ABSTRACT

STROM, CHRISTINA NELSON. Salinity Regulation of Prolactin Cell Proliferation and Apoptosis in the Euryhaline Teleost, the Tilapia (*Oreochromis mossambicus*). (Under the direction of Russell J. Borski).

The euryhaline tilapia, *Oreochromis mossambicus*, has the ability to live in both freshwater (FW) and saltwater (SW) environments. Prolactin (PRL) is the most critical hormone to promoting life in FW, and without it, tilapia loses the capacity to osmoregulate in hypotonic environments. Consistent with PRL’s actions in FW adaptation, pituitary PRL synthesis, content, secretion, and cell activity are all elevated in FW compared to SW acclimated tilapia. We found that the PRL region of a FW tilapia pituitary has a 3-fold larger volume than the PRL region of a SW pituitary. It is unclear whether this increased tissue volume is due to larger cells (hypertrophy) or more cells (hyperplasia). Therefore, we evaluated if PRL cell proliferation and apoptosis might be sensitive to salinity and could account for the greater abundance of PRL in FW versus SW fish. Freshwater tilapia were transferred to either SW or sham transferred to FW and SW fish were moved to either FW or SW over a time course of 7 days. Pituitaries were sampled over the course of salinity challenge and triple stained for determination of lactotroph cell density (nuclei staining with Hoescht dye), proliferation (BrdU labeling) and apoptosis (TUNEL assay). Lactotrophs were identified by immunostaining using tilapia specific PRL antisera. Lactotroph cell densities were 40% lower in FW than SW fish and declined when fish were transferred from SW to FW (P < 0.001). The larger volume of the PRL cell region within the pituitary area combined with
lower number of lactotrophs per unit area suggests that the cells are larger in FW than SW acclimated fish. There were negligible levels of apoptosis in lactotrophs and salinity was ineffective in regulating programmed cell death. By contrast, we found a dramatic effect of salinity on lactotroph cell proliferation. The pituitaries of FW tilapia show a higher rate of PRL cell proliferation than those of SW fish. During transfer from SW to FW proliferation increased by approximately 20-fold compared with controls (P < 0.001). When fish were transferred from FW to SW, proliferation declined within 1 day to levels observed in sham transferred SW fish. The enhanced proliferation combined with increased volume of the pituitary PRL cell region, decline in lactotroph cell density, and presumed increase in cell volume, suggests that the tilapia lactotroph undergoes hypertrophy and hyperplasia in FW environments. Apoptosis appears to play little role in regulating lactotroph density under different salinities. Overall, these results suggest that the elevated production and content of PRL critical to life in FW is mediated, in part, through enhanced lactotroph proliferation and hypertrophy.
Salinity Regulation of Prolactin Cell Proliferation and Apoptosis in the Euryhaline Teleost, the Tilapia (*Oreochromis mossambicus*).

by
Christina Nelson Strom

A thesis submitted to the Graduate Faculty of North Carolina State University
In partial fulfillment of the Requirements for the degree of Master of Science

Zoology Program

Raleigh, North Carolina

August 5, 2008

APPROVED BY:

Dr. Russell J. Borski
Committee Chair

Dr. Paul Mozdziak

Dr. Heather Patisaul
DEDICATION

To Mom and Dad for always being proud of me, always pushing me to be better, and the million other ways you have supported me. For every good quality I have, I don’t need to look far to see where I got it.
BIOGRAPHY

Christina Strom was born in Winston-Salem on November 4th, 1981. She is the only child of Michael and Susan Nelson. She was raised in Winston-Salem and graduated Bishop McGuinness Catholic High School in 2000. She went on to attend North Carolina State University in the fall of 2000 and met Prof. Russell Borski in the spring semester while taking his class. She then began working in the Borski lab the following fall on an independent honors research project. That same fall she met Patrick Strom. In the summer of 2004 she graduated with honors from NC State and married Patrick in Key Largo before beginning her graduate work in the Borski lab. Following graduation with her Masters Degree, Christina will begin working as a Research Specialist at the Lineberger Comprehensive Cancer Center at the University of North Carolina – Chapel Hill.
ACKNOWLEDGMENTS

The person who deserves the most thanks is my advisor, Russell Borski. He asked me to come to his lab to do an undergraduate project 8 years ago and I have since grown to love research. I have gone from a clueless freshman to a confident researcher under his guidance. He has given me invaluable advice on everything from lab work to life goals and I am grateful. The expertise of my committee is also essential to the success of this project.

Thank you to Paul Mozdziak for his help in planning and starting up this project. Thanks also to Heather Patisaul for giving her time in many microscope instructional sessions and for the use of her microscope. This work would not have been possible without current and former undergraduate and graduate students in the Borski lab. Thank you for help in the lab and your friendship. Fish technicians at NCSU, PAFL and the Lake Wheeler Fish Barn also need special recognition for their diligence in fish husbandry and accommodations for experiments. This work was funded by grants from the National Science Foundation.

Thank you also to all my family and friends especially Erica and Alexis for all the laughter and support. To Patrick, my husband and best friend, through all the bad days and disappointments, you make everything all right and life is better with you to balance me. You keep distracting me with real life and I appreciate it even though I never thank you enough.
# TABLE OF CONTENTS

List of Figures ............................................................................................................. vi

Introduction .................................................................................................................. 1

Background ................................................................................................................... 4

Osmoregulation ............................................................................................................ 4
Prolactin Regulation in Hydromineral Balance and Epithelial Remodeling .............. 7
Salinity Regulation of PRL Cell Activity ....................................................................... 9
Prolactin Cell Proliferation .......................................................................................... 12
Apoptosis ...................................................................................................................... 15

Objectives ................................................................................................................... 18

Hypothesis .................................................................................................................... 19

Materials and Methods ............................................................................................... 20

Volume of PRL Region ............................................................................................... 20
Salinity Challenge Experiments .................................................................................. 21
Plasma Osmolality Determination ............................................................................... 22
Detection of Proliferating Lactotrophs ....................................................................... 23
Detection of Apoptotic Lactotrophs ............................................................................. 24
Lactotroph Proliferation, Apoptosis, and Cell Density Analyses ............................... 26
Statistical Analysis ...................................................................................................... 27

Results ......................................................................................................................... 28

Volume of PRL Region ............................................................................................... 28
Plasma Osmolality During Salinity Acclimation .......................................................... 28
Lactotroph Cell Density During Salinity Acclimation .................................................. 29
Lactotroph Apoptosis During Salinity Acclimation ...................................................... 30
Lactotroph Proliferation During Salinity Acclimation ................................................ 30

Discussion ................................................................................................................... 33

Literature Cited ............................................................................................................. 41
LIST OF FIGURES

Figure 1. Volume of PRL cell region in FW and SW reared tilapia.......................... 55

Figure 2. Representatives midsagittal sections of pituitaries from six-week
FW ad SW acclimated tilapia showing PRL immunoreactive region
within the RPD ............................................................................................................... 56

Figure 3. Effect of salinity transfer on plasma osmolality in tilapia.......................... 57

Figure 4. Midsagittal section of a tilapia pituitary from a six week SW and
FW adapted fish............................................................................................................ 58

Figure 5. Lactotroph cell density of FW fish transferred to either FW or SW
or of SW fish transferred to either SW or FW for 1, 4 and 7 days............................ 59

Figure 6. Triple staining of tilapia pituitary sections for nuclei with Hoechst
33258, fluorescein, and immunoreactive PRL cells at 20X and Dnase
treated pituitary section at 10X used as a positive control........................................ 60

Figure 7. Double labeling of tilapia pituitary midsagittal sections from FW
and SW adapted fish.................................................................................................... 61
Figure 8. Lactotroph apoptosis in the pituitary of FW fish transferred to either FW or SW or of SW fish transferred to either SW or FW for 1, 4 and 7 days.................................................................................................................. 62

Figure 9. Lactotroph proliferation in the pituitary of FW fish transferred to either FW or SW or of SW fish transferred to either SW or FW for 1, 4 and 7 days in proliferation experiment 1........................................................................................................... 63

Figure 10. Lactotroph proliferation in the pituitary of FW fish transferred to either FW or SW or of SW fish transferred to either SW or FW for 1 and 7 days in proliferation experiment 2.............................................................................................................. 64

Figure 11. Lactotroph proliferation in the pituitary of FW fish transferred to either FW or SW or of SW fish transferred to either SW or FW for 1 and 7 days in proliferation experiment 3.............................................................................................................. 65

Figure 12. Double labeling of tilapia pituitary midsagittal sections from FW and SW adapted fish.......................................................................................................................... 66
INTRODUCTION

One of the most pronounced functions of prolactin (PRL) in vertebrates is control of osmoregulation (Loretz and Bern 1982). In teleosts, PRL plays an important role in freshwater adaptation (Clarke and Bern 1980). In seminal research on PRL’s actions in fish osmoregulation, Pickford and Phillips (1959) discovered that hypophysectomized killifish die after transfer to hyposmotic environments. Subsequent injection with PRL restored the fishes’ ability to live in fresh water. Even in those hypophysectomized fish species able to survive freshwater (FW) transfer, PRL still exerts hyperosmoregulatory functions where it can partially restore the decline in blood osmolality that occurs with acclimation to FW environments (Ball 1969). Euryhaline fish, such as the Mozambique tilapia (*Oreochromis mossambicus*), have the ability to withstand environments ranging from FW to seawater (SW). Upon acclimation to FW environments, fish must cope with the passive gain of water and loss of salts to its hypotonic environment. Indeed, during transfer to FW, blood osmolality initially drops and then is subsequently restored to isosmotic levels as early as 48 hours with the opposite occurring during SW adaptation (Assem and Hanke 1979; Hwang et al. 1989). Prolactin promotes FW adaptation in the tilapia as well as other euryhaline teleost fishes by acting on all osmoregulatory tissues including the gills, integument, urinary bladder, intestine, and kidney to reduce water
permeability and increase sodium and chloride uptake (Clarke and Bern 1980; Hirano 1986b).

Previous studies show that the pituitary of tilapia synthesizes and secretes two PRLs of around 177 kDa (PRL$_{177}$) and 188 kDa (PRL$_{188}$) thought to be derived from separate genes (Rentier-Delrue et al. 1989). Although the relative amount of the two PRLs appears to change with salinity, with the ratio of PRL$_{188}$/PRL$_{177}$ being higher in pituitaries of FW than SW tilapia, the secretion of both hormones appears similarly sensitive to those PRL regulatory factors examined to date (Borski et al. 2002).

Consistent with its FW-adaptive actions, pituitary synthesis, content, and secretion as well as plasma levels of both PRLs is higher in FW than SW acclimated fish. Ultrastructural features of the PRL cell also show it to be more highly active (greater number of secretory vesicles and endoplasmic reticula) in FW versus SW fish (Dharmamba et al. 1967). It appears that this heightened activity of PRL cells in vivo is likely mediated, in part, by changes in blood osmolality. Indeed, the tilapia lactotroph is directly sensitive to physiological changes in osmotic pressure as the release of both PRLs is higher during exposure to reduced osmotic pressure and declines when osmotic pressure is elevated in vitro (Seale et al. 2003b; Seale et al. 2003a). However, it is unknown whether lactotroph proliferation or apoptosis in fish generally, or of tilapia specifically, might be a contributing factor that leads to the higher pituitary PRL content
found in FW versus SW fish, and hence the hormones capacity to exert hyperosmoregulatory actions. This study evaluates the role salinity plays in regulating PRL cell proliferation and apoptosis in a model euryhaline teleost, the Mozambique tilapia.
BACKGROUND

Osmoregulation

Euryhaline fish, like the Mozambique tilapia, have the ability to withstand environments ranging from FW to full-strength SW. Upon acclimation to FW environments, fish must cope with the passive gain of water and loss of salts to its hypotonic environment. Indeed, during transfer to FW, blood osmolality initially drops and then is subsequently restored to isosmotic levels within 48 hours (Assem and Hanke 1979; Hwang et al. 1989). By contrast, during SW transfer, fish tend to lose water and gain salts due to their hypertonic environment. The rise in blood osmolality that occurs during SW transfer, from initial isosmotic values of 320-330 mOsm to as high as 450 mOsm, is then restored back to normal values (Hwang et al. 1989). Seawater fish compensate for lost water by increasing their drinking rates to up to 10 times that of FW fish, increasing intestinal absorption of water and NaCl, excreting excess salts through the gills (Evans 1987), and producing a high-salt urine. Freshwater fish, on the other hand, do not drink water thus avoiding excess water gain and produce mucus on osmoregulatory surfaces (intestine and gill) to prevent passive influx of water into the body. Freshwater fish also import ions through the gills as well as through the interrenal tissues, and so need to almost completely reabsorb all ions from filtered blood for excretion (Evans and Claiborne
2006). These physiological changes are necessary for the survival of fish in different salinities and hormones control these osmoregulatory processes.

The major osmoregulatory organs that control salt and water balance include the gill, skin, urinary bladder, interrenal (equivalent to the tetrapod kidney), and intestinal tract (Evans and Claiborne 2006). The gills are the primary organs responsible for ion-transport because of their large surface area. This is where chloride cells play a central role in salt transport and therefore differ between FW and SW fish. The chloride cell of FW fish pumps Cl\(^-\) into the blood to promote Na\(^+\) retention, and in SW fish the cells pump Cl\(^-\) out of the animal into its environment for Na\(^+\) excretion. Gill chloride cells transition from chloride-excreting cells to chloride-retaining cells when the fish move from SW or FW and the chloride-retaining cells can dedifferentiate to change to chloride-excreting cells when transitioning to a SW environment (Heijden et al. 1997). Twenty-four hours after transfer to SW, there was a change in the number, size, and morphology of the chloride cells corresponding with the start of a net efflux of Na\(^+\) and Cl\(^-\) ions (Foskett et al. 1983). The changes most likely reflect transition from the FW-type to SW-type chloride cells, with the opposite change occurring during FW acclimation (Foskett et al. 1983).
In the intestine, both water and salts are absorbed (Clarke and Bern 1980) to balance blood osmolality during salinity acclimation. The mechanism to compensate for ion loss and passive water gain in FW is suppression of the chloride pumps and reduced permeability to water. In the intestines, the opposite is observed for SW adaptation where the membranes become more permeable to salts and water and chloride is actively pumped into the blood so that water will follow down the osmotic gradient created. The esophagus and anterior region of the intestine absorb the majority of the ions in SW fish. The excess plasma salts are subsequently removed by active salt excretion from the gills. Additionally some of the excess salts are removed by interrenal tissue, creating a more concentrated, lower volume urine than that of FW fish (Stanley and Fleming 1967; Lahlou and Giordan 1970). However, fish do not have Loops of Henle as in higher vertebrates and consequently cannot make urine that is more hypertonic than the blood. The role of the esophagus and intestine is different for FW fish, as fish in a hypotonic environment do not actively drink. For FW fish, intestinal absorption of salts derived from the diet is critical and can reduce the need for active uptake of ions from the environment by the gills. This is essential in ion poor and/or acidic waters where the internal ion concentration of the fish is twice that of the environment.
Prolactin Regulation of Hydromineral Balance and Epithelial Remodeling

Among many other roles, PRL plays a central role in the FW osmoregulation of the tilapia as well as other euryhaline teleost fishes. Transfer of hypophysectomized tilapia and other fish to FW results in declining electrolyte concentrations and blood osmolarity (Pickford and Phillips 1959; Handin et al. 1964; Clarke and Bern 1980). Prolactin restores blood osmotic pressure by acting on virtually all osmoregulatory tissues including the gills, integument, urinary bladder, intestine, and kidney to reduce water permeability and increase sodium and chloride uptake (Clarke and Bern 1980; Hirano 1986b). It is not surprising, therefore, that PRL receptors are found on all osmoregulatory organs with the highest levels in kidney followed by gills, gut, skin, and liver (Auperin et al. 1994). In addition to regulating ion pumps (i.e. Na\(^+\)K\(^-\)-ATPase) and epithelial permeability (for review see Clarke and Bern 1980), PRL may also exert apoptotic or proliferative effects on osmoregulatory epithelia.

The esophagus of tilapia transferred to FW has more proliferating cells than SW-transferred fish. Prolactin receptors are colocalized and appear more abundant in these proliferating cells in FW versus SW fish (Takahashi et al. 2007). Proliferation increases in the intestine during FW adaptation and this may contribute to reducing water absorption (Takahashi et al. 2006), and therefore inhibiting the passive influx of water into the body. In the tilapia, anterior intestinal apoptosis increases during SW
acclimation and this may make the intestine wall more permeable to water. Proliferation did not play a role for the gills of the estuarine crab, *Chasmagnathus granulatus* (Genovese et al. 2000) as there was none observed during exposure to a hypotonic environment. However, consistent with its function as a FW-adapting hormone, PRL causes a de-differentiation of chloride cells in gills of SW tilapia and inhibits the active NaCl excretion function of SW chloride cells by reducing leaky pathways (Foskett et al. 1983). Prolactin also increases the number of cells responsible for the uptake of Na\(^+\) and Cl\(^-\) while working in concert with cortisol to decrease the number of cells involved in NaCl excretion in gill of SW fish (Perry 1997; Manzon 2002). Prolactin receptors were more abundant in chloride cells of SW adapted tilapia gill (Weng et al. 1997), with relatively little detected in cells of FW fish. The authors suggest the down-regulation of PRL receptors in FW chloride cells might result from higher prevailing levels of PRL found in the circulation of FW fish. The transfer of trout, *Oncorhynchus mykiss*, from FW to ion-poor FW resulted in the proliferation of chloride cells. Whether these were the FW- or SW-type is unknown (Laurent et al. 1994). Evidence suggests that salinity transitions to either SW or FW are linked to alterations in the proliferation, apoptosis or differentiation of epithelial cells, suggesting that these cellular processes are important aspects of remodeling osmoregulatory tissues and promoting salinity adaptation in fish.
Salinity Regulation of PRL Cell Activity

The pituitary can be divided into three sections; the anterior pituitary originating from the nasopharyngeal epithelium, the posterior pituitary originating from neural tissues, and the intermediate lobe (Melmed 2002). In teleosts the anterior pituitary produces eight or more hormones: follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, growth hormone, PRL, adrenocorticotropic hormone, lipotrophic hormone, and β-endorphin, with the latter two produced at relatively low levels. In the tilapia as well as other teleost fishes, PRL-producing cells or lactotrophs are segregated in the rostral pars distalis (RPD) of the anterior pituitary (Clarke and Bern 1980). The RPD of tilapia is composed of a nearly homogenous population (95%) of PRL cells with the remaining 5% constituting corticotrophs or supporting cells (Nishioka et al. 1993). The RPD of the pituitary can be easily identified and dissected. This feature makes the tilapia an excellent model for studying PRL cell function as a nearly pure population of cells can be studied in their naturally aggregated state in vitro using a completely defined culture media that lacks serum.

Among the pituitary hormones, prolactin (PRL) is the most versatile in the spectrum and number of functions it regulates (Nicoll 1981; Hirano 1986a; Lamberts and Macleod 1990; Nicoll 1993; Freeman et al. 2000). Prolactin modulates virtually every aspect of
vertebrate physiology, including osmoregulation, growth, metabolism, development, reproduction, parental behavior and immune function (Clark 1997; Bole-Feysot et al. 1998). The central biological importance of PRL highlights the need for defining those factors that govern its activity in the pituitary gland. One of the most pronounced actions of PRL in teleost fishes is in FW adaptation.

In addition to PRL’s actions in regulating osmoregulatory epithelia, the activity of the lactotroph, or its regulation, is highly sensitive to environmental salinity. The pituitary of the tilapia produces two PRLs of around 177 kDa (PRL\textsubscript{177}) and 188 kDa (PRL\textsubscript{188}). These PRLs are thought to be derived from separate genes and can be produced by the same cells (Rentier-Delrue et al. 1989). To date, there have been no conclusive studies demonstrating differential functions of the two PRLs in osmoregulation, although the smaller tPRL\textsubscript{177} may promote growth with greater efficacy than the larger hormone (Shepherd et al. 1999). Likewise, the secretion of both hormones appears similarly sensitive to those PRL regulatory factors examined to date (Borski et al. 2002). However, the relative content and \textit{in vitro} synthetic rate of each of the hormones in the pituitary may change with salinity, whereby PRL\textsubscript{188} is produced at higher levels than PRL\textsubscript{177} in FW fish (PRL\textsubscript{188}/PRL\textsubscript{177} ratio > 1) while the opposite pituitary expression pattern occurs in SW fish (PRL\textsubscript{188}/PRL\textsubscript{177} ratio < 1) (Borski et al. 1992; Yoshikawa-Ebesu et al. 1995). Although the significance of this relative expression between the two
PRLs remains unknown, they are nonetheless synthesized and released at higher levels from the pituitaries of FW compared with SW tilapia, which is consistent with PRL’s function in FW adaptation (Borski et al. 1992; Yoshikawa-Ebesu et al. 1995). The elevated synthetic rate of both PRLs in FW fish is likely due to the increased transcription as pituitary mRNA levels of both tilapia PRL$_{177}$ and PRL$_{188}$ are higher in fish transferred from ¼ SW to FW than in those kept at ¼ SW or transferred to full-strength SW (Shepherd et al. 1999). Plasma levels of both tilapia PRLs are also higher in FW- than SW- acclimated tilapia (Borski et al. 1992; Ayson et al. 1993). Ultrastructural characterization and histological analyses of the tilapia pituitary also indicates that the PRL cells of FW fish contain elevated numbers of secretory granules and more pronounced and elaborate endoplasmic reticula than those of SW tilapia (Dharmamba and Nishioka 1968).

It appears that the heightened activity of the PRL cell in FW fish might be mediated, at least in part, by changes in extracellular osmolality. In fact, the release and synthesis of PRL is inversely related to the osmotic pressure of the incubation medium. Exposure to hyposomotic media that falls well within the range of plasma osmolality seen with transfer of fish to FW, augments PRL release and synthesis in vitro (Grau et al. 1986; Borski et al. 1992; Yoshikawa-Ebesu et al. 1995). By contrast elevations in medium osmotic pressure, similar to that seen in fish acclimated to SW, reduces PRL synthesis.
and release. The increased release of PRL under hypotonic conditions is mediated, in part, by an initial large and rapid (< 5 min) cell swelling that is sustained, albeit at a lower plateau level, so long as the pituitary is exposed to reduced osmotic pressure (Seale et al. 2003a; Weber et al. 2004). The influx of extracellular Ca\textsuperscript{2+} through stretch-gated ion channels appears an important mechanism for transducing the effects of osmotic pressure on PRL release (Seale et al. 2003b; Seale et al. 2003a).

**Prolactin Cell Proliferation**

The number of cells present in the anterior pituitary, or any tissue, reflects an equilibrium between proliferating and apoptotic cells. Proliferation is the replication or reproduction of cells. Hyperplasia reflects an increase in tissue size that is governed largely by the rate of cell proliferation. On the other hand, tissue size may increase due to hypertrophy, or the increase in cell size. There are many factors that influence the proliferation of lactotrophs. In rats, estrogen (Kawashima et al. 2000), estradiol-17β (Lieberman et al. 1982; Perez et al. 1986; Kansra et al. 2005), insulin (Suzuki et al. 1999), Insulin like growth factor-I (IGF-I) (Fernandez et al. 2003; Gutierrez et al. 2007), vasoactive intestinal peptide (VIP) (Fernandez et al. 2003), epidermal growth factor (EGF) (Childs et al. 1995), and galanin (Wynick et al. 1998) stimulate proliferation and dopamine suppresses proliferation (Kelly et al. 1997; Saiardi et al. 1997; Arita et al. 1998) either in
vitro or in vivo. These hormones and neuropeptides are all present in teleosts as well as mammals and therefore could be involved in teleost lactotroph proliferation.

During pregnancy and lactation in humans, lactotrophs undergo extensive hyperplasia (Goluboff and Ezrin 1969; Stefaneanu et al. 1992; Yin and Arita 2000; Carretero et al. 2003). Then, when the lactation period ends, apoptosis of the increased population of lactotrophs follows (Ahlbom et al. 1998). Consistent with PRL’s function in lactation and the elevated proliferation of lactotrophs, levels of PRL are elevated during pregnancy (Tyson et al. 1972; Rigg et al. 1977). Pregnancy and lactation are a time of increased PRL need in the mammal, just as life in FW is a time of increased need for PRL in the euryhaline fish. Interestingly, however, relatively little is known about the dynamics in lactotroph proliferation and apoptosis under different salinities in euryhaline fish like the tilapia.

The pituitary PRL region of the swordtail is (Xiphophorus hellerii) smaller when the fish are in SW (Holtzman and Schreibman 1972). In the killifish (Fundulus heteroclitus), the PRL region of the pituitary is larger and contains more PRL cells than the RPD in a SW adapted killifish. Mitotic cells were observed via electron microscopy indicating potential proliferation of lactotrophs (Betchaku and Douglas 1980), although this was not quantified in either FW or SW fish. In embryonic zebrafish, there are more PRL cells in
pituitaries of fish reared in wells at half the osmolality (0.025% salt concentration) of routine media than in fish reared in 2X osmolality (0.1 % salt concentration) (Liu et al. 2006). These results suggest slight shifts in osmolality may alter PRL cell number during development of lactotrophs in the FW stenohaline zebrafish, although the physiological significance of testing osmolalities with ranges of salt concentrations that are below typical isosmotic environments (0.9% salt concentration) is uncertain. In the tilapia, growth hormone (GH), a related hormone in the GH/PRL/placental lactogen superfamily, is thought to play a role in SW adaptation (Borski et al. 1994). Consistent with this function, the GH staining region and GH content of the pituitary of SW reared fish is higher than that of FW tilapia (Borski et al. 1994). However, GH levels do not differ in plasma or in the pituitary depending on environmental salinity in 2 species of tilapia, the *mossambicus* and *niloticus* (Ayson et al. 1993).

We have also observed that the RPD containing predominantly PRL cells appears larger in FW versus SW tilapia, although measurements of the PRL staining region of the tilapia under different rearing salinities remains to be done. Nevertheless the higher activity of PRL cells and increased pituitary content, synthesis and secretion of PRL in the pituitary of FW than SW fish, suggests that lactotroph cell proliferation may increase in FW acclimated fish, a hypothesis that will be tested here.
Apoptosis

Apoptosis is programmed cell death. All nucleated cells have the capability to undergo the cascade of events leading to cell death, i.e. to express the proteins required for apoptosis, however the synthesis of new RNA and protein is required to activate the pathway (Weil et al. 1996). Conversely, if a cell dies due to extrinsic factors, not intrinsic, that is referred to as necrosis. These two ways in which cells die differ morphologically. In an apoptotic cell, there is shrinkage, the cell takes on an unusual shape, and the chromatin condenses breaks into fragments. The cell breaks up and neighboring cells or macrophages engulf the cellular debris (Studzinski 1999). In necrosis the cell swells and the plasma membrane dissolves, releasing its contents into the extracellular space. This is often what occurs in an inflammatory response (Studzinski 1999). The default cell pathway is programmed cell death, with positive signals required for survival, proliferation, and differentiation (Raff 1992).

The mechanisms underlying apoptosis are still largely unknown and appears highly species and cell specific. Central to the apoptotic pathway are caspases, proteins that activate cell death. They function by cleaving certain intracellular proteins, and these proteins activate proteins which are destructive to the cell and hence kill the cell quickly and efficiently (Chinnaiyan and Dixit 1996). Specific caspase blockers can inhibit programmed cell death
and so we can conclude that caspases are essential to PCD and are therefore essential to apoptosis pathway. Apoptosis is regulated by a variety of factors and cellular phosphorylation (Gajewski and Thompson 1996) and transcription of new genes are involved. (White et al. 1994).

It is these regulators of programmed cell death (ie, phosphorylation of proteins or transcription of genes) that make apoptosis a valuable tool in the control of cell populations. The first discovered proteins involved in the apoptotic cascade in invertebrates were the CED-3 and CED-4 proteins in the nematode, *Caenorhabditis elegans* (Hengartner 1996). Based on mammalian studies there may be as many as 14 caspases, which are homologous to the *C. elegans* CED and CED-like proteins. Thus, the mammalian regulation of apoptosis is likely to be substantially more complex than that of invertebrates (Chinnaiyan and Dixit 1996). There are many purposes for apoptosis in the cell; embryos and developing organisms need to sculpt structures and delete unnecessary structures, and all organisms need to control cell numbers and delete the malfunctioning, detrimental or misplaced cells (Clarke et al. 1993). Apoptosis is responsible for deleting cells of the tadpole tail during the metamorphosis to a frog. Apoptosis is also necessary for the fine formation of structures such as digits (Milligan et al. 1995). Animals deficient in CED-3-like proteins are not able to properly form digits due to inability to complete the apoptotic cascade. The default pathway for cells is to die, so that only properly functioning cells can receive signals to survive thus
helping to eliminate faulty cells (Jacobson et al. 1997). When cells are malfunctioning or functioning in a way that is harmful to the organism, these cells will likely undergo programmed cell death (PCD). For instance, lymphocytes and those T cells that do not form a useful antibody are normally terminated before there is damage to the organism (Kerr et al. 1972). Osmoregulatory epithelial cells may also undergo apoptosis under osmotic stress.

In the euryhaline mudskipper intestine, apoptosis increases during SW acclimation making the intestine wall more permeable to water (Takahashi et al. 2006). This is an additional mechanism to the formation of leaky tight junctions that allows for the passive movement of water following active transport of ions from the gut lumen into the blood. Melamed, Gur et al. discovered (Melamed et al. 1999) that estrogens and/or pituitary derived IGF-I, likely produced by gonadotrophs, may exert anti-apoptotic effects in the tilapia. These hormones reduced the observed rate of cell death in somatotrophs. Calcium ionophores applied to pituitary cells of hybrid tilapia stimulate GH and gonadotropin release, and the rate of DNA condensation and fragmentation suggesting that excessive or prolonged increases in intracellular free Ca\(^{2+}\) can promote apoptosis (Melamed and Yaron 1999). To our knowledge, studies on apoptosis and its potential regulation by osmotic stimuli in teleost lactotrophs have yet to be reported.
OBJECTIVES

Prolactin has long been known to be an important, if not essential, FW adapting hormone in teleosts. Despite evidence that the cell activity of lactotrophs and pituitary content of PRL is higher in FW than SW fish, little information exists on whether lactotroph cell proliferation and apoptosis might be contributing to the heightened amount of PRL seen in FW fish. The aim of this thesis was to determine:

1. If the volume of the PRL cell region and density of lactotrophs differ in the pituitary of FW versus SW Mozambique tilapia

2. If pituitary lactotroph cell proliferation and apoptosis might be differentially sensitive to salinity

3. The time course over which salinity might regulate lactotroph proliferation and apoptosis.
HYPOTHESES

The volume of the PRL cell region of the pituitary of FW fish is higher than that of SW fish.

The lactotrophs of FW fish show greater proliferation and reduced apoptosis than that of SW fish.

Transfer of fish to FW will increase lactotroph proliferation and reduce prolactin cell apoptosis, with the opposite occurring during SW acclimation.
MATERIALS & METHODS

Volume of PRL Region

Tilapia were reared from the period of yolk-sac absorption for 4 months in FW or SW. Male fish were killed by decapitation, and the pituitary was removed and fixed in Bouin-Hollanade fluid. After 24 hours, the pituitary was dehydrated in graded solutions of ethanol and embedded in paraffin. Serial sections cut parasagittally a 5µm were stained using the Cleveland-Wolfe (Cleveland and Wolfe 1932) technique (Cleveland and Wolfe 1932; Dharmamba and Nishioka 1968). Each section was projected through a camera lucida attached to a Nikon TMS microscope (Nikon, NY) on to a Zidas digitizer (Carl Zeiss, Thornwood, NY). The area of the entire pituitary and of the PRL cell stained region within the RPD portion of the pituitary for all serial sections of each gland was traced. The volume of the PRL region in each pituitary was determined by multiplying the area of each section by the combined thickness of all sections (5µm/section). Data were similar whether expressed as volume of PRL region alone or when normalized to the entire volume of the pituitary.
Salinity Challenge Experiments

Adult male tilapia, ranging from 40-80 grams, were preacclimated in 60 L fiberglass tanks (12 fish/tank) in either FW or artificial SW (26 ppt; Instant Ocean Aquarium Systems, Mentor, Ohio, USA) on a constant photoperiod (12hr light, 12 hr darkness) and temperature (25°C) for at least six weeks prior to all experiments. For proliferation study 1, all tilapia in the FW system were removed, placed in a holding tank, lightly sedated with MS 222 (Argent Chemical Laboratories, Redmond, WA), weighed, injected intraperitonally with 100μg BrdU/g body weight using a 15mg/ml working dilution of bromodeoxyuridine (BrdU; Sigma, St. Louis MO.) in an isotonic phosphate buffered saline at pH 7.2. BrdU is incorporated in the place of thymidine during DNA replication and hence is widely used as an index of proliferation rates. Following BrdU injection, fish were then transferred to SW or sham transferred into FW. Saltwater acclimated fish were subjected to the same procedure and then transferred to either FW or SW (sham transfer). Groups of 12 fish were sampled at 1 day, 4 days, and 7 days post-injection. The fish were sedated with MS 222, decapitated and the pituitaries were removed and placed in Carnoy’s fixative (60% EtOH, 30% glacial Acetic Acid, 10% chloroform) for proliferation analyses. In preliminary trials, we found that significant BrdU detection can be seen in tissues after 24, but not 4 hrs of initial injection. BrdU staining was also present at 7 days postinjection, although staining appeared lighter than at 24 hr sampling. An identical experiment was repeated without any injections and
pituitaries from fish following salinity challenge were placed in 10% neutral buffered formalin for apoptosis analyses.

Since it appears BrdU is highly detectable 24 hr post-injection we conducted additional salinity acclimation experiments in which fish were injected 24 hr before sampling over the 7-day time-course of salinity acclimation. For proliferation studies 2 and 3, FW tilapia were transferred to either FW (0-2ppt) or SW (24 ppt) for 1 day and 7 days. Fish sampled at 1 day were BrdU injected just prior to beginning of the transfer study. Fish sampled at 7 days were BrdU injected on day 6 of the salinity transfer experiment. A similar procedure was done for fish preacclimated to SW (24 ppt) and then transferred to either SW or FW. For sampling, fish were sedated with MS 222, decapitated and the pituitaries were removed and placed in Carnoy’s fixative (60% EtOH, 30% glacial acetic acid, 10% chloroform).

**Plasma Osmolality Determination**

Plasma osmolality of fish from the proliferation study 1 salinity challenge was analyzed to determine osmotic disturbances during the salinity acclimation process. Blood was drawn by a heparinized syringe from the caudal vein of anesthetized fish. Plasma was separated at 3000 x g for 5 min at 4°C and then stored at -20°C. Plasma osmolality was measured in duplicate using a vapor pressure osmometer (Westcor, Logan, UT).
Detection of Proliferating Lactotrophs

After fixing pituitaries for 12 hr, samples were washed three times in 100% ethanol, one time in 50% Hemo-de in ethanol, two times in 100% Hemo-de, and then placed in molten paraffin in a vacuum oven for 2 hours at 60°C. Pituitaries were sectioned at 5μm at random points within the tissue. Tissue sections were placed on glass slides, de-waxed with Hemo-de, rehydrated with a series of ethanol concentrations in water, and denatured with 2N HCl. Sections were then washed three times with PBS (pH 7.4) for 5 min, circles with a PAP-pen (Sigma, St. Louis, MO), and blocked with 10% normal goat serum (Sigma-Aldrich, St. Louis, MO) for 15 min at room temperature. Sections were dual stained with both monoclonal mouse anti-BrdU at 1:20 dilution (Beckton Dickenson, Franklin Lakes, NJ) and a polyclonal rabbit antisera to tilapia PRL at 1:2000 to identify proliferating lactrotrophs for 90 min at room temperature and then an additional 48 hr at 4°C in a humidified chamber. Negative controls were prepared identically except for the omission of the primary antibody for BrdU and the PRL antisera. Slides were washed five times for 3 min with PBS at room temperature. Secondary antibodies conjugated to fluorescein isothiocyanate (FITC) and Texas Red (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:50 dilution in PBS was then applied for 2 hr at room temperature to visualize BrdU and PRL staining, respectively. Following this dual labeling, tissues were washed three times for 5 min with PBS. For
identification of nuclei, tissues were then exposed to Hoechst 33258 (Molecular Probes Inc., Eugene, OR) at a 1:3000 dilution in PBS for 30 sec at room temperature, and then rinsed three times for 5 min in ddH₂O. Coverslips were mounted with Swartz’s mounting media (Swartz et al. 1990) containing p-phenylenediamine (70% glycerol buffered pH 8.5) and slides were maintained in the darkness at -20°C until analyses.

In preliminary qualitative studies to optimize detection, we used a range of anti-BrdU dilutions (1:10 - 1:8,000) and incubation times/temperatures and found that a dilution of 1:20 dilution during incubations of 90 min at room temperature followed by 24-48 hr at 4°C produced the best results. This protocol also worked for the polyclonal tilapia PRL antisera, although the length of the incubation was not essential for detecting PRL staining. A 1:2000 dilution of PRL antisera was optimal among the 1:500 to 1:8000 range tested. We also tried different denaturing reagents during 30 min incubations at room temperature and found that 0.07N NaOH alone and 2N HCl containing 0.1% Tween both resulted in diminished BrdU labeling compared with 2N HCl alone.

**Detection of Apoptotic Lactotrophs**

Apoptosis was determined using the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) of genomic DNA method. Terminal end nick labeling is an
effective method for detecting DNA fragmentation or DNA laddering, which is a condition exclusive to apoptotic cells. For apoptosis detection, pituitary tissue sections were dehydrated, washed, incubated and the embedded in paraffin. 5µm slices were then dewaxed, rehydrated, washed with PBS, and then blocked with 10% goat serum (Sigma-Aldrich, St. Louis, MO) for 15 min at room temperature. Sections were then incubated with tilapia PRL antisera (1:2000) for 18 hrs at 4°C, then rinsed 5 times for 3 min. with PBS. Sections were then incubated with goat anti rabbit conjugated to Texas Red for 2 hr at room temperature, and then washed 5 times with PBS for 3 min. Tissues were then labeled by an apoptosis histochemical TUNEL kit according to the manufacturer’s protocol (Promega, Madison, WI). This method was effective in determining apoptosis in rat lactotrophs (Pisera et al. 2004). Briefly, the tissues were incubated with Proteinase K to permeabilize the tissues, incubated with recombinant terminal deoxynucleotidyl transferase which labels the 3’-OH ends of the fragmented DNA with fluorescein-12, coverslipped, and then rinsed prior analysis by fluorescent microscopy. This allowed us to co-localize apoptotic cells with PRL-producing cells. A positive control was used on pituitary sections treated with DNase (10 U/ml DNase I) to confirm the TUNEL labeling method works in detecting fragmented DNA in fish lactotrophs.
Lactotroph Proliferation, Apoptosis and Cell Density Analyses

Stained pituitary sections were analyzed using a Leica 5000DM microscope fitted with 20X, and 40X objective lenses and filter cubes for DAPI, Cy3, and FITC. To quantify double label, first PRL immunostaining was observed with the Cy3 filter and photographed at 20X and 40X with a Retiga 1800 digital camera. Second, BrdU immunostaining or fragmented DNA labeling were observed in the same region with the FITC filter and photographed. The images were then merged using the MCID elite Image Analysis (Interfocus Imaging Ltd., Cambridge, England) software package. Cells immunostained for both BrdU and PRL (indicating proliferating lactotrophs) and then both fragmented DNA and PRL (indicating apoptotic lactotrophs) were then hand counted by an individual blind to the treatment groups verified by the MCID software. The DAPI and Cy3 filters were used to analyze the lactotroph density through Hoechst staining and PRL immunoreactivity. The appropriate threshold was applied to images to ensure that stained cells counted via the MCID software reflected the actual count. Three non-sequential, non-overlapping 5 um sections of pituitary was analyzed for each animal (7-10 animals/group = 21-30 pituitary sections/group). Lactotroph proliferation and apoptosis are expressed as the number of proliferating and apoptotic cells per 1000 nuclei.
Statistical Analysis

Statistical differences in proliferation, apoptosis, cell density, and blood osmolality were analyzed using a two-way analysis of variance (ANOVA) followed by the Fisher LSD test for predetermined comparisons. Data for determinations of the volume of the PRL cell region was analyzed using a one-way ANOVA test followed by the Fisher LSD test for predetermined comparisons. Statistical analyses were performed with Statistica 7.0 software (Stat Soft, Tulsa, OK). Statistical significance was set at a level of $P \leq 0.05$. All data is presented as mean $\pm$ SEM.
RESULTS

Volume of PRL Region

We investigated the volume of the PRL region of the pituitary in SW versus FW reared fish. The volume of the PRL region is 3 times greater in FW reared tilapia compared to SW tilapia (Figure 1, P < 0.001). We also observed via immunohistochemistry that the PRL stained region of the tilapia RPD appears larger in fish acclimated to FW for six weeks than those acclimated to SW (Figure 2).

Plasma Osmolality During Salinity Acclimation

Transfer of SW-acclimated fish to FW produced significant and rapid decreases in plasma osmolality by 1 day after transfer. Blood plasma osmolality levels returned to normal after 7 days (Figure 3). Conversely, transfer of FW-acclimated fish to SW produced significant and rapid increase in blood osmolality, up to nearly twice-normal levels. Levels of plasma osmolality were corrected after 7 days. The plasma osmolality levels of sham transferred FW or SW fish did not change throughout the time course.
Prior to the salinity challenge experiment, basal levels of lactotroph cell density were assessed in fish that were acclimated for six weeks and not injected with BrdU. We found that lactotroph cell density in FW and SW fish was $2.62 \pm 0.13$ and $3.40 \pm 0.12$ per 100 mm$^2$, respectively. Hence lactotrophs cell density is almost 50% less in fish acclimated to FW than those acclimated to SW (data not shown). Figure 4 shows a representative picture depicting the lactotroph density in FW versus SW fish. During the salinity challenge experiment we found an effect of treatment ($P < 0.05$) and time ($P < 0.05$) and of treatment by time ($P = 0.10$) (Figure 5A) for cell density. Similar to basal patterns prior to salinity challenge, sham transferred FW fish (FW-FW) also had around 34-51% the number of lactotrophs as sham transferred SW fish (SW-SW) throughout the entire course (day1-7) of salinity challenge ($P < 0.001$; Figures 4, 5). Sham transferred FW fish showed a slight decline in PRL cell density over time that was statistically insignificant. When SW fish were transferred to FW (SW-FW), lactotroph cell density declined over the course of the experiment by as much as 40% relative to sham transferred SW control fish. Fish transferred from FW to SW (FW-SW) showed a gradual decline in pituitary lactotroph cell density over time that was significant at day 7. Animals transferred from FW to SW were higher at all days relative FW
sham transferred control fish. Overall, FW-FW fish had lower lactotroph cell densities than SW-SW fish, and FW-SW and SW-FW fish had intermediate values to that of FW or SW fish (P < 0.05; Figure 5B).

**Lactotroph Apoptosis During Salinity Acclimation**

By triple staining tilapia pituitary sections, we were able to localize intact nuclei, fragmented nuclei and immunoreactive PRL cells (Figures 7-8). When these different cell stained images were overlaid, apoptotic PRL cells could be detected. However, we found very little apoptosis in the pituitaries of SW-SW fish, FW-FW fish, FW-SW transfers, or SW-FW transfers. There were no significant differences between any of the groups at time 0, 1 day, 4 days or 7 days after transfer or sham transfer (Figure 9). Sections that were DNAse treated showed abundant staining for Fluorescein or for fragmented DNA.

**Lactotroph Proliferation During Salinity Acclimation**

In proliferation experiment 1, we observed a detectable BrdU signal over seven days after a single injection on day 0 (Figure 9). After 1 day, there were more proliferating cells in FW (FW-FW) compared with SW (SW-SW) fish (P < 0.05). This elevation in FW fish was sustained throughout the experiment. The fish transferred from FW to SW after 1 day showed a significant reduction in lactotroph proliferation to levels similar to SW fish and
well below that of fish maintained in FW. There were no statistically significant differences in lactotroph proliferation in any of the groups at day 4, albeit a rise was observed in SW-SW, FW-SW, and SW-FW groups compared to day 1, and proliferation continued to be higher in FW-FW compared with SW-SW fish. Relative to day 1, proliferation during SW to FW transfer increased significantly by day 7. Seven day transfer of fish from SW to FW led to a 12 times heightened level of BrdU labeling. After seven days, the proliferation rate in FW fish was still higher than that of SW fish.

The trend in increased proliferation, regardless of the treatment group, and our observed loss in BrdU signal intensity over time suggested that we might have quantified sequential cell divisions from those cells that initially incorporated BrdU on day 1 following injection. To better identify or gain a snapshot of the proliferative effects of salinity at a specific time during challenge, BrdU was injected 24 hours before sampling fish at different time points during salinity challenge. Similar to our initial experiment, we observed higher levels of proliferation in FW control fish compared to SW fish (P < 0.05; Figure 10). Lactotroph proliferation declined by almost 90% when fish were transferred from FW to SW after 1 day compared with sham transferred FW control fish (P < 0.05). Pituitary lactotroph proliferation in FW-SW fish was reduced to levels similar to SW-SW fish. Relative to their SW-SW sham controls, proliferation was not altered in SW fish transferred to FW for 1 day. By day 7 of SW to FW transfer pituitary lactotroph proliferation rose by almost 20-fold,
exceeding levels of FW-FW fish (P < 0.05). Levels of proliferation remained higher in FW sham transferred fish compared to SW sham transferred fish at day 7. We lost 7-day FW to SW transfer fish due to mortalities. The study was therefore repeated and results were virtually identical to the second salinity challenge and FW animals transferred SW showed significant reductions in lactotroph proliferations by day 7 as was initially seen with day 1 post-transfer (Figure 11).
DISCUSSION

The purpose of these studies was to determine if salinity regulates lactotroph cell proliferation and apoptosis in events that might promote enhanced production and the elevated content of PRL observed in fish adapted to hypotonic environments. We show that the volume of the PRL immunoreactive region or RPD portion of the pituitary is higher in FW compared with SW tilapia. This increase in the PRL cell region is reflective in the amount of PRL measured in the pituitary with salinity. Our previous studies show that the pituitary content of PRL reared in FW for 4 months is higher than that of SW-reared fish and that this process can be reversed following 7-week acclimation of FW fish to SW and vice versa with SW to FW acclimation (Borski et al. 1992). Thus, if PRL cell region of the pituitary enlarges to accommodate the higher levels of PRL needed for fish adapting to FW.

The elevation in volume of the PRL cell region of FW fish may result from either a greater number (hyperplasia) or increase in the size of cells (hypertrophy) or both. Based on acidophilic staining of lactotrophs, cell size was shown to decline in conjunction with reduced number of secretory granules as early as 18 hr following transfer of swordtail (*Xiphophorus hellerii*) from FW to 1/3rd SW (Holtzman and Schreibman 1972). In the euryhaline killifish, PRL cell size and pituitary region appears to be large in fish maintained in FW for 21 days compared to SW fish (Betchaku and Douglas 1980). We were unable to
directly measure cell volume in our histological preparations due to ambiguity in identifying membrane boundaries. However, the lower number of definitively-identified lactotrophs measured per unit area of the pituitary of FW fish compared with that measured over similar areas in SW fish suggests that the volume of individual PRL cells may also increase in tilapia adapted to and challenged with FW. Dharmamba and Nishioka (1968), showed that the synthetic activity and number of secretory granules in PRL cells is higher in FW than SW fish, which we confirm here with electron microscopy (data not shown). Hence, the increased hypertrophy of PRL cells is likely important to accommodate the enhanced production and requirements to store PRL in lactotrophs during life in FW.

The mechanism(s) underlying potential increases and decrease in lactotroph cell volume, as ascertained through cell density measurements, in fish acclimated to FW and SW acclimation, respectively, is uncertain. The PRL cell is highly and directly sensitive to changes in osmotic pressure both in vivo (Shepherd et al. 1999) and in vitro (Grau et al. 1981). Physiologically relevant reductions in medium osmotic pressure stimulate, while elevations in osmotic pressure inhibit PRL release. It is thought that regulatory volume increases are an essential signaling component for eliciting increased PRL secretory responses to reduced osmotic pressure (Grau et al. 1994; Seale et al. 2003b; Seale et al. 2003a; Weber et al. 2004; Seale et al. 2006). Perifused PRL cells show a rapid spike and lower sustained increase in volume and intracellular Ca$^{2+}$ that is important in mediating
elevations in PRL release. The volume increase is sustained so long as medium osmotic pressure is reduced. Although these in vitro experiments were done over hours, in vivo studies using pituitaries explanted to the optic nerve show that changes in salinity or osmotic pressure can act directly, rather than through hypothalamic regulation, to act directly in regulating sustained PRL secretion. It is, therefore, possible that the reductions in osmotic pressure observed in the plasma of fish during acclimation to FW over days is mediating enhanced lactotroph hypertrophy. Indeed we found a rapid reduction in lactotroph cell density when fish were transferred from SW to FW (presumed reduction in cell volume). Interestingly, the plasma osmotic pressure of fish acclimated to FW over weeks or months is about 15 mOsmolal lower (P < 0.05) than that of long term adapted SW fish (see time 0, Figure 3: (Dean et al. 2003; Tipsmark et al. 2008)). These changes, and the more dramatic osmotic adjustments that occur with the acclimation process (Figure 3) could, therefore, be driving the differences in lactotroph cell volume that likely account, in part, for changes in the density we observed here under different salinities: lactotroph cell density declines and volume increases in FW fish with the opposite occurring in SW fish.

In addition to hypertrophy, the enhanced volume of the PRL cell region of FW relative to SW fish might also be mediated by hyperplasia. The number of cells in a tissue is governed by the combined rate of proliferation (cell division) and apoptosis (cell death). Carp epithelial cell lines preacclimated to a moderate hypertonic media (400mOsm) and then
exposed to a more severe hypertonicity (500-600 mOsm) show reduced apoptosis relative to those cells shifted directly from an isotonic (300mOsm) to severe hypertonic media *in vitro*. Cells exposed to hypotonic media did not exhibit apoptosis (Hashimoto et al. 1999). Nothing is known about regulation of lactotroph apoptosis in fish. We postulated that SW fish and/or fish acclimated from FW to SW would have higher level of lactotroph apoptosis since fish under this salinity have lower rates of PRL synthesis and secretion (Borski et al. 1992; Ayson et al. 1993; Yada et al. 1994) and cell activity (Dharmamba and Nishioka 1968), lower pituitary PRL content (Borski et al. 1992) and a reduced region of PRL cells in their pituitary (Figure 1). However, we found that there was a very low number or undetectable levels of apoptotic lactotrophs in tilapia and that salinity had no discernable effect on cell death, as measured by the TUNEL assay.

We believe that the negligible apoptosis measured via the TUNEL assay is specific to lactotrophs and reflective of the true nature of PRL cell apoptotic activity in tilapia for several reasons. First, various types of *in vitro* or *in vivo* teleost model systems show that significant apoptosis can be detected by targeting DNA fragmentation, including those using the TUNEL method (Weyts et al. 1997; Weyts et al. 1998a; Weyts et al. 1998b; Verburg-Van Demenade et al. 1999). Second, we show that treatment of pituitary sections with DNAse to fragment DNA, which is a requisite to apoptosis (Studzinski 1999), results in ample and strong apoptosis signaling (Figure 4). A series of validations were also done to demonstrate
that immuoreactive staining of PRL cells did not interfere with fluorescein labeling of fragmented DNA. Third, we found that the tilapia intestine, an organ with considerable cellular turnover, showed significant apoptosis using methods identical to that for pituitary detection (data not shown). Fourth, we found that cells outside the PRL cell region or RPD of the pituitary, including those within the proximate pars distalis show significant apoptosis. Melamed et al. (1999) demonstrated that estradiol-17β and insulin-like growth factor-I (IGF-I) can regulate tilapia somatotroph apoptosis in vitro. Clearly, our results show that under basal conditions lactotroph apoptosis is negligible and that salinity is altogether ineffective in regulating apoptosis during either acute challenge or in animals fully adapted to different salinities. Hence, it would appear that selective cell death is not a mechanism through which salinity might alter lactotroph hyperplasia and changes in the amount of PRL produced by the pituitary.

In contrast to apoptosis, salinity exerted a profound effect on proliferation of lactotrophs. In our first experimental approach lactotrophs were labeled with BrdU with an initial injection at day 0 of the salinity challenge. We then measured accumulative lactotroph proliferation over the time course of salinity challenge, when initial osmotic perturbations are evident (Figure 3). We found that 1) FW fish had substantially more proliferating cells than SW fish, 2) transfer of FW fish to SW rapidly reduces lactotroph proliferation, 3) transfer of SW fish to FW caused an almost 15-fold increase in proliferating lactotrophs by day 7, exceeding
levels of SW sham transferred fish, and 4) SW fish either sham transferred to SW or to FW showed a gradual increase in lactotroph proliferation from low initial levels found at 24 hours post transfer and BrdU post-injection (Figure 9). This latter trend in increased proliferation, regardless of treatment group, along the progressively lower BrdU intensity observed in labeled lactotrophs during salinity challenge suggests we may have quantified the accumulative effects of cellular divisions from those cells that originally incorporated BrdU on day 1 of the experiment. To reduce detection of sequential divisions of originally labeled cells during the latter periods of salinity challenge, lactotroph proliferation was instead tested in animals injected with BrdU 24 hr prior to sampling. This approach allowed for a better assessment of the time course effect of salinity on lactotroph proliferation. Similar to experiment 1 (Figure 9), we found that the level of proliferating lactotrophs is substantially elevated in FW compared with SW fish. When fish are transferred from FW to SW we again observed a rapid decline in proliferation, occurring within one day of transfer. This decline was sustained over the course of the experiment, suggesting hypertonicity inhibits lactotroph proliferation. Conversely, when fish are moved from SW to FW PRL cell proliferation increases dramatically, albeit the response is somewhat delayed as it occurred by day 7, but not day 1 of transfer. In zebrafish, slight reductions in the osmotic pressure of media accelerated proliferation of lactotrophs during embryogenesis (Liu et al. 2006). Here we show that shifts in environmental salinities that are clearly hypotonic or hypertonic to the fishes’ internal milieu elicit dramatic changes in lactotroph proliferation. Collectively, our
results are the first to demonstrate in euryhaline fish that hypotonicity activates, while hypertonicity inhibits lactotroph cell proliferation. It would appear that inhibitory effects of SW (day 1, Figures 10-11) occurs more rapidly than the stimulatory actions of FW transfer (day 7), although analyses of additional early (< 24 hr) and intermediate time points are warranted to better refine the time course over which salinity alters lactotroph proliferation.

The increase in lactotroph proliferation we observe in tilapia in FW, is reminiscent of the heightened proliferation occurring with lactation in mammals, including humans (Goluboff and Ezrin 1969; Stefaneanu et al. 1992; Yin and Arita 2000; Carretero et al. 2003), underlying the relative importance of PRL to these two processes in vertebrates. The mechanism underlying increased proliferation with FW, or vice versa with SW exposure is unknown. In rodents or pituitary cell lines, estrogens (Lieberman et al. 1982; Perez et al. 1986; Kansra et al. 2005), IGF-I (Fernandez, Sanchez-Franco et al. 2003; Gutierrez, Mukdsi et al. 2007), insulin (Suzuki et al. 1999), and other factors (vasoactive intestinal peptide, epidermal growth factor, and galanan) (Fernandez et al. 2003) induce lactotroph proliferation while dopamine suppresses proliferation (Kelly et al. 1997; Saiardi et al. 1997; Arita et al. 1998). Several of these hormones are also regulators of PRL release in the tilapia, with estradiol 17-ß and IGF-I augmenting, and dopamine inhibiting PRL release (Barry and Grau 1986; Fruchtman et al. 2000; Kajimura et al. 2002). Leptin is a cytokine recently shown to potently stimulate PRL in tilapia (Tipsmark et al. 2008). In striped bass and tilapia, IGF-I
stimulates PRL secretion and synthesis, exerts hyperosmoregulatory actions and is also produced by the pituitary, raising the possibility that it may regulate the differential sensitivity of lactotroph proliferation to salinity in fish (Reinecke et al. 1997; Fruchtman et al. 2000; Fruchtman et al. 2002). Since osmotic pressure is both a regulator of PRL synthesis and secretion and cell volume, it too might exert direct effects on lactotroph proliferation, a possibility that could be tested in vivo with explanted pituitaries or during in vitro exposures to altered media osmotic pressure. Clearly, the homogenous arrangement of PRL cells within the RPD of teleost fishes will greatly facilitate in vitro studies on the factors directly regulating lactotroph proliferation in cells under their naturally aggregated in vivo state.

In summary lactotroph proliferation is higher in tilapia maintained or exposed acutely to FW, with the opposite occurring during SW adaptation. This, along with increases in the volume of the PRL cell region, decline in lactotroph cell density, and presumed increase in cell volume, suggests that the pituitary lactotroph undergoes hypertrophy and hyperplasia during FW acclimation. Apoptosis appears to play little role in regulating lactotroph density under different salinities. Overall, these results suggest that the elevated production and content of PRL likely critical to life in FW is mediated, in part, through a concert of enhanced lactotroph proliferation and hypertrophy.
LITERATURE CITED


Yoshikawa-Ebesu, J. S. M., et al. (1995). "Effects of acclimation salinity and in vitro medium osmotic pressure on the incorporation of 3H-leucine into the two prolactins of
the tilapia, Oreochromis mossambicus." Comparative Physiology and Biochemistry 271(5): 331-339.
Figure 1. Volume of PRL region (mm$^3$ X 100 in the pituitaries of tilapia raised for 4 months in FW and SW. (mean ± SEM; N = 12). ***P < 0.001.
Figure 2. Representatives midsagital sections of pituitaries from six-week FW (A) and SW (B) acclimated tilapia showing PRL immunoreactive region within the RPD. Sections were stained with tilapia PRL antisera and detected using a secondary antibody conjugated to Texas Red. Picture shown in monochrome.
Figure 3. Effect of salinity transfer on plasma osmolality in tilapia. Freshwater fish were transferred to either FW or SW or SW fish were transferred to either SW or FW for 1, 4 and 7 days. There was a clear osmotic disturbance during salinity acclimation with transfer to FW resulting in a drop in plasma osmolality followed by recovery by day 4. By contrast transfer to SW increased plasma osmolality which recovered by day 7 of transfer. means ± SEM (N=8-10), Significant effects compared to sham controls, *P<0.05, **P<0.01, ***P<0.001.
Figure 4. Midsagittal section of a tilapia pituitary from a six week FW (left panels) and SW (right panels) adapted fish. Nuclei were stained with Hoechst 33258 (A, C). Tilapia PRL immunoreactive cells are shown in red (B, D). Note the increased density of lactotroph cells in SW versus FW fish.
Figure 5. Lactotroph cell density of FW fish transferred to either FW or SW or of SW fish transferred to either SW or FW for 1, 4 and 7 days. N = 12 fish/group/time point. Different letters denote significant differences between groups within the same time point. Asterisks denote significant differences from day 1 within a group (P < 0.05)
Figure 6. (A) Triple staining of tilapia pituitary sections for nuclei (Hoechst 33258, blue), fluorescein (green), and immunoreactive PRL cells (red) at 20X. Note the apoptotic or fluorescein-stained cells in this overlay are marked by arrowheads. (B) Dnase treated pituitary section at 10X used as a positive control.
Figure 7. Double labeling of tilapia pituitary midsagittal sections from FW (left panels) and SW (right panels) adapted fish. Lactotrophs are identified by immunoreactivity with tilapia PRL antisera (red: A, 20X; D, 2.5X). Sections were stained for apoptotic nuclei with terminal deoxynucleotidyl transferase in conjunction with fluorescein-12-dUTP (green: B 20X, E 2.5X). We identified apoptotic lactotrophs by co-localization (C 20X, F 10X) of these labels.
Figure 8. Lactotroph apoptosis in the pituitary of FW fish transferred to either FW or SW or of SW fish transferred to either SW or FW for 1, 4 and 7 days as measured by the number of cells stained with both fluorescein and PRL antisera. N = 7-12 fish/group/time point. There was a minimal level of lactotroph apoptosis in fish pituitaries during salinity acclimation and no significant differences were seen among fish acclimated to either FW or SW.
Figure 9. Lactotroph proliferation in the pituitary of FW fish transferred to either FW or SW or of SW fish transferred to either SW or FW for 1, 4 and 7 days as measured by the number of cells stained with both BrdU and PRL antisera. Values are means ± SEM, N = 7-12 fish/group/time point. All fish were injected once with BrdU at time 0. Different letters denote significant differences (P < 0.05).
Figure 10. Lactotroph proliferation in the pituitary of FW fish transferred to either FW or SW or of SW fish transferred to either SW or FW for 1 and 7 days as measured by the number of cells stained with both BrdU and PRL antisera. Values are means ± SEM, N = 7-12 fish/group/time point. All fish were injected once with BrdU 24 hours before sacrifice. Different letters denote significant differences (P < 0.05). Data for 7-day FW-SW fish is not provided due to mortalities. Figure 11 shows a similar experiment with data from all groups and time points of salinity acclimation.
Figure 11. Lactotroph proliferation in the pituitary of FW fish transferred to either FW or SW or of SW fish transferred to either SW or FW for 1 and 7 days as measured by the number of cells stained with both BrdU and PRL antisera. Values are means ± SEM, N = 7-12 fish/group/time point. All fish were injected once with BrdU 24 hours before sacrifice. Different letters denote significant differences (P < 0.05).
Figure 12. Double labeling of tilapia pituitary midsagittal sections from FW (left panels, 20X) and SW (right panels, 20X) adapted fish. Sections show PRL immunoreactive cells within the RPD region of the pituitary (red) and BrdU (green) to proliferating cells. Colocalization of PRL immunoreactivity and BrdU signify proliferating lactotrophs. Note the increased number of BrdU-labeled nuclei in FW acclimated tilapia lactotrophs compared with SW fish.