ABSTRACT

Title of Thesis: STUDIES ON THE GNRH/GTH SYSTEM OF FEMALE STRIPED BASS *(Morone saxatilis)*: EFFECTS OF GNRH AGONIST THERAPY AND COMPARISON OF REPRODUCTIVE ENDOCRINE PARAMETERS BETWEEN WILD AND CAPTIVE FISH

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In the striped bass (*Morone saxatilis*), and many other commercially important fish species, captivity results in an inability to complete final oocyte maturation (FOM), ovulation, and spawning. We hypothesize that this effect is mediated by a disruption of the hypothalamo-pituitary-gonadal (HPG) axis at the level of the gonadotropin -releasing hormones (GnRHs). To confirm this hypothesis research was conducted focused on three objectives:

First, to complement the battery of assays previously developed for analysis of the GnRH/GtH system in striped bass, an RNAse protection assay was developed to measure

specific expression of the three forms of GnRH in striped bass (salmon GnRH, chicken GnRH-II and seabream GnRH). Secondly, effects of GnRH agonist-induced ovulation on the HPG axis of captive striped bass was examined by comparison of several reproductive endocrine parameters between females sampled at four stages of oocyte development. Finally, differences were examined between the reproductive endocrine status of wild and captive female striped bass.

We conclude that sbGnRH is the most important form for the preovulatory release of pituitary GtH-II in striped bass. We suggest that captive females synthesize levels of GnRH mRNA that are comparable to their wild counterparts, however fail to release adequate quantities of bioactive GnRH within the pituitary to stimulate completion of FOM. This data may indicate that regulation of sbGnRH in striped bass occurs via post-transcriptional/translational mechanisms. Furthermore, the presence of salmon GnRH in the pituitaries of captive females may be indicative of a possible role for salmon GnRH in the regulation of FOM.

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by

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LIST OF ABBREVIATIONS

11-KT	11-ketotestosterone
20ß-S	17,20β,21-trihydroxy-4-pregnen-3-one
ARC	Aquaculture Research Center
BSA	Bovine serum albumin
BW	Body weight
CAMP	cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
cGnRH-II	Chicken gonadotropin-releasing hormone-II
Ct	Threshold cycle
DHP	17,20β-dihydroxy-4-pregnen-3-one
DNR	Department of Natural Resources
E_2	17ß-estradiol
ELISA	Enzyme-linked immunosorbent assay
ERE	Estrogen responsive element
FSH	Follicle stimulating hormone
GAP	Gonadotropin-releasing hormone associated peptide
GnRH	Gonadotropin-releasing hormone
GnRHa	Gonadotropin-releasing hormone agonist
GnRH-R	Gonadotropin-releasing hormone receptor
GRE	Glucocorticoid responsive element
GSI	Gonadosomatic index
hCG	Human chorionic gonadotropin
LH	Lueteinizing hormone
LHRH	Lueteinizing hormone releasing hormone
MIS	Maturation inducing steroid
MPF	Maturation promoting factor
MS 222	Tricaine methanesulfonate
OD	Optical density
PE	Pituitary extracts
PRL	Prolactin
RIA	Radioimmunoassay
RPA	Ribonuclease protection assay
sbGnRH	Seabream gonadotropin-releasing hormone
sGnRH	Salmon gonadotropin-releasing hormone
SL	Somatolactin
T	Testosterone
UTR	Untranslated region

CHAPTER I. GENERAL INTRODUCTION

The purpose of this project is to examine, at the molecular level, the captivity-induced reproductive dysfunction that occurs in striped bass. Specifically, we are interested in changes in levels of the brain hormone, gonadotropin-releasing hormone. This family of highly conserved decapeptide hormones are considered to be the main hormones involved in regulating reproduction in vertebrates, from the primitive agnathans through highly evolved mammals, such as humans. The following introduction will summarize our current understanding of these hormones and their relationship to striped bass reproduction. In our description we will describe primarily the situtation found in female striped bass, as females exhibit more severe reproductive dysfunction and hence are the main focus of our research.

I.A. Striped bass reproduction

I.A.1. Life History

The striped bass (*Morone saxatilis,* striper, or rockfish) is a perciform fish native to the East Coast of the United States. It wasn't until the 1870s that striped bass were introduced to the West Coast, where they have thrived. Their current range on the East Coast stretches from Canada to Florida, in the Gulf of Mexico from western Florida to Louisiana and on the West Coast from southern California up to British Columbia. Along the eastern seaboard, there are three main migratory stocks; the Hudson, the Chesapeake, and the Roanoke. Fish in Nova Scotia and below North Carolina are generally thought not to migrate.

Striped bass are anadromous, annual, group synchronous spawners. This means that they return from the open ocean to freshwater once a year to spawn a single batch of eggs or sperm. The eggs are fertilized in the water column and float downstream on the currents. As the fish hatch they congregate in nursery areas, sometimes with the congeneric white perch, in tidal sections of rivers. Juvenile fish spend the first 2-5 years of their lives in the safety of the shallows. Older, sexually mature fish spend most of the year in the ocean and only return to the tributaries to reproduce every spring. Striped bass can reach weights of 125 lbs. and live for more than 30 years. In the wild, female striped bass begin to reach sexual maturity by the age of 4 or 5, but are not fully reproductive until 6 to 8 years old. Males, on the other hand, mature slightly earlier, by their second or third year. Captivity has the effect of slightly advancing the onset of puberty in fish, but this advancement is for nought if the reproductive dysfunction is not addressed.

Ecologically striped bass are voracious predators, feeding on a wide variety of fishes, crustaceans, squids, mussels, and worms. Their tenacity has led to a popular recreational fishery which, together with an overzealous commercial fishery, brought striped bass numbers in the Chesapeake Bay to alarmingly low levels in the late 1970s and early 1980s. In Maryland, the commercial striped bass landings fell from an average 3.7 million pounds during 1958-1975 to an average of 1.3 million pounds from 1976-1984 (Murdy, Birdsong et al. 1997). These drastic declines lead to a moratorium on catching striped bass for five years, from 1985 to 1989. By the time it was lifted in the early 1990s, it was obvious from the results of government sponsored

juvenile indexes that the moratorium, combined with stock enhancement efforts, had worked and the striped bass population was rebounding.

I.A.2. Gametogenesis

As with most species of fish, striped bass perceive the changing seasons mainly, but not exclusively, by photoperiodic information and water temperature. Daylength is processed and translated by the pineal gland in the form of melatonin. Photoperiod and water temperatures increase as winter turns to spring and serve as signs for striped bass to begin migrating to their spawning grounds. Inside the female ovaries, the follicular cells have been producing low levels of testosterone since the previous summer. Testosterone is normally aromatized into 17β-estradiol (E_2) by the granulosa cells of the ovarian follicles. E_2 is the primary steroid controlling the second phase of oocyte growth or vitellogenesis. Its function is to stimulate the liver to produce vitellogenin (Kishida, Anderson et al. 1992). The recruited oocytes gradually increase in size throughout the year until late winter and early spring by accumulating vitellogenin and other yolk precursor proteins, as well as various lipids. While the females are returning to the euryhaline conditions of their spawning grounds in the Bay, E_2 levels drop, testosterone levels surge briefly, and finally give way to 17,20β-dihydroxy-4-pregnen-3-one (DHP; King et al. 1994; Mylonas et al., 1997a, Sullivan et al., 1997). DHP serves as the biosynthetic precursor 17,20β,21-trihydroxy-4-pregnen-3-one (20ß-S) which is referred to as the maturation inducing steroid (MIS) due to its actions at the follicle cell layer to induce the process of final oocyte maturation.

Fishery managers are able to examine samples of oocytes via ovarian biopsy. Using a light microscope, average oocyte diameters and estimates of the percentage of yolk droplet coalescence and lipid clearing can be estimated in the field. This is useful for timing the administration of hormone treatments, but it does not obviate the need for histological examination of the oocyte developmental stage. Only through histology can a precise understanding of the oocyte development be obtained, including such factors as the extent of yolk coalescence and the nucleus or germinal vesicle (GV) migration (see figure 1 for examples). The different stages of oocyte development become very obvious under histological examination (Figure 1). Work by Mylonas et al. (1997b) in captive reared striped bass induced to ovulate with GnRH agonist established telltale histological features which serve to delineate developmental stages in these oocytes. At the end of vitellogenesis, , or germinal vesicle (GV) is found in the middle of the oocyte, surrounded by a variety of small lipid and yolk globules (Figure 1A). As FOM begins, the GV migrates (Figure 1B) towards the periphery of the oocyte (Figure 1C) followed by the breakdown of the GV membrane (GVBD, Figure 1D). This migration is concomitant with the coalescence of the lipid droplets and is thought to be controlled by the steroids, estradiol and testosterone. Late FOM is characterized by the 'clearing' of the yolk globules and completion of GVBD and is generally faster (<24 hr) than early FOM, which can last several days to several weeks depending on temperature and other physiological factors. Late FOM is accompanied by several other characteristic changes, such as increased oocyte diameter due to water gain, cortical alveoli diffusion, etc. Late FOM is also thought to be controlled by the progestins. Both of the aforementioned hormone classes will be discussed in greater

detail later in this text. Shortly after completion of FOM, the mature egg will be ovulated (Figure 1E).

I.A.3. Hypothalamo-Pituitary-Gonadal Web

The gonadal steroids described above are part of a system referred to as the hypothalamic-pituitary-gonad (HPG) web (Figure 2). The HPG web is the dominant endocrine model for regulation of reproduction in vertebrates. At the top of this web, gonadotropin-releasing hormones are synthesized in the hypothalamus in response to a number of environmental and physiological cues. In tetrapods, gonadotropin-releasing hormones (GnRHs) are delivered to the pituitary via the hypophyseal portal system, which is absent in bony fish. Therefore, in bony fishes the GnRH peptides are transported from the hypothalamus to the pituitary through neurons which directly innervate the gonadotroph cells at the site of GnRH release. Once in the pituitary, the GnRHs are responsible for the release of the gonadotropins (GtHs). The GtHs, in turn, are secreted into the bloodstream whereupon they travel to the gonads to regulate steroidogenesis and other processes. The gonads also provide feedback directly to the pituitary and indirectly to the hypothalamus, in the form of steroid hormones and activin and inhibin. Thus the complete HPG web represents a complex and dynamic mechanism of regulating reproduction through the interaction of several key hormones and external factors, both environmental and physiological.

I.A.4. Captivity-Induced Disruption

In the estuarine spawning grounds of the Chesapeake Bay, anadromous striped bass return from the open ocean every April and May to undergo these same hormonal changes. However, many species of fish kept in captivity for extended periods lose the ability to spawn spontaneously despite the extensive efforts of culturists to reproduce the conditions encountered by wild fish (for review see Zohar 1989). The oocytes of captive fish complete vitellogenesis, but do not complete final oocyte maturation and ovulation. Instead the eggs become atretic, and are broken down for resorption. Male striped bass also experience a disruption in their ability to reproduce although it is not quite as pronounced as in the females. Very often captive males will spermiate in the springtime, but the expressible milt volume, motility, and presumably the quality of the spermatocytes, is also markedly reduced in comparison to their wild counterparts. Studies of wild male striped bass in the Chesapeake indicate that even relatively low progestogens levels are adequate to stimualte spermiation(Mylonas, Scott et al. 1997), possibly explaining why males experience less severe reproductive dysfunction in captivity. Year-round access to reliable, high quality progeny is imperative for the culture of striped bass, or any other aquaculture species, to succeed.

I.B. Hormones Influencing Reproduction

I.B.1. Gonadotropin-releasing Hormone

Fish reproduction is orchestrated by a complex interaction of many hormones. These hormones in turn are influenced by a multitude of stimuli, both external and physiological. Arguably, the most important of these players are the gonadotropin-releasing hormones. These decapeptide hormones are recognized as the key endocrine factors controlling reproduction in vertebrates. Ten highly conserved forms have been isolated from vertebrate species to date, with an additional two forms isolated from tunicates (Powell, Reska-Skinner et al. 1996). The primary structures of all twelve forms are listed in Figure 3. Excluding the two forms found in the primitive lamprey, all vertebrate forms contain modified N-terminals (pGlu) and C-terminals (NH_2) and vary only at positions five, seven, and eight with position eight being the most highly variable.

The first GnRH was isolated and characterized in 1971 from sheep and pigs (Amoss, Burgus et al. 1971). This was named mammalian GnRH (mGnRH) and established the precedent of naming new GnRH forms according to the species in which they were.discovered. Historically, it was believed that all vertebrates contained this single form of GnRH in the brain. In the two decades following this discovery, other molecular variants have been isolated from different species (Sherwood, Eiden et al. 1983). Recently, multiple forms have been discovered in the brains of single species (eg., striped bass, seabream). Of all classes of vertebrates, fishes display the highest molecular diversity

of GnRH forms, seven in total, with most species exhibiting at least two distinct forms. Even placental mammals, specifically the musk shrew (Kasten, White et al. 1996) and humans (White, Eisen et al. 1993), have now been shown to contain at least two forms, mammalian GnRH (mGnRH) and chicken GnRH-II (cGnRH-II). There is currently speculation about the presence of a third form of GnRH in mammals. Perciform fish, a group of higher teleosts (Figure 5) are increasingly being found to contain three forms of GnRH. The Zohar lab was the first to discover examples of this, originally in the gilthead seabream, *Sparus aurata* (Gothilf, Elizur et al. 1995), and then in the striped bass, *Morone saxatilis* (Gothilf, Elizur et al. 1995). Other examples include tilapia, *Tilapia sparrmanii* (King and Millar 1985), the cichlid, *Haplochromis burtoni* (White, Kasten et al. 1995) , and pumpkinseed fish, *Lepomis gibbosus* (Powell, Fischer et al. 1995).

The structure of the GnRH gene is similar to that of most other neuropeptide hormones. The gene is encoded within four exons and includes a 5' untranslated region (UTR), an N-terminal signal or leader peptide, the decapeptide, a dibasic cleavage site followed by a GnRH associated peptide (GAP), ends with a 3' UTR (Sherwood, Lovejoy et al. 1993; Yu, Lin et al. 1997). The nascent polypeptide is termed a preprohormone and includes both the signal peptide and the GAP. Processing of the preprohormone involves splicing of the signal peptide to produce the prohormone and the subsequent cleavage of the GAP to produce the active peptide hormone. The signal peptide is removed while the nascent peptide passes through the endoplasmic reticulum. Processing of the resulting prohormone occurs during transport through the Golgi apparatus and in the secretory granules into which the

hormones are stored and transported (Laycock and Wise 1996). GnRH associated peptides exhibit much lower sequence conservation between forms than do their respective GnRHs. Not only does the sequence vary between forms, but the total length of the GAPs are also seen to vary, from 56 amino acids in mGnRH to as small as 46 amino acids in several salmonid and catfish species (Sherwood, Parker et al. 1994). There is, therefore, much room for speculation on the function of these peptides. Possible roles include a structural function for the prohormone that aids in cleavage and/or transport, or even an unknown function after secretion.

For a long time it was accepted that tetrapods and other higher vertebrates possessed a single form of GnRH, however recent findings have cast doubt on that theory. In the majority of fish studied, the occurrence of a multiple GnRH system has been documented. The existence of this system has been established mainly via the use of immunocytochemical and radioimmunological studies that have localized GnRH immunoreactive (ir) cells to several key areas in the brain (Gothilf, Munoz-Cueto et al. 1996; Montero and Dufour 1996; Parhar, Pfaff et al. 1996; Amano, Urano et al. 1997; Gothilf, Meiri et al. 1997). While there are variations among different species, the general distribution is as follows. There appear to be three main concentrations of GnRH-ir cells. The anterior-most concentration of cells is observed in the olfactory bulb and terminal nerve. The next concentration of GnRH producing cells is found in preoptic area and the nucleus ventrolateralis thalami, both in the hypothalamus. This is generally found to be the species specific form of GnRH. The third and final group of GnRH producing cells is found in the midbrain tegmentum. This nucleus has been consistently shown to express chicken GnRH-II, the ancestral

form of GnRH (Figure 6). While the evidence points to the hypothalamic GnRHs, specifically those which innervate the pituitary, as being hypophysiotropic in the majority of species, the purpose and function of the other, extra-hypophysiotropic forms have yet to be fully determined. Possible roles include regulation of secondary sex characteristics, and/or behavior. There are certainly variations of this rule, but it serves as a useful rule of thumb when addressing the obvious question of the roles of multiple GnRHs in a single species.

I.B.2. Gonadotropins

Most studied teleosts exhibit a "two gonadotropin system" (Suzuki, Kawauchi et al. 1988; Hassin, Elizur et al. 1995; Elizur, Zmora et al. 1996) GtH-I and II are heterodimeric subunit glycoprotein hormones with a common α-subunit and distinct ß subunit. In mammals, these gonadotropins correspond to follicle stimulating hormone (FSH) and luetenizing hormone (LH) respectively. Using radioimmunoassays, it has been shown in male salmonids and more recently in male striped bass (Hassin, Gothilf et al. 1998) that GtH-I is expressed during the early stages of spermatogenesis and then levels subside during spermiation, at which time GtH-II is expressed. A similar idea is prevalent for the role of gonadotropins in female fish, although not as much experimental evidence is available due to the higher severity of reproductive dysfunctions commonly encountered in females. It is thought that GtH-I controls the estrogen-induced vitellogenic growth of the oocytes, while gonadotropin-II (GtH-II) controls the later stages of FOM (Kagawa, Tanaka et al. 1998). In most fish species,

GtH-I has proven difficult to purify and characterize. The subsequent lack of data leaves us only to speculate on the full range of GtH functions.

I.B.3. Gonadal Steroids

Gonadal steroids represent the final level in the hypothalamic-pituitary-gonadal web. The steroids produced by striped bass gonads are representative of steroid production in most teleosts. The most important and best studied male steroid hormones are the androgens testosterone (T) and 11-ketotestoserone (11-KT). Testosterone serves as a precursor to 11-KT and their levels in the plasma are inversely correlated in plasma samples throughout the year. Reproductive patterns of these two androgens have been examined in adult male striped bass from the Chesapeake Bay. Steady increases in plasma concentrations are seen beginning in late Summer or early Fall, reaching a peak in April and May, concurrent with the spawning season. Post-mating, or "spent", fish display the years lowest levels of all reproductive parameters, including testosterone and 11-ketotestosterone. The similarity between these two steroid hormones has made it difficult to ascribe distinct roles to either steroid, therefore their actual functions remain unclarified.

A similar profile is observed in female striped bass with regard to their major gonadal steroids, 17ß-estradiol and its precursor testosterone (Mylonas, Woods et al. 1998). One difference between males and females is that circulating levels of T and E_2 in females while measurable, remain relatively low until ovulation nears. Despite these seemingly low levels, E_2 is able to stimulate increasing production of vitellogenin throughout the year. Presumably this is accomplished by upregulation of the E_2

receptor in the liver. As mentioned earlier, 17ß-estradiol and testosterone surge briefly during the early stages of final oocyte maturation (FOM) and then recede to give way to a surge of the final major class of gonadal steroids in striped bass, the progestins.

In most fish, the processes of final oocyte maturation, spermiation and ovulation are induced by the MIS, 17,20β-dihydroxy-4-pregnen-3-one (DHP) and its close relative 17,20β,21-trihydroxy-4-pregnen-3-one (20ß-S). There is some controversy as to which of these steroids is the actual MIS, and this role likely varies between or requires both steroids in different species. The MIS is so named, in part, because of its action at the cell membrane, which results in the release of a maturation promoting factor or MPF. The MIS(s) are vital to reproduction, as ovulation and spawning are dependent upon successful completion of final oocyte maturation.

Steroids have also been shown to provide regulatory feedback to the pituitary and hypothalamus. In striped bass, several putative steroid responsive element sequences have been identified, including estrogen responsive element (ERE), glucocorticoid responsive element (GRE) and steroidogenic transcription factor-1 (Chow, Kight et al. 1998). In male tilapia, testosterone can differentially regulate GnRH expression in the brain (Soga, Sakuma et al. 1998). The seabream GnRH gene in striped bass has been shown to contain putative estrogen-responsive elements (Chow, Gothilf et al. 1995). Also, estrogen receptor mRNA and protein have been found in several areas of the rainbow trout brain, using *in situ* hybridization and immunohistochemistry (Kah, Anglade et al. 1997). Interestingly, GnRH-expressing cells could not be shown to also express estrogen receptor, however this could result could be attributed to stage

specific expression of estrogen receptor. Although not fully understood, it is clear from the results of these and many other studies that steroids provide information about the physiological condition of the gonad, not only to downstream organs such as the liver and kidney, but also to upstream organs such as the brain and pituitary.

I.C. Hormonal manipulation of spawning

I.C.1. Hypophysation

To one extent or another, reproductive dysfunction has been a problem in culturing fish since the beginning of the practice. A significant amount of research has been conducted to minimize the disruptive effects of captivity, but many problems still exist. The extent of dysfunction is different in each species and can range from a complete lack of germ cell development to lowered fecundity. The first successful approach to circumventing reproductive dysfunction was hypophysation (Houssay 1930). This involves injection of receptive broodstock with the pituitary extracts (PE) of another fish, most often carp PE. Hypophysation was soon expanded to include other cheaper, more accessible and more standardized factors, specifically human chorionic gonadotropin (hCG) and synthetic GtHs. These exogenous GtHs act upon the gonads to induce final oocyte maturation, ovulation and spawning in the same manner as the endogenous GtHs should. Hypophysation is an effective method, but has several drawbacks. Namely broodstock are temporally receptive to treatment with exogenous GtHs (i.e. for only a brief window of time). If treatment is too early or late, the exogenous GtHs are ineffective and the oocytes will not develop. In the case of the domesticated striped bass, the oocytes of most fish will not even reach the

appropriate developmental stage before atresia sets in. For this reason, GtH treatment is usually only appropriate for wild caught broodstock which have already undergone considerable oocyte maturation, far more than can be achieved by domesticated striped bass. Second, due to the large molecular size of the GtHs it is possible for the fish to develop an immune response to the treatments. This immune response, at best, will result in refraction by the fish, thus requiring larger and larger doses of GtH. At worst, repeated treatments will completely desensitize the fish to GtH treatment. In addition, the high variability between pituitary extracts can lead to problems with non-standardized doses, contaminating hormones and the spread of disease. These problems combined with high costs and labor intensity make hypophysation an effective but expensive method. From the researchers point of view, one major benefit of these treatments was the confirmation of the hypothesis that the reproductive dysfunction in captive fish is due to insufficient production and/or release of gonadotropins. Thus, the search for new methods of inducing captive fish to ovulate continued with new emphasis on the gonadotropin-releasing hormones.

I.C.2. Gonadotropin-releasing Hormone Agonists

In the late 1970's and early 1980's, the development of new drugs and delivery systems greatly advanced the field of hormonal manipulation of spawning. New therapies were devised which involve subcutaneous or intra-muscular injections of GnRH and/or its agonists (GnRHa) to receptive fish. GnRHa therapy is thought to act by stimulating the release from the pituitary of the endogenous GtHs, which in turn stimulate the gonads to synthesize the gonadal steroids that regulate final maturation

of the oocytes. In some species, GnRH(a)s have been used in combination with pimozide, a dopamine antagonist. This is designed to nullify the species-specific phenomenon of dopaminergic inhibition of GnRH-induced GtH release (see Figure 2). Agonists are synthetic peptides with minor substitutions in the amino acid sequence designed to either protect the hormone from enzymatic degradation or increase the hormone's affinity to its receptor. In the case of GnRH, the bonds between amino acids 5-6 and 9-10 were found to be the most susceptible to enzymatic degradation. In the most commonly used agonist, D•Ala^{6} Pro^{9}-NET, the glycine at position 6 was substituted with D-alanine, and the proline^{9}- glycine^{10}-NH2 bond at the C-terminus of the molecule was changed to proline^{9}-N ethylamide (see Figure 3). The modified agonists elicited superpotent *in vivo* LH release when compared to native mGnRH and sGnRH in salmon, goldfish and seabream (Zohar 1989).

Despite the efficacy with which exogenous GnRHa treatment released pituitary LH, this method was not entirely successful. Even with the structural modifications designed to increase the resistance to enzymatic degradation, the release of GtH from the pituitary was relatively short-lived, indicating that the animals were still clearing GnRH from their circulation. To circumvent this problem, several delivery systems were designed based on biodegradable biopolymers such as ethylene vinyl acetate (Langer and Zohar 1994), mixtures of cholesterol and cellulose (Crim, Sutterlin et al. 1983), or polyanhydride microspheres (Mylonas, Woods et al. 1998), which release GnRHa in a sustained manner. These systems resulted in a continuous release of LH from the pituitary and therefore allowed the oocytes of the fish to complete FOM.

By intervening in the hypothalamic-pituitary-gonad web, GnRH therapy has several advantages over GtH treatment. Primarily, GnRHs initiate an endocrine cascade which releases other less central endocrine factors in the web. These "secondary factors" are beyond immediate influence of the GtHs but may play an important role in initiating or completing reproduction. Examples include neuropeptide Y, GnRH release inhibiting factor, prostaglandins and pheromones. Second, due to their relatively small size, GnRHs are nonimmunogenic, thus this therapy can be used on the same broodstock year after year with no decrease in efficacy. Also, synthetic GnRHa can be produced affordably in highly purified and standardized batches. Finally, due to the development of the slow-release delivery technology, one application is usually sufficient to induce spawning, reducing the stress of handling for both fish and farmer. Slow-release delivery systems have also expanded the window of opportunity in which the fish are receptive to treatment. Gonadotropin-releasing hormone agonist therapy reliably results in increased milt production and quality in males. In females, results include increased frequency of synchronous spawning, higher egg quality, and therefore higher fertilization, in a wide range of fish species [eg. goldfish (Sokolowska, Peter et al. 1984); plaice (Aida, Iznmo et al. 1978); trout (Sower, Iwamoto et al. 1984); (Mylonas, Hinshaw et al. 1992); salmon (Donaldson, Hunter et al. 1981/82); seabass (Barnabe and Barnabe-Quet 1985; Harvey, Nacario et al. 1985); catfish (DeLeeuw, Goos et al. 1985); striped bass (Hodson and Sullivan 1993) and seabream (Zohar, Pagelson et al. 1987)]. The success of the GnRH treatments also supports the hypothesis of a disturbance in the brain-pituitary-gonad web as the root of the reproductive dysfunction in striped bass.

I.D. Why study reproductive endocrinology in striped bass?

The striped bass is an important fish both economically and ecologically to the Atlantic coast of the United States. In the wild, striped bass serve as near top predators whose numbers have been suffering under the heavy pressure of recreational and commercial fisheries. The combined effect of a fishing moratorium and restocking efforts by the Departments of Natural Resources of Maryland and neighboring states has allowed the striped bass fishery to recover during the past ten years. Despite current successes, continued studies of the reproductive endocrinology of striped bass are essential, not only to protect against future population declines, but also to support the burgeoning aquaculture industry. In order to operate efficiently and maximize production, striped bass farmers must have a constant, year round supply of high quality seed. To generate a year-round stock requires a complete understanding of the reproductive dysfunctions (e.g., the disruptions in the GnRH system of captive striped bass and seabream) encountered when attempting to establish a captive broodstock. Striped bass and gilthead seabream are our current models of cultured perciform fish species that express three forms of GnRH, and experience reproductive dysfunctions due to captivity. The Zohar lab, along with several other groups, has spent many years studying the reproductive endocrinology of the striped bass. As a result of this work, many of the major components of the striped bass HPG web have been either cloned, or purified, or otherwise characterized and used to examine and profile the natural endocrine cycles of the striped bass. This intense scrutiny has resulted in the striped bass becoming one of the most well studied

perciform fish species today. Current work in the Zohar lab includes the establishment of a recombinant GnRH receptor system which will allow us to study, *in vitro,* the functions and potencies of the various GnRH forms and their agonists. This project was designed to add to the already extensive knowledge of the striped bass and its reproductive endocrinology. Lessons learned from the striped bass and seabream about the reproductive endocrine web and its control can be, and have been, applied to solve reproductive problems encountered in many other species of cultured fish.

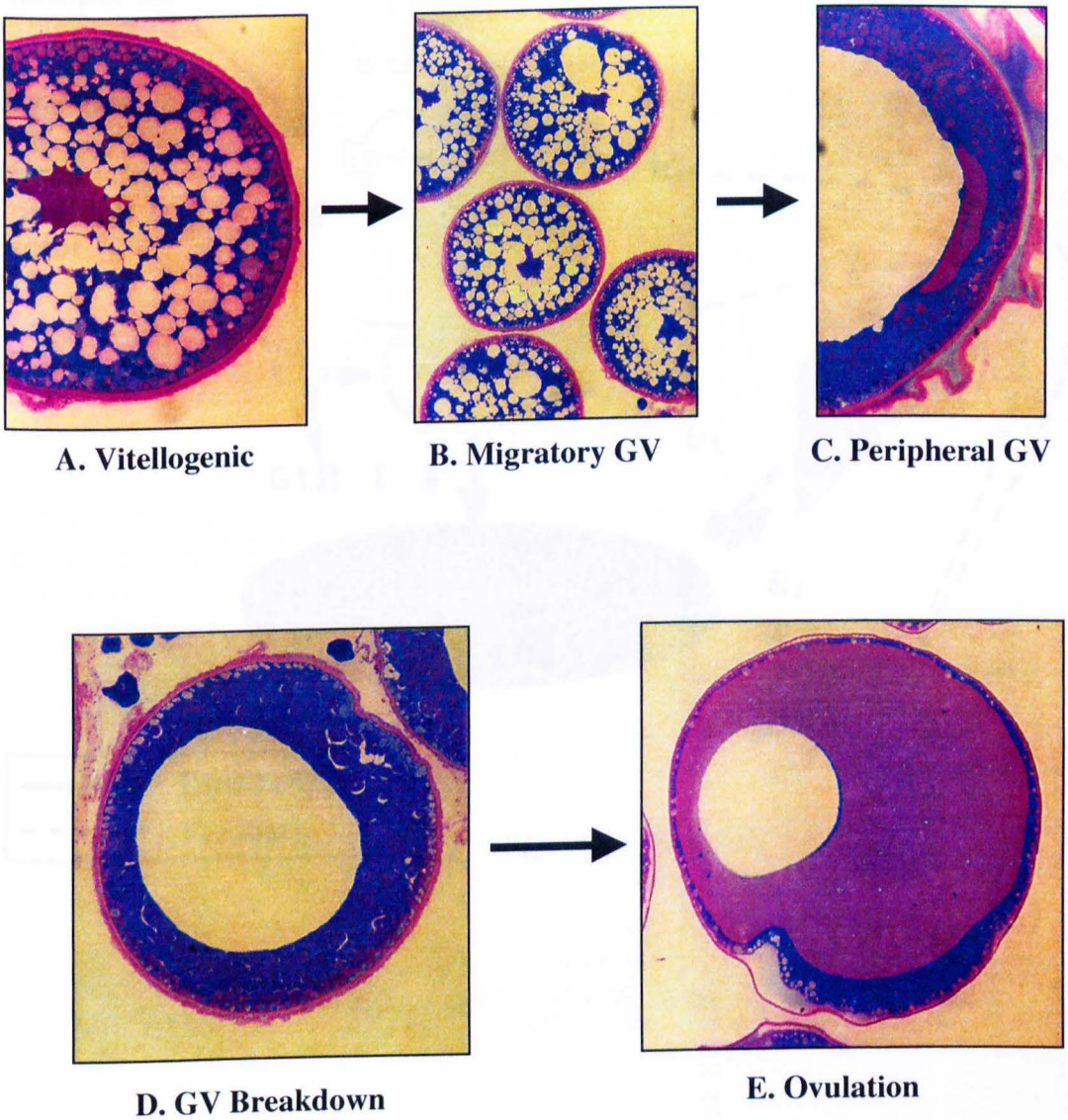


Figure 1. Histological sections of striped bass oocytes during various stages of development. Vitellogenic growth occurs throught most of the year and is the final stage accomplished by captive females without exogenous hormonal manipulation. FOM is marked by lipid droplet coalescence, and migration of the germinal vesicle (GV) to the periphery of the oocyte where it is eventually broken down prior to ovulation. Photos courtesy of Dr. C. Mylonas. Note: photographs not to scale.

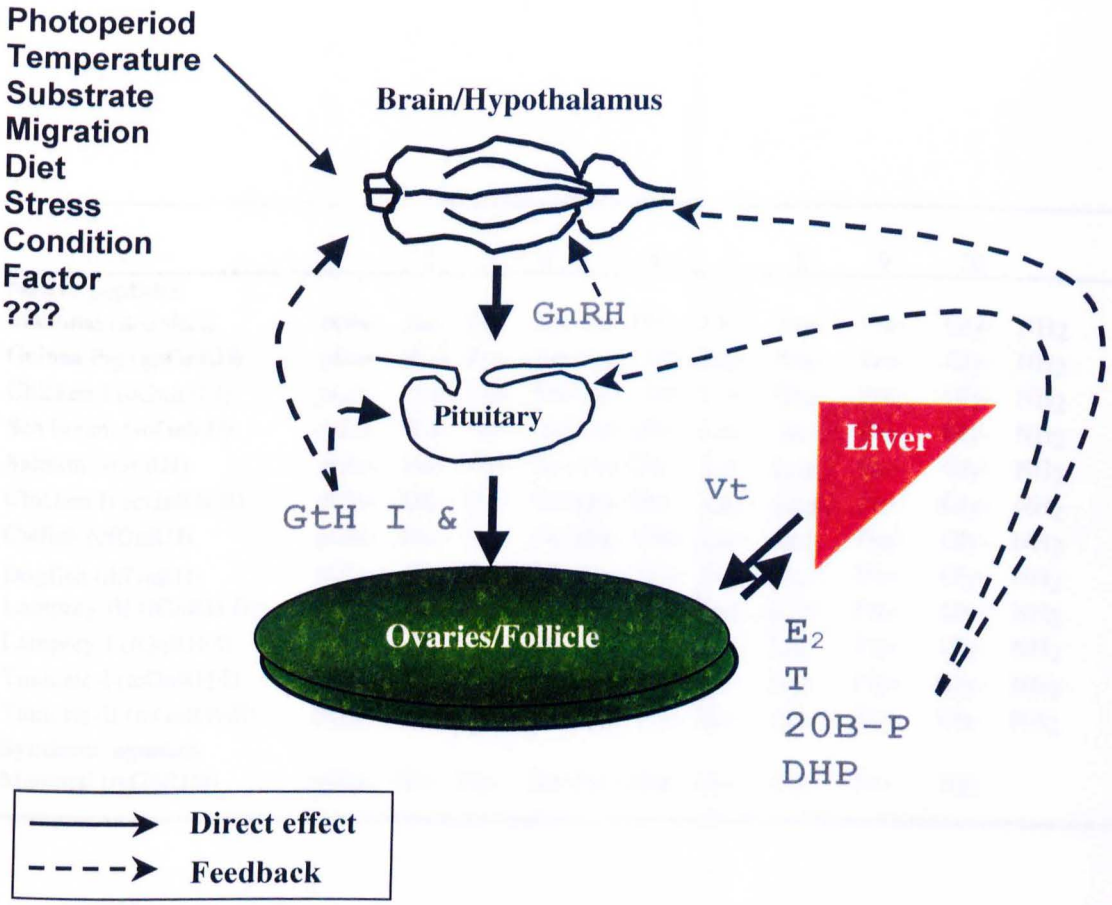


Figure 2. Schematic representation of the hypothalamic-pituitary-gonadal web in female striped bass. Gonadotropin releasing hormones (GnRHs) produced in the hypothalamus in response to a host of environmental and physiological stimuli regulate release of the gonadotropins (GtHs) from the pituitary. Gonadotropins regulate steroidogenesis in the follicular cells of the ovary. Estrogen aromatized from testosterone in the follicle cells stimulates hepatic vitellogenin (Vtg) production which fuels early oocyte growth. Late FOM is initiated by the maturation inducing steroid, either DHP or 20ß-P, which the follicle cells produce possibly in response to differential gonadotropin release from the pituitary.

	1	2	3	4	5	6	7	8	9	10	
Native peptides											
Mammal (mGnRH)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Arg-	Pro-	Gly-	NH_2
Guinea Pig (gpGnRH)	pGlu-	Tyr-	Trp-	Ser-	Tyr-	Gly-	Val-	Arg-	Pro-	Gly-	NH_2
Chicken I (cGnRH-I)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Gln-	Pro-	Gly-	NH_2
Sea bream (sbGnRH)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Ser-	Pro-	Gly-	NH_2
Salmon (sGnRH)	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Trp-	Leu-	Pro-	Gly-	NH_2
Chicken II (cGnRH II)	pGlu-	His-	Trp-	Ser-	His-	Gly-	Trp-	Gln-	Pro-	Gly-	NH_2
Catfish (cfGnRH)	pGlu-	His-	Trp-	Ser-	His-	Gly-	Leu-	Asn-	Pro-	Gly-	NH_2
Dogfish (dfGnRH)	pGlu-	His-	Trp-	Ser-	His-	Gly-	Trp-	Leu-	Pro-	Gly-	NH_2
Lamprey-III (lGnRH-III)	pGlu-	His-	Trp-	Ser-	His-	Asp-	Trp-	Lys-	Pro-	Gly-	NH_2
Lamprey-I (lGnRH-I)	pGlu-	His-	Tyr-	Ser-	Leu-	Glu-	Trp-	Lys-	Pro-	Gly-	NH_2
Tunicate-I (tcGnRH-I)	pGlu-	His-	Trp-	Ser-	Asp-	Tyr-	Phe-	Lys-	Pro-	Gly-	NH_2
Tunicate-II (tcGnRH-II)	pGlu-	His-	Trp-	Ser-	Leu-	Cys-	His-	Ala-	Pro-	Gly-	NH_2
Synthetic agonists											
Mammal (mGnRHa)	pGlu-	His-	Trp-	Ser-	Tyr-	D-Ala-	Leu-	Arg-	Pro-	NEt	

Figure 3. Amino acid sequences of the 10 forms of GnRH characterized in vertebrates and two forms from tunicates. Mammalian GnRH was the first form discovered. The three forms found in the striped bass *(Morone saxatilis)* and seabream *(Sparus aurata)* are salmon GnRH, chicken GnRH-II, and seabream GnRH. D-Ala^{6}Pro^9-NEt is the synthetic mammalian GnRH analog used in the experiments described in Chapter III.

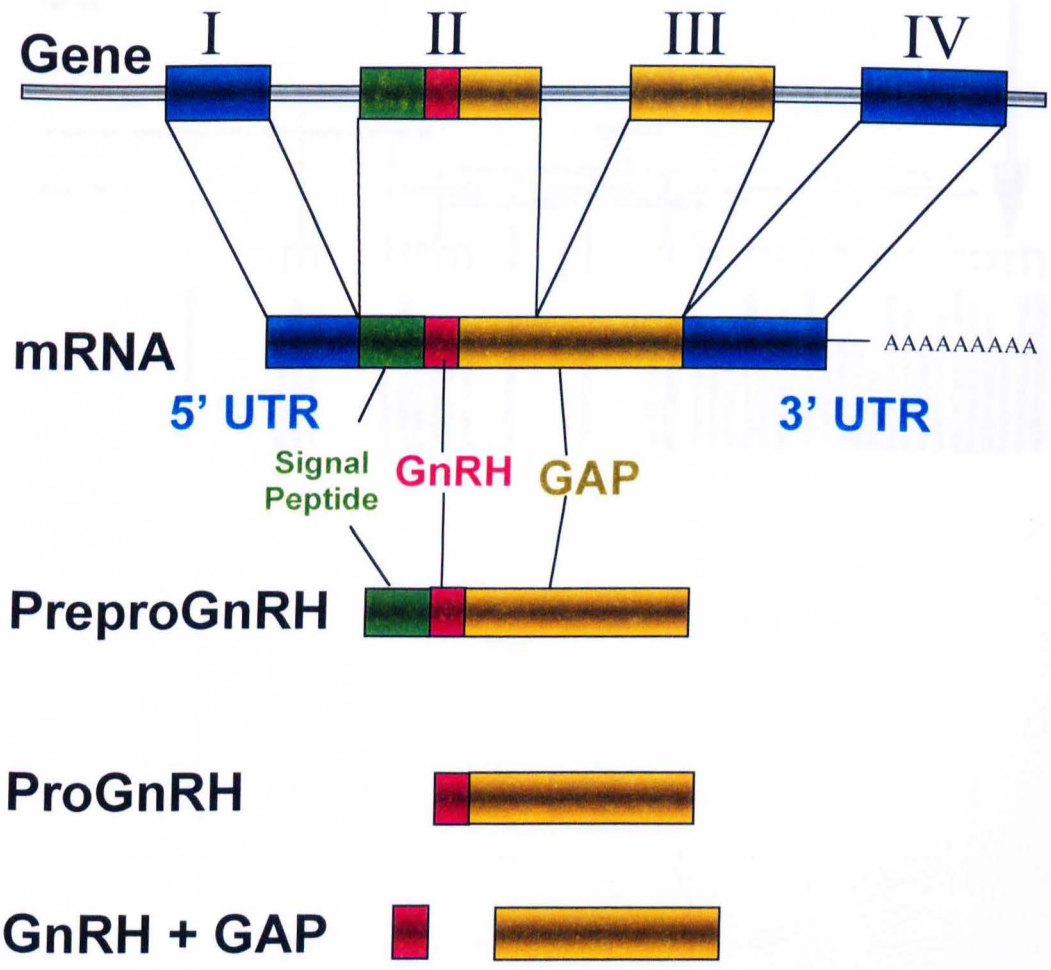


Figure 4. Gene structure and synthesis of gonadotropin-releasing hormones. The gene is comprised of four exons which encode a 5' UTR, a signal peptide, the GnRH decapeptide, the GnRH-associated peptide, and the 3' UTR. The signal peptide marks the preprohormone for packaging into secretory vesicles. Inside the secretory vesicles, the prohormone is cleaved to produce bioactive GnRH.

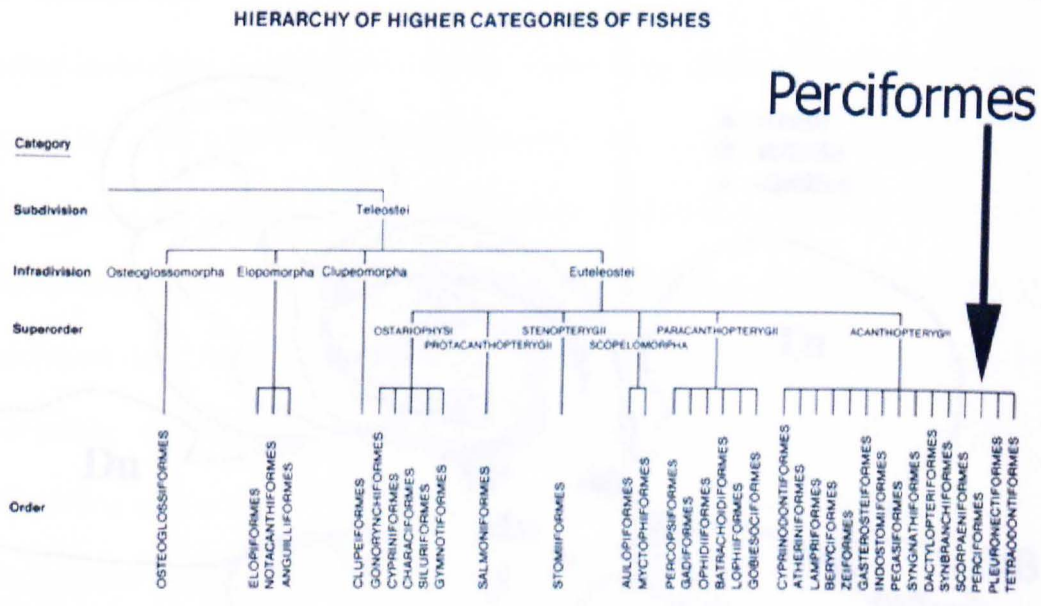


Figure 5. Proposed evolutionary tree of teleost fish. Perciform fish are third order from the right and include commercially important species such as the bass, seabream, and tilapia. From Nelson, 1984.

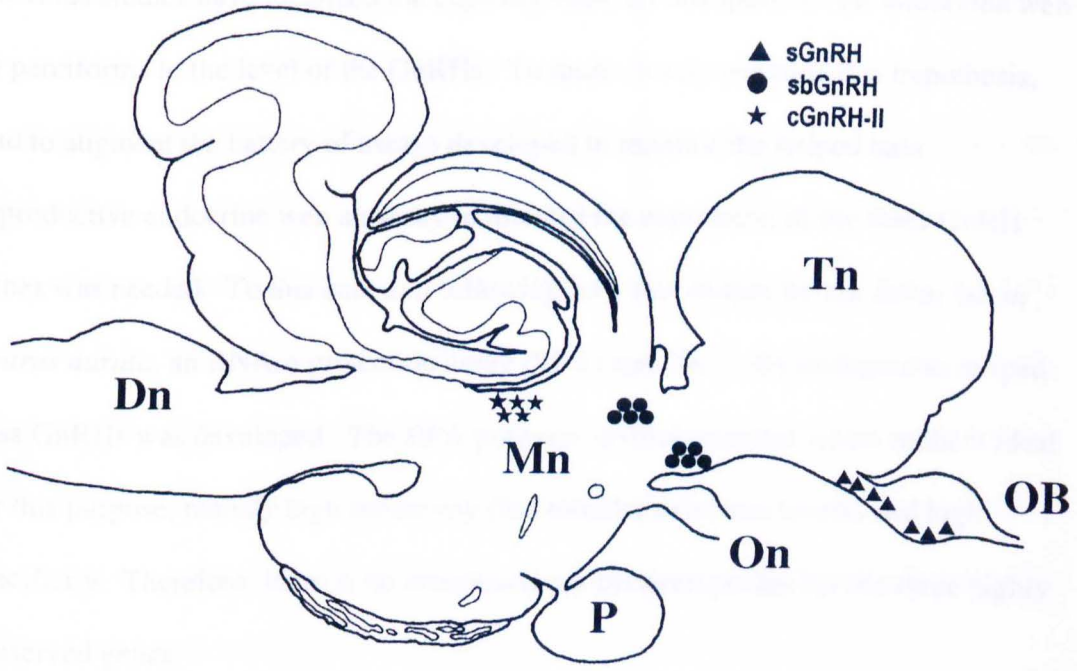


Figure 6. Distribution of the salmon GnRH, chicken GnRH-II, and seabream GnRH mRNAs in the brain of the gilthead seabream *(Sparus aurata)*. sbGnRH neurons from the pre-optic area have been shown to innervate the pituitary and thus sbGnRH is thought to be the primary hypophysiotropic form of GnRH. Dn = diencephalon, Mn = mesencephalon, Tn = telencephalon, OB = olfactory bulb, On = Olfactory nerve, P = pituitary. Adapted from Gothilf et al., 1997.

CHAPTER II: DEVELOPMENT OF THE GNRH-RNASE PROTECTION ASSAY

II.A. Introduction

Previous studies have localized the captivity-induced disruption of the endocrine web in perciforms to the level of the GnRHs. To more closely examine this hypothesis, and to augment the battery of assays developed to monitor the striped bass reproductive endocrine web an assay to monitor the expression of the three GnRH genes was needed. To this end, and following previous studies by the Zohar lab in *Sparus aurata*, an RNAse protection assay (RPA) specific to the endogenous striped bass GnRHs was developed. The RPA posesses several qualities which make it ideal for this purpose, namely high sensitivity (femtomolar detection levels) and high specificity. Therefore, there is no crossreactivity between probes for the three highly conserved genes.

II.B. Material and Methods

II.B.1. mRNA Extraction

Due to the relatively low expression of the GnRH genes in the brain, poly(A^+)-RNA, instead of total RNA, was extracted from the brains of the striped bass. This method, while longer and more tedious, gave us the ability to distinguish relatively rare target transcripts against a background of millions of other RNA molecules. The following poly(A+)-RNA procedure was adapted from the Mini RiboSep Ultra mRNA extraction kit (Collaborative Biomedical Products, Bedford, MA). Frozen brains were homogenized (Ultra-Turrax T25, IKA Labortechnik, Staufen, Germany) in a sterile

50ml conical centrifuge tube in 10ml lysis buffer (0.2M NaCl, 10mM Tris Cl, 1mM EDTA, 0.5% SDS, pH 7.5, 0.1 mg/mL proteinase K), prewarmed to 37°C to aid in solubilization of SDS, for at least 60 seconds or until no particles were visible. The homogenate is incubated at 45°C for 2 hours with light agitation (100-150 rpm; Innova Incubator-Shaker, New Brunswick, MA).

During this incubation, 0.4 mg oligo(dT)-cellulose per brain was apportioned into sterile 15ml conical centrifuge tubes. The cellulose was prepared for RNA adsorption by washing three times with 2ml of elution buffer (10mM Tris Cl, 1mM EDTA, 0.05 SDS, pH 7.5), and centrifuging at 3000 x g for 5 min between washes to pellet resin. After washing, the cellulose was equilibrated by washing twice with 1ml of binding buffer (0.5 M NaCl, 10mM Tris Cl, 1mM EDTA, 0.1% SDS, pH 7.5). Following the final wash, the cellulose remained submerged in 1ml binding buffer until the end of the two hour lysate incubation.

Prior to addition of the lysate to the cellulose, 60µl of 5M NaCl was added to the lysate to equalibrate the NaCl concentration between the two solutions. The cellulose and lysate were then mixed and incubated for 1 hr at room temperature with intermittent agitation. The cellulose was then pelleted by centrifugation (5 min at 3000 x g) and washed twice with 5ml binding buffer. After the final wash, the pellet was resuspended in 250µl of binding buffer, which was transferred to an RNAse-free microfuge column with a sterile, large-bore Pasteur pipette. The columns containing the 250µl of cellulose in binding buffer were centrifuged for 10 seconds at 5000 x g and the supernatant was discarded. The 15ml tubes were washed with 300µl binding buffer to minimize loss of cellulose. The wash buffer was centrifuged using the same

RNAse-free microfuge columns on top of the cellulose recovered from the previous centrifugation. To elute the mRNA from the column, 200µl of elution buffer was added to the column, incubated for 10 min at room temperature and then centrifuged for 10 seconds at 5000 x g. An additional 200µl of elution buffer was added to the column, incubated for 10 min and centrifuged into the microfuge containing the original 200µl of elution buffer. The mRNA is then precipitated from the elution buffer by addition of 1/10 volume 3M NaOAc (40 µl) and 2 volumes of RPA. Precipitation of the RNA is accomplished by centrifugation at 16,000 rpm for 20 min at 4°C. The supernatant was carefully removed, the microfuge tube quickly respun, and the last traces of ethanol were removed using a P20 pipetman with a sequencing gel-loading tip. The mRNA was air dried for 10 min and resuspended in 21µl of. One microliter was diluted in 99µL of RNAse-free dH_2O and used to determine the amount of RNA extracted via UV spectrophotometry. The remaining 20µl of extracted mRNA were used in the RPA.

II.B.2. PCR Amplification and Cloning of GnRH Fragments

Templates for the specific probes were cloned from the GnRH fragments PCR-amplified from a pool of striped bass brain cDNA. The PCR products were amplified using degenerate primers designed from known sequences of both striped bass and seabream GnRHs (Figure 7). The amplified fragments were blunt-end ligated into pBluescript linearized with SmaI (sGnRH, sbGnRH) or EcoRV (cGnRH-II) resulting in the plasmids depicted in Figure 8. The resulting recombinant plasmids were transformed into competent *E. coli* cells, strain JM109, using the traditional heatshock

method of bacterial transformation {Maniatis, 1989 #210}. One hundred microliters of competent cells were added to 1ng of recombinant plasmid in a sterile 1.5mL microfuge tube and incubated for 30 min on ice. The cells were then incubated for 2 min at 42°C and an additional 2 min on ice. The entire contents of the microfuge tube were transferred to a 15ml round bottom tube containing 1mL of LB media prewarmed to 37°C and incubated for 1 hr at 37°C with agitation. Following this incubation 50, 100, and 150µL of cells were plated onto LB agar plates containing 50 µg/mL ampicillin, 50 µl X-gal (20mg/mL), and 10 µl 0.5M IPTG and grown overnight at 37°C. Transformants were screened for ampicillin resistance, which is inherent on the pBluescript plasmid, and disruption of the lacZ gene by the GnRH insert. Colonies that proved both ampicillin resistant and white in coloration (ie. lac Z deficient) were grown in 5mL LB (plus 50µg/mL ampicillin) cultures for plasmid analysis. Deoxyribonucleic acid from these cultures was isolated, electrophoresed, and transferred to nylon membranes for analysis by Northern blot hybridization, using 5'-labeled oligonucleotide probes specific for the GnRH decapeptide. Inserts from clones that were positive for all three requirements (ampicillin resistant, lac Z deficient, and positive Northern blot) were sequenced and oriented on a 373A DNA Sequencer (Applied Biosystems, Foster City, CA). Positive clones for each of the GnRH forms were selected and grown in liter batches, from which plasmid stocks were purified and resuspended in RNAse-free dH_2O. Aliquots were taken from these stocks for preparation of sense- and antisense-RNA templates (Figure 9).

II.B.3. Sense RNA Standards

Sense RNA was synthesized from the recombinant plasmids to serve as standards in the RPA. Successful RNA transcription from these templates requires extensive purification of the plasmids with particular attention to contaminating RNAses. Depending upon the previously determined orientation of the GnRH insert within the multiple cloning site, the plasmids were linearized by enzymatic digestion. One hundred micrograms of recombinant plasmid were digested for at least 6 hr at 37°C in a total volume of 50μL with 10-12 units of the appropriate restriction enzyme (sGnRH and sbGnRH = BamHI; cGnRH-II = Hind III). Digestion was followed by a 30 minute incubation with 100-200μg/mL proteinase K at 50°C to eliminate any remaining RNAses. After proteinase K incubation, the plasmid was purified by phenol/$ClCH_3$ extraction followed by a second extraction using only $ClCH_3$:isoamylalcohol (IAA, 24:1). The DNA was then ethanol-precipitated and resuspended in 21μL of RNAse-free dH20. One microliter of the template was diluted in 99μL of dH20 and used to determine the OD of the sample via UV spectrophotometry. The linearized plasmid stock was then diluted to a concentration of 500ng/μL.

Using these linearized plasmids as templates, sense RNA was synthesized using the MEGAscript RNA transcription kit (Ambion, Austin, Texas). To synthesize sense RNA, 1μg (2μL) of template was incubated with 75mM of each of the nucleotide triphosphates, transcription buffer (Ambion), and 2μL of the RNA polymerase mix provided with the kit (sGnRH and sbGnRH = T7; cGnRH-II = T3) at 37°C for 6 hr in a total volume of 20μL. After the incubation, the template was removed by a

subsequent incubation with 2 units of RNAse-free DNAse at 37°C for 15 min. The reaction was terminated by addition of 115µL RNAse-free dH2O and 15µL 5M ammonium acetate. The RNA was recovered using a series of extractions (ie. one volume (150µL) phenol/$ClCH_3$ followed by $ClCH_3$/IAA and finally one volume of isopropyl alcohol) and then washing the pellet with 70% ethanol. The precipitated RNA was diluted to a stock concentration of 500ng/µL. Dilutions (0.01 to 1 fmol) were made for use as standards in the RPA.

II.B.4. Antisense RNA Probes

High specific-activity radiolabeled antisense RNA probes were synthesized for detection of GnRH mRNA in the RNAse protection assay. The antisense probes were synthesized using the same GnRH/pBluescript templates used to produce the sense RNA standards, only the alternate pBluescript promoter was used for RNA transcription. This required digestion of separate aliquots of the original template using the appropriate restriction enzymes, (sGnRH & sbGnRH = Eco RI, cGnRH-II = BamHI). Using these linearized templates and the MAXIscript RNA transcription kit (Ambion) we radiolabeled high specific-activity RNA transcripts with γ-labeled P^{32} UTP as follows.

One microgram of template was incubated for one hour at 37°C with 0.5nmoles of each of the three non-limiting nucleotide triphosphates (A,C, and G), 7.0pmole γ-labeled P^{32} UTP (800mCi/mmol), 72.5pmole γ-labeled P^{32} UTP (3000mCi/mmol), transcription buffer (Ambion), and 10 units of the appropriate RNA polymerase mix (Ambion, sGnRH & sbGnRH=T3, cGnRH-II=T7) in a total volume of 20µL. After

one hour of transcription the plasmid templates were removed by addition of 1μL of RNAse-free DNAse 1 and fifteen minutes of incubation at 37°C. Following the DNAse digestion 51μL of RNAse-free dH2O were added to the reactions, from which a one microliter aliquot was removed to a scintillation vial and served as the total counts for the subsequent calculation of nucleotide incorporation rates. The remaining 70μL of the reactions were loaded onto RNAse-free ChromaSpin-400 spincolumns and centrifuged for 5 minutes at 3000 x G. This purification removed both digested plasmid fragments as well as unincorporated nucleotides. A one microliter aliquot from the recovered products was placed into a separate scintillation vial and counted. This sample represented the amount of nucleotides incorporated into the transcription product. From the scintillation counter report concentrations of probes and specific activities were calculated. To ensure that the probes were synthesized cleanly and completely, aliquots of each of the probes were separated on a small 6% polyacrylamide/8M urea electrophoresis gel for approximately 45 min at 30mAmp (Figure 10). In the RPA, probes were used in approximately three-fold excess of the maximum value in the standard curve, which in this RPA is 1fmol. The GnRH riboprobes averaged 4-6x10^7 CPM/μg in specific activity.

II.B.5. RNAse Protection Assay

The RNAse protection assay presents a convenient and relatively simple method of quantifying the abundance of the three forms of GnRH in poly(A^+)-RNA samples from the tissues of striped bass. The manufacturer's RPA protocol from the Ambion RPA-II Kit (summarized below) was used in the following experiments.

The poly(A+)-RNA or sense RNA standards (brought to a standard volume of 100μL with 1:100 diluted yeast RNA:RNAse-free dH_2O) were coprecipitated with excess (≥ 3fmol) radiolabeled antisense RNA probes. Coprecipitation was achieved by addition of $1/10^{th}$ volume (10μL) 5M ammonium acetate and 2.5 volume (250μL) 100% ethanol, centrifugation (15 min at 14,000 rpm at 4°C), incubation on dry ice for 15 min, and centrifugation for an additional 15 min. After careful and thorough aspiration of the supernatant, the precipitated RNAs were resuspended in 20μL of hybridization buffer (80% deionized formamide, 100mM sodium citrate pH 6.4, 300mM sodium acetate pH 6.4, 1mM EDTA) and denatured in a 95°C heatblock for 3-5 min. The tubes were then transferred in the heat blocks to a 45°C cabinet incubator overnight to permit hybridization of the RNAs. Transferring the tubes while still inside the heat blocks permits the tubes to cool gradually from 95°C to 45°C and therefore aids in hybridization. Following the overnight incubation, 200μL of a 1:100 dilution of the RNAse A/RNAse T1 mixture provided with the RPA kit was added to all of the samples, excluding the non-digested control tube which received 200μL of the RNAse digestion buffer. After a 30 min incubation at 37°C, the digestions were stopped and the RNA was precipitated by addition of 300μL of the RNAse inactivation/precipitation mixture provided with the RPA-II kit. The samples were then incubated on dry ice for 15 min, centrifuged for an additional 15 minutes at 14,000rpm at 4°C and the supernatant was carefully aspirated. After air drying for approximately 10 min, the pellets were resuspended in 10μL of gel loading buffer, vortexed, immediately centrifuged, denatured at 95°C for 5 min and cooled on ice until they were loaded onto a 6% polyacrylamide/8M urea sequencing gel (38 X 50 X

0.04cm). The gel was electrophoresed for approximately 2-3 hr at 80W (800-1200V), after which it was mounted on 3MM paper (Whatman, Clifton, NJ), covered in plastic wrap, dried for 30 min at 80°C, and exposed to a phosphoscreen overnight (Kodak, Rochester, NY). Following exposure, the phosphoscreen was digitally scanned on a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA) and the resulting image analyzed (Figure 11) using ImageQuant software (Molecular Dynamics). RNA concentrations were determined by comparison of the density of the sample bands to the density of the bands of known concentration in the sense RNA standard curve run with every assay. GnRH levels were normalized to the amount of poly(A+)-RNA extracted from each brain. The rationale for using this method of standardization will be discussed in more detail in section II.C.

II.B.6. Optimization of RPA Conditions

Due to the similarity between this assay and the RPA previously developed by Dr. Yoav Gothilf (Gothilf, Meiri et al. 1997) for the same three forms of GnRH expressed in the gilthead seabream *(Sparus aurata),* no further trials were deemed necessary to optimize the conditions responsible for good sample resolution. These conditions (i.e., probe specific activity, RNAse dilutions, sample RNA concentration, and hybridization time) are discussed in detail in the RPA-II instruction manual (Ambion).

II.C. Discussion

At the time this study was initiated, the RPA was the among the most sensitive and streamlined methods available to accurately quantify the relatively low expression of multiple genes in a single sample. Recent advances have led to the development of new methods for RT-PCR quantitation of multiple mRNA species in individual samples. In addition to a more streamlined protocol, RT-PCR quantitation circumvents some of the limitations inherent to the RPA.

One of the main problems of using the RPA to detect GnRHs is assay sensitivity, which is to the femtomolar level. While this is sufficient for many purposes, it necessitates the usage of the entire sample of extracted mRNA for each assay. This means that a) the assay can be run only once, and b) expression of the three GnRHs within the different areas of the brain cannot be differentiated. The ability to make repeated measures of the same samples would not only increase the statistical power of the study, but would also encourage more comprehensive experiments which provide more data from individual samples, possibly using several different approaches (e.g., molecular endocrine, qualitative, quantitative). The ability to microdissect the brain would be especially helpful in the case of GnRHs. Microdissection would allow investigation of the hypothesized differences in expression pattern and therefore function between the GnRHs. The ability to examine GnRH mRNA fluctuations in the preoptic area of the brain, versus the olfactory bulb, will provide us greater insight into the regulation and function of the three forms of GnRH. By requiring only a small aliquot of the total mRNA sample, and having

much greater sensitivity, RT-PCR offers solutions to the major shortcomings of the RPA.

Another problem encountered when quantitating small amounts of RNA using any method, including the RPA is the wide range of methods used to standardize raw data for variances between samples. Several authors have used ß-actin levels to standardize for these differences (Gothilf, Meiri et al. 1997, Hassin, 1998 #29). ß-actin is a "housekeeping gene", supposedly free from seasonal or temporal variations. Because of this presumed stability, ß-actin levels were thought to correlate linearly with brain size, therefore serving as an appropriate normalization method for differences in brain size. However, in our experiments and in the work of others, significant fluctuations in ß-actin levels were observed between experimental groups. This observation led us to normalize the data for total mRNA extracted from the brain, which was shown to increase linearly in proportion to the weight of the brain (Figure 12).

Salmon GnRH

```
  1  AGAGCTAGAG GCAACCATCA GGATGATGGG TACAGGAGGA GTGGTGTCTC
 51  TTCCTGAAGA GGCGAGTGCC CAAACCCAAG AGAGACTTAG ACCATACAAT
101  GTAATTAATG ATGACTCCAG TCATTTTGAC CGAAAGAAAA GGTTCCCTAA
151  TAATTGAAGA GCTACAAAAA ATGAAGAAAA AACACACTGT ATTTGCATCA
201  TCATCAACAC CAGTGATGGA TCGGCCGGGT GCAGGATACT GACTT
```

Chicken GnRH-II

```
  1  TCTGGTTTTA CTGTTTGGGC TGCTTCTATG TGTGGGGGCT CAGCTGTCCA
 51  ACGCCCAGCA CTGGTCCCAT GGTTGGTACC CCGGAGGCAA GAGGGAACTG
101  GACTCTTTTG GCACGTCAGA GATTTCGGAG GAGATTAAGC TGTGTGAGGC
151  AGGAGAATGC AGCTACTTGA GACCTCAGAG GAGGAATGTT CTGAGAAATA
201  TCATTCTGGA TGCCTTAGCC AGAGAGCTCC AGAAGAGGAA GTGACAGCTC
251  TCCACCCTAT ACTGCTTTTC TACCCTCTTC TTCTGTATTC TTA
```

Seabream GnRH

```
  1  GCTGTCAGCA CTGGTCCTAT GGGCTGAGTC CAGGAGGGAA GAGGGAACTG
 51  GACGGCCTCT CAGAGACACT GGGCAATCAG ATAGTCGGGG GCTTCCCACA
101  CGTGGAGACG CCCTGCAGAG TTTTAGGCTG TGCAGTGGAA TCACCTTTCC
151  CCAAAATATA CAGAATGAAA GGATTCCTTG ACGCAGTCAC CGACAGAGAG
201  AACGGAC
```

Figure 7. Striped bass GnRH fragments cloned for use as templates in the RNAse protection assay. Degenerate primers used in polymerase chain reaction amplification are underlined and were designed using known sequences from both striped bass and seabream.

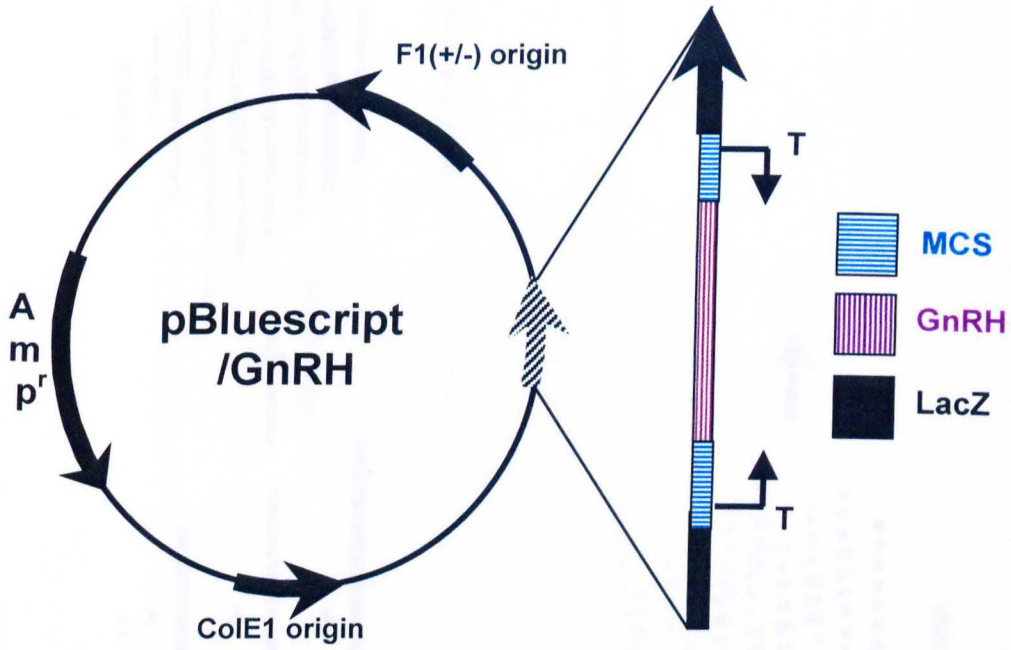


Figure 8. The cloning strategy for introducing GnRH fragments into pBluescript (Stratagene) allowed for transcription of both sense and antisense RNA for use as standards and probes, respectively, in the RNAse protection assay. Recombinant plasmids were transformed into *E. coli*, strain JM109, and selected for both ampicillin resistance and the inability to cleave X-gal, due to the disruption of the lacZ gene by the GnRH insert in the multiple cloning site (MCS) located within the lacZ reporter gene.

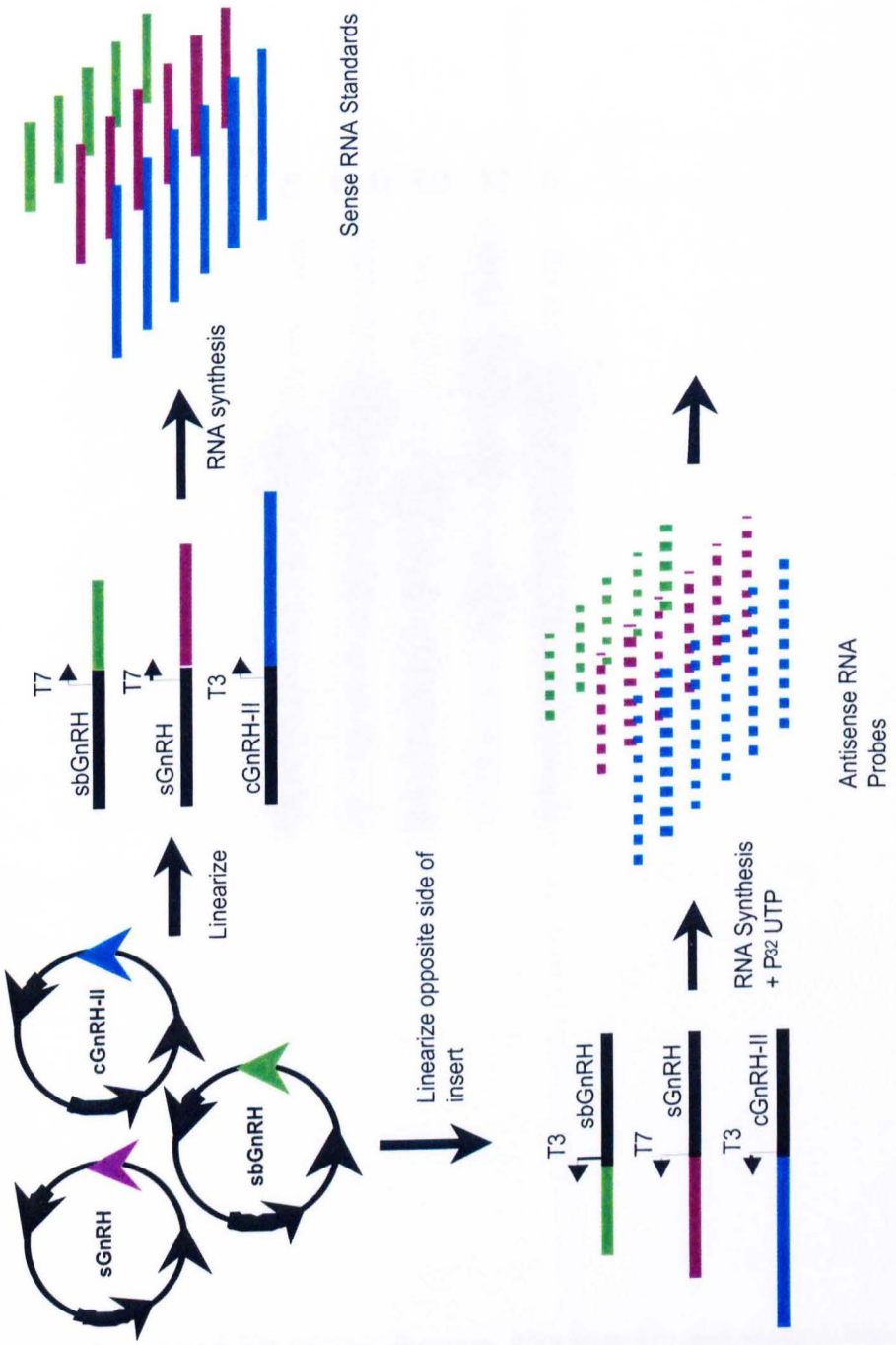
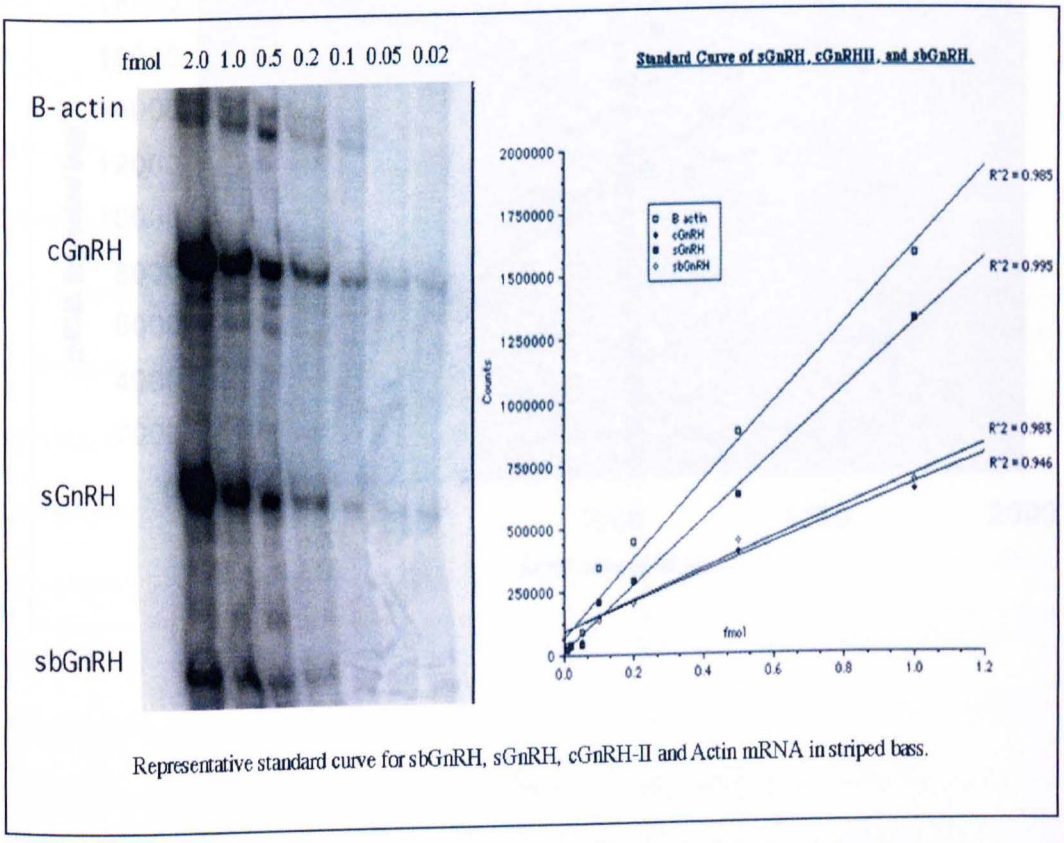


Figure 9. Production of sense and antisense RNA from GnRH fragments cloned into pBluescript. Linearizing on either side of the insert and promoting transcription with appropriate polymerases results in either high specific-activity antisense probes or non-labeled sense RNA for standards.

Figure 10. Autoradiogram of GnRH antisense riboprobes. RNA transcripts were produced from striped bass GnRH fragments PCR-amplified and cloned into pBluescript using methods described in the text. Each probe displays distinct length and sequence. (S=salmon GnRH, C-II=chicken GnRH-II, SB=sbGnRH, M=Marker, ß=ßactin)



Representative standard curve for sbGnRH, sGnRH, cGnRH-II and Actin mRNA in striped bass.

Figure 11. Representative standard curve from validated RPA for measuring the three forms of GnRH mRNA in striped bass. A digital image of a scanned gel is shown on the left. The linearity of the resulting standard curves on the right enables accurate quantitation of the mRNA levels.

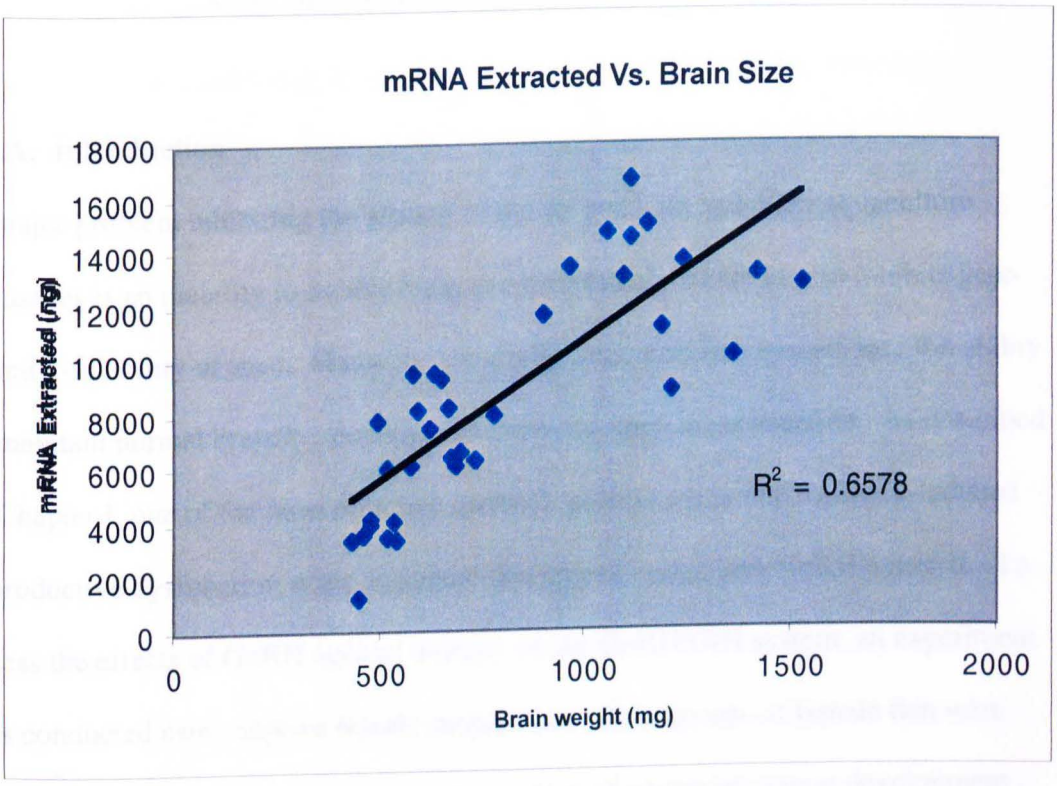


Figure 12. Messenger RNA versus brain weight. The amount of poly(A+)-RNA extracted from a brain was shown to correlate positively with the weight of the brain and therefore was used as the means of raw data normalization.

CHAPTER III. EFFECT OF GNRH AGONIST THERAPY ON THE GNRH/GTH AXIS OF CAPTIVE FEMALE STRIPED BASS

III.A. Introduction

A major problem inhibiting the growth of the striped bass and other aquaculture industries is an inability to establish captive broodstock, which in turn inhibits year-round availability of seed. Many commercially important fish species lose the ability to maintain normal breeding patterns and behavior upon domestication. As described in Chapter I, one of the most effective methods used to overcome captivity-induced reproductive dysfunction is the sustained delivery of exogenous GnRH agonists. To assess the effects of GnRH agonist therapy on the GnRH/GtH system, an experiment was conducted using captive female striped bass. Four groups of female fish were sampled, each group representing a distinct stage of advanced oocyte development and, therefore, reproductive maturity (advanced vitellogenesis, early FOM, GnRHa-induced late FOM, and atretic). By comparing and correlating the ovarian developmental stage with the GnRH mRNA levels in the brain, GnRH peptide levels in the pituitary, GtH-I and IIß subunit and GnRH-receptor (GnRH-R) mRNA levels in the pituitary, and GtH-II peptide levels in the pituitary and plasma, we attempted to discern what effect the GnRHa treatment has upon the native GnRH/GtH system during its "repair" of the reproductive dysfunction caused by captivity.

III.B. Materials and Methods

III.B.1. Experimental Design and Animal Sampling

Reproductively mature striped bass (*Morone saxatilis*, Moronidae, Teleosti) broodstock were obtained from COMB's Aquaculture Research Center (Baltimore, MD) where they had been maintained for several years in 12-foot diameter (3,500 gallons) recirculating tanks under controlled temperature and photoperiod regimes simulating their natural environment. Animals were fed a commercial trout diet (Zeigler, Gardners, PA) at the rate of 1% body weight/day (average BW = 6.5±0.25kg). All animals were maintained and treated according to protocols approved by the Center of Marine Biotechnology's Institutional Animal Care and Use Committee.

On April 22, 1999, four groups of five female fish were isolated from the captive population based on microscopic examination of the extent of ovarian development. Ovarian development was categorized using standard developmental references illustrated in by Rees and Harrell (Rees and Harrell 1990). The first group of fish displayed a majority of oocytes in the advanced stages of vitellogenesis, but which had not yet initiated final oocyte maturation (700-900μm-oocyte diameter, little to no lipid droplet coalescence, Figure 14: Group 1).

The second group of females had oocytes which were further advanced, and represented the final developmental stage reached by most captive females before the eggs undergo atresia (900-1000μm oocyte diameter; slight lipid coalescence, Figure 14: Group 2).

The third group of five females were implanted intramuscularly with 50μg/kg D•Ala^{6} Pro^{9}-NET mGnRHa when the oocytes had reached the same developmental stage as the second group of fish (900-1000μm oocyte diameter; slight lipid coalescence, Figure 14: Group 2). Upon implantation individual females were maintained separately in a six-foot diameter tank (650 gallons) with two spermiating male striped bass (implanted with approximately 20μg/kg BW of mGnRHa) per female. The water temperature was raised gradually from 15±1°C to 21±1°C over two days. These conditions were based on standard protocols used for the hormonal induction and completion of final oocyte maturation, ovulation, and spawning in captive striped bass (Smith and Whitehurst 1990). The fish were allowed to proceed with ovarian development until an estimated six to eight hours prior to ovulation as determined by ovarian biopsy (≥1000μm oocyte diameter, complete lipid coalescence, significant lipid droplet clearing, Figure 14: Group 3) and at which point the fish were sacrificed. Four of the fish reached the requisite stage and were sacrificed within 48 hours of implantation. The final fish was given a second implantation of 150μg GnRHa (≈70 μg/kg BW = total dosage) when oocyte development "stalled". The second implantation prompted resumption of oocyte development. This fish advanced to the appropriate developmental stage and was sacrificed within 72 hours of initial implantation.

The fourth group of captive female broodstock fish had reached a similar stage as the second and third groups (900-1000μm oocyte diameter; slight lipid coalescence, Figure 14: Group 2) but was allowed to proceed with "natural" oocyte development without exogenous hormonal manipulation. As with the GnRHa implanted fish, these

fish were also housed in a 6-foot tank with spermiating males at a ratio of two males to one female. The water temperature was raised from 15±1to 21±1°C over a two day period. Oocyte development was monitored via ovarian biopsy until the oocytes had become atretic (Figure 14: Group 4), at which point they were sacrificed. All five fish became atretic within two weeks of water temperature elevation, with no ovulation or spawning observed. An outline of the experimental procedure and timeline is presented in Figure 13.

The protocol for sacrificing the animals was the same for all groups. Animals were anesthetized in 0.25ppt phenoxyethanol and ovarian biopsies were drawn via catheterization. If the animal was determined to have reached an appropriate developmental stage, 10mL of blood was drawn from the caudal vasculature with a heparinized syringe. Blood samples were stored on ice with 100μL of a 3mg/mL solution of aprotinin (Sigma Chemical Co., St. Louis, MO), to prevent hormone degradation, until the plasma could be separated in the lab. Plasma was separated by centrifugation for 15 min at 4,000 x g at 4°C. From the supernatant 200μL aliquots were stored in 0.75mL centrifuge tubes at -80°C until assayed for GtH-II content.

After blood was drawn, the fish were promptly decapitated. Decapitation has been determined to be the most expedient and painless method of sacrificing the fish while keeping the brain and pituitary intact. Brains and pituitaries were rapidly dissected and flash frozen separately in liquid N_2. Ovaries and carcasses were weighed for gonadosomatic index (GSI = gonad weight/BW x 100) determination. In addition to ovary, several other major tissues (gill, heart, liver, kidney, and muscle) were sampled

and stored at -80°C for possible use as control tissues, or for use in future experiments. Ovarian tissues were removed and prepared for histology as described below.

III.B.2. Histology

During the experiment, oocyte development was monitored by light-microscopic inspection of fresh ovarian biopsies. While this method is appropriate for estimating eligibility for GnRHa treatment eligibility, a more precise and detailed analysis of oocyte development was necessary to ensure correct grouping of fish. To this end, samples of oocytes were obtained directly from the dissected ovaries, fixed in 20mL glass jars with 10mL of McDowell's solution (4% formamide, 1% glutaraldahyde) and stored at 4°C for histological analysis. Histology was performed using the JB-4Plus glycol methacrylate kit (Polysciences, Inc., Warrington, PA). The complete protocol is as follows:

McDowell's fixative was replaced every 24 hours until the tissues were completely fixed (usually ≤48 hours), at which time the samples were dehydrated in a series of 24 hour immersions in increasing concentrations of ethanol (70%, 70%, 80%, 90%, and 95%). Following the final dehydration, samples were infiltrated with and embedded in methylacrylate resin. The methacrylate blocks were cut in five-micron sections on a retracting microtome (HM430, Microm, Walldorf, Germany) with a stainless steel, D-profile knife. Tissue sections were placed in a 37°C-water bath to flatten and orient the sections, and mounted on clean microscope slides and dried on a hotplate at 40°C. The sections were stained for 120 seconds in 0.5X Polychrome I (Methylene blue/Azure II), rinsed for 15 min in dH_2O, stained for 60 seconds in 1X Polychrome II

(Basic Fuchsin), rinsed for 15 min in dH_2O, and dried at 40°C on a hotplate. After the slides were completely dry, coverslips were mounted and the sections examined microscopically to more precisely evaluate oocyte development. Stages were categorized by the most advanced oocytes present in the sample (Figure 14). Because striped bass are group synchronous spawners, the majority of recruited oocytes advance together and little asynchrony was observed.

III.B.3. mRNA and Hormonal Assays

The following assays were performed to examine the endocrine profiles of the fish at the four stages of advanced oocyte development sampled; 1) the GnRH RPA described in the previous chapter, 2) specific ELISAs to measure GnRH content of the pituitary (Holland, Gothilf et al. 1998), 3) quantitative RT-PCR for the GtH-Iß, -IIß subunits and GnRH-receptor mRNA expression in the pituitary (Alok and Zohar, unpublished communication), 4) an ELISA to measure GtH-II peptide in the pituitary (Mananos, Swanson et al. 1997), and finally 5) a radioimmunoassay to measure plasma GtH-II titer (Mylonas, Magnus et al. 1997). All of these assays were developed by the Zohar lab and validated for use with striped bass. The cited references can be obtained for further information on technical aspects of the assays, excluding the RT-PCR analysis of the gonadotropin ß-subunits and GnRH-receptor, which is in preparation. Briefly, an ABI PRISM 7700 sequence detection system (PE-Applied Biosystems, CA) was used to quantitate the relative levels of GtH-Iß, GtH-IIß, GnRH-R, and 18S ribosomal subunit gene expression in individual pituitaries. Gene specific primers were designed for the aforementioned striped bass genes using

Primer Express™ software (PE-Applied Biosystems) and used in a PCR reaction which incorporates a fluorescent dye (SYBR Green) into the dsDNA products, which are directly detected by the Taqman machine. The template for the reaction was reverse transcribed RNA, isolated from the pituitaries of experimental fish as described by Hassin (Hassin, Gothilf et al. 1998). The 18S ribosomal subunit mRNA levels were used to normalize for differences in pituitary size. Threshold cycle (Ct) values (amplification cycle at which threshold value for luminosity given off by PCR product is reached) were reported and converted to relative fold increase ($2^{-\Delta\Delta Ct}$) using Microsoft Excel which is the unit in which results are reported in this text.

III.B.4. Statistical Analysis

Statistical analysis of all the data (GSI, total body weights, GnRH mRNA, GnRH peptide, GtH ß-subunit and GnRH-R mRNA, pituitary GtH-II, plasma GtH-II) was performed on untransformed data. Significant differences between groups were detected using a one way analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test (DNMR; SuperANOVA statistical software, Abacus Concepts, CA.). Minimum significance was set to 0.05 in all cases. Results are reported as means plus or minus one standard error of the mean (SEM).

III.C. Results

III.C.1. GSI and Oocyte Development

Gonadosomatic indices or the ratio of the gonad weight to total BW was examined. The results are illustrated in Figure 15 and can be summarized as follows. GSI

showed an increasing trend as the fish matured (Figure 15B), from advanced vitellogenesis (12.210±0.755%) through early FOM (13.250±0.831%) and GnRHa-induced late FOM (17.76±2.721%). A significant decrease in GSI was observed in fish, which were allowed to go atretic (8.017±2.348%).

Measurements of average oocyte diameters were taken at the time of sacrifice using no fewer than 10 oocytes per fish. A significant increase was observed as the fish progressed from advanced vitellogenesis (900±20μm) into early FOM (1050±25μm). It is worth noting that no significant increases in oocyte diameter were observed during the latest stages of FOM in GnRHa treated fish (Figure 15A). Oocyte diameters were not measured in atretic fish because of the extensive deterioration and nonuniformity or lack of structure seen in an overwhelming majority of atretic oocytes.

III.C.2. Plasma GtH-II Peptide Levels

A specific radioimmunoassay was performed in order to examine circulating GtH-II peptide levels in the plasma of captive striped bass undergoing various stages of late oocyte development and final oocyte maturation (Figure 16A). Plasma GtH-II levels were relatively low and not significantly different in all three captive, untreated groups, including atretic fish. These values ranged from 1.94±0.208ng/mL to 2.57±0.516ng/mL. GnRHa implanted fish displayed a near twenty-fold increase in plasma GtH-II (41.33±8.354ng/mL).

III.C.3. Pituitary GtH-II Peptide Levels

GtH-II peptide levels in the pituitary were assayed via a specific ELISA (Figure 16B). GtH-II levels were highest in advanced vitellogenic fish (310±50µg/pituitary), dropped slightly but not significantly as the fish entered final oocyte maturation (210±30µg/pituitary), and remained at that level as the fish were allowed to undergo atresia (155±46µg/pituitary). GnRHa treatment significantly decreased pituitary GtH-II content, which dropped to 16±5µg/pituitary.

Total pituitary protein content was determined using a BSA-protein assay (Figure 17A), however assay results were not used to standardize pituitary GtH-II and GnRH levels in these fish. While significant differences were observed in the GnRHa treated fish, pituitary protein normalization was not deemed appropriate because the decrease was thought to be a side effect of the GnRHa treatment and, thus, was not useful for normalizing for differences in BW. In light of the fact that all fish were from the same year class and only slight differences were seen in body weight at the beginning of the study (Figure 17B), we assumed that the pituitaries were of similar size between the groups.

III.C.4. Pituitary GtH-Iß, -IIß and GnRH-R mRNA

Quantitative RT-PCR was run on total RNA extracted from the pituitaries of the four groups of captive female striped bass to determine the relative expression of the two gonadotropin-ß subunits and the GnRH receptor. Results are expressed as the fold increases over the lowest group (Figure 18). Intersample variation in total amounts of RNA reverse transcribed was normalized for by quantifying 18S ribosomal subunit

mRNA along with the three mRNAs of interest. The 18S ribosomal subunit is considered to be a housekeeping gene and therefore should correlate positively with the size of the pituitary, thus making it a useful correction factor.

In the case of GtH-Iß mRNA, the lowest expression was seen in GnRHa treated fish, therefore the other three groups are compared to this group when calculating fold increases in gene expression. GtH-Iß was most strongly expressed in the pituitaries of the advanced vitellogenic fish, and fish in early FOM (Figure 18A). Pituitaries from advanced vitellogenic fish contained nearly 23 times as much GtH-Iß subunit mRNA than the GnRHa treated fish and pituitaries from early FOM fish contained nearly 19 times as much GtH-Iß subunit mRNA as the GnRHa treated fish. Pituitaries from atretic fish also contained similarly low levels of GtH-Iß subunit.

The lowest expression of GtH-IIß subunit was seen in atretic fish, therefore the other three groups are compared to this group when calculating fold increases in gene expression. Advanced vitellogenic fish exhibited the strongest expression of GtH-IIß subunit mRNA, nearly four-fold higher compared to the atretic group. GtH-IIß subunit expression dropped significantly as the fish entered FOM, and did not significantly deviate from this level throughout late FOM and atresia (Figure 18B). GnRHa apparently had no effect on GtH-IIß subunit expression.

GnRH-receptor mRNA was found to be lowest in atretic fish, therefore the other three groups are compared to this group when calculating fold increases in gene expression. GnRH-R expression was high at the end of vitellogenesis and maintained this high level throughout FOM. Expression levels dropped only in the atretic fish.

Fold increases over the atretic group ranged from four to nearly five and a half. (Figure 18C)

III.C.5. Pituitary Content of the Three GnRH Peptides

GnRH peptide content of the pituitaries was examined using ELISAs specific for the 3 forms of GnRH in striped bass (Figure 19). Comparing levels of all three forms to each other, we observed that seabream GnRH was 10-20 times more abundant in the pituitary than chicken GnRH-II and nearly 100 times more abundant than salmon GnRH. This observation is in agreement with previous findings in striped bass and seabream (Holland, Gothilf et al. 1998) and lends support to the importance of sbGnRH in the regulation of final oocyte maturation.

Examining sbGnRH levels between the groups, we see that sbGnRH is maintained at high levels from advanced vitellogenesis (16±1.1ng/pituitary) through early FOM (18.1±2.6ng/pituitary) and into atresia (15.6±1.5ng/pituitary). GnRHa treatment affects a significant decrease in sbGnRH levels in the pituitary of captive striped bass (7.3±1.6ng/pituitary, Figure 19A).

Neither salmon GnRH nor chicken GnRH-II pituitary peptide levels fluctuated significantly over any of the developmental stages sampled. Salmon GnRH values ranged from 0.125±0.030ng/pituitary to 0.236±0.027ng/pituitary. Chicken GnRH-II values ranged from 0.519±0.132ng/pituitary to 0.748±0.133ng/pituitary.

III.C.6. Brain mRNA Levels of the Three GnRHs

Messenger RNA levels of all three forms of GnRH were measured using the RNAse protection assay described in Chapter II (Figure 20). Expression of all three GnRH genes decreased significantly as the advanced vitellogenic fish entered FOM, however no significant differences were observed in mRNA levels from early FOM through atresia. Specifically, no effect of GnRHa treatment was observed. Relative levels of the three GnRH forms to each other remained consistent throughout FOM, with sbGnRH mRNA being lowest and sGnRH and cGnRH-II nearly equal.

III.D. Discussion

III.D.1. Ovarian Effects of GnRHa Treatment

To examine the effects of GnRHa therapy on the native GnRH-GtH system of captive striped bass, we first examined what changes occurred within the ovary. In our experiments, GnRHa therapy was 100% successful in inducing completion of FOM as evidenced by several histological features, including completion of lipid droplet coalescence, GV migration and breakdown, and varying degrees of yolk clearance. It is a logical assumption that, if undisturbed, all treated fish would have proceeded to ovulate (Mylonas, Woods et al. 1998). All fish in the untreated group underwent atresia within two weeks of water temperature elevation, as expected for non-hormonally manipulated captive striped bass (Zohar, 1989, Sullivan et al., 1997, Rees and Harrell, 1990).

In agreement with previous studies in the striped bass (Mylonas, Woods et al. 1997), a significant increase in oocyte diameter was observed as the fish entered FOM. This

increase is caused mainly by hydration of the oocytes (i.e., due to creation of a hyperosmotic condition inside the oocyte by Na+/K+ ATPases and proteolytic cleavage of yolk proteins into free amino acids), as opposed to further vitellogenic growth. Interestingly, gonadosomatic indices did not increase concurrently with oocyte diameters. Rather, it was not until the fish had been treated with GnRHa and were in the later stages of FOM that an increase in GSI was observed. The increased GSI in GnRHa treated fish was accompanied by a dramatic increase in total BW, which suggests that weight was gained in some tissue or organ other than the oocytes. The average weight increase over the approximately 48-hour interval between implantation and sampling was 1.68kg, while the average body weight in that group before implantation was 6.32kg (see Fig. 17B). The average fish, therefore, increased in weight by approximately 25%, an impressive gain that can not be accounted for solely by oocyte hydration and/or increases in ovarian fluid.

One possible explanation for this weight gain lies in a hypothetical perturbance of the fish's osmoregulatory ability due to GnRHa therapy, resulting in massive uptake of water from the environment. It has long been known that in addition to its gonadotropin-releasing actions, GnRH also possesses the ability to release other pituitary hormones, including growth hormone, somatolactin (SL), and prolactin (PRL). All three factors are hormones integral to osmoregulation as well as reproduction and growth. Kakizawa et.al. (1997) demonstrated that GnRH stimulates SL release from cultured rainbow trout pituitary cells and Weber et al. (1997) showed that GnRH also stimulates release of PRL in the tilapia, *Oreochromis mosambicus*. Parhar et.al. (1996) observed associations between GnRH-ir neuronal fibers and GH

and SL cells in tilapia and sockeye salmon. Finally, Stefano et.al. (1999) localized GnRH-receptors on GtH, GH, SL and PRL-producing cells in pituitary cell cultures from the Pejerrey, *Odontesthes bonariensis,* another teleost fish. Release of GH and/or SL in our striped bass could be from direct stimulation by the GnRHa or indirectly through feedback mechanisms still related to GnRHa. One more possible explanation of this unexpected weight gain could be from the experimental procedure itself. The fish were maintained in water with a salinity of 5-6ppt, while in nature the rivers in which they spawn are basically freshwater. Finally, water uptake by the ovary may be a natural phenomenon. In conclusion, it is obvious that there is a significant effect of GnRHa on the weight of the fish, which cannot be discounted to oocyte hydration. Further investigation of this unexplained GnRHa side effect might be warranted. Massive water uptake could be a sign of osmoregulatory dysfunction caused by GnRHa treatment resulting in a major source of stress to the fish, alleviation of which may positively influence survival of both broodstock and progeny.

III.D.2. Pituitary Effects of GnRHa Treatment

Plasma and Pituitary GtH-II Peptide Levels: The next level in the GnRH-GtH axis to be examined was the pituitary. In the above-described experiments, it was observed that pituitary GtH-II content was relatively high upon completion of vitellogenesis and remained elevated as the fish entered early FOM (Figure 16B). In the GnRHa treated fish, a dramatic decrease in pituitary GtH-II content was observed, while atretic fish maintained relatively high levels of GtH-II. Combined with a significant increase in plasma GtH-II seen only in GnRHa treated fish (Figure 16A), the decrease in pituitary

GtH-II content was assumed to reflect release into the bloodstream, rather than degradation or turnover within the pituitary. A preovulatory plasma GtH-II surge is a well documented characteristic of FOM in striped bass and other teleost species, and is thought to, among other things, confer maturational competence (i.e., receptivity to the MIS, 20ß-S) to the oocytes (Sullivan, Berlinsky et al. 1997).

It is noteworthy that in the current experiment, plasma levels of GtH-II seen in GnRHa treated fish were approximately ten fold higher than previously reported (Mylonas, Scott et al. 1997) and current findings (Section IV.C.1) on plasma GtH-II levels in wild striped bass in similar reproductive stages. Our current results are also approximately four-fold higher than plasma GtH-II levels reported for captive striped bass induced to complete FOM with the same GnRHa (D-Ala^6, Pro^9•Net). The discrepancy in plasma GtH-II titers between the two GnRHa studies in captive fish may be accounted for by differences in oocyte stage at time of treatment or other, more subtle experimental variables. This evidence supports our hypothesis that the captivity-induced reproductive dysfunction in striped bass is mediated through a failure to release sufficient quantities of GtH-II from the pituitary, a failure that is overcome by GnRHa treatment. This evidence also suggests that the employed method of GnRHa treatment may result in an overstimulation of the pituitary.

Pituitary Expression of GtH-Iß, -IIß Subunit: The next pituitary parameter examined was expression of the gonadotropin-ß mRNAs (Figure 18 A,B). Little is known about the effects of GnRHa on GtH subunit expression in teleosts. Hassin et.al. (1998) demonstrated a stimulatory effect of GnRHa on GtH-Iß, IIß, and alpha

subunit expression in precocious male striped bass. This finding is in line with mammalian models, in which sex steroids regulate synthesis and secretion of both GnRHs and GtH subunits (Attardi et al., 1997, Gharib et al., 1987). For example, it has been established that gonadectomized rats exhibit increased gonadotropin subunit expression due to the combined effects of a release of negative feedback from gonadal steroids and to increased secretion of GnRH from the hypothalamus (Dalkin et al., 1990, Lalloz et al., 1988). Similar interactions between GnRHs and sex steroids have been implicated in the control of GtH biosynthesis and release in amphibians (Stamper and Licht, 1990, Stamper and Licht, 1993, Pavgi and Licht, 1989).

In the current study, GtH-Iß subunit mRNA was highest in advanced vitellogenic fish and decreased as the fish matured, regardless of whether the fish completed GnRHa-induced FOM or underwent natural atresia. This observation agrees with previous theories on the role of GtH-I in the regulation of germ cell development, (i.e., responsibility for early development and vitellogenesis and not later development and FOM.

The GtH-IIß subunit was expressed most abundantly in advanced vitellogenic fish. Levels decreased significantly as the fish entered FOM, and did not deviate significantly from this level in either GnRHa-induced fish in late FOM or nontreated atretic fish. GnRHa had no apparent affect on GtH-IIß subunit mRNA expression. This data, together with high levels of GtH-II peptide in the pituitary, suggest that failure to complete FOM, ovulation, and spawning in striped bass is not due to an inability to synthesize new GtH-II. Rather it is more a question of releasing GtH-II that had been previously synthesized and stockpiled in the "readily releasable pool".

Pituitary Expression of GnRH Receptor: Expression of GnRH-receptor mRNA in the pituitary was also examined (Figure 18C). GnRH receptor mRNA levels were maintained at a stable level from advanced vitellogenesis through GnRHa-induced, late FOM. No significant differences were seen until the fish had undergone atresia, at which point levels decreased significantly. The decrease in pituitary GnRH-R associated with atresia may be indicative of the release of the regulatory effects of native GnRHs, GnRH agonist, GtH-II, and/or estradiol, all of which have been shown to be involved in the regulation of GnRH-R expression in mammals (Yasin, Dalkin et al. 1995; Cowley, Rao et al. 1998; Norwitz, Jeong et al. 1999). Our finding does not, however, rule out the possibility that a lack of functional cell surface GnRH-receptor is at least partially responsible for the lack of GtH-II release in captive striped bass. Again, further study of this possibility should be pursued.

Pituitary Peptide Content of the Three GnRHs: The final pituitary parameter examined was peptide content of the three GnRHs. In comparison with sbGnRH, the other two forms, sGnRH and cGnRH-II, were present at relatively low levels and did not fluctuate significantly throughout any stages of oocyte development, including atresia (Figure 19). This data indicates that the sGnRH and cGnRH-II peptide levels in the pituitary do not respond to GnRHa treatment and therefore suggests that these forms of GnRH are not directly involved in controlling the release of GtH-II from the pituitary. An important observation is that seabream GnRH peptide decreased significantly in the group treated with GnRHa, a decrease not mirrored in atretic fish. The decrease in sbGnRH coincided with the decrease in pituitary GtH-II, the surge in

plasma GtH-II, and the continuation or completion of FOM. The mechanism for sbGnRH decrease can only be speculated from these results, but may be a result of positive feedback from either the pituitary or the gonads responding to the GnRHa dose.

III.D.3. Hypothalamic effects of GnRHa Treatment

Brain Expression of the Three GnRHs: The final endocrine parameter examined was expression of GnRH mRNA in the brains of the fish, as determined by specific RPA. Information on regulation of GnRH gene expression in fish is sparse. What has become obvious from the limited data from fish and the more extensive data from mammals, is the extraordinary complexity of the regulation of the multiple GnRH genes. Regulatory influences are numerous and include endocrine (e.g.GnRHs themselves, GtHs, gonadal steroids) environmental (e.g. photoperiod) and physiological (e.g. age, condition factor) elements (Botte, Lerrant et al. 1999; Dunn and Sharp 1999; Gore, Roberts et al. 1999). A factor that further complicates analysis of GnRH gene regulation is that these factors appear to be not only tissue specific (Von Schalburg and Sherwood 1999), but also temporally specific in their actions(Parhar and Iwata 1996). Much of the available research in fish focuses on the effects of gonadal steroids on immunoreactive brain GnRH content (Soga et al., 1998, Grober et al., 1991). Even less information is available on the effects of GnRH(a) on hypothalamic GnRH expression in fish. However the presence of a negative, ultrashort loop feedback has been demonstrated using GnRH agonists, both *in vivo*

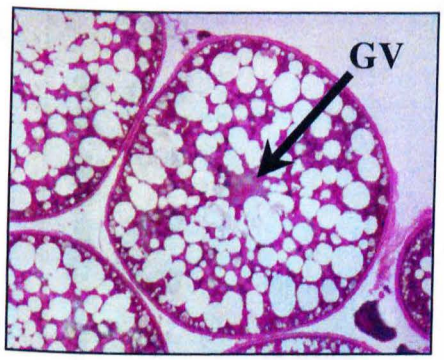
(Han, Kang et al. 1999); (Li and Pelletier 1994) and *in vitro* (Krsmanovic, Martinez-Fuentes et al. 1999) in rats.

In our experiment, a significant decrease in expression of all three forms of GnRH was observed as the fish entered FOM (Figure 20). Expression was maintained at decreased levels throughout GnRHa-induced late FOM and natural atresia. No significant changes were observed in any of the three forms in response to GnRHa treatment. A major implication of these results is the apparent absence of a negative ultrashort loop feedback on GnRH expression in female striped bass.

These results, however, may be in agreement with data collected from the gilthead seabream, *Sparus aurata*. The seabream is a multiple-batch, daily spawner in which preovulatory mRNA surges were observed in all three forms of GnRH (sbGnRH, sGnRH, and cGnRH-II) expressed in the brain (Gothilf, Meiri et al. 1997). Because the striped bass is an annual, group synchronous spawner, the daily GnRH synthesis surge that takes place approximately eight hours before spawning in the seabream may occur just once per year in striped bass, near the end of vitellogenesis. The timing of striped bass ovulation could make it such that the post-surge decrease seen in GnRH expression in the seabream four hours prior to ovulation corresponds to the entire process of FOM in striped bass. Gothilf et.al. proposed that the surge in sbGnRH expression serves to replace pituitary sbGnRH (which is responsible for the preovulatory GtH-II plasma surge), and that surges of cGnRH-II and sGnRH expression serve to replace those GnRHs when released inside the brain to stimulate courtship and spawning behavior. A correlation can be drawn in striped bass, where sbGnRH is released in the pituitary upon initiation of FOM (to stimulate preovulatory

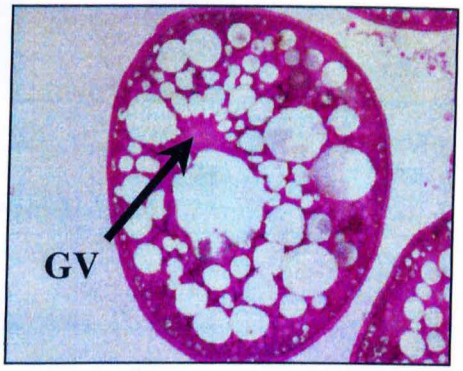
GtH-II surge) while sGnRH and cGnRH-II are released within the brain to regulate/induce spawning behavior. Because of their proposed sites of action within the brain, it may be expected that their pituitary levels would not fluctuate with FOM as sbGnRH does. Clearly this explanation would require further work for substantiation, however it does provide a working hypothesis to explain our results. The RPA does not allow for analysis of specific expression within different brain regions and/or GnRH nuclei. Despite this experimental limitation, the lack of fluctuation in whole brain GnRH expression levels throughout FOM, and similarity of expression levels between wild and captive striped bass (Section IV.C.6) suggest that the reproductive dysfunction in captive striped bass may be affecting the GnRH/GtH system at a level post-transcriptional to GnRH synthesis. There are many potential sites of post-transcriptional regulation in the biosynthesis of neuropeptide hormones such as GnRH (Figure 4). This situation with GnRH synthesis/release may be similar to that observed with the GtH-II synthesis/ release, ie. once the fish has reached final oocyte maturation it must rely on release of previously synthesized and stockpiled GnRHs, and not on *de novo* synthesis. The results of this experiment attempt to integrate data on several of the many factors controlling the complex endocrine control of final oocyte maturation in striped bass, complicated further by the "monkeywrench" thrown into the works by captivity. Clearly there is room for interpretation of the discussed data. Future works will, hopefully, support and expand upon our current findings (Section V.B)

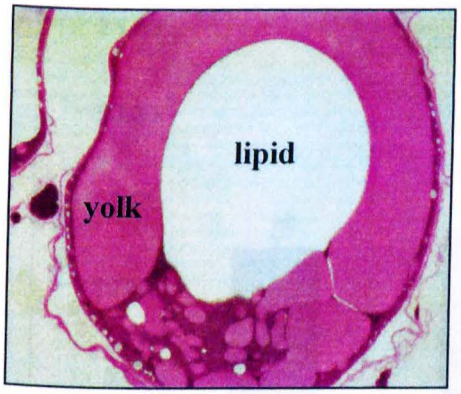
Figure 13: Timeline of experimental protocol for GnRHa effects on GnRH/GtH system of captive female striped bass. Four groups of five female fish were sacrificed at four distinct stages of oocyte development.



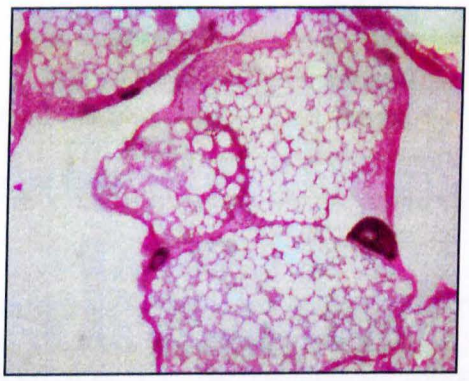
Group 1: Advanced vitellogenesis. Central germinal vesicle (GV), little to no lipid coalescence (white circles). Average oocyte diameter = 900±20μm.

Group 2: Early final oocyte maturation. Germinal vesicle migration/displacement, significant lipid coalescence. Average oocyte diameter = 1050±25μm.





Group 3: GnRHa-induced late final oocyte maturation. Completion of lipid coalescence, clearing of yolk globules (pink area), significant water uptake and volume increase. Germinal vesicle either peripheral or absent. Initiation of follicular layer separation. Average oocyte diameter = 1100±60μm.



Group 4: Atresia. Deterioration and resorption of lipid and yolk droplets, decrease in size, absent germinal vesicle, membrane breakdown.

Figure 14. Oocyte histology and descriptions of the four groups of captive striped bass sampled to assess the effects of GnRHa on their GnRH/GtH axis.

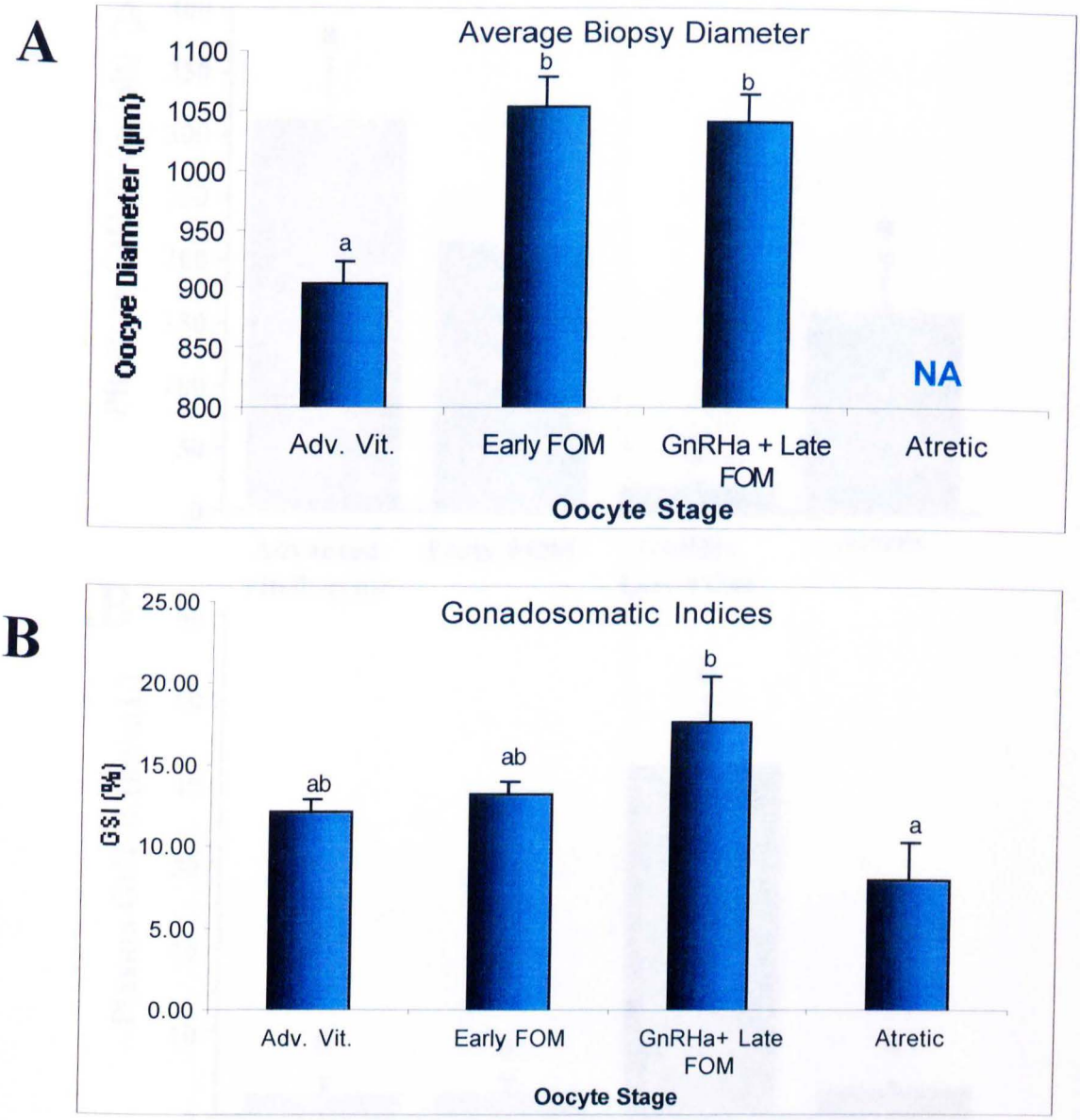


Figure 15: Gonadosomatic indices and average oocyte diameters at time of sacrifice for four groups of female striped bass. Adv. Vit. Fish were in the advanced stages of vitellogenesis. Early FOM fish advanced as far as captive fish do without hormonal manipulation. GnRHa+ Late FOM fish were treated with ~50μg GnRHa/kg BW and sacrificed during late FOM, prior to ovulation. Atretic fish were not treated with GnRHa but treated the same as GnRHa+ fish. No oocyte diameter is given for the atretic fish as the oocytes were too fragile and distorted for histology or accurate measurement.

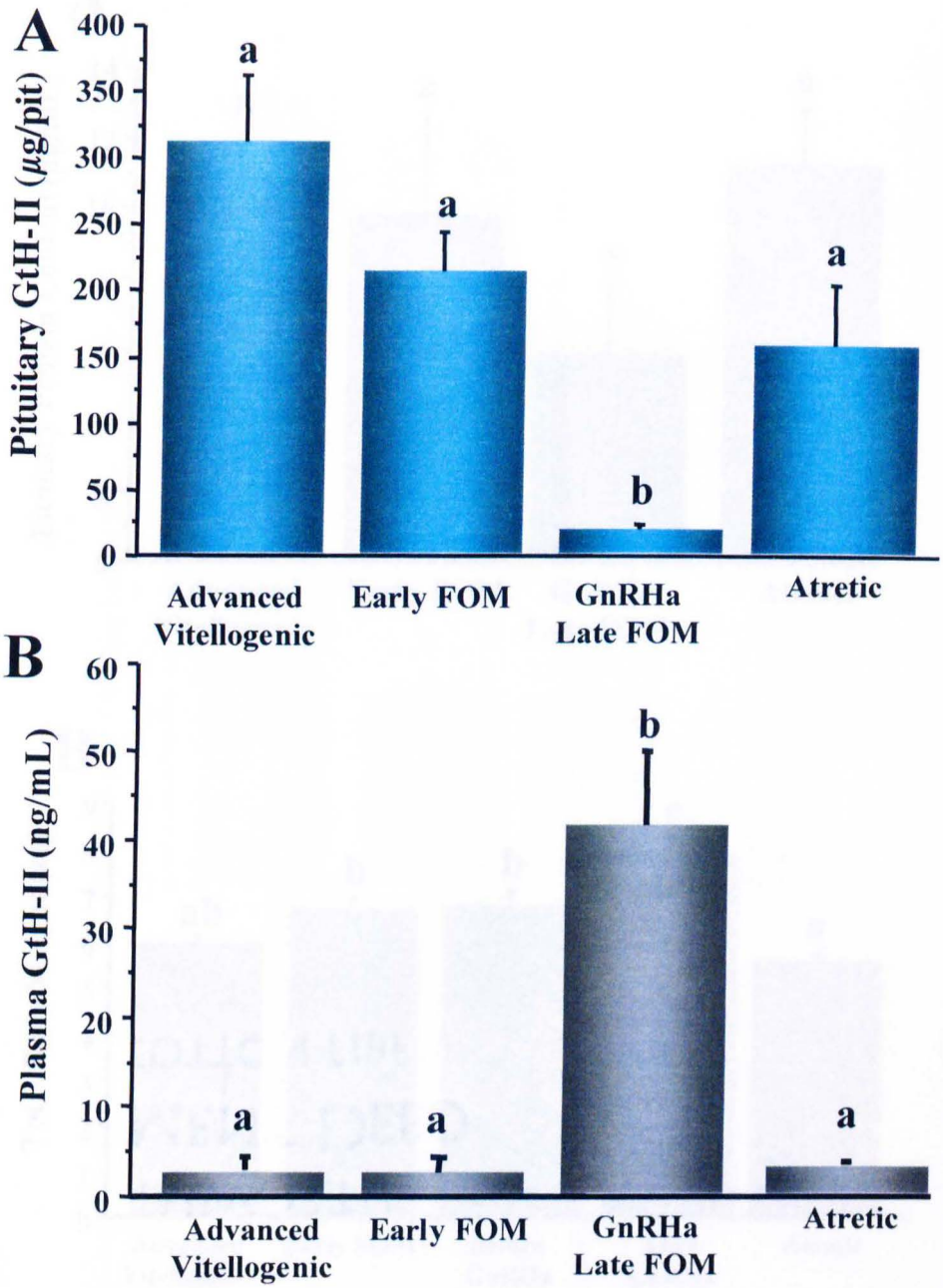


Figure 16: Pituitary and plasma GtH-II levels in captive female striped bass during four stages of late oocyte development. Pituitary GtH-II content is high in advanced vitellogenic, early FOM and atretic fish, and drops significantly in fish treated with GnRHa. These fish also exhibit a significant increase in plasma GtH-II, not seen in any other groups.

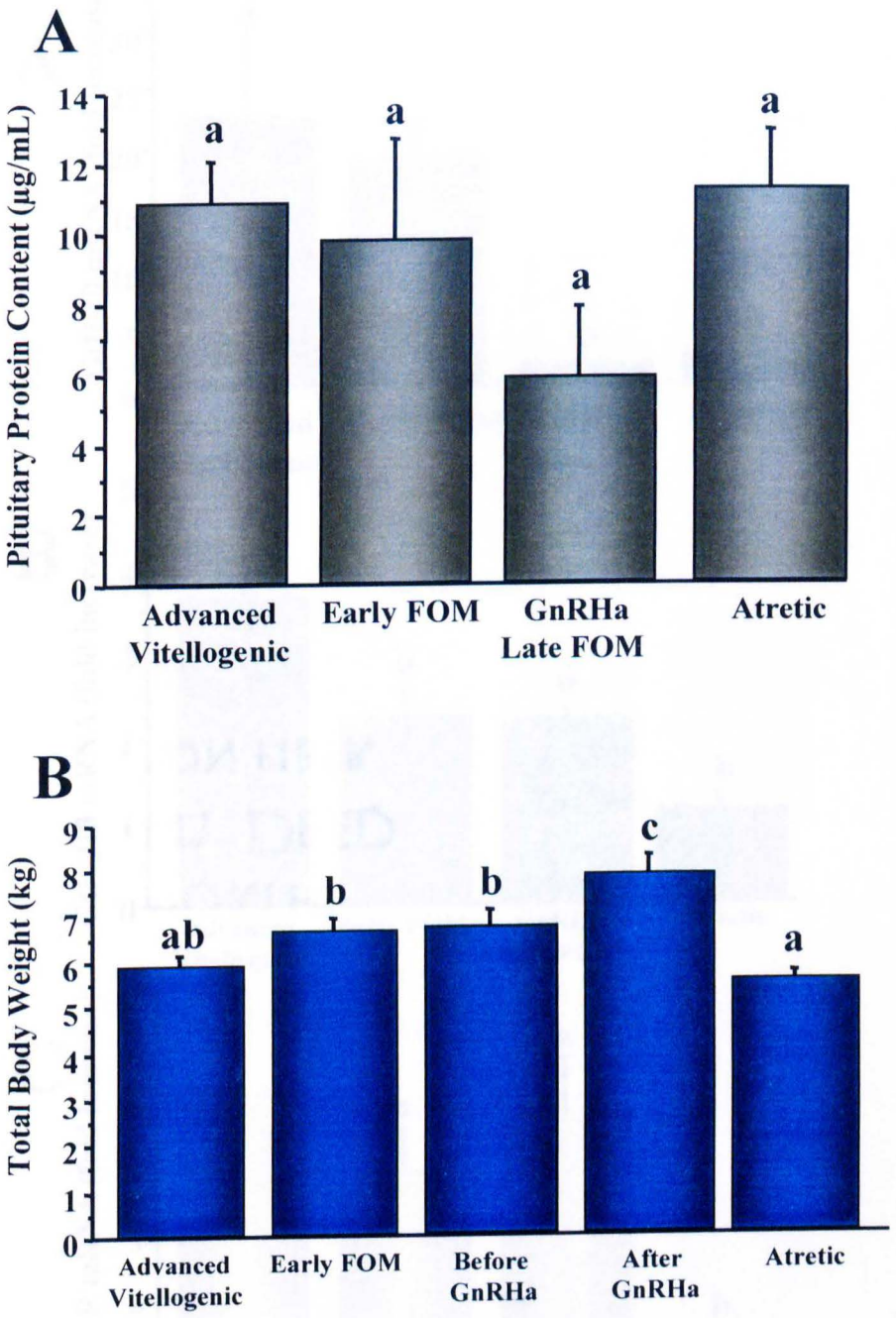


Figure 17. Pituitary protein content and total body weights before and after GnRHa treatment. A significant increase in weight was observed in the GnRHa treated fish which was not related to oocyte hydration. This increase may be related to the decrease in pituitary protein seen in the GnRHa treated fish.

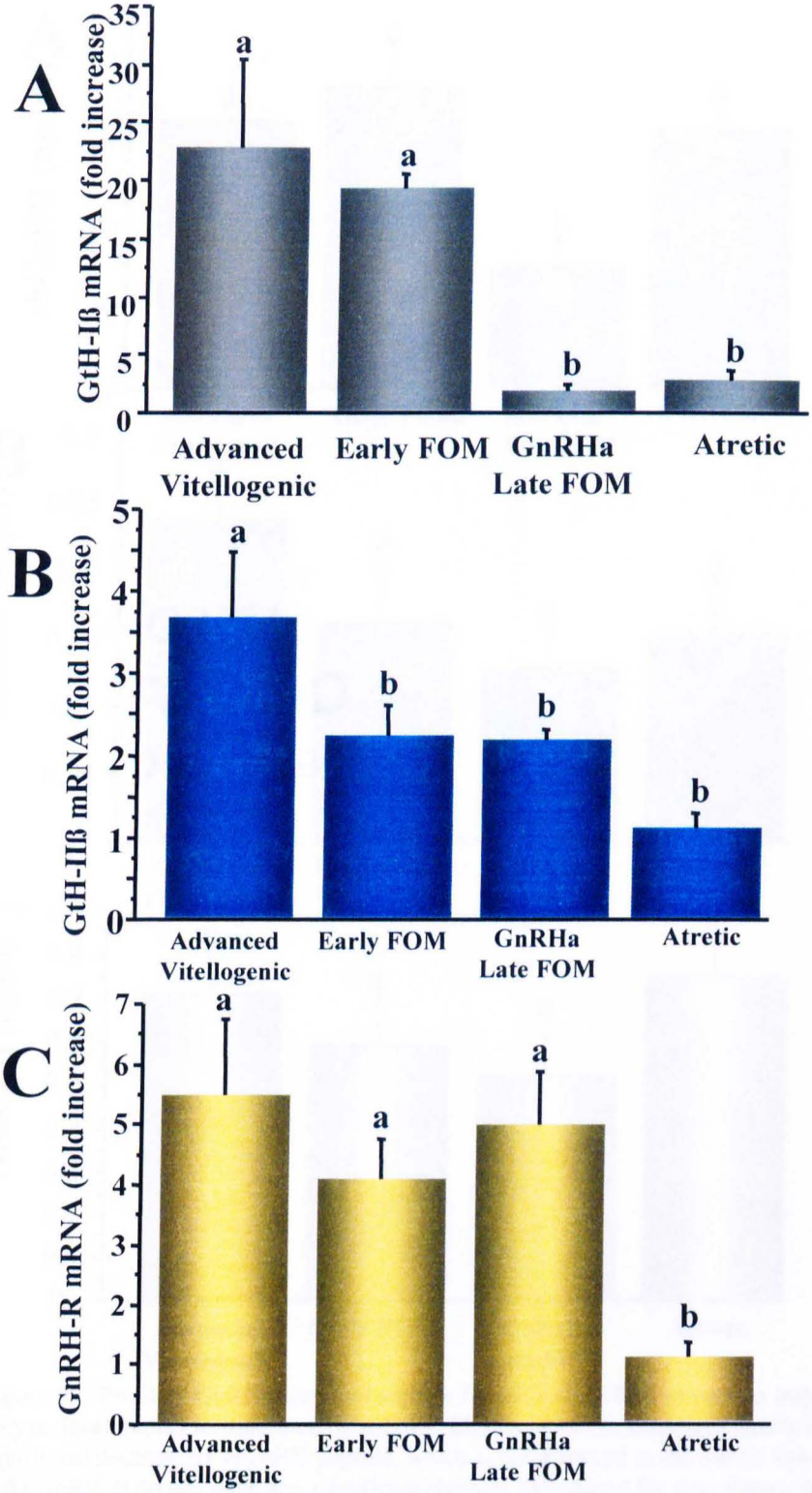


Figure 18. GtH-Iß, -IIß subunit and GnRH receptor mRNA expression in captive female striped bass at various stages of late oocyte maturation.

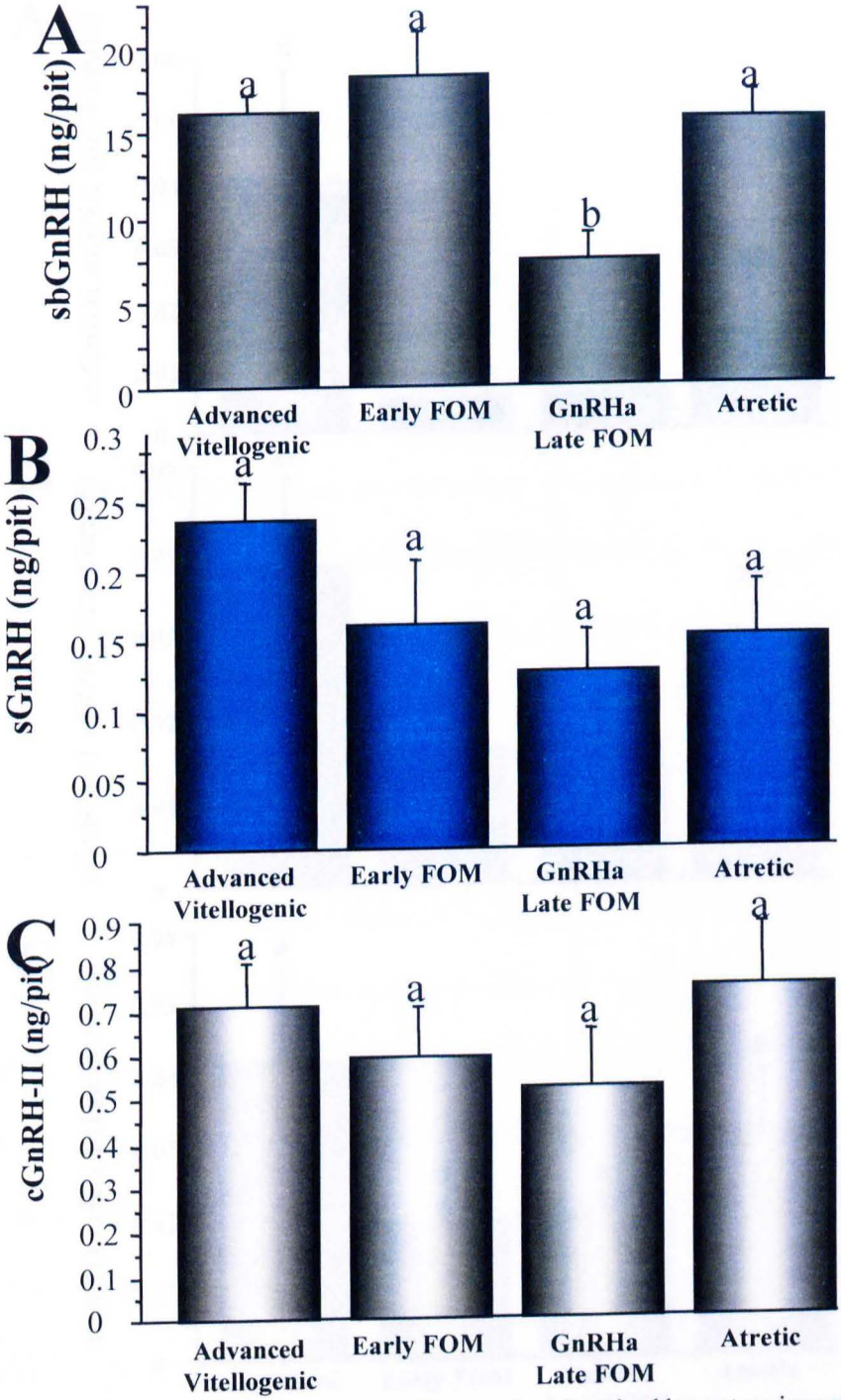


Figure 19. Pituitary GnRH content of captive female striped bass at various stages of late oocyte development as measured by specific ELISA. GnRHa treatment results in a significant decrease of sbGnRH peptide, which is not mirrored in the atretic fish. sGnRH and cGnRH-II do not show any significant changes throughout the four stages sampled.

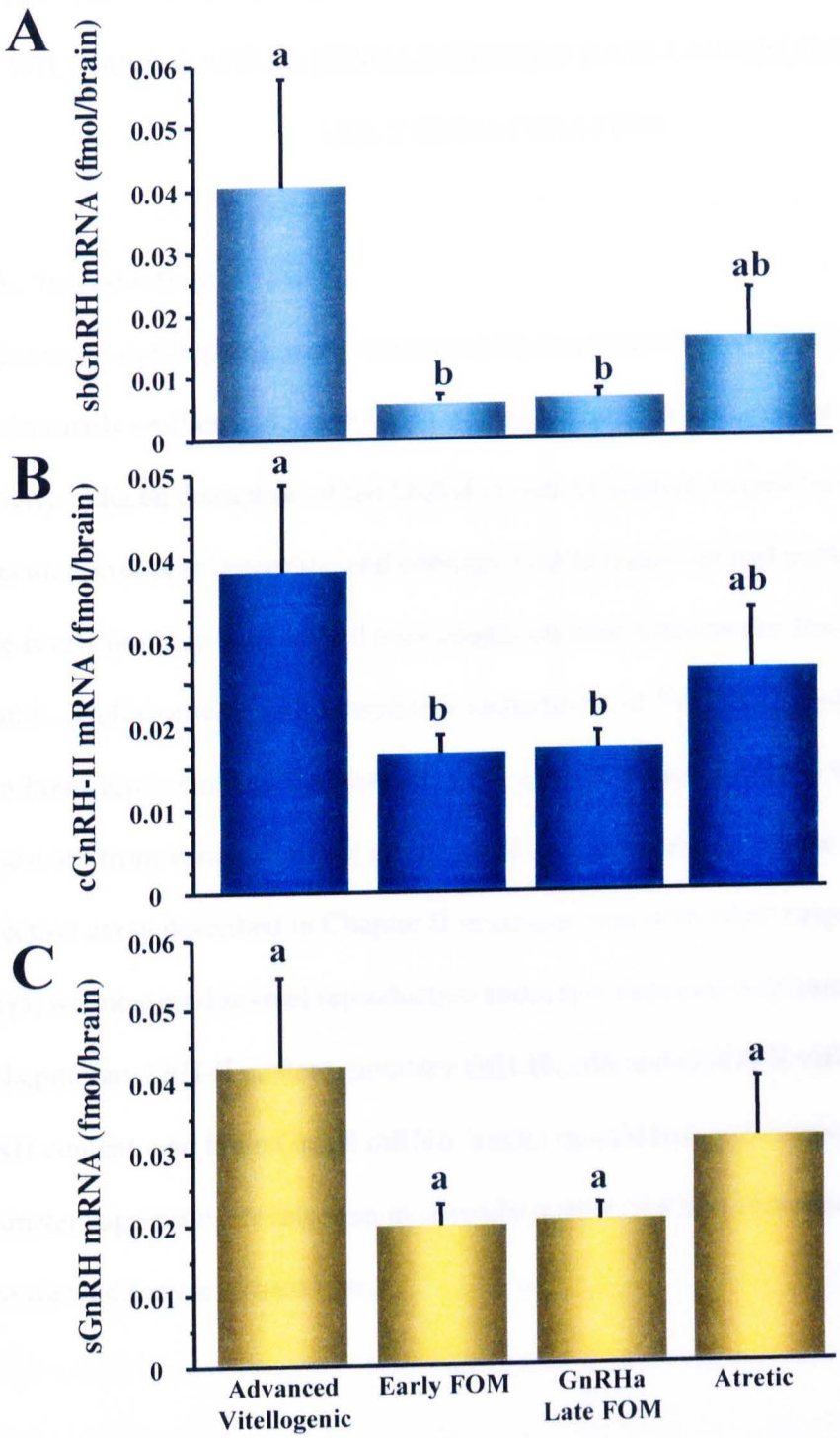


Figure 20. GnRH mRNA levels in the brains of captive female striped bass at various stages of late oocyte maturation, as determined by RNAse protection assay. No significant differences were seen between groups in any form of GnRH, suggesting that GnRHa has no effect on transcription of GnRHs.

CHAPTER IV: COMPARISON OF ENDOCRINE PARAMETERS BETWEEN WILD AND CAPTIVE FEMALE STRIPED BASS UNDERGOING FINAL OOCYTE MATURATION

IV.A. Introduction

As discussed in Chapter I, many commercially important fish species lose the ability to volitionally ovulate and spawn upon domestication. One method of confirming a captivity-induced disruption of the GnRH system of captive striped bass at the molecular level is to determine and compare GnRH transcript and peptide levels to those levels found in wild striped bass caught on their Chesapeake Bay spawning grounds. Collaboration with Maryland's Department of Natural Resources and the Horn Point Environmental Lab has provided us with unique access to wild striped bass broodstock from various parts of the Bay and its tributaries. Using the GnRH RNAse protection assay described in Chapter II in conjunction with other striped bass-specific assays, we measured several reproductive endocrine parameters (plasma GtH-II levels,pituitary GtH-II content, pituitary GtH-Iß, -IIß and GnRH-R mRNAs, pituitary GnRH content, and brain GnRH mRNA levels) in wild fish and compared these parameters against the levels seen in sexually mature, but non-reproductive, domesticated female striped bass.

IV.B. Materials and Methods

IV.B.1. Sampling Wild Striped Bass Undergoing FOM

During the Spring 1999 spawning season, nine female striped bass were sampled on their spawning grounds located in the Tuckahoe River, a tributary of the Chesapeake Bay. Sampling took place over a three-week period from April 7, 1999 through April 18, 1999, on Hogs Creek, which feeds into the Tuckahoe River, Denton, MD. Hogs Creek has been fished for several years as a source for ripe striped bass broodstock for restocking efforts conducted at the Horn Point Environmental Lab, Cambridge, MD. Freshwater streams and rivers that feed the Chesapeake, such as Hogs Creek and the Tuckahoe, represent the final destination of the spawning migration of striped bass. Oocyte maturation in these fish is timed to culminate in ovulation shortly after arrival on the spawning grounds. This situation, therefore, presents an ideal opportunity to sample relatively easily, fish that are undergoing very advanced stages of oocyte maturation.

Hogs Creek is essentially freshwater (salinity = 0 to 4ppt), and the bottom substrate is mud and silt, with an average depth of approximately 10 feet. Fishing was conducted a two to three hours following sunrise. Fish were captured via electrofishing (described in (Yeager, Van Tassel et al. 1990)) and anesthetized with tricaine methanesulfonate (MS-222) in a livewell supplemented with compressed oxygen, and ovarian biopsies were obtained via catheterization and examined. Due to the lack of working space and time on the boat, fish were maintained under anesthesia until the boat returned to the dock. Fish were then immediately sacrificed using the same protocols employed to sacrifice the captive fish in Chapter III. Briefly 10 mL of blood

was collected from the caudal vasculature and stored on ice, after which the fish were promptly decapitated. Brains and pituitaries were carefully but rapidly dissected and snap frozen in liquid nitrogen. Ovarian tissue samples were taken directly from the dissected ovaries and fixed in McDowell's solution for histological examination. A majority of the ovaries, however, were too swollen and delicate for transport back to the laboratory for weight measurement, therefore GSI data could not be obtained for the wild fish.

IV.B.2. Sampling Captive Striped Bass Undergoing Early FOM

To examine what differences exist in the reproductive endocrine status of wild and captive striped bass, captive striped bass in the latest stages of oocyte development normally seen in captivity were required. In captivity, striped bass oocytes routinely complete vitellogenesis and initiate early stages of FOM, but are not able to complete FOM or ovulation. Instead the oocytes become atretic, breakdown, and are resorbed by the fish (Zohar 1989). In April of 1999, five post-vitellogenic striped bass females in the early stages of FOM, representing the most advanced stages of normal oocyte development normally seen in captive fish (900-1000μm oocyte diameter; slight lipid coalescence) were sampled at COMB's Aquaculture Research Center. These fish were the second group of fish sampled as part of the GnRHa treatment experiment described in Chapter III. Their developmental stage also lends them perfectly to comparisons made in this study. Details on their histology are noted in Figure 14, Group 2.

IV.B.3. mRNA and Hormonal Assays

In addition to oocyte histology, the following assays, all of which were developed in the Zohar laboratory, were performed to examine the reproductive endocrine profile of wild and captive striped bass broodstock undergoing FOM; 1) the GnRH RPA described in Chapter II, 2) specific ELISAs to measure GnRH content of the pituitary (Holland, Gothilf et al. 1998), 3) real-time, semiquantitative RT-PCR mRNA analysis of GtH-Iß, -IIß and GnRH receptor expression in the pituitary (Alok and Zohar, unpublished results), 4) an ELISA to measure GtH-II peptide in the pituitary (Mananos, Swanson et al. 1997), and finally 5) a RIA to measure plasma GtH-II titer (Mylonas, Magnus et al. 1997). Additional information on technical aspects of the assays is available in the references cited.

IV.C. Results

IV.C.1. Plasma and Pituitary GtH-II Peptide Levels

Figure 22A compares the GtH-II peptide levels in the plasma of wild and captive striped bass as determined by radioimmunoassay. Wild fish undergoing mid- to late final oocyte maturation displayed a significantly higher plasma GtH-II titer (6.3±1.1ng/mL) than their captive counterparts, which were initiating early FOM (2.1±0.1ng/mL).

No significant differences were observed in pituitary GtH-II content between wild and captive striped bass in GtH-II content (Figure 22B. Pituitaries from captive fish contained an average of 28±10.3µg GtH-II/µg total pituitary protein, while wild fish undergoing mid- to late FOM averaged 36±5.83µg GtH-II/µg total pituitary protein. It

should be noted that pituitary GtH-II levels have been normalized to total pituitary protein content. Unlike the captive fish, which were all of identical year class and controlled environment, the striped bass collected from the wild were more than likely not from the same year class and even more unlikely to have shared as controlled a background as our captive fish. It is unclear how uncontrolled variables, such as diet, stress, conditional factors, and age, would have affected the GnRH/GtH system of the wild striped bass sampled. Normalizing for protein pituitary content takes into account the differences in body size and, presumably, pituitary size between the wild and captive fish, but cannot correct for the unknown effects of any number of uncontrollable environmental stimuli and genetic differences among the wild fish.

IV.C.2. Pituitary Peptide Content of the Three GnRHs

Peptide levels of the three GnRHs found in striped bass were measured in the pituitaries of wild females undergoing mid- to late FOM and captive females in early FOM via specific ELISAs (Figure 23). Seabream GnRH peptide was significantly higher in the pituitaries of captive fish (3.51±1.53ng/μg total pituitary protein) than in wild fish (0.471±0.098ng/μg total pituitary protein, Figure 23A). Chicken GnRH-II was not significantly different in the pituitaries of wild fish (0.084±0.016ng/μg total pituitary protein) and captive fish (0.123±0.059ng/μg total pituitary protein, Figure 23B). While significant amounts of sGnRH were found in the pituitaries of captive fish (0.037±0.019ng/μg total pituitary protein), sGnRH was non-detectable in the pituitaries of wild fish (Figure 23C).

IV.C.3. Pituitary GtH-Iß, -IIß Subunit and GnRH-R Expression

Expression levels of GtH-Iß, -IIß subunit and GnRH-R in the pituitaries of wild and captive striped bass were examined via real-time, semiquantitative RT-PCR analysis (Figure 24). GtH-Iß subunit mRNA was significantly higher (an approximately six fold increase) in the pituitaries of wild fish undergoing mid- to late FOM than in pituitaries of captive fish in early FOM (Figure 24A). No significant differences were seen in expression of either GtH-IIß subunit or GnRH-receptor between wild and captive fish (Figure 24B and C).

IV.C.4. Brain mRNA Levels of the Three GnRHs

The GnRH-RNAse protection assay described in Chapter II was used to compare expression of the three forms of GnRH between wild striped bass undergoing mid- to late FOM and captive striped bass initiating early FOM (Figure 25). No significant differences were observed between wild and captive fish in terms of seabream GnRH (0.008±0.003fmol/brain = wild, 0.005±0.002fmol/brain = captive), salmon GnRH (0.019±0.003fmol/brain = wild, 0.019±0.003fmol/brain = captive), or chicken GnRH-II (0.018±0.003fmol/brain = wild, 0.016±0.002fmol/brain = captive) expression levels.

IV.D. Discussion

IV.D.1. Ovarian Comparisons Between Wild and Captive Striped Bass

To compare the endocrine status of wild and captive striped bass, we must first compare the developmental stages within the ovaries between our sampled fish to establish the criteria for the comparison. Unfortunately we were unable to obtain GSI

data in the field for the wild fish. Instead, oocyte histology was performed, and a detailed analysis and comparison of the extent of oocyte development in wild and captive fish was obtained. Histological sections from the nine wild fish sampled are pictured in Figure 21. By necessity, wild fish were sampled as they were captured, and could not be maintained in the laboratory to allow their oocytes to advance to a more uniform stage. This experimental limitation led to variations, which are apparent in the histological sections, in oocyte development between the wild fish. Several of the wild fish were very close to ovulation (i.e., an estimated 10-12hr as compared with the egg development reference published by Rees and Harrell (1990). Others, while actively undergoing FOM, (lipid droplet coalescence, GV migration) were approximately 14-15 hrs prior to ovulation. The possibility of two subgroups of wild fish, displaying distinct endocrine parameters as well as oocyte histology, was entertained but dismissed. Upon further examination no significant differences between the two "subgroups" were apparent in any of the endocrine parameters examined. Therefore in subsequent discussions all nine wild fish are considered to represent a single group of wild fish undergoing mid- to late FOM

IV.D.2. Pituitary Comparisons Between Wild and Captive Striped Bass

Plasma and Pituitary GtH-II Peptide Levels: The first endocrine parameters to be compared between wild and captive striped bass were the plasma and pituitary GtH-II contents. GtH-II content was significantly higher in the plasma of wild fish (Figure 22), which correlates to the well-documented, preovulatory gonadotropin surge in teleosts. This surge is responsible for, among other things, the transition of follicular

steroidogenesis from testosterone and 17ß-estradiol to the maturation inducing steroids, which in the case of striped bass is 20ß-S, and bestowing maturational competence or increased receptivity to 20ß-S to the oocytes.

An interesting observation at this level is the similarity of pituitary GtH-II content between the wild and captive fish , expecially in light of different plasma concentrations. This is in contrast with the situation seen in captive striped bass responding to GnRHa treatment, where a significant decrease is seen in pituitary GtH-II content, concomitant with the preovulatory plasma GtH-II surge. One possible explanation for this discrepancy lies in the prospect that our current GnRHa treatment may act to over-stimulate the pituitary, resulting in a much larger than normal, or necessary, release of GtH-II. It may well be that striped bass synthesize larger quantities of GtH-II than is required, and release only an amount sufficient to stimulate steroidogenesis in the ovarian follicles. This information should be taken into account in the design of future GnRHa therapies.

Pituitary Peptide Content of the Three GnRHs: The next endocrine parameters to examine in the pituitary were the GnRH peptide levels. Examination of pituitary GnRH content leads to another interesting observation. Seabream GnRH peptide in the pituitaries of wild fish was significantly lower than in the pituitaries of captive fish. This observation is consistent with the results of the GnRHa treatment experiment from Chapter III (Section III.C.3), where fish treated with GnRHa exhibited significantly lower amounts of sbGnRH in their pituitary. These two results taken together provide additional evidence to support the hypothesis proposed in the

abstract of this thesis. Specifically, the captivity-induced reproductive endocrine failure in striped bass lies at the level of the gonadotropin-releasing hormones, especially sbGnRH, which is most responsible for GtH-II release in the pituitary and is apparently not released in sufficient quantities to stimulate completion of FOM.

No significant differences were observed in pituitary cGnRH-II peptide content between the wild and captive fish, suggesting this form may not play an important role in GtH-II release. This could be a reflection of cGnRH-IIs possible intracerebral site of action, which would be in accordance with its hypothesized role in the regulation of reproductive behavior. In light of the fact that the source(s) of pituitary cGnRH-II, and sGnRH are still unknown, it would be a mistake to discount a paracrine role for cGnRH-II during FOM solely because it's pituitary levels do not fluctuate throughout this period (Section III.C.4).

An observation that warrants further investigation was the complete and total lack of sGnRH in the pituitaries of wild fish. sGnRH was clearly present, albeit at very low levels, in captive fish. This data is reminiscent of a similar situation observed during the upstream migration of chum salmon, *Oncorhynchus keta*, which, like the striped bass, is also an anadromous teleost. All examined species of salmon are known to express only two forms of GnRH, cGnRH-II (in the midbrain), and sGnRH (in both the forebrain/olfactory region and hypothalamus/preoptic area). This is comparable to seabream, where cGnRH-II is still expressed in the midbrain, and sGnRH is apparently confined to the forebrain, but sbGnRH has evolved as the hypothalamic form (Gothilf et al., 1996). A similar situation is expected in the striped bass, but has not yet been confirmed. In an earlier study, sGnRH immunoreactivity and expression

was examined in chum salmon captured either in the coastal ocean, or upstream on their spawning grounds (Kudo, Hyodo et al. 1996). It was found that prior to migration (i.e., in the ocean), sGnRH was mainly expressed in the forebrain, olfactory nerve and olfactory bulb, and not in the preoptic area or telencephalon. The opposite situation was observed in post-migratory fish. Salmon GnRH was expressed primarily in the hypothalamus and not in the forebrain of fish sampled on the spawning grounds. This data suggests that the forebrain expression of sGnRH plays a role in regulating migratory behavior in salmon. Additional data supporting this hypothesis was provided by Parhar et.al. (1994), who demonstrated a significant increase in both immunoreactive and mRNA-expressing neurons along the nervus terminalis and in the olfactory bulb in seaward migrating chum salmon. Control of migratory behavior in striped bass could likely occur via a similar mechanism. Due to their confinement and subsequent lack of exposure to migration-associated stimuli, captive striped bass apparently express significant amounts of sGnRH as indicated by its presence in the pituitary. While we can't be certain, it is at least possible that the sGnRH in the pituitary originates from neurons in the forebrain. The fact that non-migratory captive striped bass have significant levels of salmon GnRH in their pituitaries, while post-migratory wild fish do not, could be a direct result of the environmental disparity between the two groups. Further examination of GnRH expression patterns within specific brain regions, as well as determination of the source(s) of pituitary sGnRH is required to shed more light on this intriguing result.

Literature searches resulted in little insight into the possible implications of sGnRH in the pituitary. While all forms of GnRH obviously have the ability to release GnRH,

there is preliminary evidence that multiple GnRHs can act together to exert an inhibitory effect on pituitary GtH release. Working with a recombinant African catfish GnRH receptor-expressing cell line, Tensen et.al. (1997) recently demonstrated that while both of the endogenous forms of GnRH (cfGnRH and cGnRH-II) stimulated the cyclic adenosine monophosphate (cAMP) second messenger system, exposure to certain combinations of both GnRHs actually resulted in an inhibition of cAMP levels. A mechanism for this seemingly counterintuitive result could imaginatively be formed based on competition between two (or three in the case of striped bass and seabream) GnRHs for receptors on the surface of the gonadotrophs. It has been previously shown that different forms of GnRH in striped bass having different GtH-II releasing potencies, and presumably receptor affinities (Zohar, Elizur et al. 1995). This data may help explain the presence of sGnRH in the pituitary of captive fish, and how it could function together with sbGnRH to potentiate or inhibit GtH-II release from the pituitary.

Similar to the pituitary GtH-II levels discussed above, pituitary GnRH levels were normalized for total pituitary protein content. Despite the fact that GnRHs are produced in the hypothalamus (i.e., outside of the pituitary), they are stored in the GnRH-neuron axon terminals located within the pituitary. It therefore stands to reason that, all other factors being equal, a larger fish will have a larger pituitary, which in turn will possess more axon terminals, and therefore a greater amount of GnRH than a smaller fish.

Pituitary Expression of GtH-Iß, -IIß Subunit and GnRH-R: The final endocrine parameter examined at the level of the pituitary was expression of GtH-Iß, -IIß and GnRH-R mRNAs. In this study, the only significant difference between wild and captive striped bass was seen in GtH-Iß subunit expression. Due to the lack of peptide data availability on GtH-I, we can not make conclusions regarding this finding. Neither GtH-IIß nor GnRH-R mRNA levels were significantly different between the wild and captive fish. This finding again suggests that after the fish have reached a certain developmental stage, the failure of captive fish to complete FOM arises from an inability to release previously synthesized GtH-II, and not a failure to synthesize new GtH-II. The equality of GnRH-R mRNA levels between wild and captive striped bass suggests that captive fish express sufficient amounts of GnRH-R mRNA, however it does not preclude the possibility of a shortage of functional receptors on the gonadotroph surface.

IV.D.3. Hypothalamic comparisons between wild and captive striped bass

Brain Expression of the Three GnRHs: No significant differences were observed between any of the three GnRH mRNAs expressed in the brain of captive and wild striped bass undergoing early and mid- to late FOM, respectively, as measured by RPA. This finding indicates that GnRH synthesis is not inhibited or affected by domestication in striped bass. It could also indicate that the disparities seen in pituitary GnRH content between wild and captive fish may arise from post-transcriptional regulation of GnRH.

As mentioned in the discussion of Chapter II, the RPA has now become "obsolete", replaced by Taqman semiquantitative, real-time PCR analysis of mRNA levels. When applied to the GnRHs, this new technique will allow us to examine gene expression fluctuations within specific areas of the brain. This, in turn, would permit us to more fully address the question of what role the specific GnRHs are playing within specific areas of the brain.

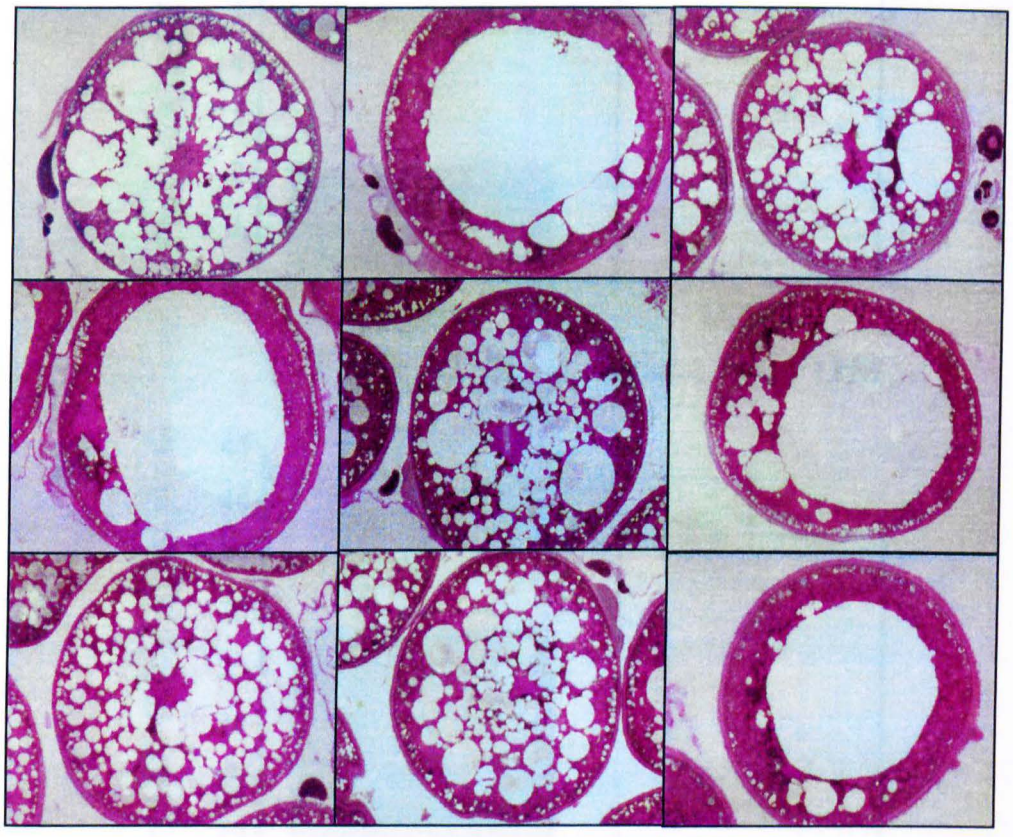


Figure 21. Oocyte histology of nine wild striped bass females sampled on their spawning grounds on the Tuckahoe River, a tributary of the Chesapeake Bay over a three week period in April 1999. All fish are in various stages of mid- to late final oocyte maturation with four being very close to ovulation and five being less advanced. No significant differences were observed between the two "subgroups" of wild fish. Therefore all fish were considered to represent mid- to late final oocyte maturation.

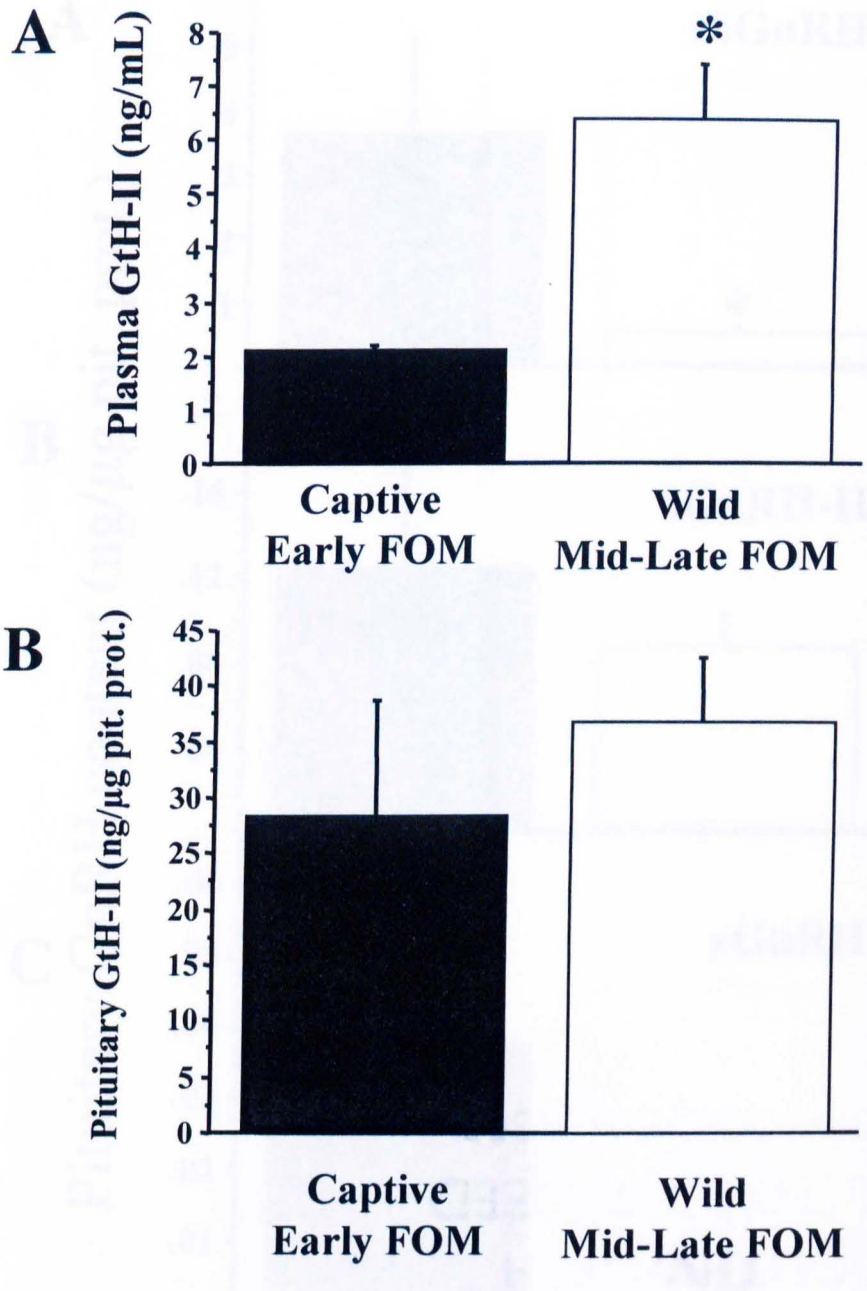


Figure 22. Pituitary and plasma GtH-II in wild during mid- to late FOM and captive striped bass in early FOM. While the levels do not differ in the pituitary, the plasma of wild fish actively undegoing final oocyte maturation contains significantly more GtH-II. Pituitary levels were normalized for total pituitary protein content to adjust for size differences between the fish.

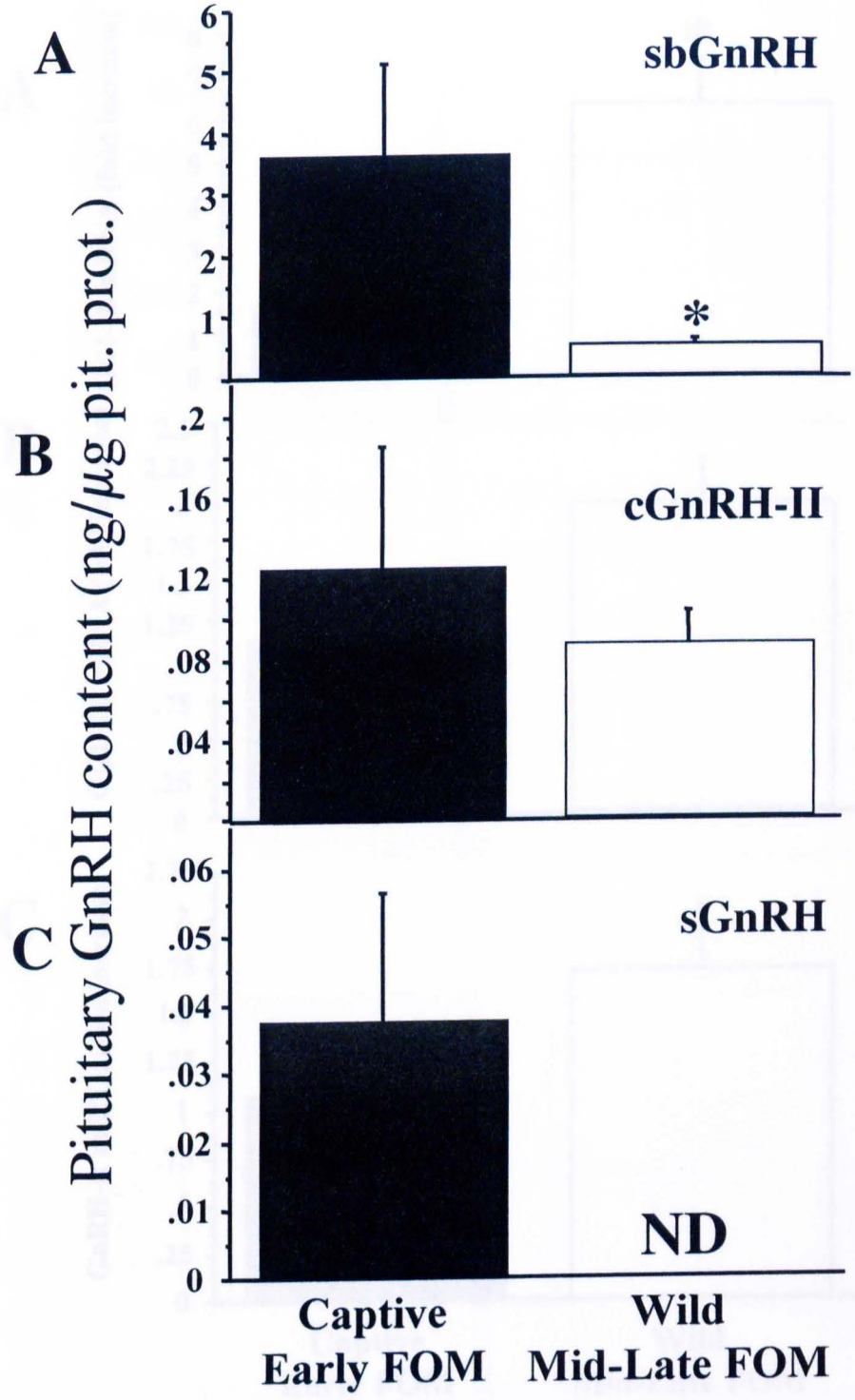


Figure 23. Pituitary GnRH content of captive fish during early FOM and wild fish undergoing late FOM. Pituitaries from wild fish contained significantly less sbGnRH, significantly more cGnRH-II, and no detectable sGnRH when compared to pituitary GnRH content in captive fish.

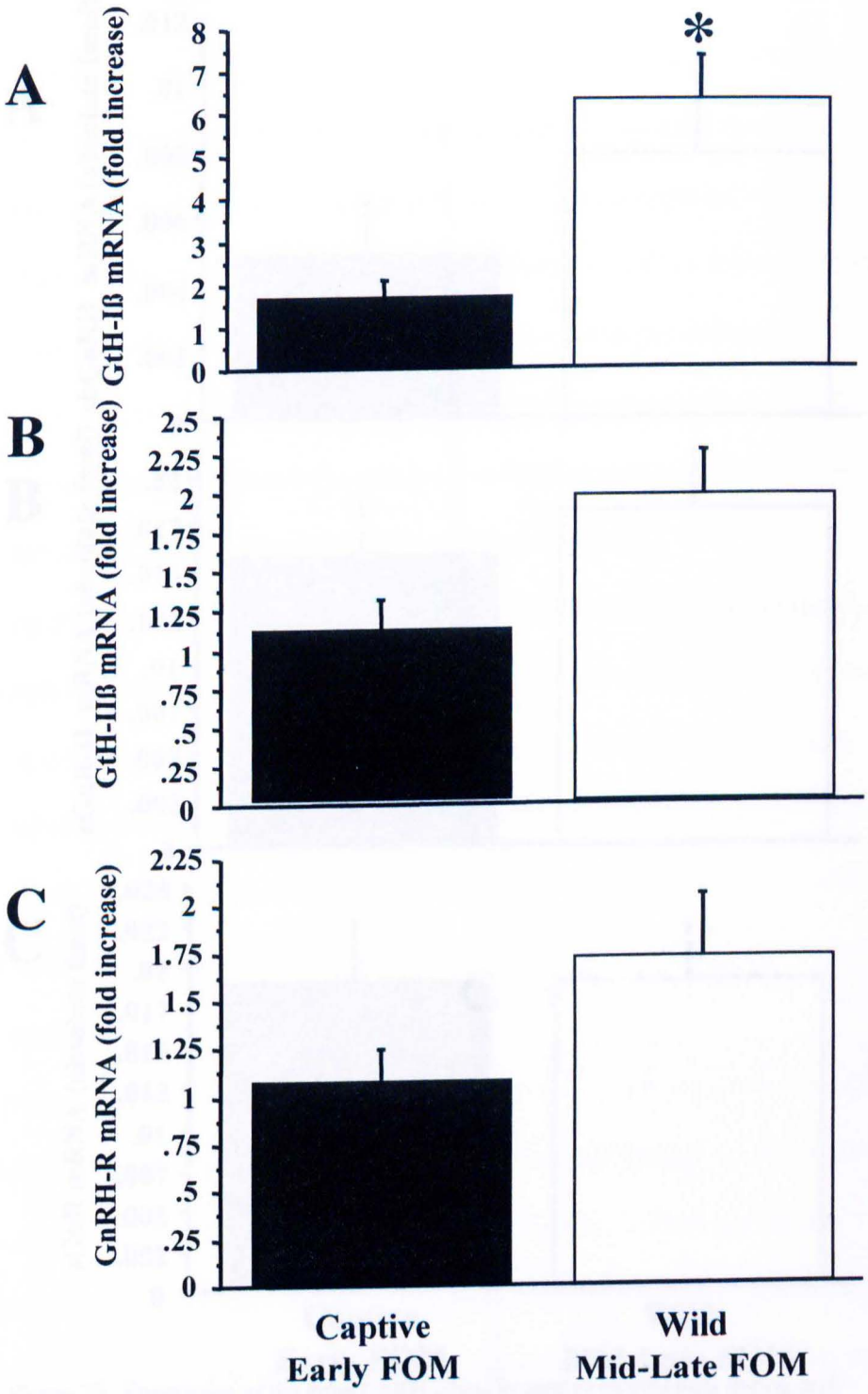


Figure 24. Expression of GtH-Iß, -IIß, and GnRH-R in the pituitaries of captive striped bass during early FOM, and wild fish undergoing mid- to late FOM. Captive fish express significantly less GtH-Iß, while no significant differences exist between wild and captive fish in either GtH-IIß or GnRH-R.

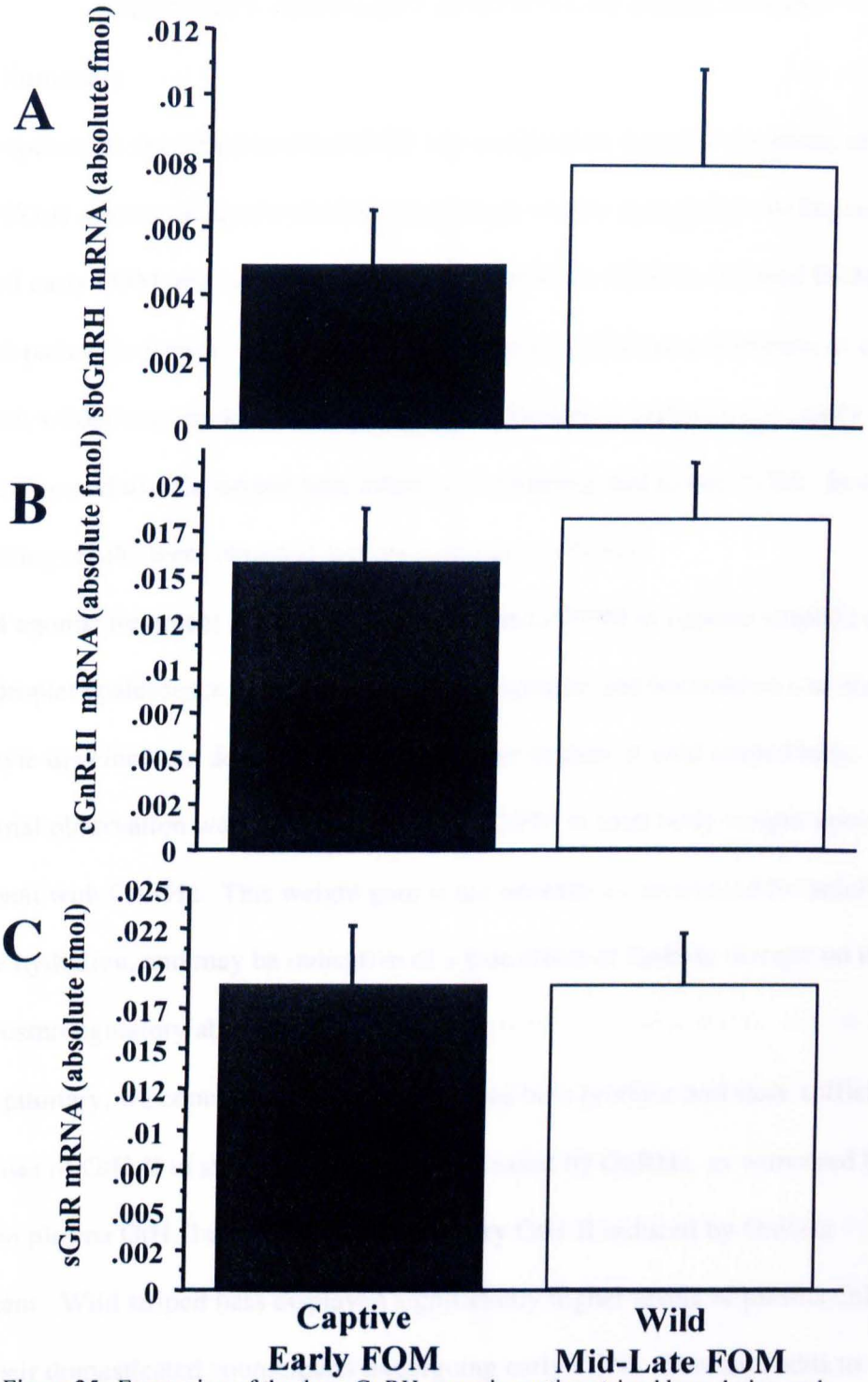


Figure 25. Expression of the three GnRH genes in captive striped bass during early FOM and wild striped bass during mid- to late FOM as measured by ribonuclease protection assay. No significant differences were apparent in the expression of any of the GnRHs, suggesting that the captivity-induced reproductive dysfunction in striped bass may occur post-transcriptionally.

CHAPTER V. SUMMARY AND FUTURE DIRECTIONS

V.A. Summary

The experiment described in Chapter III was designed to examine the status of the GnRH/GtH system of captive female striped bass as they completed vitellogenesis, entered early FOM and completed one of two pathways, GnRHa-induced FOM or the default pathway, atresia. In Chapter II, reproductive endocrine parameters of captive females, whose oocytes had advanced as far as possible in captivity (i.e., early FOM), were compared to wild striped bass actively undergoing mid to late FOM. Several interesting results were obtained and are summarized below.

GnRH agonist treatment appears to stimulate normal FOM in captive striped bass (i.e., lipid droplet coalescence, yolk clearance, GV migration and breakdown), as compared to oocyte development described in this and other studies of wild striped bass. One abnormal observation was a marked increase (~25%) in total body weight upon treatment with GnRHa. This weight gain is not necessarily accounted for solely by oocyte hydration, and may be indicative of a side effect of GnRHa therapy on the fish's osmoregulatory ability.

In the pituitary, we concluded that captive striped bass produce and store sufficient quantities of GtH-II to stimulate FOM when released by GnRHa, as witnessed by a surge in plasma GtH-II and depletion of pituitary GtH-II induced by GnRHa treatment. Wild striped bass displayed significantly higher levels of plasma GtH-II than their domesticated counterparts undergoing early FOM. This data adds to previous data implicating a lack of GtH-II as the root of the loss of reproductive competence in striped bass upon domestication. Another interesting observation was

that GnRHa treatment resulted in a near 10-fold higher concentration of plasma GtH-II than seen in wild fish undergoing natural FOM. This finding suggests that our current GnRHa therapy may overdose the pituitary, an observation that ought to be incorporated in the design of future therapies.

Pituitary transcription levels of GtH-IIß, GtH-Iß and GnRH-R mRNAs were unaffected by GnRHa treatment and similar between wild and captive fish, indicating that synthesis of the gonadotropin subunits and GnRH receptor in captive fish is probably adequate and therefore probably not a factor limiting the completion of FOM in captive striped bass.

Seabream GnRH peptide in the pituitary decreased significantly upon GnRHa treatment. Significantly lower levels of sbGnRH were also observed in the pituitaries of wild fish in comparison to captive fish, further attesting to the theorized role of sbGnRH in the release of GtH-II from the pituitary. Salmon GnRH peptide was undetectable in pituitaries of wild fish, while significant levels were observed in the pituitaries of captive fish. These levels were unaffected by GnRHa, as were cGnRH-II levels, suggesting that these two GnRH forms are regulated differently than sbGnRH. We propose that there may be an interaction between sGnRH and sbGnRH within the pituitary that negatively influences pituitary GtH-II release.

Hypothalamic mRNA expression of the three forms of GnRH decreased significantly as captive fish completed vitellogenesis. As with the gonadotropin subunits, GnRH mRNA levels also appeared to be unaffected by GnRHa treatment. Transcription levels were also similar between wild and captive fish. Again, this data suggests that the failure of captive fish to complete FOM, ovulation, and spawning is not related to

a lack of synthesis of GnRH. The possibility that captive fish do not process GnRHs correctly should be examined.

V.B. Future Directions

The above-described studies have yielded a wealth of data on the endocrine status of captive striped bass in response to GnRHa treatment and in comparison to wild fish undergoing natural FOM. As is the ultimate goal of the scientific method, each conclusion garnered from the current studies has spawned a multitude of avenues to follow up on, a few of which are presented below.

The first and most accessible research that should be conducted is *in situ* hybridization localization of the three forms of GnRH-producing neurons within the brains of striped bass. Confirmation of the expression patterns observed in seabream and determination of the source(s) of pituitary sGnRH and cGnRH-II will allow us to proceed with confidence to further studies of the GnRH/GtH system, including functions of the multiple forms of GnRH.

Despite the fact that insufficient GnRH receptor mRNA levels were not observed in captive fish, and cannot be implicated as a cause of reproductive dysfunction in captive striped bass, analysis of functional receptors within the pituitary remains a possibility which should be investigated.

Following this line of reasoning, the observation that GnRH mRNA levels were similar between wild and captive fish, and did not respond to GnRHa treatment, does not rule out the possibility of faulty post-transcriptional regulation of GnRH, resulting in insufficient levels of bioactive peptide in the pituitary. Due to direct stimulation of

pituitary gonadotrophs by GnRH-producing neurons, as opposed to transport in the hypophyseal portal system possessed by mammals, measurement of bioactive GnRH secretion rates in fish has eluded scientists. Development of hitherto unknown technologies to measure synaptic release of GnRH within the pituitary would pave the way for great advances in our understanding of the regulation of GnRH secretion in fish.

In this authors opinion, the most promising, and feasible, avenue of research would focus on interactions between sGnRH and sbGnRH within pituitary of captive fish alluded to by results of this study. Two lines of research could be followed to this end. First, it would be interesting to sample wild striped bass females at an earlier stage of their reproductive migration, and therefore oocyte developmental stage. Detection of sGnRH in the pituitaries of pre-migratory striped bass could help to confirm a situation similar to that in described in the migrating chum salmon. The second approach depends on the development of cell lines expressing recombinant striped bass GnRH receptor, work which is already underway in the Zohar lab. Such a cell line would facilitate studies on interactions between different native GnRHs and also serve as diagnostics tools for determining dosages for future GnRHa therapies. If an antagonistic interaction is indeed demonstrated between sGnRH and sbGnRH (or any of the three GnRH forms) future therapies designed to alleviate reproductive dysfunction could focus on removal or amelioration of this effect. The advent of transgenic and "gene knockout" technology raises the possibility of the generation of sGnRH-deficient striped bass strains. This technology is still in its infancy, and will

likely be developed in the zebrafish before application to striped bass and other commercially important fish species.

Clearly I've covered only a few of many questions raised by the current study. If nothing else, the results of this study exemplify the complicated and intricate nature of the neuroendocrine control of reproduction in vertebrates.

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