ISCO; Lincoln, NE). The gels were stained with Coomassie blue R-250 dissolved in a 10% methanol, 5% acetic acid solution. The gels were destained in a 10% methanol, 7% acetic acid solution until clearly discernible bands were observed and then stored in a 7% acetic acid solution. Bands of both tPRLs were quantified by using a densitometer and proprietary software (Hoefer Scientific, San Francisco, CA). For all static incubations, the release of PRL into the incubation medium was normalized as a percentage of the total PRL in the tissue and the medium.

Radioimmunoassays

Prolactins in medium collected during perifusion
incubations were measured using the homologous radioimmunoassays developed by Ayson et al., (1993) as modified by Yada et al. (1994). These radioimmunoassays were also used in studies described in following chapters for measuring the tPRLs and GH in serum and pituitaries. I will describe all three assays at this time for the sake of clarity. Hormones used as labeled-ligand were labeled with $^{125}$I using chloromine-T. The assays were performed using a double antibody method under disequilibrium conditions. Samples were run in duplicate. The tPRL$_{177}$ antiserum (P177-9-4) does not cross-react with either tPRL$_{188}$ or GH. The tPRL$_{188}$ antiserum (P188-1-3) does not cross-react with tPRL$_{177}$ and only slightly (3.1%) with GH. The GH antiserum (G-4-4) shows slight cross-reactivity with tPRL$_{177}$ (0.4%) and tPRL$_{188}$ (1.6%). Sample values were corrected for cross-reactivity using the formula:

$$\text{Corrected value} = \text{measured value} - \% \text{cross-reaction} \times \text{value of cross-reacting hormone/100}.$$ 

Repeated measurements within the same assay and in subsequent assays of a pool of FW O. mossambicus gave intra- and interassay coefficients of variation of 5.9 and 8.8% for tPRL$_{177}$, 6.3 and 9.5% for tPRL$_{188}$, and 7.8 and 10.4% for GH. The sensitivity of the assays, defined as the amount of measured hormone that can be distinguished from zero dose and calculated as twice the standard devia-
tion at zero dose, was 0.18-0.40 ng/ml for tPRL₁₇₇, 0.19-0.45 ng/ml for tPRL₁₈₈, and 0.14-0.30 ng/ml for GH when 50 µl of serum was used. Non-specific binding did not exceed 5% of total counts.

Statistical analysis
Differences among groups were determined using analysis of variance and the least significant difference test for a priori pairwise comparisons (Steel and Torrie, 1980). Experiments with only two groups were analyzed using the unpaired Student's t-test. Data are expressed as means ± SE.

RESULTS

Static Incubations
Effects of increasing medium osmolality by the addition of membrane-permeant (urea) and impermeant (mannitol) molecules on PRL release from RPD of the tilapia

The purpose of this study was to determine whether changes in medium osmolality or changes in the osmotic gradient across the cell membrane evoke sustained changes in PRL release. The release of both tPRLs was greater for tissues incubated for 18-20 hr in medium with reduced osmolality (300 mOsmolal) compared with release from
tissues incubated in medium with increased osmolality (355 mOsmolal), when the increase in osmolality is due to the addition of NaCl or mannitol (Fig. 1; P < 0.001). Increased medium osmolality did not affect PRL release when the difference in osmolality was derived from the addition of the membrane-permeant molecule, urea. Mannitol and NaCl were equally effective at reducing PRL release when added to medium to increase the osmolality of the medium.

Effects of increasing medium osmolality by the addition of the membrane-permeant molecule urea, on PRL release from RPD of the tilapia

The effect of urea on PRL release was investigated. The membrane-permeant molecule urea had no effect on PRL release when added to hyposmotic medium (300 mOsmolal), raising the osmolality 20 mOsmolals and making the medium isosmotic (320 mOsmolal), or when added to isosmotic medium, raising the osmolality 35 mOsmolals and making the medium hyperosmotic (355 mOsmolal) to tilapia plasma (Fig. 2).

Effects of increasing medium osmolality by the addition of the membrane-permeant molecule ethanol, on PRL release from RPD of the tilapia

The purpose of this study was to examine the effects
Figure 1. Effects of increasing medium osmolality by the addition of membrane-permeant (urea) and impermeant (mannitol) molecules on PRL release from RPD of tilapia. Treatment media were made by adding NaCl, mannitol or urea to hyposmotic medium (300 mOsmolal) to increase the osmolality of the media to 355 mOsmolal, numbers indicate basal osmolality of media plus additional mOsmolals contributed by mannitol or urea (mean ± SE; N = 12 per treatment; *** P < 0.001, ns = not significant at P < 0.05).
% PRL release

NaCl 300  NaCl 355  NaCl 300  NaCl 300
   + Mannitol 55  + Urea 55

ns

*** ns

\( \text{tPRL}_{188} \)
\( \text{tPRL}_{177} \)
Figure 2. Effects of increasing medium osmolality by the addition of the membrane permeant molecule urea, on PRL release from RPD of tilapia. Treatment media were made by adding NaCl or urea to hyposmotic medium (300 mOsmolal) to increase the osmolality of the media to the designated osmolalities, numbers indicate basal osmolality of media plus additional mOsmolals contributed by urea (mean ± SE; N = 12 per treatment; ns = not significant at P < 0.05).
% PRL release

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ns
of a second membrane-permeant molecule on PRL release from RPD. The addition of the membrane-permeant molecule, ethanol, to medium of varying osmolalities had no effect on PRL release from RPD (Fig. 3). Prolactin release from tissues incubated in 300 mOsmolal medium without ethanol was not significantly different from PRL release from tissues incubated in the same medium increased to 330 mOsmolal by the addition of ethanol. Prolactin release from tissues incubated in 330 mOsmolal medium without ethanol was not significantly different from PRL release from tissues incubated in the same medium increased to 355 mOsmolal by the addition of ethanol. Prolactin release from tissues incubated in 355 mOsmolal medium without ethanol was not significantly different from PRL release from tissues incubated in the same medium increased to 405 mOsmolal by the addition of ethanol.

**Perifusion Incubations**

Time-course for PRL release in response to a change in medium osmolality from hyperosmotic (355 mOsmolal) to hyposmotic (300 mOsmolal), or to hyposmotic medium made hyperosmotic by the addition of the permeant molecule urea, or the impermeant molecule mannitol

This study was aimed at investigating the time-course of possible changes in the release of the tPRLs from PRL
Figure 3. Effects of increasing medium osmolality by the addition of the membrane permeant molecule ethanol on PRL release from RPD of tilapia. Media was made by adding NaCl or ethanol to hyposmotic medium (300 mOsmolal) to increase the osmolality of the media to the designated osmolalities, numbers indicate basal osmolality of media plus additional mOsmolals contributed by ethanol (mean ± SE; N = 12 per treatment; ns = not significant at P < 0.05).
cells when medium was altered from hyperosmotic medium to either hyposmotic medium (300 mOsmolal), hyposmotic medium made hyperosmotic with the addition of the membrane-permeant molecule, urea (355 mOsmolal), or hyposmotic medium made hyperosmotic by the addition of the membrane impermeant molecule, mannitol. The insert in Figure 4 shows the changes in osmolality of the medium in response to the introduction of hyposmotic medium (300 mOsmolal) into the perifusion system, following hyperosmotic medium (355 mOsmolal). The hyposmotic medium was introduced at the beginning of fraction 1 and the drop in medium osmolality in the collection tubes occurs primarily in fraction 4, or 6-8 minutes later.

Prolactin release was not altered when the medium was switched from hyperosmotic medium to hyperosmotic medium containing mannitol (Fig. 4). Prolactin release increased when the medium was switched from hyperosmotic medium to hyposmotic medium, or to hyperosmotic medium containing urea. Prolactin release increased more rapidly when RPD were exposed to hyposmotic medium than when RPD were exposed to hyperosmotic medium containing urea. Prolactin release was greater in RPD exposed to hyposmotic medium than in RPD exposed to hyperosmotic medium containing urea, within 2 min after the onset of exposure to the new medium (fraction 4), but was no longer different at 4
Figure 4. Effects of increased medium osmolality by the addition of membrane permeant (urea) and impermeant (mannitol) molecules on tPRL$_{177}$ release (top graph) and tPRL$_{188}$ (bottom graph) from RPD of tilapia. Media was made by adding NaCl, mannitol or urea to hyposmotic medium (300 mOsmolal) to increase the osmolality of the media to 355 mOsmolal (hyperosmotic media). Tissues were incubated in hyperosmotic medium raised to 355 mOsmolal with NaCl before the media was switch to the treatment media which were 300 mOsmolal or hyposmotic medium made hyperosmotic with mannitol or urea. Fractions are 2 min fractions with numbering starting with the first sample collected at the time the treatment medium was added to the perifusion system. The insert shows the rate of osmolality change in the collected medium when hyposmotic medium is introduced into the perifusion system (mean ± SE; N = 12 per treat).
Fraction (2 min)
min (fraction 5) after the onset of exposure to the new medium. Prolactin \textsubscript{188} release was greater in RPD exposed to hyposmotic medium than in RPD exposed to hyperosmotic medium containing urea, within 2 and 4 min after the RPD were first exposed to the new medium (fractions 4 and 5), but was no longer different at 6 min (fraction 6) after the onset of exposure to the new medium. Peak PRL release was observed earlier in RPD exposed to hyposmotic medium than in RPD exposed to hyperosmotic medium containing urea. Prolactin \textsubscript{177} release peaked by 4 min after the onset of exposure to hyposmotic medium and about 10-12 min after the onset of exposure to hyperosmotic medium with urea. Prolactin \textsubscript{188} release also peaked by 4 min after the onset of exposure to hyposmotic medium and about 8-10 min after the onset of exposure to hyperosmotic medium containing urea. At 22 min after the initiation of exposure (fraction 19) to treatment media, the release of both tPRLs was similar for RPD exposed to hyposmotic medium and RPD exposed to hyperosmotic medium containing urea.

DISCUSSION

Prolactin release was high in RPD incubated for 18-20 hr in hyposmotic medium (300 mOsmolal) or medium made hyperosmotic (355 mOsmolal) with the membrane-permeant
molecules; urea or ethanol, but was low in RPD incubated in medium made hyperosmotic with NaCl or the impermeant molecule, mannitol. These data suggest that it is the osmotic gradient that leads to an increase in osmotic pressure, and not the medium osmolality per se, which accounts for the osmoreceptivity of the tilapia PRL cell. Furthermore, the greater release from tissues incubated in the medium with urea compared with mannitol and NaCl was not due to a stimulatory effect of the urea. This was demonstrated by showing that the addition of urea to media of various osmolalities, including osmolalities with reduced baseline release of the tPRLs, did not increase PRL release (Fig. 2).

Results similar to these obtained with urea were obtained with an additional permeant molecule, ethanol. Ethanol had no effect on PRL release, further supporting the notion that PRL cells of the tilapia respond to changes in osmotic pressure (Fig. 3). Contrary to our findings, Sato et al., (1991) reported isotonic ethanol solutions stimulate PRL and thyrotropin release from rat adenohypophysial cells along with inducing cell swelling. In the study by Sato and colleagues, medium at 300 mOsmo- lal was diluted 80 mOsmolal with distilled water and the osmolality restored to 300 mOsmolal with ethanol. The induced release as well as the observed swelling could
have been induced by the reduced osmolality of the diluted medium with the permeant ethanol molecules having no effect.

**Prolactin**177 and tPRL188 release from RPD incubated in hyposmotic medium and in hyperosmotic medium containing urea were the same at 22 min after the onset of exposure to these media. Furthermore, release of both tPRLs in RPD exposed to both media, were greater than the release of both PRLs in RPD exposed to hyperosmotic medium containing mannitol. These data support the results of the my static incubation experiments and the conclusion that PRL release in response to changes in osmotic pressure is sustained beyond the 10 min response observed in cells which secrete hormones that are not known to regulate the osmolarity of extracellular fluids, specifically: mammalian PRL, thyrotropin and pancreatic β-cells (Blackard et al., 1975; Sato et al., 1991; Wang et al., 1991).

The rise in release of the tPRLs upon exposure of RPD to hyposmotic medium made hyperosmotic with urea was slower than that observed after the introduction of hyposmotic medium, although PRL release in both treatments converged by 22 min after the initiation of exposure (Fig. 4). Thus, urea affected only the early phase of the response and not the later phase of the response.

The difference in response of tissues incubated in
hyposmotic medium compared with tissues incubated in hyposmotic medium made hyperosmotic with urea is consistent with urea having a diffusion rate across the cell membrane of the PRL cells that is slower than that of water. Until the urea molecules diffuse across the membrane, there is an osmotic gradient across the cell membrane. The osmotic pressure on the membrane due to the medium made hyperosmotic with NaCl or mannitol, and the medium made hyperosmotic with urea would be similar immediately after exposure and thus, PRL release would be expected to be the same. As the urea molecules diffuse across the membrane, the osmotic gradient across the cell membrane decreases as does the osmotic pressure. As this decrease in osmotic pressure occurs, PRL release increases. The time required for the urea to diffuse across the membrane is the cause of the delay in PRL release observed between the RPD exposed to hyposmotic medium and RPD exposed to hyposmotic medium with added urea. Once the urea comes into equilibrium across the membrane, only the impermeant molecules in the initial hyposmotic medium affect the osmotic pressure across the cell membrane. This is the same in both the hyposmotic medium and the hyperosmotic medium containing urea. At this point, release of the PRLs would be expected to be equal in RPD incubated in the two media, which is what is observed at
22 min after the initiation of exposure to the two media.

In summary, alterations in osmotic pressure and not osmolality are coupled to the sustained release of the osmoregulatory hormone, prolactin, in the euryhaline teleost, *O. mossambicus*. 
Chapter III

GONADOTROPIN-RELEASING HORMONE FUNCTIONS AS A PROLACTIN-RELEASING FACTOR IN THE TILAPIA, ORECHROMIS MOSSAMBICUS

INTRODUCTION

Teleosts, like most vertebrates other than placental mammals, have multiple forms of GnRH in the brain which differ in their spatial distribution throughout the brain. Three endogenous forms of GnRH have been identified in the brain of tilapia: salmon-GnRH (sGnRH), chicken-GnRH-II (cGnRH-II) and seabream-GnRH (sbGnRH) (Weber et al., 1994). The GnRH molecules are named for the first animal from which they were identified. The presence of multiple forms of GnRH together with multifarious distributions of these forms has led to speculation that separate functions may be subserved by distinct GnRH peptides. These functions may extend beyond the regulation of GtHs since GnRH has also been shown to function as a regulator of GH in several teleosts, including tilapia (Marchant et al., 1989; Melamed et al., 1995).

Growth hormone and PRL are part of the same polypeptide family and are thought to be derived from a common
ancestral gene (Niall et al., 1971). Not surprisingly, PRL and GH are regulated by many of the same factors: somatostatin, cortisol, and changes in osmotic pressure (cf. Nishioka et al., 1988). Despite these commonalities between GH and PRL, and the knowledge that GnRH regulates GH release, the effects of GnRH on PRL release in teleosts have received little attention.

There is little direct evidence to support or oppose a role for GnRH as a regulator of PRL in teleosts. In the Atlantic salmon, injections of GnRH have been shown to increase PRL cell activity, based on light and electron microscopical evidence (Ekengren et al., 1978). There was an increase in the synthetic and exocytotic activity of the cells. It was thought, however, that this effect was mediated via GtH and E2, with GnRH inducing rises in circulating concentrations of GtH and then E2 which in turn stimulated the increase in PRL cell activity. Gonadotropin-releasing hormone did not bind to PRL cells of the goldfish, suggesting that GnRH may not be a direct regulator of PRL cell function in this species (Cook et al., 1991).

In mammals, GnRH has been found to stimulate PRL release indirectly via paracrine secretions of GtH cells (Denef and Andries, 1983; Lamberts et al., 1989). There is strong evidence that angiotensin II from gonadotrophs
is a paracrine mediator of this action (cf. Saavedra, 1992; Becu-Villalobos et al., 1994). In mammals, prolactin cells are found in very close association with GtH cells (Sato, 1980). In contrast, the PRL cells in the tilapia are segregated into the anterior most region of the RPD as an almost homogeneous mass, while the GtH cells are located in the proximal pars distalis (Bern et al., 1975). This anatomical arrangement of the tilapia pituitary gland makes a paracrine-mediated effect through GtH cells less likely.

Jobin and Chang (1992) have recently shown that native GnRHs stimulate increases in [Ca\(^{2+}\)]i in GtH and GH cells of the goldfish. Borski (1993) has also recently shown that [Ca\(^{2+}\)]i is increased in PRL cells of the tilapia with exposure to reduced medium osmolality. The effects of GnRH on changes in [Ca\(^{2+}\)]i in PRL cells of a teleost have not been investigated.

The purpose of the present study was to: 1) determine whether GnRH injections can affect circulating levels of PRL; 2) determine whether GnRH can alter the release of PRL from tilapia RPD and to compare potencies of the native GnRH forms in releasing PRL; 3) characterize the effect of medium osmolality on the ability of GnRH to stimulate PRL release; 4) determine whether GnRH alters [Ca\(^{2+}\)]i and compare temporal changes in [Ca\(^{2+}\)]i with
temporal changes in PRL release; and 5) determine whether the steroid hormones, E2 or T, alter the affect of GnRH on PRL cells; both steroids stimulate PRL release.

MATERIALS AND METHODS

Animals

Tilapia, O. mossambicus, were collected from brackish water streams and maintained in FW in outdoor tanks at a temperature of 22-25°C for at least 2 months before use. The tanks were supplied with a continuous flow of FW and constant aeration. The fish were fed to satiation twice daily with Purina trout chow. All fish were mature adults and ranged in size from 70-120 g body weight.

Injection study

Mature male and female tilapia were used in the study. Fish were moved to indoor oval fiberglass tanks (60 l), 8 individuals per tank, and allowed to acclimate under a fixed photoperiod of 14L:10D to the tanks for at least 2 weeks before injections. The tanks were supplied with a continuous flow of FW and constant aeration. The fish were not fed on the day of the experiment. Fish were anesthetized with 2-phenoxyethanol at a concentration of 1 ml/l for 1 min before being weighed and given intraperito-
neal injections of an analog of mammalian-GnRH (mGnRHa; des-Gly\textsuperscript{10}, [d-Ala\textsuperscript{6}]mGnRH; Sigma, St. Louis, MO) dissolved in 0.9% saline (0.1 µg mGnRHa/g body weight) or saline for injected controls. Non-injected controls were also anesthetized. Injected animals received an injection volume of 1 µl/g body weight. Fish to be sampled at 11 hr post-injection were injected 9 hr before the other treatments so that all treatments were sampled between 3-6 PM. Fish were also anesthetized for blood collection.

Static incubations

Procedures for static incubations are described in detail in chapter II. Mature male tilapia were used in the study. The sGnRH, cGnRH-II and chicken GnRH-I (cGnRH-I) used in the study comparing these 3 forms, was purchased from Peninsula Laboratories, San Carlos, CA. The sGnRH, cGnRH-II and sbGnRH used in the study comparing these 3 forms were a gift from the Salk Institute. The GnRHs were dissolved in hyperosmotic medium to 1 mM and the steroid hormones (Sigma) were dissolved in absolute ethanol to 1 mM before dilution in medium. In studies which included steroid hormones which were dissolved in 100% ethanol, all treatments received equal volumes of ethanol.
**Perifusion incubations**

Procedures for perifusion incubations are described in detail in chapter II. Eight RPD from mature males were placed in each of 6 parallel chambers. The tissues were preincubated in control medium (355 mOsmolal) overnight. Seabream-GnRH (Salk Institute) was introduced as 5 min pulses in graded doses of 0, 0.1, 1, 10, 100, and 1000 nm with 2 hr between pulses. Ten min fractions were collected. Half of the chambers received increasing concentrations of GnRH and the other half decreasing concentrations.

**Cell dispersion for [Ca\(^{2+}\)]\(i\) measurement**

Cells from RPD were dispersed following the method described by Borski (1993). Prolactin cells of the RPD were dispersed by first placing a single tissue in 0.5 ml of a 0.25% solution of porcine trypsin 1-300 (US Biochem, Cleveland, OH) in phosphate buffered saline (PBS; pH = 7.5; 355 mOsmolal) for 45 min at 28 ± 1°C and then gently passing the tissues through a 1 ml pipette 5-10 times. The cells were centrifuged (250 X g, 5 min, 25 ± 1°C), the supernatant was decanted, and the cells were resuspended and triturated in 1 ml of trypsin free phosphate buffered saline. The cells were rinsed in this way 3 additional times and then resuspended in culture medium adjusted to
355 mOsmolal. The cells were plated onto a glass cover-slip coated with 0.1 mg/ml of poly-L-lysine. Cells were preincubated in 355 mOsmolal medium for at least 12 hr prior to the determination of \([Ca^{2+}]_i\).

**Monitoring \([Ca^{2+}]_i\) by dual microspectrofluorometry with fura-2**

Validations and procedures used in this study were described in detail by Borski (1993). The procedures are described again, briefly. Prolactin cells, plated on poly-L-lysine coated cover slips and incubated in hyperosmotic medium, were loaded with 10 \(\mu M\) of fura-2/AM (Molecular Probes, Eugene, OR), for 90 min at 28 ± 1°C. The fura-2/AM was solubilized in anhydrous dimethyl sulfoxide (DMSO; Aldrich Chemical, Milwaukee, WI) to a concentration of 10 mM prior to its final dilution to 10 \(\mu M\) (< 0.10% DMSO v/v). The fura-2/AM is cleaved to its impermeable form, fura-2 by endogenous esterases within the PRL cells. The cover slips were then mounted in a chamber that allows the cell to be perfused and the chamber was in turn mounted on a microscope stage. The PRL cells were perfused for at least 30 min in hyperosmotic medium to allow the fura-2/AM to further deesterify (Gryniewicz et al., 1985; Lewis et al., 1988).

Single cell measurements of the fura-2 ratio were
made with a dual excitation spectrofluorometer (ARCM-MIC-N, Spex Industries, Edison, NJ) interfaced with a Diaphot-TMD inverted microscope (CF 40 X oil immersion fluorite objective, Nikon). The microscope was equipped with fluorescence optics, a 50 watt halogen illuminator, epifluorescence illumination, and a quartz nose-piece (for UV). Excitation light alternated between 340 and 380 nM (narrow bandpass filters, SPEX) by a computer-controlled chopper mirror. Fluorescent emission intensity was transduced every 2 sec by a photomultiplier tube focused on a single PRL cell after it had passed through a 500 nM emission filter. A pinhole (1 mm) placed in the epiillumination path restricted the UV illumination to only the cell of interest.

All data are expressed as the relative intensity of the ratio of fura-2 fluorescence excited at 340 nm (fura-2 bound to Ca$^{2+}$) to that excited by 380 nm (free fura-2) from which background (autofluorescence) was subtracted. Shifts in this ratio (340/380) result directly from changes in [Ca$^{2+}$]$_i$ which are independent of dye concentration, cell thickness, and absolute optical efficiency of the instrument (Tsien et al., 1985; Grynkiewicz et al., 1985; cf. Poenie et al., 1986).
Incubations for measuring changes in $[Ca^{2+}]_i$ in conjunction with PRL release

Experimental media (hyperosmotic medium with or without cGnRH-II, and hyposmotic medium) were maintained at 28 ± 1°C in hanging 60 ml plastic syringes connected to an eight-point manifold perifusate selector (Hamilton Co., Reno, NV) via one-way stop cocks and polyethylene tubing. The manifold output is connected to the input port of the perifusion chamber by another piece of polyethylene tubing. The rate of perifusion through the chamber was maintained at 300 µl/min by keeping the height of the syringes and volume of all solutions in the syringes constant throughout the experiment. Chicken-GnRH-II was used in the study because it showed the widest range of stimulation in our static incubation studies. The cGnRH-II was dissolved in hyperosmotic medium (0.1 pM cGnRH-II, 355 mOsmolal). Hyposmotic medium (300 mOsmolal) was introduced to the system at the end of each run.

The amount of the tPRLs released from the dispersed cells was insufficient to measure tPRL release into the media. For this reason, in 2 runs of the experiment, 4 RPD were placed in a chamber taken from the perifusion system described earlier and placed inline after the chamber with the dispersed cell preparation used for $[Ca^{2+}]_i$ measurements. The experimental medium flowed...
through the chamber with the cells for $[Ca^{2+}]_i$
measurements and then through polyethylene tubing to the
chamber with the 4 RPD, and finally to a fraction
collector. One min fractions were collected and tPRL in
the media were measured as described for the perifusion
system earlier.

Quantification of PRLs

Prolactin release was quantified using a combination
of gel electrophoresis and densitometry for 18-20 hr
cultures and radioimmunoassay for 3 hr cultures and serum.
The electrophoresis and radioimmunoassay procedures are
described in detail in chapter II. For perifusion incuba-
tions, the data are expressed as percent of baseline
release which is the sum of the concentrations of hormone
in each of the three fractions following and including the
introduction of the GnRH ($b_1, b_2, b_3$), divided by the sum of
the hormone in the three fractions preceding the introduc-
tion of the GnRH ($a_1, a_2, a_3$), expressed as percentage
$((b_1+b_2+b_3)/(a_1+a_2+a_3) \times 100)$.

Statistical analysis

Differences among groups were determined using analy-
sis of variance and the least significant difference test
for a priori pairwise comparisons (LSD) analysis. Due to
the much greater response to GnRH than to the steroid hormones in the experiment comparing these responses, the mean sum of squares used in the LSD analysis was derived using only values from groups being compared (Snedecor and Cochran, 1980). Experiments with only two groups were analyzed using the unpaired Student's t-test. Data are expressed as means ± SE.

RESULTS

The effects of mGnRHa injections on serum concentrations of PRL

Intraperitoneal injections of 0.1 µg mGnRHa/g body weight elevated serum tPRL_{188} and tPRL_{177} concentrations of mature male and female tilapia at 1 hr after injection, over concentrations observed in vehicle-injected and non-injected controls (Fig. 5; P < 0.01). Serum hormone concentrations were not significantly different from controls at 11 hr after injection. Injections did not alter GH serum concentrations.

The effects of mGnRHa on the in vitro release of PRL from tilapia RPD incubated in isosmotic medium

To determine the response of PRL cells to GnRH, RPD were incubated for 18-20 hr in isosmotic medium.
Figure 5. Effects of intraperitoneal injections of 0.1 μg/g body weight of mGnRHa on serum PRL levels in male (M) and female (F) O. mossambicus at 1 and 11 hrs post-injection (non-injected, black bars; vehicle injected, open bars; mGnRHa injected, diagonal bars) (mean ± SE, N = 10-12 per treatment from 2 replicate experiments, ** P < 0.01 and *** P < 0.001, comparisons are to injected controls).
(320 mOsmolal) containing graded concentrations of mGnRHa ranging from 0.01 nM to 1 µM. Tissues were incubated in isosmotic medium (320 mOsmolal) which elicits moderate baseline PRL release so that either a stimulatory or inhibitory effect of GnRH on PRL release could be observed. Incubation with mGnRHa stimulated the release of tPRL177 and tPRL188 with the greatest response elicited at a concentration of 10 nM mGnRHa (Fig. 6; \( P < 0.01 \)).

Release is variable with isosmotic medium. For this reason, data from each of two replicate experiments were normalized as a percent of controls before being combined.

The effects of medium osmolality on mGnRHa stimulated release of PRL from tilapia RPD

To determine whether GnRH could overcome the effects of medium osmolality on PRL release, RPD were incubated for 18-20 hr in hyposmotic medium (300 mOsmolal), isosmotic medium (320 mOsmolal), or hyperosmotic medium (355 mOsmolal), with graded concentrations of mGnRHa. The release of both PRLs showed a typically inverse correlation with medium osmolality in the absence of GnRH (Fig. 7). Release of both PRLs was stimulated by mGnRHa at a concentration of 100 nM when incubated in isosmotic and hyperosmotic medium (\( P < 0.01 \) and 0.001 respectively). There was no significant difference among treatments.
Figure 6. Prolactin release from RPD of *O. mossambicus* during 18-20 hr incubations in isosmotic medium (320 mOsmolal), over a range of mGnRHα concentrations. Due to variability of baseline release in isosmotic medium, the data are expressed as a percent change from the control values for each replicate of the experiment (mean ± SE, N = 10-12 per treatment from 2 replicate experiments, **P < 0.01**).
Figure 7. Prolactin release from RPD of *O. mossambicus* during 18-20 hr incubations with mGnRHα, in hyposmotic medium (300 mOsmolal), isosmotic medium (320 mOsmolal) and hyperosmotic medium (355 mOsmolal; mean ± SE, N = 10-12 per treatment from 2 replicate experiments, ** P < 0.01 and *** P < 0.001).
exhibiting the greatest stimulation for each osmolality, possibly because stimulation was near maximal under each condition. Better separation of treatment effects was observable with hyperosmotic medium due to a lower baseline release. For this reason, subsequent studies were conducted in hyperosmotic medium.

The relative potency of various GnRH molecules on the release of PRL from tilapia RPD

The potency of the GnRH forms were compared using 18-20 hr static incubations of RPD in hyperosmotic medium. These incubations differ from the previous incubations in that the tissues were preincubated in hyperosmotic control medium overnight and the medium was changed and tissues rinsed 3 times before the treatment medium was added. This long preincubation reduced baseline release and provided a better separation of responses. The response of tPRLs were similar to each other in both studies. In the first set of experiments (Fig. 8), cGnRH-I, a form of GnRH which was not found in the tilapia, was the least potent form of GnRH tested. Chicken-GnRH-I did not eliciting a significant increase in the release of either PRL until a concentration of 1000 nM. Both native forms of GnRH, sGnRH and cGnRH-II were more potent than cGnRH-I. A significant response with sGnRH was observed with 1 nM
Figure 8. Prolactin release from RPD of *O. mossambicus* during 18-20 hr incubations in hyperosmotic medium (355 mOsmolal), over a range of cGnRH-I, cGnRH-II and sGnRH concentrations (mean ± SE, N = 10-12 per treatment from 2 replicate experiments, * P < 0.05 and *** P < 0.001).
sGnRH. Chicken GnRH-II was found to be the most potent of the three forms tested. A significant increase in the release of both tPRLs was observed with the lowest concentration tested, 0.01 nm cGnRH-II. All three forms evoked the maximum response observed. When three forms of GnRH that are native to the tilapia were compared (Fig. 9), cGnRH-II was again found to be the most potent form of the native GnRHs, followed by sGnRH. Seabream GnRH was the least potent for stimulating the release of both tPRLs.

Effects of sbGnRH on PRL release from tilapia RPD in perifusion incubation

Single short exposures (5 min pulse) of RPD to sbGnRH stimulated the release of both PRLs in a dose-related manner at concentrations similar to that observed with the 18-20 hr static incubations (Fig. 10). A stimulation was first observed at a concentration of 10 nM sbGnRH (P < 0.05).

The effects of cGnRH-II on \([Ca^{2+}]_i\) and PRL release

Chicken GnRH-II and hyposmotic medium evoked increases in \([Ca^{2+}]_i\) and PRL release (Figs. 11, 12, 13). Chicken GnRH-II was effective at a concentration as low as 0.1 pM. An increase in \([Ca^{2+}]_i\) was observed within 1 min of exposure to cGnRH-II or hyposmotic medium. A reduction
Figure 9. Prolactin release from RPD of *O. mossambicus* during 18-20 hr incubations in hyperosmotic medium (355 mOsmolal), over a range of cGnRH-II, sGnRH, and sbGnRH concentrations (mean ± SE, N = 6 per treatment, * P < 0.05, ** P < 0.01 and *** P < 0.001).
Figure 10. Prolactin release from RPD of *O. mossambicus* during perifusion incubation in hyperosmotic medium (355 mOsmolal), in response to a range of sbGnRH concentrations delivered in 5 min pulses. Fractions were collected at 10 min intervals and the data are presented as the percent of total hormone released into the medium in the 3 fractions following the introduction of the sbGnRH, compared to the total hormone in the 3 fractions preceding the introduction of the sbGnRH, at each concentration (mean ± SE, N = 10 RPD per column and 6 columns per GnRH form, * P < 0.05 and ** P < 0.001).
PRL release (% basal)

Log sbGnRH (nM)

- tPRL_{188}
- tPRL_{177}

**

*
Figure 11. Changes in $[\text{Ca}^{++}]_i$ from dispersed PRL cells of *O. mossambicus* during perifusion incubation in hyperosmotic medium (355 mOsmolal), in response to a range of cGnRH-II concentrations and a switch to hyposmotic medium (300 mOsmolal). The $[\text{Ca}^{++}]_i$ is expressed as the relative intensity of the ratio of fura-2 fluorescence excited at 340 nm (fura-2 bound to Ca$^{++}$) to that excited by 380 nm (free fura-2).
Figure 12. Prolactin release from 4 RPD and changes in \([\text{Ca}^{++}]_i\) from dispersed PRL cells of *O. mossambicus* during perifusion incubation in hyperosmotic medium (355 mOsmolal), in response to a range of decreasing cGnRH-II concentrations and a switch to hyposmotic medium (300 mOsmolal). Fractions were collected at 1 min and the PRL data are presented as ng/ml of medium. The \([\text{Ca}^{++}]_i\) is expressed as the relative intensity of the ratio of fura-2 fluorescence excited at 340 nm (fura-2 bound to \(\text{Ca}^{++}\)) to that excited by 380 nm (free fura-2).
Control

GnRH 0.01 nM 355 mOsm

GnRH 0.0001 nM 355 mOsm

Control 355 mOsm

Control 300 mOsm

Serum concentration (ng/ml)

Fractions (1 min)

Minutes

Ratio (340/380)

Minutes
Figure 13. Prolactin release from 4 RPD and changes in $[\text{Ca}^{++}]_i$ from dispersed PRL cells of *O. mossambicus* during perifusion incubation in hyperosmotic medium (355 mOsmolal), in response to a range of increasing cGnRH-II concentrations alternating with hyperosmotic medium without cGnRH-II and a switch to hyposmotic medium (300 mOsmolal). Fractions were collected at 1 min and the PRL data are presented as ng/ml of medium. The $[\text{Ca}^{++}]_i$ is expressed as the relative intensity of the ratio of fura-2 fluorescence excited at 340 nm (fura-2 bound to Ca$^{++}$) to that excited by 380 nm (free fura-2).
in $[\text{Ca}^{2+}]_i$ was observed within 1 min after exposure to control medium (hyperosmotic medium without cGnRH-II, Fig. 13). Prolactin release in response to both GnRH and hyposmotic medium appeared to be biphasic. An initial, large response was followed by lower levels that were still above baseline, even though $[\text{Ca}^{2+}]_i$ remained high (Figs. 12 and 13). A dose response to cGnRH-II was not observed for $[\text{Ca}^{2+}]_i$. Repeated exposures to cGnRH-II with 10 min breaks between exposures evoked increases in $[\text{Ca}^{2+}]_i$ that were similar in magnitude to the first response but PRL release did not appear to be restimulated (Fig 13). Exposure to hyposmotic medium was able to evoke an increase in PRL release following a failure in repeated GnRH exposures even though both elicited similar responses in $[\text{Ca}^{2+}]_i$ (Fig. 13).

The effects of T and E$_2$ on sGnRH induced PRL release

Treatment with sGnRH (100 nm), T (10 nM), and E$_2$ (10 nM) stimulated the release of both PRLs from RPD during 3 hr incubations in hyperosmotic medium, following an overnight preincubation in control medium (Fig. 14; all $P < 0.05$). Combined treatments resulted in greater release of both PRLs than either sGnRH or steroid treatments alone ($P < 0.05$ for comparisons for each individual treat-
Figure 14. Prolactin release from RPD of *O. mossambicus* during 3 hr incubations in hyperosmotic medium (355 mOsmolal), with 10 nM E₂, 10 nM T, 100 nM sGnRH or a combination of the sGnRH and the steroid hormones (mean ± SE, N = 10-12 per treatment from 2 replicate experiments, * P < 0.05, ** P < 0.01 and *** P < 0.001).
ment compared with treatments in which they were used in combination with one of the other hormones).

DISCUSSION

Effects of GnRH injections on PRL release

Injections of the tilapia with the GnRH analog, mGnRHa, elevated serum concentrations of the tPRLs. These data provide evidence that GnRH either stimulates the PRL cells to release PRL directly, or stimulates less direct pathways that lead to PRL release from PRL cells. The injections almost certainly lead to an increase in GtH release which in turn leads to an increase in the release of steroids from the gonads. I present data that confirm earlier findings that T and E₂ stimulate PRL release in the tilapia (Barry and Grau, 1986; Borski et al., 1991). Taken together, the rise in circulating concentrations of PRL in the tilapia in response to GnRH injections, could have been mediated via GtH and then steroid hormones. Ekengren and colleagues (1978) suggested this pathway to explain how GnRH injections increased PRL cell activity in the Atlantic salmon.

The rise in circulating PRL levels within 1 hr of a single injection of mGnRHa (0.1 μg/g body weight) occurred within a time frame consistent with a GtH and steroid
mediated response. Intraperitoneal injections of catfish-GnRH (0.125 μg/g body weight) elevated plasma concentrations of GtH-II and 11-ketotestosterone in African catfish, (Clarias gariepinus), within 1 hr post-injection, and cGnRH-II injections (dosage as low as 0.002 μg/g body weight) elevated plasma GtH-II concentrations within 0.5 hr (Schulz et al., 1993). Each of these time points represent the earliest time points examined. Both forms of GnRH are found in the pituitary of the African catfish with catfish-GnRH being 37-fold more abundant than cGnRH-II.

The rapid elevation in steroid concentrations in response to GnRH injections makes in vivo studies of the effects of GnRH on PRL release difficult to interpret in intact sexually mature fish. A stimulation of PRL levels in gonadectomized fish in response to GnRH injections may be a better indication that GnRH directly stimulates PRL release, however, the GnRH response may be mediated via other factors. In the rat, GnRH has been shown to stimulate PRL release via paracrine action involving GtH cells (Denef and Andries, 1983). For these reasons, in vitro examinations of the effects of GnRH on PRL cells was emphasized in my study.

The rise in serum PRL concentrations induced by GnRH injections demonstrates that increases in circulating GnRH
can affect PRL levels, regardless of the mechanism. Thus, the interpretation of in vivo treatments with GnRHs, intended to elevate GtH or GH, should consider the effects of changes in PRL levels on the observed responses to the treatments.

*In vitro* evidence that GnRH functions as a PRL-releasing factor

All three forms of GnRH that are native to the tilapia stimulated the release of $tPRL_{188}$ and $tPRL_{177}$ from RPD *in vitro*. Concentrations of GnRH found to stimulate PRL release were well within the range for receptor-mediated action. Furthermore, the range of concentrations of GnRH capable of stimulating PRL release was similar to those shown to stimulate GtH and GH release from pituitary fragments of teleosts including tilapia (Levavi-Sivan and Yaron, 1989; Peter et al, 1990; Melamed et al., 1995). These data, and the fact that the RPD is an almost homogeneous mass of PRL cells, suggest that GnRH is a direct regulator of PRL cell activity in the tilapia, *O. mossambicus*.

Consistent with the notion that GnRH's effects on PRL release are direct and receptor mediated, treatment with cGnRH-II evoked an increase in $[Ca^{2+}]_i$ within 1 min of exposure and an elevation in PRL release within 2 min.
These data suggest that calcium is operating as a second messenger in mediating the effects of GnRH on PRL release.

The pathway of GnRH action on PRL cells of the tilapia clearly differs from that of the rat. In the rat, GnRH has been shown to stimulate PRL release via paracrine action involving GtH cells (Denef and Andries, 1983). The RPD of the tilapia does not contain either GtH cells or GH cells. Growth hormone cell have also been to respond to GnRH in teleosts, including the tilapia (Marchant et al., 1989; Melamed et al., 1995).

The pathway of GnRH action on PRL cells of the tilapia may also differ from that of the goldfish. Cook and colleagues found negligible binding of a sGnRH analog to prolactin cells of the goldfish compared with binding to GtH and GH cells. Either the PRL cells of the goldfish do not respond to GnRH or the receptors are only expressed during certain periods, for example, discrete stages of development or reproduction. Prolactin is a FW-adapting hormone in teleosts and may always be expressed in euryhaline teleosts such as the tilapia (Grau et al., 1994). Alternatively, the direct regulation of PRL cells by GnRH may be a late development in the evolution of teleosts. Direct regulation of PRL cells by GnRH may exist in highly derived teleosts as the tilapia but not in lower teleosts as the goldfish or in mammals. An investigation of GnRH
receptors on PRL cells of the tilapia needs to be conducted as well as studies on the effects of GnRH on PRL release in other fishes, including the goldfish.

**Potencies of the 3 native forms of GnRH**

The tilapia and seabream have the same 3 native forms of GnRH (Powell et al., 1994; Weber et al., 1994). Interestingly, their order of potency are the same for in vivo stimulation of GtH-II release in seabream (Zohar et al., 1995) and in vitro stimulation of PRL release in tilapia. Seabream GnRH is by far the most abundant form in the pituitary of the tilapia (N. Sherwood, personal communication) and seabream (Powell, et al., 1994). Seabream-GnRH is also the least potent in stimulating PRL or GtH release, and cGnRH-II is the most potent in both circumstances. The cause for differences in potency in stimulating PRL release is at the pituitary level. Differences in peripheral degradation by specific peptidases at the liver or kidney, as listed among the possible factors contributing to the same order of potency in seabream, can not be a factor in my in vitro studies.

Reasons for the differing potencies of the various GnRH molecules include differences in receptor affinities, differences in intracellular degradation rates of the GnRH molecules within the pituitary, and activation of differ-
ent signal transduction cascades. A single type of GnRH receptor has been found in the pituitary of all fish examined to date with the exception of the goldfish. This suggests that the different GnRH forms are competing for the same receptor. Fish found to have a single type of GnRH receptor in the pituitary include the African catfish (Leeuw et al., 1988), winter flounder (Crim et al., 1988), stickleback (Andersson et al., 1989), and seabream (Pagelson and Zohar, 1992). More than one type of receptor has been identified in the goldfish (Habibi et al., 1987).

The native GnRH peptide with the greater GtH-II-releasing activity has been found to have greater receptor affinity in African catfish (cGnRH-II > catfish-GnRH; Schulz et al. 1993) and goldfish (cGnRH-II > sGnRH; Peter et al. 1990; Habibi, 1991). Nevertheless, in the same study in the goldfish, sGnRH was more active in releasing GH.

Gonadotropin-releasing hormone is degraded within the pituitary cells of teleosts (Goren et al., 1990; Zohar et al., 1990). The pituitary cells posses cytosolic peptidases that degrade GnRH after it has been internalized. Differences in biological activities of various GnRH molecules have been shown to be attributable to their differing susceptibility to degradation by these peptidases (Goren et al., 1990; Zohar et al., 1990).
Chang and colleagues (1993) have demonstrated that different native GnRH forms activate different signal transduction pathways in GtH cells of the goldfish. They suggest that binding of the two GnRH molecules to the same receptor leads to distinct receptor-conformation changes and different G-protein linkages. These different pathways may result in different potencies for the different GnRH molecules.

The order of potency appears to parallel the evolution of the GnRH peptides. The cGnRH-II peptide is present in cartilaginous fishes, sGnRH first appears in early teleosts and sbGnRH in late teleosts (cf. Sherwood et al. 1994). The presence of a single GnRH receptor in most teleosts suggests the peptides may have evolved independently of the receptor or receptors and therefore the older peptides may have a greater affinity to the receptor or receptors. On the other hand, if the pituitary cells which respond to GnRH have evolved specific peptidases to break down the biologically relevant GnRH form, then the biologically relevant GnRH peptide would have the shorter half life and lower biological activity (Goren et al., 1990; Zohar et al., 1990). Either possibility is consistent with sbGnRH being the biologically relevant regulator of GtHs, GH and PRLs in the pituitary. Furthermore, since sbGnRH is delivered by
neurosecretory fibers directly to the cells of the pituitary, sbGnRH can be delivered in high concentrations, and thus, a greater potency may not be necessary for the hormone to have its effect.

Roles for the different forms of GnRH

The dominance of sbGnRH in the pituitaries suggests that sbGnRH may be the GnRH form which is the regulator of GtHs, GH and PRLs. Nevertheless, examinations of pituitary and circulating levels of native GnRHs under many varying physiological and developmental conditions are needed before regulation of pituitary cells by other GnRH forms can be dismissed. Schulz and colleagues (1993) found catfish-GnRH to be in greater abundance than cGnRH-II in the pituitaries of the African catfish, *Clarias gariepinus*, with cGnRH-II being the more potent stimulator of GtH-II release *in vivo* and *in vitro*. The researchers present the possibility that catfish-GnRH may be the regulator of moderate levels of circulating GtH and the more potent cGnRH-II may be the regulator of GtH surges, such as those associated with spawning.

Injections and implants of native forms of GnRH and analogs have been shown to stimulate GtH and GH release. Thus, pituitary tissues can respond to GnRH in the blood. Whether circulating GnRH has biological significance in
most teleosts has yet to be determined. Gonadotropin-releasing hormone and a GnRH binding protein have been identified in the circulation of the goldfish. Furthermore, the concentrations of GnRH in the circulation were within a range found to stimulate GtH and GH release in vitro in the same species (Huang and Peter, 1988; Peter et al., 1990). In preliminary studies GnRH was not detected in the serum of tilapia (N. Sherwood, personal communication). Nonetheless, my in vitro studies suggest that cGnRH-II, which is endogenous in the tilapia (Weber et al., 1994), may be able to stimulate PRL release at concentrations which are below the detectable limits of the radioimmunoassay used to measure the GnRH.

Effects of T and E₂ on GnRH stimulated PRL release

The steroid hormones, E₂ and T, are able to enhance the response of PRL cells to GnRH, increasing percent release over 3-fold. The steroids may regulate the sensitivity of the PRL cells to GnRH stimulation during the reproductive cycle. Alternatively, GnRH molecules may regulate the sensitivity of PRL cells to T and E₂ stimulation. The potentiation of the response of PRL cells by GnRH and thyrotropin-releasing hormone by sex steroids supports the suggestion by Barry and Grau (1986)
that there may be a shift in the control of PRL secretion with changes in the reproductive state of the tilapia.

_Hypothalamic stimulators of PRL cells in the tilapia_

Prolactin secretion in teleosts is thought to be predominantly under inhibitory control, however, evidence had suggested that a hypothalamic prolactin-releasing factor was involved in the control of PRL secretion in fish (see reviews, Clarke and Bern, 1980; Ball, 1981). Barry and Grau (1986) have shown that thyrotropin-releasing hormone stimulates PRL release in the tilapia but only following E₂ preincubation. Until the present study, thyrotropin-releasing hormone was the only hypothalamic factor shown to have the capability to stimulate PRL release in the tilapia.

**Summary**

Gonadotropin-releasing hormone appears to function as a PRL-releasing factor in the tilapia, _O. mossambicus_, and this effect appears to be direct. All endogenous GnRH molecules stimulate the release of the two tPRLs, tPRL_{177} and tPRL_{188}, from RPD with the following order of potency: cGnRH-II > sGnRH > sbGnRH. Furthermore, the data suggest that calcium is operating as a second messenger in mediat-
ing the effects of GnRH on PRL release and the sex steroids, \( E_2 \) and \( T \), potentiate the response of PRL cells to GnRH.
Chapter IV

CHANGES IN SERUM AND PITUITARY LEVELS OF PROLACTIN AND GROWTH HORMONE WITH FASTING IN THE TILAPIA, OREOCHROMIS MOSSAMBICUS

INTRODUCTION

Prolactin and GH have effects on protein, carbohydrate, and lipid metabolism in mammals (see reviews, Nicoll, 1974; Fain, 1980; Davidson, 1987). The roles of PRL and GH in metabolism in teleosts are not well characterized. Prolactin has been shown to be either lipolytic or lipogenic depending on many factors including temperature, photoperiod, circadian rhythms, development and circulating levels of other hormones (review see, Baker and Wigham, 1979; Sheridan, 1986). Interestingly, plasma PRL concentrations are not altered by 30 days of fasting in Kokanee salmon, (Oncorhynchus nerka; McKeown et al., 1975). This is the only study to measure circulating PRL levels during fasting in a teleost. Nevertheless, Rodgers and colleagues (1992) found both pituitary concentrations of PRL and basal PRL release from RPD in vitro to be decreased after 2 weeks of fasting in O. mossambicus. In this same study, PRL release in vitro was negatively
correlated with essential amino acids concentration but was unaffected by D-glucose concentration in the medium. Although actions of PRL in metabolism appear to be conserved, evidence for a change in secretion rate with fasting is limited.

The roles of GH in metabolism and fasting in teleosts have received more attention than those of PRL, but are not without controversy. Growth hormone treatment has been shown to increase circulating levels of free fatty acids (FFAs) in the rainbow trout and goldfish and to mobilize lipid reserves from the liver of rainbow trout and coho salmon (Minick and Chavin, 1970; Leatherland and Nuti, 1981; Sheridan, 1986). In addition, GH injections increase liver glycogen and muscle FFAs in Kokanee salmon, but not plasma FFAs or glucose (McKeown et al., 1975). Growth hormone injections do not appear to stimulate lipolysis in the eel, Anguilla japonica, and tilapia, O. mossambicus, but instead appear to stimulate the mobilization of amino acids and glucose. Injections of bovine-GH in hypophysectomized eels increase serum amino acid concentrations and do not alter plasma lipid concentrations (Inui et al., 1985). Injections of bovine-GH stimulate the release of glycogen and not lipid from liver reserves and increases serum concentrations of amino acids and glucose but not protein, lipid or cholesterol in the
tilapia (Leung et al., 1991). Consistent with this elevation in circulating amino acids and glucose, Rodgers and colleagues (1992) found an increase in GH release from the proximal pars distalis of tilapia in response to reductions in essential amino acids and glucose in the medium. Together these studies suggest that GH cells in the tilapia act in the regulation of, and are directly responsive to, changes in the blood concentrations of amino acids and glucose. Thus, GH would be expected to increase with fasting in the tilapia, as in mammals.

Increases in circulating and pituitary levels of GH have been observed in fasted teleosts. Increases in plasma GH concentrations in rainbow trout have been observed within 3 weeks of fasting in studies by Wagner and McKeown (1986), and within 1 week in studies by Sumpter et al. (1991a). No change in plasma GH concentrations was observed after 30 days of fasting in Kokanee salmon (McKeown et al., 1975). Farbridge and Leatherland (1992) observed a bi-modal response to fasting in rainbow trout, first exhibiting a decline in plasma GH concentrations at 2 weeks compared with fed controls, followed by an elevation at 4 weeks. Plasma GH concentrations in the fed controls may not have been different from initial values and plasma GH levels were elevated at 2 weeks of fasting in a repeat of the study. Melamed (1993) observed an
increase in plasma GH concentrations within 16 days of fasting in "sub-optimally" fed tilapia hybrids (O. niloticus X O. aureus). Also in tilapia, O. mossambicus, pituitary content of GH was elevated with 2 weeks of fasting (Rodgers et al., 1992).

Farbridge et al. (1992) found that feeding rainbow trout only once every five days resulted in reduced plasma GH and suggested that GH concentrations are depressed by low feeding rates. This is not consistent with the study on the tilapia hybrid, by Melamed (1993). The tilapia at the start of the experiment by Melamed, were described as "sub-optimally fed". When the fish were switched to an increased feeding rate, plasma GH concentrations fell within 8 days. Clearly, the nutritional state of the animal prior to fasting and changes in nutritional state during fasting affects the hormonal response to subsequent fasting.

In order to learn more about the roles of PRL and GH in tilapia metabolism, I have characterized changes in serum concentrations and pituitary content of the tPRLs and GH with 21 and 31 days of fasting in well-fed tilapia with a high condition factor (CF; fat tilapia) and tilapia with a low CF (thin tilapia). The fasting should lead to an alteration in metabolic state requiring the mobilization of energy stores. If the tPRLs or GH are involved in
the mobilization of these energy stores, then their serum concentrations or content in the pituitary may be altered. Changes in body weight (BW), standard length (SL), gonad weight, and liver weight were recorded to gauge the effects of fasting on the metabolic state of the animals.

MATERIALS AND METHODS

Animals

Tilapia, *O. mossambicus*, were collected from brackish water streams and maintained in FW in outdoor tanks (22-25°C), for at least 3 months before use. The fish were fed to satiation twice daily with Purina trout chow. Fish were not fed on the day of sampling.

Fish were transferred to an indoor facility with a fixed photoperiod of 14L:10D. Fish were placed in oval fiberglass tanks (60 l). The tanks were outfitted with a continuous water flow (~23°C) and constant aeration. The fish ranged in size from about 60-100 g. Fish were allowed to acclimate to the tanks for at least 2 weeks before the start of the experiments.

Fish were anesthetized with 2-phenoxyethanol (1 ml/l) for blood collection. Blood was drawn from the hemal arch within 5 min of introduction to the anesthesia, using a 28-gauge needle attached to a 1-ml disposable syringe.
Blood was stored on ice and the serum was separated from the formed elements by centrifugation and then stored at -80°C until it was analyzed for PRLs and GH by radioimmunoassay. The radioimmunoassays are detailed in chapter II. Anesthetization with 2-phenoxyethanol did not affect serum PRL and GH levels (Fig. 15).

Additional measurements and collections of tissues varied with experiments. Body weight, SL, gonad weight and liver weight were recorded and pituitaries were collected and placed in 20 µl of distilled water in 1.5-ml microcentrifuge tubes and stored at -80°C until they were analyzed for PRLs and GH by radioimmunoassay. Pituitaries were sonicated in phosphate buffered saline and diluted in phosphate buffer with 1% bovine serum albumin (Sigma). Protein content was determined in the undiluted samples using a Bio-Rad (Richman CA) protein assay kit. Data were expressed as micrograms of hormone per pituitary (µg/pit.) as opposed to micrograms of hormone per milligram of protein for 2 reasons. First, the fish in each study were similar in size and therefore should have similar size pituitaries. Secondly, pituitary proteins; PRLs, GH, and GtHs would be expected to change dramatically under the conditions of the studies, possibly affecting pituitary protein content. For example, PRL content will appear to decrease if there is a large increase in pituitary GH
Figure 15. Effects of 2-phenoxyethanol (1 ml/l) on prolactin and growth hormone serum concentrations in the tilapia, Oreochromis mossambicus (mean ± SE, N = 10-15 per treatment, ns = not significant at P < 0.05).
content and PRL is normalized to pituitary protein.

Gonadosomatic index (GSI; ((gonad weight (g)/(g)) X 100)
hepatosomatic index (HSI; ((liver weight (g)/BW (g)) X
100) and condition factor (CF; ((BW (g)/(SL (cm))^3) X 100)
were calculated. When sampling required killing the
animals, the fish were quickly decapitated following
anesthetization.

Statistical analysis

Differences among groups were determined using analy­
sis of variance and the least significant difference test
for a priori pairwise comparisons analysis (Steel and
Torrie, 1980). Comparisons between two means were per­
formed using the unpaired Student's t-test. Data are
expressed as means ± SE.

Study 1

Two fasting studies were conducted. The first study
examined the effects of 10 and 21 days of fasting on
female and 21 days of fasting on male tilapia with a high
condition factor (~3.6). The study was replicated in two
trials. Body weights, SL and gonad weight measurements
were recorded for the second trial of the study. In both
trials of the study fish were placed 5 to a tank and there
were two tanks per treatment (10 treatments). Fasting
started on a staggered basis so that all fish were sampled on the same day between 1300 and 1600. Fish were fed to satiation twice daily with Purina trout chow when not fasting.

Study 2

The second study examined the effects of 10, 21, and 31 days of fasting on male tilapia with a low condition factor (~3.2). Study 2 was conducted following the same protocol as Study 1 except 6 fish were placed in each tank and were fed once daily when not fasted as part of the study. The fish used in the second study were fed a limited ration once daily for 2 months prior to the start of the study. Body weights, SL, gonad weight and liver weight measurements were recorded for the study.

RESULTS

The effects of fasting on serum concentrations and pituitary content of the PRLs and GH in male and female tilapia with a high condition factor

Serum levels of tPRL$^{177}$ increased from 1.8 ± 0.3 ng/ml in fed female tilapia, to 6.2 ± 1.3 ng/ml with 10 days of fasting (P < 0.001) and 5.3 ± 0.5 ng/ml with 21 days of fasting (Fig. 16; P < 0.01). A similar trend
Figure 16. Changes in serum concentrations of prolactin and growth hormone during fasting in male (M) and female (F) tilapia Oreochromis mossambicus with a high condition factor (mean ± SE, N = 10-12 per treatment; * P < 0.05, ** P < 0.01, *** P < 0.001).
was not significant for tPRL_{188} (ANOVA P = 0.059). Serum tPRL_{177} and tPRL_{188} concentrations were elevated in male tilapia fasted for 21 days. Prolactin_{177} concentrations increased from 1.7 ± 0.2 to 6.7 ± 1.3 ng/ml with 21 days of fasting (P < 0.01) and tPRL_{188} serum concentrations increased from 7.5 ± 0.8 to 12.6 ± 2.1 ng/ml (P < 0.05). Serum GH concentrations were not affected by 21 days of fasting in either sex.

Pituitary content of tPRL_{177} was elevated in female tilapia fasted 21 days compared with females fasted 10 days or fed (Fig. 17; P < 0.01). Fasting did not affect pituitary tPRL_{177} content in male tilapia or tPRL_{188} content in male or female tilapia. Pituitary content of GH increased from 3.9 ± 0.6 μg/pit. in fed female tilapia to 6.1 ± 0.6 μg/pit. in female tilapia fasted for 21 days, and increased from 3.9 ± 0.2 μg/pit. in fed male tilapia to 5.4 ± 0.4 μg/pit. in male tilapia fasted for 21 days (P < 0.001 and P < 0.01 respectively).

The effects of fasting on condition factor, body weight, standard length and GSI in male and female tilapia with a high condition factor

At the start of the study, CFs were similar for male and female tilapia, ranging from 3.58 to 3.77 (Table 1).
Figure 17. Changes in pituitary content of prolactin and growth hormone during fasting in male (M) and female (F) tilapia Oreochromis mossambicus with a high condition factor (mean ± SE, N = 10-12 per treatment; ** P < 0.01, *** P < 0.001).
Table 1. Effects of fasting up to 21 days on condition factor, body weight (g) and standard length (cm) in male and female tilapia with a high condition factor (CF > 3.6; mean ± SE).

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<th>SL-E</th>
<th>%CH</th>
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</tbody>
</table>

(B, beginning; E, end; BW, body weight; SL, standard length; %CH, % change; t-T, t-Test comparisons; * P < 0.05, ** P < 0.01; ns, not significant)
Condition factors were reduced by 21 days of fasting in males compared with values for the same animals at the start of the study (P < 0.05), and with fed males at the end of the study (P < 0.05). The CFs for females in all treatments were lower at the end of the study compared with initial values (all P < 0.01). Condition factors were not different among treatments with females, at the end of fasting. There were no significant changes in BW or SL with treatment. Nevertheless, there was a significant drop in GSI for females fasted for 21 days (Fig. 18; P < 0.01). The follicles of fasted fish were soft, suggesting that atresia was beginning. The GSI for males did not change with fasting.

The effects of fasting up to 31 days on serum concentrations and pituitary content of the PRLs and GH in male tilapia with a low condition factor

I did not see an expected increase in serum GH with 21 days of fasting with fish that had a high CF. For this reason, the study was repeated with animals with a lower CF, and fasting was extended to 31 days.

Serum concentrations of tPRL_{177} and tPRL_{188}, but not GH, were elevated in tilapia fasted for 10 days (Fig. 19; both P < 0.05). Serum tPRL_{177} increased from 0.6 ± 0.1 ng/ml to 9.0 ± 2.8 ng/ml and tPRL_{188} increased from
Figure 18. Changes in gonadosomatic index (GSI) during fasting in male (M) and female (F) tilapia Oreochromis mossambicus with a high condition factor (mean ± SE, N = 10 per treatment; ** P < 0.01).
Days Fasted

Males

Females

GSI

Days Fasted

0 21 0 10 21
Figure 19. Changes serum concentrations of prolactin and growth hormone during fasting in male tilapia Oreochromis mossambicus with a low condition factor (mean ± SE, N = 9-10 per treatment; * P < 0.05, ** P < 0.01, *** P < 0.001).
Day 0 10 21 31

Serum concentration (ng/ml)

\[\text{tPRL}_{177} \quad \text{tPRL}_{188} \]

Days Fasted

GH

\[** \quad ** \quad *** \]
5.7 ± 0.3 ng/ml to 12.2 ± 1.3 ng/ml, comparing fish that had been fed to fish that had been fasted. Serum PRL concentrations were elevated in fish fasted for 21 and 31 days, compared with fed fish. Growth hormone concentrations were less than 1 ng/ml in fish fasted up to 21 days and then increased to 3.5 ± 0.7 ng/ml in fish fasted for 31 days (P < 0.001).

Pituitary tPRL<sub>188</sub> levels were not different among fed animals and animals fasted for 10 and 21 days. Pituitary content of tPRL<sub>188</sub> decreased with day 31 of fasting (Fig. 20; P < 0.01). Pituitary content of tPRL<sub>177</sub> was not significantly affected by fasting. Pituitary GH content increased with fasting. Growth hormone rose from 7.9 ± 0.8 μg/pit. in fed animals, to 13.0 ± 1.9 μg/pit. in animals fasted for 21 days (P < 0.05), and 14.3 ± 2.1 μg/pit. in tilapia fasted for 31 days (P < 0.01).

The effects of fasting up to 31 days on condition factor, body weight, standard length, GSI and HSI in male tilapia with a low condition factor

Body weight and SL were measured at the start of the experiment, at the beginning of the fasting period, and at the end of the fasting period (Table 2). Changes in BW, SL and CF were compared from the start of the fasting period to the end of the fasting period, as well as from
Figure 20. Changes pituitary content of prolactin and growth hormone during fasting in male tilapia Oreochromis mossambicus with a low condition factor (mean ± SE, N = 9-10 per treatment; * P < 0.05, ** P < 0.01).
Table 2. Effects of fasting up to 31 days on condition factor, body weight (g) and standard length (cm) in male tilapia with a low condition factor (CF > 3.2; Mean ± SE)

<table>
<thead>
<tr>
<th>Start of fasting and end of fasting</th>
<th>Days</th>
<th>BW-B</th>
<th>BW-E</th>
<th>%CH t-T</th>
<th>SL-B</th>
<th>SL-E</th>
<th>%CH t-T</th>
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<th>CF-E</th>
<th>%CH t-T</th>
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</table>

ANOVA * *** * ** n ***

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<td>0.36</td>
<td>3.21</td>
<td>2.88</td>
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</table>

ANOVA ns *** ns ** ns ***

(B, beginning; E, end; BW, body weight; SL, standard length; %CH, % change; t-T, t-Test comparisons; * P < 0.05, ** P < 0.01, *** P < 0.001; ns = not significant)
the start of the experiment to the end of the fasting period, and the start of the experiment to the start of the fasting period. The CFs did not differ among treatment groups from the start of the experiment to the start of fasting. This consistency in CF suggests that the nutritional status of the animals remained unchanged throughout the period before fasting was initiated.

Condition factor was reduced from 3.11 ± 0.05 to 2.86 ± 0.04 (P < 0.01) with 21 days of fasting, and from 3.21 ± 0.05 to 2.88 ± 0.04 (P < 0.001) with 31 days of fasting.

The difference (P < 0.05) in BW and SL among treatment groups at the start of the fasting period is due to the slightly smaller size of the fish in the group to be fasted for 31 days at the start of the study, combined with the growth of the fish during the interval between the start of the experiment and the beginning of the fasting period in the other groups. Body weight, SL and CF decreased as fasting increased (P < 0.001, P < 0.01, P < 0.001 respectively). There was a significant increase in BW in the group fasted for 10 days that occurred between the start of the experiment and the start of the fasting period (P < 0.05).

Hepatosomatic index was reduced at 10 days of fasting (Fig. 21; P < 0.001). The HSI of fed fish was 1.72 ± 0.07 compared with 0.97 ± 0.05 for fasted fish. There was no
Figure 21. Changes in hepatosomatic index (HSI) during fasting in male tilapia Oreochromis mossambicus with a low condition factor (mean ± SE, N = 10 per treatment; *** P < 0.001 ).
further reduction in HSI as fasting continued. There were no differences in GSI among treatments at the end of the study.

DISCUSSION

Serum concentrations and pituitary content of GH and serum concentrations of tPRL$_{177}$ and tPRL$_{188}$ were elevated in response to fasting in *O. mossambicus*. These data suggest that GH and the tPRLs have roles in the regulation of intermediary metabolism in the tilapia, *O. mossambicus*. Furthermore, serum concentrations of the tPRLs increased earlier in response to fasting than GH, and thus, the tPRLs and GH may have different roles in regulating metabolism.

An increase in circulating GH appeared later in response to fasting in *O. mossambicus* than has been observed in most other teleosts. Serum GH concentrations were elevated at 31 days of fasting in male tilapia with a low CF. Serum concentrations of GH were unchanged for the first 21 days of fasting in female *O. mossambicus*, with a high CF, and in males with a high or a low CF. Increases in circulating GH concentrations were observed within 1-3 weeks in rainbow trout (*Oncorhynchus mykiss*; Wagner and McKeown, 1986; Sumpter et al., 1991) and within 16 days in
a tilapia hybrid (O. niloticus X O. aureus; Melamed, 1993). On the other hand, McKeown et al. (1975) did not see an elevation in plasma GH concentrations in Kokanee salmon fasted for 30 days.

The difference in the timing of the increase in circulating GH levels among the studies may be due to a variety of factors. Among these factors are differences in basal metabolic rates of the different animals. These differences may be due simply to species or species differences combined with differences in developmental stage or environment. For example, in my study there was only a ~31% difference in BW change between male tilapia that had been fed and male tilapia that had been fasted for 31 days. The rainbow trout in the study by Wagner and McKeown (1986) that had been fed were ~250% greater in BW than the fish that had been fasted for 21 days. The greater weight loss observed in the trout compared with the tilapia is likely due to differences in metabolism related to the tilapia being a herbivore and the trout being a carnivore. A second factor contributing to the timing of an increase in circulating GH levels in response to fasting is the metabolic state of the animals at the start of the fasting period. The tilapia hybrid used in the fasting study by Melamed (1993) were described as "sub-optimally" fed. There is no further description of
the condition of the animals. In that study, the plasma GH concentrations started at over 200 ng/ml and increased to over 500 ng/ml following 16 days of fasting, or dropped to 15-20 ng/ml following 16 days of "optimal feeding". In my study, fish with low and high CFs had serum GH concentrations below 2 ng/ml before fasting. The elevated GH levels prior to fasting in the tilapia hybrids may suggest that their energy reserves were more depleted than any of the fish in the present study. Thus, the hybrid tilapia may have required an increase in GH sooner after a further reduction in feeding, to mobilize additional energy stores or protect essential resources from catabolism. Alternatively, the difference in response between the 2 tilapias may be due to species differences in the hormonal regulation of metabolism. The hybrid is selected for fast growth and has higher baseline GH levels. Regardless of the differences in the timing of the increases in circulating GH levels, circulating concentrations of GH were elevated in the tilapia in response to fasting as in most species of teleosts examined.

The pituitary content of GH was elevated at 21 days of fasting in all groups in both studies; male and female tilapia that had a high CF (~3.6) prior to fasting, and male tilapia that had a low CF (~3.2) prior to fasting. The pituitary content of GH was not elevated at 10 days of
fasting in any of these groups. This is consistent with a previous report by Rodgers and colleagues (1992) that pituitary GH content was elevated with 2 weeks of fasting in this same species. Increased pituitary GH content was observed prior to an increase in serum GH concentration in male tilapia fasted for 31 days. These data suggest that pituitary GH may increase before release from the pituitary is increased.

An earlier change in pituitary GH content with fasting, than serum concentration with fasting, suggests that changes in pituitary GH content may be the better indicator of changes in GH cell activity with changes in metabolic state accompanying fasting. Consistent with this notion, pituitary GH content, but not serum concentration, was elevated at 21 days of fasting, and at 21 days of fasting there were signs of changes in metabolic state in the tilapia which had been fasted. In both studies CF was reduced in male tilapia fasted for 21 days compared with fed fish and compared with values from the same fish prior to fasting. In addition, HSI was reduced by 10 days of fasting in male tilapia suggesting that liver energy stores were mobilized. The GSIs of female tilapia fasted for 21 days were reduced compared to those of fed fish, suggesting ovarian growth was compromised.

Serum tPRL concentrations were increased with 10
days of fasting in male and female tilapia and in tilapia with a low and a high CF. This is the first report of an increase in circulating PRL concentrations in response to fasting in a teleost. Prolactin$_{177}$ appears more sensitive to fasting than tPRL$_{188}$. Unlike serum tPRL$_{177}$ concentrations, serum tPRL$_{188}$ concentrations were not elevated in female tilapia with a high CF, which had been fasted for 10 or 21 days. In the same study, serum tPRL$_{177}$ concentrations were increased 3 fold and tPRL$_{188}$ levels were increased by about 70% in male tilapia at 21 days of fasting compared with fed tilapia. Serum tPRL$_{177}$ and tPRL$_{188}$ concentrations were elevated with 10 days of fasting in male tilapia with a low CF.

Rodgers and colleagues (1992) found that RPD removed from tilapia (O. mossambicus) fasted for 2 weeks had reduced spontaneous release of PRL. The pituitary content of PRL together with the hormone released into the medium during 20 hr incubations, was also lower for the fasted O. mossambicus (sex not identified). The increase in serum PRL concentrations I observed in response to fasting may at first appear to be inconsistent with the reduction in spontaneous PRL release from RPD with fasting, reported by Rodgers and colleagues (1992). It is possible that the reduction in PRL release from RPD of fasted fish, observed by Rodgers and colleagues, was the result of a depletion.
of stored PRL in the RPD, prior to removal of the tissues. This interpretation is consistent with the increase in circulating tPRLs I observed with fasting and the reduced PRL content in the pituitaries of the fasted tilapia in the studies by Rodgers and colleagues.

Pituitary content of tPRL$_{177}$ was elevated at 21 days of fasting in female tilapia with a high CF whereas pituitary content of tPRL$_{177}$ in males and tPRL$_{188}$ in males and females were not altered by fasting. On the other hand, pituitary content of tPRL$_{188}$ was reduced in male tilapia with a low CF at 31 days of fasting while there was no change in pituitary tPRL$_{177}$ content. The effect of fasting on pituitary PRL content appears to be sex specific, however, more data are needed before this notion can be confirmed.

The differences in serum concentration and pituitary content of PRL and GH among fed and fasted tilapia observed in this study are assumed to be in response to a need to mobilize or protect energy reserves in response to a switch from anabolic to catabolic activities, or at least an increase in catabolic activities with fasting. Several lines of evidence support that there were changes in metabolic state with the imposed fasting. In the second study with tilapia with a low CF, there was a decline in CF at 21 and 31 days of fasting. Also in the
second study, there was a dramatic decrease in HSI by day 10 of fasting suggesting a mobilization and possible depletion of available energy reserves in the liver. In the first study, there was a decline in CF for males but no decline in CF related to fasting for females. The females may not have been in the same nutritional state at the end of the study even though there were no differences in CF between fed and fasted female tilapia. Although not significant, there appeared to be a trend towards a decrease in BW and SL with increased fasting time. There may have been reduced linear growth with fasting keeping pace with any decline in body weight, thus, a decline in CF with fasting was not observed. There may not have been sufficient resources for maintaining linear growth in fish which started with a low CF in Study 2 and therefore changes in nutritional state were more easily detected in changes in CF with fasting. A decline in GSI together with an apparent softening of the follicles suggests that the females in Study 1 were suffering from a nutritional deficit, enough to impact ovarian development.

Although there were differences in changes in CF in response to fasting between the two studies, suggesting that there were differences between the two studies as to the changes in metabolic states, there were no discernible differences in hormone response. In both studies, one
with low CF (~3.2) and one with high CF (~3.6), pituitary
content but not serum concentrations of GH increased with
21 days of fasting. In addition, serum concentrations of
tPRL$_{177}$ were increased by 10 days of fasting in both
studies and serum concentrations of tPRL$_{188}$ were elevated
in males with 21 days of fasting in both studies. The
consistency of the responses may be due to the limited
sampling. Serum PRL concentrations may have increased
earlier within the first 10 days of fasting, and pituitary
GH content may have increased earlier between days 10 and
21 of fasting in the tilapia with a lower CF than in the
tilapia with a higher CF. On the other hand, the
metabolic or nutritional state of the tilapia at the start
of fasting may be of less importance than a change in
nutritional state.

The differences in response to fasting by PRL and GH
suggest the hormones may have disparate actions. Serum
tPRL$_{177}$ increased much earlier than serum GH concentra-
tions. Prolactin may be mobilizing energy stores from the
liver which undergoes most of its weight loss within 10
days. Growth hormone may then rise to mobilize other
energy reserves or to inhibit the mobilizing or cataboliz-
ing of needed resources. Further study is needed to
understand the roles of PRL and GH in metabolic processes
associated with fasting in the tilapia. Among these
studies are kinetic studies to see if there are changes in the metabolic clearance rates of the tPRLs or GH associated with fasting and studies on changes in body composition and blood metabolites in response to fasting and homologous hormone treatment. Even then, these studies must be conducted under different physiological conditions and at different developmental stages.

In summary, serum concentrations of tPRL\textsubscript{177} and tPRL\textsubscript{188} increased soon after the initiation of fasting while changes in pituitary content of GH and serum concentrations of GH were not observed until late in fasting. Prolactin\textsubscript{177} appeared to be more sensitive to changes in nutritional state than tPRL\textsubscript{188}. Changes in pituitary content of GH were observed before changes in serum concentrations of the hormone while the opposite was true with PRL. In addition, the PRL response to fasting may be sex specific. Prolactin\textsubscript{177} content in the pituitary increased with fasting in female tilapia with no change in male tilapia. A decline in pituitary tPRL\textsubscript{188} content was observed in males with a low CF at 31 days of fasting but there were not females in the experiment for comparison. These studies provide evidence that the tPRLs and GH are active in the regulation of metabolism in the tilapia, \emph{O. mossambicus} and that the tPRLs and GH may have disparate functions.
Chapter V

CHANGES IN SERUM CONCENTRATIONS AND PITUITARY CONTENT OF PROLACTIN AND GROWTH HORMONE DURING THE REPRODUCTIVE CYCLE OF FEMALE TILAPIA, OREOCHROMIS MOSSAMBICUS ADAPTED TO FRESH WATER AND SEAWATER

INTRODUCTION

Prolactin and GH have actions in osmoregulation, metabolism, and reproduction (Nicoll, 1974; Clarke and Bern, 1980; Loretz and Bern, 1982; Hirano, 1986). These processes are intertwined within the reproductive habits of the tilapia, O. mossambicus. Oreochromis mossambicus can reproduce in both FW and SW. Furthermore, feeding is reduced in the female tilapia when it broods offspring in its buccal cavity. Brooding lasts for approximately 3 weeks. The subject of this study was the roles of the tPRLs and GH in the regulation of these disparate though overlapping processes during reproduction in the female tilapia.

Prolactin and GH may regulate reproductive functions in the female tilapia. Prolactin has been implicated in parental care in teleosts including cichlids. Behaviors shown to be elicited by PRL are expressed in female tila-
pia. These behaviors include "calling movements" to fry and reduced feeding behavior which may prevent the cannibalism of young (Noble et al., 1938; Neil, 1964; Blum and Fielder, 1965; Blum, 1966). Prolactin has also been shown to induce mucus secretion as a source of nutrition for developing fry (see review, Nicoll, 1974).

Fish PRLs and GHs have been shown to stimulate ovarian growth and steroidogenesis in teleosts (Singh et al., 1988; Tan et al., 1988; Danzmann et al., 1990; Van Der Kraak et al., 1990). In the tilapia, tPRLs have been shown to alter steroid release from testicular tissues of courting and non-courting males in vitro (Rubin and Specker, 1992). The tPRLs stimulate T production from testis fragments of courting males but have no effect on testicular tissues from non-courting males. The tPRLs augment the response of testicular tissues from courting males to ovine-luteinizing hormone but inhibit this response in testis from non-courting males. In addition, tPRL188 has been shown to stimulate E2 production by vitellogenic oocytes of the guppy, Poecilia reticulata (Tan et al., 1988). Further supporting a direct action of PRL and GH in regulating steroidogenesis in teleosts is the identification of PRL and GH binding sites in the ovary. Prolactin binding sites have been identified in the ovary of the tilapia and GH receptors have been iden-
tified in the ovaries of salmonids (Edery et al., 1984; Mourot et al., 1992).

Reproductive hormones have been shown to stimulate or modulate the release of PRL and GH in tilapia. The steroid hormones, E₂ and T have been shown to stimulate PRL cells (Nagahama et al., 1975; Wigham et al., 1977; Barry and Grau, 1986; Borski et al., 1991) and modulate the response of PRL and GH cells to hypothalamic factors including thyrotropin-releasing hormone, GnRH and GHRH (Barry and Grau, 1986; Melamed et al., 1995).

Prolactin and GH regulate osmoregulatory functions in the tilapia and may be active in these roles depending on the salinity in which the tilapia is reproducing (cf. Nishioka et al., 1988; cf. Grau et al., 1994). Pituitary content and blood concentrations of the tPRLs are greater in FW- than in SW-adapted tilapia (Nicoll et al., 1981; Borski et al., 1992; Ayson et al.; 1993, Yada et al., 1994). Furthermore, Borski and colleagues (1992) have shown that the two tPRLs are differentially regulated by environmental salinity (Borski et al., 1992). Pituitary content of GH is greater in SW- than in FW-adapted tilapia (Borski et al., 1994). Yada et al. (1994) observed an increase in circulating GH shortly after transfer of male O. mossambicus from FW to 70%-SW but no change in females. In the same study, GH levels in the plasma were reduced.
shortly after transfer from SW to FW in both sexes, compared to animals at the same time point which had been transferred from SW to SW. Interestingly, circulating levels of GH were no longer different from controls, in either direction of transfer and either sex, by 7 days after transfer. Sakamoto and colleagues (1994) also observed an increase in plasma GH following transfer of O. mossambicus from FW to SW whereas plasma GH was not altered following transfer from SW to FW. Furthermore, the increase in plasma GH was observed in both males and females and at the end of the study, day 14 after transfer, plasma GH was elevated in males but not in females (Sakamoto, personal communication).

Finally, the tPRLs and GH may regulate metabolic activities in the brooding female tilapia which experiences a reduction in food intake. I have shown in chapter IV that serum and pituitary levels of the tPRLs and GH increase in response to fasting. Changes in hormone levels observed during the reproductive cycle of the female tilapia may therefore derive from changes in nutritional state. Sumpter and colleagues (1991a,b) have determined that changes in circulating GH concentrations during the reproductive cycle of male and female rainbow trout are a consequence of changes in metabolic state and
not due to reproduction *per se*. A similar situation may exist with the tilapia.

The foregoing discussion suggests that reproductive, metabolic and osmoregulatory requirements of the female tilapia may contribute to PRL and GH cell activity during the reproductive cycle. In chapter IV, I have described changes in serum concentrations and pituitary content of the tPRLs and GH that occur with changes in metabolic state induced by fasting. In the studies described in the present chapter I characterized changes in serum concentrations and pituitary content of the tPRLs and GH that occur over the course of the reproductive cycle of the female tilapia. I also examined the effects of the salinity on the patterns of changes in the tPRLs and GH in the serum and pituitary, observed during the reproductive cycle. Changes in BW, SL, CF, GSI and HSI that occur over the course of the reproductive cycle were also examined in an attempt to evaluate the contribution of changes in metabolic state to hormone changes. The aim was to gain insight into how the tilapia balances the regulation of different functions by the same hormones, by comparing hormone patterns for female tilapia reproducing in FW and SW, and hormone patterns observed during fasting.

Comparing changes in hormones during the reproductive cycle of fish maintained under various conditions may help
to clarify which aspects of the hormone patterns are
intrinsic to the reproductive cycle. Three studies were
conducted. Study 1 was conducted in an outdoor tank with
FW animals. This study was conducted to examine changes
in serum concentrations and pituitary content of the tPRLs
and GH in tilapia that are reproducing in an environment
that is conducive to their natural behavior. Studies 2
and 3 were conducted in indoor tanks. The fish and envi­
ronmental conditions for studies 2 and 3 were similar
except that in Study 2, the fish were adapted to FW and in
Study 3 the fish were adapted to SW. Study 2 was also
designed to expand and refine sampling to include earlier
stages of the reproductive cycle. The purpose of Study 3
was to provide for a comparison of serum and pituitary
patterns of PRL and GH between FW- and SW-adapted tilapia.

MATERIALS AND METHODS

Animals

Tilapia, O. mossambicus were collected from brackish
water streams and maintained in FW in outdoor tanks at a
temperature of 22-25°C for at least 2 months before use.
The fish were fed to satiation twice daily with Purina
trout chow. Fish were handled and sampled using proce­
dures described in chapter IV. Prolactins and GH in serum
and pituitaries were measured using the homologous radioimmunoassays developed by Ayson et al., (1993) and modified by Yada et al. (1994) as described in chapter III. Pituitaries were collected and prepared for measurement by radioimmunoassay as described in chapter IV.

The reproductive stage of the animals was classified using two criteria. Follicle mass of the largest clutch of follicles in the ovary was used to classify non-brooding females, and stage of larval development of the young was used to classify brooding females. Follicle mass was determined by collecting and weighing a group of 20 follicles from the most advanced clutch of follicles within the ovary. Follicle growth was found to be linear over time starting from spawning in *O. mossambicus* (Weber et al., 1992). Non-brooding females were grouped according to follicle mass, to the nearest mg. Follicle mass and brooding stage were recorded to investigate changes in ovarian growth during the brooding phase of the reproductive cycle. The days from spawning for each stage were estimated from the stage of development of the eggs and larvae, based on previous studies that examined the rate of larval development in *O. mossambicus* (Weber et al., 1989; Okimoto et al., 1993).

Initially, brooding females were classified into 10 stages based on the developmental stage of the young.
These stages are: early-egg, eyes not pigmented; mid-egg, eyes pigmented but no spinal movement; late-egg, spinal movement; early-yolksac, up until 50% of the larvae can swim in the water column; mid-yolksac, up until pigmentation covers the sides of the larvae and yolk can only be seen through the bottom of the fish; late-yolksac, up until yolk can not be found in the larvae; females brooding post-yolksac larvae. In most of the present studies, these stages have been collapsed into just 3 brooding stages: females brooding eggs, females brooding yolksac larvae, and females brooding post-yolksac larvae. Females designated as simply non-brooding females were females with follicles greater than 4 mg. Four mg follicles are not found in females which are brooding. Follicle mass does not usually exceed 3 mg in brooding tilapia (Fig. 22). Follicles weigh about 6 mg at ovulation.

Statistical analysis

Differences among groups were determined using analysis of variance and the least significant difference test for a priori pairwise comparisons analysis (Steel and Torrie, 1980). Comparisons between two means were performed using the unpaired Student's t-test. Data are expressed as means ± SE.
Study 1

Fish were transferred to an outdoor vinyl-lined broodstock tank (18 ft diameter) with a slow, continuous flow of FW. The fish were not sampled for at least 2 months after transfer to allow the females time to begin reproducing. Females averaged about 150-200 g. Water temperature was dependent on ambient temperatures and ranged from ~22-26°C. An algal bloom was maintained in the tank as a dietary supplement by controlling water exchange rate. The tank also accumulated detritus upon which the tilapia can feed. Detritus-like matter was seen in the gut of brooding and non-brooding tilapia. The females in this tank were observed to stay near the center of the tank with the males establishing nest cites around the perimeter.

Females were sampled from the outdoor tank on 3 occasions, once each in July, November and April, for a total of 126 fish. Fish were collected by hand net and placed immediately in anesthesia. Blood samples were collected from all fish, and pituitaries were collected in April and November as described in chapter IV. Also in November; SL and liver weight was recorded for all females for the determination of HSI and CF, and follicle mass was recorded for brooding females for the examination of
ovarian growth during the brooding phase. Only females with follicles larger than 4 mg were identified as non-brooding females to insure that they were not brooding females which had released their clutch before capture. During the April collection, the time of capture was recorded. Prolactin and GH levels were not correlated with time from the start of collection, suggesting that the collection method did not affect hormone levels (data not shown).

**Study 2**

Fish were transferred to an indoor facility with a fixed photoperiod of 14L:10D. Fish were distributed among 3 fiberglass tanks (500 l) fitted with a plexiglass window. In each tank, 20 females were housed with 2 males. Females weighed 60-100 g. The tanks were outfitted with a continuous flow of FW, constant aeration, and submersible heaters that maintained the water temperature at 26.5 ± 0.5°C. The indoor tanks had limited detritus buildup. The fish were not sampled for at least 8 weeks following their introduction to the tanks. At the time of sampling, females that were brooding were identified through the windows of the tanks and removed first. This prevented the females from releasing the clutches of young they were brooding before I had a chance to identify the females as
brooding females. Females which had not been brooding but were in stages earlier than late vitellogenesis can not be distinguished with certainty from brooding females with similar size follicles that had been brooding but released their clutch prior to capture. Therefore, sampling of females during the early stages of ovarian growth required that all females be identified as brooding females or non-brooding females prior to collection.

Only non-brooding females whose follicle mass exceeded 1.5 mg were included in the study. This is because females with a follicle mass of less than 1.5 mg may not have been reproductively mature or may have terminated brooding prematurely. A mass of 1.5 mg was chosen as the lower limit because FW females brooding post-yolksac larvae were found to have follicles with a mass of just under 1.5 mg (Fig. 22). Samples were collected on 5 occasions from July through December, from a total of 177 females. Body weight, SL, liver weight and gonad weight were recorded during the final sample collection for determination of HSI, CF and CF with gonad weight subtracted from BW.

Study 3

Study 3 was the same as Study 2 except the fish were adapted to SW. Fish were introduced into the tanks as in
Study 2 and the salinity of the water was slowly increased to full-strength SW over a 1 week period. The tanks were then supplied with a continuous flow of SW. The fish were not sampled for at least 8 weeks following this acclimation to SW. The non-brooding females were all females which had a follicle mass greater than 4 mg. Follicle mass was determined for all brooding SW fish for the examination of ovarian growth during the brooding phase. Samples were collected on 2 occasions in October and 1 in November from a total of 53 females. Body weight, SL and liver weight were recorded during the final sample collection for the determination of HSI and CF.

RESULTS

Follicle growth during the brooding phase of the reproductive cycle

Follicle growth during the brooding phase of the reproductive cycle was examined in fish collected in Study 1 (FW, outdoor tank) and Study 3 (SW, indoor tank). Follicles in fish from Study 1 increased in size from 0.22 ± 0.07 mg in females brooding early stage eggs to 1.31 ± 0.17 mg in females brooding post-yolksac larvae (Fig. 22). Follicles in fish from Study 3 increased in size from 0.30 ± 0.01 mg in females brooding early stage eggs to
Figure 22. Changes in follicle mass during the brooding period of the tilapia Oreochromis mossambicus. For each study, points marked by different letters are significantly different ($P < 0.05$). Points without letters are $N=1$ and were not included in the analysis (mean ± SE; study 1 $N$: EE=3, ME=5, LE=1, EY=6, MY=1, LY=6, PY=6, 28 fish total; study 3 $N$: EE=3, ME=4, LE=2, EY=3, MY=3, LY=5, PY=9, 29 fish total). EE, early-egg; ME, mid-egg; LE, late-egg; EY, early-yolksac larvae; MY, mid-yolksac larvae; LY, late-yolksac larvae; PY, post-yolksac larvae brooding females.
2.34 ± 0.31 mg in females brooding post-yolksac larvae. Follicles in females brooding late-yolksac larvae were similar in size to follicles in females brooding post-yolksac larvae in both studies.

Changes in condition factor and hepatosomatic index with brooding

The CF of FW females maintained in the outdoor tank (Study 1) did not significantly change with brooding stage. The HSI was reduced in females carrying early-yolksac larvae, about day 5-6 after spawning, compared with non-brooding females (Fig. 23; P < 0.05). Detritus-like matter was seen in the gut of brooding and non-brooding tilapia in Study 1 but not in any of the brooding fish in studies 2 or 3. Condition factor and HSI were reduced in FW (Study 2) and SW (Study 3) females brooding post-yolksac larvae compared with non-brooding females (Fig. 24; P < 0.05). The drop in CF in FW fish (Study 2) was significant even when gonad weights were subtracted from body weights before CF was calculated (P < 0.05).
Figure 23. Changes in condition factor (CF) and hepatosomatic index (HSI) during the brooding period of the tilapia Oreochromis mossambicus. For each subject, points marked by different letters are significantly different (P < 0.05). Points without letter are N=1 and were not included in the analysis (mean ± SE; study 1 N: NB=8, EE=3, ME=5, LE=1, EY=6, MY=1, LY=6, PY=6, 28 fish total; study 3 N: NB=7, EE=3, ME=4, LE=2 EY=3, MY=3, LY=5, PY=9, 36 fish total). NB, non-brooding; EE, early-egg; ME, mid-egg; LE, late-egg; EY, early-yolksac larvae; MY, mid-yolksac larvae; LY, late-yolksac larvae; PY, post-yolksac larvae brooding females.
Approximate day from spawning (Stage)
Figure 24. Changes in condition factor (CF), CF calculated without gonad weights included in body weight values (CF-GW), and hepatosomatic index (HSI) in non-brooding (NB) female tilapia Oreochromis mossambicus and female tilapia brooding post-yolksac-larvae, adapted to fresh water (FW) and seawater (SW) (mean ± SE, FW: N = 7-9 per time point; SW: N = 4; * P < 0.05, ** P < 0.01, *** P < 0.001).
Serum concentrations and pituitary content of PRL and GH during the brooding cycle in FW-adapted female tilapia, maintained in outdoor tanks (Study 1)

This study was conducted to examine changes in serum concentrations and pituitary content of the tPRLs and GH in tilapia that are reproducing in an environment that is conducive to their natural behavior. Serum tPRL_{177} concentrations but not tPRL_{188} concentrations were elevated in female tilapia at the end of the brooding phase over levels in non-brooding females (Fig. 25). Serum concentrations of tPRL_{177} were elevated in females brooding post-yolksac larvae over those observed in non-brooding females or in females brooding either eggs or yolksac-larvae (P < 0.05). Serum concentrations of tPRL_{188} remained at about 3-4 ng/ml throughout the brooding cycle. Serum GH concentrations were also elevated in female tilapia at the end of the brooding phase compared with concentrations in non-brooding females. Serum GH concentrations were below 1 ng/ml in non-brooding females and in egg-brooding females before increasing to 2.3 ± 0.3 ng/ml in females brooding yolksac larvae (P < 0.01) and 4.9 ± 0.7 ng/ml in females brooding post-yolksac larvae (P < 0.001).

Pituitary content of tPRL_{177} changed over the brooding cycle whereas tPRL_{188} did not (Fig. 26). Pituitary
Figure 25. Changes in serum concentrations of prolactin and growth hormone during the brooding period of the tilapia Oreochromis mossambicus in fresh water.
(mean ± SE, N = 30-47 per treatment for serum levels, N = 24-35 for pituitary levels; 126 fish total; * P < 0.05, ** P < 0.01, *** P < 0.001). N, non-brooding females; E, females brooding eggs; Y, females brooding yolksac larvae; PY, females brooding post-yolksac larvae.
Figure 26. Changes in pituitary content of prolactin and growth hormone during the brooding period of the tilapia Oreochromis mossambicus in fresh water. (mean ± SE, N = 30-47 per treatment for serum levels, N = 24-35 for pituitary levels; 126 fish total; * P < 0.05, ** P < 0.01, *** P < 0.001 ). N, non-brooding females; E, females brooding eggs; Y, females brooding yolksac larvae; PY, females brooding post-yolksac larvae.
content of tPRL\textsubscript{177} increased from 4.3 ± 0.3 μg/pit. in non-brooding females to 6.0 ± 0.5 μg/pit. in females brooding eggs (P < 0.01), and 5.8 ± 0.3 μg/pit. in females brooding yolksac-larvae (P < 0.05). Pituitary tPRL\textsubscript{177} content in females brooding post-yolksac larvae was not different from those at any other stage. Pituitary content of tPRL\textsubscript{188} remained at about 8-10 μg/pit. throughout the brooding cycle. Pituitary content of GH increased from 10.5 ± 1.3 μg/pit. in non-brooding females to 17.6 ± 1.1 μg/pit. in females brooding post-yolksac larvae (P < 0.001).

Serum PRL and GH concentrations during the reproductive cycle of the female tilapia adapted to FW and maintained in indoor tanks (Study 2).

The purpose of this study was to expand and refine sampling to include earlier stages of the reproductive cycle. Serum tPRL\textsubscript{177} concentrations were lowest at the end of ovarian growth, when the follicles average 5-6 mg (4.6 ng/ml). Serum tPRL\textsubscript{177} then increased to 7.8 ± 0.6 ng/ml in egg-brooding females (P < 0.01) and remained elevated throughout the remainder of the brooding phase (Fig. 27). The concentrations were not different among the non-brooding stages or between the early ovarian growth stages and the brooding stages. A significant
Figure 27. Changes in serum prolactin and growth hormone concentrations during the reproductive cycle of the female tilapia Oreochromis mossambicus (mean ± SE, N = 12-48 per time point, 177 fish total, for each hormone, points marked by different letters are significantly different at P < 0.05). Stages are classified by follicle mass (mg) and developmental stage of brooded young. E, females brooding eggs; Y, females brooding yolksac larvae; PY, females brooding post-yolksac larvae.
effect of reproductive stage with \( tPRL_{188} \) concentration was not detected by analysis of variance (\( P = 0.14 \)), however, the pattern for \( tPRL_{188} \) appeared similar to that of \( tPRL_{177} \). Growth hormone concentrations were low throughout the ovarian growth phase in non-brooding females (<1.5 ng/ml), and increased with the continuation of brooding to reach 2.5 ± 0.3 ng/ml in females brooding yolksac larvae (\( P < 0.001 \) compared with egg-brooding females) and 3.3 ± 0.5 in females brooding post-yolksac larvae (\( P < 0.05 \) compared with yolksac larvae brooding females).

Serum concentrations and pituitary content of PRL and GH during the brooding cycle in SW-adapted female tilapia, maintained in indoor tanks (Study 3)

The purpose of this study was to provide for a comparison of serum and pituitary patterns of PRL and GH between FW- and SW-adapted tilapia. Only those non-brooding females with follicles larger than 4 mg were included in the study, for the reasons discussed above.

The acclimation of tilapia to SW for a minimum of 8 weeks reduced circulating \( tPRL_{177} \) to undetectable levels. Serum \( tPRL_{188} \) concentration was reduced to ~4 ng/ml, from ~9 ng/ml measured in FW tilapia under similar conditions (from Study 2; Fig. 28). Serum \( tPRL_{188} \) in non-brooding
Figure 28. Changes in serum concentrations of prolactin and growth hormone during the brooding phase of the reproductive cycle in SW-adapted female tilapia, Oreochromis mossambicus, maintained in indoor tanks; (mean ± SE, N = 6-20 per treatment, 53 fish total; * P < 0.05, *** P < 0.001 compared with non-brooding females unless otherwise indicated). N, non-brooding females; E, females brooding eggs; Y, females brooding yolk sac larvae; PY, females brooding post-yolk sac larvae.
females was not significantly different from females at any stage of the brooding phase. Serum tPRL188 concentrations were increased in females brooding post-yolksac larvae over levels in females brooding eggs. Serum GH concentrations increased as brooding continued. Serum GH increased from 1 ng/ml in non-brooding and in egg brooding females to 1.5 ± 0.2 ng/ml in females brooding yolksac larvae (P < 0.05) and 3.7 ± 0.9 ng/ml in females brooding post-yolksac larvae (P < 0.001, compared with non-brooding females). Serum GH concentrations and changes in concentrations were similar in all studies.

In SW, pituitary content of tPRL177 and tPRL188 did not differ among females at various stages of the brooding cycle (Fig. 29). Growth hormone content was elevated in females brooding post-yolksac larvae compared with all other stages (P < 0.05).

DISCUSSION

Patterns of changes in the tPRLs in serum and pituitaries of the female tilapia, O. mossambicus, during the reproductive cycle were affected by the salinity to which the fish were adapted. Patterns of changes in GH, on the other hand, were not affected by salinity. Furthermore, the patterns for the tPRLs and GH observed
Figure 29. Changes in pituitary content of prolactin and growth hormone during the brooding phase of the reproductive cycle in SW-adapted female tilapia, Oreochromis mossambicus, maintained in indoor tanks; (mean ± SE, N = 6-20 per treatment, 53 fish total; * P < 0.05 compared with non-brooding females). N, non-brooding females; E, females brooding eggs; Y, females brooding yolksac larvae; PY, females brooding post-yolksac larvae.
Pituitary content (μg/pituitary)

Stage

N E Y PY N E Y PY N E Y PY

177
during the reproductive cycle had characteristics that were both similar to and distinct from patterns observed during fasting as described in chapter IV. These findings suggest that changes in metabolic state may influence tPRL and GH cell activity during the reproductive cycle although they can not account for all the changes in serum concentration and pituitary content of the hormones.

Overall, serum PRL concentrations were highest in females brooding post-yolksac larvae in each of the three studies. Nonetheless, changes in serum and pituitary levels with the reproductive cycle appeared to be small and there were differences among studies as to when PRL levels increased during the brooding phase. The observed patterns of serum concentration and pituitary content of the tPRLs do not support the notion that changes in serum concentrations of the tPRLs are driving parental care behavior or ovarian function in the female tilapia. On the other hand, the patterns of serum concentration and pituitary content of GH during the reproductive cycle were consistent in all three studies. Serum GH concentrations increased during the brooding phase of the reproductive cycle followed by an increase in pituitary content of the hormone. These changes in serum and pituitary GH levels appear to be a characteristic component of the reproductive cycle of the female tilapia and suggest that GH has
functions intrinsic to reproduction. In the following segments, I will first discuss changes in the physiology of the female tilapia during the reproductive cycle and then relate these changes to the patterns of serum and pituitary tPRLs and GH levels observed during the reproductive cycle.

Ovarian growth during the brooding phase of the reproductive cycle

Ovarian follicles grew in size throughout most of the brooding phase in the 2 studies in which it was examined, Studies 1 and 3. Follicles reached their maximum weight when the females were brooding late-yolksac larvae, about day 12 days post-spawning, and then stopped growing throughout the remainder of the brooding phase. Follicles increased in weight from less than 0.4 mg the day after spawning up to 1.3 and 2.3 mg by the end of the brooding phase in Study 1 and Study 3 fish respectively. The difference in growth between Study 1 and Study 3 may be due to a combination of factors including salinity, water temperature, and size of the fish. The females in Study 1 were adapted to FW, maintained in at outdoor tank at ambient temperatures (~22-26), and were about 150-200 g. The females in Study 3 were adapted to SW, maintained indoors in tanks heated to 26.5 ± 0.5°C, and were
What is of interest is that the follicles stopped growing at about the same stage of the reproductive cycle in both studies and not at the same size.

Smith and Haley (1987, 1988) gave a similar account of ovarian growth during the brooding phase of this species following a histological examination of ovarian development. They reported that ovarian growth was arrested between days 15-25 after spawning. In addition, they found that ovarian growth did not cease in females which did not brood, even when fasted for 20 days. The oocytes of some of the fasted females showed softening, an early sign of atresia. I also observed a reduction in GSI and softening of follicles in fasted female tilapia (chapter IV). Smith and Haley (1988) suggested that the interruption of oocyte growth is not due simply to a reduction in food intake. This notion is supported by the consistency in the timing of the interruption in oocyte growth in both the studies by Smith and Haley (1987, 1988) and in my studies; and the finding that oocyte growth is interrupted at a particular stage, regardless of follicle size. The size of the follicles and the stage of the brooded young were not recorded in the studies by Smith and Haley (1987, 1988).

Smith and Haley (1988) showed that E$_2$ and T were elevated during brooding and suggested that these steroids
may be involved in either brooding behavior or in protecting the oocytes from atresia. Furthermore, Smith and Haley (1987) found that postovulatory follicles are steroidogenic and persist longer in brooding than non-brooding females. They suggested that these structures may also be involved in either brooding behavior, protecting the oocytes from atresia or inhibiting oocyte growth in the latter part of the brooding phase. I have found that PRL and GH are elevated during the brooding phase, and thus, may participate in these actions. Moreover, PRL and GH may have roles in regulating circulating concentrations of levels of E₂ and T, and in prolonging the life of the postovulatory follicles. Alternatively, PRL and GH may have metabolic roles during the brooding phase, mobilizing and directing energy reserves.

Changes in condition factor and hepatosomatic index with brooding in FW-and SW-adapted tilapia

Condition factor and HSI declined in females as brooding continued. These data suggest that nutritional status declined during the brooding phase of the reproductive cycle. The decline in CF between non-brooding females and females brooding post-yolksac larvae was significant for animals sampled from the indoor tanks (Studies 2 and 3) but not for females from the outdoor
tank (Study 1). One possible reason CF was not reduced in females brooding post-yolksac larvae in the outdoor tank is that the females had resumed feeding. The outdoor tank had detritus on the bottom and the fish had detritus-like matter in their guts. Furthermore, there was more space in the outdoor tank, possibly allowing the brooding females to release their clutches and feed. I think the females in Study 1 did undergo changes in condition during brooding that I was unable to detect. Although CF did not decline significantly in females in Study 1, the mean CF in females brooding yolksac larvae from the outdoor tank (Study 1) was 12% lower than in non-brooding females. This is the same difference in mean CF for non-brooding females and females brooding post-yolksac larvae in studies 2 and 3, where those differences were significant. The 12% decline in CF observed during the brooding phase is equal to or greater than reductions in CF that occurred over the course of 21 days of fasting in female tilapia (7.5%) and 31 days of fasting in male tilapia (10.5%; chapter IV).

In the present study, HSI was reduced from 2.3 in non-brooding females to below 1.5 in females brooding post-yolksac larvae, in all studies. A significant reduction was observed in females brooding early-yolksac larvae, about 6 days after spawning, (Study 1). The early
decline in HSI with brooding is comparable to a decline in HSI observed with 10 days of fasting (chapter IV). The drop in HSI during brooding was most likely due to nutritional demands rather than a decline associated with a decrease in vitellogenin synthesis activity. In support of this notion, HSI was reduced from 2.4 in non-brooding females to 1.7 in females brooding early-yolksac larvae, even though oocyte growth continues beyond this stage (Study 1). In addition, studies suggest that tilapia produce vitellogenin which is secreted into mucus as a source of nutrition for developing post-yolksac larvae (Kishida and Specker, 1994).

Effect of salinity on serum and pituitary patterns of tPRL during the brooding cycle

Salinity affected baseline serum concentrations and the patterns of the tPRLs in serum and pituitaries during the brooding cycle of the tilapia. Circulating titers of the tPRLs were greatly reduced in SW fish (Study 3) compared with FW fish (Study 2) held under similar conditions. Prolactin_{177} was usually not detectable in the serum of the SW-adapted tilapia. Reduced serum concentrations of the tPRLs in the SW tilapia compared with levels in FW tilapia is consistent with previous findings in O. mossambicus (Nicoll et al., 1981; Borski et al., 1992;
Ayson et al., 1993; Yada et al., 1994). Differences in levels and patterns of the tPRLs in serum and pituitaries with salinity suggest that the tPRLs perform osmoregulatory actions during the reproductive cycle of female tilapia adapted to FW. Nevertheless, large differences in serum tPRL concentrations in FW- compared with SW-adapted tilapia do not imply that the hormones do not have actions other than in osmoregulation. Low levels of the tPRLs may be adequate for the actions of the tPRLs in reproduction or metabolism.

Changes in serum tPRL$_{188}$ were also different in the FW- and SW-adapted tilapia during the reproductive cycle. In FW, serum concentrations of tPRL$_{188}$ did not change over the reproductive cycle. In SW, by contrast, serum tPRL$_{188}$ concentrations were elevated in females that were brooding post-yolksac larvae over levels in females brooding eggs. No changes in pituitary content of tPRL$_{177}$ or tPRL$_{188}$ were observed in the SW-adapted tilapia. No changes in pituitary content of tPRL$_{188}$ were observed in the FW fish from the outdoor tank (Study 1), however, pituitary tPRL$_{177}$ content was elevated in females brooding eggs and females brooding yolksac larvae, over levels in non-brooding females.
Effect of salinity on patterns of GH in serum and pituitaries during the brooding cycle

Salinity did not influence patterns of changes in serum or pituitary GH during the brooding cycle of the tilapia. Increases in circulating GH concentrations, in response to transfer from FW to SW, have yet to be observed in the tilapia, *O. mossambicus*, to last longer than 14 days. Yada et al. (1994) observed a transient increase in plasma GH concentrations in male tilapia after transfer from FW to 70%-SW but no change in females. Sakamoto and colleagues (1994) also observed an increase in plasma GH following transfer of *O. mossambicus* from FW to SW, whereas plasma GH was not altered following transfer from SW to FW. Furthermore, the increase in plasma GH following transfer to SW was observed in both males and females, and at the end of the study, day 14 after transfer, plasma GH was elevated in males but not in females (Sakamoto, personal communication). Transient increases in blood GH occur after SW transfer in coho salmon and rainbow trout. These rises are accompanied by increases in metabolic clearance rate and secretion rates of GH (Sakamoto et al., 1990, 1991). Whether there are salinity-specific differences in metabolic clearance and secretion of GH in the tilapia remains to be deter-
mined. Borski and colleagues (1994) found that pituitary GH content was elevated in *O. mossambicus* reared in SW for 7 months and in tilapia transferred from FW to SW for 49 days. Nonetheless, in the present studies, changes in serum and pituitary levels of GH during the brooding cycle were similar in FW- and SW-adapted tilapia.

A final consideration is that female tilapia may not respond to changes in salinity as do males. Only males were used in the studies by Borski and colleague (1994). Furthermore, Yada et al. (1994) did not observe changes in plasma GH concentrations in female tilapia following transfer from FW to SW despite seeing increases with males.

*Changes in serum concentrations and pituitary content of PRL during the reproductive cycle of the female tilapia*

Overall, serum PRL concentrations were highest in females brooding post-yolksac larvae in each of the three studies. In the FW-adapted fish, serum concentrations of tPRL$_{177}$ increased in brooding females over levels in those in the later stages of vitellogenesis. Nonetheless, the patterns of change in tPRL serum and pituitary levels varied with rearing condition. In fish reared in the outdoor tank, serum tPRL$_{177}$ concentrations were not significantly elevated until late in the brooding phase, that
is when females were brooding post-yolksac larvae. Serum concentrations of tPRL$_{177}$ were elevated early in the brooding phase when tilapia were held in indoor tanks.

Serum concentrations of tPRL$_{188}$ did not change significantly in tilapia adapted to FW. In SW, by contrast, serum concentrations of tPRL$_{188}$ were elevated when females were brooding post-yolksac larvae above those measured in females brooding eggs. Prolactin$_{177}$ was not detectable in most of the serum samples taken from the SW-adapted tilapia. For this reason, it could not be determined whether serum tPRL$_{177}$ concentrations might vary in a physiologically important manner.

The pattern of serum tPRL concentrations in non-brooding tilapia during the oocyte growth phase may be a continuation of the pattern observed in females brooding post-yolksac larvae (Study 2). Female $O. mossambicus$ can spawn at 22 to 40 day intervals with oocyte growth immediately following the termination of brooding (Neil, 1966; Smith and Haley, 1987). Serum tPRL concentrations may increase with brooding and then, following the termination of brooding, slowly decrease as ovarian growth progresses. Changes in serum tPRL$_{188}$ in the FW fish reared in the indoor tanks appear to parallel changes in tPRL$_{177}$. Thus, the same factors which control tPRL$_{177}$ levels may also govern tPRL$_{188}$.
Few conclusions can be drawn from the pituitary patterns of the tPRLs observed during the reproductive cycle. Pituitary content of tPRL₁₇₇ was elevated in FW females brooding eggs or brooding yolksac larvae compared with levels in non-brooding females, late in vitellogenesis. On the other hand, there were no changes in tPRL₁₇₇ content in the pituitaries from SW tilapia. There were no changes in pituitary content of tPRL₁₈₈ in FW or SW animals during the brooding cycle.

Although serum PRL concentration was highest in females brooding post-yolksac larvae in all three studies, these changes were relatively small compared with changes observed with fasting (chapter IV) and transfer between FW and SW (Yada et al., 1994). There was also an inconsistency regarding when serum PRL concentrations begin to increase in the brooding phase. This suggests that changes in serum PRL concentrations do not drive parental care or ovarian function in the female tilapia. This view is supported by the conclusions of Wendelaar Bonga and colleagues (1984) who found that PRL cell activity did not vary between non-brooding and brooding female tilapia, whether adapted to FW or SW. Their conclusions were based on ultrastructure morphometry and incorporation rates of [³H]-lysine into PRL cells.

This conclusion does not preclude PRL from having
roles in reproduction in the tilapia, including actions in parental care or ovarian function. It is not necessary that there be large changes in PRL for PRL to have affects. Changes may be occurring in other aspects of the physiology of the animals associated with reproduction that makes target tissues either less receptive or more receptive to PRL stimulation. This may include changes in PRL receptors in target tissues or changes in hormones that either potentiate or antagonize the actions of PRL.

Changes in serum concentrations and pituitary content of GH during the reproductive cycle of the female tilapia

The patterns of change in serum and pituitary GH were consistent in all 3 studies. Serum GH concentrations increased beginning when females were brooding yolksac larvae and continued to increase further in females brooding post-yolksac larvae. Pituitary GH content was elevated in females brooding post-yolksac larvae both in SW and in FW. This suggests that an increase (> 3 fold) in serum GH during the brooding stages of the reproductive cycle is a characteristic component of the reproductive cycle of the female tilapia.
Factors affecting PRL and GH cell activity during the reproductive cycle of the female tilapia

Prolactin and GH cell activity is likely to be influenced by many factors during reproduction. Among these are salinity, steroid hormone concentrations, and nutrition.

Salinity appeared to influence baseline PRL serum and pituitary levels as well as the patterns in which serum and pituitary PRL changed over the reproductive cycle. Salinity did not influence the patterns of changes in serum and pituitary GH over the reproductive cycle. Both $E_2$ and $T$ are elevated in brooding tilapia (Smith and Haley, 1988). The steroid hormones, $E_2$ and $T$, have been shown to stimulate PRL release directly and potentiate the stimulatory effects of GnRH and thyrotropin-releasing hormone on PRL release in the tilapia (Barry and Grau, 1986; Borski et al., 1991; chapter III). These same steroid hormones have also been shown to potentiate the stimulatory actions of GnRH and GHRH on GH release in a tilapia hybrid (Melamed 1993).

Changes in serum tPRL concentrations that occur over the course of the reproductive cycle in female tilapia vary with rearing condition. This may suggest that PRL serves functions other than, or in addition to, regulating aspects of reproduction, that are nonetheless tied to the
reproductive cycle. This may include metabolic actions. Changes in CF and HSI observed during the reproductive cycle were similar or greater than changes observed with 21 days of fasting in studies described in chapter IV. In those studies, serum concentrations of the tPRLs were increased with 10 days of fasting in male and female tilapia. Thus, changes in metabolic state during the reproductive cycle may be contributing to the changes in serum tPRL concentrations observed during the course of the reproductive cycle.

Sumpter et al. (1991a,b) found increases in circulating GH concentrations were inversely correlated with changes in CF during fasting and CF during the reproductive cycle of male and female rainbow trout. They concluded that changes in GH observed during the reproductive cycle were due to a loss of condition and not a consequence of reproduction per se. Reductions in CF and HSI during the brooding phase of the reproductive cycle in tilapia suggest that a similar situation may exist in this species.

The 12% decline in CF observed during the brooding phase is equal to or greater than reductions in CF that occurred over the course of 21 days of fasting in female tilapia (7.5%) and 31 days of fasting in male tilapia (10.5%; chapter IV). Pituitary content of GH were elevat-
ed in the female tilapia fasted for 21 days compared with females which were fed, and serum concentrations of GH and pituitary contents of GH were elevated in the male tilapia fasted for 31 days. Overall, the decreases in HSI and CF during the brooding phase of the reproductive cycle are consistent with the idea that serum and pituitary levels of GH are elevated in response to changes in metabolic state occurring during the brooding phase of the reproductive cycle.

Although tPRL and GH serum concentrations and pituitary content were elevated during brooding, the patterns were different than with fasting. Serum tPRL

concentrations were elevated prior to increases in tPRL

in pituitaries of female tilapia during fasting. By contrast, pituitary tPRL

levels were elevated prior to an elevation in serum levels during the brooding cycle. Serum concentrations of tPRL

appeared to change in a manner which is similar to tPRL

during fasting and the reproductive cycle. In both instances, these changes were not statistically significant (chapter IV).

Fasting caused elevations in serum and pituitary levels of GH in O. mossambicus (chapter IV). Increases in pituitary content of GH were first observed at 21 days of fasting in female tilapia and in females brooding post-yolksac larvae (about 20 days post-spawning). Thus,
changes in pituitary content of GH occurred over a similar
time-course in both fasting and brooding female tilapia.
Nevertheless, serum levels of GH increased before pitui-
tary levels in females during the brooding cycle whereas
the reverse was found during fasting. These differences
in patterns between fasting and brooding animals suggest
that the changes in serum concentration and pituitary
content of the tPRLs and GH observed during the brooding
phase of the reproductive cycle are not due simply to a
decrease in food intake.

Possible actions of PRL and GH during the reproductive
cycle of the female tilapia

The present study suggests that the tPRLs have ac-
tions in osmoregulation during the reproductive cycle in
FW adapted-tilapia. The present study also suggests that
the tPRLs and GH may have actions associated with the
brooding phase of the reproductive cycle. It is unclear,
however, whether these actions are more closely aligned to
the regulation of metabolism or reproduction.

Prolactin and GH may have metabolic roles closely
associated with reproduction, ushering resources to or
away from ovarian growth and vitellogenin production. The
tPRLs and GH may release metabolites for vitellogenin
production or protect energy stores and protein from
utilization during this period when food intake is low.

The tPRLs and GH may have actions in parental behavior, steroidogenesis, or maintenance of postovulatory follicles. Possible actions in parental care include "calling movements" to fry, reduced feeding behavior to prevent feeding on young, and mucus production. Wedelaar Bonga and colleagues (1984) dismissed a role for PRL in parental care in the tilapia due to the absence of a change in PRL cell activity. I have shown that PRL levels are elevated in FW tilapia at the end of the brooding period. Furthermore, as I have argued previously, large changes in PRL release may not be necessary for PRL to have actions. Changes in target tissue receptors and in interacting hormones which may modulate PRL's actions, may change during the reproductive cycle.

I have mentioned previously that, Smith and Haley (1988) have shown that \( E_2 \) and T levels are elevated during the brooding phase and steroidogenic postovulatory follicles persist longer in brooding females than in non-brooding females. The tPRLs or GH may be involved in regulating these events. Fish PRLs including tPRLs, and GHs have been shown to stimulate ovarian growth and steroidogenesis in teleosts (Singh et al., 1988; Tan et al., 1988; Danzmann et al., 1990; Van Der Kraak et al., 1990; Rubin and Specker, 1992).
Clearly, more work is required before the actions of PRL and GH during the reproductive cycle of the female tilapia are clear. Studies should include: 1) metabolic clearance studies for the tPRLs, GH and steroid hormones; 2) studies on the efficacy of native hormones to elicit or maintain parental behavior and mucus production; 3) in vitro studies on the effects of the tPRLs and GH on steroidogenesis from ovarian tissues, including postovulatory follicles; 4) studies on the efficacy of the tPRL and GH to maintain postovulatory follicle function in non-brooding tilapia; and 5) studies to determine whether tPRLs, GH or steroid hormones can protect oocytes from atresia during fasting.
Chapter VI

CONCLUSIONS

Consistent with PRL's role in FW osmoregulation, PRL cells of the tilapia release PRL in response to small changes in medium osmolality that are well within the range of plasma osmolalities observed in vivo (Nagahama et al., 1975; Wigham et al., 1977; Grau et al., 1981, Kelly et al., 1988). I have shown that alterations in osmotic pressure and not osmolality are coupled to the sustained release of the osmoregulatory hormones, tPRL_{177} and tPRL_{188}. This is the first study to describe a direct response to osmotic pressure by a cell with a clearly demonstrated osmotic output. Prolactin release was high in RPD incubated for 18-20 hr in hyposmotic medium (300 mOsmolal) or medium made hyperosmotic (355 mOsmolal) with the membrane-permeant molecules, urea or ethanol, but was low in RPD incubated in medium made hyperosmotic with NaCl or the membrane-impermeant molecule, mannitol. These data suggest that it is the osmotic gradient across the cell membrane that leads to an increase in osmotic pressure, and not the medium osmolality per se, which accounts for the osmoreceptivity of the tilapia PRL cell. In perifusion studies, tPRL_{177} and PRL_{188} release was the same from RPD incubated in hyposmotic medium and in hyperosmotic
medium containing urea, at 22 min after the onset of exposure to these media. Furthermore, release was greater in both media compared with tPRL release from RPD incubated in hyposmotic media made hyperosmotic with the membrane-impermeant molecule, mannitol. These data provide additional evidence that the increase in tPRL release in response to reductions in osmotic pressure is sustained.

I have shown that injections of mGnRHa elevate serum concentrations of the tPRLs and incubation of RPD in medium containing GnRH molecules that are native to the tilapia, increases PRL release into the medium. These data provide evidence that GnRH functions as a PRL-releasing factor in the tilapia, *O. mossambicus*. The forms of GnRH endogenous to the tilapia stimulated the release of the tPRLs from RPD with the following order of potency: cGnRH-II > sGnRH > sbGnRH. This action appears to be directly on the PRL cells. Support for this notion comes from several lines of evidence. First, RPD are a nearly homogeneous population of PRL cells (95-99% pure), GtH and GH cells which have been shown to respond to GnRH are not present in the RPD. Second, the range of GnRH concentrations used in the studies were compatible with receptor mediated action. Thirdly, cGnRH-II evoked an increase in [Ca\(^{2+}\)]\(_i\) within 1 min of exposure and an elevation in PRL release within 2 min. These data suggest that
calcium operates as a second messenger in mediating the effects of GnRH on PRL release.

Prolactin secretion in teleosts is thought to be predominantly under inhibitory control, however, evidence had suggested that a hypothalamic prolactin-releasing factor was involved in the control of PRL secretion in fish (reviewed by Clarke and Bern, 1980; Ball, 1981). Barry and Grau (1986) have shown that thyrotropin-releasing hormone stimulates PRL release in the tilapia but only following E₂ preincubation. Until the present study, thyrotropin-releasing hormone was the only hypothalamic factor shown to have the capability to stimulate PRL release in the tilapia. Furthermore, the steroid hormones E₂ and T are able to potentiate the response of PRL cells to GnRH, increasing percent release over 3 fold. The steroids may regulate the sensitivity of the PRL cells to GnRH stimulation during the reproductive cycle. Alternatively, GnRH molecules may regulate the sensitivity of PRL cells to T and E₂ stimulation. The potentiation of the response of PRL cells to GnRH and thyrotropin-releasing hormone by sex steroids supports the suggestion by Barry and Grau (1986) that there may be a shift in the control of PRL secretion with changes in the reproductive state of the tilapia.

Serum concentration and pituitary content of GH and
serum concentrations of tPRL$_{177}$ and tPRL$_{188}$ were elevated in response to fasting in *O. mossambicus*. These data suggest that GH and the tPRLs have roles in the regulation of intermediary metabolism in the tilapia, *O. mossambicus*. This is the first report of changes in circulating PRL levels in response to fasting in a teleost. Furthermore, serum concentrations of the tPRLs increased earlier in response to fasting than GH, and thus, the tPRLs and GH may have different roles in regulating metabolism.

Patterns of changes in serum and pituitary tPRL levels observed during the reproductive cycle of the female tilapia, *O. mossambicus*, were affected by the salinity to which the fish were adapted, whereas GH patterns were not affected by salinity. The patterns for the tPRLs and GH observed during the reproductive cycle had characteristics that were both similar to and distinct from patterns observed during fasting. In addition, changes in CF and HSI observed during the brooding phase of the reproductive cycle were similar in magnitude to changes observed with fasting which were accompanied by changes in serum tPRL and GH concentrations and GH pituitary content. These findings suggest that changes in metabolic state may influence tPRL and GH cell activity during the reproductive cycle although they can not ac-
count for all the changes in serum concentrations and pituitary content of the hormones.

Overall, serum PRL concentrations were highest in females brooding post-yolksac larvae. Nonetheless, changes in serum and pituitary levels with the reproductive cycle appeared small and there were discrepancies among studies as to when serum PRL concentrations increased during the brooding phase. The observed patterns of serum concentrations and pituitary content of the tPRLs do not support the notion that changes in serum concentrations of the tPRLs are driving parental care behaviors or ovarian function in the female tilapia. On the other hand, the pattern of serum and pituitary GH levels during the reproductive cycle was consistent among three studies. Serum GH concentrations increased during the brooding phase of the reproductive cycle, followed by an increase in pituitary content of the hormone. These changes in serum and pituitary GH levels appear to be a characteristic component of hormone changes during the reproductive cycle of the female tilapia and suggest that GH has functions intrinsic to reproduction.
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