ABSTRACT

Title of Dissertation: GONADAL AND STEROID FEEDBACK REGULATION OF THE HYPOTHALAMUS-PITUITARY AXIS IN STRIPED BASS (*Morone saxatilis*)

Ulrike Klenke, Doctor of Philosophy, 2006

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The objective of the present study was to expand our understanding of the mechanisms of gonadal steroid feedback regulation of the hypothalamus-pituitary (HP) axis during several reproductive stages (juvenile, pubertal, adult) throughout the life cycle of the striped bass. Towards this end, we investigated effects of bilateral gonadectomy and steroid replacement on the endocrine correlates of the HP axis *in vivo*. We also developed a brain-slice culture method and utilized pituitary cell cultures to investigate direct effects of estrogen on these correlates at the level of the brain and the pituitary *in vitro*. Our findings indicate that: 1) During their development, the gonads play an important role in providing feedback to the HP axis. These feedback patterns change during the transformation from the juvenile to the adult and throughout the adult

reproductive cycle. The pathways involved use both non-steroidal and steroidal pathways as regulatory mechanisms. 2) Gonads, through their steroids, become more involved in regulating the HP axis during reproductive development and their main feedback target appears to be gene transcription in the pituitary. 3) The observed changes in gonadal feedback throughout the adult reproductive cycle probably reflect the physiological requirements of gametogenesis. 4) The responsiveness of the HP axis towards steroids initially appears during puberty and further increases in adult females. In adults, steroids solely affect the pituitary in early stages of gametogenesis, while in later stages GnRH expression in the brain is also regulated by steroids. However, the nature of the feedback is dependent on estrogenic and/or androgenic pathways. 5) Our *in vitro* studies showed that estrogens act directly at the levels of the brain and the pituitary in female adult fish. Based on these findings, it appears that the activity along the endocrine reproductive web of striped bass intensifies with age and that prior cycles of oocyte development may prime the HP axis to respond faster and more vigorously in subsequent years. This study has provided an improved resolution and a broader perspective on mechanisms involved in gonadal steroid feedback regulation of GnRH neural activity and its targets at the level of the pituitary in striped bass.

GONADAL AND STEROID FEEDBACK REGULATION OF THE HYPOTHALAMUS-PITUITARY AXIS IN STRIPED BASS (*MORONE SAXATILIS*)

by

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DEDICATION

This dissertation is dedicated to the memory of my father,

Günther Klenke.

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ABBREVIATIONS

Hormones and Proteins

11-KA	11-ketoandrostenedione
11-KT	11-ketotestosterone
17α,20β-P	17α,20β-dihydroxy-4-pregnen-3-one
20β-S	17α,20β,21,trihydroxy-4-pregnen-3-one
AR	Androgen receptor
DA	Dopamine
E_2	17β-estradiol
ER	Estrogen receptor
FSH	Follicle-stimulating hormone
GABA	γ-aminobutyric acid
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GnRHa	GnRH agonist
GnRH-R	Gonadotropin-releasing hormone receptor
GtH	Gonadotropin
KP	Kisspeptin
LH	Luteinizing hormone
MIS	Maturation-inducing steroid hormones
NPY	Neuropeptide Y
P	Progesterone
StAR	Steroidogenic acute regulatory protein
T	Testosterone
TGF	Transforming growth factor
Vg	Vitellogenin
VLDL	Very-low-density lipoproteins

Experimental Methodology

GC	Gonadectomized fish, treated with the vehicle only
gdx	Gonadectomized
GE	Gonadectomized fish, treated with E_2
GK	Gonadectomized fish, treated with 11-KA
GT	Gonadectomized fish, treated with T
SE	Sham operated fish, treated with E_2
SK	Sham operated fish, treated with 11-KA
s-o fish	Sham operated fish
ST	Sham operated fish, treated with T

Other

ANOVA	Analysis of variance
CV	Coefficients of variations
ddw	Double distilled water
dps	Days post-surgery
ELISA	Enzyme-linked immunosorbent assays
GSI	Gonado-somatic index
GVBD	Germinal vesicle break-down
HP axis	Hypothalamus-pituitary axis
HPG axis	Hypothalamus-pituitary-gonadal axis
MBH	Medio-basal hypothalamus
MT	Midbrain tegmentum
OB-TN	Olfactory bulb-terminal nerve
PCR	Polymerase chain reaction
PIT	Passive integrated responder
POA	Preoptic area
RIA	Radioimmuno assay
SEM	Standard error of the mean

SPECIES

African catfish	*Clarias gariepinus*
African cichlid	*Haplochromis burtoni*
Atlantic salmon	*Salmo salar*
Black carp	*Micropogonias undulatus*
Black porgy	*Acanthopagrus schlegeli*
Bluehead wrasse	*Thalassoma bifasciatum*
Chinese loach	*Misgurnus anguillicaudatus*
Coho salmon	*Oncorhynchus kisutch*
Common carp	*Cyprinus carpio*
Dwarf gourami	*Colisa lalia*
European eel	*Anguilla anguilla*
European sea bass	*Dicentrarchus labrax*
Gilthead seabream	*Sparus aurata*
Goldfish	*Carassius auratus*
Japanese eel	*Anguilla japonica*
Masu salmon	*Oncorhynchus masou*
Platyfish	*Xiphophorus maculatus*
Rainbow trout	*Oncorhynchus mykiss*
Red seabream	*Pagrus major*
Striped bass	*Morone saxatilis*
Tilapia	*Oreochromis mossambicus*
	Oreochromis niloticus
White bass	*Morone chrysops*

CHAPTER 1. GENERAL INTRODUCTION

PROJECT RELEVANCE

Fish comprise more than 50% of known vertebrate species, making them the largest vertebrate group on earth. Due to the large habitat differences in salinity, temperature, pH, nutrition and photoperiods, different fish species have evolved a variety of morphological and physiological characteristics. One very important difference is the extreme diversity of reproductive strategies used to guarantee species survival. The regulation of reproduction in all vertebrates occurs via the hypothalamus-pituitary-gonadal (HPG) axis.

During the life of fish, distinct maturational stages can be identified that display different characteristics in the HPG axis. Juvenile animals must undergo the process of puberty to become sexually mature and reproduce. In adults, gamete development occurs in reproductive cycles, the length and number depending on the fish species.

One of the key factors controlling reproductive processes in fish is the feedback exerted by the gonads to the HP-portion of the axis via steroids or gonadal peptides. Despite extensive and ongoing effort, this is still a poorly understood mechanism. In particular, the mechanisms of the estrogenic versus androgenic pathways and their direct targets (brain and/or pituitary) in female models, is poorly researched. Further, the functional significance of multiple forms of gonadotropin-releasing hormones (GnRH) in vertebrate brains is not clearly elucidated to date. Especially, their regulation through pathways exerted by the gonads is not well known.

Understanding and controlling the reproductive cycle of fish is not only of great importance for basic biology and fish reproduction, but also essential for the aquaculture industry to develop domesticated broodstock and manipulate maturation and spawning to support continued growth in fish farming. However many fish species, including the striped bass, do not spawn spontaneously when raised in captivity. There is good evidence that the failure to undergo the stages of final gamete maturation, ovulation and spawning reflects a lack of gonadotropin (GtH) release from the pituitary (Zohar et al., 1995; Zohar et al., 1989). This is likely the result of a disruption in synthesis or release of GnRH occurring at the level of the brain. Consequently, final oocyte maturation, ovulation and spawning of captive fish can be induced by exogenous administration of synthetic GnRH analogues (Zohar et al., 1995; Zohar, 1996; Mylonas et al., 2001).

Another problematic factor in fish farming is the timing and/or completion of puberty. Many fish (especially salmonids) undergo precocious puberty, which leads to stunted growth, decreased flesh quality, and early mortalities (Gaillard et al., 2004). On the other hand, other species mature only after multiple years and quickly become a commercial burden due to the cost of maintenance and feeding prior to their productivity as broodstock (Okuzawa, 2002). Therefore, it is necessary to better understand the regulation and mechanisms governing the onset and progression of puberty, in order to be able to either delay puberty or induce spawning in captive fish and increase the commercial value of this industry.

Increased knowledge of the neuroendocrine mechanisms underlying puberty and the adult reproductive cycle will help pave the way for developing new techniques for maturation regulation and spawning induction to benefit aquaculture. Furthermore, it will

provide crucial insights into the basic regulation of reproduction in vertebrates and will facilitate the effort to better understand the plasticity of the HP axis.

REPRODUCTION IN FISH

Reproduction in vertebrates involves hormonally regulated processes such as growth and maturation of gonads and germ cells and insures the survival of a species.

Fish have evolved a wide variety of reproductive strategies including synchronous hermaphroditism, protandrous (sex change from male to female) and protogynous (sex change from female to male) hermaphroditism and gonochorism (Jalabert, 2005). The patterns of ovarian development vary depending on the reproductive strategies of the species and the following distinctions can be made: oviparous (external fertilization, embryo develops outside the mother), viviparous (internal fertilization, the embryo develops inside the mother, providing the growing embryo with nutrition), and ovoviviparous (internal fertilization, mothers lay yolky eggs which are retained in the oviduct until hatching) (Tyler et al., 1996). In the oviparous and ovoviviparous species, all nutrients are contained within the egg. This includes protein, lipids, energy, enzymes, but also vitamins and minerals necessary for embryo survival and development until the feeding stage (Tyler et al., 2000).

A common principle for all fish, however, is oogenesis, the production of large yolky eggs through the development of the oocyte (Tyler et al., 1996). Oocyte development can be divided into four main phases: oogonial proliferation, primary growth, in which oocytes arrest in the first meiotic prophase (Nagahama, 1983), including the alveolus stage and initiation of zonagenesis, secondary growth, involving

vitellogenesis and oocyte maturation, at which stage the reinitiation and completion of the first meiotic division and subsequent progression to metaphase II occurs, and ovulation (Tyler et al., 2000; Patino and Sullivan, 2002). However, these phases are only approximate and can easily overlap, since subsequent stages may need preparation in the ongoing stage (e.g., mRNA synthesis, see below) (Selman and Wallace, 1989).

Reproductive development in female fish

In adult fish, the ovaries are generally paired structures attached to the body cavity on either side of the dorsal mesentry (Nagahama, 1983). The structure of the growing ovarian follicle is remarkably similar in most fishes. The developing oocyte is located in the center of the follicle and is surrounded by the acellular layer, the *zona radiata* (*zr*) and further by a single layer of granulosa cells and a thin theca layer consisting of connective tissue, blood, and lymph capillaries (Figure 1). Granulosa and thecal cells play an integral role in steroidogenesis (Figure 2) and help modulate the transfer of yolk precursors into the oocyte during growth phases, oocyte maturation and ovulation (Nagahama et al., 1994).

The onset of primary oocyte growth is marked by synthesis of cytoplasmic organelles (e.g., mitochondria, multivesicular bodies, endoplasmic reticulum, and golgi elements) and an increased production of RNA in the nucleus, coding for proteins required for further oocyte growth, like vitellogenin receptors and yolk processing enzymes (Prat et al., 1998; Perazzolo et al., 1999). The synthesis of the *zona radiata* is also initiated during the primary growth phase (Hyllner et al., 1994). Sources of the *zr*-proteins are either the ovary or the liver, depending on the fish species, and their induction of synthesis appears to be E_2 regulated (Tyler et al., 2000). Cortical alveoli are

formed containing polysialoglycoproteins, which after fertilization will be released into the perivitelline space and harden, ultimately combining with the *zr* to form the protective vitelline envelope (eggshell). In the final stages of primary growth, lipid bodies appear and continue to amass throughout the subsequent growth phases (Wiegand, 1996).

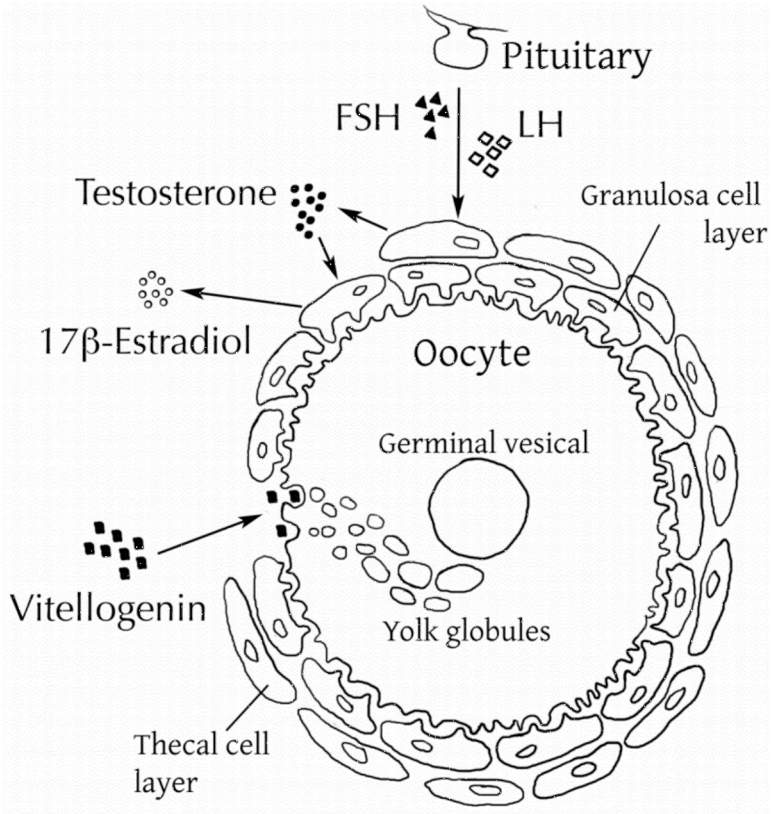

Figure 1: Generalized diagram of an ovarian follicle in fish. The follicle includes the testosterone producing theca cell layer and the estrogen synthesizing granulosa cell layer, which are regulated by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secreted by the pituitary. Hepatic vitellogenin is taken up by the follicle and is converted into yolk granules inside the oocyte (adapted from Nagahama et al., 1994).

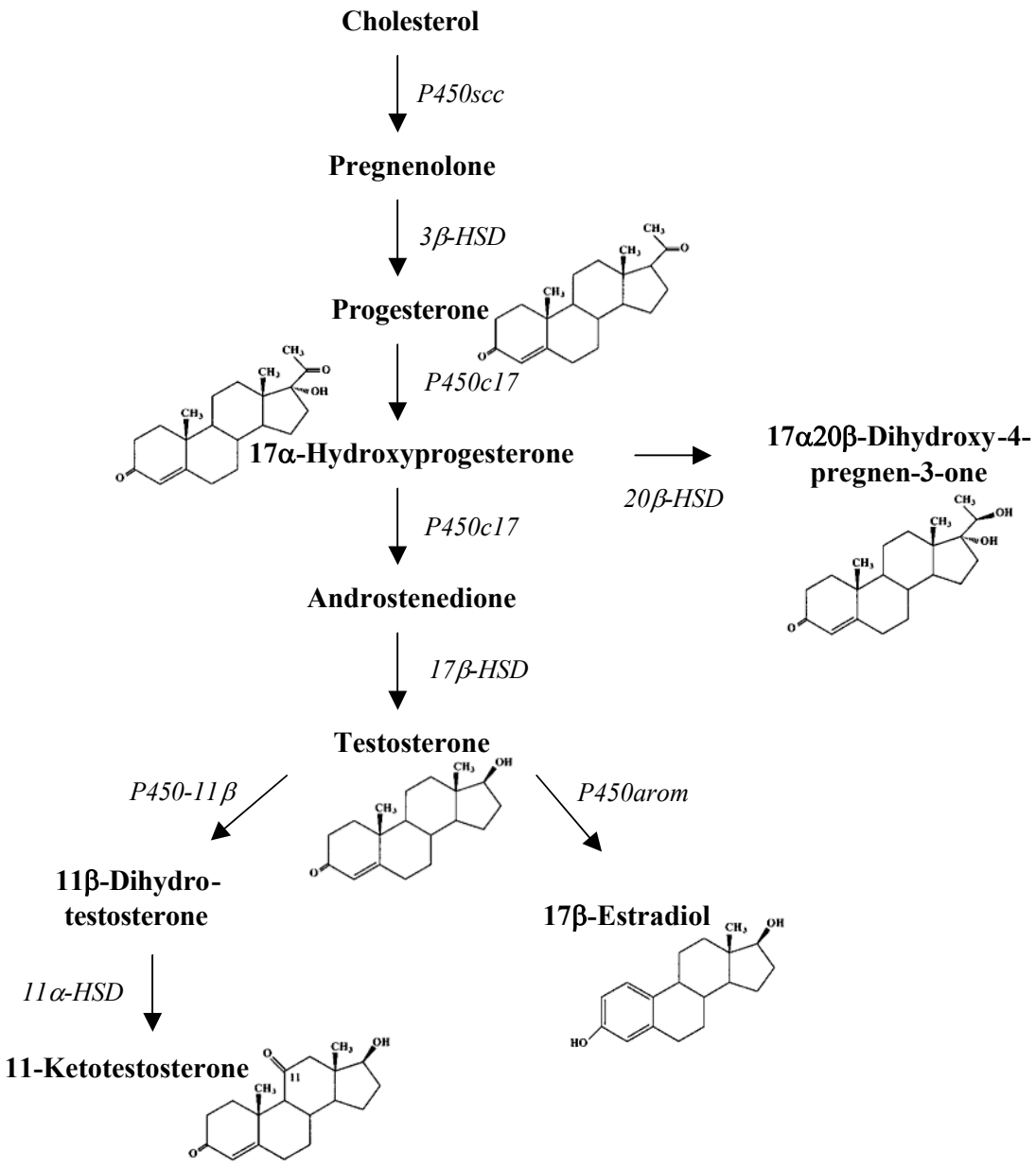

Figure 2: Steroidogenic pathways of androgens, estrogens and progestins in teleost. The key steroids are shown as chemical structure. *P450scc*: side-chain cleavage cytochrome; *3β-HSD*: 3β-hydroxysteroid dehydrogenase; *P450c17*: 17α-hydroxylase/C17-20 lyase cytochrome; *20β-HSD*: 20β-hydroxysteroid dehydrogenase; *17β-HSD*: 17β-hydroxysteroid dehydrogenase; *P450arom*: aromatase cytochrome; *P450-11β*: cytochrome P450-11β-hydroxylase; *11α-HSD*: 3α-hydroxysteroid dehydrogenase (adapted from Nagahama et al., 1994).

The secondary oocyte growth, vitellogenesis, is the stage responsible for the enormous increase in the size of oocytes in most teleost and can account for up to 90% of the final egg size (Tyler et al., 1991). Depending on the species pattern of ovarian

development, the duration of vitellogenesis can last for as little as 5 days (asynchronous spawner) or up to nine months (e.g., salmonids) (reviewed by Tyler et al., 1996). This stage primarily depends on yolk accumulation from vitellogenin (Vg) uptake. Vgs are synthesized in the liver under the endogenous stimulation of estrogens produced by granulosa cells (Sullivan et al., 1997; Tao et al., 1993) and released into the bloodstream. Vg binds to the oocyte surface and is internalized via receptor-mediated endocytosis (Wallace and Selman, 1990). These specific oocyte Vg receptors are clustered in clathrin-coated pits. Coated vesicles fuse with golgian lysosomes in the outer ooplasm of the oocytes and form multivesicular bodies (Le Menn et al., 2000). The golgian lysosomes contain cathepsin D, which breaks down Vg into its main components (e.g., lipovitellins, phosvitins and phosvettes) and processes them into yolk proteins (Carnevali et al., 1999; Carnevali et al., 2006). The number, types and forms of yolk proteins differ between teleost species. However, they are all typically stored in the form of globules or granules (Tyler et al., 2000). Teleost Vgs contain ~20% lipids (mostly triacylglycerols & phospholipids), which are the main component of membranes and serve as energy sources and intracellular mediators. In pelagic spawning teleosts, the mature oocyte contains oil droplets, which coalesce into one oil globule for buoyancy purposes (Tyler et al., 2000; Yaron and Sivan, 2006). In order for fertilization to occur, the oocytes must complete the first meiotic division. Full-grown oocytes will resume their first meiotic division under the appropriate hormonal and environmental stimulation (Yaron and Sivan, 2006). First meiotic division involves the breakdown of the germinal vesicle (GVBD), chromosome condensation, assembly of the first meiotic spindle, and extrusion of the polar body. Secondary oocytes then undergo the second meiotic division to become

mature eggs. Ovulation, that is, the rupture of the follicle and the release of the eggs into the ovarian lumen (Patino and Sullivan, 2002), follows.

The endocrine regulation of reproduction

Gonadotropins and ovarian steroid hormones regulate oocyte growth and maturation in teleosts and other vertebrates (Nagahama, 2000). Environmental changes (e.g., temperature and photoperiod) together with internal signals provide cues to the central nervous system that trigger the maturation processes. In response, the hypothalamus secretes GnRH, which is the central regulator of the reproductive hormonal cascade, and stimulates the release of GtHs from the pituitary. Two GtHs are secreted from the teleost pituitary. They are heterodimeric glycoproteins, which share an identical α-subunit and distinct β-subunits that confer the biological specificity of the respective GtH. Structurally, the glycoproteins are FSH-like and LH-like and are therefore referred to as FSH and LH in the teleost literature (Querat et al., 2001). LH appears to regulate final oocyte maturation in all fish studied thus far, while the relative roles of FSH and LH in regulating steroidogenesis during secondary oocyte growth varies with species (Yaron et al., 2003; Swanson et al., 2003). However in the salmonid model, FSH stimulates the uptake of Vg *in vivo* (Tyler et al., 1991) and has been shown to recruit oocytes into the process of vitellogenesis (Tyler et al., 1997).

GtH secretion is regulated by a feedback mechanism via E_2 and T (Peter and Yu, 1997). E_2 is the major estrogen in female teleost, but large amounts of the androgen T is also produced by the ovary. In the ovarian two-cell model, the theca cells synthesize T (see Figure 1), which is subsequently aromatized by cytochrome P450 aromatase to E_2 by the granulosa cells (Kagawa et al., 1982; Nagahama, 2000). E_2 stimulates the production

of Vg and *zr*-proteins in the liver of female fish (Hyllner et al., 1991; Oppen-Berntsen et al., 1992a; Oppen-Berntsen et al., 1992b). Ovarian steroidogenesis is also stimulated by GH, somatolactin, and IGF-I, which have also been shown to play important roles in regulating ovarian development in a variety of fish species [GH (Singh et al., 1988; Van der Kraak et al., 1990); somatolactin (Planas et al., 1992); IGF-I (Le Gac et al., 1993; Maestro et al., 1997; Kagawa et al., 2003)].

Key-Regulatory Elements of the Reproductive Axis

GnRH

The decapeptide gonadotropin-releasing hormone plays, as mentioned above, a key role in reproduction. This neuropeptide is synthesized in the hypothalamus and regulates synthesis and release of the GtHs. GnRH also acts as a neuromodulator in the brain and as a paracrine factor in peripheral tissues, especially the gonads (Habibi et al., 1994a; Peng et al., 1994). To date, 23 different forms of GnRH have been structurally identified (Table 1) (Millar, 2005).

It has been well established that all vertebrates, including humans, express two forms of GnRH in the brain (Lescheid et al., 1997; White et al., 1998). One is the highly conserved and ubiquitous form of GnRH II (formerly called chicken GnRH II); the other form, GnRH I, is species-specific and more divergent. Highly evolved fish, such as perciforms were reported to have three forms of GnRH (Powell et al., 1994; Gothilf et al., 1995; White et al., 1995; Okuzawa et al., 1997). The three variants are derived from three distinct genes (White and Fernald, 1998; Chow et al., 1998) and are expressed in

different areas of the fish brain as shown by Gothilf et al. (1996) in the gilthead seabream: GnRH I (seabream GnRH) is expressed in the preoptic area (POA),

Table 1: Amino acid sequences of naturally occurring GnRHs. The boxed regions show the conserved NH_2 and COOH terminal residues, which play important functional roles. Here the GnRHs are named according to the species in which they were first discovered and they may be represented in more than one species. However, a new terminology based on the location of expression uses the terms GnRH I, II and III to describe the variants found in a species and is used in the text. (adapted from Millar, 2005).

	1	2	3	4	5	6	7	8	9	10	
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	NH_2
Guinea Pig	pGlu	Tyr	Tyr	Ser	Tyr	Gly	Val	Arg	Pro	Gly	NH_2
Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly	NH_2
Rana d.	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly	NH_2
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly	NH_2
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly	NH_2
Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly	NH_2
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly	NH_2
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly	NH_2
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly	NH_2
Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	NH_2
Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly	NH_2
Lamprey I	pGlu	His	Trp	Ser	His	Glu	Trp	Lys	Pro	Gly	NH_2
Chelyosoma I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly	NH_2
Chelyosoma II	pGlu	His	Trp	Ser	Leu	Cus	His	Ala	Pro	Gly	NH_2
Ciona I	pGlu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	Gly	NH_2
Ciona II	pGlu	His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	Gly	NH_2
Ciona III	pGlu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	Gly	NH_2
Ciona IV	pGlu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	Gly	NH_2
Ciona V	pGlu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	Gly	NH_2
Ciona VI	pGlu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	Gly	NH_2
Ciona VII	pGlu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	Gly	NH_2
Octopus	pGlu	Asn	Tyr	His	Phe	Ser	Trp	His	Pro	Gly	NH_2

GnRH II in the midbrain tegmentum (MT), and GnRH III (salmon GnRH) in the olfactory bulb-terminal nerve area (OB-TN). The same pattern of distribution was also observed in other perciform species [tilapia (*Oreochromis mossambicus*) (Parhar et al., 1998); European sea bass (*Dicentrarchus labrax*) (Gonzalez-Martinez et al., 2001)]. In teleosts, GnRH neurons from the POA innervate the pituitary directly, and fibers either

synapse on the gonadotrophs or end in close vicinity to them (Anglade et al., 1993; Mousa and Mousa, 2003). In mammals, one of the crucial characteristics of GnRH release activity is a coordinated, pulsatile secretion of the peptide simultaneously from many axon terminals located in the median eminence of the mediobasal hypothalamus (Hotchkiss and Knobil, 1994). Although pulsatility of GnRH is believed to be evident in all vertebrates (Dellovade et al., 1998), there is no conclusive evidence in teleost fish at present.

Regulation of GnRH

Many physiological factors, e.g., photoperiod, sexual behavior, stress and gonadal maturation, influence the GnRH system of vertebrates in the brain in order to control reproduction appropriately. Mediators for these effects include various substances that are involved in regulation of GnRH synthesis and release, such as glutamate and γ-aminobutyric acid (GABA) (Trudeau et al., 2000), dopamine (DA) (Dufour et al., 2005), serotonin (Khan and Thomas, 1993; Senthilkumaran et al., 2001) neuropeptide Y (NPY) (Li et al., 1999), leptin (Cunningham et al., 1999; Foster and Nagatani, 1999) and steroid hormones. Although this is not an exhaustive list, it does show that many regulatory factors are known to influence the reproductive process at the GnRH level (for mammals reviewed by Herbison, 2006). However, this research will focus on elucidation of mechanisms of steroid regulation of GnRH expression and release.

In mammals, ovariectomy increases GnRH I secretion and estrogen replacement reverses this effect (Terasawa, 1995; Levine et al., 1995). The preovulatory GnRH I surge, on the other hand, is induced by a positive feedback of E_2 and progesterone (P) (Caraty et al., 1995; Terasawa, 1995; Levine et al., 1995). In parallel with the increased

GnRH I secretion prior to ovulation, there is an increase in GnRH I gene expression (Zoeller and Young, 1988; Gore and Roberts, 1995). The involvement of gonadal steroids in the regulation of the GnRH I gene expression in mammals is controversial. Castration of male rats typically resulted in an increase in GnRH I mRNA levels (e.g., Toranzo et al., 1989; Malik et al., 1991) and T treatment induced a decrease (Toranzo et al., 1989; Li and Pelletier, 1995). However, following ovariectomy in the female, GnRH I mRNA levels have been reported (in obvious disagreement) to decrease (Kim et al., 1989), increase (Roberts et al., 1989; Toranzo et al., 1989) and to be unaffected (Kelly et al., 1989), and E_2 treatment was shown to decrease (Zoeller et al., 1988; Toranzo et al., 1989) or increase these mRNA levels (Roberts et al., 1989; Kim et al., 1989; Rosie et al., 1990; Weesner et al., 1993; Petersen et al., 1996). Differences in results may arise from the use of different techniques to measure GnRH gene expression or peptide levels, differences in species or strains, the reproductive stage, and/or the age of the animal. In addition estrogen, for example, exerts both negative and positive feedback effects on the GnRH I-producing neurons. Therefore, the discrepancies in results may be due to the ability of estrogen to stimulate or inhibit the neuroendocrine axis, depending upon the experimental model.

The regulation of GnRH II, which has been reported in the brain of rodents, primates and humans (Lescheid et al., 1997; White et al., 1998; Chen et al., 1998; Gestrin et al., 1999), is less clear. Studies in the musk shrew showed that GnRH II immunoreactivity was increased after ovariectomy, suggesting that ovarian steroids may modulate the release of GnRH II (Rissman and Li, 1998). Recent studies by Chen et al., (2002) in human neuronal medulloblastoma cells (TE-671) and Khosravi & Leung (2003)

in human granulosa luteal cells, demonstrated a differential regulation of GnRH I and II by estrogen. E_2 treatment increases endogenous GnRH II mRNA levels and decreases endogenous GnRH I mRNA levels, suggesting that regulation of GnRH I and II by E_2 in humans may be similar in the brain and the ovary.

Studies of the effects of gonadal steroid feedback on the GnRH system in fish are still few in number, even though they are some of the most probable candidates for the regulation of the GnRH system in teleosts. Reports on positive feedback in male fish are the most common. Treating male and female bluehead wrasse with 11-ketotestosterone (11-KT), the major androgen in teleosts, induced an increase in the number of GnRH I immunoreactive cells in the preoptic area (Grober et al., 1991). In male masu salmon, treatment with 17α-methyltestosterone induced precocious puberty and a three-fold increase in GnRH I mRNA-expressing cells in the POA, while GnRH III cell number in the olfactory bulb or terminal nerve was not affected (Amano et al., 1994a). In male African catfish, T and E_2 exerted a positive influence on the amount of GnRH I (Dubois et al., 1998; Dubois et al., 2001). T treatment of castrated male tilapia reduced GnRH I mRNA levels, elevated GnRH III mRNA levels, and had no effect on expression of GnRH II (Soga et al., 1998). The same researchers treated immature, castrated male tilapia with E_2, 11-KT and thyroid hormone and found that 11-KT had no effect on the expression of any GnRH form. E_2 increased GnRH I mRNA levels, but had no effect on GnRH II and III (Parhar et al., 2000). However, a study done in immature and mature male red seabream, showed that implantation with 11-KT increased brain GnRH I mRNA levels, while implants with E_2 and T had no effect (Okuzawa et al., 2002). Studies in female fish, on the other hand, are rather scarce. In female silver eels, E_2 induced the

increase in the level of brain and pituitary GnRH I, but had no effect on GnRH II levels (Montero et al., 1995). In prepubertal female red seabream, E_2 and T (the two major sex steroids of females) do not affect the brain GnRH I mRNA expression, whereas 11-KT, which is found in low concentrations in the serum, exerts a positive feedback on GnRH I expression (Okuzawa et al., 2002). A recent study in male black porgy showed that the injection of GnRH antagonist abolished E_2 stimulation of LH release *in vivo*, clearly demonstrating that the E_2 effect was mediated by GnRH control of LH release (Yen et al., 2002).

Since the GnRH neurons are able to react to steroids, it is presumed that GnRH neurons contain estrogen and/or androgen receptors (ERs and ARs). Both steroid receptors were identified in fish brain (Pakdel et al., 2000; Touhata et al., 1999), but have not been shown to co-localize with the GnRH neurons (Navas et al., 1995; Gelinas & Callard 1997).

In mammals, GnRH neurons have been shown to express functional estrogen receptor β (ERβ) (Hrabovszky et al., 2000; Hrabovszky et al., 2001; Kallo et al., 2001), providing evidence for possible direct regulation by estrogen. However, receptor expression levels are fairly low. Rapid actions via this receptor have been reported by Abraham et al. (2003), who showed phosphorylation of cAMP-response element binding protein in mouse GnRH neurons in response to E_2.

Therefore it is still presumed that gonadal steroids mainly regulate GnRH neuron activity indirectly through trans-synaptic transmission: ERα-expressing neurons located in the preoptic area, as well as within the mediobasal hypothalamus and caudal brainstem, project to GnRH cell bodies and regulate GnRH synthesis and release in

response to estrogen. The neuro-chemical identity of the estrogen-receptive primary afferents to GnRH neurons includes GABA, glutamate, NPY, and norepinephrine. However, this is not an exclusive list. Many neurons in the brain exhibit rapid nongenomic response to E_2 and may be primary afferents within the GnRH network (reviewed by Herbison, 2006). Kisspeptin (KP) and its receptor GPR54 have been recently reported to be a new regulator in the neuroendocrine control of the HPG axis. KP is critically important in many mammalian species for the release of GnRH, and appears to act directly via the GnRH neurons. In addition, recent data suggests that KP-secreting neurons are the 'missing link' between gonadal steroids and the GnRH neuronal network, facilitating both positive and negative feedback control depending on the location of the KP-expressing neurons (reviewed by Smith et al., 2006). Furthermore, the expression of GPR54 receptor in GnRH neurons has been shown in tilapia by Parhar et al. (2004), giving evidence for a similar system in teleost fish. However, fish kisspeptins have not been reported to date.

A third mechanism through which estrogen feedback can exert control over the GnRH network is via glial and endothelial cell transmission, since it has been shown that glial cells participate in gonadal steroid-dependent plasticity (Garcia-Segura and McCarthy, 2004). Glial cells may control directly the connectivity of GnRH neurons and even subtle modulations of the physical relationship between glial cells and GnRH neurons may exert substantial impact on GnRH neuron connectivity. Evidence has been reported for seasonal (Jansen et al., 2003), pubertal (Witkin et al., 1997), and gonadal hormone-dependent (Witkin et al., 1991) correlations between glial cell apposition and GnRH neuron activity. Furthermore, GnRH terminals are enveloped by tanycytic end-

feet, which build a physical barrier influencing GnRH secretion by allowing more/less GnRH to reach the portal vasculature (Prevot, 2002). This mechanism appears also to be involved in estrous cycle regulation of GnRH release (Prevot et al., 1999b). Moreover, estrogen-stimulated growth factor secretion by glial cells plays an active role in determining GnRH secretion (Melcangi et al., 2002; Prevot, 2002; Ojeda et al., 2003b). These pathways include epidermal growth factor family members, like transforming growth factor (TGF)-α, neuregulins, and TGF-β family members.

Another possibility is that gonadal steroids influence the membrane excitability of GnRH neurons in a direct manner (El-Etr et al., 1995; Lagrange et al., 1995, Herbison and Pape, 2001). For example, Kelly et al. (2003) showed a steroid modulation of potassium channel activity in GnRH neurons that resulted in hyperpolarization and activation of a variety of signal transduction pathways. Estrogen modulation of GnRH neuron firing patterns (Nunemaker et al., 2002; Temple et al., 2004), as well as involvement of the nitric oxide signaling pathways (Prevot et al., 1999a) were also observed.

The GnRH-Receptor

GnRH exerts its effects through binding to the GnRH receptor (GnRH-R), a plasma membrane-bound receptor belonging to the family of G protein-coupled receptors. Among the signal transduction mechanisms activated by the receptor are phosphatidylinositol hydrolysis and mobilization of intracellular Ca^{2+} stores, as well as production of diacylglycerol and activation of protein kinase-C (Kraus et al., 2001). To date, cDNAs encoding GnRH-R have been cloned from several vertebrates including teleosts. The known receptors can be grouped into three distinct classes based on

phylogenetic tree analysis (Types I, II, and III). To date, all three cognate receptor types have been found in amphibians. Only Type I and II have been found in mammals, and only Type I and III have been found in perciform fish (Millar et al., 2004; Levavi-Sivan and Avitan, 2005). However, contrary to the mammalian GnRH-R Type I, all piscine GnRH receptors have a C-terminal cytoplasmic tail (Blomenrohr et al., 2002) that is involved in GnRH binding and desensitization through rapid internalization of the GnRH-R (Lin et al., 1998). Of the endogenous GnRHs present in fish, GnRH II exhibits the highest potency in activating the GnRH-R second messenger systems, with GnRH I and III also showing significant potency (Alok et al., 2000; reviewed in Weltzien et al., 2004). In the pituitary, *in situ* hybridization and immunocytochemistry studies have demonstrated the GnRH receptor in gonadotroph, somatotroph and lactotroph cells, depending on the fish species investigated (reviewed by Levavi-Sivan and Avitan, 2005). However, information available to date regarding the regulatory mechanisms of the expression of GnRH-R in teleosts is only limited.

In mammals, pulsatile GnRH I stimulation is essential for appropriate GnRH-R expression levels, at the same time avoiding receptor down-regulation due to continuous hormonal stimulation (Liu et al., 2003). The changes in GnRH I pulse frequency and amplitude vary during puberty, the estrous cycle and menopause, which results in changing GnRH-R levels (Norwitz et al., 1999). Therefore, it appears that the regulation of GnRH-R expression is mainly exerted via estrogen and GnRH itself (Hapgood et al., 2005). These two substances had synergistic effects on ovariectomized ewes, in which the pituitary gland was disconnected from the hypothalamus, resulting in an increased number of GnRH-R and increased GnRH-R mRNA (Kirkpatrick et al., 1998). Thus, E_2

may increase GnRH-R by increasing GnRH release from the hypothalamus and/or by directly influencing GnRH-R at the anterior pituitary gland. These two mechanisms for increasing GnRH-R mRNA concentrations, i.e., a direct stimulatory effect and augmentation of responses to pulsatile GnRH, were also shown for both male and female rats by Yasin et al. (1995). Contradicting these results, GnRH-R mRNA levels were not influenced by steroid treatment for 7 d in cows (Looper et al., 2003). Differential GnRH-R expression patterns throughout the estrous cycle were reported in sheep and rats. Concentrations of GnRH-R mRNA increased during the preovulatory period in sheep (Brooks et al., 1993; Turzillo et al., 1994) and rats (Bauer-Dantoin et al., 1993). Bauer-Dantoin et al. (1995) suggested that enhanced GnRH-R mRNA levels on the day of proestrous are largely due to the facilitatory actions of E_2, exerted indirectly through hypothalamic routes. But the duration of exposure to steroids influences mRNA expression levels of GnRH-R. Acute treatment of ovariectomized ewes with E_2 increased GnRH-R mRNA (Hamernik et al., 1995; Turzillo et al., 1995). In contrast, chronic treatment with E_2 for 7 d decreased GnRH-R mRNA in ovariectomized rats (Kaiser et al., 1993) and also significantly decreased the GnRH-R transcript levels in human ovarian surface epithelial cells (Kang et al., 2001). GnRH-R promotor experiments suggest that transcription is the predominant mechanism of estrogen up-regulation of the GnRH-R numbers in the pituitary (Hapgood et al., 2005). Besides steroids, other gonadal factors like activin and inhibin also modulate GnRH-R synthesis and numbers in the pituitary. Activin-A increases GnRH-R synthesis in rat pituitary cell cultures (Braden and Conn, 1992) and decreases receptor numbers in sheep pituitary cultures (Gregg et al., 1991). Inhibin was found to prevent the stimulation of receptor synthesis by GnRH I in rat

pituitary cultures (Braden et al., 1990), but increases GnRH-R mRNA levels (Wu et al., 1994) and receptor numbers (Gregg et al., 1991) in sheep pituitary cultures.

Research conducted with teleost models showed that GnRH agonist treatment of immature juvenile red seabream caused a significant increase in GnRH-R mRNA levels, compared to control groups, suggesting that GnRH itself is a positive regulator of its own receptor (Okuzawa et al., 2002). These observations are supported by work done by Levavi-Sivan et al. (2004), who showed that GnRH up-regulates its own receptor *in vivo* and *in vitro* in tilapia. The same study also reported that GnRH-R levels were higher in females than males, suggesting higher estrogen levels in the female as an explanation for the sex difference. Moreover, they observed that dopamine has a negative effect on GnRH-R mRNA levels. Steroid treatment of juvenile red seabream with E_2 or 11-KT decreased levels of GnRH-R mRNA, compared to controls, suggesting that steroids exert a negative effect on GnRH-R expression (Okuzawa et al., 2002). In the African catfish, castration in males resulted in a two-fold increase in the pituitary GnRH-R binding capacity, and replacement with androstendione reversed the increase of the binding capacity (Habibi et al., 1989).

The GtH-System

The regulation of GtH biosynthesis and release has been the focus of intense study in the reproductive biology field. However, many of the mechanisms controlling this step in the reproductive axis are still not well understood. *In vivo* and *in vitro* approaches have been used to elucidate the differences between direct regulatory effects at the level of the pituitary or the indirect regulation via the hypothalamic GnRH network.

Gonadal feedback regulation is a primary control mechanism of GtH release *in vivo*. Both androgen and estrogen maintain an inhibitory effect on LH and FSH secretion through the inhibition of GnRH pulsatile release, although direct effects on gonadotrophs have also been observed. The gonadal peptides, inhibin, activin, and follistatin, predominantly regulate FSH secretion, producing both stimulatory (activin) and inhibitory (inhibin, follistatin) effects and may be important in the differential regulation of LH and FSH (Jeong and Kaiser, 2006).

Removal of the gonads in female rats results in a slow rise of LH plasma levels, which are not significant until Day 2 or 3 post-surgery (Dalkin et al., 1993; Burger et al., 2001). At the same time, α-subunit and LHβ mRNA levels start to increase, continuing their rise through day 21. However, FSH appears to be differentially regulated with a different magnitude and time course of changes. FSH plasma and transcript levels increase rapidly after ovariectomy, with plasma levels doubling after 8 hours and FSHβ expression by 30–60 minutes, continuing to rise until day 7 (Dalkin et al., 1990; Dalkin et al., 1993; Burger et al., 2001). The increase in GnRH secretion after ovariectomy is an important regulator of subunit transcription. Suppression of GnRH by administering either GnRH antagonist or estradiol *in vivo*, resulted in a significant decrease in α-subunit and LHβ mRNA synthesis rates, but had no effect or only a modest negative effect on FSHβ in long-term ovariectomized rats (Shupnik et al., 1988; Shupnik, 1990; Fallest et al., 1995).

While the α-subunit and LHβ expression after ovariectomy appear primarily to be controlled by GnRH, regulatory mechanisms of FSH transcription are more complex. Gonadal peptides may play an important role, especially in light of data showing that

inhibin suppresses FSHβ transcription (Burger et al., 2001). Moreover, this process occurs faster than the decrease in FSHβ mRNA levels after GnRH antagonist administration, suggesting that inhibin may also affect FSHβ mRNA stability.

The rise in LHβ mRNA levels after ovariectomy can be rapidly suppressed by administration of estrogen (Shupnik et al., 1988). This post-ovariectomized rise in LHβ transcription was also abolished by treatment with GnRH antagonist. Additional treatment with E_2 had no additive effect. These results suggest that E_2 regulates LHβ via the GnRH system in the hypothalamus (Dalkin et al., 1993; Shupnik and Fallest, 1994; Fallest et al., 1995; Burger et al., 2001). However, estrogens also exert direct action on the pituitary. E_2 rapidly increases LHβ mRNA synthesis in pituitary cells from ovariectomized rats (Shupnik et al., 1989a; Shupnik, 1996), and in pituitary cells from cycling rats, with the greatest effect in cells from rats in proestrous (Shupnik et al., 1989a).

The focus on steroid effects on FSHβ transcription has not been as intensive as the studies on LHβ regulation. Nevertheless, it appears that regulation of FSHβ differs between species and involves other factors besides GnRH and sex steroids (Jeong and Kaiser, 2006). Studies *in vivo* have shown that E_2 suppresses the post-gonadectomy rise in FSHβ mRNA levels in the rat (Shupnik et al., 1988). This also occurs via the GnRH system, since co-treatment with GnRH antagonist prevented suppression by estrogen (Shupnik and Fallest, 1994). However, this steroid had no effect on FSHβ transcription in female pituitary fragments (Shupnik et al., 1989a). In contrast to the rat, estrogen decreased both steady-state FSHβ mRNA levels and synthesis in cultured ovine pituitary cells (Phillips et al., 1988; Baratta et al., 2001). E_2 also inhibited the activity of an ovine

FSHβ promotor-LUC construct transfected into ovine pituitary cells (Miller and Miller, 1996). Overall, estrogen has been conclusively shown to be a primary player in steroid feedback at the pituitary level throughout vertebrates.

Extensive research has been conducted to elucidate the steroid regulation of GtHs using fish as an *in vivo* model. Results from these studies are somewhat contradictory and seem to vary depending on the fish species, the reproductive stage, gender and other experimental characteristics. Dickey and Swanson (1998) showed that FSH plasma levels in coho salmon decreased in response to T and E_2, but 17α,20β-dihydroxy-4-pregnen-3-one was ineffective. In contrast, Borg et al. (1998) found a positive testicular feedback on FSH and LH in Atlantic salmon (*Salmo salar*) male parr, in which pituitary levels decreased after castration and steroid replacement with 11-KT increased plasma and pituitary FSH. T, however, suppressed FSH before the spawning season (July) and increased FSH during the spawning season (November). T implants used after the spawning season prevented a decrease in plasma LH levels, but had no effect on FSH (Antonopoulou et al., 1999), indicating that steroid feedback effects on FSH can be positive or negative depending on the reproductive stage of the fish. This phenomenon was also observed in sexually recrudescent and preovulatory female common carp and Chinese loach, in which T potentiates LH release in response to GnRHa (Trudeau et al., 1991). In an *in vitro* approach using isolated primary pituitary cells from female goldfish at different reproductive stages, exposure of T overnight did not affect basal LH release but did increase GnRH-stimulated LH release (Lo and Chang, 1998). Another important consideration for the study of steroid feedback is that gonadotrophs may have aromatizing capabilities (Melamed et al., 2000) and therefore T may be aromatized to E_2

in treated fish, making it difficult to determine if the effects are derived through an androgenic or estrogenic pathway. This control again is species-specific. For example, in the protanderous black porgy, LH levels in the control and T treatment were similar, but in E_2-injected fish the LH levels were significantly higher (Lee et al., 1999; Du et al., 2001).

Regulation of the GtH subunits by steroids has also been a focus of many studies. Treatment with T and other aromatizable androgens or E_2 increased LHβ mRNA levels in goldfish (Kobayashi et al., 2000), coho salmon (Dickey and Swanson, 1998), European eel (Querat et al., 1991; Schmitz et al., 2005), and European sea bass (Mateos et al., 2002). In agreement with this data, treatment with T or E_2 increased pituitary LH levels in rainbow trout (Breton et al., 1997), Atlantic salmon parr (Antonopoulou et al., 1999), coho salmon (Dickey and Swanson, 1998), and European sea bass (Mateos et al., 2002). In goldfish, physiological doses of E_2 stimulated alpha, FSHβ and LHβ *in vivo* and *in vitro*, during all stages of gonadal development (Huggard-Nelson et al., 2002). In contrast to this data, both steroids, aromatizable T and E_2, decreased FSHβ mRNA levels in coho salmon (Dickey and Swanson, 1998) juvenile or recrudescing goldfish (Sohn et al., 1998; Kobayashi et al., 2000), the European sea bass (Mateos et al., 2002) and the European eel (Schmitz et al., 2005). Detailed studies in tilapia indicated that regulation of FSHβ expression is very sensitive to steroid levels. Sex steroid levels that were too low or too high caused low levels of FSHβ, and only fish with moderately low levels (T/E_2 of 1 ng/ml) have high levels of FSHβ mRNA. Fish with high steroid levels coincided with high levels of LHβ, and in regressed males a positive correlation between circulating T and LHβ mRNA levels was seen (Melamed et al., 2000). *In vitro* work using tilapia

pituitary cells showed that in immature males FSHβ increased after incubation with T (10 nM) for 36-48 hr. In maturing males, low T doses (10 – 100 nM) caused an increase in FSHβ mRNA (Melamed et al., 1997). At the end of the spawning season, higher doses of steroids (T/E_2; 1 – 100 nM) decreased FSHβ mRNA levels and an addition of T or E_2 (10 pM – 100 nM) did not change LHβ mRNA levels (Melamed et al., 2000). Exposure of regressed tilapia to T led to a dose-dependent increase in LHβ mRNA, but no change in FSHβ mRNA was seen; exposure to 11-KT had no effect on FSHβ/LHβ mRNA levels (Melamed et al., 1998). These studies show that T has a similar effect to that of E_2 on the transcription of GtH subunits and that non-aromatizable androgens lack such an effect, thus suggesting that T exerts its effects through aromatization to E_2.

In most fish species, the effects of sex steroids are thought to be exerted by E_2 in both, males and females. Evidence of this is found via experiments where non-aromatizable androgens or aromatase inhibitors abolish the steroid effect (Melamed et al., 1998; Antonopoulou et al., 1999; Rebers et al., 2000; Cavaco et al., 2001). In contrast to this hypothesis, DHT caused an increase in α-subunit and LHβ mRNA levels and decreased FSHβ expression, which was the same result observed after T and E_2 treatments in European sea bass (Mateos et al., 2002). Moreover, treatment with T and non-aromatizable androgens elevated LHβ and LH in the European eel, whereas E_2 had no effect (Huang et al., 1997). These results support the hypothesis of an androgen-specific effect. To date, there is a large amount of available information regarding steroid effects on LH and FSH in fish, but it is partially contradictory. Most work is done *in vivo* and therefore distinctions between the direct effects of steroids at the level of the pituitary and indirect effects mediated by hypothalamic (GnRH) or other agents are difficult to

distinguish. Furthermore, the difference in gender, species and reproductive stage of the various model organisms in these studies makes it difficult to compare the results and gain a meaningful understanding of the complex role of steroid regulation of the gonadotropins.

PUBERTY IN MAMMALS AND FISH AND ITS ENDOCRINE CONTROL

In vertebrates, the phase of acquiring maturational competence and successfully reproducing for the first time is called puberty (Terasawa and Fernandez, 2001). This involves the activation of the HPG axis, which controls the process of gonad maturation in the adult. The key regulator at the level of the hypothalamus is GnRH, which controls synthesis and release of FSH and LH through its receptor on gonadotroph cells.

In mammals, GnRH is released in a pulsatile fashion, which is required for GtH secretion (Plant, 2002). The anatomical development and synthetic capacity of the GnRH neuronal network occurs very early in life (reviewed by Ebling, 2005), but is followed by a period of quiescence. The onset of puberty reactivates the GnRH pulse generator, but the neurobiological mechanisms triggering this event are not fully understood yet (Plant and Witchel, 2006). It is believed that the GnRH neuronal system is a passive entity in the pubertal initiation of GnRH release and that changes in neuronal and glial networks connected to the GnRH neurons are the cause for the onset (Ojeda et al., 2003a).

The inhibition of GnRH release in the juvenile phase appears to be primarily due to the neurotransmitter GABA (Plant, 2001). GABA is the primary inhibitory neurotransmitter in the mammalian brain and declines with the onset of female puberty (Mitsushima et al., 1996). During the pubertal process the inhibitory tone of GABA

declines and, concomitantly, GnRH release-stimulating neurotransmitters, like glutamate and norepinephrine (Terasawa et al., 1999; Terasawa and Fernandez, 2001) increase. Moreover, the GnRH system acquires stimulatory synaptic input via structural remodeling by the surrounding glial cells. Glial ensheathment, the extension and withdrawal of astrocytic processes, serve to modulate synaptic contacts of the GnRH cell bodies, axons and terminals (Mahesh et al., 2006), and they also directly regulate GnRH neurons through growth factors like transforming growth factor (TGF) alpha/beta and neuregulin (Ojeda and Terasawa, 2002). This structural plasticity plays a permissive role in mediating the loss or gain in synaptic input to GnRH neurons observed at the onset of puberty.

The timing of puberty in mammals and fish depends on many internal and external factors controlling the reactivation of the HPG axis. Information about growth, body fat/composition, stress, annual change in photoperiod, circadian time, gonadal steroids, energy status and olfactory cues has to be conveyed to the GnRH neuronal system to induce the onset of puberty (Okuzawa, 2002; Ebling, 2005). Therefore many neurotransmitters have been implicated to play a role in triggering puberty: growth hormone (GH), insulin-like growth factors (IGFs), leptin (Cheung et al., 2001), kisspeptins (Smith et al., 2006).

The specific trigger and timing for the onset of puberty has not been identified in any vertebrate as of yet (Schulz and Goos, 1999), but two conceptual frameworks have been proposed to explain the initiation of puberty. In the gonadostat concept, low levels of sex steroids released by juvenile gonads exert negative feedback, which is responsible for the inhibition of GnRH release prior to puberty in mammals. At puberty, the GnRH

neuronal system looses the sensitivity to this feedback and an increase in GnRH release leads to an activation of the PG axis and subsequent maturation of the gonads (Ryan et al., 1988). The missing-link concept refers to the inability of one or more components of the HPG axis to respond/function prior to puberty (Goos, 1993). However, many studies in fish have shown that gonadal steroids stimulate all levels of the HPG axis in juvenile and adult animals (reviewed by Dufour et al., 2000; Montero and Dufour, 1996; Okuzawa, 2002). These observations suggest that the gonadostat concept is not relevant to fish and that the production of sex steroids and/or the expression of their receptors are part of the missing link for the initiation of puberty (Schulz and Goos, 1999).

In the African catfish, *Clarias gariepinus*, sex steroids have been proposed to be essential for the puberty-associated activation of the HPG axis (Schulz and Goos, 1999). Dufour et al. (2000) suggests that positive steroidal feedback may play an important role as an "amplifier", but is not a trigger for the onset of puberty. Steroid feedback effects on the HP axis are very complex and vary greatly between teleost species, depending on maturational stage, and the sex of the fish (Dufour et al., 2000). Treatment with estrogens and/or testosterone increased GnRH neuron numbers in the POA of male masu salmon, *Oncorhynchus masou*, (Amano et al., 1994b) and male tilapia, *Oreochromis niloticus*, (Parhar et al., 2000). Further, steroids positively influenced the hypothalamic GnRH content in male African catfish, (Dubois et al., 1998; Dubois et al., 2000) and female silver eel, *Anguilla anguilla*, as well as pituitary content in the eel (Montero et al., 1995). In juvenile male and female red seabream, *Pagrus major*, implantation with 11-ketotestosterone for one month increased hypothalamic GnRH mRNA levels, while E_2 and T had no effect (Okuzawa et al., 2002).

Studies examining steroid feedback on FSH in juveniles have produced various results, depending on the model. In prepubertal coho salmon, *Oncorhynchus kisutch*, steroids exerted a negative effect on FSH *in vivo* (Dickey and Swanson, 1998) and a similar effect was observed in juvenile rainbow trout, *Oncorhynchus mykiss*, (Saligaut et al., 1998) and the Japanese eel, *Anguilla japonica*, (Yoshiura et al., 1999). No *in vitro* effects were observed in the rainbow trout (Xiong et al., 1994), but positive effects were reported in tilapia (Melamed et al., 1997).

Positive feedback effects on LH synthesis and release in juvenile teleosts have been reported in numerous studies: in platyfish (Schreibman et al., 1986), goldfish (Huggard et al., 1996), European eel (Querat et al., 1991), black carp (Gur et al., 1995) and the European sea bass (Mateos et al., 2002). Moreover, steroids enhance LH release in response to GnRH in goldfish (Trudeau et al., 1991) and thus may contribute to the onset of puberty, however the FSH levels in these species are not known, which makes an evaluation much less meaningful.

The initiation of puberty is also dependent on the size of the fish and its body composition. Le Gac et al. (1993) provided an in depth discussion of the close relationship between growth and reproduction. Metabolic hormones and growth factors act on the entire HPG axis (Ojeda and Skinner, 2006). It has been shown in pituitary cells from juvenile female European eel, that insulin-growth factor-I (IGF-I) stimulates synthesis and release of LH (Huang et al., 1998). It also increased the FSH content and GnRH-stimulated FSH release from juvenile coho salmon *in vitro* (Baker et al., 2000a), and enhanced the sensitivity to GnRH-induced FSH and LH release in rainbow trout pituitary cells (Weil et al., 1999). The hormone leptin signals the state of lipid storage to

the hypothalamus and may be important in the onset of puberty in mammals (Ebling, 2005). Even though endogenous leptin has not been identified in fish at this point, studies using "human leptin" have been conducted in fish. In the European sea bass (Peyon et al., 2001) and rainbow trout (Weil et al., 2003), positive effects on FSH and/or LH release have been shown *in vitro*. However, no clear *in vivo* effects were observed in juvenile coho salmon (Baker et al., 2000b).

Similar to mammalian inhibition by GABA, studies conducted in juveniles suggest that dopamine (DA) may play an inhibitory role in the control of puberty (Dufour et al., 2005). Studies in the juvenile silver eel showed that only the combination of GnRHa, T and pimozide caused significant increases in LH synthesis and release. Therefore, it appears that in the prepubertal eel the removal of DA inhibition is required to trigger GnRH-stimulated LH synthesis and release, as well as ovarian development (reviewed in Dufour et al., 2005). However, studies by Holland et al. (1998b) examining combined treatments with testosterone (T), GnRH agonist and a DA-D_2 receptor antagonist (pimozide) in juvenile striped bass provided no evidence of DA involvement in the control of puberty in this species. In the red seabream, administration of GnRHa alone was able to induce precocious puberty, with no further effect of DA antagonist, also indicating that DA does not play a discernable role in puberty (Kumakura et al., 2003). Earlier work in salmonids induced precocious sexual maturation using GnRHa and steroid treatments, thus providing further evidence for a minor or nonexistent involvement of DA inhibition in the control of puberty (Crim and Evans, 1983).

Understanding the regulation of puberty in fish is very important. Even though many studies have been conducted to investigate the regulation and timing of teleost

puberty, the general mechanisms controlling the onset of puberty are still not known (Okuzawa, 2002). Besides understanding the basic biology of fish reproduction, puberty plays an essential role in aquaculture and fish farming (Goos, 1993). Several commercially important species (sturgeon, striped bass, carp, eel), take many years to mature, which involves financial costs for feed, broodstock maintenance, and time until these species are ready to produce gametes for harvest purposes. Acceleration of puberty and maturation at an earlier stage would improve cost-efficiency greatly. Moreover, preventing precocious puberty in farmed fish species, like salmonids, which results in stunted growth and decreased flesh quality (Gaillard et al., 2004), would also be a great benefit to the aquaculture industry.

STRIPED BASS AS A MODEL

The striped bass (*Morone saxatilis*) is an anadromous, oviparous perciform fish. This teleost exhibits a group-synchronous pattern of ovarian maturation in which a single clutch of oocytes is recruited through secondary growth, final maturation and ovulation comprising a single annual spawning event (reviewed by Sullivan et al., 1997). These animals spawn once during the spring of each year, and thus there is a distinct cyclic annual pattern of ovarian development with its endocrine correlates. The timing of reproduction in striped bass, as in other teleost, is triggered by photothermal cues such as diurnal and seasonal changes in temperature and day length (Blythe et al., 1994b; Blythe et al., 1994a).

The recruitment of primary oocytes into early secondary growth occurs during August/September and determines the recrudescent stage. Small oocytes [diameter

< 250 μm; (Sullivan et al., 1997)] and low plasma levels of steroid hormones are observed during this phase (Holland et al., 2000). During vitellogenesis, the oocyte grows (250 ~ 750 μm) by storing lipid droplets and egg yolk, which are vitellogenin-derived products (Tao et al., 1993). Concomitantly, steroid hormone production increases to stimulate vitellogenin uptake (Berlinsky and Specker, 1991; Sullivan et al., 1997) (Figure 3).

In striped bass, sustained low levels of E_2 and T characterize most of the oocyte growth. However, these levels are consistently greater that the basal non-detectable levels seen in reproductively quiescent animals just prior to gonadal recrudescence in immature fish (Tao et al., 1993; Sullivan et al., 1997; Mylonas et al., 1998; Holland et al., 2000). In all *Morone* species examined to date, there is an abrupt and sustained rise in circulating E_2 and T levels in the month prior to spawning (Mylonas et al., 1998). This late-cycle surge of plasma steroid levels may be involved in feedback to the HP axis, inducing synthesis and release of LH, possibly at the expense of FSH production (Sullivan et al., 1997; Hassin et al., 1999). However, since we still lack an assay to measure circulating FSH in *Morone* species, there is no support for this hypothesis. Maturation of competent follicles involves a shift in steroidogenic synthesis products, from production of E_2 and T to production of C_{21} steroids (Sullivan et al., 1997; Mylonas et al., 1997a; Weber and Sullivan, 2000; Patino et al., 2001; Weber and Sullivan, 2001), which include the final maturation-inducing steroid (MIS) hormones, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) and 17α,20β,21,trihydroxy-4-pregnen-3-one (20β-S). The MIS bind to specific receptors on the oocyte surface and induce maturation via the induction of a maturation-promoting factor (Nagahama et al., 1994).

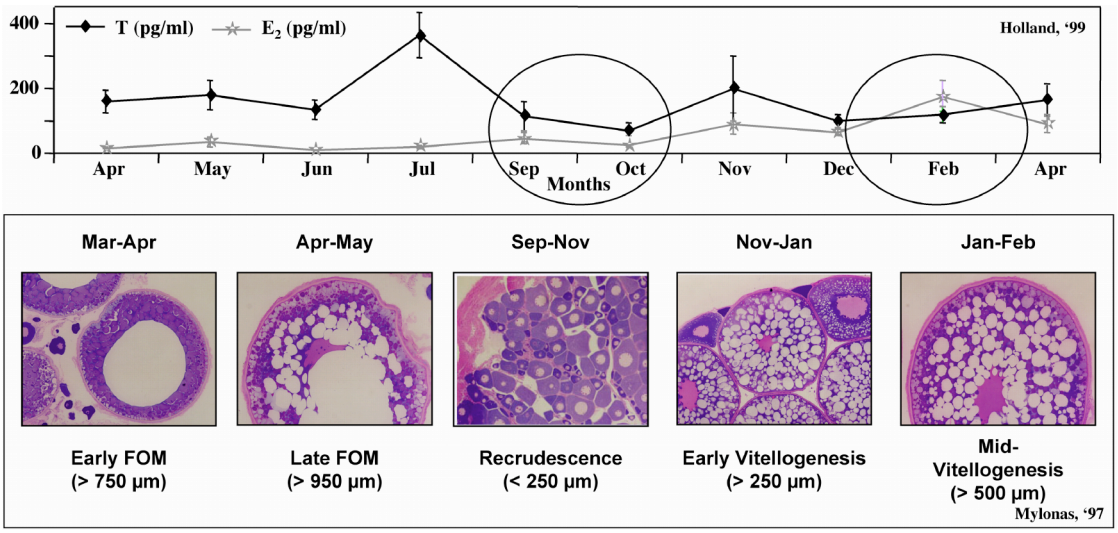

Figure 3: The top shows the estrogen and testosterone plasma profiles of adult female striped bass over the duration of one reproductive cycle (data adapted from Holland, 1999). The bottom part depicts histological sections of striped bass oocytes undergoing different stages of the reproductive cycle, with corresponding oocyte diameters indicated beneath each stage (adapted from Mylonas et al., 1997d). The circles in the hormone profile represent the experimental timepoints chosen for our investigation (Chapter 4 and 5) and the hormone levels typically observed during the stages of recrudescence and midvitellogenesis.

The endocrine axis has been well studied in striped bass. Three forms of GnRH and their distribution have been described by Gothilf et al. (1995) and the gonadotropin genes for FSH and LH have been cloned (Hassin et al., 1995). With this information, assays for GnRH peptides, LH protein and FSHβ and LHβ transcription levels have been developed and used to describe the endocrine processes occurring during puberty in this species. Striped bass females display a relatively late puberty. The majority of captive females initiate gonadal development at 3 years of age. Although oocyte diameter increases during the third year, full maturity may not be reached until 1 or 2 years later, indicating that complete maturation may take several years in this species (Holland et al., 2000).

GnRH I is the dominant form found in striped bass pituitaries and shows, together with GnRH II, a positive correlation to oocyte diameter and GSI during development

(Holland et al., 2001). In adolescent fish, GnRH I and II correlated to a greater extent to GSI and LH pituitary content than to FSHβ and LHβ mRNA levels, even though they had been shown to be directly stimulated by GnRH in adult striped bass (Hassin et al., 1998). These results suggest that gonadotropin function and gonadal development in striped bass is regulated by GnRH I and/or GnRH II. However, it appears that the availability of GnRH in the pituitary is not limiting the onset of puberty: GnRH I and II were abundantly present in 1-year-old females and immature and mature siblings (2- and 3-year-old females) were similar, even though gonadal growth was only observed in maturing animals (Holland et al., 2001). Therefore, the timing of puberty may depend on other maturational processes in the HPG axis.

The elevated GnRH levels in 1-year-old females did not result in an increase in FSHβ or LHβ transcription, thereby demonstrating that there is no responsiveness at the level of the pituitary to the hypothalamic GnRH stimulation. However, FSHβ and LHβ mRNA and LH protein levels were elevated in 2-year-old female fish, which suggests that pituitary activation has occurred and that the increased gonadotropin activity was the result of an increase in GnRH release (Hassin et al., 1999; Holland et al., 2001).

FSH appears to play a pivotal role during puberty in female striped bass. Levels of FSHβ mRNA were significantly lower in adolescent 3-year-olds compared to maturing 4-year-old females. Furthermore, 3-year-old maturing fish displayed significantly higher levels than their immature siblings. These results suggest that the maturation state depends on gonadotropic activity, especially that of FSHβ expression (Hassin et al., 1999). The developing gonad may also be a key player in the transformation process from juvenile to adult animal. Low levels of FSHβ and LHβ in juveniles, together with

the lack of seasonality in the oocyte growth profiles, suggest that the early developmental phase is not under pituitary gonadotropin control (Hassin et al., 1999; Holland et al., 2000). This is supported by observations *in vitro*, where ovaries of 2-year-old females displayed limited responsiveness to gonadotropin stimulation (Holland et al., 1998b).

The striped bass and its hybrids are important fish for aquaculture in the USA. However, these species exhibit a major reproductive dysfunction in culture. Adult females will complete vitellogenesis, but fail to reach maturational competence and ovulation. The GnRH system was identified as the cause for the block in reproduction in captivity. Disruption of the system can be reversed by exogenous administration of synthetic GnRH analogues, which induces final ooctye maturation, ovulation and spawning in captive fish (Mylonas et al., 2001). Due to the popularity of this species in aquaculture, many aspects of its reproductive physiology have been studied (see above), however, there are still several mechanisms underlying the neuroendocrine control of the reproductive cycle that are largely unknown. Additional investigations are needed to further our understanding of these mechanisms and to develop improved techniques for spawning induction.

PROJECT OBJECTIVES AND ORGANIZATION OF THE STUDY

The primary objectives of this study were: 1) to measure the endocrine correlates of the hypothalamus-pituitary axis after gonadectomy and steroid replacement in juvenile, pubertal and adult female striped bass, in order to better understand the ontogeny of gonadal steroid feedback regulation occurring during different stages of the life and reproductive cycle in our model organism, 2) to determine the direct

responsiveness of the GnRH network in the brain and that of its receptor and the GtH system in gonadotroph cells in the pituitary towards estrogen regulation *in vitro*.

The first objective is addressed in Chapters 2, 3 and 4 (see Table 2). A detailed time series study of the response of the adult female GnRH-GtH system to gonad removal and estrogen replacement over an experimental period of 28 days is reported in Chapter 2. Based on the results obtained in this study, the experimental set-up, used in the following studies (Chapter 3 and 4), was designed. In Chapter 3, the reaction of the HP axis to gonadal feedback and steroid treatment in the juvenile and pubertal striped bass is described and discussed, in view of the processes occurring during the transformation from the immature to the adult animal. This study was comprised of two experiments. The first was conducted with juvenile female striped bass at three different stages of their development (13-, 19-, 23-months old) and the effects of gonadectomy and steroid replacement with E_2 and 11-KA biodegradable microspheric delivery systems, as well as treatment of intact animals with the same steroids, were determined. In the second experiment, pubertal female and male striped bass, 28-months old, were gonadectomized and also treated with E_2 and 11-KA replacement therapy. In both experiments, the endocrine correlates such as expression levels of the three GnRH variants, the GnRH-R, FSHβ and LHβ subunits, as well as LH protein levels, were examined. To analyze steroid feedback control and steroid regulation in the adult female, two different stages of the reproductive cycle were chosen, in order to elucidate distinctive changes of the GnRH-GtH system. These results are reported in Chapter 4. Two experiments were conducted at the stages of recrudescence and midvitellogenesis of ovarian development. Effects of gonadectomy and steroid replacement on GnRH and GtH hormone synthesis and release

and expression of the GnRH-R were investigated and discussed by comparing the results obtained for the two reproductive stages. The second objective was addressed in Chapter 5 (Table 2). A brain-slice culture method was developed to determine the direct effects of steroids (estrogen) on GnRH neurons in the brain. Further, a dispersed pituitary cell culture method was used to discern effects of steroid feedback directly at the level of pituitary gonadotrophs. These *in vitro* experiments were performed twice: the first time using tissue obtained from recrudescent females and the second with tissues from midvitellogenic females, matching the stages investigated *in vivo* (Chapter 4). Results obtained for the three variants of GnRH, the GnRH-R and FSHβ and LHβ subunit expression were compared between the two stages of oogenesis and also to the results reported *in vivo*, thereby providing an examination of direct versus indirect effects of steroid regulation of the HP axis. Finally, in Chapter 6 the major findings of these studies are summarized and discussed in relation to the maturational processes of female striped bass going through puberty and the adult reproductive cycle. A model of endogenous gonadal feedback and responsiveness of the HP axis to exogenous steroid stimulation was developed and is described.

Table 2: Organizational summary of study components. Individual rows provide an overview of, respectively, the type of studies, treatment regimes and model organisms (stages) used. Columns reflect the chapter in which the pertinent results are reported.

Chapter	2	3		4		5	
Type of Study	*in vivo* times series gonadectomy	*in vivo* gonadectomy		*in vivo* gonadectomy		*in vitro* brain-slice culture pituitary cell culture	
Treatment Regime	estrogen	estrogen 11-ketandrostenedione		estrogen 11-KA	testosterone	estrogen	
Model	midvitellogenic female hybrid bass	juvenile female striped bass	pubertal male and female striped bass	recrudescent female striped bass	midvitellogenic female striped bass	recrudescent female striped bass	midvitellogenic female striped bass

CHAPTER 2. STEROID FEEDBACK REGULATION OF THE HYPOTHALAMUS-PITUITARY AXIS OF ADULT FEMALE HYBRID STRIPED BASS: A TIME SERIES STUDY

ABSTRACT

The goal of our study was to establish a baseline for the response of the hypothalamus-pituitary axis to the removal of gonadal feedback over an extended period of time in a female teleost fish. To determine the effects of bilateral gonadectomy and 17β-estradiol (E_2) replacement, we monitored GnRH synthesis and release, as well as pituitary GnRH-R and GtH subunit expression, and further pituitary/plasma LH protein levels. Adult female hybrid striped bass undergoing midvitellogenesis were divided into two gonadectomized (gdx) groups and one sham group. One gdx group received E_2 (2 mg/kg) via microspheric delivery systems; the other was given a vehicle injection. Groups were sampled on days 3, 6, 9, 14, and 28 post-surgery (dps). The synthesis of all three forms of GnRH was not regulated by an acute steroidal feedback, however, long-term effects were seen in decreasing GnRH I and III transcript levels in both gdx and E_2 treatment groups. Due to gonad removal, GnRH I peptide levels decreased after 9 dps until the end of the experiment, however, this effect was rescued by estrogen. GnRH II peptide concentration decreased after 9 dps in the E_2 replacement group only, suggesting a negative regulation by this steroid. No effects on GnRH III peptide levels were observed. Expression of the GnRH-R was suppressed in both treatment groups starting at 9 dps, suggesting the removal of a positive feedback (gdx group) as well as a negative regulation by estrogen (E_2 replacement group). FSHβ transcription continually increased

in the gdx group during the experimental period. On the other hand, estrogen dramatically reduced FSHβ mRNA levels compared to the sham group, indicating an estrogenic negative feedback exerted on this GtH subunit during midvitellogenesis. Neither treatment had an effect on LHβ synthesis until 28 dps, when E_2 suppressed LHβ synthesis, suggesting an absence of gonadal feedback control of LHβ transcription at this stage. LH protein levels in the pituitary were only affected at the end of the experiment (28 dps, decreased in the E_2 replacement group). After gdx, LH plasma levels increased 9 dps and remained elevated until 28 dps and this effect was restored in the E_2 replacement group, indicating that LH release is controlled by a negative estrogenic feedback. In conclusion, both, positive and negative gonadal feedback regulation of the HP axis is observed during midvitellogenesis, depending on the hormone and in view of synthesis or release. Based on our results, it appears that after 9 dps, a relatively stable steady-state in the hormonal response is achieved. Thus, future experiments will be terminated around that timepoint.

INTRODUCTION

It has been well established that gonadal steroid hormones exert critical regulatory feedback actions on the activity of the hypothalamic-pituitary (HP) axis in vertebrates. They may provide either positive or negative feedback signals modulating the synthesis and release of reproductive hormones. However, the mechanisms through which gonadal steroids exert their modulatory actions on the HP axis are not completely clear. In the hypothalamus the key target for steroid regulation is the gonadotropin-releasing hormone (GnRH) neuronal network (Okuzawa et al., 2002; Sagrillo et al., 1996).

GnRH, a decapeptide, is the neuroendocrine link between environmental influences and the reproductive hormonal cascade. All vertebrates express two forms of GnRH in the brain (White et al., 1998) and highly evolved fish, such as perciforms, have three forms of GnRH (Okuzawa et al., 1997). The three variants are derived from three distinct genes (Chow et al., 1998) and are expressed in different areas of the fish brain (Gothilf et al., 1996): GnRH I is expressed in the preoptic area (POA), GnRH II in the midbrain tegmentum (MT), and GnRH III in the olfactory bulb-terminal nerve area (OB-TN). All three forms induce the release of gonadotropins (GtH) from the pituitary when administered endogenously (reviewed by Van der Kraak et al., 1998), however not all variants are directly involved in pituitary regulation. Further, in fish, the GnRH neurons of the POA directly innervate the anterior pituitary (Kah et al., 1993), and therefore it is hypothesized, that GnRH I, which is expressed in these neurons, regulates the gonadotrophs. Differences in the regulatory function of the multiple GnRHs are based on their location of expression (Oka, 2002).

Even though many studies have been conducted in mammals to investigate the role of gonadal steroid feedback to the GnRH-GtH system the results are very contradictory. Reason for the conflicting results reported, may be the differences in species and gender or in the experimental design (reviewed by Sagrillo et al., 1996). Studies investigating the effects of steroid feedback on GnRH expression and release in fish show similar discrepancies. Though there are only a small number of studies available, results are contradictory showing positive (Dubois et al., 1998; Dubois et al., 2001; Okuzawa et al., 2002; Parhar et al., 2000) or negative effects (Soga et al., 1998) or

no influence at all (Okuzawa et al., 2002; Parhar et al., 2000; Soga et al., 1998) of steroids.

Furthermore, steroid hormones play a pivotal role in modulating the synthesis and number of the GnRH receptor (GnRH-R) in the pituitary (Hapgood et al., 2005; Levavi-Sivan and Avitan, 2005). The responsiveness of gonadotroph cells to GnRH depends on the number of GnRH-Rs on the cell surface. Gonadal steroid feedback also targets the expression and release of the GtHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), themselves (Yaron et al., 2003; Burger et al., 2004). These proteins are either regulated directly at the level of the pituitary or indirectly via the hypothalamic GnRH system. But despite intensive research, the detailed feedback mechanisms by which they are controlled are still unknown. Most studies to date have only focused on a small part of the HP-axis, studying either the GnRH system (synthesis and/or release), focused on the GnRH-R transcription and/or receptor numbers on the gonadotroph cells, or studied GtHs synthesis and/or secretion.

A central issue in the field is to better understand the complex response of the multiple key players of the HP axis to steroid feedback regulation. Furthermore, a comparison between short-term and long-term effects of steroid removal on the HP axis has never been reported in fish. Therefore, we investigated the timely response of the GnRH-GtH system to gonad-removal and steroid replacement in a teleost fish model, the female hybrid striped bass [a cross between striped bass, *Morone saxatilis*, and the white bass, *M. chrysops*, (Mylonas et al., 2001)] to establish a baseline study for further investigations. The hybrid bass is an important species for fisheries and aquaculture

(Harrell, 1997). Females, undergoing synchronous gametogenesis, spawn annually once maturity is reached at about 2 years of age.

MATERIAL AND METHODS

Experimental Animals

Hybrid striped bass were purchased from Hallwood Fisheries, Hallwood, VA in fall 2002 and transferred to the Center of Marine Biotechnology's Aquaculture Research Center, Baltimore, MD, when the fish were 2 years old. The fish were maintained in 6 foot diameter recirculation systems, at 8 ppt salinity, under a simulated natural photo- and thermoperiod regime. The animals were kept under these conditions until the beginning of the experiment. All animals at the Aquaculture Research Facility were maintained and sampled according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland Biotechnology Institute.

Treatment Protocols

Bilateral gonadectomies were carried out according to procedures modified from Billard et al. (1977). Fish were anesthetized (70 ppm phenoxyethanol, Baker Inc., Phillipsburg, NJ), bled, weighed and placed on a surgical table, and their gills continuously irrigated with oxygenated water containing anesthesia. Using a scalpel, a 2–4 cm incision was made along the midline between the pectoral and pelvic fins. The ovaries were detached from the abdominal membranes and removed. The incision was sutured using 2.0 silk thread (Burns Veterinary Supply, Inc., Owings Mills, MD) and closed with tissue glue (Burns Veterinary Supply, Inc.). Post-operation, fish were

returned to their original tank systems. The extracted gonads were weighed for calculation of the gonado-somatic index (GSI; [gonad weight/body weight] x 100%). Control fish were given a sham-operation (s-o) receiving an incision and suture, but the ovaries were not removed.

In November 2002, female fish undergoing midvitellogenesis [average fluke length 58 cm, average weight 1.4 kg, GSI 4.2%, average oocyte diameter 650 μm, see (Figure 3)] were divided into three groups (Figure 4). The first group (n = 30) was given a sham-operation and the other two groups (n = 47 / per group) were gonadectomized. After surgery, the intact s-o females and one gonadectomized group were given a vehicle injection (Sham; GDX), the other gonadectomized group received E_2 steroid replacement (GDX+E_2; 2 mg/kg) via microspheric delivery systems (modified from Holland et al., 1998b). A commercial Finfish Bass Broodstock diet (Zeigler, Gardners, PA) was fed twice daily. Fish from each group were sacrificed on days 3, 6, 9, 14 and 28 post-surgery.

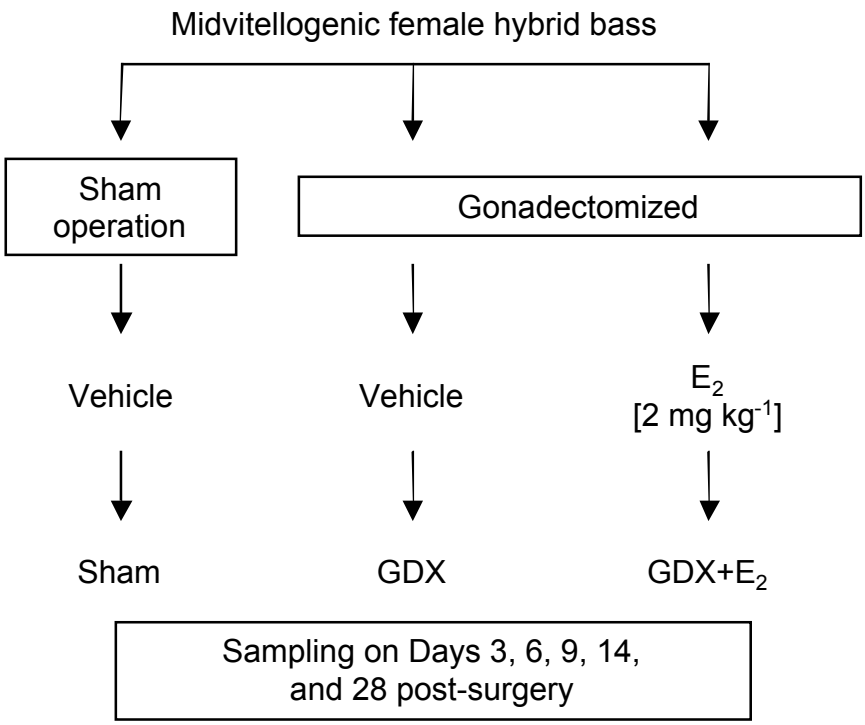

Figure 4: Midvitellogenic female hybrid time series study. Diagram of the experimental design.

Upon sacrifice, brains and pituitaries were removed and snap-frozen in liquid nitrogen. Blood samples were collected using heparinized syringes and immediately mixed with aprotinin (Sigma-Aldrich, St. Louis, MO) at the final concentration of 0.84 TIU/ml. After centrifugation, plasma was collected and all samples were stored at –80 ˚C until further analysis.

Quantification of Transcript Levels of GnRHs, GnRH-R, FSHβ, and LHβ

Transcript levels of the three GnRHs in the brain, as well as for the GnRH-R, FSHβ, and LHβ in pituitaries were measured using real-time fluorescence-based quantitative RT-PCR assays.

To generate RNA standards, plasmids containing each of the above-mentioned relevant cDNAs, were linearized and gene-specific RNA was transcribed. The reaction

mixture (20 µl) included 1 µg of linearized template cDNA, 1 mM of ATP, GTP, CTP and UTP, 10 mM DTT, 20 units RNAse inhibitor and T3 or T7 RNA polymerase (Invitrogen, CA, USA). Plasmid DNA was removed after 2 hours of incubation at 37 ˚C, by 1 unit RNase-free DNase (Promega, WI, USA). Addition of 40 µl of 5 mM EDTA (pH 8.0) stopped the reaction. RNA was then purified through a size exclusion column, (Chroma Spin-400; BD Biosciences, CA). For 18s standards, total RNA was isolated from 20 pituitaries, using Tri-reagent (MRC Inc., Cincinnati, OH), a modified acid-phenol extraction method, followed by DNAse treatment. RNA was pooled post-extraction. The amount of each RNA standard was measured using RiboGreen RNA quantification kit (Molecular Probe, Eugene, OR).

Total RNA, isolated from whole brains or pituitaries using Tri-reagent was quantified spectrophotometrically. RNA standards and total RNA from each sample were reverse-transcribed into cDNA using random hexamers and MMLV reverse transcriptase (Promega). Duplicate cDNA aliquots (1 ng of total RNA for 18s and LHβ RNA; 10 ng for FSHβ and GnRH-R; 50 ng for all GnRH transcripts) from each sample served as templates in PCR. The reaction was performed using SYBR Green PCR core reagent (Applied Biosystems, Forster City, CA) containing 200 mM gene-specific primers (Table 3). Primers were designed to span intron/exon boundaries in order to avoid amplification of genomic DNA. Amplification reactions were carried out in a ABI Prism® 7700 Sequence Detection System at 50 ˚C for 4 min, 95 ˚C for 10 min, and 40 cycles of 95 ˚C for 15 s and 60 ˚C for 60 s. Copy numbers in unknown samples were determined by comparing C_T, the fractional cycle number at which fluorescence passes a baseline

threshold value (Fink et al., 1998), to the specific standard (run in every plate) and normalized to the amount of 18s RNA in each sample.

Table 3: List of oligonucleotides used as specific primers in RT-PCR assays.

	Primer	Nucleotide sequence	Ampliconsize (bp)	Reference GeneBank
GnRH I	UK 210	5′-GGAACGGACGGCCTCTCA-3′	56	AFO56314
	UK 2	5′-GTGGGAAGCCCCCGACTA-3′		
GnRH II	UK 211	5′-AAGCAGCCCAAACAGTAAAACC-3′	127	AF056313
	UK 212	5′-CTGACCAACTGAAGGACAAGCA-3′		
GnRH III	UK 207	5′-TGGAGTCATCATTAATTACATTGTATGG-3′	134	submitted
	UK 208	5′-GAAGAGAAGTGTGGGAGAGCTAGAG-3′		
FSHβ	UK 215	5′-TGGCCTCACCGAGGTCAT-3′	113	L35070
	UK 216	5′-CAGTCTCCGTTACAGATTCTCTGTTC-3′		
LHβ	UK 161	5′-CTTGGGACAGCCCTCCTTCT-3′	111	L35096
	UK 162	5′-CTGGGAGCCACATCTGACAT-3′		
GnRH-R	UK 167	5′-GGGATGAGTGTTGTGCTGTCA-3′	156	AF218841
	UK 168	5′-CCACGTGTTGTACACTGAGTGAAG-3′		
18s RNA	TSB 18sF	5′-ACCACCCACAGAATCGAGAAA-3′	98	AY587263
	TSB 18sR	5′-GCCTGCGGCTTAATTTGACT-3′		

Quantification of Peptide Levels of GnRHs and LH Protein Levels

In pituitaries, all GnRHs were measured using ELISAs that have been developed to specifically measure peptide levels in perciform fish (Holland et al., 1998b). These GnRH ELISAs are classical competitive enzyme immunoassays, which are highly specific, with very little or no cross-reactivity of antibodies (0.005 – 3.7%), sensitivities up to around 10 pg/well, and intra- and interassay coefficients of variations (CV) below 10% (Holland et al., 1998b). Briefly, pituitaries were sonicated on ice in 200 µl phosphate buffer. After sonication, 140 µl of homogenate were extracted with an equal volume of 4 N acetic acid. Samples were frozen, lyophilized and reconstituted in assay buffer. The samples or appropriate standards were incubated with antiserum and specific GnRH tracer in a microtiter plate well for 72 h at 4 °C. Colorimetric reagent was added

and after 3 h of incubation the optical density was read in an automatic plate reader at 405 nm (Thermomax, Molecular Devices, Menlo Park, CA).

Pituitary LH content was measured using a homologous ELISA designed to specifically quantify the striped bass LHβ subunit (Mananos et al., 1997). The range of the standard curve is 0.78 to 10 ng/ml, and the intra- and interassay CVs for this assay are 7.7% and 8.7% respectively. The antigen-antibody complexes were detected by addition of goat anti-rabbit IgG horseradish peroxidase conjugate (GAR-HRP, affinity purified EIA grade, BioRad) and TMB peroxidase substrate (KPL, Inc., Gaithersburg, MD). Absorbencies were read at 450 nm, using an automatic microplate reader (Thermomax, Molecular Devices).

Quantification of Plasma LH and Estrogen Levels

Blood samples from experimental fish were immediately centrifuged, and plasma was separated and stored at –80 °C until further processing for steroid hormone quantification. Plasma LH content was measured using a homologous radioimmunoassay (RIA) according to Blaise et al. (1996). Levels were measured in duplicate and all plasma samples were run in a single RIA to eliminate interassay variation. The intra-assay CV for the LH RIA is 4.6%.

Plasma E_2 was measured using a commercially available solid-phase RIA that measures total amount of hormone in unextracted, heparinized plasma (DPC, Los Angeles, CA). This assay has been validated for use with striped bass plasma and assay characteristics have been previously described (Mylonas et al., 1997b).

Statistical Analysis

The data on brain GnRH transcripts, GnRH peptides in pituitary, and transcripts of GnRH-R, FSHβ, and LHβ, as well as protein levels of LH in pituitary and plasma E_2 levels, were subjected to Analysis of Variance (ANOVA) to determine the existence of changes over time or between experimental groups (one-way and two-way ANOVA). To specifically identify statistical differences among means of each of the above parameters, the means were further compared using Scheffe'e post hoc test. Statistical significance was set at $P < 0.05$.

RESULTS

There were no mortalities in any of the control or experimental groups. The completeness of gonadectomy was verified in each fish upon sacrifice. In none of the fish remaining gonadal tissue was observed.

Estrogen Levels in the Plasma

Estradiol levels in s-o fish ranged between 118.52 ± 24.6 up to 266.27 ± 61.65 pg/ml over the 28 day long experimental period, with no statistical difference detected. In the GDX group estradiol levels had dropped below the detection limit of the RIA after 3 dps and did not change until day 28 post-surgery. Levels of estradiol in the GDX+E_2 group reached values of 2.33 ± 0.3 ng/ml and maintained this level until the end of the experiment (range between 2.30 ± 0.12 to 2.57 ± 0.14 ng/ml). These levels are normally seen in striped bass undergoing stages of late vitellogenesis (Mylonas et al., 1997c) and are therefore considered to be in a physiological range for this model organism.

GnRH Expression Levels in the Brain

To investigate the effects of gonadectomy and in vivo E_2 replacement therapy on the transcripts of the three GnRHs, GnRH-R, FSHβ and LHβ, quantitative PCR assays were established and used for measuring changes in the mRNA levels of each gene. In the assays, the basal line was set between 3 and 15 cycles (default setting) for all transcripts except for 18s RNA (between 3 and 8 cycles). The correlation coefficient for each standard curve in each reaction plate was between 0.95 and 0.99. Negative controls (ddw) showed no significant amplification. The level of detection for each transcript was set at the lowest standard point with a significantly different amplification (significantly lower C_T value) than the controls. The setting for each transcript was 480 copies/reaction for all three GnRHs and 600/6000 copies for FSHβ, LHβ and the GnRH-R. After normalization with the amount of 18s RNA, the results were presented as copy number of transcript per ng total RNA.

Neither gonadectomy nor E_2 replacement had an effect on GnRH I mRNA levels after 3, 6, 9 or 14 dps (Figure 5A). After 28 days, however, GnRH I expression was significantly down regulated in both treatment groups. Expression of GnRH II was not influenced by either of the treatments for the duration of the experiment (Figure 5B). After 14 days post surgery mRNA levels of GnRH III were significantly reduced in the GDX+E_2 group until the termination of the experiment. Gonadectomy also reduced GnRH III mRNA levels significantly, but only in the long-term gonadectomized animals (28 dps) (Figure 5C).

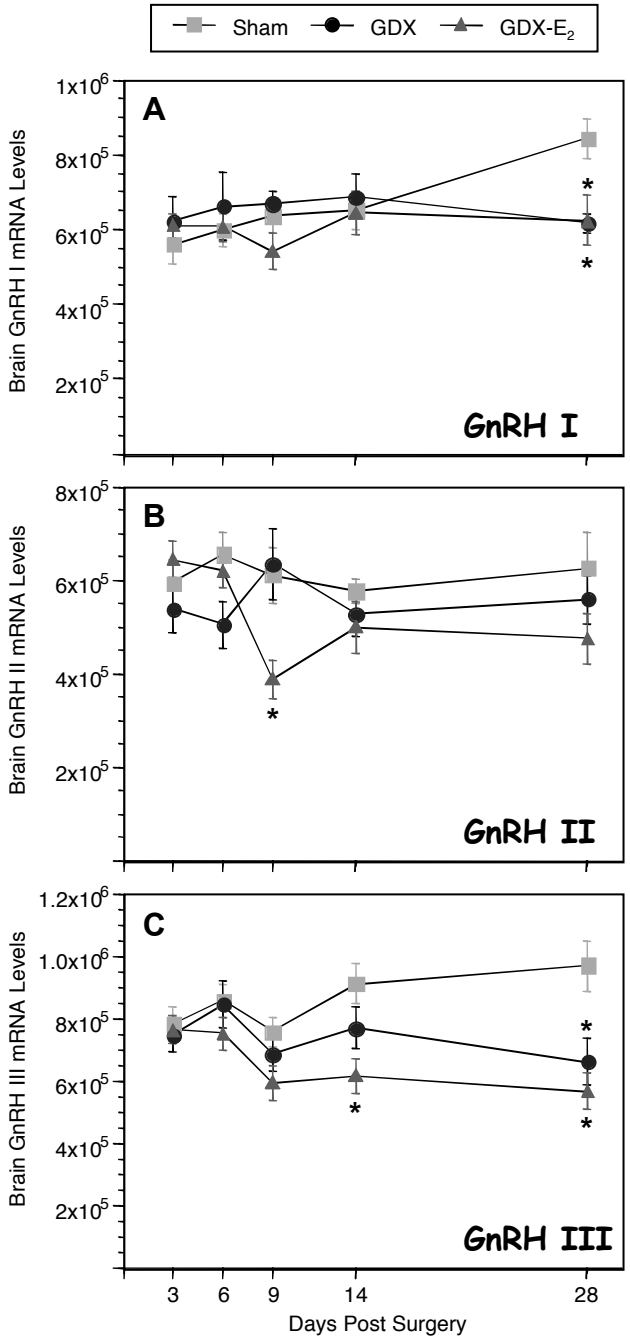

Figure 5: Transcript levels of GnRH I, II and III: Effects of gonadectomy and estrogen replacement. Female hybrid striped bass were either given a sham operation (Sham), gonadectomized (GDX), or gonadectomized and received E_2 (2 mg/kg) steroid replacement via microspheric delivery systems. Fish from each group were sacrificed on days 3, 6, 9, 14 and 28 post-surgery. Total RNA was extracted from brains and reverse transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Shown are mRNA levels as the mean ± SEM in copy number/ng of total RNA, *$P < 0.05$.

GnRH Peptide Levels in the Pituitary

To determine the effects of gonadal steroid removal and estrogen replacement on GnRH peptide levels in the pituitary, pituitary extracts of each sample were analyzed in duplicates using GnRH peptide specific ELISAs. Results are presented as μg/pituitary for GnRH I and ng/pituitary for GnRH II & III.

GnRH I peptide levels in the pituitary were significantly decreased in the GDX group after 9, 14 and 28 dps (Figure 6A). Estrogen replacement restored GnRH I levels to those found in s-o animals. No changes were found in GnRH II content of the pituitary in the GDX group (Figure 6B), but E_2 replacement significantly decreased the peptide levels by 9 dps until the end of the experiment. Gonadectomy had no effect on the pituitary content of GnRH III peptide (Figure 6C), and even though there was a significant increase of GnRH III 6 dps in the E_2 replacement group, no further differences could be detected for the remainder of the experiment.

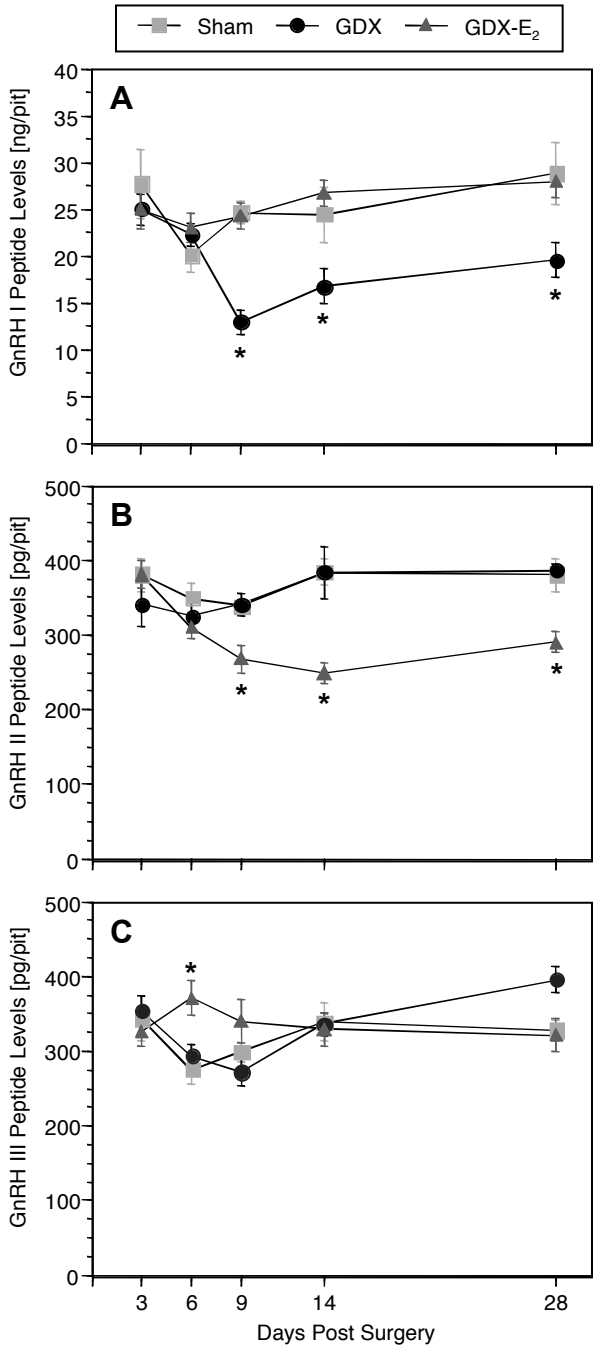

Figure 6: Pituitary GnRH I, II and III peptide levels: Effects of gonadectomy and estrogen replacement. Female hybrid striped bass were either given a sham operation (Sham), gonadectomized (GDX), or gonadectomized and received E_2 (2 mg/kg) steroid replacement via microspheric delivery systems. Fish from each group were sacrificed on days 3, 6, 9, 14 and 28 post-surgery. GnRH peptides were extracted from pituitaries and measured using peptide-specific ELISAs. Shown are peptide levels as the mean ± SEM in pg/pituitary, *$P < 0.05$.

GnRH-R Expression Levels in the Pituitary

Real time PCR analysis was performed to obtain mRNA levels of the GnRH-R in the bass pituitary. Removal of the gonads significantly reduced GnRH-R mRNA levels on day 3, 9 and 28 post-surgery, with the same trend to be found on day 6 and 14 post-surgery (Figure 7). Replacement with E_2 did not show any effect in the first two time points but also inhibited receptor expression on day 9,14 and 28 post-surgery. Analysis of the data using a two-way ANOVA to account for the effects of treatment as well as time, showed that both treatments significantly down regulated GnRH-R mRNA levels at 14 and 28 dps.

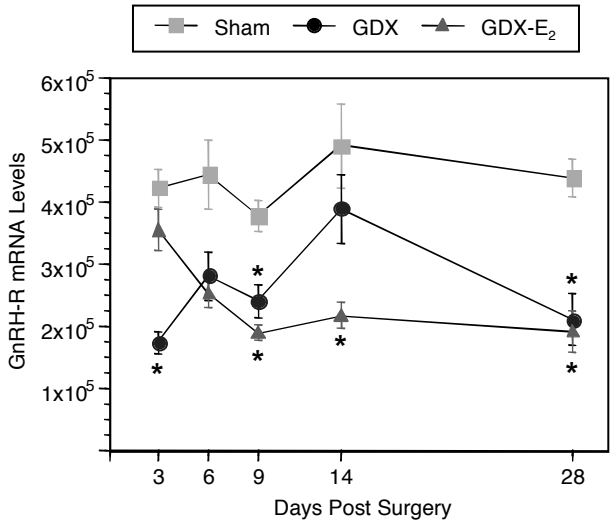

Figure 7: Pituitary GnRH-R mRNA levels: Effects of gonadectomy and estrogen replacement. Female hybrid striped bass were either given a sham operation (Sham), gonadectomized (GDX), or gonadectomized and received E_2 (2 mg/kg) steroid replacement via microspheric delivery systems. Fish from each group were sacrificed on days 3, 6, 9, 14 and 28 post-surgery. Total RNA was extracted from pituitaries and reverse transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Shown are mRNA levels as the mean±SEM in copy number/ng of total RNA, *$P < 0.05$.

GtH Subunit Expression Levels in the Pituitary

Real time PCR was also used to analyze GtH subunit mRNA levels in response to the experimental procedures. FSHβ subunit expression increased significantly in the

GDX group starting on day 3 post-surgery (Figure 8A). This increase in synthesis was maintained until the termination of the experiment 28 dps. On the contrary, replacement with E_2 significantly decreased FSHβ mRNA levels after 6 dps and inhibited expression further for the duration of the experiment compared to the control group.

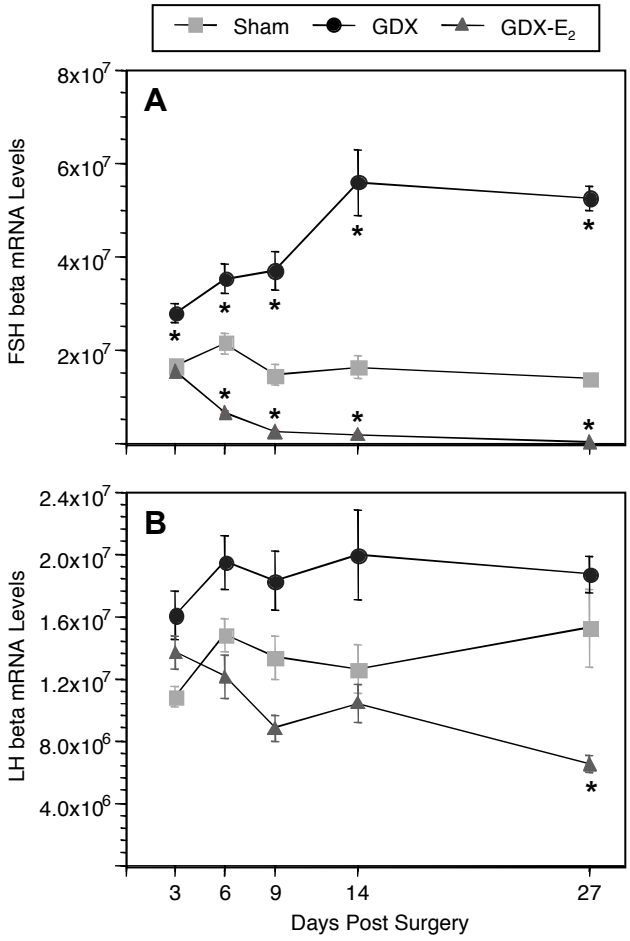

Figure 8: Pituitary FSHβ and LHβ expression levels: Effects of gonadectomy and estrogen replacement. Female hybrid striped bass were either given a sham operation (Sham), gonadectomized (GDX), or gonadectomized and received E_2 (2 mg/kg) steroid replacement via microspheric delivery systems. Fish from each group were sacrificed on days 3, 6, 9, 14 and 28 post-surgery. Total RNA was extracted from pituitaries and reverse transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Shown are mRNA levels as the mean±SEM in copy number/ng of total RNA, *P < 0.05.

On the short-term, LHβ mRNA levels were not affected by the removal of the gonads, but after 28 dps synthesis had significantly increased (Figure 8B). E_2 replacement had no short-term effect either but after 28 days of exposure decreased transcript levels significantly.

LH Protein Levels in the Pituitary and Plasma

To investigate the changes in LH protein levels in the pituitary, we analyzed the samples in duplicates using a specific homologous ELISA. The results are presented in μg/pituitary. LH protein levels did not change in the GDX group for the duration of the experiment, when compared to the s-o control animals (Figure 9A). LH content of the pituitary decreased significantly in the GDX+E_2 group after 3 and 28 dps, but no difference was detected after 6, 9 or 14 dps.

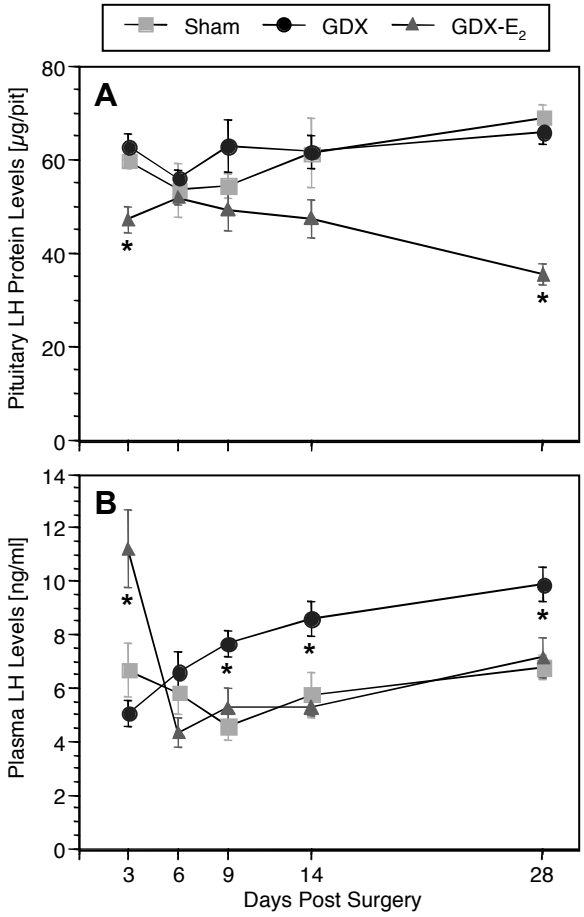

Figure 9: Pituitary (A) and plasma (B) LH protein levels: Effects of gonadectomy and estrogen replacement. Female hybrid striped bass were either given a sham operation (Sham), gonadectomized (GDX), or gonadectomized and received E_2 (2 mg/kg) steroid replacement via microspheric delivery systems. Fish from each group were sacrificed on days 3, 6, 9, 14 and 28 post-surgery. LH protein was extracted from pituitaries and measured using a specific ELISA. Plasma LH was determined using a striped bass specific RIA. Shown are protein levels as the mean±SEM in µg/pituitary (A) or ng/ml (B), *$P < 0.05$.

Plasma LH was measured in duplicate using a specific RIA. Results are presented in ng/ml plasma. 9 dps after the gonadectomy a significant increase in LH plasma levels was detected (Figure 9B). These levels remained elevated compared to the control group until the end of the experiment 28 dps. In the GDX+E_2 group LH plasma levels had increased significantly at 3 dps, but dropped to control levels after 6 dps and showed no further changes until the end of the experiment.

DISCUSSION

The goal of this study was to establish a baseline of the response of the HP-axis to different durations of gonadectomy and E_2 replacement. In view of these results, further experiments were to be designed to develop a better understanding of the role of steroid feedback in regulating the GnRH/GtH system during different stages of sexual maturation (puberty) and the adult reproductive cycle. We monitored the expression of three GnRHs in the hypothalamus, and their peptide contents in the pituitary. We also measured mRNA levels of the GnRH-R and the gonadotropin subunits, FSHβ and LHβ in the pituitary, as well as LH protein content. Further, LH and E_2 plasma levels were analyzed. The experiments were terminated after 3, 6, 9, 14, and 28 days post-surgery to be able to differentiate between short-term and long-term responses of the HP axis. This sampling regime was chosen based on evidence in mammals that shows that the HP axis responds to gonadectomy during the 3 - 4 weeks post surgery and than stabilizes (reviewed in Sagrillo et al., 1996). We find in our study that in most aspects of the HP axis, a stable state is reached after 9 days post-surgery. The majority of significant changes in hormone synthesis and /or release had taken place by this time period and did not change thereafter. However, some significant differences in the response to the experimental procedures developed only 28 days post-surgery, which was the last sampling date. This shows that the HP axis consists of a very plastic system, in which changes occur at different levels at different points in time. Careful consideration of experimental design is necessary when evaluating the effects of gonadectomy and steroid replacement on the reproductive axis.

GnRH Expression Levels in the Brain

During the first few weeks, no changes were observed in expression levels of the hypophysiotrophic GnRH I between treatment groups. However, after 28 dps GnRH I transcript levels were significantly lower in the gonadectomized and estrogen replacement groups compared to controls. These results are contradictory to other studies conducted in fish. In female silver eels, E_2 induced an increase in the level of brain and pituitary GnRH I (Montero et al., 1995), and E_2 treatment of immature, castrated male tilapia also increased GnRH I mRNA levels (Parhar et al., 2000). However, in agreement with our results is a study done by Okuzawa et al. (2002), in which implantation with E_2 did not change transcript levels of GnRH I. In mammals, ovariectomy in females has variously been reported to decrease expression (Kim et al., 1989; Roberts et al., 1989), have no effect (Kelly et al., 1989), or increase transcript levels (Toranzo et al., 1989). Treatment with E_2 also has ambiguous results, leading either to an increase (Kim et al., 1989; Roberts et al., 1989) or decrease (Toranzo et al., 1989) in GnRH I gene expression. Given the diversity of effects of gonadal steroid regulation on GnRH I neurons, the plurality of the results in this study are not totally unexpected. Surprising is the decrease in transcript levels in both treatment groups after 28 days. The absence of gonadal input, not estrogen in this case, may be responsible for cytological changes of the GnRH I neurons, where a decrease in neuronal numbers, as well as soma size, could result in a decrease in GnRH I transcript levels. White et al. (2002), have shown in the territorial African cichlid that social signals can alter GnRH I expression, as well as soma size and cell numbers of GnRH I neurons. These changes can take place between 3 days and 3 weeks depending on the direction of the social switch.

Expression levels of GnRH II were not changed by removal of gonadal feedback or estrogen replacement in this study. These results agree with findings in other teleost fish. Immature, castrated male tilapia, showed no response in GnRH II transcript levels after treatment with E_2 (Parhar et al., 2000). Estrogen also had no effect on GnRH II level in female silver eels (Montero et al., 1995). But our results are in discrepancy with the few mammalian studies to date that have investigated the steroidal effect on GnRH II expression. Studies in the musk shrew showed an increase in GnRH II production and release after ovariectomy, suggesting a negative feedback control of gonadal steroids (Rissman and Li, 1998). Densmore and Urbanski (2004) found GnRH-II mRNA expression to be significantly more abundant in ovariectomized rhesus macaques treated with E_2 than in ovariectomized animals. *In vitro* studies by Chen et al., (2002) in human neuronal medulloblastoma cells (TE-671) and Khosravi and Leung (2003) in human granulosa luteal cells, demonstrated, that E_2 treatment increases endogenous GnRH II mRNA levels. Giving evidence that in mammals the GnRH II gene is capable of being regulated by estrogen in different cell types. Differences in estrogenic control of the GnRH II neuronal system may be due to species specificity or the reproductive timing of the experiment. Our model organism was undergoing stages of midvitellogenesis during our experimental period, and since there are implications of GnRH II being involved in sexual behavior (Volkoff and Peter, 1999; Kauffman and Rissman, 2004a), there could be no feedback regulation of the GnRH II synthesis apparatus by gonadal factors necessary at this reproductive stage. Further it has been shown, that *in vitro* results do not always concur with findings of *in vivo* systems, due to the absence of the complete signaling array provided by the whole organism.

During our study, transcript levels of GnRH III significantly decreased after two weeks in the E_2 replacement group and after 4 weeks in the gonadectomized group. Only a few studies in fish have investigated the effect of steroids on GnRH III neurons. Immature, castrated male tilapia, treated with E_2, showed no effect on the expression of GnRH III (Parhar et al., 2000), while T treatment of castrated male tilapia elevated GnRH III mRNA levels (Soga et al., 1998). In male masu salmon, treatment with 17α-methyltestosterone had no effect on GnRH III cell number in the olfactory bulb or terminal nerve (Amano et al., 1994a). These controversial results can be explained by the difference in model species, sex, and experimental design, including length of treatments and dosage. The late decrease of GnRH III transcript in both treatment groups could also be explained by cytological changes of the neuronal system in the terminal nerve area due to missing gonadal stimuli. Since this effect is more pronounced in the E_2 treatment group, involvement of intermediary factors, that are regulated by estrogen and in turn regulate the GnRH system, like GABA or dopamine (Kah et al., 2000), could explain the inhibitory effect and its earlier onset compared to the gonadectomized group.

The synthesis of all three forms of GnRH is not regulated by an acute steroidal feedback during midvitellogenesis in female hybrid bass. However, the observed long-term effects in GnRH I and III transcript levels may be due to the removal of a positive gonadal influence, which may cause changes in the respective neuronal cytology (see above).

GnRH Peptide Levels in the Pituitary

In order to distinguish between the effects of gonadal steroids on GnRH synthesis and release, we determined the levels of all three GnRHs in the pituitary. The amount of

GnRH I peptide decreases significantly after 9 dps and remains suppressed throughout the experiment. E_2 replacement reversed the GnRH I levels to those found in the s-o animals. This is in agreement with early studies conducted in mammals, showing that ovariectomy reduced levels of GnRH I in the mediobasal hypothalamus (Kalra, 1976; Wheaton, 1979), primarily due to the reduction in content of GnRH I in the median eminence (Kalra, 1976; Kobayashi et al., 1978). This effect could also be reversed by estrogen administration (Kalra, 1976). Later studies, measuring proGnRH I or GnRH I, found that ovariectomy for 2-3 weeks decreased the peptide levels in female rats compared to levels in control animals (Ackland et al., 1988; Kelly et al., 1989; Roberts et al., 1989). Peptide content is reported to increase following several days of estrogen (Roberts et al., 1989) or estrogen and progesterone treatment (Ackland et al., 1988). In fish, studies of GnRH peptide levels in response to gonadal manipulations are scarce. Early studies were done using antisera in RIA systems that recognized all GnRH forms present and are therefore difficult to compare. Using specific antisera, Montero et al. (1995) showed that in female silver eels, chronic exposure of E_2 induced the increase in the level of brain and pituitary GnRH I. Estrogen also exerted a positive influence on the amount of GnRH I levels in male African catfish (Dubois et al., 1998; Dubois et al., 2001). Since we did not observe any changes in mRNA levels for GnRH I, the decrease observed in the pituitary must be a result of changes in post-transcriptional processes. A possibility would be that gonadectomy inhibits enzymatic processing of the different GnRH peptide forms (proGnRH / mature GnRH peptide) or inhibits their release, or alternatively inhibits an intermediary neuronal system (e.g., GABA or dopamine), which causes the decrease in peptide levels in the pituitary. However, E_2 replacement promotes

GnRH release either by reinstating positive feedback towards the intermediary messenger or by promoting the enzymatic processes needed to convert GnRH to its mature peptide form.

We did not observe a change in GnRH II peptide levels in the gonadectomized group compared to the intact control animals during this study. However, peptide levels in the estrogen-treated animals decreased significantly after 9 days post-surgery and remained low until the end of the experiment (28 dps). This result is in contrast to the few other studies measuring GnRH II peptide levels. Female silver eels showed no changes in brain and pituitary GnRH II levels after treatment with E_2 (Montero et al., 1995). In mammals, the focus on GnRH II has been on expression rather than peptide levels. These studies showed a positive regulation of GnRH II transcript levels (Chen et al., 2002; Densmore and Urbanski, 2004), which hypothetically would not result in a decrease in peptide levels in the median eminence/pituitary. There are two possible explanations for the decrease of GnRH II peptide in the pituitary. On one hand, the estrogen levels induced by the implanted microspheres were significantly higher compared to the levels observed in the intact controls. Even though levels remain physiological, they compared more closely to levels found in this species towards the end of oogenesis, near the time of ovulation (Mylonas et al., 1997c). Therefore, the HP axis of the gonadectomized animals was not competent to respond to such high levels of estrogen at this stage. Since it has been shown that the effects of estrogen can be biphasic (Sagrillo et al., 1996), it can be hypothesized that this high dose causes a negative feedback, whereas a lower dose may have a positive impact. On the other hand, intermediary neuronal systems, controlled by

the higher levels of estrogen, may have a negative regulatory effect on the GnRH II release.

Pituitary levels of the third form of GnRH remained unchanged in the gonadectomized group compared to the s-o controls. The same result was observed in the E_2 replacement group, despite the decrease in GnRH III transcript levels in the hypothalamus. This observation further supports the conclusion that hormone transcript levels are not necessarily directly correlated with hormone release.

GnRH-R Expression Levels in the Pituitary

To understand a central part in the HP-axis, knowledge about the signals and mechanisms that regulate the expression of GnRH-Rs is essential. Responsiveness to GnRH depends on the number of GnRH-Rs on the cell surface of the gonadotrophs. There is evidence that indicates that the number of GnRH-Rs is partially dependent upon the level on GnRH-R transcript (Wu et al., 1994). Therefore we studied the levels of expression of the GnRH-R in the pituitary. In gonadectomized fish, GnRH-R transcript levels decreased significantly early in the experiment and remained suppressed for the duration. This is in contrast to what has been shown for mammals, where GnRH-R mRNA levels rose after ovariectomy (Kaiser et al., 1993; Turzillo et al., 1998). The rise in transcript levels may be due to direct effects of gonadal factors, such as steroids and activin, on pituitary gonadotrophs. Alternatively, modulations occurring in the hypothalamic GnRH pulse frequency or amplitude could indirectly be responsible in mammals (Kaiser et al., 1993; Brooks and McNeilly, 1994; Wu et al., 1994; Yasin et al., 1995; Turzillo et al., 1998). GnRH I regulates the receptor transcript in a biphasic manner with initial exposure to hormone leading to an increase in receptor expression, whereas

prolonged exposure leads to a receptor down-regulation (Norwitz et al., 1999). The reason for the down-regulation of receptor transcript in our study may be the result of a synergy of an acute direct effect, as well as long-term indirect regulation. Missing gonadal factors, which exert at this reproductive stage a positive/stimulatory feedback, cause an acute (after 3 days) decrease in receptor mRNA levels. One candidate factor is activin, which in mammals has been shown to increase GnRH-R mRNA levels (Rispoli and Nett, 2005). After 9 days, and lasting until the end of the experiment, a decrease in GnRH I peptide levels is observed in the pituitaries of gonadectomized animals. Since it has been shown in mammals, as well as teleost fish, that GnRH regulates the transcriptional level of its own receptor in gonadotrophs (Kaiser et al., 1993; Brooks and McNeilly, 1994; Yasin et al., 1995; Okuzawa et al., 2002; Levavi-Sivan et al., 2004; Jodo et al., 2005), this observed decrease in GnRH I peptide could be responsible for the indirect regulation, via the hypothalamus, of the long-term decrease in receptor expression. To our knowledge, no study in fish to date has determined the effect of gonadectomy on expression levels of the GnRH-R.

E_2 replacement treatment also caused a decrease in GnRH-R transcript levels in our study. This is in agreement with observations made in mammalian studies, where estrogen decreased the post-castration rise (Kaiser et al., 1993; Looper et al., 2003). There is also evidence from *in vitro* data, where E_2 inhibits GnRH-R mRNA expression in a dose- and time-dependent manner in ovarian carcinoma OVCAR-3 cells via a receptor-mediated mechanism (Kang et al., 2001). A similar suppressing effect of E_2 on the GnRH-R mRNA level has also been observed in primary-cultured granulosa-luteal cells (Nathwani et al., 2000). In fish it has been shown that GnRH-R expression varies

during the reproductive cycle (Jodo et al., 2005) and also that there are sex differences between males and females (Levavi-Sivan et al., 2004; Jodo et al., 2005), which the authors attributed to the changes and differences in sexual steroids. Further, studies in juvenile red seabream, treated with E_2 or 11 KT showed a decrease in GnRH-R mRNA levels compared to control fish, suggesting also a negative steroid effect on receptor transcript levels (Okuzawa et al., 2002).

GtH Subunit Expression Levels in the Pituitary

To study the direct and indirect effects of gonadal feedback on GtH subunit expression, we determined transcript levels of FSHβ and LHβ in the pituitary. In the gonadectomized group, we detected an immediate and significant rise in FSHβ transcript levels (3 dps), which continued increasing until day 14 and remained at that level until day 28 post-surgery. E_2 replacement suppressed FSHβ mRNA levels significantly on day 6 and inhibited expression almost completely by the end of the experiment.

In mammals, both serum FSH and FSHβ mRNA increase rapidly after ovariectomy. An acute response in transcription levels occurs after 30-60 min resulting in increased serum FSH at 8 hours post-surgery (Dalkin et al., 1993). Levels continue to rise through day 7 (Dalkin et al., 1990; Dalkin et al., 1993; Burger et al., 2001) and remain elevated up to 30 days (Shupnik et al., 1988). The effects of E_2 on FSHβ transcription appear to be different among mammalian species. *In vivo*, E_2 suppressed the post-ovariectomy increase in FSHβ mRNA synthesis in the rat (Shupnik et al., 1988), but not in GnRH antagonist-treated animals (Shupnik and Fallest, 1994), suggesting hypothalamic actions of E_2. Furthermore, estrogen had no effect on FSHβ mRNA synthesis in female rat pituitary fragments (Shupnik et al., 1989a). In contrast to the rat,

E_2 suppressed mRNA synthesis in cultured ovine pituitary cells (Baratta et al., 2001), and also the activity of an ovine FSHβ promotor-LUC construct transfected into ovine pituitary cells (Miller and Miller, 1996). In sheep, E_2 inhibits FSHβ transcription, but activin is clearly involved in the effects caused by steroids (Nett et al., 2002).

In goldfish, physiological doses of E_2 stimulated FSHβ *in vivo* and *in vitro*, during all stages of gonadal development (Huggard-Nelson et al., 2002). In contrast to this data, steroids (T/E_2) decreased FSHβ mRNA levels in coho salmon (Dickey and Swanson, 1998) juvenile and recrudescing goldfish (Sohn et al., 1998; Sohn et al., 2000; Kobayashi et al., 2000) and the European sea bass (Mateos et al., 2002). Detailed studies in tilapia indicated that regulation of FSHβ expression is very sensitive to steroid levels. Sex steroid levels that were too low or too high caused low levels of FSHβ, and only fish with moderately low levels (T/E_2 of 1 ng/ml) have high levels of FSHβ mRNA (Melamed et al., 2000).

Based on these observations, FSHβ transcript levels seem to be under a negative feedback in most teleost fish, which is in agreement with our results. The immediate increase of expression in the gonadectomized group suggests the removal of an acute, direct negative feedback at the level of the pituitary, which either does not involve the hypothalamic GnRH system or masks any effect due to changes in GnRH levels. As seen above, FSHβ expression is very sensitive to steroidal regulation. Because the circulating E_2 levels in gonadectomized and estrogen replaced fish were much higher than endogenous levels in the controls, it is not surprising that the estrogen replacement did not mimic the feedback situation on FSHβ mRNA levels, but rather suppressed the expression further.

In gonadectomized fish, transcript levels of LHβ show an increasing trend from day 3, yet become significantly different from controls only at day 28 post-surgery. In the E_2 replacement group the opposite is observed, with levels decreasing from day 3 but only attaining significance at the end of the experiment.

In mammals, serum LH rises slowly after ovariectomy and is not significantly elevated until days 2 or 3 (Dalkin et al., 1993; Burger et al., 2001). Coincident with the increase in GnRH, LHβ mRNA expression begins to rise about day 3, and continues to rise through day 21 (Dalkin et al., 1990; Dalkin et al., 1993; Burger et al., 2001). Estrogens rapidly suppress the post-ovariectomy increase in LHβ mRNA levels (Shupnik et al., 1988), but this effect was abolished by GnRH antagonist administration (Dalkin et al., 1993; Shupnik and Fallest, 1994; Fallest et al., 1995; Burger et al., 2001), and additional treatment with E_2 was no more effective than antagonist alone (Shupnik and Fallest, 1994), suggesting hypothalamic control of E_2 actions. However, estrogens also exert direct action on the pituitary, rapidly increasing LHβ mRNA synthesis in pituitary cells from ovariectomized rats (Shupnik et al., 1989a; Shupnik, 1996).

In contrast to mammals, estrogenic steroids have a positive effect on LHβ subunit expression in teleost fish. Treatment with T and other aromatizable androgens or E_2 increased LHβ mRNA levels in goldfish (Kobayashi et al., 2000), coho salmon (Dickey and Swanson, 1998), European eel (Querat et al., 1991; Schmitz et al., 2005), and European sea bass (Mateos et al., 2002). In agreement with this data, treatment with T or E_2 increased pituitary LH levels in rainbow trout (Breton et al., 1997), Atlantic salmon parr (Antonopoulou et al., 1999), coho salmon (Dickey and Swanson, 1998), and European sea bass (Mateos et al., 2002). In goldfish, physiological doses of E_2 stimulated

alpha, FSHβ and LHβ *in vivo* and *in vitro*, during all stages of gonadal development (Huggard-Nelson et al., 2002). Similar in the African catfish pituitary, T or E_2 stimulated synthesis of LHβ transcripts and peptide *in vitro* (Rebers et al., 2000).

This slow rise in LHβ mRNA levels is therefore in agreement with the mammalian results but is contrary to what has been observed in other fish species. LHβ transcription in hybrid bass, at this reproductive stage, appears to be regulated by a negative feedback exerted by gonadal factors. Data suggests that this action is not mediated through the GnRH system, since changes in pituitary GnRH I peptide levels only appear after 9 days. Furthermore, the observed decrease in GnRH I would rather cause a decrease in LHβ mRNA levels, as suggested by the literature.

Surprisingly, E_2 replacement has the tendency to decrease LH transcript levels, reaching significance after 28 days. This is the complete opposite of what has been shown in other fish species, but conforms to most studies conducted in mammals. Estrogen seems to be responsible for the negative feedback exerted by the gonads in hybrid striped bass undergoing stages of midvitellogenesis. However, the induced estrogen levels were significantly higher than those found in the control animals, and more comparable to what females would experience near the time of ovulation. The observed response may therefore not be the physiologically appropriate response for this reproductive stage.

LH Protein Levels in the Pituitary and Plasma

To evaluate the effects of gonadal steroids on the synthesis and release of LH in the pituitary, we determined LH protein levels in the pituitary and plasma. We observed no changes of pituitary LH content in the gonadectomized animals, which is in contrast to

studies conducted in fish where gonadectomy caused a decrease in both pituitary and plasma LH (reviewed by Yaron et al., 2003). In mammals, pituitary LH content increases and coincides with a rise in LH plasma levels after ovariectomy (Sagrillo et al., 1996). This post-ovariectomy rise may be mediated by increased amplitude and / or frequency of GnRH pulses from the hypothalamus, where GnRH neuronal activity presumably increases in the absence of inhibitory steroid feedback (Sagrillo et al., 1996). We observed a slight increase in LHβ transcription rate in the gonadectomized group and no changes in pituitary LH protein levels during our study. But we find a significant increase in LH plasma levels 9 days post-surgery, which remains elevated compared to the control group until the end of the experiment. It is therefore not surprising that with an increase in synthesis, as well as release, we find a steady state level of pituitary LH.

In the estrogen replacement group, we observed a decrease in levels of LH protein over time, reaching significance after 28 dps. Plasma levels were unchanged after an initial drop from day 3 to 6 post-surgery. Even though the animals experience a decrease in LHβ expression and a decrease in stored pituitary LH, plasma LH levels remain unchanged. Therefore the decrease in pituitary LH levels may be due to an enzymatic breakdown of the stored LH protein. However, the aforementioned high levels of estrogen, which are not typically experienced by the organism at this stage, could be responsible for this unusual physiological response.

In conclusion, the data shown in this study give evidence to support a negative estrogenic feedback on the GnRH network in the hypothalamus in female hybrid striped bass that are undergoing stages of midvitellogenesis. The regulatory action, however, occurs not at the level of GnRH synthesis, but rather may involve enzymatic processing

of the GnRH peptide and/or its release into the pituitary. GnRH receptor transcript levels appear to be influenced by an acute positive gonadal feedback in the short-term, which is not estrogenic in nature, and a synergistic regulation by gonadal feedback and hypothalamic GnRH in the long-term.

The effects of gonadal feedback on pituitary gonadotropins observed in this experiment appear to be exerted directly, rather than via the hypothalamus. This is shown by the fact that the changes in GtH subunit transcription and LH release do not follow the pattern of synthesis and release of the hypothalamic GnRH system.

In summary, this study, for the first time to our knowledge, examined multiple sites of the hypothalamic-pituitary axis in its response to removal of gonadal feedback and estrogen replacement, considering GnRH synthesis, its release, as well as GnRH-R transcript levels at the pituitary. We further evaluated the response of the GtHs, by determining synthesis, storage and release patterns. This overview gives an insight in regulatory actions of gonadal feedback at the level of the hypothalamus and pituitary at subsequent time points for an extended period of time. Based on our results, we determined that a fairly stable steady-state in hormonal changes due to treatment is reached after 9dps and that further significant changes appear only after 28dps. Thus, all future experiments using similar treatment paradigms will be terminated after 10 dps, to examine the effects of gonad removal and steroid replacement on the HP axis in teleost fish.

CHAPTER 3. STEROID FEEDBACK REGULATION OF THE HYPOTHALAMUS-PITUITARY AXIS OF JUVENILE AND PUBERTAL STRIPED BASS

ABSTRACT

To identify the role of gonadal steroid feedback in the pubertal development of the hypothalamo-pituitary-gonadal axis in striped bass (*Morone saxatilis*), we investigated the effects of bilateral gonadectomy and steroid replacement treatment, using 17ß-estradiol (E_2) and 11-ketoandrostenedione (11-KA), on GnRH, GnRH-R and GtH gene expression, as well as LH protein levels in the pituitary. At three time points (2 spring, 1 fall) during juvenile development, females were divided into 6 groups. Three gonadectomized (gdx) and 3 sham-operated (s-o) groups were established. One sham and one gdx group received E_2 (2 mg/kg) and 11-KA (2 mg/kg) respectively via microspheric delivery systems, the remaining two groups were given vehicle injection. Pubertal males and females were each divided into 4 groups, with 1 sham and 3 gdx groups. The gdx groups were treated with the same steroid paradigm as above, while one gdx and the sham group received vehicle only. Ten days post-surgery, mRNA levels of the three GnRH forms, as well as GnRH-R, FSHβ and LHβ, were determined using real-time quantitative PCR normalized to 18 S mRNA levels. Pituitary LH protein levels were measured with a specific ELISA. Plasma steroid levels were measured using RIAs. Expression levels of all three GnRH variants did not change during the juvenile stage in females. The same result was observed in pubertal females, however, treatment with 11-KA increased all three GnRH transcript levels in pubertal male fish. The expression of

the GnRH-R is positively regulated via an estrogenic pathway during both the pre- and pubertal stage, showing a seasonal pattern of regulation in the juvenile females. FSHβ mRNA levels are suppressed by steroids and gonad removal during the spring, but not in the fall. Generally, LHβ transcription was not affected by steroids and the same was true for LH protein levels in the pituitary. The obtained data suggests that the response of the HP axis to gonadal feedback and steroid replacement gains in complexity during the maturational development from juvenile to pubertal animal, becoming more capable to regulate reproductive processes.

INTRODUCTION

Puberty is the developmental process by which a sexually immature juvenile transforms into a mature adult, with its full capacity to successfully complete gametogenesis (Schulz et al., 2000). The onset of puberty is dependant on age, endogenous circannual rhythms, body growth and nutritional status, as well as external factors such as photoperiod, water temperature, and social interactions (e.g., predators, population density) (reviewed in Schulz and Goos, 1999; Okuzawa, 2002; Ojeda and Skinner, 2006).

In fish, sexual development can be accompanied by a reduction in body growth and deteriorated flesh quality. Therefore, precocious puberty, which is observed in some commercially important fish species like salmonids is undesirable (Gaillard et al., 2004). However, other key aquaculture species take years to mature, which elevates the total cost of maintaining broodstock animals prior to their use for gamete production. For this reason, it is very important to be able to control sexual maturity in fish.

The mechanisms of the onset of puberty in vertebrates are very complex and not well understood. The identification of the trigger sites along the HPG axis that control the onset of sexual maturation is crucial for identifying factors that could be used to manipulate these sites. The GnRH neuronal system in the hypothalamus is one of the key elements in regulating reproduction in vertebrates. In mammals, it appears that at the onset of puberty a central, gonad-independent inhibition suppresses the pulsatile GnRH release during the juvenile period and/or an increase in excitatory input to GnRH neurons (Terasawa and Fernandez, 2001; Ojeda and Skinner, 2006; Plant and Witchel, 2006) is activated by external and internal cues, leading to the production and release of gonadotropins and subsequent initiation of gonadal development.

Although suggested as the paradigm for all vertebrates (Dellovade et al., 1998), the pulsatile release pattern of GnRH has not been yet identified in teleost fish. To explain the onset of puberty in fish, the missing-link concept has been proposed by Goos (Goos, 1993), referring to the inability of one or more components of the HPG axis to respond/function before puberty.

The activity of the HPG axis in fish is low prior to puberty. GnRH expression in the brain and GnRH peptide levels in the pituitary are lower in juvenile fish and correlate well with gonadal development in many fish species (Amano et al., 1992; Amano et al., 1993; Amano et al., 1995; Amano et al., 1997; Dubois et al., 1998; Senthilkumaran et al., 1999; Okuzawa et al., 2003). The activation of the GnRH neuronal system may be the trigger for the onset of puberty, however in some fish species an increase in GnRH peptide levels was observed pre-puberty (Rodriguez et al., 2000; Holland et al., 2001),

suggesting it could be a secondary phenomenon caused, for example, by positive feedback of gonadal steroids.

FSH plays a more pivotal role during early puberty than LH in most fish. Pituitary and plasma FSH levels are higher than LH levels in juvenile coho salmon (Swanson et al., 1991) and rainbow trout (Breton et al., 1998; Gomez et al., 1999). Results obtained in striped bass also suggest the importance of FSH in pubertal development, with increasing FSHβ transcript levels during early puberty, while LHβ mRNA did not rise until late puberty, coinciding with the late stages of the reproductive cycle. However, studies in the European sea bass indicate that LH may be partly involved in early puberty, although the lack of available FSH data for this species dilutes the meaning of these results (Rodriguez et al., 2000).

During pubertal development, production of gonadal steroids increases along with other gonadal peptides like activin (Ge, 2000; Holland et al., 2000; Schulz and Miura, 2002). Sex steroids have been proposed as a natural inducer of puberty in male African catfish (Schulz and Goos, 1999) or to provide a permissive amplifier-action through positive feedback (Dufour et al., 2000). Steroid feedback is very complex and differs among species, gender and maturational stage. However, in juveniles steroid treatment effects are observed at the level of the hypothalamus, as one of the foremost stimulators of the GnRH system, as well as at the level of the pituitary, generally as a negative regulator of FSH and a positive regulator of LH (reviewed by Okuzawa, 2002).

Another phenomenon of pubertal development is the so-called ‘dummy-run’. In some fish species (rainbow trout, striped bass), an incomplete cycle of gametogenesis is accompanied by elevated pituitary or plasma GtH and brain/pituitary GnRH levels.

Typically, this run occurs in the season before 'true' puberty occurs (Prat et al., 1996; Hassin et al., 1999; Holland et al., 2001). In view of this, it was suggested that the HP component of the axis was fully developed, responding to external stimuli, while the gonads were still too immature (Holland et al., 2001). Therefore, the primary site of the HPG axis responsible for the onset of puberty is still unknown in fish and further investigations are required.

Our model, the striped bass (*Morone saxatilis*) is a commercially important, late maturing fish species. Captive females initiate gonadal development at 3 years of age, however pubertal development may take several years to be completed in this species (Holland et al., 2000). Studies of physiological changes that occur before and during puberty in striped bass (Holland et al., 2000; Holland et al., 2001; Hassin et al., 1999) have shown that pituitary GnRH I and II peptide levels in 1-year-old females were elevated. However, FSH and LH synthesis was at basal levels, indicating the lack of responsiveness at the level of the pituitary to hypothalamic GnRH. In contrast, FSH and LH mRNA and LH protein levels in 2-year-old female fish were elevated and it was suggested that increased gonadotropin activity was the result of an increase in GnRH release (Hassin et al., 1998; Hassin et al., 1999; Holland et al., 2001). The fact that pubertal females exhibit lower GtH gene expression levels compared to mature females (4-year-old) and that FSH mRNA levels are significantly lower in immature females compared to their maturing 3-year-old siblings, leads to the conclusion that the maturation state depends on gonadotropic activity (Hassin et al., 1999). Alternatively, the gonads may play an important role in the timing as well, since the low levels of FSH/LH observed in juveniles and the lack of seasonality in oocyte growth profiles suggests that

the early developmental phase is not under GtH control (Hassin et al., 1999). Holland et al. (1998b) further supports this theory with assay data showing that ovaries of 2-year-old females display limited responsiveness to GtH stimulation.

The involvement of T and E_2 in the regulation of puberty in striped bass seems unlikely because the onset of sexual maturation was not associated with changes in the plasma levels of these steroids (Holland et al., 2000). However, steroids do play an important role in regulating different parts of the HP axis in many juvenile fish (Okuzawa, 2002) and may be a critical factor in delaying or advancing puberty.

The objective of the present work was to analyze the response of the juvenile and pubertal hypothalamus-pituitary axis to the removal of gonadal feedback and steroid replacement, as well as steroid treatment of the intact animal. Expression levels of three GnRH variants in the hypothalamus, transcription levels of the GnRH-R, and LH and FSH were measured, as well as LH pituitary content. We were unable to study FSH protein levels, since there is still no quantitative assay available.

MATERIAL AND METHODS

Experimental Animals and Treatment Protocol

Striped bass were acquired from UMCES Horn Point Laboratory, Cambridge, MD in fall 2000 and transferred to the Center of Marine Biotechnology's Aquaculture Research Center, Baltimore, MD, when fish were 4 month old. The fish were maintained in 6 and 12 foot diameter recirculation systems, at 8 ppt salinity, under a simulated natural photo- and thermoperiod regime. The animals were maintained as a stock population in the above conditions until selected for the following experiment. All

animals at the Aquaculture Research Facility were maintained and sampled according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland Biotechnology Institute.

Treatment Protocols

Bilateral gonadectomies were carried out according to procedures modified from Billard et al. (1977). Fish were anesthetized (70 ppm phenoxyethanol, Baker Inc., Phillipsburg, NJ), bled, weighed and placed on a surgical table, and their gills continuously irrigated with oxygenated water containing anesthesia. Using a scalpel, a 2–4 cm incision was made along the midline between the pectoral and pelvic fins. The ovaries were detached from the abdominal membranes and removed. The incision was sutured using 2.0 silk thread (Burns Veterinary Supply, Inc., Owings Mills, MD) and closed with tissue glue (Burns Veterinary Supply, Inc.). Post-operation, fish were returned to the experimental tank systems. The extracted gonads were weighed for calculation of the gonado-somatic index (GSI; [gonad weight/body weight] x 100%). Control fish were given a sham-operation (s-o) receiving an incision and suture, but the ovaries were not removed.

Experiment 1: The Effect of Gonadectomy and Steroid Replacement on the Reproductive Axis in Juvenile Striped Bass.

Based on results reported by Holland et al (2000) on striped bass gonadal development and plasma steroid levels during pubertal development, we chose the following time points to conduct our experiments: May 2001, November 2001 and March 2002. At each of these months, 60 fish of average size were selected (May: fluke length

19 cm, weight 83 g, GSI 0.5%; November: fluke length 33 cm, weight 540 g, GSI 0.3%; March: fluke length 36 cm, weight 630 g, GSI 0.4%) from a mixed sex population and divided into 2 groups of 30 fish each (Figure 10).

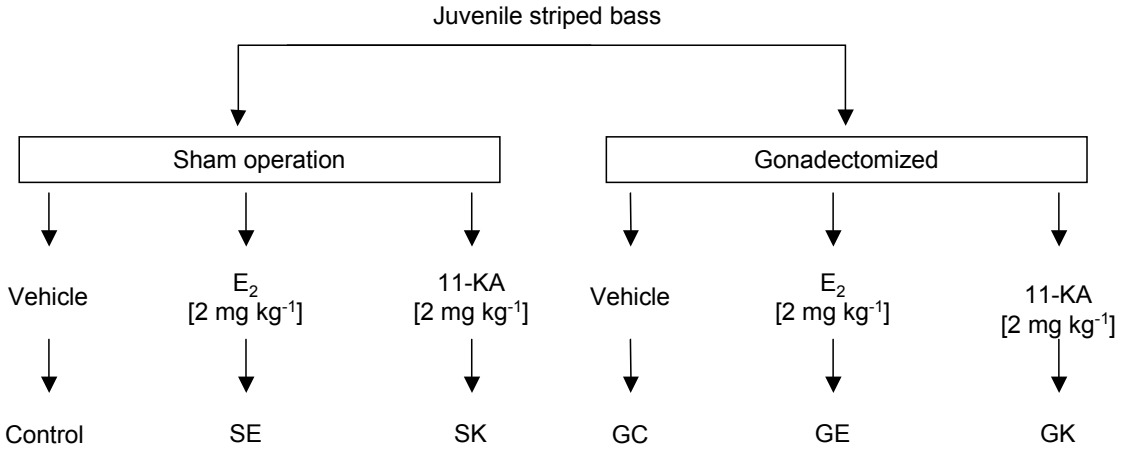

Figure 10: Juvenile striped bass study (experiment 1). Diagram of the experimental design.

The first group was given a sham operation, the other group was gonadectomized. After surgery, the s-o fish were divided into 3 groups of 10 fish each. One group was given a vehicle injection (control), the second group received E_2 steroid treatment (SE; 2 mg/kg) via microspheric delivery systems (modified from Holland et al., 1998b), and the third group received 11-ketoandrostenedione (11-KA) steroid treatment (SK; 2 mg/kg) via microspheric delivery systems. 11-KA is the precursor of 11-ketotestosterone, the primary androgen in fish (Scott et al., 1980). Moreover, 11-KA or 11-KT are non-aromatizable androgens and therefore cannot be converted into estrogen. Consequently, treatment with 11-KA will only affect the HP-axis via the androgen receptor pathway. This will help to clearly identify the differences between estrogenic and androgenic feedback. The gonadectomized group was also split into 3 groups, which received the same treatment paradigms (GC, GE, GK). A commercial Finfish Bass diet (Zeigler,

Gardners, PA) was fed twice daily. Fish were sacrificed on day 10 post-surgery based on results obtained in previous studies (Chapter 2). Upon sacrifice, brains and pituitaries were removed and snap-frozen in liquid nitrogen. Blood samples were collected using heparinized syringes and immediately mixed with aprotinin (Sigma-Aldrich, St. Louis, MO) at the final concentration of 0.84 TIU/ml. After centrifugation, plasma was collected and all samples were stored at –80 ˚C until further analysis.

Experiment 2: The Effect of Gonadectomy and Steroid Replacement on the Reproductive Axis in Pubertal Striped Bass.

Based on results reported by Holland et al (2000) on striped bass gonadal development and plasma steroid levels during pubertal development, we determined that fish were initiating puberty at the end of their third year of life. Therefore, we conducted this experiment in August 2002. Fish (n = 70) of average size were selected (fluke length 40 cm, weight 820 g, GSI females 0.6%, GSI males 0.3%) from a mixed sex population and divided into 4 groups (Figure 11).

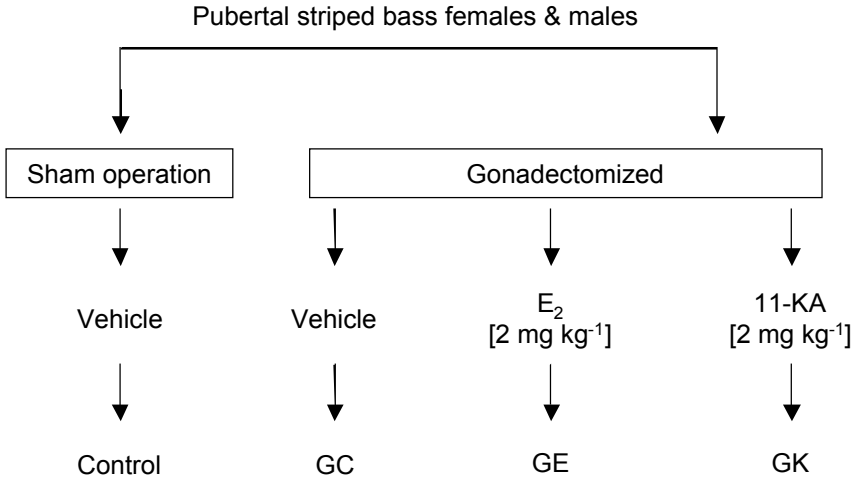

Figure 11: Pubertal striped bass females and males study (experiment 2). Diagram of the experimental design.

The first group was given a sham operation (16 females, 12 males), the other 3 groups were gonadectomized. Post-surgery, fish were marked with passive integrated responder (PIT) tags (Biosonics, Inc.) and returned to 6' diameter tank systems (one tank per treatment) for the duration of the experiment. After surgery, the s-o fish were given a vehicle injection (control). The first gonadectomized group was also given a vehicle injection (GC; 7 females, 7 males), the second group received E_2 steroid replacement (GE; 2 mg/kg; 7 females, 6 males) via microspheric delivery systems (modified from Holland et al., 1998b), and the third group received 11-KA steroid replacement (GK; 2 mg/kg; 8 females, 7 males) via microspheric delivery systems. A commercial Finfish Bass diet (Zeigler, Gardners, PA) was fed twice daily. Fish were sacrificed on day 10 post-surgery based on results obtained in previous studies (Chapter 2). Upon sacrifice, brains and pituitaries were removed and snap-frozen in liquid nitrogen. Blood samples were collected using heparinized syringes and immediately mixed with aprotinin (Sigma-Aldrich, St. Louis, MO) at the final concentration of 0.84 TIU/ml. After centrifugation, plasma was collected and all samples were stored at –80 ˚C until further analysis.

Quantification of Transcript Levels of GnRHs, GnRH-R, FSHβ, and LHβ

Transcript levels of the three GnRHs in the brain, as well as for the GnRH-R, FSHβ, and LHβ in pituitaries were measured using real-time fluorescence-based quantitative RT-PCR assays.

Total RNA, isolated from whole brains or pituitaries using Tri-reagent (MRC Inc., Cincinnati, OH) was quantified spectrophotometrically. RNA standards and total RNA from each sample were reverse-transcribed into cDNA using random hexamers and MMLV reverse transcriptase (Promega, Madison, WI). Duplicate cDNA aliquots (1 ng of

total RNA for 18s and LHβ RNA; 10 ng for FSHβ and GnRH-R; 50 ng for all GnRH transcripts) from each sample served as templates in PCR. The reaction was performed using SYBR Green PCR core reagent (Applied Biosystems, Forster City, CA) containing 200 mM gene-specific primers (Chapter 2). Primers were designed to span intron/exon boundaries in order to avoid amplification of genomic DNA. Amplification reactions were carried out in a ABI Prism® 7700 Sequence Detection System at 50 ˚C for 4 min, 95 ˚C for 10 min, and 40 cycles of 95 ˚C for 15 s and 60 ˚C for 60 s. Copy numbers in unknown samples were determined by comparing C_T, the fractional cycle number at which fluorescence passes a baseline threshold value (Fink et al., 1998), to the specific standard (run in every plate) and normalized to the amount of 18s RNA in each sample.

Quantification of LH Protein Levels

Pituitary LH content was measured by using a homologous ELISA (Mananos et al., 1997). Briefly, pituitaries were sonicated on ice in 200 µl phosphate buffer. After sonication, 10 µl of the homogenate was aspirated and diluted in 1 ml of 100 mM PBS containing 0.05% Tween 20. The range of the standard curve is 0.78 to 10 ng/ml, and the intra- and interassay CVs for this assay are 7.7% and 8.7% respectively. The antigen-antibody complexes were detected by addition of goat anti-rabbit IgG horseradish peroxidase conjugate (GAR-HRP, affinity purified EIA grade, BioRad) and TMB peroxidase substrate (KPL, Inc., Gaithersburg, MD). Absorbencies were read at 450 nm, using an automatic microplate reader (Thermomax, Molecular Devices, Menlo Park, CA). To avoid differences due to interassay variation, all samples from one experiment were run in the same ELISA.

Quantification of Estrogen and Testosterone Levels

Blood samples from experimental fish were immediately centrifuged, and plasma was separated and stored at –80 °C until further processing for steroid hormone quantification. Plasma E_2 and T were measured using commercially available, specific solid-phase RIAs that measure total amount of the respective hormone in unextracted, heparinized plasma (DPC, Los Angeles, CA). These assays have been validated for use with striped bass plasma and assay characteristics have been previously described (Mylonas et al., 1997a).

Statistical Analysis

The data on brain GnRH and pituitary GnRH-R, FSHβ, and LHβ transcripts, as well as protein levels of LH in pituitary and plasma E_2 and T levels, were subjected to Analysis of Variance (ANOVA) to determine the existence of changes between experimental groups. To specifically identify statistical differences among means of each of the above parameters, the means were further compared using Scheffe's post hoc test. Statistical significance was set at $P < 0.05$.

RESULTS

Experiment 1

No mortalities occurred in any of the three experiments conducted with juvenile striped bass. All gonadectomies were complete, which was visually verified at the termination of the experiments. However, due to the fact that the fish were selected from a mixed population and that we could not determine the sex of the young animals before

conducting the experiment, we had considerably more females per group than males. Reports by Holland et al. (1998b; Holland et al., 2000) have shown that levels of reproductive hormones vary between male and female fish during pubertal development, which made pooling samples in our experiments impossible. Since we had, with minor exceptions, more females per group than males, we omitted the data obtained for all males and analyzed only the female samples for this report. All groups were included in the statistical analysis, except the GK group of the March 2002 experiment, which had only 2 female fish in the group. The number of female fish per group is shown under each graph.

Plasma steroid levels in control juvenile striped bass were near detection levels for estrogen and below the detection limit for T. Levels in the GC were below levels of the control group (E_2) or non-detectable (T) as well. Steroid treatment increased plasma levels by 10 to 15-fold for estrogen. T was only detectable in the GK and SK groups and ranged between 105.2 ± 26.6 and 795.5 ± 130.1 pg/ml.

GnRH Expression Levels in the Brain

To determine the responsiveness of the GnRH neuronal network to steroid feedback regulation in juvenile striped bass, we measured the effects of gonadectomy, estrogen and androgen treatment on expression levels of the three GnRHs found in our model species. Transcripts of each gene were measured using quantitative PCR assays (Chapter 2). The level of detection for all GnRH transcripts was 480 copies/reaction and the results are normalized to the amount of 18s RNA. For better visualization of the changes happening in response to the different treatments, the data is presented as the

fold-change in mRNA levels compared to the corresponding control groups of the experiments.

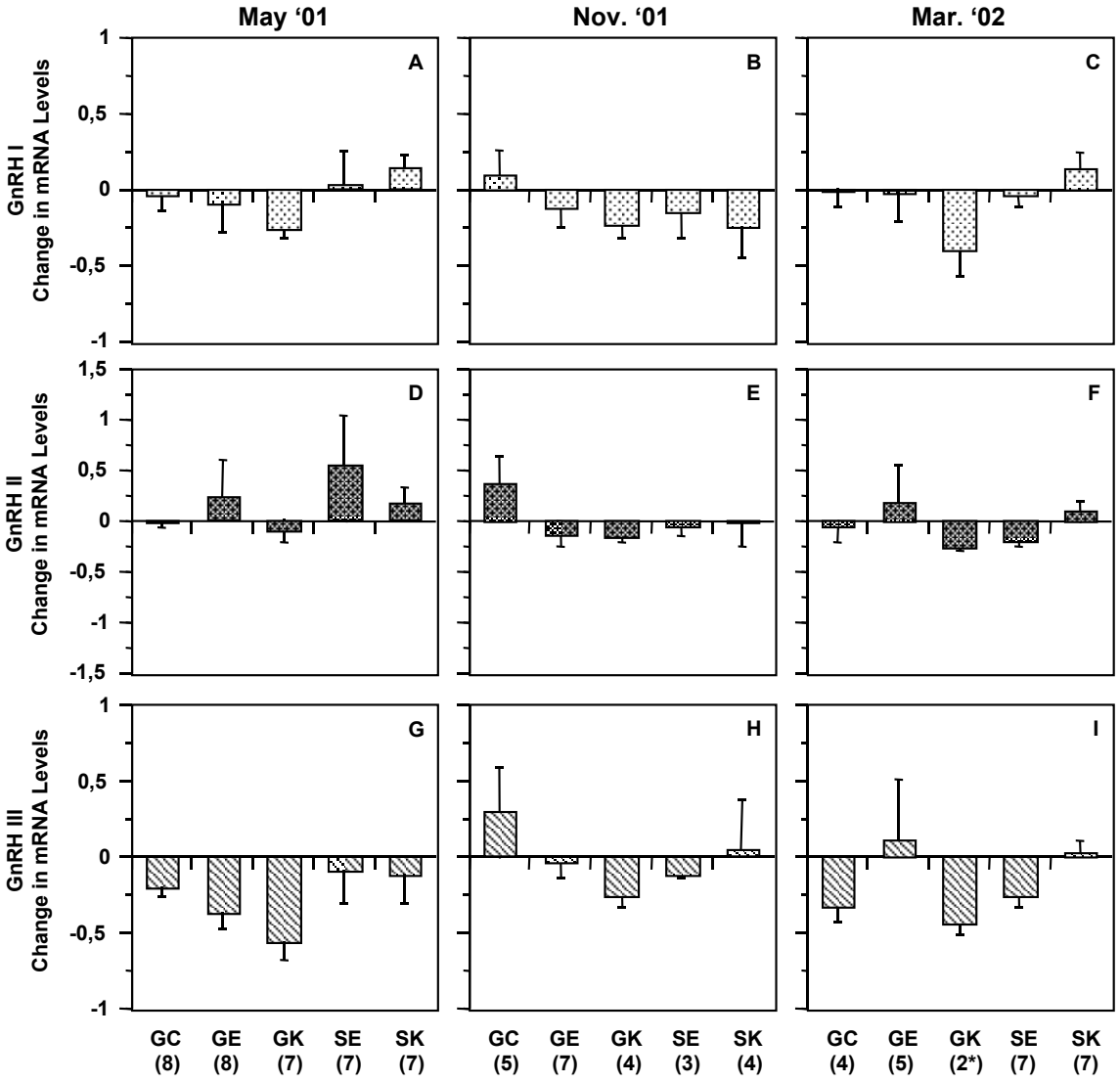

Figure 12: Transcript levels of GnRH I, II and III; Effects of gonadectomy and steroid treatment. In May 2001, November 2001 and March 2002, juvenile striped bass were gonadectomized (GC), or gonadectomized and treated with E_2 (GE) or 11-KA (GK) steroid replacement via microspheric delivery systems (2 mg/kg dose for both, E_2 and 11-KA). Intact s-o fish were also treated with the same steroids (SE and SK). Fish were sacrificed on day 10 post-surgery. Total RNA was extracted from brains and reverse-transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$. The numbers beneath the treatment groups indicate the number of fish per group (* this group was not included in statistic evaluation of the results due to its low n).

Figure 12 represents the results we obtained for the GnRH expression levels in experiment 1. At no time during juvenile development, from May 2001 until March 2002, did we observe changes in expression levels of GnRH I, II or III in any of the 5 treatment groups. Removal of the gonads, replacement with either estrogen or 11-KA, or treatment of s-o intact females with E_2 or 11-KA did not result in a significant response from the GnRH neurons.

GnRH-R Expression Levels in the Pituitary

To investigate the effects of steroid feedback at the level of the pituitary in juvenile striped bass, we focused on alterations in GnRH-R expression. With a level of detection of 600 copies/reaction for the GnRH-R, the same real-time PCR analysis as mentioned above was employed to quantify the amounts of GnRH-R mRNA in the pituitary. The results were normalized to 18s RNA and are presented as the fold-change in mRNA levels compared to control. As can be seen in Figure 13, neither gonadectomy nor replacement or treatment of the s-o intact fish with 11-KA caused significant changes in GnRH-R synthesis during any of the three time points of juvenile development. However in May 2001, E_2 significantly up-regulated GnRH-R mRNA levels in the intact females (Figure 13A). Similar results were observed in March 2002 (Figure 13C), when estrogen replacement and treatment of the s-o females elevated receptor transcript levels significantly by more than 5 to 10-fold compared to controls. The same treatment groups showed an increasing trend in the November 2001 experiment, but failed to reach the level of statistical significance.

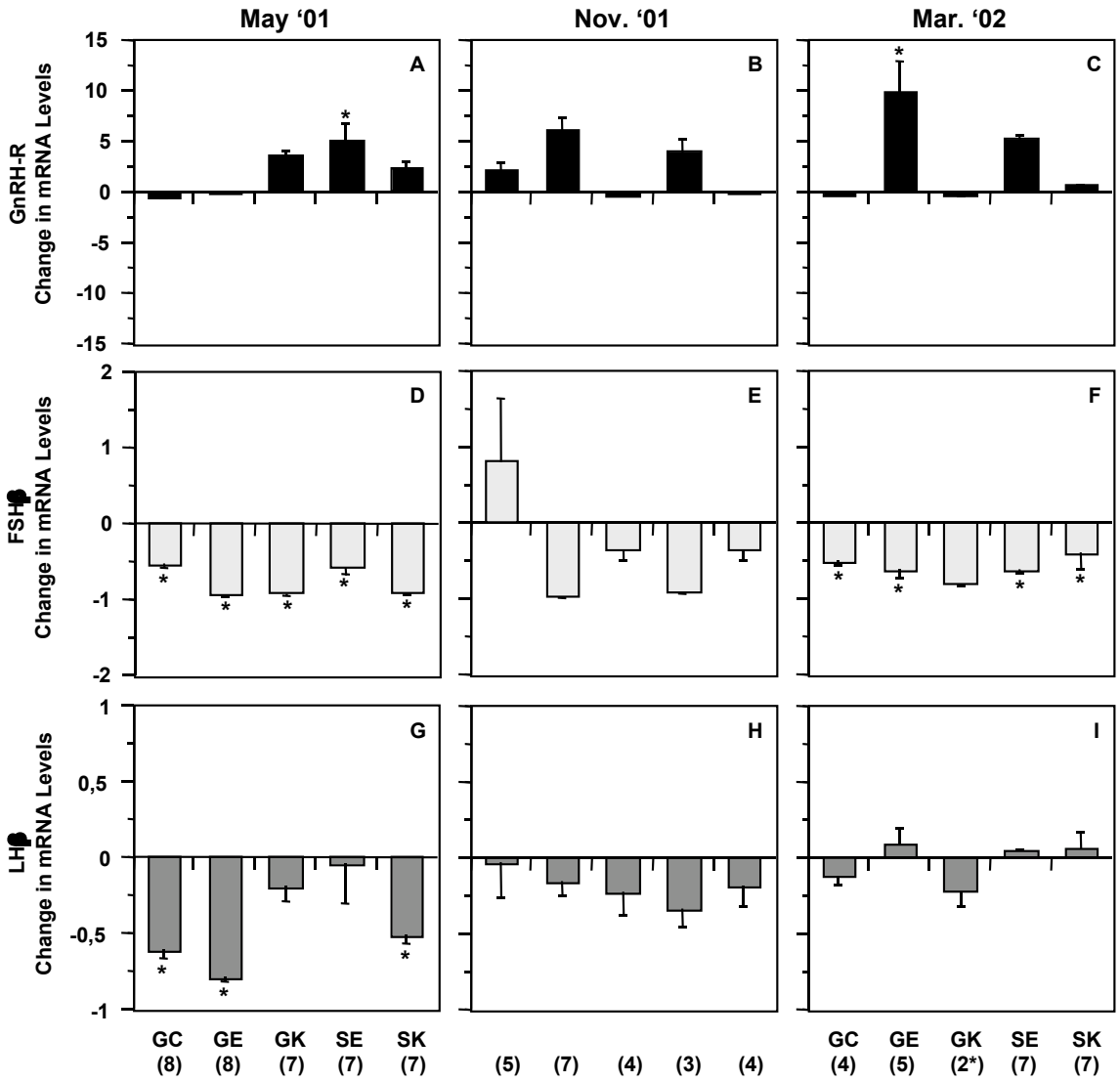

Figure 13: Pituitary GnRH-R, FSHβ and LHβ mRNA levels: Effects of gonadectomy and steroid treatment. In May 2001, November 2001 and March 2002, juvenile striped bass were gonadectomized (GC), or gonadectomized and treated with E_2 (GE) or 11-KA (GK) steroid replacement via microspheric delivery systems (2 mg/kg dose for both, E_2 and 11-KA). Intact s-o fish were also treated with the same steroids (SE and SK). Fish were sacrificed on day 10 post-surgery. Total RNA was extracted from pituitaries and reverse-transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$. The numbers beneath the treatment groups indicate the number of fish per group (* this group was not included in statistic evaluation of the results due to its low sample number).

GtH Subunit Expression Levels in the Pituitary

In addition to the GnRH-R, the gonadotropins FSH and LH are primary targets of gonadal steroids in the pituitary. Therefore, we analyzed the GtH subunit mRNA levels in

response to the experimental treatments using real-time PCR. The levels of detection for FSHβ and LHβ were 600 copies/reaction. The data is also presented as the fold-change in mRNA levels compared to control, after normalization to18s RNA. In May 2001, all treatments resulted in a significant decrease in FSHβ transcription (Figure 13D). Moreover, similar reductions in mRNA levels were observed in the experiment conducted in March 2002 (Figure 13F), where only the response of the GK treatment group was not significant due to the low number of fish (n = 2). However, FSHβ transcription was not affected by gonadectomy or steroid administration in gonadectomized or s-o females during the November 2001 experiment (Figure 13E). LHβ expression decreased significantly after removal of the gonads in May 2001 and the same response was observed in the E_2 replacement group, as well as 11-KA treatment of s-o fish. However, no changes were observed in the GK or SE groups (Figure 13G). Neither of the two subsequent experiments in November 2001 or March 2002 showed a treatment effect on LHβ synthesis (Figure 13H & Figure 13I).

LH Protein Levels in the Pituitary

To investigate the changes in LH protein levels in the pituitary, we analyzed the samples in duplicate using a specific homologous ELISA. The results are presented as the fold-change of protein levels compared to control. Figure 14 shows the results obtained for the three experiments conducted with juvenile striped bass females. There were no significant changes in LH protein levels observed in the pituitary at any time point during juvenile development.

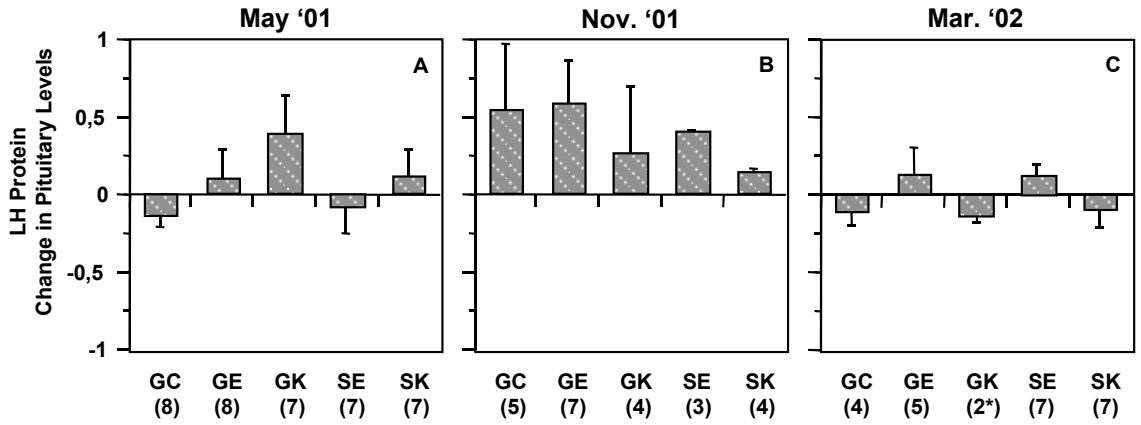

Figure 14: Pituitary LH protein levels: Effects of gonadectomy and steroid treatment. In May 2001, November 2001 and March 2002, juvenile striped bass were gonadectomized (GC), or gonadectomized and treated with E_2 (GE) or 11-KA (GK) steroid replacement via microspheric delivery systems (2 mg/kg dose for both, E_2 and 11-KA). Intact s-o fish were also treated with the same steroids (SE and SK). Fish were sacrificed on day 10 post-surgery. LH protein was extracted from pituitaries and measured using a specific ELISA. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$. The numbers beneath the treatment groups indicate the number of fish per group (* this group was not included in statistic evaluation of the results due to its low sample number).

Experiment 2

The second experiment was conducted during the pubertal phase of striped bass development based on prior reports from Holland et al. (1998b; Holland et al., 2000). In this experiment, we evaluated the response of the male and female HP axis to gonadectomy and steroid replacement therapy using E_2 and 11-KA microspheres for delivery. No mortalities occurred during the experiment, which lasted 10 days. The completeness of the gonadectomy was verified at the time of termination by visually surveying the gonadal cavity for signs of gonadal tissue.

Plasma steroid levels in control pubertal striped bass were near detection levels for estrogen and below the detection limit for T. Likewise, levels in the GC were below levels of the control group (E_2) or non-detectable (T). Steroid treatment increased plasma levels by 10 to 15-fold for estrogen. T was only detectable in the GK and SK groups and ranged between 105.2 ± 26.6 and 795.5 ± 130.1 pg/ml.

GnRH Expression Levels in the Brain

In August 2002, an investigation of the effects of steroid feedback regulation of GnRH synthesis in the hypothalamus of pubertal males and females was undertaken. The effects of gonadectomy, and estrogen and androgen replacement therapy, on GnRH I, II and III mRNA levels were measured using the same real-time PCR assay as mentioned above. As shown in Figure 15, none of the treatments had any effect on expression levels of GnRH I, II and III in females at this stage of the reproductive cycle. In males, neither gonadectomy nor E_2 replacement changed the synthesis of any of the GnRH forms. However, replacement with the non-aromatizable 11-KA elevated GnRH transcript levels of all three forms of GnRH significantly (Figure 15B, D & F).

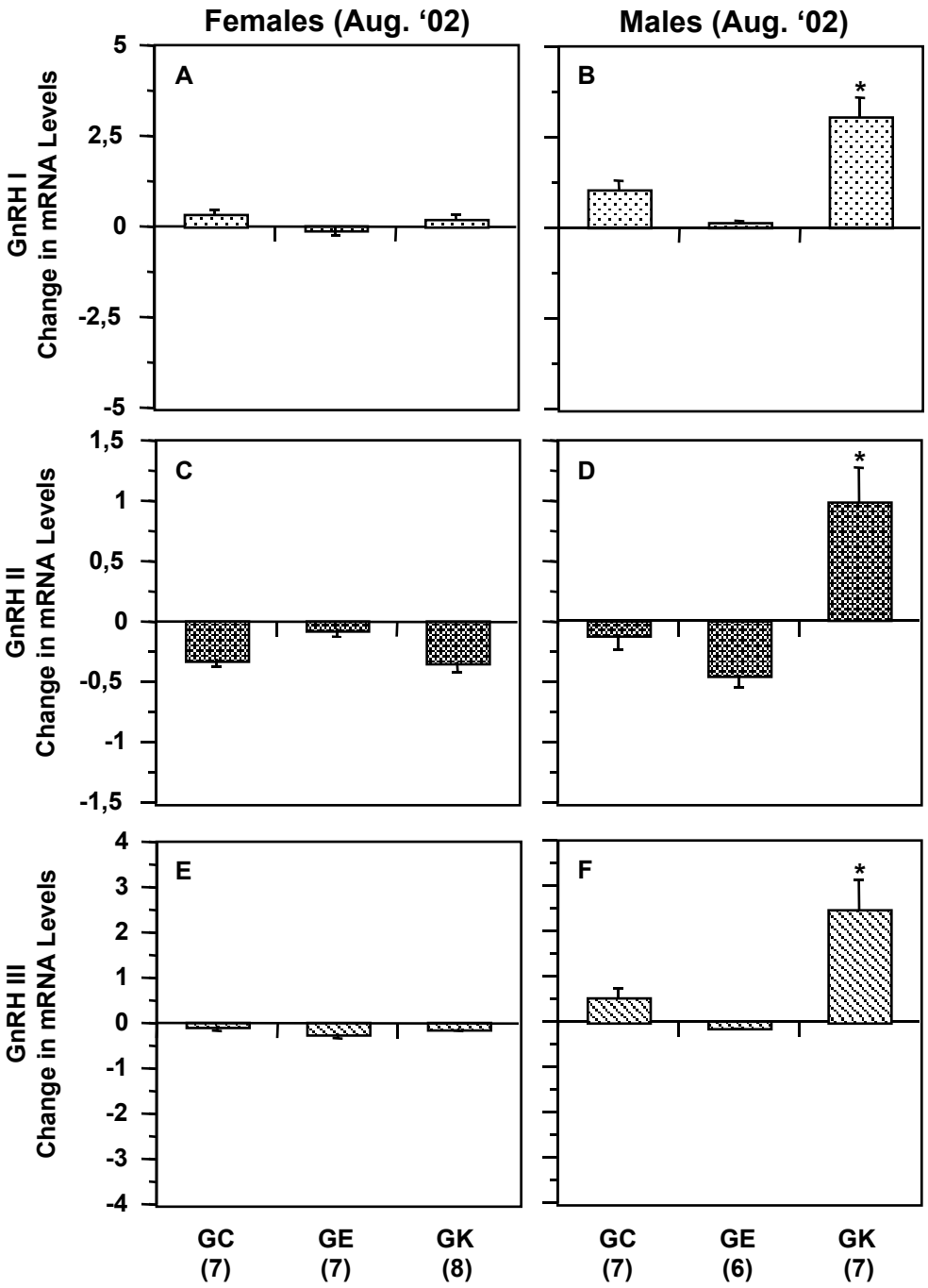

Figure 15: Transcript levels of GnRH I, II and III: Effects of gonadectomy and steroid treatment. In August 2002, pubertal male and female striped bass were gonadectomized (GC), or gonadectomized and received either E_2 (GE) or 11-KA (GK) steroid replacement via microspheric delivery systems (2 mg/kg dose for both, E_2 and 11-KA). Fish were sacrificed on day 10 post-surgery. Total RNA was extracted from brains and reverse-transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$. The numbers beneath the treatment groups indicate the number of fish per group.

GnRH-R Expression Levels in the Pituitary

At the level of the pituitary, we investigated alterations in GnRH-R transcription due to steroid removal or replacement using real-time PCR. In both sexes, only replacement with E_2 elevated expression levels of the receptor significantly, whereas gonadectomy or 11-KA treatment had no effect (Figure 16A & B).

GtH Subunit Expression Levels in the Pituitary

In order to understand the responsiveness of the pubertal gonadotropin system towards steroid actions, we analyzed pituitary FSH and LH subunit expression using a real-time PCR assay. Gonadectomy in females had no effect on FSHβ transcript levels. However, steroid replacement with both E_2 and 11-KA decreased subunit expression significantly (Figure 16C). In males, removal of steroid feedback elevated FSHβ mRNA significantly and E_2 and 11-KA replacement returned expression levels back to control levels (Figure 16D). LHβ synthesis did not change in response to either removal of the gonads nor to E_2 or 11-KA replacement in either sex (Figure 16E & F).

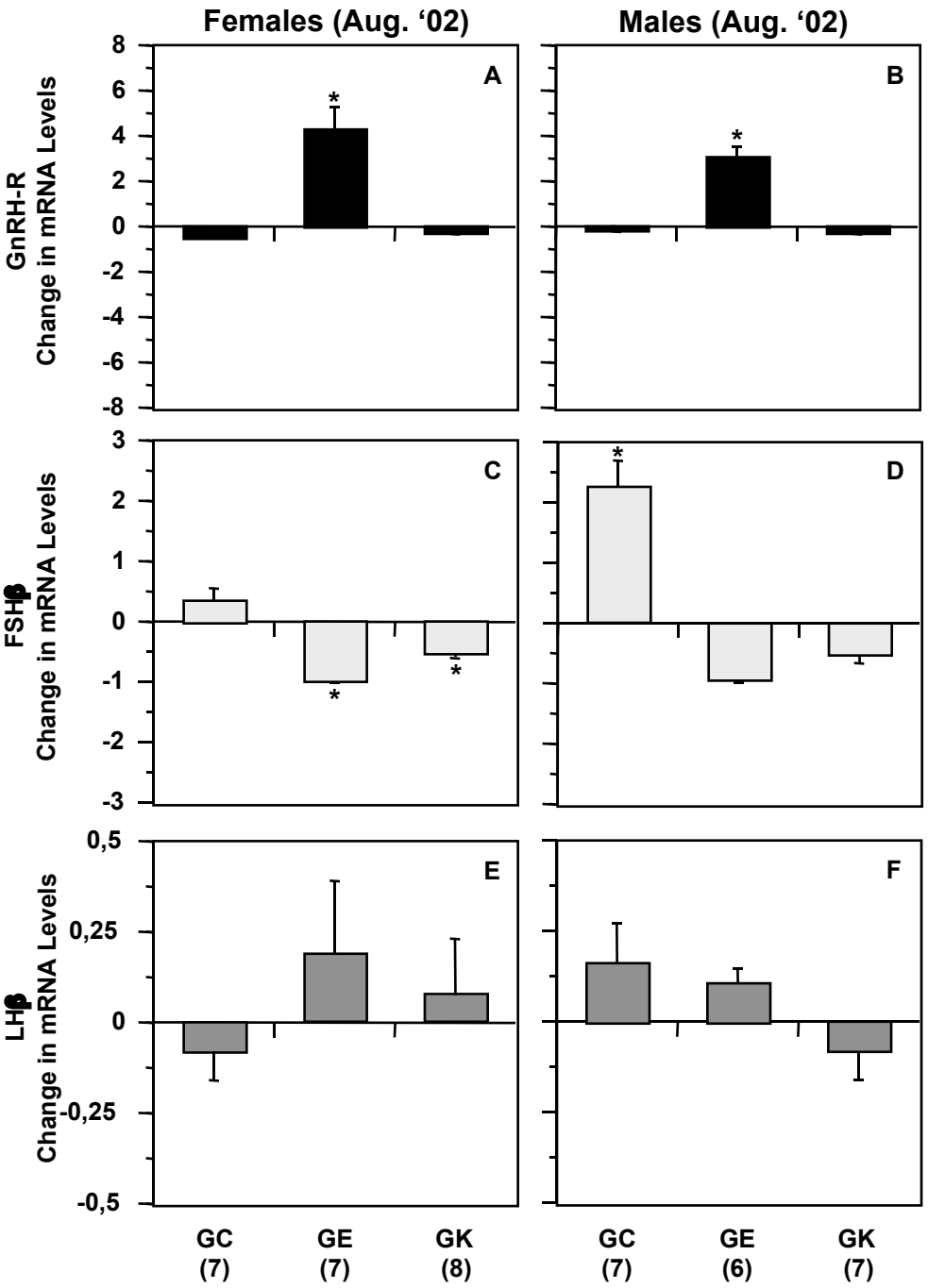

Figure 16: Pituitary GnRH-R, FSHβ and LHβ mRNA levels: Effects of gonadectomy and steroid treatment. In August 2002, pubertal male and female striped bass were gonadectomized (GC), or gonadectomized and received either E_2 (GE) or 11-KA (GK) steroid replacement via microspheric delivery systems (2 mg/kg dose for both, E_2 and 11-KA). Fish were sacrificed on day 10 post-surgery. Total RNA was extracted from pituitaries and reverse-transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$. The numbers beneath the treatment groups indicate the number of fish per group.

LH Protein Levels in the Pituitary

Using a specific homologous ELISA, we measured pituitary LH protein content to study the steroidal effect on LH release. In females, the amounts of LH did not change in response to any of the three treatment paradigms (Figure 17A). However, in males androgen replacement significantly reduced LH levels in the pituitary, but gonadectomy and E_2 replacement had no effect (Figure 17B).

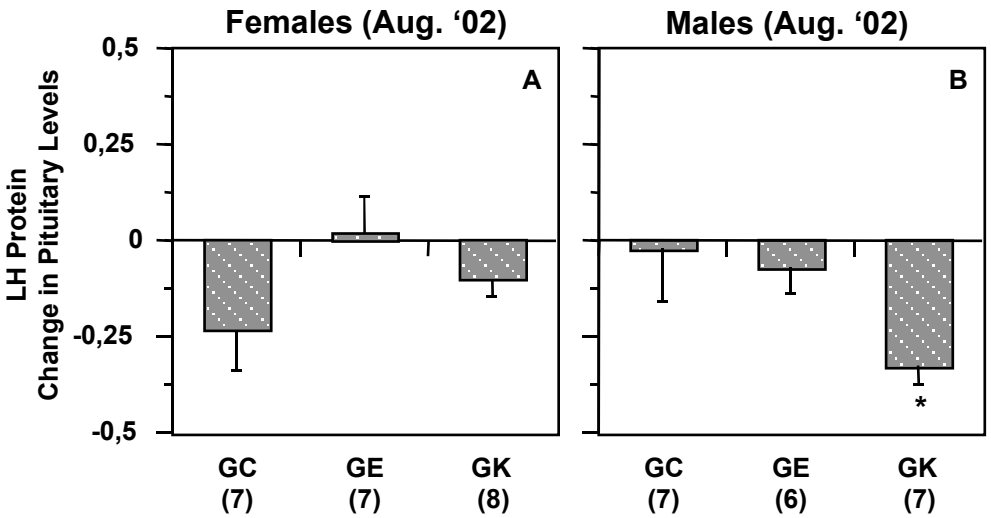

Figure 17: Pituitary LH protein levels: Effects of gonadectomy and steroid treatment. In August 2002, pubertal male and female striped bass were gonadectomized (GC), or gonadectomized and received either E_2 (GE) or 11-KA (GK) steroid replacement via microspheric delivery systems (2 mg/kg dose for both, E_2 and 11-KA). Fish were sacrificed on day 10 post-surgery. LH protein was extracted from pituitaries and measured using a specific ELISA. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$. The numbers beneath the treatment groups indicate the number of fish per group.

DISCUSSION

The involvement of steroids in the onset and timing of puberty is still very controversial. For some teleost species, gonadal steroids appear to be absolutely necessary for puberty to occur [African catfish, (Schulz and Goos, 1999)]. For other teleosts, they are permissive or an enhancer in the initiation of the maturation process (Dufour et al., 2000). However, in still other species, their regulatory role seems to come

into play only during the adult reproductive cycle. Work done by Holland et al. (2000) and Hassin et al. (1999) in striped bass has suggested that endogenous levels of E_2 and T did not play a role in the initiation of puberty in the HP axis. In this model organism, however, nothing is known of the effects of gonad removal and steroid replacement, nor of the effects of steroid treatment on GnRH synthesis in the hypothalamus or GnRH-R transcription in the pituitary in juvenile and pubertal fish. Therefore, this study focused on four time points during juvenile and pubertal development to investigate the role of steroids in the regulation of the HP axis in striped bass. The studied phases were chosen based on previous work (Hassin et al., 1999; Holland et al., 2000; Holland et al., 2001) and on the seasonality of the reproductive cycle (recrudescent phase in the fall, vitellogenic stage in spring).

GnRH Transcription in the Hypothalamus

In juvenile females, the GnRH neuronal network is not responsive to gonadal steroids in the second and third year of development. Neither gonadectomy, nor replacement with estrogen or the non-aromatizable androgen 11-KA, which is converted in the fish body to 11-KT, the main androgen found in male fish (Scott et al., 1980), have an effect on the synthesis of any form of GnRH found in striped bass. Moreover, even treatment of intact s-o animals with the same dosages of steroids did not change transcription levels of GnRH I, II or III. This is an indication that exposure of the gonads to steroids for 10 days does not change the 'composition' of factors in the gonadal feedback [e.g. peptides like activin (Ge, 2000)] or that possible changes have no effect on GnRH expression in the juvenile brain.

Ovariectomy and steroid replacement with E_2 or 11-KA in the early stages of the reproductive cycle had no effect on GnRH expression levels in the pubertal striped bass female. However, steroid replacement with 11-KA in castrated male striped bass elevated GnRH transcription of all three forms significantly, showing an active response of the GnRH neuronal network to steroid treatment in pubertal males. The fact that no effect on GnRH expression in the castrated group was observed suggests that there is no relevant feedback effect of endogenous steroids at this stage. Estrogen replacement also had no effect, which in pubertal males indicates that androgens are of more importance as a regulator of the GnRH neuronal network in the hypothalamus via an androgen receptor specific pathway. The finding that GnRH expression is up-regulated by 11-KA treatment in male pubertal fish demonstrates that there is a responsiveness of the GnRH system to gonadal steroids in pubertal striped bass.

Our results obtained in male pubertal striped bass are in full agreement with studies conducted in male masu salmon, where treatment with 17α-methyltestosterone increased the GnRH neuron number in the preoptic area of the hypothalamus (Amano et al., 1994a). Moreover, in the male African catfish steroid treatment with testosterone and E_2 exerted a positive influence on hypothalamic GnRH content (Dubois et al., 1998; Dubois et al., 2000) and E_2 also increased the number of POA GnRH neurons in male tilapia (Parhar et al., 2000), indicating that the steroidogenic pathway (ER versus AR) is dependent on the species rather than the gender. Similar results were also observed in juvenile male and female red seabream, where 11-KT implantation for one month increased hypothalamic GnRH mRNA levels. However, E_2 and testosterone treatment had no effect in either sex (Okuzawa et al., 2002). Montero et al. (1995) observed an

increase in GnRH levels in the brain and pituitary of female silver eel, whereas we did not observe any response to treatment in female pubertal striped bass. This contrast may be due to the fact that the response of the GnRH system appears to be very species-specific, as well as dependent on the maturational stage of the animals during the experimental phase.

The insensitivity of the juvenile GnRH neuronal network to stimulation by steroids shown in this report concurs with the general view of the mammalian juvenile period, in which the decrease in GnRH secretion in the neonatal animal and the primary activation of GnRH secretion in the onset of puberty are processes that are independent of gonadal steroid feedback (Ebling, 2005). This has been demonstrated by the decrease in GtH secretion after the neonatal period and subsequent increase in the pubertal period in an agonadal primate model (Plant, 1985). However, the reactivation of GtH release occurs earlier in these animals, suggesting that steroid feedback mechanisms play a role in the later stages of pubertal maturation (Rapisarda et al., 1983).

Ontogeny studies in mammals and fish have shown that the anatomical development of the GnRH system becomes established early in life and that the synthetic capacity is present well before puberty [mammals: (reviewed in Terasawa and Fernandez, 2001; Ojeda and Terasawa, 2002; Ebling, 2005); fish: (Feist and Schreck, 1996; Parhar, 1997; Chiba et al., 1999; Wong et al., 2004)]. Thus, the common findings across many mammalian studies is that increases in GnRH gene expression and translation precede the increases in GtH secretion that drive gonadal maturation (reviewed in Plant, 2001). A similar situation is found in fish, where levels of GnRH are low, but present, in juvenile fish and increase during sexual maturation (Amano et al., 1992; Amano et al., 1993;

Holland et al., 1998a; Holland et al., 2001). It therefore appears that the availability of GnRH in the pituitary is not limiting the onset of puberty in fish, but rather other maturational processes in the pituitary or gonad may initiate pubertal processes.

It appears that in mammals the inhibition of the adult secretory pattern of the GnRH system during the juvenile phase is probably due to the immaturity of transsynaptic input regulating GnRH neurons before puberty. This is caused by either insufficient excitatory neuronal input to GnRH neurons (e.g., glutamate, norepinephrine), or by the suppressing activity of inhibitory neuronal input to GnRH neurons (e.g., GABA, NPY) (Terasawa and Fernandez, 2001; Plant, 2002). Of further importance for the timing of puberty is the concept of 'structural plasticity', which refers to the respective acquisition or loss of stimulatory or inhibitory synaptic inputs to the GnRH neuronal system. Glial cells surrounding GnRH neurons undergo plastic rearrangements and establish direct cell-cell communication, which requires the participation of growth factors like transforming growth factor beta 1 and 2 (TGFβs) or insulin-like growth factor I (IGF-I) (Ojeda et al., 2003b), and thereby stimulate GnRH release.

In teleost species, GABA has been suggested to have a positive effect on GnRH and GtH synthesis/release (reviewed in Trudeau et al., 2000; Kah et al., 2000) during the juvenile phase. However, studies conducted in juvenile teleosts, and primarily in the European eel, suggest that DA may play an inhibitory role in the control of puberty (Dufour et al., 2005). Yet, Holland et al. (1998b) showed in juvenile striped bass that combined treatments with T, GnRH agonist and a DA-D_2 receptor antagonist (pimozide) had no indication of DA involvement in the control of puberty. Moreover, administration of the GnRHa alone was able to induce precocious puberty in the red seabream, with no

further effect of DA antagonist, indicating no role for DA in puberty (Kumakura et al., 2003).

In striped bass, steroid feedback is a very important regulator of the HP axis in the adult reproductive cycle (Chapter 4), nonetheless during the juvenile phase no response to steroidal stimulation is observed. Even though the known inhibitory systems governing puberty in other teleost species or mammals appear not to be involved in the inhibition of the GnRH neuronal network in juvenile striped bass, there may be other external or internal factors responsible.

Sexual maturation in teleost is controlled by photoperiod and temperature, which both can act to accelerate or delay the timing of puberty (Bromage et al., 2001). The mechanisms underlying the transduction of the external information to the reproductive axis are still unclear, but involve neurotransmitters like melatonin or clock genes (Kah et al., 2000). Reproduction is also tightly connected to the somatic growth axis (Le Gac et al., 1993) and there has been growing evidence that growth factors influence the HPG axis on multiple levels during the initiation of puberty in vertebrates (Ojeda and Terasawa, 2002). There are indications as well that IGF-I may play an important role during puberty. Huang et al. (1998) demonstrated that IGF-I regulated the synthesis and release of LH in pituitary cells from female silver eels, while Weil et al. (1999) showed an increase in sensitivity of FSH and LH GnRH-induced release in cells from rainbow trout. *In vivo* evidence includes increased IGF-I plasma levels during puberty in rainbow trout (Le Gac et al., 2000) and higher levels of FSH in the pituitary and increased FSH release induced by GnRH in juvenile coho salmon (Baker et al., 2000a). Another metabolic signal controlling reproduction and puberty in mammals is leptin, which is

produced by adipose cells and generates information about the fat content of the body (Fruhbeck et al., 1998). This hormone has not been identified in fish to date, although some studies have been conducted to investigate the effects of leptin as a modulator of the GnRH-GtH complex and puberty initiation in fish (Baker et al., 2000b; Peyon et al., 2001; Weil et al., 2003). Leptin has a direct positive effect on GtH production and release, which seems to be dependent on the sexual status of the fish. Additionally, a permissive role for this hormone in the initiation of puberty, once its levels surpass a critical threshold (Weil et al., 2003), has been proposed. Further studies are required to better understand the mechanisms that connect the growth and reproductive axis and their involvement in the onset of puberty.

The recent discovery of the importance of kisspeptin and its receptor GPR54 in the regulation of GnRH neurons, and its possible role as a mediator of steroid feedback in mammals, elevate the need to incorporate this peptide in our understanding of steroid regulation at the onset of puberty. Originally, kisspeptin (metastin) was identified as the natural peptide ligand for GPR54, a G protein-coupled receptor that when mutated in humans, is associated with the failure to progress through puberty (de Roux et al., 2003; Seminara et al., 2003). Recent studies have identified the kisspeptin/GPR54 system as a mediator for negative and positive steroid feedback on the GnRH neuronal network in adult mammals (Smith et al., 2005a; Smith et al., 2005b). Furthermore, several lines of evidence suggest that an increase in the efficacy of the kisspeptin-GPR54 signaling accompanies the onset of puberty in mammals, although the mechanisms they initiate seem to be species dependent. Thus, the activities of both kisspeptin and its receptor appear to increase during pubertal development. Albeit the factors that set this process in

motion are still unknown, increased kisspeptin system activity appears to be a prerequisite for puberty to occur (Dungan et al., 2006). Recently GPR54 mRNA expression has been reported in GnRH neurons in tilapia (Parhar et al., 2004), but kisspeptins have not been identified thus far. The presence of the GPR54 in fish GnRH neurons provides a basis for the hypothesis that a system similar to the kisspeptin/GRP54 arrangement in mammals is present in fish and could provide the missing link between gonadal steroid feedback and the regulation of GnRH neurons during puberty, as well as during the adult reproductive cycle.

The lack of response of the GnRH neuronal network to steroids during the juvenile and early pubertal phase in female striped bass may be caused by absence of excitatory neuronal input. Based on the structural plasticity theory, synaptic connections needed for relaying steroid feedback (through kisspeptin or growth factors) to GnRH neurons may not yet be in place. Furthermore, receptors (GPR54) for these stimulatory transmitters may also be missing at this stage of development. A slow increase in steroid levels during the juvenile/pubertal development may be stimulating these structural rearranging processes to occur and may therefore be permissive for the onset of sexual maturation. More comprehensive studies are needed to better understand the mechanisms through which steroid feedback is regulating the GnRH neuronal network during the initiation of puberty and adulthood.

GnRH-R Transcription in the Pituitary

Appropriate expression of the GnRH-R in gonadotrophs is critical for GnRH signaling and hence for gonadotropin secretion and sexual development. One key regulator of GnRH-R transcription during the adult reproductive cycle is gonadal steroid

feedback (Hapgood et al., 2005). To understand the control exerted by sex steroids on GnRH-R transcription during the juvenile and pubertal phase, we studied GnRH-R mRNA levels in response to gonadectomy and steroid treatments.

Removal of the gonads had no effect on GnRH-R expression in juvenile females suggesting that no feedback is exerted by gonadal factors during this developmental period. Replacement with 11-KA or treatment with the same steroid of intact s-o females did not result in any changes in transcription levels either. However, E_2 treatment did have positive effects on receptor transcription in the experiments conducted during spring. In 13-month old, intact s-o females (May 2001) estrogen elevated receptor mRNA levels almost 5-fold above control and a year later E_2 replacement increased receptor transcription by 10-fold in 23-month old females (March 2002). In the fall (November 2001), 19-month old juveniles showed a positive trend in GnRH-R expression in E_2-treated fish, but did not reach significance. These results suggest that GnRH-R expression in the pituitary is responsive to sex steroids in the juvenile fish. The different effects observed in spring versus fall experiments were not surprising considering the stages of the adult reproductive cycle the animals would experience. In pubertal males and females, 28-months of age (August 2002), expression of the GnRH-R was also not under gonadal feedback control. However, E_2 replacement significantly increased GnRH-R transcription in both sexes, whereas 11-KA had no effect.

To date, few studies have investigated GnRH-R expression during the juvenile and pubertal stages in vertebrates. In the female rat, data on the ontogeny of GnRH-R expression shows maximal rates during infancy, which are kept at a minimum during the juvenile prepubertal period. At the initiation of puberty, receptor levels rise

concomitantly with increased GnRH release, indicating that the developmental activation of the GnRH-R gene expression is entirely GnRH dependent (Zapatero-Caballero et al., 2003). Similar results were reported in juvenile female red seabream, where Kumakura et al., (2003) showed that implantation with GnRHa resulted in a significant increase in GnRH-R transcription levels in the pituitary, suggesting that GnRH itself is a positive regulator of its own receptor in teleost. Steroid treatment with E_2 and 11-KT of juvenile mixed sex red seabream decreased GnRH-R expression, suggesting a negative feedback effect on GnRH-R transcription by steroids in juveniles (Okuzawa et al., 2002). These results are contradictory to our findings in female striped bass, where estrogen had a positive effect, while 11-KA had none. This may be due to the fact that Okuzawa et al. (2002) observed these changes in a mixed sex population, where 11-KT may have been acting on GnRH-R mRNA levels in males only. Moreover, the red seabream has a very different gonadal development. Since they have bi-potential gonads and asynchronous gamete development, their response of the HP axis could be very different from our model. Thus, it appears that steroid regulation of GnRH-R expression is dependent on species, sex and maturation stage of the animal.

Elucidating the manner in which GnRH-R synthesis is regulated may be a key to understanding the process of puberty in vertebrates, and in fish specifically. In our model, the juvenile striped bass, transcription of GnRH-R is not influenced by endogenous gonadal factors. However, administration of exogenous estrogen results in a positive regulation of the receptor mRNA levels, which becomes stronger with the onset of puberty. But even though the receptor synthesis is responding to steroid regulation, the endogenous steroid levels are probably too low to exert any feedback effects on pituitary

receptor expression during the juvenile and pubertal stage of development. As there is only scarce information about the seasonal expression and physiological regulation of the GnRH-Rs, the logical next step towards understanding how the HPG axis is controlled are more studies on the involvement of the receptor in the process of sexual maturation.

FSH and LH Transcription and LH Protein Levels in the Pituitary

Gonadal steroids regulate biosynthesis of gonadotropins in the pituitary in adult fish. Studies on steroid feedback on FSH in juveniles have produced various results depending on the species of the model. In prepubertal coho salmon, steroids exerted a negative effect on FSH *in vivo* (Dickey and Swanson, 1998) and a similar effect was observed in juvenile rainbow trout (Saligaut et al., 1998) and the Japanese eel (Yoshiura et al., 1999). No effects were observed in the rainbow trout *in vitro* (Xiong et al., 1994) and positive effects were reported in tilapia (Melamed et al., 1997). We therefore determined the expression levels of FSH and LH in the pituitary in response to gonadectomy and steroid treatment.

In both spring experiments (May 2001, March 2002), the spawning season of adult striped bass, FSHβ transcription was significantly inhibited by all treatment paradigms. However, in November 2001, the recrudescence stage of adult females, no significant down-regulation of FHSβ mRNA levels was observed, even though there was a negative trend in the estrogen replacement/treatment groups. Our results support the finding in salmonids (see above), where a negative regulation of FSHβ transcription in response to steroids was observed. Of interest is the seasonality of the steroid feedback effect observed in juvenile female striped bass. Hassin et al. (1999) reported in juvenile females of the same species a cyclic expression pattern for FSHβ mRNA levels with

rising levels in the fall, its peaks around November and a decline in the spring. The observed negative regulation by steroids in the spring could be an explanation for the decreased levels found in this earlier study. Further the reported rise in FSHβ levels during the fall appears not to be regulated by gonadal factors and even exogenous steroids had no significant effect (this study). These results suggest that there is a shift of mechanisms in the control of FSHβ expression between the seasons of the (adult) reproductive cycle, which is already apparent in juvenile females. During the early phase of gametogenesis, FSH transcription is not under gonadal regulation, which changes in the spring, where negative feedback control is exerted.

In fall 2002, ovariectomy of pubertal females had no effect on FSHβ transcription, suggesting that no gonadal feedback control was removed. However, steroid replacement with E_2 and 11-KA significantly decreased FSHβ mRNA levels showing a sensitivity of this GtH for negative steroidal feedback during recrudescence. These results are in contrast to observations made in juveniles, where steroids had no effect in the fall (see above). This discrepancy may be due to the ongoing maturation of the HPG axis and the establishment of the adult reproductive pattern, which is more sensitive to steroid feedback, during the recrudescent stage, than in the juvenile animal. In males, gonadectomy significantly increased FSHβ transcription, which could be reversed by steroid replacement with both E_2 and 11-KA. These results suggest that a negative feedback regulation exerted from the gonads, via both steroidogenic pathways (estrogenic and androgenic), on FSHβ expression is in place in pubertal males during the early stages of the reproductive cycle.

Gonadal regulation of FSHβ biosynthesis is not only exerted via steroids. In juveniles, gonadectomy alone also significantly decreased FSHβ mRNA levels in the spring experiments, suggesting that another gonadal factor, e.g. activin, may play an important role. Activin is part of the TGFβ superfamily (Massague, 1987) and it has been well documented in mammals to be critical in the regulation of FSH but not LH biosynthesis (Phillips, 2005; Phillips and de Kretser, 1998). Studies conducted in goldfish have shown that recombinant activin B and human activin A significantly stimulated FSH expression and suppressed LH transcription (Yuen and Ge, 2004). Application of follistatin (activin binding protein) to pituitary cells blocked the effect of exogenous activin on expression levels of FSH and LH and even caused inverse changes of basal FSH and LH mRNA levels, which were opposite of those of activin (Yuen and Ge, 2004). In tilapia however, human activin A stimulated the expression of both FSH and LH *in vitro* (Yaron et al., 2001), suggesting that regulation of GtH subunits by activin is dependent on the fish species and probably on the stage of sexual maturation.

Our results suggest that FSH synthesis in juveniles during spring is under positive gonadal regulation, which is not steroidogenic in nature but rather due to activin or other unidentified factors. However, the pituitary system is responsive to exogenously administered sex steroid stimulation during the juvenile phase. During the onset of puberty no gonadal effects are observed in females, even though decreased synthesis as a response to steroid treatment is shown. In males however, negative steroid feedback appears to be in place, based on increasing FSH mRNA levels after gonadectomy and reestablishment of control levels after steroid replacement (via estrogenic and androgenic pathways).

Changes in LHβ transcription levels in response to our treatment paradigms were observed in the prepubertal phase only in the May 2001 experiment, when fish were 13-months old. At this time, ovariectomy significantly decreased LHβ mRNA levels, suggesting the removal of a positive gonadal derived feedback at this stage of sexual maturation. This feedback is not estrogenic in nature, since a decrease in LHβ expression was also observed in the E_2 replacement group. Treatment of intact s-o females with estrogen had no effect. However, replacement with 11-KA reestablished LHβ mRNA levels to those found in controls, indicating that the gonadal factor could be an androgen. Surprisingly though, 11-KA treatment of intact s-o females significantly decreased synthesis of this GtH. These contradictory results may be explained with the fact that 11-KA acts on the gonad in the intact s-o females and causes changes in the 'factor-cocktail' produced by the gonads, which appears to have a different effect on LHβ transcription than 11-KA alone. Similar observations were made in the juvenile male African catfish, where treatment of intact animals with 11-KT had an inhibitory effect on the amount of pituitary LH (Cavaco et al., 1995).

Experiments conducted in November 2001 and March 2002 showed no effect in response to any treatment paradigm, suggesting that LHβ synthesis regulating mechanisms employed by steroids were either inactive or not in place during this developmental period. Similar observations were made in female and male pubertal fish, where LHβ transcription did not change as a result of gonadectomy and steroid replacement (E_2 and 11-KA) during early gametogenesis (August 2002). These results were surprising, since positive feedback effects on LH synthesis and release in juvenile teleost have been reported in numerous studies: platyfish (Schreibman et al., 1986),

goldfish (Huggard et al., 1996), European eel (Querat et al., 1991), black carp (Gur et al., 1995) and the European sea bass (Mateos et al., 2002). Moreover, steroids enhance LH release in response to GnRH in goldfish (Trudeau et al., 1991b), however FSH levels in these species are not known, which makes it difficult to determine which GtH plays what role during the sexual maturation process. Hassin et al. (1999) reported in juvenile striped bass that FSHβ mRNA levels were lower than LHβ, but increased at the onset of gonadal development, suggesting a role in the onset of gamete development for FSH. LHβ transcription levels did not increase until late puberty, the final stages of the reproductive cycle, indicating an involvement of this GtH in the later stages of gamete development. In light of this hypothesis, the missing steroidal regulation of LHβ during the juvenile and early pubertal phase is not as surprising. However, an involvement of LH in the early juvenile phase can not be dismissed, based on gonadal regulation of LHβ transcription in 13 months-old in this study and the higher levels of LHβ compared to FSHβ transcription reported by Hassin et al. (1999) in the same developmental period.

Steroid treatment of immature fish with T or E_2 increased pituitary LH protein levels in rainbow trout (Breton et al., 1997), Atlantic salmon parr (Antonopoulou et al., 1999), coho salmon (Dickey and Swanson, 1998), and European sea bass (Mateos et al., 2002). However, in all three experiments conducted in juvenile striped bass females, neither gonadectomy nor steroid replacement/treatment had any effect on pituitary LH content. The same result was observed in pubertal females. In pubertal males however, even though gonadectomy and estrogen replacement left the amount of LH unchanged, 11-KA significantly decreased LH protein levels. This result suggests, that gonadotroph

cells in pubertal males acquired the ability to respond to steroid stimulation of LH release.

In summary, all three forms of GnRH are not responsive to steroid regulation during the juvenile stage in female striped bass. With the onset of puberty, in the recrudescent phase of gametogenesis, no regulation of GnRH neurons by sex steroids was observed in females. However, in males the expression of all three GnRHs is positively influenced by 11-KA, suggesting an increasing sensitivity to steroid regulation of the hypothalamus in pubertal male striped bass. Expression of the GnRH-R is positively regulated via an estrogenic pathway during both the pre- and pubertal stage, showing a seasonal pattern of regulation in the juvenile females. FSHβ mRNA levels are suppressed by steroids and gonad removal during the spring, but not in the fall, indicating a seasonal regulation by gonadal factors over the reproductive cycle that is already apparent in the prepubertal females. Besides inhibitory effects on LHβ expression in the youngest females (13-months old), steroids had no effect on transcription of this GtH, or on the amount of LH protein in the pituitary. However, whether or not these results obtained for the regulation of GtH biosynthesis occur via direct regulatory mechanisms exerted at the pituitary, or indirectly via the GnRH system, cannot be determined due to missing information about GnRH release from the hypothalamus.

CHAPTER 4. STEROID FEEDBACK REGULATION OF THE HYPOTHALAMUS-PITUITARY AXIS AT DIFFERENT STAGES IN THE REPRODUCTIVE CYCLE OF ADULT FEMALE STRIPED BASS

ABSTRACT

Regulation of the reproductive cycle in adult striped bass (*Morone saxatilis*) occurs via the hypothalamo-pituitary-gonadal (HPG) axis. In this study, we wanted to identify the effects of gonadal feedback on the HP components of the axis at two distinctly different stages of the reproductive cycle: recrudescence and midvitellogenesis. Therefore, the effects of bilateral gonadectomy and steroid replacement/treatment on GnRH, GnRH-R and GtH gene expression, as well as pituitary GnRH peptide, and pituitary/plasma LH protein levels were monitored. During recrudescence, females were divided into 6 groups. Three gonadectomized (gdx) and 3 sham-operated (s-o) groups were established. One sham and one gdx group received E_2 (2 mg/kg) and 11-KA (2 mg/kg) respectively via microspheric delivery systems, the remaining two groups were given vehicle injection. Midvitellogenic females were divided into four groups. Two gdx and two s-o groups were established. Half of the gdx and s-o animals received T (4 mg/kg) via microspheric delivery systems, the remaining fish received vehicle only. Ten days post-operation, mRNA levels of the three GnRHs, GnRH-R, FSHβ and LHβ were determined, using real-time quantitative PCR normalized to 18 S mRNA levels. Pituitary GnRH peptide and LH protein levels were measured using specific ELISAs. Plasma LH steroid levels were measured using RIAs. Synthesis of either GnRH form was not under gonadal steroid feedback regulation at the investigated stages, however GnRH

neuronal sensitivity to exogenous administered steroids increased during midvitellogenesis. The GnRH-R is under positive gonadal feedback during recrudescence, however, this effect is not observed in the subsequent stage. Gonadal feedback regulation of FSHβ transcription changes from an inhibitory effect during recrudescence to a stimulatory influence during midvitellogenesis. LHβ is not under the regulatory influence of gonadal feedback at either reproductive stage. Furthermore, LH protein levels were stable after ovariectomy in both experiments, suggesting no gonadal regulation of LH release. These results suggest that the main target for gonadal feedback is gene transcription of the GnRH-R and FSHβ during recrudescence and only FSHβ during midvitellogenesis. Furthermore, results obtained in the steroid treated groups demonstrated an increased responsiveness of the HP axis to steroids during the later stage of the reproductive cycle.

INTRODUCTION

Sex steroids exert a key role in the neuroendocrine control of reproduction. Discrepant effects of estrogen on the hypophysiotrophic form of gonadotropin-releasing hormone (GnRH) neurons were observed in mammals, where GnRH I biosynthesis is inhibited while GnRH I secretion can be either stimulated, reduced or remain unaffected. These results suggest that the regulatory role of sex steroids is very complex since it involves direct and indirect effects through genomic and/or non-genomic mechanisms (Matagne et al., 2005; Herbison, 2006). Glial cells are another very important player involved in these regulatory mechanisms; in fact, steroid hormones and growth factors act in an synergistic manner at the level of hypothalamic astrocytes to control GnRH I

neurons (Galbiati et al., 2003). Estradiol targets multiple mechanisms (potassium currents, excitability) to alter GnRH I neuron firing patterns, and the balance of stimulatory and inhibitory actions determine whether the integrated response is to increase or to decrease its release (reviewed by Moenter et al., 2003).

The GnRH-R plays a central role in the pituitary by relaying the information generated by the hypothalamus to the gonadotrophs and subsequently the regulation of synthesis and release of FSH and LH. GnRH-R gene expression is influenced by a multitude of factors, including gonadal steroid hormones, inhibin, activin and perhaps most importantly GnRH I itself (Rispoli and Nett, 2005). In mammals, estrogen has been shown to tightly regulate GnRH-R transcription in the pituitary. An increase in GnRH-R mRNA levels in rat pituitary has been observed after ovariectomy, and replacement therapy of the gonadectomized animals with E_2 can markedly decrease GnRH-R transcription (Kaiser et al., 1993). However, E_2 also exerts a positive feedback action on pituitary GnRH-R gene expression, presumably by enhancing hypothalamic GnRH I secretion, which is important for sensitizing gonadotrophs to GnRH during the preovulatory LH surge (Bauer-Dantoin and Jameson, 1995).

Synthesis and secretion of gonadotropins in mammals are also regulated by gonadal steroids. Both androgens and estrogens maintain an inhibitory effect on LH and FSH secretion. This action of gonadal steroids is primarily through the inhibition of GnRH pulsatile release, although direct effects on gonadotrophs have also been observed (reviewed by Jeong and Kaiser, 2006). Removal of the gonads results in a rise in LH and FSH secretion and expression of both gonadotropin subunits is increased after gonadectomy. This is likely due to lack of negative steroid feedback on GnRH I, but the

magnitude and time course of changes for each subunit vary (Jeong and Kaiser, 2006). While the increase in LH expression can be blocked by E_2 administration to ovariectomized animals, the rise in FSHβ mRNA levels is only partially suppressed by E_2, suggesting that the loss of non-steroidal factors from the gonads, such as activin and inhibin, are likely to regulate FSHβ transcription as well.

Sex steroid modulation of the HP axis in teleosts has been the focus of many studies. Several reports have indicated that steroids regulate the GnRH neuronal systems in fish. Most evidence related to positive feedback on hypothalamic GnRH I comes from male fish. Androgen or estrogen treatment caused an increase in GnRH I preoptic cell numbers (Grober et al., 1991; Amano et al., 1994a; Parhar et al., 2000) or exerted a positive influence on GnRH I amount (Dubois et al., 1998; Dubois et al., 2001). The few studies conducted in females reported a positive estrogen influence on GnRH I peptide levels in the hypothalamus and pituitary of European eels (Montero et al., 1995) and seasonal studies in female red seabream showed a positive correlation of the fluctuations in plasma E_2 and T levels with GnRH I mRNA levels, suggesting a positive gonadal feedback mechanism (Okuzawa et al., 2002).

Expression of the GnRH-R is inhibited by sex steroids (E_2 and 11-KT) in juvenile mixed sex red seabream. However, GnRH-R mRNA levels were higher in tilapia females than males, suggesting an association with the higher E_2 plasma levels in females (Levavi-Sivan et al., 2004). Furthermore, Alok et al. (2000) reported higher levels of GnRH-R mRNA in pituitaries of advanced female striped bass compared to those with less developed ovaries, also indicating seasonal regulation of GnRH-R mRNA.

Sex steroid hormones have both positive and negative effects on FSH and LH synthesis and secretion, depending on the mode of administration, the steroid, and the reproductive stage of the animal. In general, in most teleosts, aromatizable androgens or E_2 stimulate LH synthesis, LH pituitary content, and release *in vivo*. Furthermore, these steroids increase pituitary responsiveness to exogenous GnRH stimulation. Transcription of FSHβ appears to be negatively regulated by sex steroids, however the effect of steroids on FSH release can be positive and negative depending on the reproductive phase and if the experiments were conducted *in vivo* or *in vitro* (reviewed by Yaron et al., 2003). Moreover other gonadal peptides, like activin play a major role in the regulation of FSH synthesis and release (Yam et al., 1999b; Yuen and Ge, 2004).

The results obtained in animals of various species, examining expression levels of either GnRH I, the GnRH-R and/or GtHs across the normal reproductive cycle, and following castration and steroid replacement, have not always produced consistent results in mammals or in teleosts. Thus, the exact role of gonadal steroids in the control of the HP axis and their mechanisms of action, remain controversial and need further investigation.

Consequently, we designed the present study to gain a better understanding of the regulatory role of steroid feedback on the HP axis during two distinct stages of the reproductive cycle. Our model species is the anadromous striped bass, which has an annual reproductive cycle with group synchronous ovarian development (Specker et al., 1987). In this species, three forms of GnRH are present in the brain, all of which have been described previously (Gothilf et al., 1995). GnRH I is the only form to be expressed in preoptic neurons in the hypothalamus, which project directly to the pituitary (Kah et

al., 1993). In females, characteristic stages of oocyte development can be classified in sequence as primary growth, early secondary growth, vitellogenic and final maturation (Sullivan et al., 1997). The recruitment of primary oocytes into early secondary growth occurs during August/September and determines the recrudescent stage. Small oocytes [diameter <250 μm; (Sullivan et al., 1997)] and low plasma levels of steroid hormones are observed during this phase (Holland et al., 2000). During vitellogenesis, the oocyte grows [250 ~ 750 μm] by storing lipid droplets and egg yolk, which are vitellogenin-derived products (Tao et al., 1993). Concomitantly, steroid hormone production increases to stimulate vitellogenin-uptake (Berlinsky and Specker, 1991; Sullivan et al., 1997). Steroid levels peak at the beginning of final oocyte maturation (Mylonas et al., 1997d; Mylonas et al., 1997a) and may be the signal for the conclusion of vitellogenesis (Jackson and Sullivan, 1995). Based on these characteristics, the objective of the present study was to investigate changes in expression and peptide levels of the discussed reproductive hormones and receptors of the HP axis in response to gonadectomy and steroid replacement during the recrudescent and midvitellogenic stages of the reproductive cycle in female striped bass. This study complements earlier reports on gonadal steroid feedback regulation of the juvenile and pubertal striped bass (Chapter 3) and serves to elucidate the changes in gonadal regulatory mechanisms throughout the life cycle of the striped bass.

MATERIAL AND METHODS

Experimental Animals

Striped bass, 4 years of age (year class 1997) were acquired from the Crane Aquaculture Facility (University of Maryland), Baltimore, MD in summer 2001 for experiment 1. Fish, used in experiment 2, were acquired from the Aquaculture Research and Ecology Laboratory at Horn Point (UMCES), Cambridge, MD in fall 1996, when fish were 4 months old. All fish were transferred to the Center of Marine Biotechnology's Aquaculture Research Center, Baltimore, MD. The fish were maintained in 12 foot diameter recirculation systems, at 8 ppt salinity, under a simulated natural photo- and thermoperiod regime. The animals were maintained as a stock population in the above conditions until selected for the following experiment. All animals at the Aquaculture Research Center were maintained and sampled according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland Biotechnology Institute.

Treatment Protocols

Bilateral gonadectomies were carried out according to procedures modified from Billard et al. (Billard et al., 1977). Fish were anesthetized (70 ppm phenoxyethanol, Baker Inc., Phillipsburg, NJ), bled, weighed and placed on a surgical table, and their gills continuously irrigated with oxygenated water containing anesthesia. Using a scalpel, a 2–4 cm incision was made along the midline between the pectoral and pelvic fins. The ovaries were detached from the abdominal membranes and removed. The incision was sutured using 2.0 silk thread (Burns Veterinary Supply, Inc., Owings Mills, MD) and

closed with tissue glue (Burns Veterinary Supply, Inc.). Post-operation, fish were returned to their original tank systems. The extracted gonads were weighed for calculation of the gonado-somatic index (GSI; [gonad weight/body weight] x 100%). Control fish were given a sham-operation (s-o) receiving an incision and suture, but the ovaries were not removed.

Experiment 1: Steroid Feedback Regulation in Recrudescent Female Striped Bass

In September 2001, 60 females of average size were selected [fluke length 54 cm, weight 2.1 kg, GSI 1%, average oocyte diameter 250 μm, see (Figure 3)] from a mixed sex population and divided into 2 groups of 30 fish each (Figure 18). One group was given a sham-operation, the other group was gonadectomized. After surgery, the s-o fish were divided into 3 groups of 10 fish each. One group was given a vehicle injection (control), the second group received E_2 steroid treatment (SE; 2 mg/kg) via microspheric delivery systems (modified from Holland et al., 1998b), and the third group received 11-ketoandrostenedione (11-KA) steroid treatment (SK; 2 mg/kg) via microspheric delivery systems. 11-KA is the precursor of 11-ketotestosterone, the primary androgen in fish (Scott et al., 1980). Moreover, 11-KA or 11-KT are non-aromatizable androgens and therefore cannot be converted into estrogen. Consequently, treatment with 11-KA will only affect the HP axis via the androgen receptor pathway. This will help to clearly identify the differences between estrogenic and androgenic feedback. The gonadectomized group was also split into 3 groups, which received the same treatment paradigms: vehicle (GC), E_2 steroid replacement (GE; 2 mg/kg), and 11-KA steroid replacement (GK; 2 mg/kg). A commercial Finfish Bass Broodstock diet (Zeigler, Gardners, PA) was fed twice daily. Fish were sacrificed on day 10 post-surgery based on

results obtained in previous studies (Chapter 2). Upon sacrifice, brains and pituitaries were removed and snap-frozen in liquid nitrogen. Blood samples were collected using heparinized syringes and immediately mixed with aprotinin (Sigma-Aldrich, St. Louis, MO) at the final concentration of 0.84 TIU/ml. After centrifugation, plasma was collected and all samples were stored at –80 ˚C until further analysis.

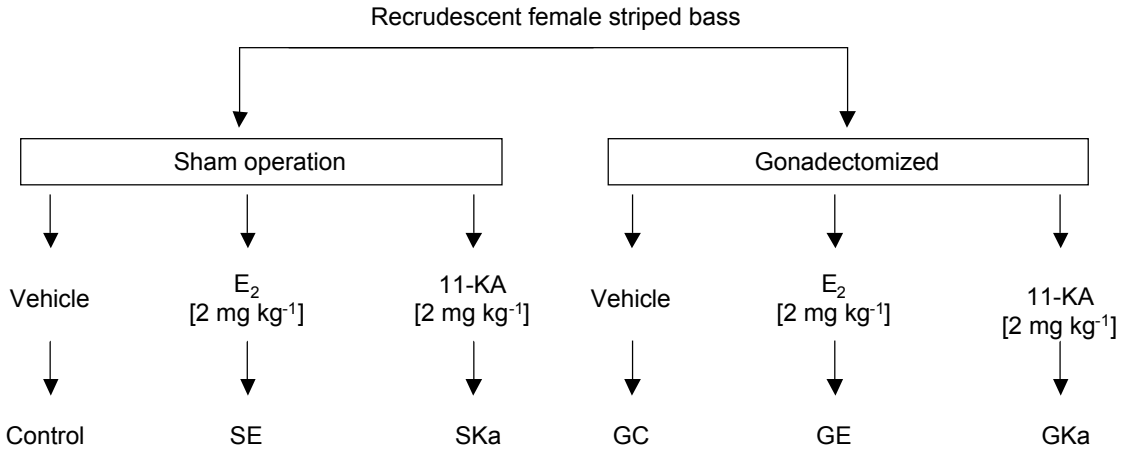

Figure 18: Recrudescent female study. Diagram of the experimental design.

Experiment 2: Steroid Feedback Regulation in Midvitellogenic Female Striped Bass

In late January 2000, 40 females of average size were selected [fluke length 42 cm, weight 2.5 kg, GSI 2%, average oocyte diameter 540 µm, see (Figure 3)] from a mixed sex population and divided into 4 groups of 10 fish each. The first 2 groups were given a sham-operation, the other 2 groups were gonadectomized (Figure 19). After surgery, the intact s-o groups were given either a vehicle injection (control), or received a T steroid treatment via microspheric delivery systems (ST; 4 mg/kg) (Holland et al., 1998b). Both gonadectomized groups received the same treatment paradigms, vehicle injection (GC) and T replacement (GT). A commercial Finfish Bass Broodstock diet

(Zeigler, Gardners, PA) was fed twice daily. Fish were sacrificed on day 10 post-surgery based on results obtained in previous studies (Chapter 2). Upon sacrifice, brains and pituitaries were removed and snap-frozen in liquid nitrogen. Blood samples were collected using heparinized syringes and immediately mixed with aprotinin (Sigma-Aldrich, St. Louis, MO) at the final concentration of 0.84 TIU/ml. After centrifugation, plasma was collected and all samples were stored at –80 ˚C until further analysis.

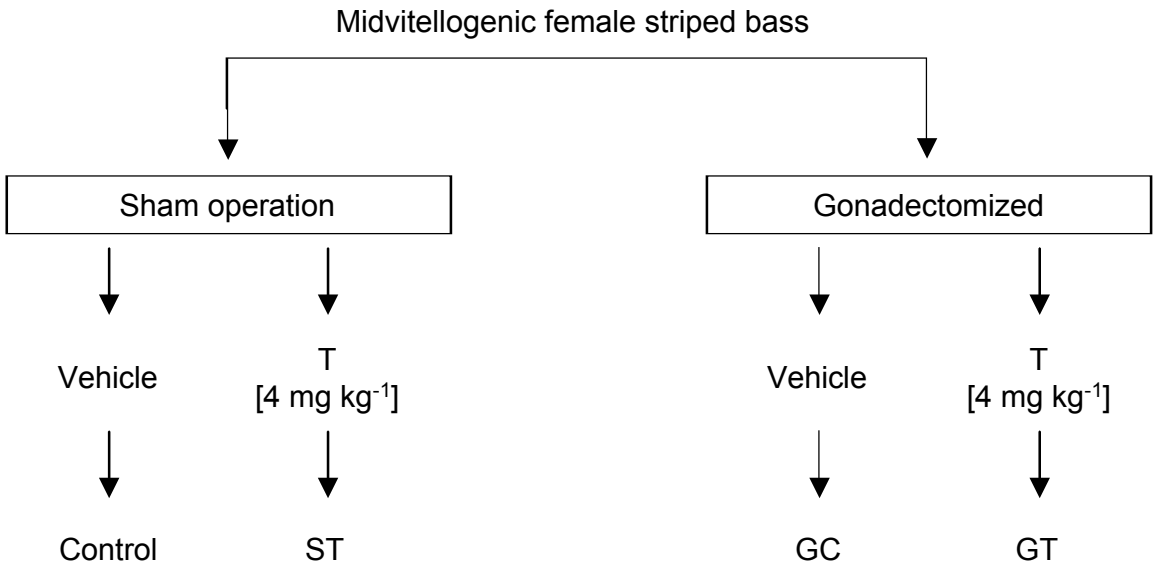

Figure 19: Midvitellogenic female study. Diagram of the experimental design.

Quantification of Transcript Levels of GnRHs, GnRH-R, FSHβ, and LHβ

Transcript levels of the three GnRHs in the brain, as well as for the GnRH-R, FSHβ, and LHβ in pituitaries were measured in both experiments using real-time fluorescence-based quantitative RT-PCR assays recently developed in our laboratory (Chapter 2).

Total RNA, isolated from whole brains or pituitaries using Tri-reagent (MRC Inc., Cincinnati, OH) was quantified spectrophotometrically. RNA standards and total RNA

from each sample were reverse-transcribed into cDNA using random hexamers and MMLV reverse transcriptase (Promega, Madison, WI). Duplicate cDNA aliquots (1 ng of total RNA for 18s and LHβ RNA; 10 ng for FSHβ and GnRH-R; 50 ng for all GnRH transcripts) from each sample served as templates in PCR. The reaction was performed using SYBR Green PCR core reagent (Applied Biosystems, Forster City, CA) containing 200 mM gene-specific primers (Chapter 2). Primers were designed to span intron/exon boundaries in order to avoid amplification of genomic DNA. Amplification reactions were carried out in a ABI Prism® 7700 Sequence Detection System at 50 ˚C for 4 min, 95 ˚C for 10 min, and 40 cycles of 95 ˚C for 15 s and 60 ˚C for 60 s. Copy numbers in unknown samples were determined by comparing C_T, the fractional cycle number at which fluorescence passes a baseline threshold value (Fink et al., 1998), to the specific standard (run in every plate) and normalized to the amount of 18s RNA in each sample.

Quantification of Peptide Levels of GnRHs and LH Protein Levels

In pituitaries from experiment 2, all GnRHs were measured using ELISAs that have been developed to specifically measure peptide levels in perciform fish (Holland et al., 1998a). These GnRH ELISAs are classical competitive enzyme immunoassays, which are highly specific, with very little or no cross-reactivity of antibodies (0.005 – 3.7%), sensitivities up to around 10 pg/well, and intra- and interassay coefficients of variations (CV) below 10% (Holland et al., 1998a). Briefly, pituitaries were sonicated on ice in 200 µl phosphate buffer. After sonication, 140 µl of homogenate were extracted with an equal volume of 4 N acetic acid. Samples were frozen, lyophilized and reconstituted in assay buffer. The samples or appropriate standards were incubated with antiserum and specific GnRH tracer in a microtiter plate well for 72 h at 4 °C. Colorimetric reagent was added

and after 3 h of incubation the optical density was read in an automatic plate reader at 405 nm (Thermomax, Molecular Devices, Menlo Park, CA).

Pituitary LH content was measured using a homologous ELISA (Mananos et al., 1997) in samples from both experiments. Briefly, pituitaries were sonicated on ice in 200 µl phosphate buffer. After sonication, 10 µl of the homogenate was aspirated and diluted in 1 ml of 100 mM PBS containing 0.05% Tween 20. The range of the standard curve is 0.78 to 10 ng/ml, and the intra- and interassay CVs for this assay are 7.7% and 8.7% respectively. The antigen-antibody complexes were detected by addition of goat anti-rabbit IgG horseradish peroxidase conjugate (GAR-HRP, affinity purified EIA grade, BioRad) and TMB peroxidase substrate (KPL, Inc., Gaithersburg, MD). Absorbencies were read at 450 nm, using an automatic microplate reader (Thermomax, Molecular Devices). To avoid differences due to interassay variation, all samples from the experiment were run in the same ELISA.

Quantification of Plasma LH, Estrogen and Testosterone Levels

Blood samples from experimental fish were immediately centrifuged, and plasma was separated and stored at –80 °C until further processing for steroid hormone quantification. Plasma LH content was measured using a homologous radioimmunoassay (RIA) according to Blaise et al. (1996) in samples obtained in experiment 1. Levels were measured in duplicate and all plasma samples were run in a single RIA to eliminate interassay variation. The intra-assay CV for the LH RIA is 4.6%.

Plasma E_2 and T was measured in samples of both experiments using commercially available, specific solid-phase RIAs that measure total amount of the respective hormone in unextracted, heparinized plasma (DPC, Los Angeles, CA). These

assays have been validated for use with striped bass plasma and assay characteristics have been previously described (Mylonas et al., 1997a).

Statistical Analysis

The data on brain GnRH and pituitary GnRH peptides, GnRH-R, FSHβ, and LHβ transcripts, as well as protein levels of LH in pituitary and plasma LH, E_2 and T levels, were subjected to Analysis of Variance (ANOVA) to determine the existence of changes between experimental groups. To specifically identify statistical differences among means of each of the above parameters, the means were further compared using Fisher's post hoc test. Statistical significance was set at $P < 0.05$.

RESULTS

There were no mortalities recorded during either of the experiments. All gonadectomized females were visually examined for remaining gonadal tissue upon sacrifice, to verify the completeness of the surgery. No remaining tissue was observed in any of the experimental animals, therefore all females were included in the analysis of the results.

Experiment 1: Estrogen and Testosterone Levels in the Plasma

During the oocyte recruitment phase, in the early stage of the reproductive cycle, estrogen production is very low in the ovaries, as detected in corresponding plasma levels (Table 4). Gonadectomy abolished estrogen levels completely in the GC group and reduced the levels in the GK group to almost the limits of detection. Similar low levels were observed in the s-o group treated with 11-KA. Estrogen replacement in the

ovariectomized group and treatment of the intact animals, via microspheres, elevated plasma levels up to 10-fold compared to control animals. These levels are still beneath the estrogen plasma levels measured in female striped bass undergoing vitellogenesis [2500 pg/ml; (Mylonas et al., 1997a)]. Testosterone levels were below the detection limit (50 pg/ml) in the control fish, as well as in the GC, GE and SE groups. Plasma levels in both androgen-treated groups were around 350 pg/ml. Due to the cross-reactivity of the T antibody with 11-KT (16%), it is quite plausible that the results represent an increase in 11-KT in response to the 11-KA microsphere treatment.

Table 4: Plasma steroid levels of recrudescent female striped bass in s-o control fish (SC), after gonadectomy (GC), after steroid replacement therapy with E_2 (GE) or 11-KA (GK), and after steroid treatment of intact s-o females with E_2 (SE) or 11-KA (SK).

Steroid	SC	GC	GE	GK	SE	SK
E_2 [pg/ml]	135.7 ± 20	n.d.	1203.7 ± 220	17.7 ± 1	1300.6 ± 272	23.2 ± 3
T [pg/ml]	n.d.	n.d.	n.d.	345.1 ± 41	n.d.	365.9 ± 104

Experiment 1: GnRH Expression Levels in the Brain

During early recrudescence, primary growth oocytes are being recruited into the pool of maturing gametes, entering the secondary growth phase. In this stage of the reproductive cycle, steroid levels are relatively low. To investigate the responsiveness of the GnRH neuronal network to steroid feedback regulation at this stage, we determined the effects of gonadectomy, estrogen and androgen treatment on expression levels of the three GnRHs. Transcript levels of each gene were measured using quantitative PCR assays (Chapter 2). The level of detection for all GnRH transcripts was 480 copies/ reaction and the results are normalized to the amount of 18s RNA.

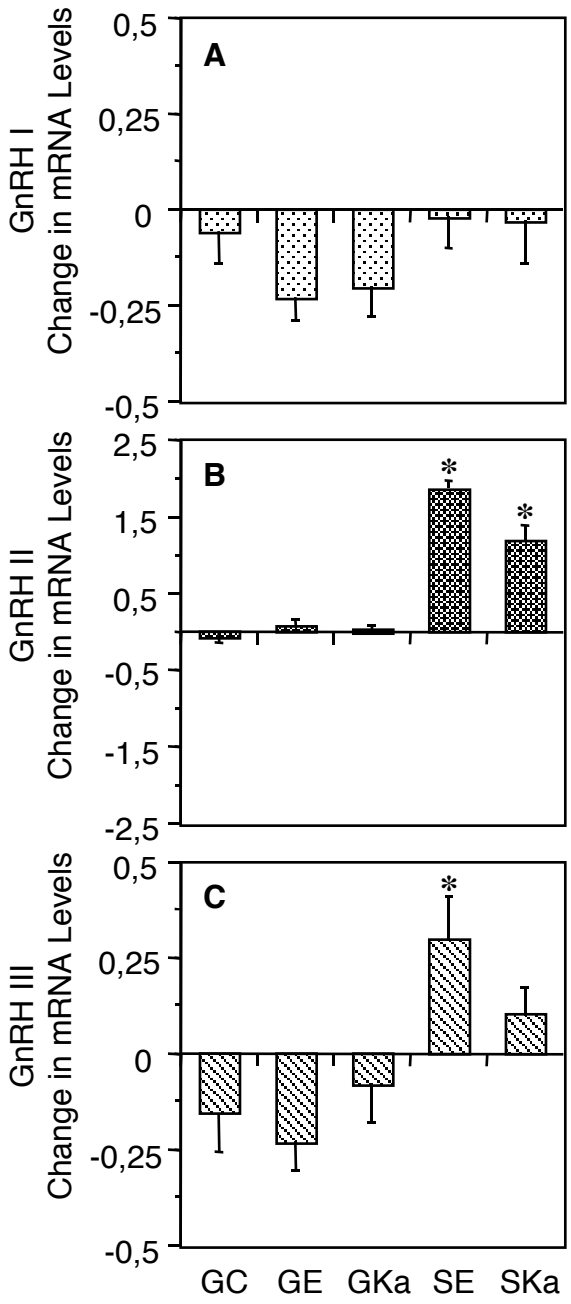

Figure 20: Transcript levels of GnRH I, II and III: Effects of gonadectomy and steroid treatment. Recrudescent female striped bass were gonadectomized (GC), or gonadectomized and treated with either E_2 (GE) or 11-KA (GK) steroid replacement via microspheric delivery systems (2 mg/kg dose for both E_2 and 11-KA). Intact s-o fish were also treated with the same steroids (SE and SK). Fish were sacrificed on day 10 post-surgery. Total RNA was extracted from brains and reverse-transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$.

For better visualization of the changes occurring in response to the treatments, the data is presented as the fold-change in mRNA levels compared to the control group. The removal of the major steroid-producing organ had no effect on the synthesis of any of the three GnRH forms (Figure 20).

The same result was observed in the steroid replacement groups, where neither E_2 nor 11-KA caused significant changes. However, steroid treatment of the s-o animals with E_2 significantly increased expression of GnRH II and III, but had no effect on GnRH I mRNA levels. 11-KA also elevated GnRH II transcript levels, but failed to have an effect on GnRH I and III synthesis.

Experiment 1: GnRH-R Expression Levels in the Pituitary

One of the main targets for steroid feedback in the HPG axis is the GnRH-R at the level of the pituitary. Therefore, we focused on alterations in GnRH-R expression in response to our treatment paradigms. With a level of detection of 600 copies/reaction for the GnRH-R, real-time PCR analysis was employed to quantify the amounts of GnRH-R mRNA in the pituitary. The results were normalized to 18s RNA and are presented as the fold-change in mRNA levels compared to control. As can be seen in Figure 21A, gonadectomy significantly decreased GnRH-R synthesis. E_2 replacement, as well as E_2 treatment of the s-o females, elevated GnRH-R transcript levels significantly. Injection of 11-KA caused a decreasing trend in the ovariectomized group, but lowered mRNA levels significantly in the intact s-o females.

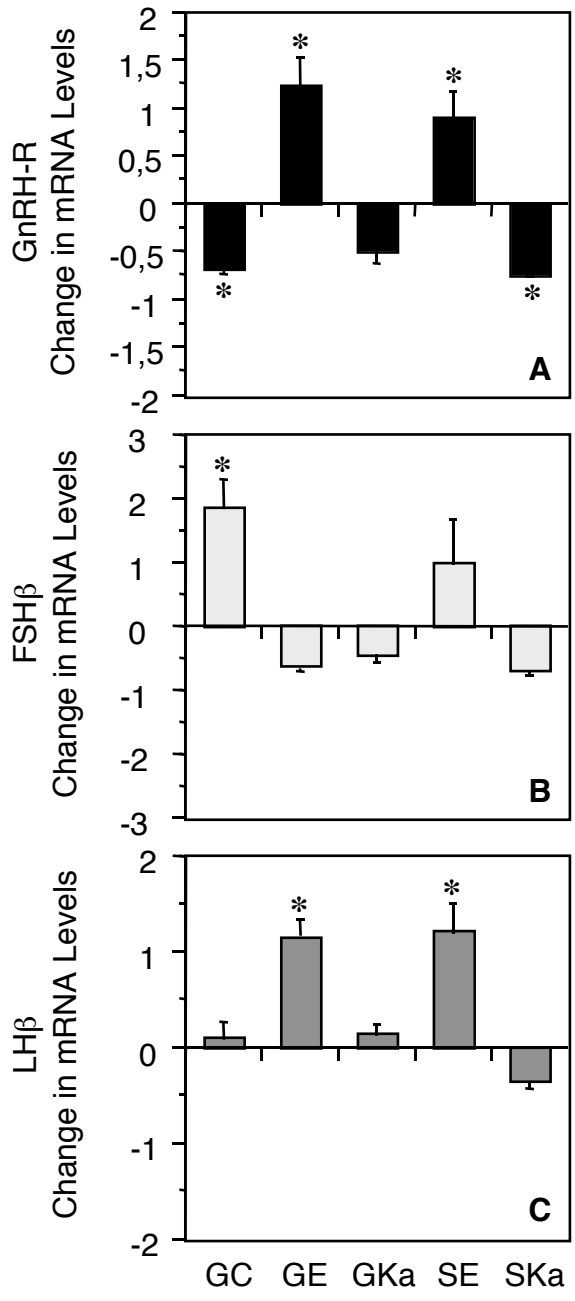

Figure 21: Pituitary GnRH-R, FSHβ and LHβ mRNA levels: Effects of gonadectomy and steroid treatment. Recrudescent female striped bass were gonadectomized (GC), or gonadectomized and treated with either E_2 (GE) or 11-KA (GK) steroid replacement via microspheric delivery systems (2 mg/kg dose for both E_2 and 11-KA). Intact s-o fish were also treated with the same steroids (SE and SK). Fish were sacrificed on day 10 post-surgery. Total RNA was extracted from pituitaries and reverse-transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$.

Experiment 1: GtH Subunit Expression Levels in the Pituitary

Estrogen and androgen control of gonadotropin synthesis is an important regulatory mechanism of the HPG axis. Therefore, we analyzed FSHβ and LHβ subunit mRNA levels in response to the experimental treatments using real-time PCR. The levels of detection for FSHβ and LHβ were 600 copies/reaction. The data is also presented as the fold-change in mRNA levels compared to control, after normalization to 18s RNA. Ovariectomy resulted in a significant elevation of FSHβ transcription (Figure 21B). Both steroid replacements maintained FSHβ synthesis at control levels and the same response was observed in steroid-treated intact females. Neither gonadectomy nor any treatment with 11-KA had an effect on LHβ subunit expression (Figure 21C). However, both estrogen-treated groups responded with an increase in mRNA levels.

Experiment 1: LH Protein Levels in Pituitary and Plasma

To investigate the changes in LH protein levels in the pituitary, we analyzed the samples in duplicate using a specific homologous ELISA. LH plasma levels were measured using a RIA. The results are presented as the fold-change of protein levels compared to control. Figure 22 shows the results obtained for recrudescent females. There were no significant changes in LH protein levels observed in the ovariectomized or E_2-treated animals. Androgen treatment decreased the pituitary LH content significantly in the gonadectomized animals as well as in the s-o groups. LH levels in the plasma of the ovariectomized and intact estrogen-treated groups did not change compared to controls. However, 11-KA lowered plasma levels in both gonadectomized and s-o groups and replacement with E_2 also caused a decrease in plasma LH.

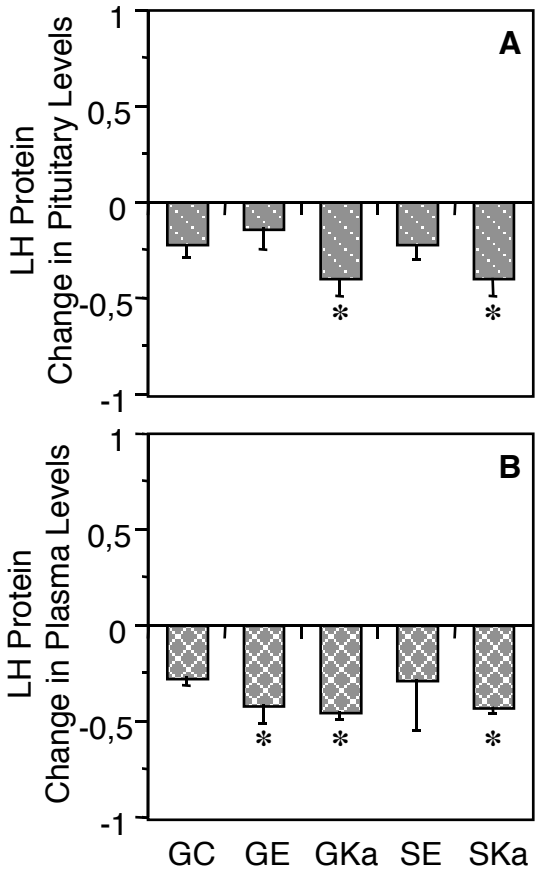

Figure 22: Pituitary (A) and plasma (B) LH protein levels: Effects of gonadectomy and steroid treatment. Recrudescent female striped bass were gonadectomized (GC), or gonadectomized and treated with either E_2 (GE) or 11-KA (GK) steroid replacement via microspheric delivery systems (2 mg/kg dose for both E_2 and 11-KA). Intact s-o fish were also treated with the same steroids (SE and SK). Fish were sacrificed on day 10 post-surgery. LH protein was extracted from pituitaries and measured using a specific ELISA. Plasma LH was determined using a striped bass specific RIA. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$.

Experiment 2: Estrogen and Testosterone Levels in the Plasma

During midvitellogenesis, the steroid levels rise slowly, due to increased production by the growing oocytes. Results for E_2 and T plasma levels are presented in Table 5. All three treatments, gonadectomy, as well as application of T to gonadectomized or s-o intact females, abolished E_2 plasma levels in the experimental animals. Plasma T remained at control levels in the GC group but increased by almost 150-fold due to T microsphere treatment in the GT and ST groups.

Table 5: Plasma steroid levels of midvitellogenic female striped bass in s-o control fish (SC), after gonadectomy (GC), after steroid replacement therapy with T (GT), and after steroid treatment of intact s-o females T (ST).

Steroid	SC	GC	GT	ST
E_2 [ng/ml]	0.12 ± 0.02	n.d.	n.d.	n.d.
T [ng/ml]	0.13 ± 0.02	0.10 ± 0.03	18.5 ± 2.5	19.5 ± 1.6

Experiment 2: GnRH Expression Levels in the Brain

To determine changes in the synthesis of the three forms of GnRH in response to changing steroid levels over the reproductive cycle, we investigated the effect of gonad removal and T replacement therapy, as well as treating s-o intact females with T. Transcript levels of each gene were measured using quantitative PCR assays (see above). GnRH I mRNA levels did not change in the GC group (Figure 23A). However, T replacement significantly elevated GnRH I expression. T administration had no effect in s-o intact females. In the GC group, no response in GnRH II mRNA levels was observed (Figure 23B), whereas T treatment decreased GnRH II expression in both groups, GT and ST, significantly. The third form of GnRH was also not affected by gonad removal (Figure 23C). However, T replacement caused a significant drop in GnRH III transcript levels. A similar trend was observed in s-o females, but it was not statistically significant.

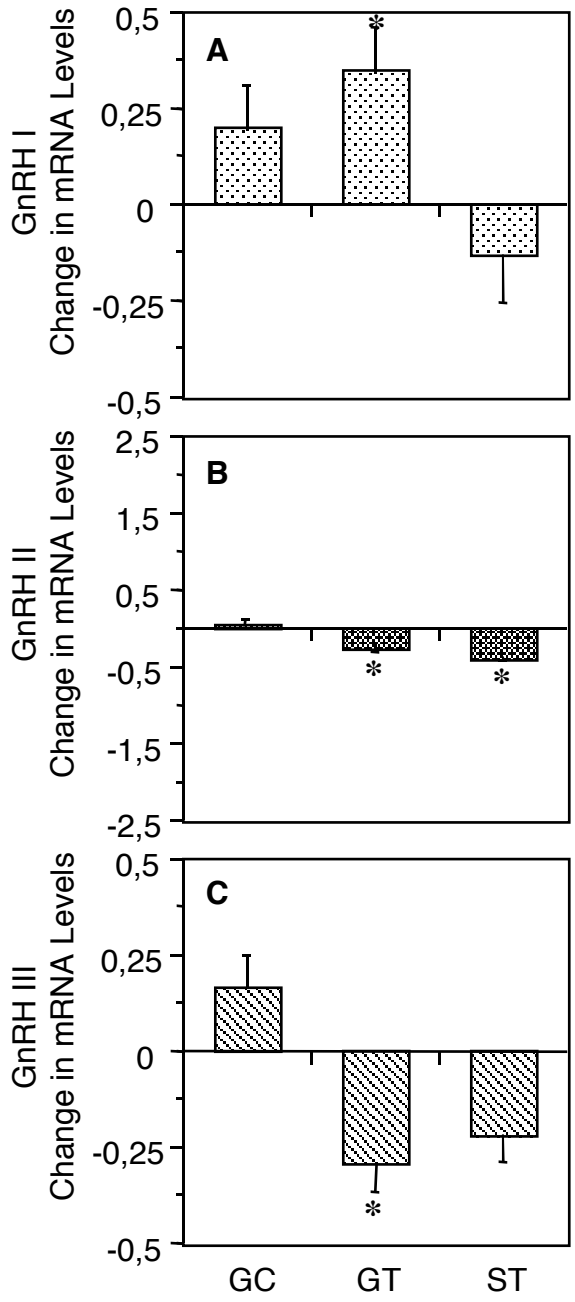

Figure 23: Transcript levels of GnRH I, II and III: Effects of gonadectomy and steroid treatment. Midvitellogenic female striped bass were gonadectomized (GC), or gonadectomized and treated with T (GT) steroid replacement via microspheric delivery systems (4 mg/kg). Intact s-o fish were also treated with T (ST). Fish were sacrificed on day 10 post-surgery. Total RNA was extracted from brains and reverse-transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$.

Experiment 2: GnRH Peptide Levels in the Pituitary

To be able to understand the relationship between GnRH synthesis and release in response to steroid feedback, we measured the actual GnRH peptide levels in the pituitary. Samples were measured in duplicate using a specific homologous ELISA. The results are presented as the fold-change of peptide levels compared to control. As presented in Figure 24, ovariectomy had no effect on the pituitary peptide content of any GnRH form. GnRH I levels dropped significantly after T replacement and T treatment of s-o intact animals, however there were no changes observed in GnRH II and III peptide content.

Experiment 2: GnRH-R Expression Levels in the Pituitary

Steroid feedback at the level of the pituitary alters GnRH-R expression over the reproductive cycle. To investigate possible feedback regulation of GnRH-R transcription during midvitellogenesis, we quantified the amounts of GnRH-R mRNA in the pituitary using real-time PCR (see above). As can be seen in Figure 25A, gonadectomy had no effect on GnRH-R synthesis. However T replacement, as well as T treatment of the s-o females increased transcript levels dramatically up to 18-fold.

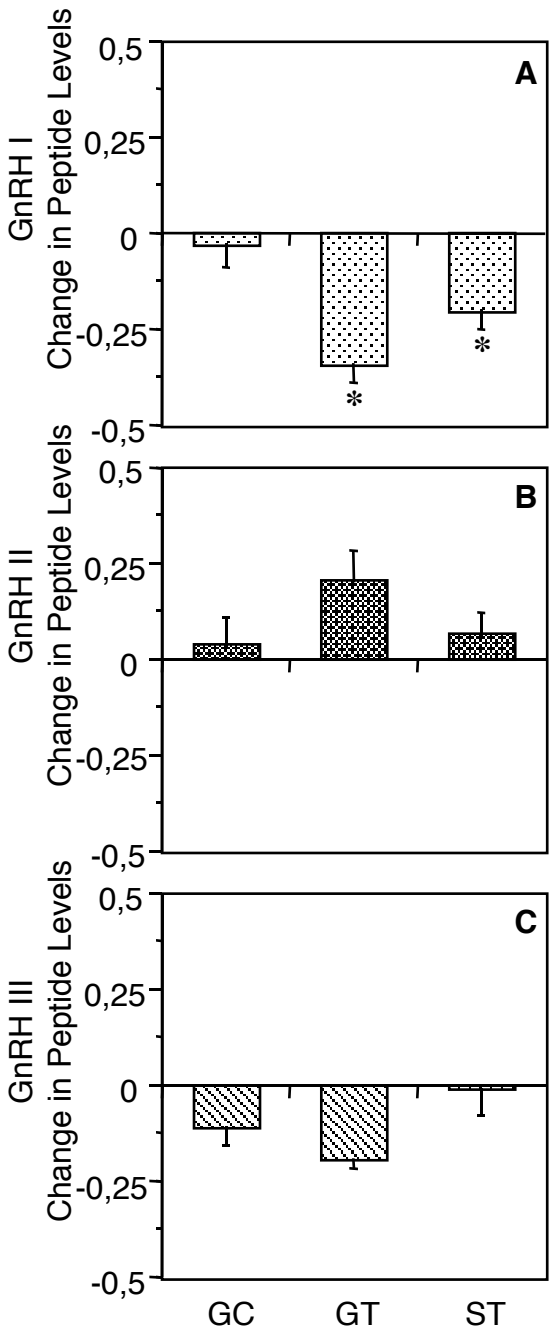

Figure 24: Pituitary GnRH I, II and III peptide levels: Effects of gonadectomy and steroid treatment. Midvitellogenic female striped bass were gonadectomized (GC), or gonadectomized and treated with T (GT) steroid replacement via microspheric delivery systems (4 mg/kg). Intact s-o fish were also treated with T (ST). Fish were sacrificed on day 10 post-surgery. GnRH peptides were extracted from pituitaries and measured using peptide-specific ELISAs. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$.

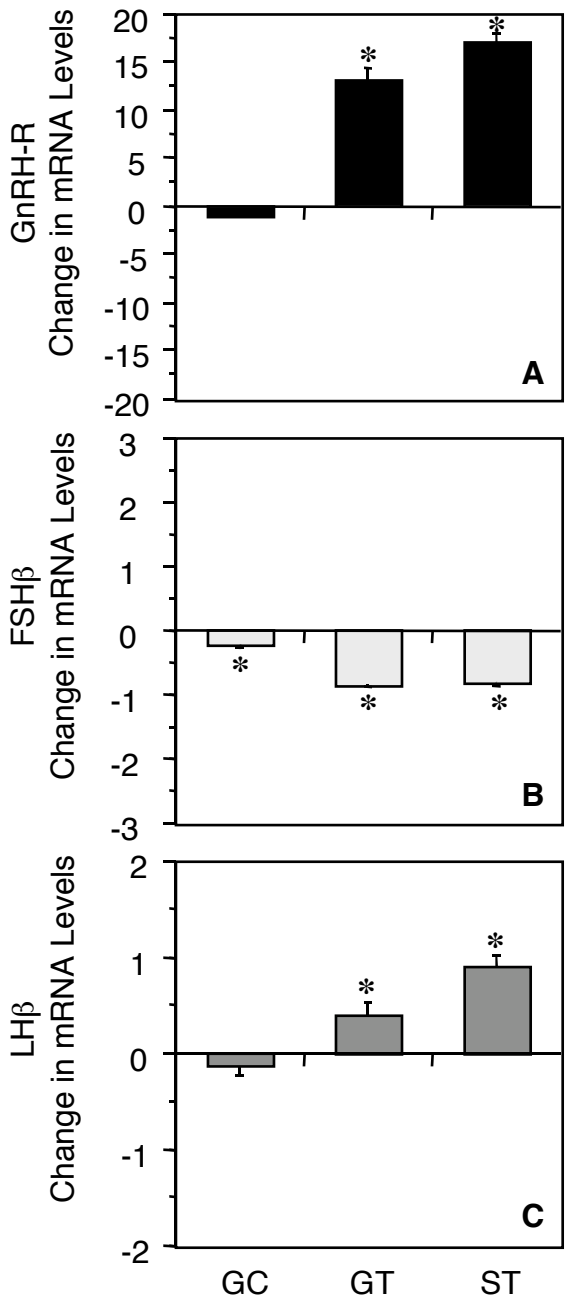

Figure 25: Pituitary GnRH-R, FSHβ and LHβ mRNA levels: Effects of gonadectomy and steroid treatment. Midvitellogenic female striped bass were gonadectomized (GC), or gonadectomized and treated with T (GT) steroid replacement via microspheric delivery systems (4 mg/kg). Intact s-o fish were also treated with T (ST). Fish were sacrificed on day 10 post-surgery. Total RNA was extracted from brains and reverse-transcribed to cDNA. Expression levels were measured using quantitative real-time PCR. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$.

Experiment 2: GtH Subunit Expression Levels in the Pituitary

Another important target of steroid regulation at the level of the pituitary is the synthesis of the gonadotropins, FSH and LH. Therefore, we analyzed FSHβ and LHβ subunit mRNA levels, in response to our experimental treatments, using real-time PCR (see above). All three treatments, ovariectomy, T replacement and T treatment of s-o intact females, resulted in a significant decrease of FSHβ transcription (Figure 25B). Gonadectomy had no effect on LHβ mRNA levels, whereas T significantly increased LHβ subunit expression in gonadectomized and s-o females (Figure 25C).

Experiment 2: LH Protein Levels in the Pituitary

To investigate the changes in LH protein levels in the pituitary, we analyzed the samples in duplicate using a specific homologous ELISA. Figure 26 shows the results obtained for midvitellogenic females. There were no changes in LH protein levels observed in any of the three treatment groups compared to control.

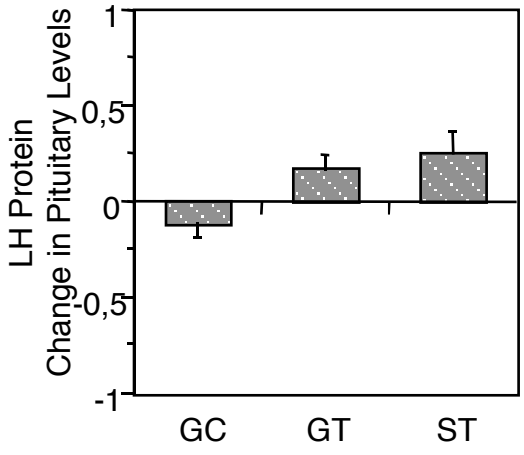

Figure 26: Pituitary LH protein levels: Effects of gonadectomy and steroid treatment. Midvitellogenic female striped bass were gonadectomized (GC), or gonadectomized and treated with T (GT) steroid replacement via microspheric delivery systems (4 mg/kg). Intact s-o fish were also treated with T (ST). Fish were sacrificed on day 10 post-surgery. LH protein was extracted from pituitaries and measured using a specific ELISA. Results are shown as the fold-change in mRNA levels compared to control, $P < 0.05$.

DISCUSSION

Sex steroids have been reported to have both positive and negative feedback effects on the different levels of the HP axis, depending on the maturational stage and on the level at which regulation takes place, transcription, translation or secretion. Many studies have been conducted to understand sex steroid regulation of synthesis and release of gonadotropins in a variety of species, however less is known about steroid control of the different GnRH variants and the GnRH-R, especially in females. In this study, we investigated for the first time the effects of gonadal steroid feedback on the entire GnRH-GtH system. We determined the response of GnRH biosynthesis in the brain, as well as changes in GnRH peptide levels, GnRH-R, FSHβ and LHβ transcription, and LH protein levels in the pituitary gonadotrophs after the removal of gonadal feedback and subsequent steroid replacement. Based on the fact that feedback effects change due to the maturational stage, we chose two distinctly different stages in the reproductive cycle of female striped bass, recrudescence and midvitellogenesis, to compare similarities and differences occurring during oocyte development. This study was conducted to complement previous work (Chapter 3), which focused on the gonadal feedback mechanisms and their ontogeny throughout juvenile and pubertal stages of our model organism, in order to determine the ontogeny of gonadal steroid feedback throughout the life cycle of the striped bass.

GnRH Expression in the Brain

Of the three variants of GnRHs found in the striped bass brain, the preoptic GnRH I is believed to be the hypophysiotrophic form, which is responsible for regulating gonadotropin synthesis and release. This area of the hypothalamus directly innervates the

pituitary in fish (Kah et al., 1993) and GnRH I is the dominant form found in the pituitary of many teleost species (Powell et al., 1994; Weber et al., 1997; Rodriguez et al., 2000; Holland et al., 2001). Moreover, seasonal changes in GnRH I expression and levels in the pituitary correlating with gonadal development have been observed in seabream species (Gothilf et al., 1997; Okuzawa et al., 2003). Therefore, the distinct absence of endogenous gonadal feedback regulation of GnRH I synthesis at both stages of the reproductive cycle was somewhat unexpected. At the beginning of gametogenesis, GnRH I neurons appear to lack sensitivity to steroidal (E_2 and 11-KA) regulation, since none of the steroid treatments in the ovariectomized and s-o intact animals had an effect on transcription levels. This finding is supported by *in vitro* studies conducted at the same reproductive stage, where only the highest dose of E_2 (100 nM) with the longest incubation period (6 hours) provoked an increase in GnRH I expression (Chapter 5). However, during midvitellogenesis T replacement increased GnRH I mRNA levels significantly, indicating that GnRH I neurons acquired the capacity to respond to steroid regulation. Further support for this hypothesis comes from *in vitro* studies in midvitellogenic females, where brain slices incubated with E_2 showed an increase in GnRH I expression (Chapter 5). The presence of similar effects on GnRH I transcription after treatment with aromatizable T *in vivo* and E_2 *in vitro* suggests that this steroid regulation occurs via an estrogenic pathway. Administration of T to the s-o intact females had no effect on GnRH I expression. This may be the result of changes occurring at the levels of the gonads leading to a different feedback 'cocktail of factors'. For example, it has been shown that steroid treatments have profound effects on gonadal steroidogenesis

by decreasing steroid enzyme expression (Baron et al., 2005) and that androgens mediate growth of oocytes (Lokman et al., 2003) in fish.

The evidence for positive steroid influence on the GnRH I neuronal system in this report is supported by findings in other teleost species, where positive steroidal influence on GnRH I cell numbers and peptide content in the brain and pituitary, have been demonstrated before (Grober et al., 1991; Amano et al., 1994a; Montero et al., 1995; Dubois et al., 1998; Parhar et al., 2000; Dubois et al., 2001).

In most mammals, the regulation of GnRH I transcription by sex steroids, especially estrogen, has been shown to change between a positive feedback during the mid- to late follicular phase of the cycle to induce the GnRH/LH surge required for ovulation and a suppressive feedback during the other stages of the cycle (Herbison, 2006). It appears that three different mechanisms are involved in estrogen regulation of GnRH neurons: 1) A direct effect on steroid receptors present in GnRH neurons themselves. Although, no ERα protein or mRNA has been shown to exist in GnRH neurons to date (Herbison, 1998; Herbison, 2006), ERβ was co-localized with GnRH neurons *in vivo* and proven functional (Skynner et al., 1999; Hrabovszky et al., 2000; Kallo et al., 2001) and therefore could be a mediator of estrogen feedback. 2) An indirect effect via interneuronal systems possessing steroid receptors and transferring the information via classical neurotransmitters or neuropeptides (e.g., GABA, glutamate, NPY) (Herbison, 2006). Very recent studies suggest that kisspeptins and their receptors (GPR54), which are located on GnRH neurons (Irwig et al., 2004), are the long-sought link between peripheral sex steroids and GnRH synthesis and release (Smith et al., 2006). 3) An indirect effect on glial cells via activation of glial cell steroid receptors, resulting in

alterations in glial cell morphology concurrent with changes in GnRH neuron connectivity or via changes in glial growth factor secretion pattern (Prevot, 2002). However, exactly which of these mechanisms (or a combination thereof) is responsible for relaying the positive or negative feedback effects of estrogen is still unknown.

To date, no estrogen receptor subtypes have been demonstrated in fish GnRH neurons. It is therefore believed that sex steroids exert their effects indirectly via interneuronal systems, involving neurotransmitters like GABA, dopamine and/or NPY in steroid feedback (reviewed by Kah et al., 2000). The recent report of GPR54 expression in GnRH neurons in a cichlid fish (Parhar et al., 2004) opens up new possibilities of an existing kisspeptin/GPR54 system in fish brains, providing a relay for information of circulating sex steroids levels to GnRH neurons and closing the regulatory gap in steroid feedback in fish as well.

There has been no convincing evidence for a GnRH II role in regulating reproductive processes at the level of the pituitary, and it has been suggested by Oka (2002) that this peptide serves as a neurotransmitter in the brain. Its regulation by gonadal steroids has been studied in fish. However, most reports gave no evidence for steroid effects exerted on GnRH II expression (Montero et al., 1995; Soga et al., 1998; Parhar et al., 2000). In our study, we observed no endogenous steroid regulation of GnRH II transcription in either of the two stages of oogenesis. Steroid replacement in ovariectomized females with estrogens or androgens had no effect during recrudescence, however T replacement in midvitellogenic females caused a significant decrease in GnRH II mRNA levels. This result indicates an increased responsiveness of GnRH II neurons to steroid regulation. Our *in vitro* findings in the same species and maturational

stages showed that E_2 treatment during early gametogenesis had no effect on GnRH II transcription, but in the mid-cycle, expression was increased (Chapter 5). The different results observed *in vivo* (decrease) and *in vitro* (increase) may be due to the fact that T can act via both, the estrogen and androgen receptor pathways and therefore produce dissimilar outcomes.

GnRH II expression significantly increased in s-o intact recrudescent females treated with E_2 and 11-KA, providing evidence that the GnRH neuronal network is capable of responding to gonadal feedback. However, this response is unlikely due to direct effects of sex steroids on the HP axis. Steroid treatment effects on the gonads may have induced changes in production of gonadal factors, which resulted in the observed response of the GnRH II neurons. In addition, the nature of the steroid effect changes between the reproductive stages, i.e., stimulatory during early ovarian development and inhibitory in the midvitellogenic females.

A functional role of GnRH II in mammalian reproduction has been shown in the musk shrew, where GnRH II administration resulted in modulation of reproductive behavior and food intake (Temple et al., 2003; Kauffman and Rissman, 2004b). A recent study in marmoset monkeys also reported that GnRH II stimulates female sexual behavior (Barnett et al., 2006). However, information about steroid regulation of GnRH II biosynthesis and release is scarce. Densmore and Urbanski (2004) reported that ovariectomy and estrogen replacement in female rhesus macaques increased GnRH II mRNA levels in the medio-basal hypothalamus (MBH) significantly. Furthermore, the authors showed that ~50% of GnRH II neurons in the MBH expressed ERβ-immunoreactivity, suggesting that E_2 can directly exert a positive influence on GnRH II

gene expression in the MBH in primates. *In vitro* studies using the human GnRH promotor showed that there was a significant activation of GnRH II gene expression by estrogen in human neuronal medulloblastoma cells (TE-671). However, promotor sequence analysis revealed only a partial putative ERE (Chen et al., 2002). Further analysis revealed an SP1 site and a cyclic adenosine monophosphate-response element site. Since these elements are known to interact with ER (Xie et al., 1999; Sabbah et al., 1999), it is possible that E_2 mediates its stimulatory effect through these sites (Chen et al., 2002).

In this study, we observed a positive influence of estrogen on GnRH II gene expression in female striped bass. The above reports from the mammalian literature further support our hypothesis that estrogen positively regulates GnRH II transcription.

GnRH III neurons are localized rostral along the terminal nerve, projecting their axons throughout the various brain loci, and have been shown to have neuromodulatory functions (Oka, 2002; Saito et al., 2003). Despite the observation of GnRH III peptide in pituitaries of some fish species, it is questionable, whether this form is involved in regulating the reproductive axis. In this study, we report that transcription of GnRH III is also not regulated by an endogenous steroid feedback mechanism at the investigated stages of the reproductive cycle. However T replacement in the later stage caused a decrease in mRNA levels. Only estrogen treatment of s-o intact females at the beginning of gametogenesis evoked a positive effect, suggesting that estrogen administration to developing ovaries induces and/or inhibits hormone production, which results in a different feedback effect on the HP axis than exerted by E_2 alone in the ovariectomized group. This could be either a dosage effect of the combined endogenous and exogenous

steroids or the consequence of a different gonadal factor (e.g., activin or androgens). These results are in agreement with evidence generated in our *in vitro* study, where E_2 triggered a positive response in brain slices obtained from recrudescent females and not in the midvitellogenic stage (Chapter 5). The response to T replacement during midvitellogenesis we observed, gives further evidence for the hypothesis of an increased sensitivity of the GnRH neuronal network during the process of gonadal maturation in the female striped bass.

Only a few studies in fish have investigated the effect of steroids on GnRH III neurons. E_2 had no effect on GnRH III expression in immature, castrated male tilapia (Parhar et al., 2000), while T treatment of castrated male tilapia elevated GnRH III mRNA levels (Soga et al., 1998). In male masu salmon, treatment with 17α-methyltestosterone had no effect on GnRH III cell number in the olfactory bulb or terminal nerve (Amano et al., 1994a). These reports, together with our results, demonstrate how variable the response of GnRH III biosynthesis and cell numbers to steroid regulation can be. Additionally, they highlight the degree to which the response depends on the species, sex and maturational stage of the investigated fish model.

The results of this study show that, in female striped bass, biosynthesis of the GnRH neuronal network is not controlled by endogenous gonadal steroid regulation at the investigated stages of the reproductive cycle. However, we observed an increased responsiveness of GnRH neurons to replacement steroid treatment during midvitellogenesis, indicating a change in sensitivity from the onset of ovarian development to the midvitellogenic phase. There could be several mechanisms responsible for this observed change in response. First, a central inhibition by

intermediary neurotransmitters could be the reason for a non-reactive GnRH neuronal system in the beginning of oogenesis. Second, as suggested for mammals, glial-neuron cyto-structural connections, which facilitate steroid regulation, may be disengaged during the early phase and lead to a 'quiescence' of the GnRH neurons, which may not need to be active at this stage of gonadal development. Since GnRH neurons only express low levels of ERβ, but not ERα, it is believed that estrogen control is relayed via intermediate neurons, which express ERα (Herbison, 1998). Thus, another possible scenario would be that these intermediate factors are not yet 'in place' and feedback is not transmitted to the GnRH neuronal network. The observed increase in sensitivity could be the result of slowly increasing steroid levels produced by the ovary during gonadal development, acting as a primer of the GnRH network by either removing the central inhibition, causing restructuring of the glial ensheathment of GnRH neurons or induce production of intermediary transmitter systems. Nonetheless, the recrudescent brain is not completely unresponsive to gonadal regulation, as seen in the s-o intact females treated with exogenous steroids. The 10-day steroid exposure resulted in increased GnRH II and III expression. The explanation for this response may lie within changes happening at the level of the gonads. The ovaries respond to exogenous steroids with acceleration and/or inhibition of developmental processes and, as a result, the composition of ovarian hormone production changes. Therefore, gonadal feedback in these females is very different from the 'straight' hormone treatment we administered (i.e., dosage, steroids, peptides such as activin), and may be responsible for the changes in GnRH transcription.

GnRH Peptide Levels in the Pituitary

The processes of GnRH biosynthesis and its secretion are not necessarily regulated by the same steroidal mechanisms (Sagrillo et al., 1996). Therefore it is possible that even though no endogenous feedback regulation occurred at the level of the brain, GnRH release in the pituitary may be very well regulated by steroid feedback during the earlier stages of the reproductive cycle. No changes in GnRH I peptide levels were found in ovariectomized midvitellogenic females, indicating no gonadal regulation of GnRH release. However, T replacement decreased GnRH I levels in the pituitary significantly in both treatment groups. Thus, the exogenously increased levels of steroids did have the capacity to cause GnRH I release from the nerve terminals located in the pituitary and also demonstrate the responsiveness of the GnRH network to steroidal regulation at this stage.

Neither removal of the gonads nor steroid replacement or treatment had an effect on pituitary levels of GnRH II or III. Thus, steroids appear not to regulate release of these two GnRH variants during midvitellogenesis. It is not surprising to find that only GnRH I release is affected by steroid treatment, since this GnRH has been proposed to be the hypophysiotrophic form, regulating GtH synthesis and release in the pituitary (Holland et al., 1998a; Holland et al., 2001; Okuzawa et al., 2002). The functions of multiple GnRHs found in the pituitary are still unclear. However, it has been hypothesized, that they play additional regulatory roles in teleost species, involving regulation of growth hormone (Le Gac et al., 1993) or prolactin release (Weber et al., 1997), which may not require steroid regulation.

GnRH-R Expression in the Pituitary

One of the key players in the hormonal cascade controlling reproduction in vertebrates is the GnRH-R, located on the gonadotroph cells in the pituitary. Through this receptor, the hypothalamic signal is transduced and converted as part of the synthesis and secretion processes of FSH and LH. Steroid regulation of GnRH-R expression has been shown to play an important role in vertebrate reproduction (reviewed by Hapgood et al., 2005; Jeong and Kaiser, 2006), however not many physiological studies have been done in teleost fish to date.

Expression of the GnRH-R is under a positive gonadal influence during recrudescence, as shown by the decrease in mRNA levels after gonadectomy. Estrogen replacement and treatment of the s-o intact females caused a significant increase in transcription, suggesting that E_2 has a positive effect on receptor expression. However, it is questionable whether the endogenous feedback is exerted via estrogens, since circulating plasma levels during this stage are fairly low (Berlinsky and Specker, 1991; King et al., 1994) compared to the levels induced by exogenous steroid administration in this study. Activin has been shown to increase GnRH-R expression in mammals *in vitro* and *in vivo* (Braden and Conn, 1992; Fernandez-Vazquez et al., 1996; Norwitz et al., 2002), and therefore could be a good candidate for positive regulation of the receptor at this stage. Transcription levels of the GnRH-R decreased after 11-KA treatment, indicating that androgens, at this stage, exert an inhibitory effect on GnRH-R expression in the pituitary. A similar result was observed in juvenile red seabream, where treatment with 11-ketotestosterone significantly decreased receptor mRNA levels (Okuzawa et al., 2002). However, the same study also reported an inhibitory influence of estrogen on

GnRH-R transcription, which is contradictory to our observations, but may be related to differences in species and reproductive strategy. Nonetheless, a positive estrogenic influence on GnRH-R expression has also been observed *in vitro* in pituitary cell cultures obtained from female striped bass undergoing recrudescence (Chapter 5), giving further evidence for a direct regulation of the receptor by E_2.

During midvitellogenesis, we did not observe an endogenous feedback mechanism regulating GnRH-R transcription, however T administration to both ovariectomized and s-o intact fish, increased receptor mRNA levels up to 17.5-fold. There is a high probability that this effect is reached via an estrogenic pathway, after the aromatization of testosterone, acting directly at the pituitary level and supported by GnRH release from the hypothalamus. This hypothesis is based on a) *in vitro* results from female striped bass undergoing midvitellogenesis, where E_2 treatment significantly elevated receptor mRNA levels directly in dispersed pituitary cells throughout all treatment groups (Chapter 5), and b) the fact that GnRH I peptide levels decreased in the pituitary after T treatment. GnRH has been shown to up-regulate its own receptor in the pituitary in mammals and fish (Kaiser et al., 1993; Yasin et al., 1995; Okuzawa et al., 2002; Levavi-Sivan et al., 2004). Furthermore, positive effects of estrogen on receptor transcription occur indirectly via an increase in GnRH release (Yasin et al., 1995). This appears to be true in our study also, where the decrease in GnRH I levels indicate a release of this peptide which, in turn, could 'help' in the up-regulation of the GnRH-R. This synergized effect of estrogen and GnRH may be responsible for the extremely high levels of receptor transcription observed after T treatment.

GtH Subunit Expression in the Pituitary

Regulation of GtH synthesis and release occurs primarily via the GnRH system in the hypothalamus, however, gonadal steroids have also been shown to play a pivotal role in controlling these processes at the level of the pituitary. This study provides further evidence that sex steroids have regulatory functions in the FSHβ and LHβ biosynthesis and LH release in fish.

After ovariectomy of recrudescent females, we observed an increase in FSHβ transcription, demonstrating the removal of a negative gonadal feedback. Steroid replacement, with both estrogen and androgen, restored control levels, suggesting a negative influence of these hormones on FSHβ expression at this stage. However, the nature of the gonadal feedback changed during ongoing ovarian development, since gonadectomy in the midvitellogenic females resulted in the removal of a positive gonadal feedback. Moreover, T replacement and treatment of s-o intact animals during this stage further suppressed FSHβ expression. Therefore, it is probable that the positive feedback is not exerted via steroids, but through other gonadal factors. As demonstrated for mammals and fish (Carroll et al., 1989; Rivier and Vale, 1991; Yam et al., 1999b; Welt et al., 2002; Yuen and Ge, 2004), activin positively influences FSHβ transcription in the pituitary and may be responsible for the positive influence observed in this study. In view of the reproductive stage, it is not surprising that the ovaries exert a positive influence on FSHβ transcription. For salmonids, it has been suggested that FSHβ stimulation of the ovary, together with E_2 secretion, regulates the growth of the previtellogenic ovary (Gomez et al., 1999; Campbell et al., 2003), requiring positive feedback regulation. In both stages investigated, steroids showed a suppressive effect on FSHβ mRNA levels.

However, it is unlikely that this occurs via a direct regulation at the level of the pituitary. Work in dispersed pituitary cells from striped bass females of the same stages, demonstrated no influence of estrogen on expression levels of this gonadotropin (Chapter 5). We therefore hypothesize that the regulation of the subunit synthesis occurs either indirectly via hypothalamic neurotransmitter or involves other pituitary compounds, which are regulated by steroids.

The evidence given in this study for a negative regulation of FSHβ by steroids is supported by reports from other fish species. Recently, Schmitz et al. (2005) showed an inhibitory effect of T on FSHβ mRNA levels, while E_2 had no effect in female European eel. Steroids (T/E_2) also decreased FSHβ mRNA levels in coho salmon (Dickey and Swanson, 1998), juvenile or recrudescing goldfish (Sohn et al., 1998; Sohn et al., 2000; Kobayashi et al., 2000) and the European sea bass (Mateos et al., 2002). Furthermore, detailed studies in tilapia indicated that regulation of FSHβ expression is very sensitive to steroid levels (Melamed et al., 2000).

Our experiments demonstrated that LHβ subunit expression is not regulated via gonadal feedback during recrudescence and midvitellogenesis in female striped bass. However, estrogen positively influences LHβ transcription, whereas 11-KA has no effect at the onset of oogenesis. In the mid-cycle, treatment with aromatizable testosterone also increases LHβ mRNA levels, further supporting a positive (estrogenic) regulation of this gonadotropin subunit. This steroid effect appears to be mediated (at least partially) via indirect pathways, as demonstrated for FSHβ subunit transcription. *In vitro* work showed no changes in LHβ transcription in response to E_2 exposure in recrudescent pituitary cells and only long term exposure (18 hours) caused an increase in LHβ expression in cells

obtained from midvitellogenic females (Chapter 5). This may suggest an indirect regulation via GnRH or other neurotransmitters regulating LHβ transcription [e.g., GABA/glutamate (Trudeau et al., 2000)] during the early phase of ovarian development. However, during vitellogenesis a combination of multiple control mechanisms is probable. Direct effects after long-term exposure *in vitro* and an increase in GnRH *in vivo* suggest that the positive influence exerted by E_2 is the sum of both pathways. Our data shows that estrogen positively influences LHβ transcription. However, circulating E_2 levels are not elevated enough during the two investigated reproductive stages to result in an increase in transcription *in vivo*. Closer to the spawning season, circulating plasma steroid levels will rise in females, as reported by Berlinsky and Specker (1991) and Mylonas et al. (1998), and will cause the LH surge inducing the final stages of oocyte maturation.

The positive influence of estrogen on LHβ subunit transcription, both *in vivo* and *in vitro*, has also been demonstrated in many other teleost species (reviewed in Yaron et al., 2003). In mammals, estrogen suppresses the post-castration rise of LHβ mRNA via negative feedback to the hypothalamic GnRH system (Fallest et al., 1995; Burger et al., 2001). *In vitro*, however, E_2 rapidly increased LHβ mRNA synthesis in pituitary cells from ovariectomized and cycling rats (Shupnik et al., 1989b; Shupnik, 1996), suggesting a positive regulatory effect directly on gonadotroph cells.

LH Protein Levels in Pituitary and Plasma

There have been many reports on the increase of LH plasma levels, and concomitant decrease in pituitary LH, as a response to gonadectomy in teleost and mammalian species (reviewed in Yaron et al., 2003; Burger et al., 2004; Jeong and

Kaiser, 2006). However, in our experiments, we did not observe changes in LH pituitary levels after gonadectomy at either reproductive stage in female striped bass. This suggest that gonadal feedback is not regulating LH release from the pituitary during this part of the reproductive cycle in our model and supports the hypothesis that LH is not involved in regulating ovarian development during the earlier stages, as has been suggested for the salmonid model (Swanson et al., 1991). During recrudescence, pituitary LH levels decrease only after androgen treatment. Surprisingly, LH plasma levels also decrease in the same treatment groups. This phenomenon may be explained either by the fact that the 10-day release of LH from the pituitary may have depleted the LH stores or the possibility that the observed LH decrease in the pituitary does not reflect a release, but rather degradation. On the other hand, E_2 treatment had no effect on LH protein levels. However, a decrease in plasma levels did occur. In view of increasing LHβ expression in these groups, this result was very unexpected. One explanation for this discrepancy may be that LHβ mRNA is not translated into protein and/or that LH release is inhibited. Both scenarios would render the levels of LH in the pituitary unchanged compared to controls. In midvitellogenic females, pituitary LH levels do not change in response to T treatment. Since we also observed an increase in transcription levels, it appears that the hypothesis proposing an inhibition of LHβ mRNA translation may still be in effect at this stage of ovarian development. However, the absence of LH plasma data do not allow for further speculation regarding these results.

In conclusion, we reported in this study that gonadal feedback does not regulate GnRH synthesis or release during the earlier stages of the reproductive cycle in female striped bass. However, we observed an increase in sensitivity on the GnRH neuronal

network to steroid exposure during midvitellogenesis. Expression of the GnRH-R is under gonadal regulation during recrudescence, but this effect was lost in the later stage. Estrogen may be responsible for the feedback regulation, due to its positive influence on receptor expression levels, however, this effect was also shown during midvitellogenesis. This observation, together with the fact that endogenous circulating estrogen levels are low in the early stages of oocyte development, suggests that steroids are not responsible for the endogenous gonadal feedback. Gonadal feedback regulation of FSHβ transcription in the pituitary changes from an inhibitory effect during recrudescence to a stimulatory influence during midvitellogenesis. Steroid treatment during early gametogenesis restores the negative feedback and thus, may be responsible for the effect. However, testosterone administration in mid-cycle inhibited FSHβ transcription further, suggesting that steroids are not involved in the positive feedback exerted by the gonads at the studied stages of ovarian development. The second gonadotropin, LHβ, is not under the regulatory influence of gonadal feedback. A stimulatory estrogenic effect was observed in both reproductive stages after exogenous steroid administration. Surprisingly, pituitary LH protein levels were stable after ovariectomy in both experiments, suggesting no gonadal regulation of LH release during the earlier stages of the cycle. However, our herein reported results confirm the hypothesis that LH is of more importance in the advanced stages of oogenesis and FSH is more involved in regulating vitellogenesis in oocytes.

CHAPTER 5. DIRECT ESTROGEN EFFECTS ON THE HYPOTHALAMUS-PITUITARY AXIS OF ADULT FEMALE STRIPED BASS: AN *IN VITRO*-STUDY

ABSTRACT

To improve our understanding of the neuroendocrine regulation of fish reproduction, an *in vitro* approach was developed using a fish brain-slice culture method. One of the key regulators of the complex control of the fish reproductive axis is gonadal steroid feedback. Therefore, we investigated the regulatory effect of estradiol (E_2) on GnRHs, GnRH-receptor and GtH gene expression at different stages of the reproductive cycle. Striped bass brain-slice cultures obtained from either recrudescent or midvitellogenic females were prepared and incubated with a low (1 nM) or high (100 nM) dose of E_2 for 3 and 6 hours. Dispersed pituitary cell cultures from the same animals were prepared and also treated with low or high doses of E_2 for 3, 6, and 18 hours. Tissue and cells were extracted for total RNA. Transcript levels of all three GnRHs, GnRH-R, FSHβ and LHβ were determined. During recrudescence, only the high dose of E_2 increased GnRH I mRNA levels after 6 hours in the brain-slice cultures. Expression of GnRH II was not altered by treatment, whereas GnRH III mRNA levels were significantly increased by only the low dose of E_2 after 6 hours. In contrast, during midvitellogenesis, both doses of E_2 stimulated expression of GnRH I compared to control, with significant differences after 3 hours for the low dose and after 6 hours for the high treatment dose. 1 nM E_2 also significantly increased GnRH II mRNA levels at both sampling times. No response was observed for GnRH III mRNA levels. The primary

target for direct steroid regulation in the pituitary is the GnRH-R. During both stages of the reproductive cycle, increased expression was shown after 3 and 6 hours of incubation with both E_2 doses. In recrudescent females, GnRH-R mRNA returned to control levels after 18 hours of incubation, however, transcription was not decreased after this time in midvitellogenic pituitary cell cultures. FSHβ transcription was not affected at any time. LHβ expression in the midvitellogenic pituitary cells was stimulated by both E_2 doses only after 18 hours. These data indicate that E_2 directly regulates GnRH transcription at the level of the brain in a positive feedback mechanism. The target GnRH and responsiveness of its neuron population is dependent on the stage of the reproductive cycle. The GnRH-R is under direct positive regulation by this gonadal steroid, while the GtH subunits do not appear to be controlled by estrogen. These results support our findings of *in vivo* studies conducted in females of the same species (Chapter 4).

INTRODUCTION

The mechanisms involved in steroid feedback action on the GnRH neuronal network, especially those of estrogen (E_2), are still not completely clear. A main controversy is whether steroid hormones act directly in the GnRH neuron or indirectly through intermediary neurons or glial cells to regulate GnRH output. Until recently, many studies demonstrated that GnRH neurons lacked steroid hormone receptors (reviewed in Herbison, 1998), which supported the prevailing hypothesis that steroid hormones indirectly affect GnRH neurons. But reports of estrogen receptor beta (ERβ) expression in GnRH neurons (Hrabovszky et al., 2001; Kallo et al., 2001; Abraham et al., 2003) have challenged this general perception. Moreover, E_2 has been shown to act directly on

GnRH neurons by increasing intracellular calcium oscillations in GnRH neurons (Temple et al., 2004), and also to alter the excitability of GnRH neurons through the modulation of voltage-gated ion channels (DeFazio and Moenter, 2002). Further, Abraham et al. (2003) demonstrated an increase of cAMP response element-binding protein phosphorylation in GnRH neurons in response to E_2 stimulation via a non-genomic, ERβ-dependent mechanism. To date, no ER isoforms have been reported in teleost GnRH neurons. This could be due to technical difficulties in detecting low levels of expression or the possibility that the receptor is only briefly expressed at specific points in the reproductive cycle.

Specific targets of steroid feedback at the level of the pituitary include the GnRH receptor (GnRH-R), as well as the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In mammals, regulation of GnRH-R expression appears to be exerted primarily via estrogen and GnRH itself. Reports have suggested that E_2 may increase GnRH-R by increasing GnRH release from the hypothalamus and/or by directly influencing GnRH-R expression at the anterior pituitary gland (Kirkpatrick et al., 1998; Yasin et al., 1995). E_2 regulation of the GtHs is multilayered and appears to be mediated by different pathways (Burger et al., 2004). *In vivo*, LHβ gene expression is inhibited indirectly by suppression of GnRH (Shupnik and Fallest, 1994). In contrast, FSHβ regulation by steroids is more complex and involves other factors such as activin and inhibin (Gregory and Kaiser, 2004). *In vitro* studies have shown, however, that estrogen also directly influences gonadotropin gene expression, thus stimulating LHβ transcription and down-regulating FSHβ expression (Burger et al., 2004).

Steroidal regulation of the GnRH-GtH axis in fish has been extensively investigated (reviewed by Yaron et al., 2003), but most studies have been done using *in vivo* models. This experimental approach cannot accurately target the site of the steroid effect or its mode of action. Therefore, reports of steroid effects, for example, on GtH content in the pituitary or mRNA expression levels achieved *in vitro* may not match, and may even contradict results obtained *in vivo* (Huang et al., 1997; Rebers et al., 2000; reviewed by Yaron et al., 2003). *In vitro* studies examining GnRH content and/or release from the hypothalamus are scarce in fish (Yu et al., 1991; Yu and Peter, 1992; Senthilkumaran et al., 2001), and do not include steroid effects. Reports on GnRH mRNA levels *in vitro* are virtually nonexistent. There are also only a few published reports on the regulation of the GnRH-R in fish. It has been shown that GnRH agonist treatment of immature juvenile red seabream caused a significant increase in GnRH-R mRNA levels, suggesting that GnRH itself is a positive regulator of its own receptor (Okuzawa et al., 2002). Juvenile fish treated with E_2 or 11-KT had significantly lower levels of GnRH-R mRNA than controls, indicating that steroids exert a negative effect on GnRH-R expression. Steroid regulation of the gonadotropins in fish generally increases LHβ transcript levels, whereas FSHβ gene expression is reduced when cells are exposed to high steroid levels (Kah et al., 2000; Yaron et al., 2003).

The goal of this study was to locate the main target of estrogenic feedback in the HPG axis and elucidate the specific effects exerted. In order to achieve this goal, we developed a primary brain-slice culture method and employed primary pituitary cell culture, which has been used successfully in teleost fish (Levavi-Sivan and Yaron, 1992; Melamed et al., 1999). The central focus of this investigation was the estrogenic

regulation of gene expression of the three forms of GnRH in the perciform brain, as well as direct regulation of transcription of the GnRH-R and the gonadotropins in the gonadotroph cells of the pituitary.

Our model, the female striped bass (*Morone saxatilis*), is a late maturing perciform fish. They are seasonal breeders, spawning once a year during the spring. The synchronous gametogenesis of this species over a year-long period has led to the characterization of well-defined stages of gonadal development, where the ovary consists of a homogenous population of gametes (Specker et al., 1987; Woods III and Sullivan, 1993). Changes in the hormone profiles over the reproductive cycle have been reported (Berlinsky and Specker, 1991; Mylonas et al., 1998; Holland et al., 2000), which provides the basis for studying the steroid feedback control of the HP axis in this model organism.

MATERIAL AND METHODS

Experimental Animals and Treatment Protocol

Striped bass were acquired from UMCES Aquaculture and Ecology Research Lab at Horn Point, Cambridge, MD in fall 2000 and transferred to the Center of Marine Biotechnology's Aquaculture Research Center, Baltimore, MD, when fish were 4 month old. The fish were maintained in 6 foot and later transferred to 12 foot diameter recirculation systems, at 8 ppt salinity, under a simulated natural photo- and thermoperiod regime. The animals were kept under these conditions until the beginning of the experiment in October 2004. All animals at the Aquaculture Research Facility were

maintained and sampled according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland Biotechnology Institute.

In October 2004/February 2005, 24 female fish undergoing early recrudescence [average fluke length 61 cm, average weight 3.5 kg, GSI 1.7%, average oocyte diameter 230 μm (see Figure 3)], or midvitellogenesis [average fluke length 57 cm, average weight 3.4 kg, GSI 11.8%, average oocyte diameter 530 μm], respectively, were divided into 4 groups (n = 6). Fish were anesthetized (70 ppm phenoxyethanol, Baker Inc., Phillipsburg, NJ), weighed, measured and decapitated. Brain and pituitary tissue was extracted and kept in media on ice.

In Vitro Culture of Brain Slices

Upon sacrifice, brain and pituitaries were removed and kept on ice until sectioning. Brains were quickly bisected along the medial axis, and each resulting hemi-brain was cut into 300 μm thick slices using a McIlwain mechanical tissue chopper (The Vibratome Company, O'Fallon, MO). All slices obtained from each hemi-brain were pooled, washed three times with incubation media and then transferred into 6-well sterile cell culture plates (Falcon, Lincoln Park, NJ, USA) containing 2 ml of media per well (Figure 27). The incubation media consisted of Hanks' salt solution (pH 7.2; Gibco/Invitrogen Corp., Grand Island, NY) buffered with 25 mM HEPES (Sigma, MO, USA) and supplemented with 1 mg/ml glucose (Gibco/Invitrogen Corp.), 0.1% (w/v) bovine serum albumin (BSA, fraction V, Sigma). Ascorbic acid (50 μM; Sigma) and bacitracin (50 μM; Sigma**)** were added to prevent oxidation and degradation of the GnRHs. The incubation was carried out in an environmental chamber at 18 °C. For each individual fish, pooled slices from one hemi-brain were incubated with either a 1 or 100

nM dose of E_2. Slices from the second hemi-brain, serving as control, were incubated in media without E_2. Media was replaced after every 60 min, with fresh solution containing the same steroid concentrations. The incubation was terminated after either 3 or 6 h and the slices were snap-frozen for analysis of GnRH transcript levels.

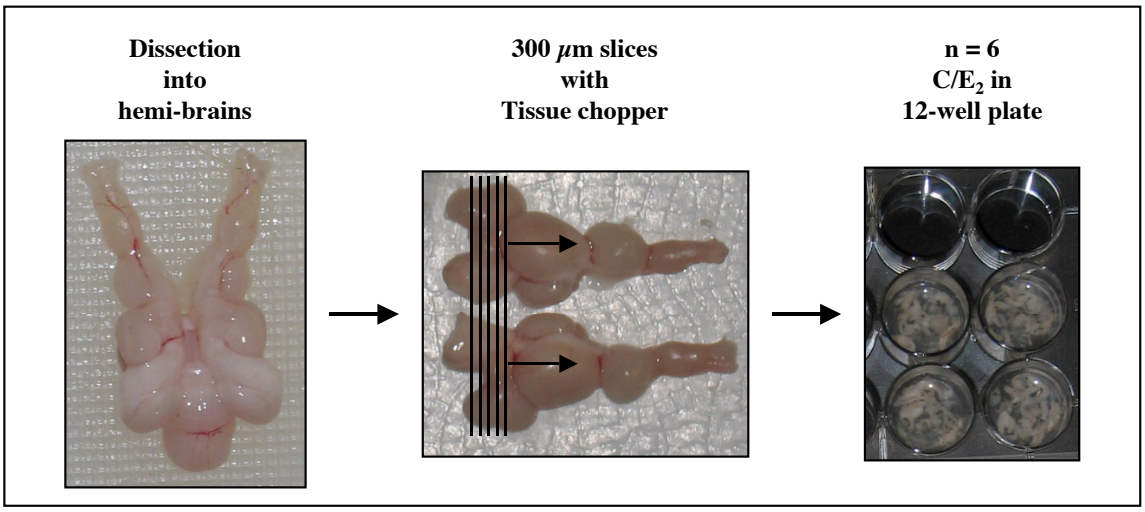

Figure 27: Preparation of fish brain slices for the *in vitro* study. For explanation see text. Control hemi-brain (C) and treated hemi-brain (E_2) are prepared in 12-well plates.

In Vitro Pituitary Cells

Pituitaries were washed in culture medium consisting of phenol-red free LB-15 medium (pH 7.44, osmolarity 350 mmole/kg; Gibco/Invitrogen Corp.) containing 20 mM HEPES (Sigma), 9 mM sodium bicarbonate (Sigma), 0.15% BSA and penicillin / nystatin / streptomycin (Gibco/Invitrogen Corp.) at 100 units, 0.1 mg and 12.5 units per ml, respectively. Pituitaries were then cut into small fragments and incubated in 5 ml Trypsin solution B [0.25% Trypsin (Gibco/Invitrogen Corp.); 0.01% glucose (Sigma)] and 0.5 mM EDTA (pH 8; Sigma) for an incubation period of 5 min. Mechanical triturating, starting with a Pasteur pipette and finishing with a syringe fitted with an 18G needle, was utilized to support the enzymatic dispersion process. The dispersion was terminated by

addition of 1 ml fetal calf serum (FCS; Gibco/Invitrogen Corp.). The percentage of viable cells was estimated by staining with trypan blue following cell counts on a hemocytometer. Cells were plated in 12-well tissue culture plates (Falcon) at a density of 2×10^5 cells/2 ml incubation media/well (basic medium containing 10% FCS) and incubated at 20 °C (Figure 28). After a recovery and attachment period of three days, each well was washed twice with 2 ml incubation media. Media was than added containing either a low (1 nM) or high (100 nM) dose of E_2 or a control solution (without E_2), for incubation periods of 3, 6 and 18 hours (n = 5 wells/treatment). Media was collected and frozen for subsequent analysis of LH content. Total RNA was extracted from cells using TRI-reagent (MRC Inc., Cincinnati, OH) to analyze GnRH-R and GtH subunit transcript levels.

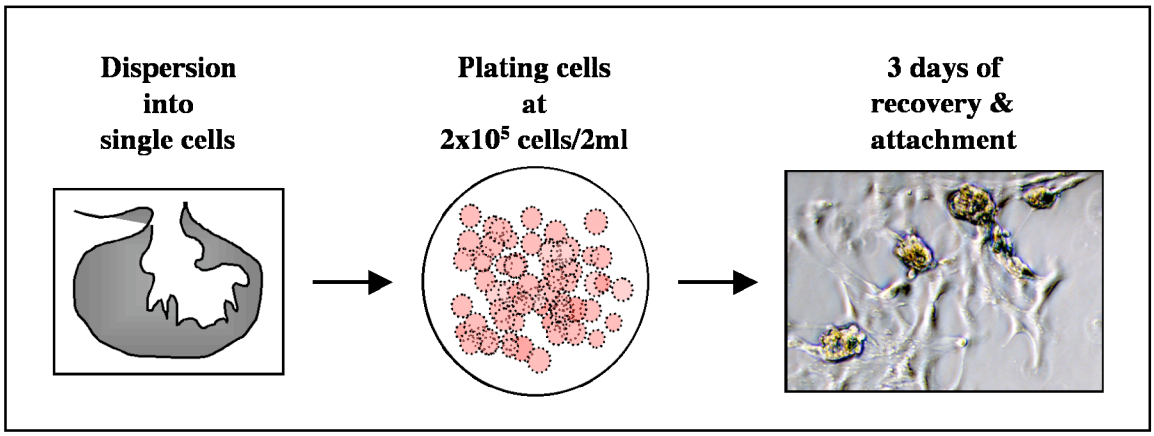

Figure 28: Schematic diagram of the *in vitro* pituitary experiment. Picture to the right shows gonadotroph cells after three days in culture. For explanation see text.

Quantification of Transcript Levels of GnRHs, GnRH-R, FSHβ, and LHβ

Transcript levels of the three GnRHs in the brain, and for the GnRH-R, FSHβ, and LHβ in pituitaries were measured using real-time fluorescence-based quantitative RT-PCR assays.

Total RNA isolated from whole brains or pituitaries using Tri-reagent was quantified spectrophotometrically. RNA standards and total RNA from each sample were reverse-transcribed into cDNA using random hexamers and MMLV reverse transcriptase (Promega, Madison, WI). Duplicate cDNA aliquots (1 ng of total RNA for 18s and LHβ RNA; 10 ng for FSHβ and GnRH-R; 50 ng for all GnRH transcripts) from each sample served as templates in PCR. The reaction was performed using SYBR Green PCR core reagent (Applied Biosystems, Forster City, CA) containing 200 mM gene-specific primers (Chapter 2). Primers were designed to span intron/exon boundaries of the corresponding target genes in order to avoid amplification of genomic DNA. Amplification reactions were carried out in an ABI Prism® 7700 Sequence Detection System at 50 ˚C for 4 min, 95 ˚C for 10 min, and 40 cycles of 95 ˚C for 15 s and 60 ˚C for 60 s. Copy numbers in unknown samples were determined by comparing C_T, the fractional cycle number at which fluorescence passes a baseline threshold value (Fink et al., 1998), to the specific standard (run in every plate) and normalized to the amount of 18s RNA in each sample.

Statistical Analysis

The data on transcript levels of the 3 GnRHs, GnRH-R, FSHβ, and LHβ were subjected to Analysis of Variance (ANOVA) to determine the existence of changes between experimental groups. To specifically identify statistical differences among means of each of the above parameters, the means were further compared using Scheffe'e post hoc test. Statistical significance was set at $P < 0.05$.

RESULTS

Effects of E_2 on GnRH Transcript Levels

To determine the direct effects of estrogen on GnRH neurons in the hypothalamus, we measured (using real-time PCR) synthesis of GnRH transcripts in brain-slice cultures, obtained at different stages of the reproductive cycle, which were exposed to different E_2 concentrations and various incubation lengths. Figure 29 illustrates changes in transcript levels of the three forms of GnRH identified in striped bass, in response to different E_2 challenges at the two reproductive stages investigated.

During early recrudescence, GnRH I transcript levels are not affected by either the low or high dose of estrogen after three hours of incubation (Figure 29A). After six hours, GnRH I mRNA levels are significantly increased by treatment with 100 nM estrogen. However, the low dose caused no increase in GnRH I synthesis. Closer to the spawning season, while oocytes were undergoing the stages of midvitellogenesis, E_2 appeared to up-regulate the GnRH I mRNA levels in a time and dose-dependent manner with significant effects at 1 nM E_2 after 3 hours and 100 nM E_2 after 6 hours (Figure 29B).

Expression levels of GnRH II were not affected by any estrogen challenges during early gametogenesis (Figure 29C). Conversely, during the latter stages of gonadal development, the low dose of E_2 (1 nM) significantly increased GnRH II transcript levels by 2.5-fold after 3 hours and almost 6-fold after 6 hours of incubation. The high dose of estrogen had no effect at either time point (Figure 29D).

After 3 hours of estrogen treatment of the recrudescent brain-slice culture, a decreasing trend in expression levels of GnRH III could be observed (Figure 29E).

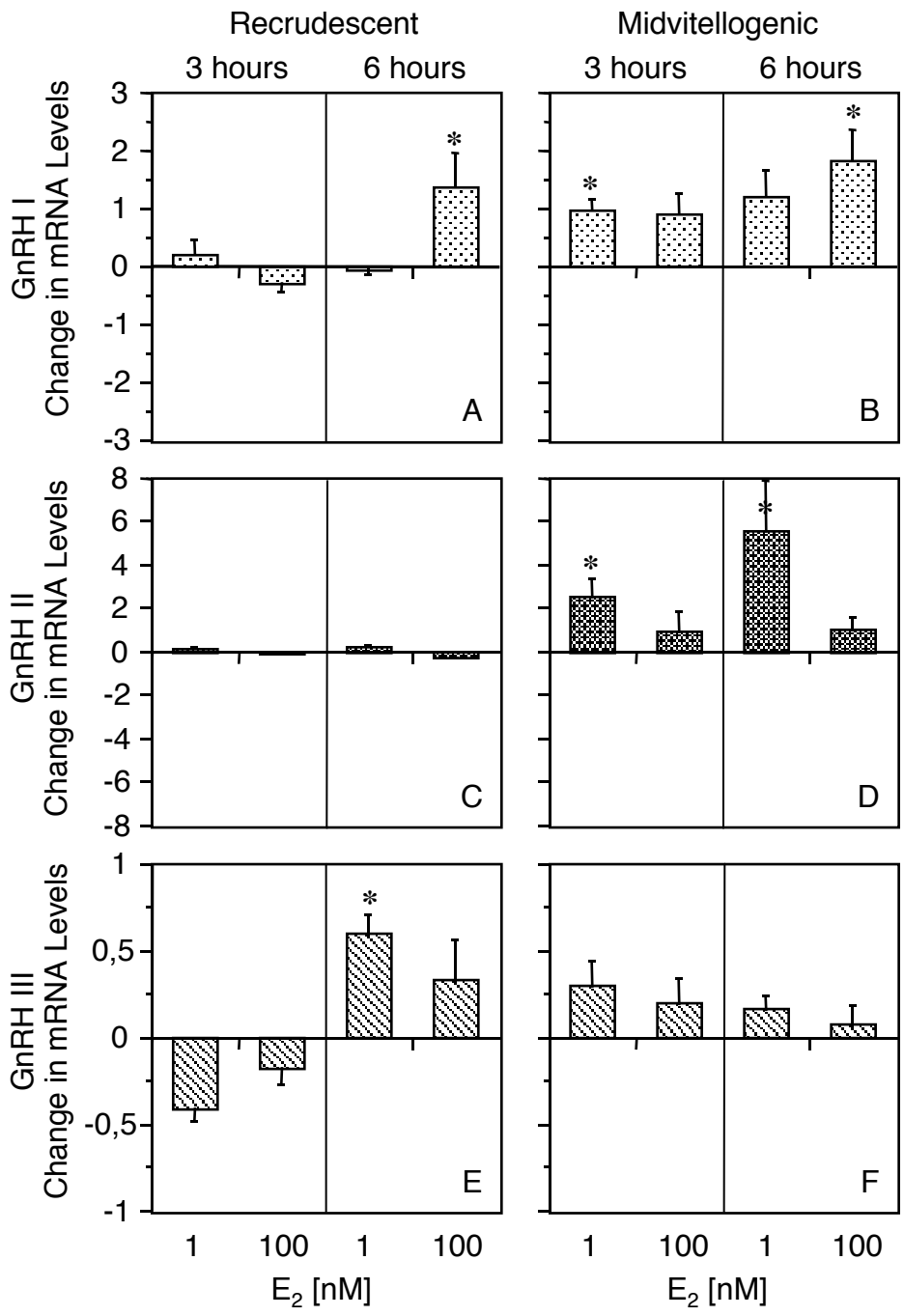

Figure 29: Effects of 17β-estradiol on GnRH I, II and III mRNA levels in brain-slice cultures obtained from females undergoing recrudescence (A, C and E) or midvitellogenesis (B, D and F). Hemi-brains were cut into 300 µm thick slices, washed and incubated with either a 1 or 100 nM dose of E_2. Slices from the corresponding hemi-brain served as control and were incubated with media only. Media was replaced every 60 min and the incubation was terminated after 3 or 6 h. Total RNA was extracted and reverse-transcribed into cDNA. The expression levels of GnRH I, II and III were measured by quantitative real-time PCR. Data are shown as the fold-change in mRNA levels compared to control and are represented as the mean ± SEM (n = 6), *P < 0.05 vs. control.

This, however, was reversed after 6 hours of incubation, during which the 1 nM dose of E_2 caused a significant increase in mRNA levels (Figure 29F). An increase was also noticed in the 100 nM treated group but this was not significant. During midvitellogenesis, the GnRH III system did not show any response to either the dosage of estrogen or length of incubation time.

GnRH Receptor and GtH Subunit Expression Levels

The main targets of estrogen feedback at the level of the pituitary are the GnRH-R, as well as the GtH subunits FHSβ and LHβ. To investigate direct estrogen effects on these three genes, we determined their expression levels in primary pituitary cells, obtained from female striped bass undergoing early recrudescence or midvitellogenesis, and treated with two E_2 dosages over 3 and 6 hours of incubation. Figure 30 shows the results obtained by real-time PCR for the transcript levels of the receptor and the GtHs.

During early gonadal development, GnRH-R mRNA levels are significantly increased after estrogen treatment at both dosages after 3 and 6 hours of incubation (Figure 30A). This effect appeared to be abolished by 18 hours in both E_2 treatment groups. However, pituitary cells obtained from females undergoing midvitellogenesis maintained a significant up-regulation of GnRH-R transcript at all three time points for both treatment dosages of E_2 (Figure 30B).

No changes were observed in FSHβ expression in pituitary cells from recrudescent females after treatment with either E_2 dosage at any time points of the experiment (Figure 30C). Similar results were obtained for the latter stages of gonadal development, with the exception of the 1 nM E_2 treatment at 3 hours, during which a significant decrease in FSHβ mRNA levels was detected (Figure 30D). However, there is

no detectable trend for E_2 regulation of FSHβ expression at either of the two reproductive stages.

LHβ mRNA levels were not changed by any estrogen treatment in pituitary cells, obtained from females undergoing early recrudescence (Figure 30E). However, long-term incubation with either the low or high dose of E_2 caused a significant increase in transcript levels during midvitellogenesis (Figure 30F).

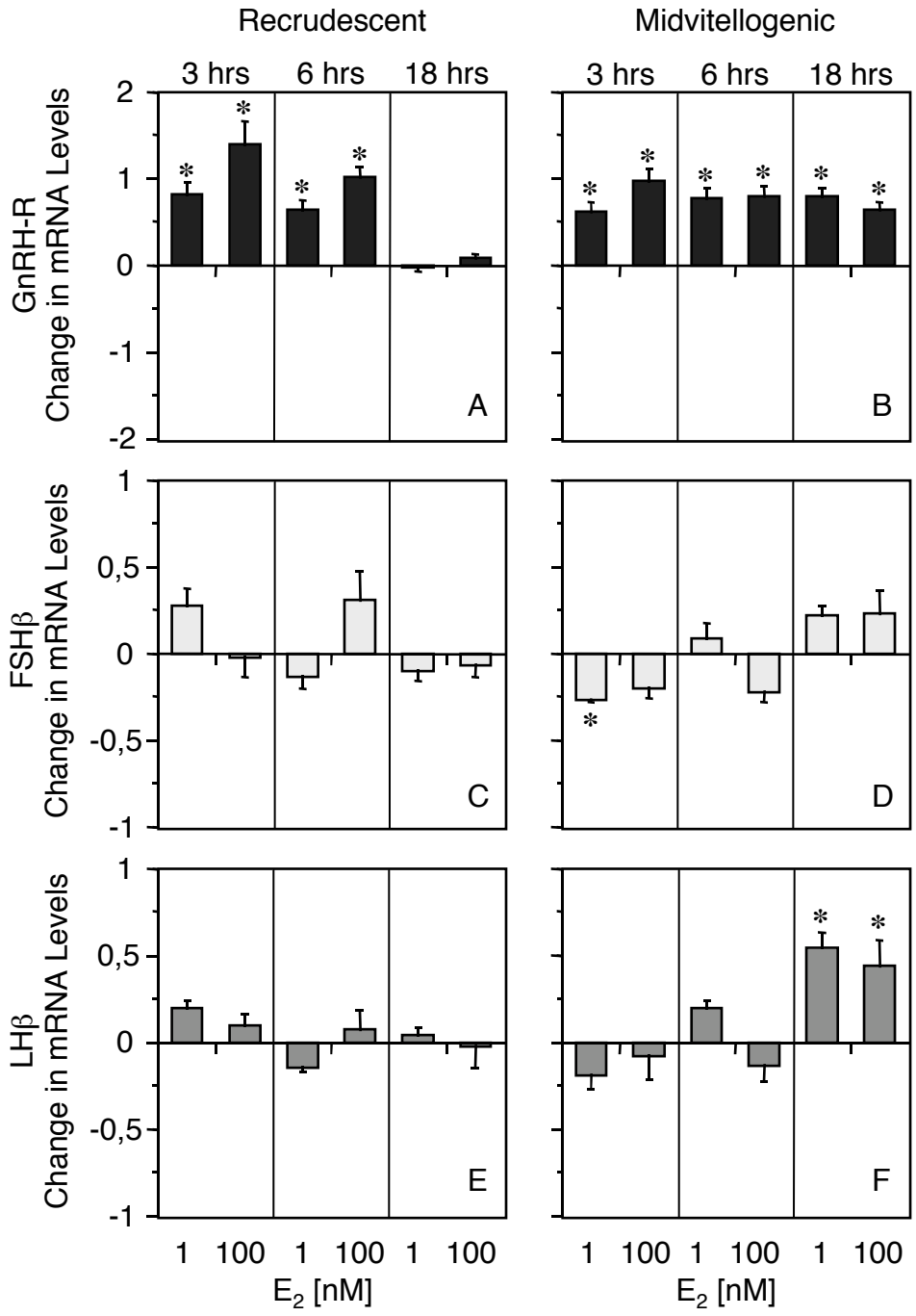

Figure 30: Effects of 17β-estradiol on GnRH-R, FSHβ and LHβ mRNA levels in pituitary cell cultures obtained from females undergoing recrudescence (A, C and E) or midvitellogenesis (B, D and F). Pooled pituitaries (n = 6) were dispersed and plated at a density of 2×10^5 cells/2 ml incubation media/well. After a 72 h recovery and attachment period, cells were washed and incubated with either a 1 or 100 nM dose of E_2 or a control solution (devoid of E_2) for incubation periods of 3, 6 and 18 hours (n = 5 wells / treatment). Total RNA was extracted and reverse-transcribed into cDNA. The expression levels of GnRH-R, FSHβ and LHβ were measured by quantitative real-time PCR. Data are shown as the fold-change in mRNA levels compared to control and are represented as the mean ± SEM, *P < 0.05 vs. control.

DISCUSSION

Effects at the Level of the Brain

This *in vitro* study demonstrates that estrogen differentially regulates the GnRH-expressing neuronal populations at the level of the brain throughout the reproductive cycle. During the early phase of gonadal recrudescence, only exposure to the higher dose of E_2 and the longest incubation time increased expression of the hypophysiotrophic GnRH I. However, in the relatively advanced reproductive stage of midvitellogenesis, even the lowest dose and shortest incubation time induced a significant rise in mRNA levels of this GnRH form. Estrogen regulation of GnRH I mRNA *in vivo* is controversial in mammals, where studies support both an increase and a decrease of expression (Zoeller et al., 1988; Kim et al., 1989; Toranzo et al., 1989; Roberts et al., 1989; Rosie et al., 1990; Weesner et al., 1993; Petersen et al., 1996). These differences in results may arise from the use of different techniques to measure expression levels, differences in species, reproductive stage, age of the animals and/or experimental design. In addition, estrogen exerts both negative and positive feedback effects on GnRH I-producing neurons (Herbison, 1998). Therefore, the discrepancies in the results may also be due to the ability of estrogen to stimulate or inhibit the neuroendocrine axis, depending upon the experimental model. *In vitro* studies in mammals focusing on estrogen regulation of GnRH I expression reported largely inhibitory effects. Exposure of GT1-7 cells to E_2 resulted in reduction of GnRH I mRNA expression (Roy et al., 1999). Furthermore, estrogen was shown to down-regulate mRNA levels of GnRH I in a neuronal cell line, TE-671 (Chen et al., 2002).

In vivo studies, conducted in fish, to determine the effect of estrogen on GnRH I transcription, have also reported diverse results. E_2 had a positive influence on GnRH I mRNA in immature, castrated male tilapia (Soga et al., 1998; Parhar et al., 2000) but had no effect in immature and mature male or prepubertal female red seabream (Okuzawa et al., 2002). Treating ovariectomized or sham-operated adult female striped bass undergoing gonadal recrudescence with estrogen *in vivo*, we observed no effect on GnRH I mRNA levels. However, in our experiment conducted during midvitellogenesis using the same model species, treatment with T caused a significant increase in transcript levels (Chapter 4). Our *in vivo* findings are in good agreement to what we observed in this *in vitro* study, where no response of GnRH I neurons is seen during early gametogenesis, but during midvitellogenesis a very clear dose and time-dependent increase occurs.

Incubation of the female brain with estrogen during the early stages of oogenesis had no effect on transcript levels of GnRH II. However, a pronounced increase of GnRH II mRNA expression (up to 6-fold) was observed during midvitellogenesis, but only after treatment with the lower dose of E_2. That E_2 exerts positive effects on GnRH II gene expression has also been shown in other experimental systems, like human neuronal medulloblastoma cells (Chen et al., 2002) and human granulosa luteal cells (Khosravi and Leung, 2003). Furthermore, *in vivo* studies in ovariectomized primates showed that E_2 replacement enhanced GnRH II mRNA levels well above those observed in intact animals (Densmore and Urbanski, 2004). However, midbrain GnRH II expression was not altered by estrogen in either the cichlid or European silver eel (Parhar et al., 1996; Parhar et al., 2000; Montero et al., 1995). Our studies utilizing gonadectomy followed by steroid replacement in adult female striped bass resulted in no changes in GnRH II

transcription during early recrudescence. However, during midvitellogenesis, we observed that T treatment had an effect on GnRH II mRNA levels (Chapter 4). Our *in vitro* data for GnRH II expression levels in this study support our findings in recrudescent females *in vivo* and indicate that GnRH II neurons are not responsive to estrogen during recrudescence. Furthermore, these neurons change their ability to respond to steroids in the subsequent stages, which we have demonstrated here *in vitro* and *in vivo* (Chapter 4). The increase of GnRH II mRNA levels after E_2 treatment and the observed decrease after T treatment *in vivo* (Figure 23) gives an indication about the distinct pathways (androgenic versus estrogenic) for gonadal regulation of the GnRH II neurons.

The GnRH III neuron population in adult female striped bass appears not to be under the direct regulation of estrogen. Brain-slice cultures prepared from both reproductive stages showed no changes in expression, except after a 6 hour incubation with 1 nM estrogen during early recrudescence, where an increase of about 0.6-fold was observed. This is supported by experiments we have done *in vivo*, where ovariectomized female striped bass treated with estrogen also showed no response to estrogen replacement during recrudescence (Chapter 4). Likewise, *in vivo* studies conducted by Parhar et al. (2000) demonstrated no changes in GnRH III mRNA levels after estrogen treatment of immature cichlids. Although the evidence is limited, the terminal nerve GnRH III neuronal system appears not to be under estrogenic regulation. In the literature, the GnRH III neuron population has been implicated as a neuromodulator, based of the fact that its neurons project their axons widely in the brain (Oka, 2002). This is supported by reports from the dwarf gourami, where GnRH III modulates the pacemaker activity of GnRH III neurons in an autocrine fashion (Abe and Oka, 2000; Abe and Oka, 2002).

Furthermore, GnRH III appears to be involved in regulating the response of retinal ganglion cells to light in goldfish (Walker and Stell, 1986; Umino and Dowling, 1991). The neuromodulatory functions of GnRH III would not logically be under the influence of gonadal steroids. Based on studies in cichlids, Soga et al. (1998) hypothesized that GnRH III neurons influence GnRH I mRNA synthesis via terminal nerve projections found in close proximity to POA GnRH I neurons. This would suggest that GnRH III, together with gonadal steroids, regulates the hypophysiotrophic GnRH, but is itself under the control of other factors.

The responsiveness of both the GnRH I and II systems changes over the course of gamete development. During the early stages of recrudescence, when circulating levels of estrogen are relatively low (Mylonas et al., 1998), GnRH synthesis is not under estrogenic control (as shown *in vitro* and *in vivo*). However with rising E_2 levels, produced by the vitellogenic oocytes, both neuron populations show positive feedback regulation of GnRH synthesis by the gonadal steroid. These results lead us to the working hypothesis that during the early stages of the reproductive cycle, GnRH I and II neurons are either inhibited by other factors (neurotransmitters/missing stimulants) or unresponsive due to missing transsynaptic connections.

A similar situation is found during the onset of puberty in mammals, where the GnRH network needs to be activated and multiple events are known to prompt the process. Changes in transsynaptic and glial inputs to the GnRH neuronal network are necessary to initiate mammalian puberty. An increase in glutamatergic neurotransmission (Ojeda and Terasawa, 2002) follows the changes to network input, ultimately exerting a major role in control of GnRH release. In addition, the inhibitory influence during

prepubertal development of GABAergic neurons, acting via $GABA_A$ receptors (Mitsushima and Kimura, 1997), must be removed for the system to become competent. The regulation of GnRH secretion by the astro-glial network occurs via two related mechanisms, plastic rearrangements and direct cell-cell communication, involving growth factors produced by the glial cells (Ojeda et al., 2003a) and also plays a role during the reproductive cycle in adult mammals. Parkash and Kaur (2003a) showed in adult rats that the polysialylated form of neural cell adhesion molecule (PSA-NCAM), which is a known marker of neuronal plasticity, is enhanced by estrogen. Furthermore, they observed greater expression of PSA-NCAM associated with GnRH terminals in the proestrous phase than during the diestrous phase of female rats. The same molecule has been shown to promote rearrangement of cells in close vicinity to the GnRH neurons and their terminals (Viguie et al., 2001) and may be able to integrate input of the extracellular environment via facilitatory or inhibitory neuronal-glial remodeling events. More evidence for estrogenic influence was demonstrated by Hoyk et al. (2001), showing that changing circulating E_2 levels reversibly reduced the number of axo-somatic GABA synapses, along with changing the ensheathment of neuronal somata by astrocytes. Similar results were obtained by Garcia-Segura et al. (1999), who also demonstrated that E_2 induces a transient disconnection of axo-somatic inhibitory synapses during preovulatory and ovulatory stages of the estrous cycle in rodents. In addition, astrocytes have the potential to release GnRH-stimulating neuroactive factors such as transforming growth factor-β1 (Melcangi et al., 1995; Galbiati et al., 1996; Ojeda et al., 2000; Buchanan et al., 2000; Zwain et al., 2002).

Thus, it may be necessary for the GnRH network in the adult female striped bass to undergo similar changes over the course of gamete development in order to be able to respond to gonadal steroid feedback. The rising levels of estrogen throughout the course of oogenesis may be responsible for the rearrangement of the GnRH neuronal network and, based on these changes, the increased responsiveness of GnRH I and II neurons to positive steroid feedback.

Despite our data supporting estrogenic regulation of the GnRH neuronal network and differential regulation of the three distinct GnRH neuron populations in the striped bass brain, we cannot elucidate the pathway by which these actions are directed. These effects could be related either directly via ERβ, which has been shown to be expressed in GnRH neurons in mammals (Hrabovszky et al., 2001; Herbison and Pape, 2001; Kallo et al., 2001; Abraham et al., 2003), or indirectly by stimulation of GnRH synthesis and secretion via afferent E_2-sensitive neuronal populations (Herbison, 1998; Goubillon et al., 1999; Chappell and Levine, 2000; Goubillon et al., 2002). Our newly developed brain-slice culture system could facilitate the determination of the actual pathways involved. With this now-established experimental approach, identification of specific neurotransmitters via the use of their inhibitors, in conjunction with estrogen treatment, may be possible. Further studies are certainly necessary to follow up on these questions.

Effects at the Level of the Pituitary

The regulation of hormone synthesis and release in the gonadotroph cells of the pituitary is a central issue in understanding how the reproductive axis works. Besides hypothalamic control, feedback from the gonads is a key factor regulating reproduction at the level of the pituitary. Therefore, determination of the direct effects of estrogen on

GtH and GnRH-R synthesis and release and the eventual changes occurring over the reproductive season was essential to fully understand feedback control at the pituitary level. In this study, we observed that during early recrudescence E_2 up-regulated GnRH-R transcript levels after 3 and 6 hours of incubation in a dose-dependent manner. However, this effect was lost after 18 hours of incubation with either 1 or 100 nM estrogen. Repeating this experiment with pituitary cells obtained from females undergoing characteristic stages of midvitellogenesis, we demonstrated a positive feedback effect of E_2 for all three incubation times and both dosages. These results support our *in vivo* experiments using the same experimental model. Estrogen replacement after ovariectomy in recrudescent females resulted also in a significant increase of GnRH-R mRNA levels compared to controls (Chapter 4). Furthermore, we demonstrated that during midvitellogenesis, treatment with T resulted in a dramatic increase of GnRH-R transcript levels to almost 13-fold. This rise in receptor expression in response to T may have been caused by utilizing both androgenic and estrogenic pathways (Chapter 4). However, our results conflict with other reports of estrogen effects on GnRH-R expression. In mammals, E_2 replacement decreased the post-castration rise of GnRH-R (Kaiser et al., 1993; Looper et al., 2003). *In vitro* work in different cell systems, like ovarian carcinoma and primary-cultured granulosa-luteal cells, also showed that estrogen inhibits GnRH-R expression (Kang et al., 2001; Nathwani et al., 2000). Furthermore, evidence collected in fish, demonstrated a decrease in GnRH-R mRNA levels after E_2 treatment *in vivo* (Okuzawa et al., 2002). This evidence indicates that estrogen regulation of GnRH-R gene expression is dependent on species, maturational stage and experimental procedure.

Our results in this report support the hypothesis that estrogen regulates GnRH-R expression directly at the level of the pituitary, thus sensitizing the pituitary to hypothalamic signals. The decrease of GnRH-R mRNA levels after 18 hours of incubation with E_2, compared to control, is not consistent with our results observed *in vivo*. This may be due to the absence of other stimulatory factors, which *in vivo* are secreted from the brain and provide an additive effect, in the pituitary cell culture. GnRH itself is one of the key regulators of GnRH-R expression (reviewed by Hapgood et al., 2005), therefore the estrogen-mediated increase in expression *in vitro* is not further supported by a GnRH signal from the hypothalamus, which may be the reason for the observed decrease of receptor expression after 18 hours of incubation. However, during the later stages of gametogenesis, GnRH-R transcript levels remained elevated. This result may be related to the rising levels of steroids, as well as gonadal factors such as activin or inhibin (Gregory and Kaiser, 2004), over the course of the reproductive cycle. In essence, these factors may serve to 'prime' the gonadotroph cells. This prolonged steroid exposure could induce cytological and structural changes in the gonadotroph cell connectivity, as well as changes in stimulatory and/or inhibitory neurotransmitter systems. This theory is supported by the fact that in our pituitary cell cultures obtained from females undergoing midvitellogenesis, the GnRH-R mRNA levels did not decrease throughout the long-term incubation.

The control of the synthesis and release of FSH and LH throughout the reproductive cycle has been a center of attention for many years. In addition to GnRH, the primary hypothalamic regulator, gonadal factors such as sex steroids, activin and inhibin, regulate gonadotropin production. Moreover, specific changes in the anatomical

relationship between gonadotrophs and other pituitary cell types may contribute to this regulation by modifying local paracrine signals from one cell type to another (Cheung, 1983). In this study, we report that in pituitary cells from recrudescent female striped bass, neither E_2 treatment dose had an effect on FSHβ expression levels. A similar result was observed later in the reproductive cycle, with the exception of a significant decrease in transcription after exposure to 1 nM E_2 for 3 hours. However, we observed in our *in vivo* experiments in the same model that FSHβ transcription was under positive gonadal feedback during recrudescence and negative control during midvitellogenesis (Chapter 4). These opposing *in vivo*/*in vitro* results may be explained by an indirect gonadal regulation of FSHβ gene transcription *in vivo* via the GnRH system or other hypothalamic neurotransmitters.

In vitro work in other teleost fish showed a differential regulation of FSHβ by steroids. Studies in male tilapia pituitary cells in general indicated that regulation of FSHβ expression is very sensitive to steroid levels. Too low/high doses caused a decrease in FSHβ mRNA levels. Only the addition of moderately low levels resulted in an increase in FSHβ transcript (Melamed et al., 2000). Furthermore, cells from immature and early maturing fish showed that low T doses elevated FSHβ mRNA. However, in cells from late maturing fish, T depressed FSHβ transcript levels and was ineffective in cells from regressed fish (Melamed et al., 1998). The discrepancy in these results compared to our study may partly be due to our use of a female model, with a different reproductive strategy. However, *in vivo* reports from many different fish species showed that steroid treatments (T/E_2) also decreased FSHβ mRNA levels [coho salmon (Dickey and

Swanson, 1998); juvenile or recrudescing goldfish (Sohn et al., 1998; Kobayashi et al., 2000); European sea bass (Mateos et al., 2002)], which supports our *in vivo* findings.

Similar to the data in fish, *in vivo* and *in vitro* studies in other vertebrates display varying results with regard to regulation of FSHβ transcription. In general support of our findings, E_2 had no effect on FSHβ mRNA synthesis in female rat pituitary fragments (Shupnik and Fallest, 1994). However, the same steroid suppressed mRNA synthesis in cultured ovine pituitary cells (Phillips et al., 1988; Baratta et al., 2001). *In vivo*, E_2 also exerts a negative feedback, suppressing the post-ovariectomy increase in FSHβ mRNA levels in the rat (Shupnik et al., 1988). This effect was reversed when co-treated with a GnRH antagonist, demonstrating that the site of E_2 action in rat is the hypothalamus (Shupnik and Fallest, 1994).

Our *in vitro* studies at the pituitary level showed that exposure of gonadotroph cells to estrogen has no observed regulatory effect on FSHβ transcription at either stage of the reproductive cycle investigated. This may be the result of having missed the effective treatment dosage, since the FSHβ subunit appears to be very sensitive to steroid levels. However, estrogen seems to target the hypothalamus, and specifically the GnRH neurons, to regulate the subunit, rather than exerting a direct effect at the level of the pituitary. Our findings in striped bass can certainly be interpreted in support of this theory.

LHβ subunit expression was also determined in our pituitary cell culture experiments. At the beginning of gametogenesis, we did not observe any changes in transcription in response to either E_2 treatment dose or incubation time. In pituitary cells collected from midvitellogenic females, neither 3 nor 6 hours of incubation with either

dose of E_2 had an effect. However, long-term incubation with both dosages of estrogen for 18 hours significantly increased LHβ mRNA levels. *In vivo*, LHβ expression in female striped bass was stimulated by steroids at both investigated stages (Chapter 4). The *in vitro* and *in vivo* results together support the hypothesis that LHβ transcription regulation occurs indirectly via a hypothalamic pathway.

Our data is supported by *in vitro* work using male tilapia pituitary cells from different gonadal stages. Treating these cells with T or E_2 had no effect on LHβ mRNA levels (Melamed et al., 2000). The only exception was observed in cells obtained from regressed fish, where T increased LHβ transcript levels in a dose-dependant manner (Melamed et al., 1998). However, in the African catfish exposure of pituitary cells to E_2 elevated LHβ transcript (Rebers et al., 2000). These differences may be explained by the different sex of the model and their different reproductive strategies.

In vitro work in mammals has shown that estrogens exert a direct action on the pituitary. E_2 rapidly increased LHβ mRNA synthesis rates in pituitary cells from OVX rats (Shupnik et al., 1989a; Shupnik, 1996), and in pituitary cells from cycling rats, with the greatest effect in cells obtained from rats in proestrous (Shupnik et al., 1989a). The latter study provides evidence that the response of pituitary cells is dependent on the cellular environment experienced in the animal before extraction. Changes in gonadal steroid feedback on the pituitary will change the pituitary 'set-up' or 'prime' the tissue. This may include anatomical changes in gonadotrophs or between gonadotrophs and other pituitary cell types. In several species, it has been shown that the gonadotrophs synthesize and secrete both FSH and LH (reviewed by Childs, 1997). Evidence in rodents has suggested that gonadotrophs may also express GH (Childs et al., 1994; Childs, 1997;

Nunez et al., 2003) and that the proportion of GH mRNA-expressing cells in the rat is significantly influenced by the stage of the estrous cycle (Childs, 2000). These findings were supported by Mignot & Skinner (2005), who detected LH/GH co-expressing cells in ovine pituitaries, with the ratio being significantly influenced by the stage of the estrous cycle. Childs (2002) proposed that somatotrophs may function as co-gonadotrophs, which is based on a population of multihormonal/multipotential cells existing in the anterior pituitary to support the extra needs of the gonadotroph and somatotroph population over the reproductive cycle.

The observed increase in LHβ mRNA levels in our study may be evidence for the recruitment of monohormonal gonadotrophs to switch to production of LHβ transcripts, induced by the long-term incubation with E_2. Another possible scenario would be that somatotroph cells are recruited to produce GnRH-R transcript and also LHβ mRNA. Evidence for the latter theory includes the increased expression of GnRH-R mRNA after 18 hours in pituitary cells from midvitellogenic females.

This study has shown that over the reproductive cycle in female striped bass, a differential regulation of the distinct GnRH neuron populations by estrogen occurs. GnRH I and II expression levels are under positive control by E_2 in the later stages of the reproductive cycle. However, no influence by this gonadal steroid was observed during early gametogenesis, leading to the hypothesis that the GnRH neuronal system is not responsive to gonadal steroids at this stage. GnRH III does not appear to be regulated by this sex steroid at any time throughout the reproductive cycle. These results are in good agreement with our findings in the same species *in vivo* (Chapter 4; Figure 20; Figure 23). At the level of the pituitary, direct estrogenic effects were observed only on GnRH-R

transcription levels during both reproductive stages investigated. Our results support the hypothesis that estrogen regulates GnRH-R expression directly at the level of the pituitary, sensitizing the pituitary to hypothalamic signals. Direct regulation of the GtH subunits by estrogen appears to occur only in advanced stages of the reproductive cycle and, based on our findings, its primary target is suggested to be LHβ transcription. The *in vitro* observations made at the level of the pituitary indicate that the changes in GtH transcription in response to gonadal steroid feedback observed *in vivo* are the result of indirect regulation occurring via the hypothalamic pathway.

CHAPTER 6. GENERAL DISCUSSION

This chapter serves as a summary of all major findings from the present study. Throughout this dissertation, the study results have been examined with respect to individual levels of the HP axis. This chapter approaches the results from a more holistic viewpoint, examining the data with aspect to the entire HPG axis. In addition, the data from our studies have been incorporated with relevant information from the literature to form a model describing the ontogeny of gonadal steroid feedback regulation of the HP axis throughout the juvenile and pubertal phase of sexual development, as well as during two specific stages of the adult reproductive cycle in female striped bass. Figure 31 depicts the changes in positive and/or negative endogenous gonadal feedback on the HP axis at the investigated stages, while Figure 32 represents the corresponding effects observed after steroid replacement. The results, in which no effects of treatment were observed, are not represented in these graphics.

THE TIMELY RESPONSE OF THE HP AXIS TO GONADECTOMY AND STEROID REPLACEMENT IN A LONG-TERM EXPERIMENT: BASELINE INFORMATION

Gonadal steroid feedback regulation of the hypothalamus-pituitary axis plays a key role in reproduction in all vertebrate species. Analyzing the mechanisms involved in controlling the hormonal cascade and the timely response of the target systems to feedback is essential for an integrated understanding of the reproductive axis.

Effects on the HP axis in response to gonadectomy and steroid replacement have been extensively studied in many vertebrate models with varying results. For example,

GnRH expression and peptide levels have been reported to increase, decrease or remain unaffected following gonadectomy. Furthermore, controversial reports have been provided regarding steroid modulation of the GnRH neuronal network (reviewed by Sagrillo et al., 1996). Studies that focus on GnRH-R expression have produced similar discrepancies. The same holds true for FSH and LH, which have been shown to be differentially regulated over the course of time after gonadectomy and steroid replacement (reviewed by Burger et al., 2004). However, experimental outcomes depend largely on methods, experimental duration and timing, treatment dosage and/or steroid application, as well as the experimental model. We decided therefore, to first conduct a detailed study of the timely response of the HP axis to a gonadectomy and steroid replacement experiment (Chapter 2) to better understand and evaluate previous study results, and further to aid in designing our subsequent experiments (Chapter 3 and Chapter 4). This was done in view of understanding the development of gonadal steroid feedback throughout the life stages (juvenile, pubertal, adulthood) of our primary model, the female striped bass.

For our baseline study, we determined the response of the HP axis 3, 6, 9, 14, and 28 dps by evaluating its endocrine correlates. Significant changes in feedback regulation occurred, at most levels, 9 days after the initiation of the experiment and lasted until termination (Table 6). For example, a negative estrogenic feedback on GnRH I release was revealed 9 dps and remained unchanged. Furthermore, alterations in GnRH II peptide levels, GnRH-R expression and LH plasma levels were also observed at this critical time of 9 days post-surgery. Only FSHβ transcription was affected in a more acute way, displaying increased levels already 3 dps and remaining elevated throughout the

experiment. Changes at the level of the brain were only observed in the long term, when GnRH gene expression decreased at 28 dps.

Based on these results, we determined that at 9 days after gonadectomy and steroid replacement most endocrine correlates of the HP axis have established a steady-state level at which they remain until at least 28 dps. Therefore we concluded that an experimental length of 10 days will be optimal for further experiments investigating gonadal steroid feedback in striped bass females.

Table 6: Changes in measured endocrine correlates of the HP axis in response to gonadectomy and steroid replacement throughout our time series study (simplified results, for details see Chapter 2; yellow: no change; red: decrease; green: increase).

	3 days	**6 days**	**9 days**	**14 days**	**28 days**
GnRH I mRNA	⇔	⇔	⇔	⇔	⇓
GnRH II mRNA	⇔	⇔	⇓	⇔	⇔
GnRH III mRNA	⇔	⇔	⇔	⇔	⇓
GnRH I peptide	⇔	⇔	⇓	⇓	⇓
GnRH II peptide	⇔	⇔	⇓	⇓	⇓
GnRH III peptide	⇔	⇑	⇔	⇔	⇔
GnRH-R mRNA	⇓	⇔	⇓	⇓	⇓
FSHβ mRNA	⇑	⇑	⇑	⇑	⇑
LHβ mRNA	⇔	⇔	⇔	⇔	⇓
Pituitary LH protein	⇓	⇔	⇔	⇔	⇓
Plasma LH protein	⇔	⇔	⇑	⇑	⇑

THE JUVENILE HP AXIS

In juvenile female striped bass, gonadal feedback occurs in a seasonal pattern with regulatory functions observed only during the spring, when animals are 13- and 23-months old. The primary target of positive endogenous feedback was FSHβ gene expression, however, based on the results observed in the steroid replacement groups, the nature of the feedback was determined to be non-steroidal (Chapter 3). Furthermore, LHβ transcription was positively controlled by a non-steroidal, gonadal feedback, but only

during the spring of the first year (Figure 31). These results indicate that FSH is an important regulator of seasonal gonadal events during the juvenile period of development in our model and may play a major role in early gametogenesis, as has been suggested in salmonids (Swanson et al., 1991). LH appears to be of particular importance for gonadal development during the first year of life but not in subsequent juvenile stages. Increased synthesis of LHβ in the juvenile phase was also shown by Hassin et al. (Hassin et al., 1999) using the same model. However, the role LH plays in this developmental stage remains to be determined.

Based on our results, we are not able to conclude whether this gonadal regulation of the gonadotropins is exerted via an indirect hypothalamic pathway involving neuro-compounds or via direct effects on gonadotroph cells. However, this regulation appears to follow an annual rhythm that is associated with a) developmental events in the growing oocytes and b) seasonally changing GnRH levels, which were observed in juvenile and maturing female striped bass in studies done by Holland et al. (2000, 2001). These investigators observed rising pituitary levels of GnRH I and II in the fall, which peaked in the spring and declined thereafter. The presence of GnRH in juvenile pituitaries lends credence to the speculation that the endogenous gonadal feedback may be conveyed via GnRH release from the hypothalamic nerve terminals in the pituitary, thereby increasing GtH gene transcription. This hypothesis is supported by reports that the gonadal peptide activin has been shown to release GnRH (reviewed by Gregory and Kaiser, 2004)) and therefore may be responsible for the positive non-steroidal effect observed in our model (Figure 31).

Removal of the gonads in juvenile animals had no effects on GnRH gene expression in the brain or GnRH-R transcription and LH protein levels in the pituitary (Chapter 3), indicating that at this developmental stage no feedback control of these endocrine correlates is necessary for ovarian development.

It is not surprising that the feedback in juvenile females is non-steroidal in nature, since endogenous steroid levels are non-detectable or near detection limits (Chapter 3). However, replacement with estrogen or androgen resulted in inhibitory effects on GtH expression (primarily FSH), and E_2 stimulated GnRH-R expression in 23-month old females in the spring (Figure 31). Our *in vitro* studies in adult females showed that FSHβ transcription is not directly regulated by estrogen (Chapter 5), supporting the theory that the observed inhibition may be the result of an indirect pathway via hypothalamic neurotransmitter systems or other pituitary compounds. These results indicate that even though internal steroid levels are low or non-existent, components of the HP axis are capable in this early stage of conveying steroid feedback at the level of the pituitary. The mechanism underlying this feedback or its possible physiological significance remains elusive. However it appears unlikely that the GnRH neuronal network is involved, based on the complete absence of regulation (gonadal/steroidal) during this developmental phase.

THE PUBERTAL HP AXIS

In the fall, 28-month old pubertal females displayed no gonadal feedback regulation of the HP axis, which mirrored the results obtained in 19-month old juvenile animals during the previous fall (Chapter 3; Figure 31). These results indicate that

gonadotropins originating from the pituitary may not be as necessary during this phase of gonadal development as at later stages. However, this more mature HP axis showed a completely different response to steroid replacement than what was observed in the younger animals the previous year (Figure 32). Estrogen replacement stimulated GnRH-R mRNA levels, while inhibiting FSHβ transcription, which also was negatively regulated by 11-KA. The same pattern of regulation observed in the spring experiment is still present in the following fall, suggesting that components of the HP axis remained active in this subsequent stage. However, as in juveniles, GnRH biosynthesis was not affected by our treatments (Figure 32), suggesting an ongoing unresponsiveness of the female GnRH neuronal system to steroid regulation. The reasons for this "quiescence" may be an ongoing, central, non-gonadal inhibition exerted via other neuronal transmitter systems, such as GABA (Herbison, 2006) or dopamine (Dufour et al., 2005). In addition, stimulatory inputs (glutamate, Kisspeptin, NPY) may not yet be in place, perhaps because necessary cytostructural rearrangements via glial cells remain incomplete. In other words, the infrastructure for connecting and/or disconnecting GnRH neurons (Ojeda et al., 2003a) may not yet be established.

In contrast to the females, we observed endogenous gonadal feedback in pubertal male striped bass, which, for the first time, was steroidal in nature (Chapter 3; Figure 31). The primary target is FSHβ biosynthesis, which was negatively influenced via estrogenic and/or androgenic pathways and, based on our *in vitro* results (Chapter 5), it likely occurred indirectly involving other neurotransmitter compounds. One of the main roles of FSH in male fish is regulation of spermatogenesis (reviewed by Weltzien et al., 2004), however our results suggest that this early stage of gametogenesis is FSH-independent.

Nevertheless, transcription levels do not necessarily mirror circulating hormone levels and this must be taken into account when considering the physiological relevance of gene expression levels (Schulz et al., 2001). Thus for a more complete understanding of the physiological role played by FSH, development of a quantitative assay for FSH protein in striped bass is necessary.

More prominent results were observed after steroid replacement in the pubertal male (Chapter 3; Figure 32). Inhibition of FSHβ transcription by E_2 and 11-KA and a positive influence exerted on GnRH-R expression by E_2 alone were seen in males, as was also shown in females. In addition, 11-KA also decreased LH protein levels in the pituitary, either by inducing release of LH or increasing LH degradation in gonadotroph cells. Some of these effects may be regulated via the release of GnRH in the pituitary, however, missing data on GnRH peptide levels in the pituitary prevent a full test of this hypothesis. Nonetheless, the remarkable results for increased GnRH gene expression observed in the brain of pubertal males lend strong support to this ongoing assumption. While estrogen had no effects on GnRH gene expression, 11-KA increased transcription of all three variants, demonstrating a steroid responsiveness of the GnRH neurons in the brain at this stage of reproductive development (Figure 32).

THE ADULT HP AXIS

In our model, the HP axis in recrudescent, adult female striped bass is regulated via gonadal feedback (Chapter 4), which is also steroidal in nature, as in pubertal males (Figure 31). At this stage of early oogenesis, the ovary inhibits FSHβ synthesis via an estrogenic or androgenic pathway. This gonadal regulation does not occur via direct

modulation at the level of the gonadotrophs, which has been shown *in vitro* in pituitary cells obtained from females of the same reproductive stage (Chapter 5). Rather it involves indirect control of FSH expression exerted via other neurotransmitter systems. The inhibition of FSHβ transcription indicates that gonadal development at this stage may decrease the circulating levels of GtH, however, as mentioned above, transcription levels may not follow hormone release patterns, and therefore this hypothesis remains unsupported. Recent findings by Wong and Zohar (2004) have shown that all three GtH subunits are expressed in primary and secondary oocytes in the gilthead seabream (*Sparus aurata*), also a perciform fish. Furthermore, GnRH expression has likewise been reported in female gonads (Habibi et al., 1994b; Nabissi et al., 1997; Pati and Habibi, 1998), providing evidence that a fully functional 'HP axis' exists in the ovary itself. This system may be controlling the earliest stages of gametogenesis, making regulation via the HP axis unnecessary. We also observed a positive estrogenic feedback mechanism up-regulating GnRH-R mRNA levels during this stage (Chapter 4, Figure 31). This effect is directly exerted at the level of the gonadotrophs as revealed by our *in vitro* results (Chapter 5). An increase in GnRH-R transcription suggests an increased sensitivity of the pituitary for hypothalamic GnRH signaling at this stage and indicates that the GnRH-R plays a very important role in gonadal steroid feedback regulation of the HP axis. However, whether the enhanced sensitivity for GnRH signaling is utilized remains to be validated. Our studies showed that no regulation of GnRH biosynthesis occurred and coupled with missing GnRH peptide data, this question remains unanswered.

The changes between endogenous gonadal feedback observed in recrudescent females and pubertal females of the previous year are quite remarkable (Figure 31). The

missing feedback in pubertal females (Chapter 3) may be an indication that the recruited oocytes at this stage were still too small to produce the necessary steroids or other factors for a 'mature' pattern of gonadal feedback. Holland et al. (2000) showed that only 70% of 3-year-old females had oocytes that reached the critical size for recruitment into the subsequent developing pool. However, most of these oocytes will not reach the critical size needed for initiating maturation at the end of vitellogenesis and fail to spawn that year (so called 'dummy-run'). It is therefore possible that our experimental population of pubertal females belonged to this group and therefore displayed no feedback, reflecting a non-functional endocrine reproductive axis.

Steroids exert more effects on the HP axis in the recrudescent females than in females a year younger (Figure 32). While estrogen directly increases GnRH-R expression in the pituitary, its suppressive effect on FSHβ mRNA levels occurs via indirect regulation of this GtH (Chapter 5). The newly observed stimulation of LHβ transcription by E_2 also occurs indirectly at this stage (Chapter 5) and may involve GnRH release initiated by this steroid. However, biosynthesis of GnRH in the brain is not affected by any steroid treatment, indicating that GnRH neurons are not sensitive to steroids in terms of transcription regulation. 11-KA suppresses FSHβ gene expression and also decreases GnRH-R mRNA levels (Chapter 4), providing evidence that hormone regulation by estrogens or androgens can differ greatly in their outcome. LH pituitary levels decrease after 11-KA treatment (Chapter 4), indicating that either LH release or degradation mechanisms are very sensitive to androgen regulation. However, in analyzing the physiological relevance of these results we need to be aware that 11-KA is not found in female striped bass, where T is the usually dominant androgen. Nonetheless,

11-KA is non-aromatizable and was therefore chosen in these experiments, since it can only act through the androgen receptor, enabling a more direct examination of the actions occurring via this pathway. Whether the documented results occur directly or indirectly remains unresolved, since we did not include androgens in our *in vitro* study.

The second investigated stage during the adult reproductive cycle was midvitellogenesis. In this phase the females are committed to undergo a full and functional cycle and are, at the end of January/beginning of February, two to three months away from ovulation and spawning. In these females, gonadal feedback regulation has changed compared to the recrudescent animals (Figure 31). GnRH-R gene expression is no longer under gonadal influence, and the previously observed suppression of FSHβ transcription has switched to a stimulatory feedback that is non-steroidal in nature (Chapter 4). The increased synthesis of FSHβ during this stage supports the hypothesis that FSH plays a role in regulating the vitellogenesis phase of oocyte development. In other fish models, especially salmonids, FSH has been proposed to be involved by increasing E_2 production in granulosa cells, which in turn increases vitellogenin production in hepatic cells and enhances subsequent Vg uptake by the oocytes. Therefore, a positive feedback regulation of the HP axis by the gonads at this point would support this scenario. The gonadal factor exerting this non-steroid feedback is likely to be activin, which is produced by fish gonads and has been shown to stimulate FSH expression and release from the pituitary (Yam et al., 1999a; Ge, 2000). Moreover, activin also releases GnRH from the terminal nerve endings of GnRH neurons (Gregory and Kaiser, 2004), supporting the hypothesis that this increase in FSHβ expression is regulated via the indirect release of GnRH in the pituitary. However, the elevation of

GnRH-R synthesis in response to gonadal feedback (observed in the previous stage) is not occurring at this stage, indicating that increased sensitivity to the hypothalamic message is not necessary at this point. Furthermore, no feedback is exerted on the GnRH neuronal network (Figure 31), providing further evidence for this theory.

Steroid replacement during this stage induces very complex changes along the HP axis, implying that the sensitivity of the axis to steroids has increased throughout oogenesis (Figure 32). Testosterone inhibits FSHβ transcription, while stimulating LHβ and GnRH-R expression in the pituitary. Regulation of the GtH subunits is likely to occur indirectly involving other neurotransmitters, while the increase in GnRH-R synthesis may be achieved directly via T aromatization to E_2, based on our *in vitro* results (Chapter 5). It appears that T administration has 'accelerated' the axis and the observed response is more likely to occur during later stages of oogenesis. The increase in receptor expression elevates the sensitivity of the pituitary to GnRH regulation. Furthermore, the increase in LHβ synthesis may be in preparation for the LH surge, essential for inducing oocyte maturation and spawning. FSHβ transcription is suppressed by the increased steroid levels and may be unnecessary at this time point. This scenario is supported by the decreasing levels of GnRH I peptide in the pituitary (Chapter 4), which may suggest a release of GnRH. This released GnRH in turn may be responsible for the observed up-regulation of GnRH-R and LHβ expression levels. More evidence is provided by the response of all three GnRH neuron populations to T replacement in the brain (Figure 32), where GnRH I synthesis is stimulated while GnRH II and III mRNA levels are suppressed (Chapter 4). The mechanism regulating the increase in GnRH I synthesis seems to be mediated via an estrogenic pathway, based on the stimulatory results

observed after estrogen treatment of brain-slice cultures obtained from females undergoing midvitellogenesis (Chapter 5). However, we are not able to determine if E_2 directly affects GnRH neurons through estrogen receptors, whether the regulation occurs via an intermediary neurotransmitter system, or if glial cells are involved. GnRH II expression is inhibited by T *in vivo* (Chapter 4) and an increase in mRNA levels is observed after a low dose treatment with E_2 *in vitro* (Chapter 5). There are two possible explanations for this result: 1) the estrogen levels reached after the conversion of T to E_2 were too high and had inhibitory effects, like the high dose of estrogen *in vitro* (Chapter 5), or 2) the regulation involved AR pathways, which may result in a decrease in transcription.

GENERAL CONCLUSIONS

The comparison of the different developmental stages (juvenile, pubertal, adult) in the female yielded extremely interesting observations, unique in the field, related to the effects of steroids on the GnRH neuronal network during midvitellogenesis. These observations support the theory that the GnRH system in the brain is unresponsive to steroid regulation in the female until the adult reproductive stage. Our model reflects the absence of steroid regulation up to this point. This is the first observation of this kind reported in teleost fish. One explanation for the change in activity may be the rising circulating steroid levels, which may exert a priming effect on the GnRH neuronal network. Steroid levels of juvenile animals are near or below the detection limit and are still low in pubertal animals (Chapter 3), while the basal levels have increased in recrudescent females and slowly rise during vitellogenesis. Therefore, the increased

exposure to rising levels of circulating estrogen throughout oogenesis may result in plastic neuronal rearrangements, such as in glial cells, and changes in inhibitory and/or stimulatory input to the GnRH neurons that are necessary for a response to gonadal steroid feedback regulation. These interactions appear to require temporal coordination to ensure that the system is ready to initiate the LH surge at the time when oocytes need to mature. Therefore, our observations in midvitellogenic females after T treatment present the HPG axis in preparation for the final stages of oogenesis.

Also of great interest is that gonadal feedback in juveniles targets only the pituitary in a seasonal pattern to stimulate GtH subunit expression in the first year and subsequently only FHSβ in the second year. The non-steroidal nature of the feedback is not surprising, considering the non-detectable steroid plasma levels. However, steroids do evoke responses in the HP axis in this early age (Figure 32), which has not been demonstrated previously. That the key target is FSH strongly suggests an involvement of this gonadotropin in regulation of gonadal development in the juvenile phase of striped bass development.

In the pubertal male, we observed gonadal steroid feedback for the first time, as well as steroid regulation of the GnRH neuronal network. These results indicate an increased responsiveness of the HP axis towards steroid regulation and also confirm previous results that the male endocrine gonadal axis matures earlier than in females of the same age (Hassin et al., 2000; Holland et al., 2000). Based on these distinctive findings, it appears that the reproductive performance of female striped bass, i.e., the complexity of gonadal feedback and steroid effects, increases with age and that prior

cycles of oocyte development may prime the HP axis to respond faster and more vigorously in subsequent years.

In summary, our model reflects several new and interesting pieces of information on gonadal feedback regulation of the HP axis. Major findings incorporated in this model are: 1) Gonadal feedback patterns change during the transformation from the juvenile to the adult and the throughout the adult reproductive cycle, but the primary target of feedback appears to be the pituitary. 2) The responsiveness of the HP axis towards gonadal sex steroids seems to develop during puberty, which leads to the observed increase in responses to steroids in adult females compared to the juvenile animals. 3) Gonadal steroids appear not to be responsible for the onset of puberty in striped bass, which supports the previously stated hypothesis by Holland et al. (2001). 4) The different feedback patterns observed in recrudescent and midvitellogenic females are due to the reproductive needs of hormonal regulation. FSH appears to be more involved in vitellogenesis than LH, however, it may not be needed during recrudescence, which seems to be independent of pituitary GtH. The missing responsiveness of the GnRH system during early oogenesis, together with the very distinct response in the subsequent stage, may be due to priming effects of rising steroid levels and reflect the entry of the ovaries into advanced and final reproductive stages. The addition of data on other stages will broaden our understanding of the HP axis regulation throughout the entire life cycle and may lead to novel therapies and mechanisms by which the axis can be manipulated. Nevertheless, this study has provided an improved resolution and a broader perspective on the mechanisms involved in gonadal steroid feedback regulation of GnRH neural activity and its targets at the level of the pituitary.

FUTURE DIRECTIONS

This study has provided, for the first time, insight into gonadal steroid feedback at many different stages throughout the juvenile and pubertal development of striped bass, as well as the adult reproductive cycle. However, some developmentally significant stages are still in need of investigation. The additional information on these stages would expand our model and provide a more comprehensive picture of the 'evolution' of gonadal feedback regulation during the entire striped bass life cycle. For example, it would be of interest to determine the response of the HP axis in pubertal females during spring, especially in view of comparing those females going through the 'dummy-run' with their immature siblings (Chapter 3). Another very important stage not investigated during this study is the maturational phase of oogenesis, during which the proposed LH surge induces maturation and spawning. The herein proposed hypothesis that gonadal steroid feedback may regulate the entire HP axis, including GnRH synthesis in the brain at this important endocrine stage, could be evaluated via this experiment (Chapter 4). Information about both these stages will facilitate our understanding of the reproductive cycle and pubertal development in female striped bass and the involvement of steroids in these processes. Based on this knowledge, new techniques to manipulate reproduction and especially the onset of puberty in commercially valuable fish may be designed for use in the aquaculture industry.

Our development of an innovative brain-slice culture technique and its validation in this study (Chapter 5) may answer many questions about the regulation of the GnRH system in the teleost fish brain and as an experimental tool is easily applied to other fish species. Our model would also be enhanced by determining the effects exerted by

androgens on GnRH gene expression of the three variants, to clarify the differences between estrogenic and androgenic feedback. Furthermore, it would be of great significance to identify the transsynaptic factors involved in relaying steroid regulation to the GnRH neuronal network, by treating the cultures with estrogen and various neurotransmitter antagonist/inhibitors and observing the response in GnRH gene expression. Our in vitro system provides a powerful tool for testing the multiple factors involved in GnRH regulation. In addition, the hypothesis that GnRH III may regulate GnRH I in response to external and environmental stimuli would be testable by including only the POA area of the brain in the slice culture and treating them with exogenous GnRH III. Finally, regulation of GnRH III by factors such as pheromones could be tested with this new tool. These possible paths of investigation would elucidate many signaling mechanisms and the regulatory responses involved and help in the effort to understand the plasticity of the HP axis and its control of vertebrate reproduction.

Figure 31: Description see next page.

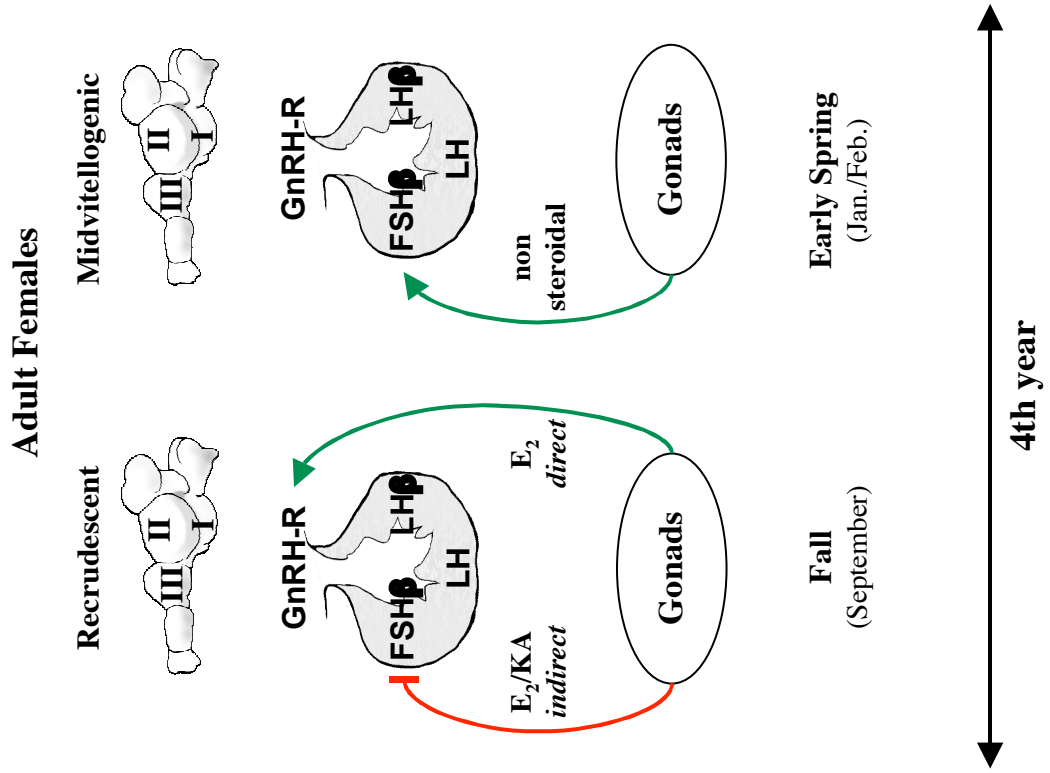

Figure 31: (cont'd) Schematic representation of a conceptual model of the gonadal feedback regulation of the endocrine correlates of the HP axis at different stages of striped bass development: juvenile, pubertal, and adulthood. For explanation see text. The numerals I, II, and III in the brain designate the respective GnRH gene transcript. Green arrows indicate positive feedback, while red lines denote negative feedback, no feedback effects are not represented in this graphic.

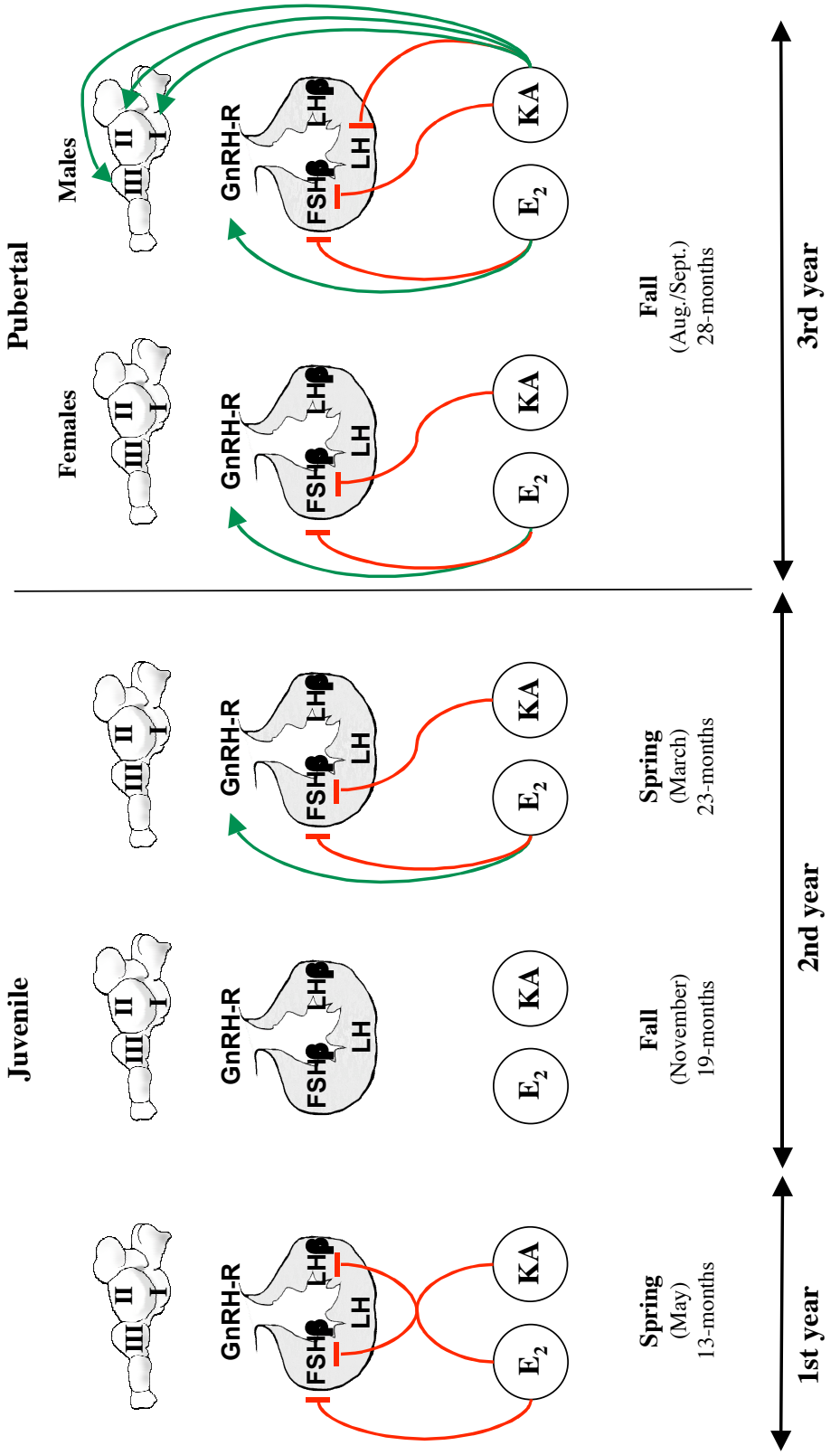

Figure 32: Description see next page.

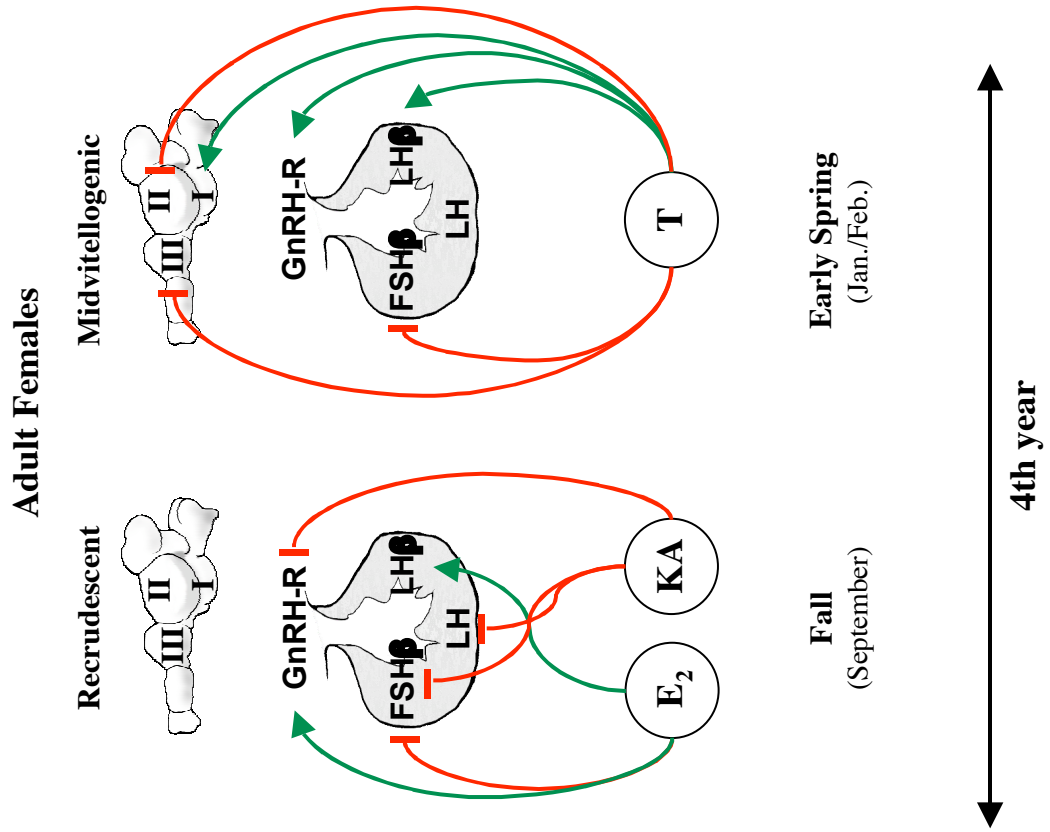

Figure 32: (cont'd) Schematic representation of a conceptual model of steroid replacement effects on the endocrine correlates of the HP axis at different stages of striped bass development: juvenile, pubertal, and adulthood. For explanation see text. The numerals I, II, and III in the brain designate the respective GnRH gene transcript. Green arrows indicate positive feedback, while red lines denote negative feedback, no feedback effects are not represented in this graphic.

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2002–2004:	Ph. D. student, Graduate research assistant, Center of Marine Biotechnology, University of Maryland
1999–2002:	Research scholar, Center of Marine Biotechnology, University of Maryland in affiliation with a Ph.D. program Carl von Ossietzky University, Oldenburg
1996–1998:	Research assistant at the Alfred-Wegener-Institut for polar and marine research, Bremerhaven, Germany
1996, Feb–June:	Internship at the Terramare Research Center, Wilhelmshaven, Germany

SYPOSIUM ORAL PRESENTATIONS

Sep., 2005:	Annual MEES Colloquium, Baltimore, USA
May, 2005:	7th International Marine Biotechnology Conference, St. Johns, Canada
May, 2003:	7th International Symposium on Reproductive Physiology of Fish, Mie, Japan
July, 2002:	Society for the Study of Reproduction 35th Annual Meeting, Baltimore, USA
Jan., 1998:	2nd Annual TASC Meeting Bremerhaven, Germany

SYPOSIUM POSTER PRESENTATIONS

June, 2005:	15th International Congress of Comparative Endocrinology, Boston, USA
Sep., 2004:	5th International Symposium on Fish Endocrinology, Castillion, Spain
Sep., 2003:	6th International Marine Biotechnology Conference, Chiba, Japan
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AWARDS

Sep., 2003: Travel Grant received from University of Maryland, College Park to participate in the 6th International Marine Biotechnology Conference, Chiba, Japan

2000-2001: Center of Marine Biotechnology Graduate Fellowship

PUBLICATIONS

1. **U. Klenke** (1998). The reproduction biology of *Calanus finmarchicus* during a time series in the Norwegia Sea in relation to biotic and abiotic factors; master thesis, pp 1 - 99. *Thesis for receiving the Diploma (M.Sc.) of the Department of Biology, Carl v. Ossietzky University Oldenburg.*

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6. U. Klenke and Y. Zohar (2003). Gonadal regulation of gonadotropin subunit expression and pituitary LH protein content in femal hybrid striped bass. Fish. Physiol. Biochem. 28(1-4): 25-27.

7. G.L. Taranger. E. Vikingstad. U. Klenke. I. Mayer. S.O. Stefansson. B. Norberg. T. Hansen. Y. Zohar. E. Andersson (2003). Effects of photoperiod, temperature and GnRHa treatment on the reproductive physiology of Atlantic salmon (*Salmo salar* L.) broodstock. Fish. Physiol. Biochem. 28(1-4): 403-406.

MANUSCRIPTS IN PREPARATION

1. **U. Klenke** & Y. Zohar (2006). Steroid feedback regulation of the hypothalamus-pituitary axis of female hybrid striped bass: a time series study.

2. **U. Klenke** & Y. Zohar (2006). Steroid feedback regulation of the hypothalamus-pituitary axis of juvenile and pubertal striped bass (*Morone saxatilis*).

3. **U. Klenke** & Y. Zohar (2006). Steroid feedback regulation of the hypothalamus-pituitary axis of recrudenscent and midvitellogenic female striped bass (*Morone saxatilis*.

4. **U. Klenke** & Y. Zohar (2006). Estrogen regulation of the hypothalamus-pituitary axis *in vitro*: A brain-slice study.

5. **U. Klenke**, T.T. Wong & Y. Zohar (2006). Steroid feedback regulation of the GnRH/GtH system in male gilthead seabream (*Sparus aurata*) during early gametogenesis.

6. **U. Klenke**, T.T. Wong & Y. Zohar (2006). Steroid feedback regulation of the GnRH/GtH system in male gilthead seabream (*Sparus aurata*) during the spawning season.