ABSTRACT

Title of Thesis:	STUDIES ON REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY IN A PRIMITIVE TELEOSTEI, THE AMERICAN SHAD (*Alosa sapidissima*)
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American shad is an anadromous fish that displays asynchronous ovarian development. In an effort to enhance our understanding of the reproductive cycle of this primitive Teleostei species, and provide better management tools for the Chesapeake fishery, we have conducted studies of its gonadal and hormonal cycles. We developed various assays to measure reproductive endocrine factors including: 17,20β-dihydroxy-4-pregnen-3-one (DHP) and GnRH. Fish were collected from the Susquehanna River during their spawning migration. One group of fish was sacrificed on site to assess reproductive parameters of wild shad. A second group was transported to a Maryland State hatchery and treated with gonadotropin-releasing hormone agonist (GnRHa) using several delivery systems. These treatments were followed during a two-week period by measurement of various hormonal levels. In addition, fecundity and fertilization of the hatchery groups were measured daily. Our results shed light on the reproductive physiology and endocrinology of the American shad and lay the foundation for usage of GnRHa to induce shad spawning in captivity.

STUDIES ON REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY IN A PRIMITIVE TELEOSTEI, THE AMERICAN SHAD (*Alosa sapidissima*)

By

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

20β-S	17,20β,21-trihydroxy-4-pregnen-3-one
Ab	Antibody
ANOVA	Analysis of variance
AT	Atresia
BSA	Bovine serum albumin
C	Celsius
cDNA	Complementary deoxyribonucleic acid
cfGnRH	Catfish gonadotropin releasing hormone
cGnRH II	Chicken gonadotropin releasing hormone (Type II)
CPM	Counts per minute
DHP	17,20β-dihydroxy-4-pregnen-3-one
DNR	Department of Natural Resources
E_2	17β-estradiol
ELISA	Enzyme-linked immunosorbent assay
EVAc	Ethyl vinyl acetate copolymer
FOM	Final oocyte maturation
FSH	Follicle stimulating hormone
GAP	Gonadotropin releasing hormone associated peptide
GH	Growth hormone
gpGnRH	Guinea pig gonadotropin releasing hormone
GnRH	Gonadotropin releasing hormone
GnRHa	Gonadotropin releasing hormone analogue
GSI	Gonadosomatic index
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
GVM	Germinal vesicle migration
HPG axis	Hypothalamo-pituitary-gonado axis
HPLC	High-pressure liquid chromatography
hrGnRH	Herring gonadotropin releasing hormone
LH	Luteinizing hormone
mGnRH	Mammalian gonadotropin releasing hormone
MIS	Maturation inducing steroid
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
PG	Primary growth
PI	Post-injection
Pit	Pituitary
Pre-Vg	Pre-vitellogenesis
RACE	Rapid Amplification of cDNA Ends
rGnRH	Rana gonadotropin releasing hormone
RIA	Radioimmunoassay
RT	Room temperature

Shad	American shad (*Alosa sapidissima*)
sGnRH	Salmon gonadotropin releasing hormone
T	Testosterone
Vg I	Vitellogenesis I
Vg II	Vitellogenesis II
VTG	Vitellogenin
wfGnRH	Whitefish gonadotropin releasing hormone

Chapter I General introduction

The purpose of this study was to examine several aspects of American shad (Wilson, 1811) reproduction. The American shad is an important species in the Chesapeake estuary, but its population has declined severely over the past century. The overall goal of this work was to gather basic information regarding shad reproductive physiology and endocrinology and to study the use of hormonal manipulation of spawning in captive shad, with the aim of establishing a successful hatchery-based seed production and stock enhancement program.

I.A. American shad

I.A.1 Life history

The American shad (*Alosa sapidissima)* is an anadromous fish of the Clupeidae family as well as the largest species of the Herring family. Native to the east coast of the United States, the main estuaries in which the shad reproduces are the Hudson and the Chesapeake. In 1871, shad were introduced to the Pacific and are believed to have a current range from Baja California in the winter to Alaska in the summer. The life history of the Atlantic American shad is not completely or adequately known, it has been established that adults spend most of the year along the Atlantic seaboard from New Brunswick, Canada to northern Florida. Shad schools migrate based on water temperature. During the summer months they are found off the shore of Quebec and during the winter they migrate to the Florida seaboard (Leggett et al., 1972).

In early spring, the shad assemble at staging grounds at the mouth of estuaries in a temporal pattern related to their geographic location (earlier to the south). The cue for the staging event seems to be temperature and photoperiod mediated. Thus, shad that enter rivers in the south to spawn do so earlier. Shad to the north enter the estuaries progressively later, based on latitude. Leggett et al., (1972) reported that 90% of shad participating in the spawning migration along the east coast do so when the river temperature is between 16.0 and 19.5° C.

The shad maintain a genetically distinct staging assembly based on area of origin (Nolan et al., 1991). This genetic distinction is maintained due to the fact that shad return to their natal river to spawn (Hill et al., 1957), thus river populations are reproductively discreet.

American shad reach sexual maturity three to five years post-hatch, spawning migration, also known as "spawning run", occurs in the Chesapeake Bay during the months of May-July. Despite this prolonged spawning migration, the peak of spawning activity lasts only approximately three weeks. The timing of this peak can vary depending on water temperatures. Spawning occurs in fresh water mainly in the upper sections of tributaries. Some shad die at the spawning grounds and others return to the Atlantic to spawn again during the next season. It seems that there is a tendency of "southern" Shad to be semelparous (produce all offspring during one spawning season), whereas to the north they become increasingly iteroparous (produce offspring repetitively over several spawning seasons) (Benzen et al., 1989). This difference is most probably due to the higher energy levels that are expended by the "southern" shad during the return from the spawning migration, which typically occurs at higher water temperatures. In turn, it

appears, based on limited fecundity data, that the fecundity per spawn is higher in "southern" shad and lower in "northern" shad. Thus the average lifetime fecundity is constant throughout the Atlantic coast shad populations.

The spawning pattern is presumed to be one of batch spawning, however verification of this (beyond speculation based on gonadal morphology or other indicators) requires observation of repeated spawning events in the same female. Therefore the exact frequency of spawning is not clear, but it has been hypothesized that a batch of eggs is spawned every two to four days (Mylonas et al., 1995b; Olney et al., 2001). Following hatching, young shad remain in the rivers until they reach a size of 7-15 cm, the typical size at which juveniles enter the ocean. The young shad remain in the ocean until their first spawning migration at the age of three to five years.

I.A.2 Ecology

Historically, American shad was a very important commercial species in the eastern United States. In 1907, Meehan wrote, "There is little that the people living along the line of the Delaware Valley are more interested in or guard more jealously than the shad industry in the Delaware River". Shad landings during the spring months were in the thousand of millions of tons and a thriving fishery existed in many rivers.

However, commercial landings of American shad in America's eastern rivers and estuaries have dramatically decreased over the last 100 years (Hattala, 1997; Atlantic States Marine Fisheries Commission, 1998), a result of pollution, overfishing, construction of steam-electric power plants and hydroelectric dams (that obstruct or disrupt the fish's migration pattern), and other stresses (Limburg, 2001).

For example, in 1896 the total Chesapeake landings of shad were 16,712,000 lb, in 1941 2,659,500 lb (Joint State Government Commission, 1949) and 500,000 lb in 1992 (http://www.fws.gov/r5cbfo/SHAD.HTM). In less than 100 years, the shad fishery and industry have all but disappeared in the Chesapeake.

In spite of restocking programs and a moratorium in Maryland since 1980 and in Virginia since 1992, shad fisheries in several Atlantic coast states (e.g., Maryland, Virginia, Carolinas) are facing the grave problem of declining populations. If further steps are not taken, a final collapse of any remaining American shad runs is eminent in multiple areas (http://www.dnr.cornell.edu/hydro2/fishpart.htm).

I.A.3 Captivity-induced spawning

Due to the steep decline in shad populations, the Maryland Department of Natural Resources (DNR) initiated a program to restock parts of the Chesapeake with shad through a hatchery-based rearing program. This program is based on catching broodstock shad during their spawning migration and then spawning them in a hatchery. Larvae are grown in the hatchery for 6-12 days and then transported and released into select riverine locations within the Chesapeake estuary. Some larvae are stocked into ponds and raised for 30 days before being released as juveniles, although the majority of shad stocked are at the larval stage. From the onset of the program, however, it was clear that captive spawning of broodstock would be a bottleneck. As is the case with many other fish species, American shad do not spawn spontaniously in a hatchery. The solution was to

induce the shad to spawn at the hatchery using GnRHa. This program was initiated in 1994 and the results were initially promising (Mylonas et al., 1995b). However, Maryland Department of Natural Resources (DNR) hatchery personnel have been reporting a decline during the last several years, both in viable eggs per female and in fertilization (Federal Aid Report #NA66FA0208, 1997). In view of the reduced level of success during recent GnRHa-induced spawnings, in spring 2002 the hatchery staff shifted from a GnRHa implant-based induction method to strip spawning of ovulated females at the collection sites.

The strip spawning technique has several major disadvantages, including being labor intensive and damaging or lethal to the migrating (and physiologically-stressed) animal. Maybe the most important aspect is that by strip spawning, we are only obtaining one of multiple spawning batches that could be utilized in a hatchery setting.

In spite of these problems, approximately 12 million American shad larvae and juveniles were stocked into the Chesapeake Bay from 1994 to 2001, of which 8.67% were juveniles and the rest were at the larval stage (Minkkinen, 2001). These shad were stocked into several rivers including the Patuxent and Choptank. All stocked shad were marked prior to release using oxytetracycline. The initial sampling results of mature shad reveal that a very large percentage of juvenile and mature shad are of hatchery origin and only a minority are wild. In 2000, for instance, 91% of all mature shad sampled during the spawning migration in the Patuxent River were of hatchery origin, similar results have been obtained in years since then. This data indicates a problematic situation of a very small wild shad population, but on the other hand also emphasizes the success and impact of the hatchery-based rearing program.

I.B. American shad reproduction

I.B.1 Reproductive strategy

The American shad is an anadromous fish, yet unlike most anadromous fish such as salmon, striped bass, and trout, which spawn only once during each reproductive cycle (synchronous and group synchronous spawning), the shad is a batch spawner (multiple spawning events during each reproductive cycle). This means that the shad spawn several times during the spawning migration. However, the number of batches and the interval between spawning episodes have not been firmly established. The total annual fecundity is also unknown, the number of eggs spawned per batch is estimated to be between 25,000 and 80,000 (Olney et al., 2001) and the interval between spawns is approximated to be two to four days.

As with all batch spawning fish, the shad ovarian morphology is asynchronous. This morphology is one in which the ovary contains, at any given time during the spawning migration, oocytes at different stages of development. This type of morphology allows a sequence of events in which the most progressed oocytes that are scattered throughout the ovary are in effect the next cohort of oocytes to be ovulated and spawned. Subsequently the next cohort of oocytes progresses to maturation and is spawned.

The "batch" strategy of reproduction has several advantages, the most apparent being that each spawn is released at a different location thus increasing chances of survival and each batch is presumably fertilized by a different male thus increasing genetic diversity.

I.B.2 Ovary morphology

As in other anadromous species, the development of the shad ovary in preparation of the spawning season is triggered by environmental cues. These cues are believed to be photoperiod and temperature. As the days become longer and the water temperature rises, the shad begin migrating towards the spawning grounds and the gonads begin maturing. The result is rapid development and enlargement of the ovary. A certain percentage of the oogonia in the ovary begin developing faster, the production of vitellogenin in the liver is stimulated by E_2 and transferred to the developing oocytes. When the shad start their migration into the estuary, and throughout the spawning period, the ovaries contain oocytes at various stages of development. The final maturation of oocytes, their ovulation and spawning itself takes place in a repeated sequence. During the spawning migration, the ovaries continue to develop and can reach a gonad somatic index (GSI) of approximately 34% (Olney et al., 2001).

Ovarian development can be assessed by several parameters; one is GSI, which is based on the percentage of body weight that the ovaries comprise. This index is commonly used to assess ovarian development in many fish species. Another parameter used is a simple macroscopic assessment of the ovary morphology, including color, size, blood vessels present, and whether or not the ovary contains hydrated oocytes and/or ovulated oocytes (Olney et al., 2001). The third method is using a microscopic analysis of histologically-prepared oocytes from the ovary, which are sectioned and stained. Mylonas (1995b) staged oocyte development in the shad based on the morphology of the oocytes. In this study oocytes were divided into five developmental stages. The five stages are: primary growth (PG), pre-vitellogenesis (pre-Vg), vitellogenesis I (Vg-I), which is the

early stage of vitellogenesis, vitellogenesis II (Vg-II), which is the late stage of vitellogenesis, and atresia (AT).

I.B.3 Hypothalamo-Pituitary-Gonad axis

The HPG axis is the main endocrine-reproductive axis in vertebrates. In all vertebrates, this axis controls the development and maturation of the gametes and their release. The HPG axis cascade is triggered by environmental cues. These signals are transduced into a hormonal signal, which in turn causes the release of the hypophysiotropic form of GnRH from the hypothalamus to the pituitary. A more detailed discussion of GnRH and other reproductive hormones is provided in section I.C. In mammals, the GnRH is delivered to the pituitary via a hypophyseal blood portal system. In fish this system does not exist, the GnRH neurons directly innervate the pituitary. The binding of GnRH to GnRH receptors in the pituitary elicits the release of gonadotropins from the pituitary to the blood, specifically, luteinizing hormone (LH) and follicle stimulating hormone (FSH). These two hormones travel through the blood stream to the gonads where they trigger steroidogenesis, gametogenesis and other processes.

Various gonadal hormones and factors such as E_2, T activin and inhibin feedback to the pituitary and the hypothalamus thus up-regulating or down-regulating further transcription, translation and release of GnRH and gonadotropins (Nagahama, 1994).

When this web functions properly, and given sufficient environmental and behavioral cues, this cascade culminates in oocyte ovulation and spawning in the female and in sperm maturation and release in the males.

I.C. Hormones influencing reproduction

I.C.1 Gonadotropin releasing hormones

GnRH is a decapeptide that plays a central role in reproduction. Its central reproductive function is to induce the production and release of gonadotropins from the pituitary (Yaron et al., 2003). However many species have multiple forms of GnRH that may or may not participate in regulation of reproduction.

All vertebrate species studied to date have multiple forms of GnRH present in the brain. However, these forms have distinct locations within the central nervous system. To date, 14 distinct forms of GnRH have been identified in vertebrates, most of whom have at least one form of GnRH in common, the cGnRH-II. However, this form is located mainly in the midbrain region and is therefore thought to be a non-hypophysiotropic form (Adams et al., 2002; Gothilf et al., 1996). Based on research done in several species of birds and mammals, it is hypothesized that this form is connected to reproductive behavior (Temple et al,. 2003; Millar and Rissman., 2003), and probably not gonadal development.

In teleosts, either two or three forms of GnRH are present in the brain. The common form in all teleosts is cGnRH-II. As noted above, this form has been localized mainly to the mid and hindbrain. In teleosts that have two forms of GnRH, the second form is the form that is most abundant in the hypothalamus and pituitary and is therefore considered to be the hypophysiotropic form. In most ancient teleosts, this form is the mGnRH (eel, sturgeon). In teleosts that evolved later, the prevalent second form is sGnRH (zebrafish, salmon, medaka) (Steven et al., 2003; Powell et al., 1996; Amano et al., 2002).

In teleosts, which have three forms of GnRH, the hypophysiotropic form is the "species-specific" form, i.e., not sGnRH or cGnRH-II, but a third unique GnRH form such as sbGnRH, wfGnRH, and pGnRH (Gothilf et al., 1995; Adams et al., 2002; Lethimonier et al., 2004).

In American shad, the forms of GnRH present in the brain were not known. Based on HPLC analysis, Carolsfeld et al. (2000), described in Pacific herring, which is closely related to the American shad, three forms of GnRH. The three forms that were found were cGnRH-II, sGnRH and hrGnRH, which is a novel form of GnRH. However these GnRHs were not sequenced. Based on the above paper, the hypophysiotropic form of GnRH in the Pacific herring is the species-specific hrGnRH. This was concluded based on the fact that the hrGnRH was found in the pituitary in much higher concentrations than the other two GnRH forms (Carolsfeld et al., 2000).

GnRH is a fairly conserved decapeptide, the different forms vary mainly in amino acid positions five, seven and eight. The structure of the GnRH gene in vertebrates consists of four exons separated by three internal introns. Exon 1 contains a 5' untranslated region, exon 2 encodes the GnRH decapeptide and part of the GAP region, exon 3 encodes an additional segment of the GAP and the last exon encodes the final region of the GAP along with a 3' UTR (Lin et al., 1998; Gothilf et al., 1995).

I.C.2 Gonadotropins

In teleosts, as well and in other vertebrates, gonadotropins are the primary mediators of oocyte and sperm maturation. Teleosts, like higher vertebrates, have two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Sekine

et al., 1989; Elizur et al., 1996; Suzuki et al., 1988). These two hormones are heterodimeric glycoproteins that have a common α-subunit and distinct β-subunits (Rathnam and Saxena., 1971; Yaron et al., 2003).

The gonadotropins are produced in the pituitary and released into the blood stream in response to several cues, the most important of which is the hypophysiotropic GnRH form. Upon reaching the ovaries, both gonadotropins stimulate the production of gonadal steroids and MIS. The "classical" view of gonadotropin regulation of ovary development in teleosts is that FSH is at higher levels during early vitellogenesis, whereas LH is at higher levels during FOM and ovulation (Kagawa et al., 1998; Prat et al., 1996). Thus, it is hypothesized that the primarily FSH activates early oocyte development, including synthesis of vitellogenin and zona-radiata proteins in the liver, and their incorporation in the ovary. LH is more active during FOM, including germinal vesicle migration, breakdown and oocyte ovulation. However, variations upon this general scheme of events can be found in different teleost species.

I.C.3 Gonadal steroids

Gonadal steroids are synthesized and secreted from the gonads; they are the final level of regulation in the HPG axis. In females, there are several steroids that take part in the process of oocyte growth and maturation. Testosterone (T) serves mainly as a precursor for E_2, although in some species it has been shown to effect LH secretion by increasing pituitary responsiveness to GnRH (Yaron et al., 2003). In several fish species, T can be detected in the plasma at various levels throughout oogenesis (Mylonal et al., 1997a; Fitzpatrick et al., 1986). In other species, T levels in the plasma seem to be at low

and constant levels throughout ovary maturation (Gothilf et al., 1997). E_2 plays a central role in oocyte growth, it is produced in the oocyte granulosa cells and released into the blood where it up regulates and binds to E_2 receptors in the liver mediating vitellogenin production (Mosconi et al., 2002). In many teleosts, a brief surge in both T and E_2 occurs during early oocyte FOM. This surge gives way to an elevation of the maturation inducing steroids (MIS), which mediate GV migration and GV breakdown, during late stages of FOM.

There are two primary MIS in teleosts, 17,20β-dihydroxy-4-pregnen-3-one (DHP) and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) (Nagahama, 1997). Both the thecal and the granulosa cell layers of the oocyte follicular layer are required for MIS production. While T and 17α-hydroxyprogesterone are produced in the thecal cells, E_2 and both DHP and 20β-S are biosynthesized in the granulose cells from these two hormones (respectively). The up-regulation and production of the MIS are triggered mainly by LH. Once released, the MIS induces the resumption of meiosis, marked by GV migration and breakdown. The MIS induces this effect by binding to MIS receptors on the oocyte plasma membrane, which in turn causes intracellular activation of maturation-promoting factors such as cdc2, kinase and cyclin B. It is important to note that not both MIS are needed for FOM in all fish species. In most species, one MIS form is dominant and elicits oocyte response (Nagahama, 1997; Nagahama, 1994; Ohta et al., 2002).

The gonadal steroids were also shown to have various levels of regulatory feedback effect on the pituitary and the hypothalamus, although these effects seem to be to a large extent species-specific. Breton and Sambroni (1996) showed that both T and E_2 have a positive feedback effect on sGnRH levels in the brain of rainbow trout, and also

induced changes in LH levels. Other studies have shown that the lack of gonadal steroids (caused by gonadectomy) produces a negative feedback effect, both on LH and FSH levels, in which LH and FSH plasma levels are decreased. However, this effect varies based on gonadal developmental stage (Larsen and Swanson, 1997). E_2 and T have also been directly connected to regulation of gonadotropin levels. In coho salmon, exogenous T and E_2 decreased FSH plasma levels, but had a positive effect on LHβ mRNA levels (Dickey and Swanson, 1998).

I.D Hormonal manipulation of spawning

I.D.1 Hatchery-based spawning

The need for spawning fish in a controlled environment is not new. Since the beginning of aquaculture thousands of years ago in China, capturing fingerlings in rivers or in the sea and transporting them to ponds or reservoirs has been a difficult task. With the large growth of aquaculture during the last decades, it has been imperative to develop a reliable method of obtaining fingerlings, not only by sporadically catching wild spawning females, but rather by continual production from a reliable source. For this reason, many attempts have been made to spawn fish in captivity (Zohar, 1986; Zohar and Mylonas, 2001).

This endeavor has been partially successful, some fish species spawn in captivity without external intervention and other species do not. The exact reasons for this lack of success are not clear, however the underlying reason is lack of natural environmental conditions. The abnormal hatchery condition causes a failure to release GnRH from the hypothalamus, which in turn prevents the release of LH/FSH from the pituitary, thus denying normal gonad development and spawning. Several approaches to circumventing

this problem have been undertaken in the past. The first approach was injection of pituitary extracts, which contain large quantities of the necessary gonadotropins. This approach had some success but also many drawbacks including, among others, the variability in pituitary LH content, the presence of various unwanted hormones in pituitary extracts, and the transfer of disease.

Another approach taken was the usage of human chorionic gonadotropin (hCG), which is purified from the urine of pregnant females. However, when using this method the timing of the injections must be precise. In addition the fish recipient can develop an immune response to the large gonadotropin proteins. The best approach to date is the administration of exogenous GnRH peptide. Initially this method also presented severe problems (see I.D.2), however due to manipulations of the GnRH peptide, it has become a widely used method (Zohar and Mylonas, 2001; Zohar, 1986).

At present, many fish species are spawned in captivity, both in ponds and in tanks. Moreover, by manipulating photoperiod and temperature, year round spawning, or an extended spawning season, has been achieved in many cases. These advances allow a reliable supply of fingerlings both to aquaculture facilities and for the purpose of ecological programs aimed at restocking endangered fish species back to their natural environment (Zohar and Mylonas, 2001).

I.D.2 Gonadotropin-releasing hormone agonist

When the first attempts to use exogenous GnRH to induce spawning were made, a serious obstacle was encountered. The GnRH was found to have a very short half-life due to peptide degradation by enzymes (Goren et al., 1990; Zohar et al., 1990b) and therefore repetitive GnRH injections were necessary. These repetitive injections place an additional

stress on the brood and were not always successful. Native GnRH was found to be most susceptible to enzymatic degradation at bonds between amino acids 5-6 and 9-10 (Goren et al., 1990; Zohar, 1986). To circumvent this problem, a GnRH analog with a longer half-life was developed. The most commonly used agonist is a GnRH analog (GnRHa) in which a glycine at position six is replaced by a dextrorotatory alanine, and a carboxy terminus proline^{9}-glycine^{10}-NH2 with a proline^{9}-N ethylamide (D Ala^{6} Pro^{9} –NET). This modified GnRH has been shown to be superior to other GnRHs in inducing gonadotropin release and due to its modifications has a substantially longer half-life than native GnRHs (Zohar, 1990a; Zohar et al., 1990b; Zohar, 1986). However, despite the substantially longer half-life of the analog, repetitive injections were still necessary to induce successful spawning.

In the early 1980's, great strides were made in developing methods of sustained drug delivery. These methods entail subcutaneous or intramuscular injections of the drug, which is combined with a polymer (Rhine et al., 1980). Thus, the drug is released slowly over time into the blood stream. This method is utilized for the purpose of sustained delivery of GnRH to the blood stream of captive fish. The GnRH implant sustains a prolonged release of GnRH into the blood, and this GnRH acts to stimulate gonadotropin release from the pituitary. This, in turn, results in normal FOM and spawning in females and elevated spermiation in males (Mylonas et al., 1995a; Mylonas et al., 1995b; Hassin et al., 1998).

By using GnRH analog together with a polymer-based release system, multiple fish species have been successfully induced to spawn in captivity (Mylonas et al., 1998; Mylonas et al., 1997a; Vermeirssen et al., 1998; Zohar, 1986; Zohar and Mylonas, 2001).

Chapter II Assay development

II.A. Introduction

One of our objectives was to obtain basic shad endocrine reproductive data during the spawning season and in response to GnRHa treatment. As no hormonal assays have been developed for shad, our first step was to develop such assays.

In view of obtaining a preliminary understanding of the workings of the shad HPG axis, we decided to focus on developing assays for three central players of this axis: GnRHs, LH, and MIS, as these hormones are crucial during the final stages of gametogenesis. Rather than developing these assays de novo, we attempted to validate existing assays for use with shad hormones. To assist us in developing these assays, sequencing of the shad GnRH forms and the shad LHβ was necessary.

Establishing which forms of GnRH the shad possesses was a crucial step, both in establishing a GnRH assay and in attempting to clarify the temporal relationship between GnRH levels and gonad development. As specified previously, the forms of GnRH in shad were assumed to be identical to the three forms of GnRH previously isolated from Pacific herring by Carolsfeld et al., (2000). These three forms were sGnRH, cGnRH II and a novel form, hrGnRH. These three forms were detected using HPLC methods, therefore only the amino acid sequence of these peptides was known. Sequencing of these forms was necessary to verify their presence in shad and is important both to facilitate future brain localization studies, and to better understand the evolutionary development and importance of GnRH multiplicity. The American shad is a primitive teleost from the Clupeiform order, which evolved before the Euteleosts. It is also the earliest evolved teleosts known to possess three forms of GnRH. For these reasons, it is a good candidate

to study the evolution of the "three form GnRH system", and the divergence from the two-form system found in primitive teleosts.

As discussed earlier, another central hormone in the reproductive axis is LH. As specified in section II.C, due to unsuccessful attempts at developing a plasma LH assay, we sequenced the shad LHβ so as to establish which LHβ Ab would be most suitable for use in a shad LHβ RIA assay. The higher the homology between the shad LHβ and the LHβ against which the Ab was generated, the higher the chances of detection and cross reactivity. In addition, sequencing the LHβ cDNA could assist greatly in studying both shad and other alosa species. Using the LHβ sequence, we can determine mRNA levels of LHβ in the pituitary, and conduct sequence homology comparisons to other species. As the shad LHβ mRNA and amino acid sequence was not known, we used the known Pacific herring LHβ sequence (Power at al., 1997) to construct primers.

II.B Cloning of GnRH and LHβ cDNA

II.B.1Materials and methods

II.B.1a RNA extraction

American shad RNA was extracted from brains of female shad using a procedure based on the Mini-Ribosep Ultra mRNA isolation kit (Collaborative Biomedical Products, Bedford, MA). The brains were extracted in the field from fish caught at Conowingo Dam (Hartford County, MD) and immediately placed in liquid nitrogen. Brain samples were later stored in a –80°C freezer pending analysis. The frozen brains were homogenized (Ultra-Turrax T25, IKA Laboratechnik, Staufen, Germany) in a sterile 15ml tube containing 10ml of lysis buffer (0.2M NaCl, 10mM Tris Cl, 1mM EDTA, 0.5% SDS, pH 7.5, 0.1 mg/ml proteinase K) for 60 seconds. The homogenate was incubated while agitating lightly at 45°C for 2 hours.

During incubation, oligo(dT)-cellulose was prepared for RNA binding by washing it three times with 2ml of elution buffer (10mM Tris Cl, 1mM EDTA, 0.05 SDS, pH 7.5), between washes the oligo(dT)-cellulose was centrifuged for 5 min at 3000g. Next the cellulose was equilibrated by washing twice with 1ml of binding buffer (0.5 M NaCl, 10mM Tris Cl, 1mM EDTA, 0.1% SDS, pH 7.5). 0.4 mg of the prepared cellulose was left submerged in each tube, in 1ml of binding buffer until the end of lysate incubation. Prior to mixing the lysate with the cellulose, 60µl of 5M NaCl was added to the lysate. The cellulose and the lysate were mixed, amid intermittent agitation, and left at room temp for 1 hour. The cellulose was subsequently pelleted by centrifugation (5min at 3000 g) and washed twice with 5ml binding buffer. The pellet was suspended in 250µl of binding buffer, which was transferred to a microfuge column. We centrifuged the column

(10 sec at 5000 g) and discarded the supernatant. The 15ml tube was washed again with 300µl of binding buffer and centrifuged again, to minimize loss of cellulose, and the process was repeated. The column was eluted with 200µl of elution buffer. The elutant was incubated for 10min at RT and centrifuged (10 sec, 5000 g). An additional 200µl were added to the column, which was again incubated for 10 min and centrifuged. We then eluted the mRNA from the buffer by adding 0.1 volume of 3M NaOAc (40µl) and 2 volumes ethanol. The mRNA was extracted by centrifuging (5000g) for 20 min at 4°C, subsequently removing the supernatant. The mRNA was air-dried and re-suspended in 20µl of RNAse-free H_2O and stored at -80°C.

II.B.1b PCR amplification and cloning

The mRNA from the shad brain was transformed into cDNA by reverse transcription using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA). The resulting cDNA was amplified using the same kit with degenerate and specific primers, as well as specific primers supplied with the kit (UPM, NUP).

The degenerate primers and degenerate-nested primers for GnRH were constructed based on conserved regions in the decapeptide region of the specific GnRH forms from multiple teleost species. This technique was used to amplify the cGnRH-II and sGnRH cDNAs, as sequences for these GnRHs are known from other species. The species used for this alignment were goldfish, zebrafish, cichlid, medaka, salmon, catfish and sea bream.

There were no existing hrGnRH sequences from other species, therefore the known hrGnRH amino acid sequence was used to construct degenerate primers.

The primers for the shad LHβ were constructed based on two regions of the Pacific herring LHβ sequence (Power et al., 1997) that are conserved in the LHβ sequence of multiple teleost species.

These primers (Table 1) were used to amplify the cDNA of the three hypothetical shad GnRHs. The PCR product was run on a polyacrylamide gel and selected bands were cut out and purified (QIAquick gel extractor kit, Qiagen, Valencia, CA), the cDNA sequences were ligated into a pGEM-T vector (Promega, Madison, WI). The construct was then transformed into competent cells (DH5α) which were grown on Ampicillin-XGal-IPTG plates (based on pGEM-T protocol). White colonies were selected and grown in liquid media, and examined for incorporation of the DNA fragment using PCR with sequence and plasmid-specific primers. Following verification of incorporation of the plasmids in the bacteria, cells were grown in liquid media and the plasmids were extracted (QIAprep spin miniprep kit, Qiagen, Valencia, CA) and sequenced.

After an initial segment of the GnRH was sequenced, specific primers were constructed and a 5'/3' RACE protocol was used (SMART RACE cDNA amplification kit, clontech, Palo Alto, CA) to sequence the complete GnRH mRNA sequence.

Table 1

GnRH	Primer name	Race direction	Sequence
hrGnRH	HRDF1	3’	CARCAYTGGTCICAYGGNCTNTC
	HRDF2	3’	CARCAYTGGTCICAYGGNCTNAG
	HRDF3	3’	TTRAGYCCIGGHGGVAARAG
cGnRH-II	CDF1	3’	CACTGGTCYCAYGGYTGGTA
	CDF2	3’	GTAYCCYGGAGGMAAGAG
	CDR1	5’	TAGCTGCATTYYCCYGCYTCRCA
sGnRH	SDF1	3’	CAGCAYTGGTCITAYGGDTGGCT
	SDF2	3’	CCNGGHGGRAARAGAAGYGT
	SDF3	3’	GTKGGDGARITIGAGGCMAC
LHβ	LHF	3’	CTGCCCGAGGTGCCTGGTGT
	LHR	5’	GGGTCCACCCCATCAGCACA
	LHR-2	5’	CACGAAAGGGTCCACGCCATC
	LHR-3	5’	TTCAGGCCCCAGGCTCTCCA

- Y=C/T, M=A/C, R=A/G, H=T/C/A, V=C/A/G, N/I=C/A/T/G.

Table 1

Sequences of degenerate and specific primers used to clone the shad GnRHs and LHβ.

II.B.2 Results

II.B.2a hrGnRH sequence

The first fragment amplified was a partial hrGnRH sequence. This sequence was used to construct specific primers and the RACE method was used to sequence the complete hrGnRH mRNA sequence (Fig 1). The predicted decapeptide portion of the sequence was consistent with the known hrGnRH amino acid sequence published by Carolsfeld et al., (2000).

Figure 1

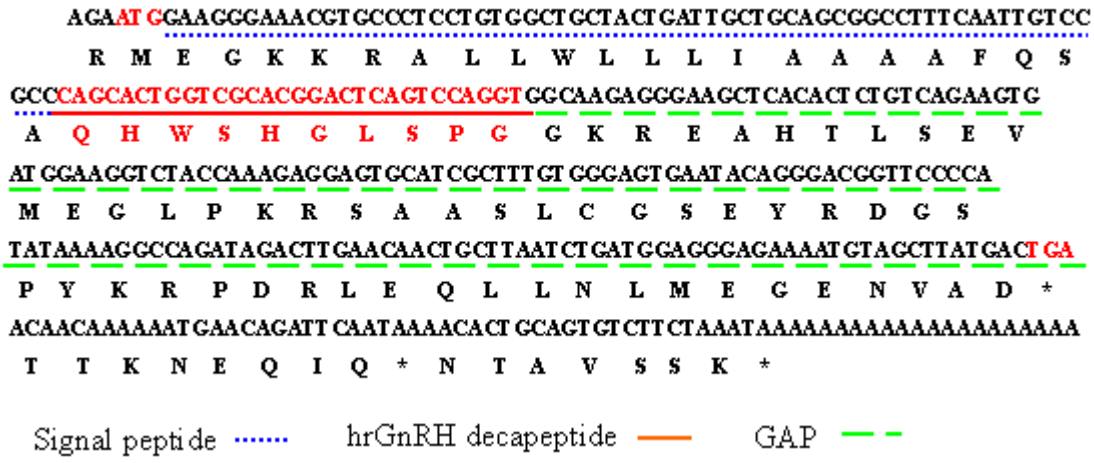


Figure 1

American shad hrGnRH prepro cDNA sequence (GeneBank, accession - AF536381).

* are placed at stop codon sites.

The hGnRH mRNA is identical in structure to other vertebrate GnRHs (Lin et al., 1998). Following transcriptional start signal, the first section of the sequence is a signal peptide. This region possesses the conserved hydrophobic core of leucine found in most GnRH signal peptides. The next portion of the sequence is the GnRH decapeptide itself,

which, as specified earlier, confirms the amino acid sequence of hrGnRH published by Carolsfeld et al., (2000).

As in most vertebrate GnRH forms discovered to date, the shad decapeptide amino acid sequence is relatively conserved. Specifically, positions 1-4, 6 and 9-10 are identical in all forms discovered (excluding lamprey forms). The hrGnRH differs from the sbGnRH only at position 5, which consists of a histidine instead of a tyrosine (Gothilf et al., 1995). When comparing the hrGnRH to mGnRH, the fifth position and the eighth positions differ.

Downstream of the decapeptide, is a GnRH GAP. The functional role of the GAP is not clear, it has been suggested to assist in folding the precursor for processing or to regulate prolactin and gonadotropin release. There is low homology between GAP regions of different GnRH forms (Lin et al., 1998).

The newly sequenced hrGnRH can be instrumental in gaining additional information regarding the evolution of the various GnRH forms and GnRH multiplicity. As mentioned in the introduction, all species investigated to date, possess two or three forms of GnRH, and several invertebrates also seem to possess multiple GnRH forms. There have been several propositions as to the evolutionary development of GnRH multiplicity, one of these is the suggestion that there are two main lineages in the GnRH tree: The cGnRH-II lineage and the mammalian lineage. Dubois (2002), suggests that from the mGnRH lineage an additional lineage arose that includes the cGnRH-I, gpGnRH, rGnRH and the "fish" GnRHs including the sGnRH and the species specific GnRHs.

In our analysis of the phylogeny of different GnRH forms (Fig 2) we used the Entrez Nucleotides database sequences of the full peptide sequence of various GnRH forms. We conducted a clustalW sequence alignment (Thompson et al., 1994) followed by creating an un-rooted cladogram tree, using the neighbor Joining method (Saitou and Neil, 1987).

Figure 2

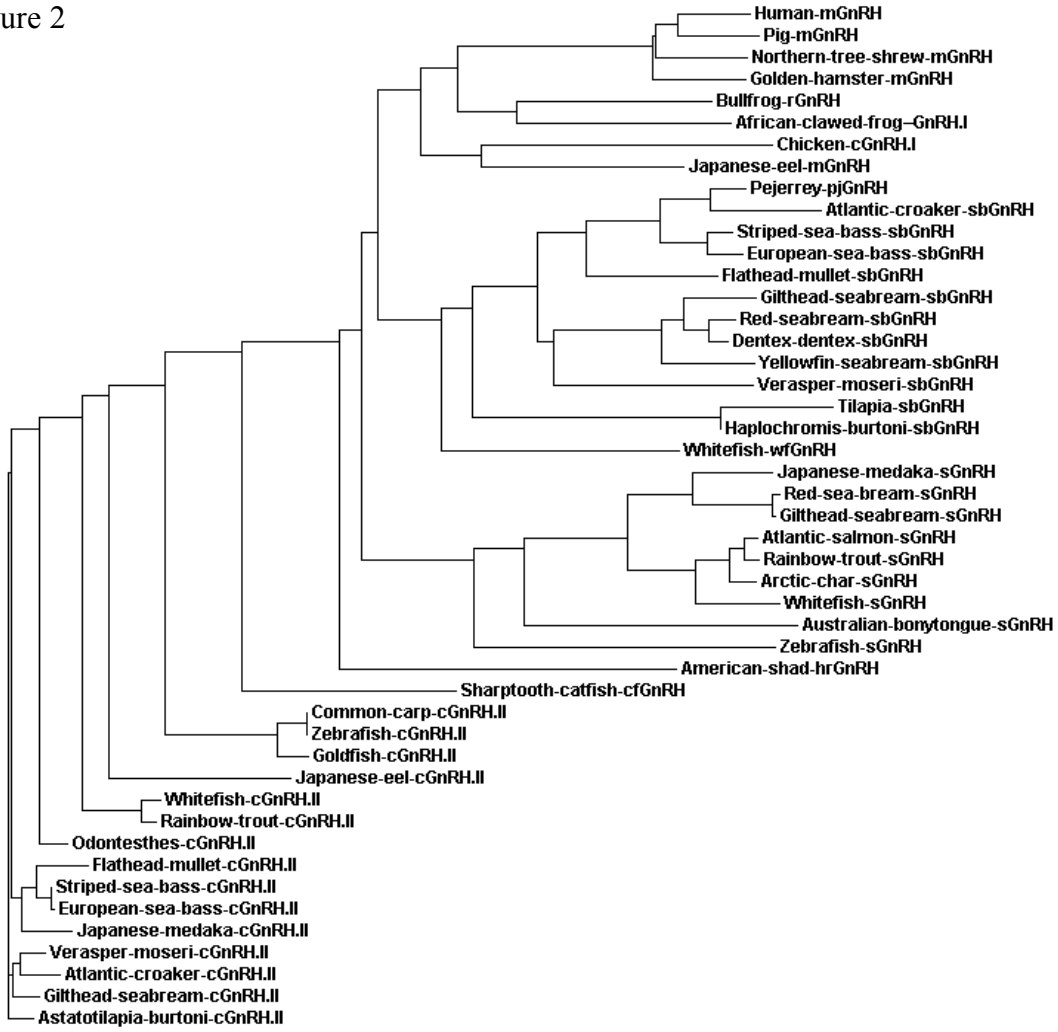


Fig 2

Phylogenetic analysis of GnRH forms. Based on pre-pro peptide sequences of GnRH peptide sequences gathered using the Entrez search and retrieval system. Tree is a cladogram based on neighbor-joining analysis.

II.B.2b cGnRH-II and sGnRH sequences

We followed the same procedure as described previously to clone and sequence a partial fragment (893bp) of the cGnRH-II mRNA (not shown), thus verifying that shad has the cGnRH-II form.

In spite of attempts to clone the shad sGnRH using degenerate primers, we were not able to clone this form from the shad brain. We hypothesize that this is due to the fact that its levels are very low, and that it is found primarily in the olfactory bulbs. However, most teleosts possess the sGnRH form and we therefore assume that it is also present in the shad. Moreover, the Atlantic herring, which is closely related to the shad, and like the shad, has the hrGnRH form, also possesses the sGnRH form. Ultimately, using GnRH ELISA, we were able to detect the sGnRH form (see II.C.1).

II.B.2c LHβ sequence

The shad LHβ mRNA sequence (Fig 3) has low homology to LHβ mRNA sequences of other species. However, when translating the mRNA sequence to a deduced amino acid sequence, the shad LHβ shows a relatively high homology to LHβ of several species. The highest homology (86% identity) is to Atlantic herring. Also, several species of carp have identities ranging from 74-72% and channel catfish has a 70% homology to the shad LHβ amino acid sequence. As with hrGnRH, the shad LHβ shares the highest homology to species that are phylogenetically related to it, such as carp and catfish. The deduced peptide amino acid sequence shares conserved elements that have been found in

LHβ sequences of other species, such as 12 cysteine residues and conserved regions (Yoshiura et al., 1997).

Figure 3

CGC GGG GAC ACT TAA CTC TCT ACC AAC TGG ACT GAC TGA CTG ACT GAC TGG AGG

ATG GCC CGT ACC CCA CAG TGC ACT ATT CTG CTC TTT CTG TCG GTA CTG GCT GTG

CCA TCA CAG TGC TTC CAC CTG CAG CCC TGT GTA CTG GTC AAT GAG ACT GTG TCC
F H L Q P C V L V N E T V S

GTG GAG AAA GAG GGC TGC CCG AGG TGC CTG GTG TTC CAG ACC ACC ATC TGC AGT
V E K E G C P R C L V F Q T T I C S

GGA CAC TGC CTG ACC AAA GAG CCT GTG TAC AAA AGC CCA TTC TCC ATG GTG TCC
G H C L T K E P V Y K S P F S M V S

CAG CAT GTG TGC ACA TAT GGC AAC TTC CGT TAT GAG ACG GTA CGT CTG CCT GAC
Q H V C T Y G N F R Y E T V R L P D

TGT GCT GAT GGC GTG GAC CCT TTC GTG AGC TAC CCA GTG GCC CTG AGC TGT AAG
C A D G V D P F V S Y P V A L S C K

TGT AGT TTG TGC CCC ATG GAT ACA TCT GAC TGC TCC CTG GAG AGC CTG GGG CCT
C S L C P M D T S D C S L E S L G P

GAA TTT TGC ATG AGT GAG AGA ATG CAT GCC TAT GAG AGT CAG AGA CTG CCT CAC
E F C M S E R M H A Y E S Q R L P H

TAT GAC TAT TAG CTG CCT GTG TAG ACA GTT TGT GCT GAA TTC CAA TTT CAT GCA
Y D Y * L P V *

TTT TAT TGA TTA ATA AGC ACC CCA GTA GAG TAG TGT TGT GCC AAT GGA TGT AAT

GGT TTT TTN TCT CCA ATA AAT GTC CCT CCA AAT AAA TTA AAA AAA AAA AAA AAA

Figure 3

American shad LHβ cDNA and deduced amino acid sequence, as sequenced from shad pituitary RNA. Asterisks represent stop codons. Putative polyadenylation site is underlined by a solid line, putative mature peptide is underlined by a intermittent line.

I.B.3 Discussion

In an effort to develop tools and assays to further explore the reproductive endocrinology of American shad, we attempted to sequence the shad GnRH forms and it's LHβ. Our initial hypothesis, regarding which forms of GnRH are present in shad, was based on the forms found in the Pacific herring. However, to verify that the shad indeed has the same three forms (cGnRH-II, sGnRH, and hrGnRH), we attempted to clone and sequence their cDNA. We successfully cloned and sequenced the full-length hrGnRH mRNA and a partial cGnRH-II mRNA sequence. Based on the fact that the shad has hrGnRH (like Pacific herring) and that sGnRH was detected in our ELISA, we concluded that shad has three forms of GnRH – sGnRH, cGnRH II and hrGnRH.

Sequencing shad hrGnRH and the partial cGnRH-II sequence will have multiple additional benefits. First, these sequences can be used to localize the different GnRH forms in the brain of *Alosa sapidissima* and other Alosa species. In addition, the hrGnRH sequence will be instrumental in studying the evolution and action of GnRH multiplicity.

At present, many vertebrates including humans have been shown to have two or three forms of GnRH. It is hypothesized that all vertebrates have either two of three forms of GnRH. Humans have been shown to possess both mGnRH and cGnRH-II (Neill, 2002). However little is known about the function of GnRH multiplicity, apart from the fact that in vertebrates, one form of GnRH in each species is the hypophysiotropic form and is responsible for inducing the secretion of gonadotropin from the pituitary.

It has been hypothesized that cGnRH-II is active in regulating sexual behavior. this has been documented in various birds and mammals. It has also been suggested that cGnRH-II acts in the CNS as a general neurotransmitter (Maney et al., 1997; Temple et

al., 2003). The sequence and localization of every additional GnRH form brings us closer to understanding the reason and function for GnRH multiplicity.

The shad is a primitive Teleostei species, and to date is the most primitive species of teleost known to have three forms of GnRH. Osteichthyes species, and some Teleostei that evolved earlier than the shad, possesses only two GnRH forms. For this reason, studying the sequence of shad GnRH forms, and especially the hrGnRH sequence and its homology to other GnRH forms, is central to understanding GnRH evolution and action.

Based on the analysis of our cladogram it seems that indeed there are two main lineages in the GnRH tree: the cGnRH-II and the mGnRH which is in phylogenetic proximity to the sbGnRH and sGnRH branches which include several species specific GnRH's such as pjGnRH , rGnRH, and wfGnRH. This observation is in agreement with the hypothesis given by Dubois (2002) regarding GnRH evolution. However the hrGnRH and the cfGnRH are not part of the cGnRH-II or mGnRH lineages but seem to have diverged from an ancestral form at a certain time point, possibly directly from the cGnRH-II form. These two species are relatively ancient and as mentioned the herring family is the most primitive family to date in which three GnRH forms have been found. Based on this data we hypothesize that the hrGnRH, the cfGnRH and possibly additional GnRH forms yet to be discovered, represent an additional lineage in the GnRH phylogenetic tree, which is distinct from the cGnRH-II and the mGnRH lineages. It is also interesting to note that the eel mGnRH, which evolved prior to the mammalian mGnRH is not clusters with the mammalian mGnRHs but with the cGnRH-I and the rGnRH forms.

As mentioned, the shad LHβ sequence was found to have a high homology to carp and catfish LHβ. This finding reinforces the idea that in many cases, phylogeneticaly-close species have specific genes that show a high sequence homology.

II.C Assay development

II.C.1 GnRH ELISA

GnRHs are key regulatory factors in the vertebrate HPG axis. More specifically, the hypophysiotropic forms of GnRH have been shown to have central role in the regulation of vertebrate reproduction. Therefore measuring GnRH levels in the pituitary was an important step towards understanding basic reproductive mechanisms and dysfunction in Alosa species. Based on the Sherwood et al. (2000) paper on Pacific herring GnRHs and our own sequencing of the shad GnRHs, we knew that three GnRH forms were present in shad – cGnRH-II, sGnRH and hrGnRH.

Extracts from pituitaries collected during the 2001 spawning migration were used to measure the GnRHs. Frozen (–80°C) pituitaries were thawed and after the addition of 200µl of H_2O sonicated for 20sec (duty cycle 100%, output #3, Sonifier 250, Branson Inc., Danbury, CT).

ELISA for the cGnRH-II and the sGnRH have been well established in our laboratory for a variety of fish species. We validated and used the established GnRH ELISA for gilthead sea bream (Holland et al, 1998), since a specific ELISA to detect the hrGnRH has never been developed.

To detect hrGnRH, we attempted to use a polyclonal antibody (Ab) against mGnRH. This polyclonal Ab has been shown to bind specifically to the sbGnRH without

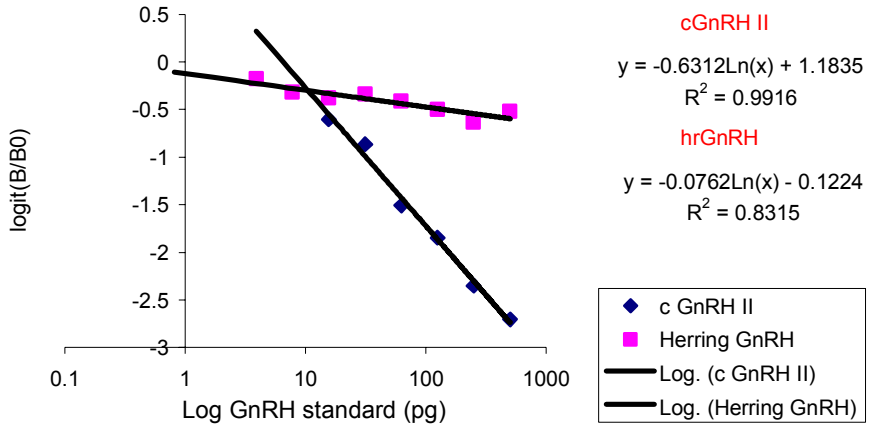
cross-reacting with sGnRH or cGnRH-II, therefore it is used in the sea bream ELISA to detect the sbGnRH (Holland et al, 1998). As hrGnRH and sbGnRH differ only by one amino acid, we attempted to use the mGnRH Ab to detect the hrGnRH.

To assure that there is no cross-reactivity between the sGnRH/cGnRH-II Abs and the hrGnRH peptide, as well as to examine the feasibility of using the mGnRH Ab for detecting hrGnRH, we conducted cross-reactivity experiments using purified sGnRH, cGnRH-II and hrGnRH (obtained courtesy of Dr. J. Rivier, Salk Institute) peptides as standards with the corresponding Ab.

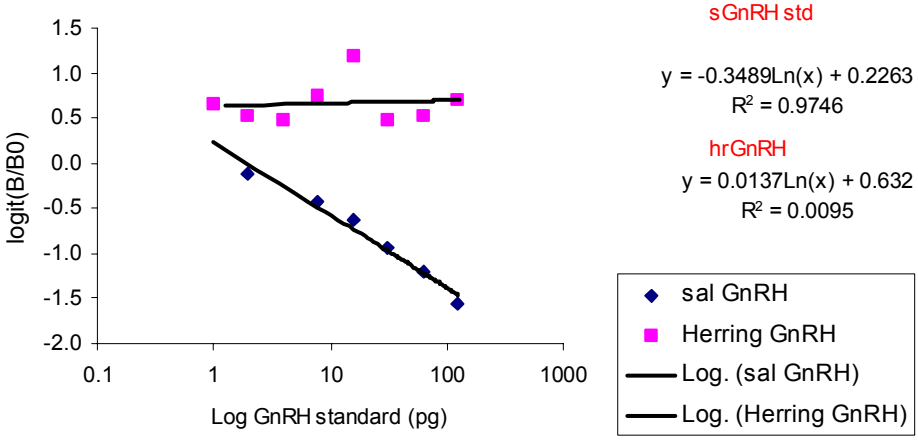
As can be seen in figure 4, there was no cross-reactivity between the hrGnRH and the cGnRH-II/sGnRH Ab. There was also good parallelism in binding between serial dilutions of mGnRH and purified hrGnRH using the mGnRH Ab (Fig 4c).
This indicated that the existing seabream ELISA, using the mGnRH Ab to detect hrGnRH levels, can be used successfully to measure the three GnRH peptide levels in shad pituitary extracts without cross-reactivity or specificity problems.

Figure 4

a.


0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
logit(B/B0)
0.1
1
10
100
1000
Log GnRH standard (pg)
cGnRH II
y = -0.6312Ln(x) + 1.1835
R2 = 0.9916
hrGnRH
y = -0.0762Ln(x) - 0.1224
R2 = 0.8315
c GnRH II
Herring GnRH
Log. (c GnRH II)
Log. (Herring GnRH)

b.


1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
logit(B/B0)
0.1
1
10
100
1000
Log GnRH standard (pg)
sGnRH std
y = -0.3489Ln(x) + 0.2263
R2 = 0.9746
hrGnRH
y = 0.0137Ln(x) + 0.632
R2 = 0.0095
sal GnRH
Herring GnRH
Log. (sal GnRH)
Log. (Herring GnRH)

c.

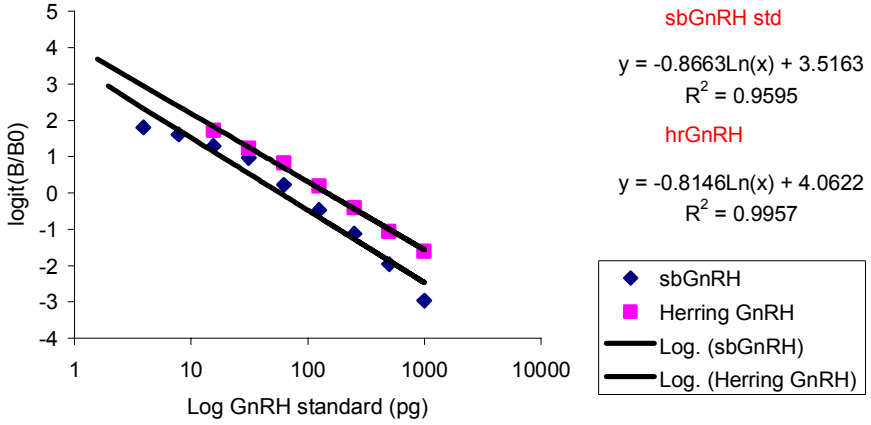

5
4
3
2
1
0
-1
-2
-3
-4
logit(B/B0)
1
10
100
1000
10000
Log GnRH standard (pg)
sbGnRH std
y = -0.8663Ln(x) + 3.5163
R2 = 0.9595
hrGnRH
y = -0.8146Ln(x) + 4.0622
R2 = 0.9957
sbGnRH
Herring GnRH
Log. (sbGnRH)
Log. (Herring GnRH)

Figure 4

Displacement curve for shad GnRHs generated via ELISA. Serial dilutions of GnRH standards were incubated with specific GnRH Abs, to examine cross-reactivity/parallelism between the GnRH Ab's and hrGnRH.

a. Serial dilutions of cGnRH and hrGnRH as detected by cGnRH Ab. No significant cross-reactivity between the cGnRH Ab and hrGnRH was detected.
b. Serial dilutions of sGnRH and hrGnRH detected by sGnRH Ab. No significant cross-reactivity between sGnRH Ab and hrGnRH was detected.
c. Serial dilutions of sbGnRH and hrGnRH detected by mGnRH Ab. Parallelism exist between sbGnRH and hrGnRH in binding to the mGnRH Ab.

These findings indicated that we could use the established cGnRH/sGnRH established assay to detect these two peptides without their corresponding Ab's cross-reacting with the hrGnRH in the samples. Moreover, our findings convincingly demonstrate that we could use the mGnRH Ab to accurately detect hrGnRH levels.

II.C.2 LHβ RIA

Three assays were examined for potential use in quantifying plasma shad LH levels. The striped bass LHβ RIA has been shown to work well in several fish species (Mananos et al., 1997; Mylonas et al., 1997). However, using the striped bass LHβ assay, no cross-reactivity was found to be present between the striped bass LHβ Ab and shad plasma or pituitary extracts (data not shown).

Attempts to use other Ab's, based on existing LHβ RIA's from other fish species, were expected to have little success unless the specific Ab was generated for an LHβ

with high homology to the shad LHβ. However, shad LHβ had not been sequenced, thus our first step was to sequence its mRNA (see II.B.2c). Using the cloned shad LHβ sequence, a homology search revealed a high degree of homology between the deduced sequence of the shad LHβ peptide and LHβ peptides of several species: Atlantic herring (86% identity), silver carp (74% identity), and coho salmon (67% identity). Therefore, we obtained the coho salmon LHβ and its Ab (courtesy of Dr. P. Swanson) and conducted a serial dilution RIA experiment using pituitary extracts (Fig 5) and plasma samples (not shown) to determine whether the coho LHβ Ab cross-reacts with the shad LHβ protein (Larsen and Swanson, 1997). The cross-reactivity between the coho Ab and the shad LHβ was found to be low, and therefore not practical for our purposes.

Figure 5.

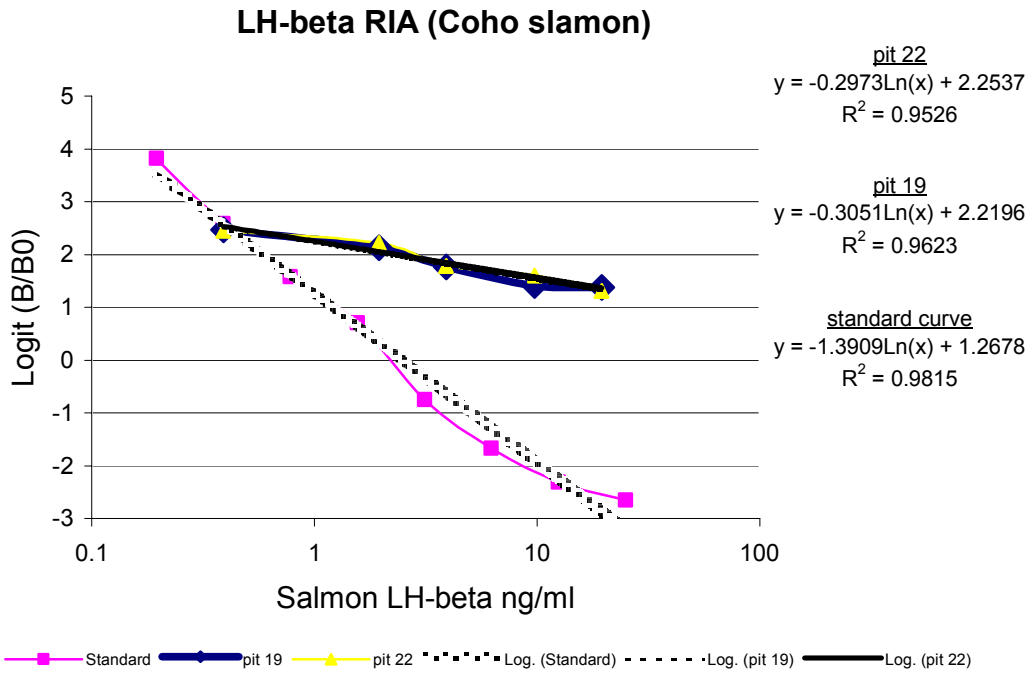


Figure 5

LHβ RIA assay using shad LH pituitary extracts, salmon LHβ Ab, and salmon LHβ as standard. No parallelism was found between the salmon LHβ standard curve and the serial dilution curves of shad pituitary extracts.

This result indicates that despite the relatively high homology between the shad and salmon LHβ amino acid sequences, the salmon LHβ RIA assay could not be used to measure LH levels in shad.

As the carp LHβ peptide sequence was also found to have a high homology to the American shad LHβ sequence, we sent samples of shad plasma and pituitary extracts to Dr. Chang (University of Alberta, Canada) for analysis using the carp LHβ RIA assay. Unfortunately, this assay was also not capable of detecting the shad LHβ. In conclusion, none of the above LH assays (striped bass, coho salmon and common carp) were capable of detecting LH in American shad, despite the high sequence homology between these LHβs. In the absence of a shad LHβ-specific Ab, we were not able to measure LH levels in shad plasma or pituitary extracts.

II.C.3 MIS RIA

An integral aspect of our efforts to clarify the mechanism and cycle of shad oocyte development was determining the presence and fluctuations of the shad maturation-inducing steroid (MIS). The Pacific herring, which is also of the Clupidae family and has the same GnRH forms as the Alosa sapidissima, was used as a reference species in determining the relevant shad MIS. Carolsfeld et al. (1996) determined that the

main MIS in Pacific herring was the 17α, 20β-dihydroxyprogesterone. Therefore, we developed a RIA for this steroid in shad. The first step was to produce tritiated 17alpha-20beta-DHP which would be used as a tracer. This was done by conducting a conversion of 17α-hydroxyprogesterone to 17α, 20β-dihydroxyprogesterone (DHP) using the 3α,20β-hydroxysteroid-dehydrogenase enzyme (based on methods of Dr. J. Trant). After producing the tritiated steroid, which was used as the "tracer" we proceeded to conduct the DHP RIA assay, based on the published protocols of Dr. A. Scott.
The 50% binding of the tracer to the DHP Ab (supplied by Dr. A. Scott) was found to be at an Ab dilution of 1:5000. We subsequently used this concentration in the assay.

Prior to determining MIH plasma levels, it was necessary to establish that there is no interference by plasma components with the MIH-Ab binding. Plasma samples from a female shad were therefore spiked with three concentrations of DHP. The endogenous level of DHP in the plasma, as well as the DHP levels in the spiked plasma, were calculated via RIA. An examination of the resulting data (Fig. 6) reveled that total DHP levels, as measured in the spiked plasma, corresponded with the known endogenous level of DHP plus the amount of exogenous DHP added. In short, there is no interference of Ab-DHP cross-reaction due to plasma proteins. Thus, this assay can be used to accurately measure Alosa plasma DHP levels without extracting DHP from the plasma.

Figure 6.

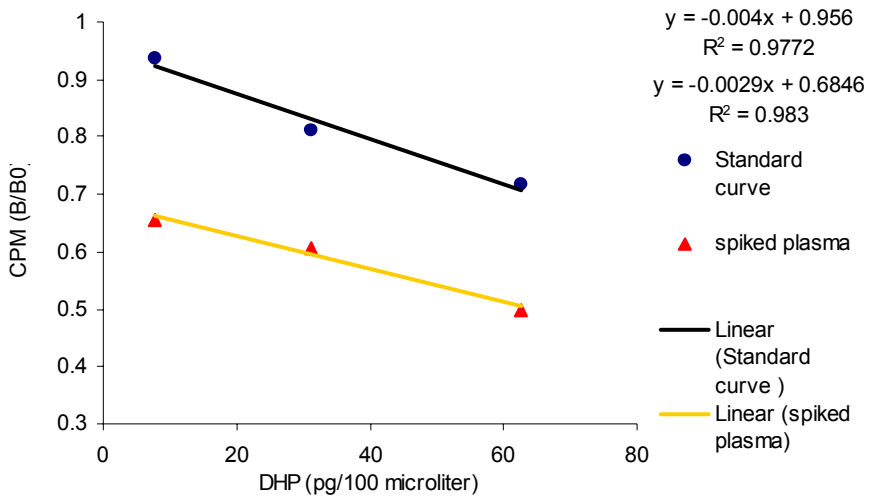


Figure 6

A partial standard curve of DHP compared to plasma spiked with three concentrations of exogenous DHP. The standard curve is in blue; the spiked plasma samples are in red. The spiked points correspond to an addition of 7.8, 31.25 and 62.5 pg of DHP to plasma samples. The cpm of the spiked plasma corresponds to the amount of the endogenous DHP in the sample + the amount of DHP exogenously added. This indicates that there are no components in the shad plasma that interfere with the reliable detection of DHP levels.

II.C.4 Discussion

Developing the GnRH and DHP hormone assays was a crucial step in this research. Starting to unravel the HPG cascade and the cross talk between the various organs and glands of the HPG axis requires at the very least sensitive assays for the hypophysiotrophic GnRH and the MIS, in addition to assays to quantify E_2 and T.

By coupling the shad's ovarian developmental stage with the hypophysiotropic GnRH and MIH levels, we can begin to unravel the shad's HPG axis dynamic as well as study the effects of GnRHa treatment on the HPG axis.

In addition, the hrGnRH assay, will allow us to conduct GnRH studies in different Alosan species in which the hrGnRH is the hypophisiotropic form. Moreover, the development of this assay gives us some interesting insight into the Ab specificity to structurally similar GnRHs in which only 2-3 amino acids vary.

We were unsuccessful in our attempt to use existing LH RIAs created for other species to detect the shad LH in the plasma or pituitary. This approach did not work despite the fact that the shad LHβ has high homology to the LHβ of the species whose assays were used. This points to the complexity of the LHβ secondary and tertiary structure, which seems to prevent polyclonal Ab recognition. However, as we were able to develop an assay that measures the MIH levels, the combination of gonadal developmental stage, GnRH levels, DHP levels, T and E_2 levels, allows us to gain insight into the workings of the shad HPG axis.

Future development of a shad-specific LH assay is nonetheless important. To develop such an assay, relatively large amounts of purified shad/herring LH will be needed as part of the process of producing an Ab. Two approaches can be used to obtain

this goal. One approach would be collection of shad/herring pituitaries and use of HPLC to purify LH, however, A large number of pituitaries are needed using this approach. Another alternative would be to produce recombinant LHβ protein, based on the known shad LHβ sequence. This can be done by using state of the art techniques to produce large amounts of specific recombinant proteins in eukaryotic cell cultures.

Chapter III. Spawning in captivity

III.A. Introduction

As emphasized previously, hatchery spawning is a crucial aspect of any restocking program. Strip spawning of fish collected on the spawning grounds is limited by time and manpower, inefficient, and detrimental to the brood fish. Moreover, strip spawning does not take advantage of the fact that shad are repeat spawners, thus by using this technique one loses the majority of the eggs that may otherwise be available.

A program to conduct hatchery-based shad spawning was initiated by the Maryland DNR in 1995. From the onset, it was clear that without external stimulation the shad do not reproduce well in captivity, a phenomenon known to occur in many fish species (Zohar et al., 1986; Zohar et al., 1990a; Zohar and Mylonas, 2001). In many cases the underlying reason for this infertility is inadequate stimulation of gonadotroph cells by GnRH. Delivering exogenous GnRHa in a repeated or sustained release method reverses this condition, causing an elevation in plasma gonadotropin levels (Zohar et al., 1990a; Mateos et al., 2002; Hassin et al., 1998) resulting in successful reproduction in captivity.

The GnRHa implant method was adopted in American shad by Mylonas et al. (1995b) who conducted preliminary GnRH implant experiments at the DNR Manning hatchery. In this study, female and male shad that were caught at Conowingo dam during the spawning migration were injecting with GnRH analogue EVAc implants. This initial induction trial was successful and resulted in the shad spawning in captivity. However, in subsequent trials several years later, in spite of usage of similar methods, the fecundity and egg quality of the captive shad seemed to be declining (Federal aid report #NA66FA0208, 1997; S. Minkkinnen, personal communication). In view of the above,

our goal was to reassess the action of GnRHa on the fecundity and fertilization of hatchery shad and to couple these results to reproductive spawning and hormonal profiles. Assessing these parameters would empirically establish whether there is indeed a reduction in GnRHa effectiveness relative to past experiments and, if so, would also define the hormonal mechanism underlying this problem.

III.B. Materials and methods

III.B.1 Experimental design

We developed three experimental designs aimed at three objectives. Our first objective was to assess the shad response to GnRHa treatment using various delivery methods. We did this by dividing the shad into three groups, one group was injected with GnRHa microspheres, one with GnRHa implants and last with sham implants containing no GnRHa. These groups were placed in separate tanks and monitored for spawning fecundity and fertilization. Our second goal was to elucidate endocrine patterns in these three groups. To achieve this goal, we sacrificed on days two and four post-treatment, six females from the implanted and the sham groups in view of further analysis of plasma, pituitary, and gonads.

Our third goal was to clarify the shad's spawning cycle. In this experiment, we placed single females with two males in individual tanks. Thus, we were able to monitor spawning activity, fecundity and fertilization in single females.

Figure 7

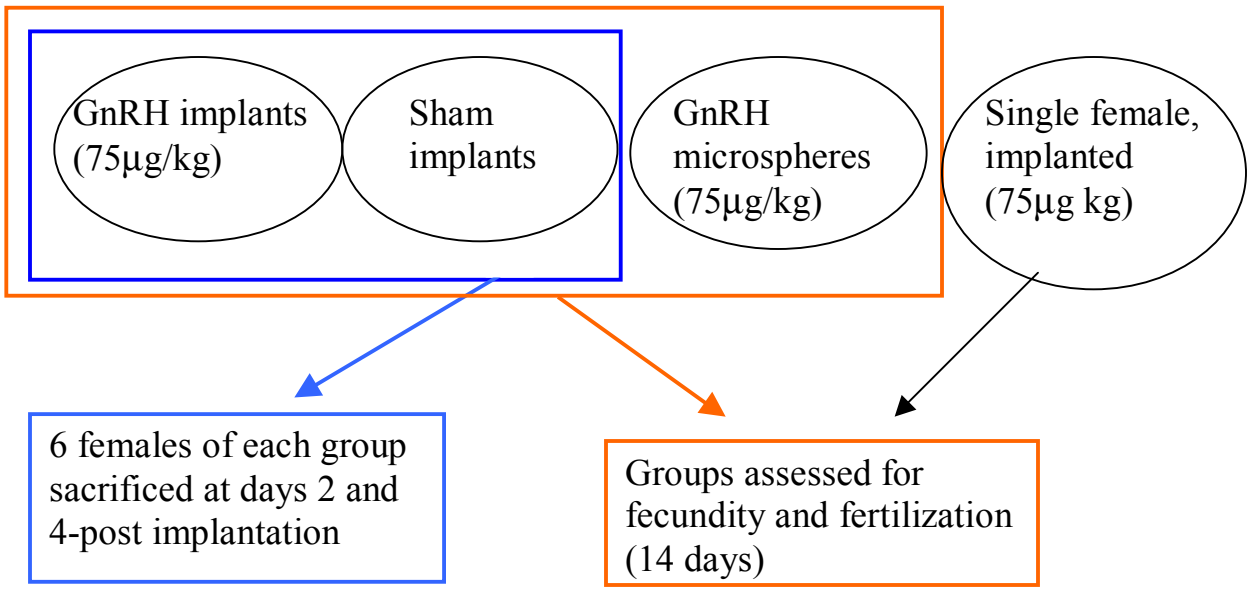


Figure 7

A schematic of experiments conducted on hatchery female shad. Fish were treated with one of three treatments: implants, microspheres or sham implants. These groups were assessed for fecundity and fertilization. In two of these groups (GnRH implants and sham implants) six femeles were sampled at days 2 and 4. A fourth group consisted of individual females treated with GnRH implants that were monitored for fecundity and fertilization.

III.B.2 Animal collection and treatment

Male and female adult American shad were obtained from the Susquehanna River, Hartford County Maryland on April 29th, 2001. Shad conducting the spawning migration were caught on the day prior to sampling in the dam's fish lift, these fish were kept over night in a flow through holding tank supplied with water from the river. On the day of sampling, the fish were placed in a 37,800 l tank, which was mounted on a vehicle. The water in the tank was river water at 16°C, aerated, and supplemented with NaCl to a concentration of 3ppt. The shad were

driven to the Manning hatchery in Waldorf, MD where the following procedures were conducted:

Fish were anesthetized using 2-phenoxyethanol at a concentration of 1ml/l. The shad ovary was biopsied at the hatchery prior to GnRHa administration, to assess ovary developmental stage. Unlike some perciforms (e.g. striped bass, sea bream), shad oocytes cannot be "cleared" using a 1:1:1 MeOH formaldehyde-acetic acid solution to assess internal oocyte structure. Thus, assessments of shad biopsies in the field were based on oocyte size and translucency. Only shad that were at the middle stages of oocyte maturation were included in the experiment, since the GnRHa induction works best when initiated at a mid-developmental stage. We obtained a biopsy of the ovary by inserting a 4mm catheter through the oviduct. The sample was placed on a slide and examined under a dissecting microscope. If large translucent oocytes (hydrated) were seen, the female was not used. The presence of large, hydrated, translucent oocytes would suggest that the animal is at an advanced stage FOM and therefore not a suitable candidate for exogenous GnRHa stimulation of the ovary. Only females that had no such oocytes were selected. These females had oocytes at various stages of vitellogenesis, but no hydrated oocytes.

The selected females were allocated into three groups, which were injected either with GnRHa ethyl-vinyl acetate copolymer (EVAc) implants (Zohar et al., 1990) containing GnRHa ([Des-Gly^{10} , D-Ala^{6} , Pro^{9}] GnRH, Ethyl Amide) at mean doses of 0 µg/kg (sham) or 83 (± 28) µg/kg (using 150µg implants) or with polyanhydride microspheres (Mylonas et al., 1995a) containing GnRHa at a dose of 75 µg/kg. All males were treated with GnRHa EVAc implants at a mean dose of 68 (± 25) µg/kg of GnRHa (using 75µg implants). These GnRHa delivery systems were injected intramuscularly. The GnRHa and sham implants were injected into the

muscle using a hypodermic 12-gauge needle. The microspheres, suspended in 100µl of vehicle, were injected into the muscle using a 18-gauge needle. Tissue glue (3M Vetbond, 3M Inc., St. Paul, MN) was applied to seal the wound.

We placed the fish by treatment group into seven circular tanks of two volumes (7 m^3 or 4.6 m^3), so that each tank contained between 6 and 16 females and between 12 and 26 males (~1:2 ratio). We collected spawned eggs daily for 14 days from egg collectors. Eggs were counted to assess fecundity and then moved to MacDonald hatching jars. 24h post-collection we examined the eggs under a stereoscope to determine fertilization percentage based on observation of embryo development.

III.B.3 GnRH implant and microsphere production

GnRHa implants were produced according to Zohar et al., (1990). Briefly, the GnRHa used was [Des-Gly^{10},D-Ala^{6},Pro^{9}]-LH-RH, Ethyl Amide (Bacham, H-4070). The peptide is mixed into a BSA-inulin-water mixture and freeze dried (Labconco Freezone 12, Labonco Inc.). The residue is then ground using a glass pestle and subsequently 18ml of ethylene-vinyl acetate/$MeCl_2$ solution is added. This mixture is vortexed and poured into an aluminium cast that was pre-chilled using dry ice. The resulting polymer plate is left to evaporate at –20°C for 3 days and then desiccated for 24h.

The implants are then punched from this plate; the implant size is 2mm by 1mm. Each implant contains a specified amount of GnRHa, based on the initial amount of GnRHa used for that plate. Previous work in the lab has shown that the hormone is distributed uniformly throughout the implant plate (Mylonas, unpublished). For the work

reported in this text, we used implants that contained 0, 75 or 150µg of GnRHa per implant.

Microspheres containing GnRH were produced as described by Mylonas (1995a). Briefly, GnRHa is dissolved in distilled water containing gelatin. This solution is kept at 65°C until addition of $MeCl_2$ containing p[FAD-SA] (fatty acid dimmer and sebacic acid), to this cold (4 °C) 1% polyvinyl alcohol (PVA) saturated with $MeCl_2$ was added and the solution is vortexed. The solution was poured into a beaker containing (4 °C) 1% PVA and stirred for 4 h. After evaporating the $MeCl_2$ the microspheres are filtered through a 250µm sieve, and a 25µm sieve, rinsed, and lyophilized. Prior to injection the microspheres are placed into a viscous vehicle solution and subsequently injected at the appropriate dose into the muscle.

III.B.4 Hatchery Conditions

The experiments took place at the DNR Manning hatchery in Waldorf, MD. The fish were maintained indoors in flow-through tanks of various sizes (7^3 and 4.6^3). Tanks were supplied with water from a local spring at an exchange rate of 25% daily. The water temperature ranged from 16-20°C and NaCl was added periodically to maintain a salinity of 3ppt. The fish were not fed during the duration of the experiment.

The spawned eggs were automatically transported to the egg collector boxes through an airlift system. The egg collectors were supplied with the same water as the tank. Twice daily, in the morning and in the evening, the eggs were removed from the collectors, using a mesh screen, a sample was counted and egg number was estimated based on this count and total volume. Eggs were then placed into modified McDonald

hatchery jars, with an approximate exchange rate of 2L/min, for the purpose of larval rearing and release. Once daily an anti fungal prophylactic formalin treatment was administered to the eggs (600:1 formalin for 20 min). At 24h post-collection, the eggs were assessed for fertilization by taking a sample and estimating the number of eggs with visible cell division.

III.B.5 Ovarian sampling

Ovary samples were collected from females that were sacrificed at days 2 and 4. In these fish, we extracted the whole ovary, weighed it, and collected an equal amount of oocytes from random locations in both lobes of the ovary. This oocyte biopsy was placed in a 4% formaldehyde, 1% gluteraldehyde fixative (4F:1G) (McDowell and Trump, 1976) for later histological examination.

When addressing ovary stage in this chapter, the method of determining ovary stage for each female was as follows: Multiple histological sections (see IV.B.3) of oocytes from each female were examined by compound microscope. Based on the morphology and diameter of the oocytes (IV.B.3 and IV.C.2), we determined the stage of the most advanced oocytes in the ovary. Each of these developmental stages received a number (Table 2). This number became the indicator for the developmental stage of that specific female.

Table 2

Stage of most advanced oocytes	Rank
Vitellogenesis I	1
Vitellogenesis II	2
Germinal vesicle migration	3
Germinal vesicle breakdown	4
Atresia	5

Table 2

Oocyte developmental stages ranking

III.B.6 Histology

The fixed oocytes were dehydrated in a 75-90% ethanol series over a period of 3 days and then embedded in glycol methacrylate plastic (JB-4 Mini Kit, Polysciences, Inc., Warrington PA, USA). The embedded tissue was cut into 3-4µm serial sections on a microtome (Microm, HM 340, Portsmouth, NH) and the sections were placed onto glass slides.

The sections were then stained with methylene blue/basic fucasin (Bennett et al., 1976), dried, and inspected using a compound microscope at 4-10x magnifications.

III.B.7 Animal sampling

The plasma and pituitary from 12 sham and 12 GnRHa-implanted female fish were used to measure hormonal levels. Six fish were sacrificed from each group on days 2 and 4 post-implantation. The fish were anesthetized in 1ml/l of 2-phenoxyethanol and

3ml of blood was drawn from the caudal vasculature using a heparinized syringe. The blood was placed on ice in vials containing 100 µl of a 3mg/ml solution of aprotinin (Sigma Chemicals Co., St. Louis, MO) to prevent blood clotting and protein degradation. Plasma was separated by centrifuging the blood in the lab for 15 min at 4000 x g at 4°C, the plasma was then stored in 200µl aliquot's at –80°C.

Following the extraction of blood, the animals were weighed, decapitated, and the pituitary and brain were removed. The pituitary and brain were put into separate tubes and snap-frozen in liquid nitrogen. Once in the lab, the pituitary and brain were placed into –80°C. The abdomen of the fish was then opened and the ovary was removed, weighed, and a sample of oocytes was taken from both lobes of the ovary for histological analysis as described above.

III.B.8 Hormone measurements

The hormones measured were GnRH peptide levels in the pituitary, and testosterone, E_2, and DHP in the plasma. The measurements of the GnRHs and the DHP were carried out according to the assay procedures specified in chapter IIB. E_2 and T were measured using commercial E_2 and T kits for plasma hormone mesurments (Coat-A-Count Total Testosterone/Estradiol, Diagnostic Products Corporation, Los Angeles, CA).

III.B.9 Statistics

To conduct the GnRH levels and E_2, DHP and GSI comparisons, one-way ANOVA for independent samples was applied, followed by a Tukey's HSD post hoc test.

Comparisons between two groups were done using either t tests or the ordinal Mann-Whitney test. The graphs presenting data, which were analyzed using a non-parametric test, include the standard deviation, although this deviation was not a part of the statistical test. The letters (a) and (b) above the bars indicate statistically significant differences in mean.

III.C. Results

III.C.1 Fecundity

Mean fork length and weight (± standard deviation) for females was 48.4 (± 2.8) cm and 1.8 (± 0.3) kg, respectively, and for males 43.5 (± 4.2) cm and 1.1 (± 0.2) kg, respectively. We assessed the three treatment groups for fecundity and fertilization over a two-week period following treatment. As can be seen in figure 8, we observed three distinct peaks of spawning in the GnRHa implanted females, these three peaks were accompanied by substantial spawning on days preceding and following the peak. In the sham and the GnRHa microsphere-treated fish we observed only two days of spawning, no substantial additional spawning took place in these two groups.

The total fecundity of the GnRHa implant-treated group was over three-fold the total fecundity of the control (0 µg GnRH) group; the fecundity of GnRHa microsphere-treated fish was approximately the same as the sham-control group (Fig. 8). The average fecundity of each female in the GnRHa implant group for the duration of the experiment was approximately 32,000 eggs/kg, compared to 6000 eggs/kg for the microsphere group and 9500 eggs/kg for the sham group.

Three peaks of spawning on days 3, 6 and 9, characterized the spawning of the GnRHa implant-treated fish. The sham and microsphere-treated fish did not display a three day spawning

cycle, however their spawning peaks did occur at distinct intervals and not on successive days (Fig. 8).

Figure 8

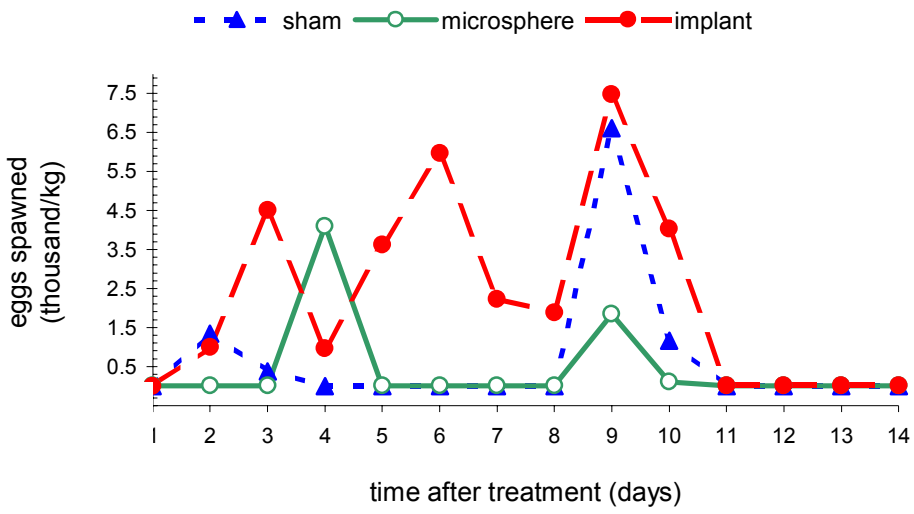


Figure 8

Fecundity of three American shad treatment groups: sham implants, GnRHa microspheres, and GnRHa implants. Fecundity is based on eggs spawned per kg body weight of the female, measured twice daily over a period of 14 days.

Day 9 marked the maximum daily fecundity for both the GnRHa implant and sham groups, after day 11 no significant spawning took place.

III.C.2 Fertilization

Fertilization was assessed by examining a sample of the eggs that were spawned, 24h post-collection. The parameter for fertilization was detection of embryo development as explained in the methods section. The fertilization rates varied significantly within all

treatment groups. On numerous occasions spawning occurred, but when assessing the eggs for development we found that no eggs were fertilized. The maximum fertilization percentage recorded was 54%. The average fertilization rate for significant spawning events (above 1000 eggs/kg) for the sham group was 35.8%, for the microsphere group was 35.2%, and for the GnRHa implant group was 10.2%.

On day 9, there was a rise in fertilization success in all groups. This day marked the maximum fertilization rate for all groups. This fertilization peak coincided with a peak in spawning for both the GnRHa implant and sham groups. After day 9, none of the groups spawned in any significant amount, therefore fertilization rates after day 9 are null (Fig 9).

Figure 9

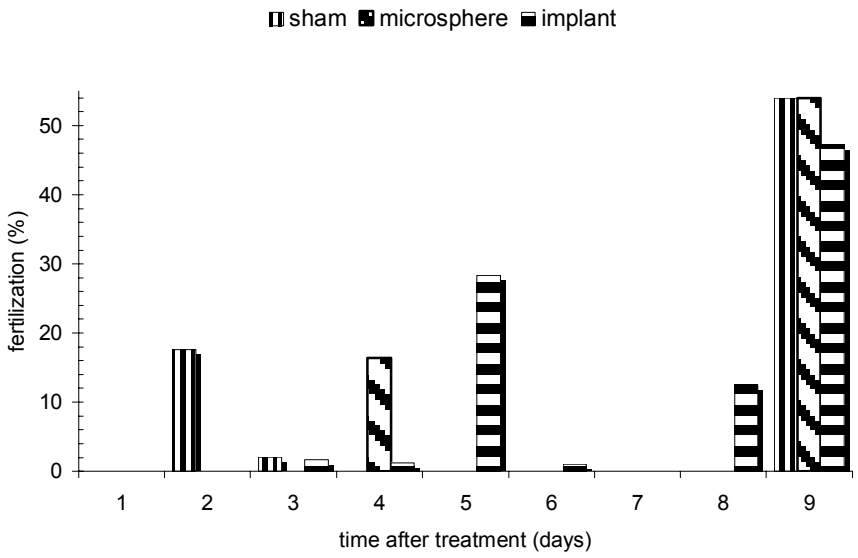


Figure 9

Fertilization percentage of shad eggs is determined 24 hours post-collection. Fertilization is displayed according to treatment groups and as measured daily for 14 days (no fertilization on day 10 and no spawning past day 10).

III.C.3 Gonadotropin releasing hormone

In view of establishing which of the three GnRHs is the hypophysiotropic form, we examined which of the three GnRHs is expressed at the highest overall level in the pituitary of all females sampled, regardless of treatment. Specific ELISAs were used to detect the levels of the three GnRHs (see methods). When observing the total average levels of the GnRHs in the captive females, with or without GnRHa treatment, it became apparent that the hrGnRH was expressed at much higher levels in the pituitary than the sGnRH or the cGnRH-II. The average hrGnRH level in the pituitary of all females was 7.7 (±2.6) ng/pituitary, the sGnRH was 1.9 (±0.6) and the cGnRH-II 0.16 (±0.07). Thus, hrGnRH pituitary levels were significantly higher than the other GnRHs, approximately 4-fold higher than the sGnRH and 48-fold higher than the cGnRH-II (Fig 10). The same trend was apparent when analyzing the GnRH levels of each treatment group separately. No statistically significant difference was found between the sGnRH and cGnRH-II levels.

Figure 10

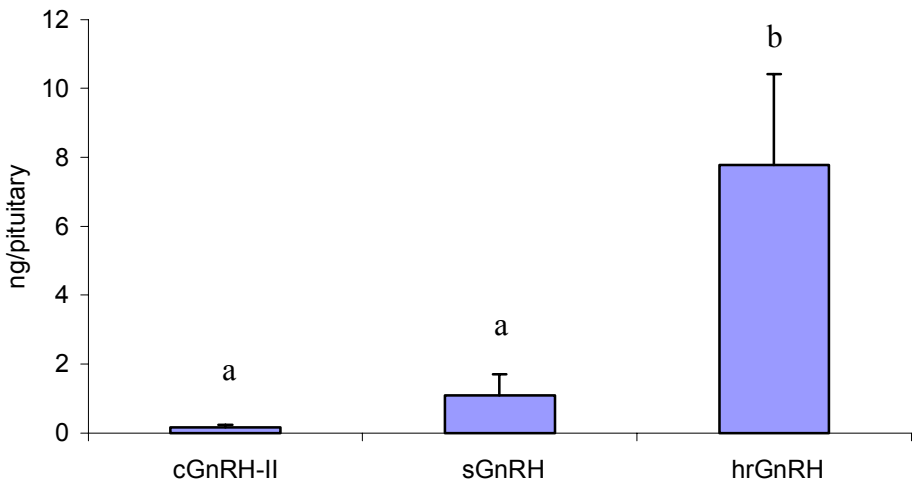


Figure 10

Mean (± SD) of GnRH peptide levels in the captive female shad pituitary (N=24). Sampling occurred on days 2 and 4 post-implantation in both sham and GnRHa-treated groups. Significant differences in mean from the hrGnRH were found for both cGnRH-II and sGnRH (ANOVA, $P<0.01$).

No significant differences were found in the mean of any of the GnRHs between the GnRHa implant-treated group and the sham group. Nor were any significant correlations found between hrGnRH level and gonadal stage or GSI.

III.C.4 Estradiol

As discussed in I.C.2, E_2 levels provide an indication of gonadal development. Therefore assessing E_2 levels provided us with an indication of the efficacy of GnRHa treatment on ovary development. When comparing the GnRHa implant treatment (pooled time points)

to sham treatment, significantly higher levels of E_2 in the plasma of the GnRHa-treated females (Fig 11) is observed. The same result is obtained when separating both time points (data not shown). It should be noted that there was significant variation of E_2 levels within each treatment group.

Figure 11

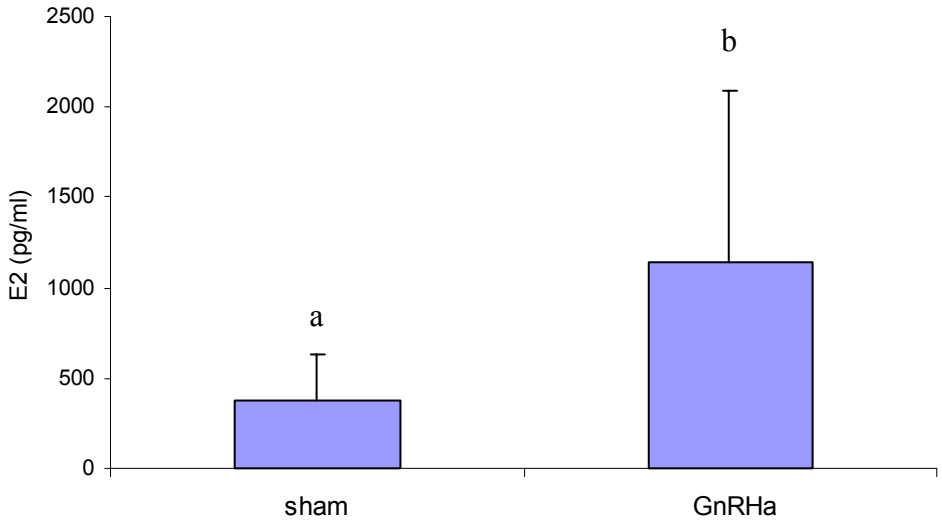


Figure 11

Mean E_2 plasma levels in the sham and GnRHa implant-treated females (days 2, 4 PI grouped, n=12). The E_2 plasma levels in the GnRHa-treated group were statistically significantly higher than in the sham group (Mann-Whitney, $p<0.01$).

The E_2 levels were also found to correlate to the ovary developmental stage. When the most advanced oocytes in the ovary were at vitellogenic stages (Vg I and Vg II), E_2 plasma levels were relatively low (153±0 and 330±169 pg/ml respectively). The same is true for stage 3 (GV migration), 266±140 pg/ml. However plasma E_2 levels rose significantly when the advanced oocytes were at the GVBD and atretic stages

(1472±1062 and 1038±792 respectively). This was true when examining E_2 levels in all females, regardless of treatment or time point (Fig 12).

Figure 12

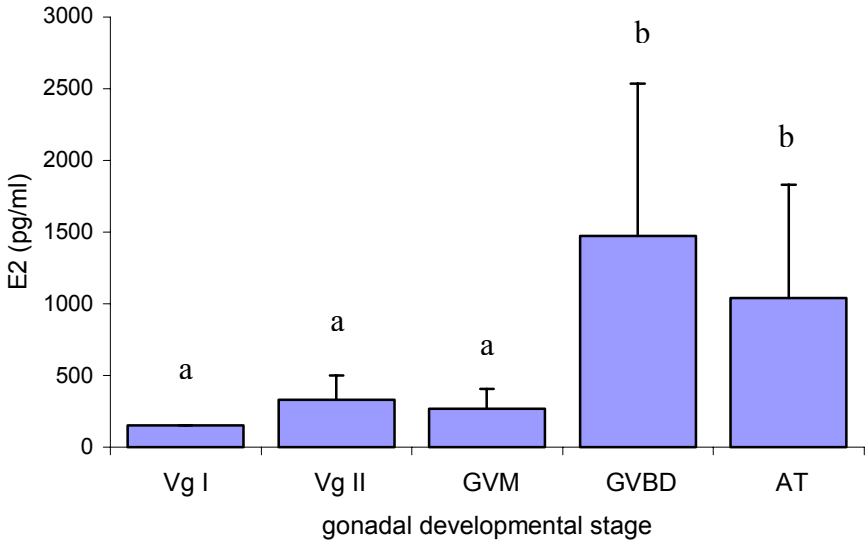


Figure 12

Gonadal stage vs. E_2 plasma levels in hatchery females treated with either GnRHa implants or sham implants (days 2,4 PI). Significant differences in means were found between stages 1, 2, 3 and stages 4,5 ($p<0.05$, ANOVA).

III.C.5 Testosterone

Plasma testosterone levels were measured in shad female plasma in the two treatment groups (sham and GnRHa) at the two time points (2, 4 days PI). In all cases, T levels were below detection threshold (20pg/ml).

III.C.6 Maturation inducing steroid

MIS is an indicator of oocyte maturation, as discussed in I.C.3, the MIS is produced in FOM oocytes. The MIS measured was DHP, as this has been shown to be the predominant MIS in the Pacific herring, which is closely related to the shad. The mean DHP level measured in the GnRHa-treated group was significantly higher than the mean of the sham group; the means of the two groups were 5024 pg/ml and 1800 pg/ml respectively (Fig 13). The variation within the two groups was significant, which is why a non-parametric test was used (Mann-Whitney). When examining the overall correlation in both groups, i.e., between DHP levels and gonad stage, a significant correlation between these two factors was found. The plasma DHP level rose significantly only when the most advanced oocytes in the ovary were at the atretic stage. At his stage the plasma DHP level was 7426 pg/ml compared to an average of 1851 pg/ml in all other stages (Fig 14).

Figure 13

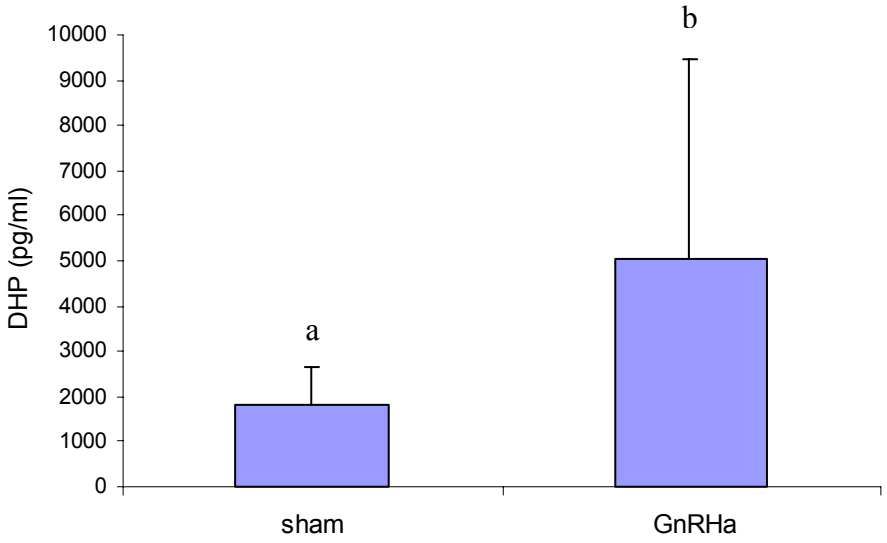


Figure13

Means of the DHP plasma levels in the sham and GnRHa-treated females (days 2,4 PI grouped, n=12). The DHP plasma levels in the GnRHa-treated group were statistically significantly higher than in the sham group (Mann-Whitney, $p<0.01$).

Figure 14

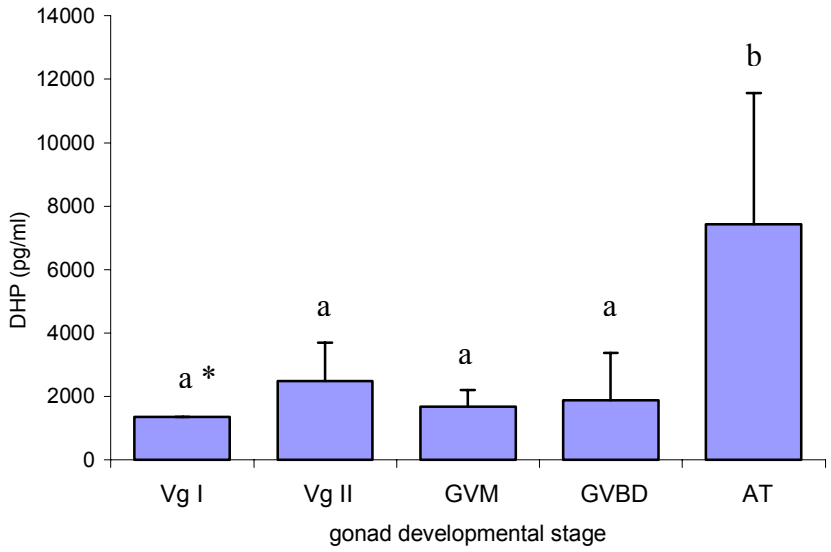


Figure 14

Gonadal stage based on the developmental stage of the most advanced oocytes in the ovary vs. DHP plasma levels in hatchery females treated with either GnRHa or sham implants (days 2, 4 PI). Significant differences in means were found between stages 1, 2, 3, 4 and stage 5. ($p<0.05$, *- $p<0.01$, ANOVA).

III.C.7 Gonadal stages

The assessment of gonadal stage in the hatchery fish was based on microscopic analysis of histology samples taken from the ovary. According to this method (IV.C.2), the assessment of the ovary stage is based on the developmental stage of the most

advanced oocytes in a given ovary. Based on this method, the mean gonad stage was significantly higher in fish treated with the GnRHa implants than in the sham fish (Fig 15). This finding is in agreement with the fact that the GnRHa-treated group had significantly higher fecundity during the hatchery experiment. When comparing the GSI data of the two groups, no significant difference was found. However when comparing GSI and gonadal stage, a significant difference was found, GSI was significantly higher during the GVBD stage (Fig 16). This finding is in agreement with Olney et al., (2001) and with our observations. There were no significant differences of GSI between the other stages, but a trend can be seen in which GSI rises as the ovarian stage progresses, reaches a peak at GVBD (at which point the most advanced cohort of oocytes is hydrated and ovulated), and then starts declines at the advent of the atretic stage.

Figure 15

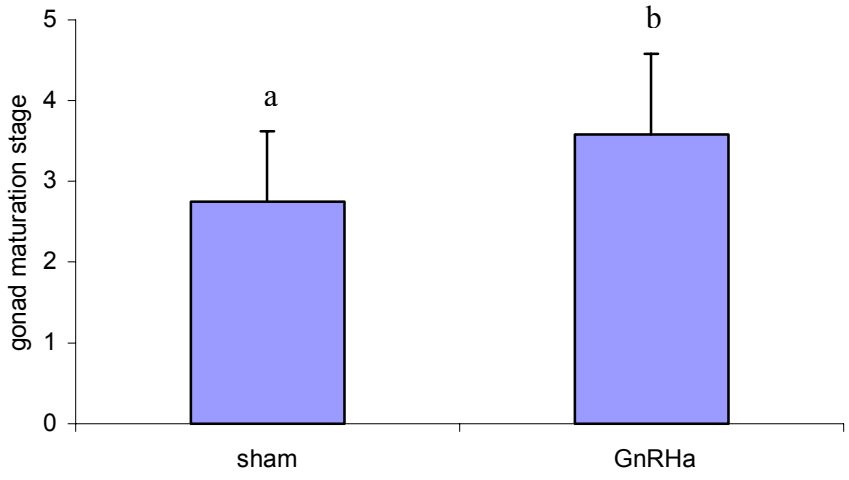


Figure 15

Mean (± SD) of gonadal stage of the captive female shad (n=12) sampling took place at days 2 and 4 post-implantation in both sham and GnRHa-treated groups.

A significant difference in gonadal stage was found between the two groups. (t-test, $P<0.05$).

Figure 16

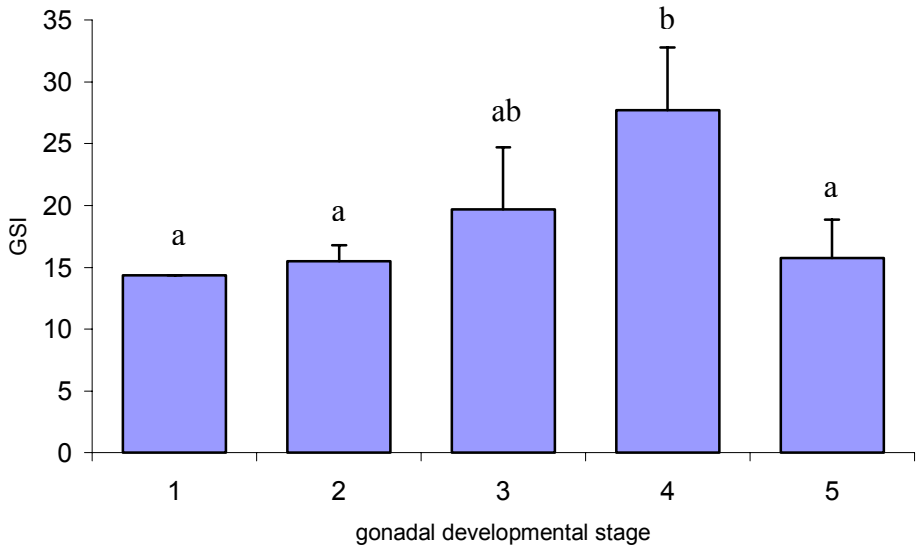


Figure 16

Gonadal stage based on the developmental stage of the most advanced oocytes in the ovary vs. GSI of hatchery females treated with either GnRHa or sham implants (days 2, 4 PI, N=23). Significant differences in means were found between stages 1, 2, 3, 5 and stage 4. ($p<0.05$, ANOVA).

III.C.8 Single female experiment

Establishing the nature of the shad spawning cycle was an additional goal of the hatchery experiments. Mylonas et al. (1995b) and Olney et al. (2001) hypothesize that

shad spawning kinetics consists of batch spawning, as is suggested by the asynchronous ovary morphology, and the kinetics of group spawning. To assess this aspect of reproduction, we placed single females in separate tanks with 2 males. Four of seven females spawned only once during the 10 days of the experiment, the other three females each spawned twice, but in these cases the first spawning event was relatively large, and the second small (Fig 17). The average fecundity per kg body weight (excluding one outlier) was 23,730, which is 75% of the average fecundity in the group setting.

Figure 17

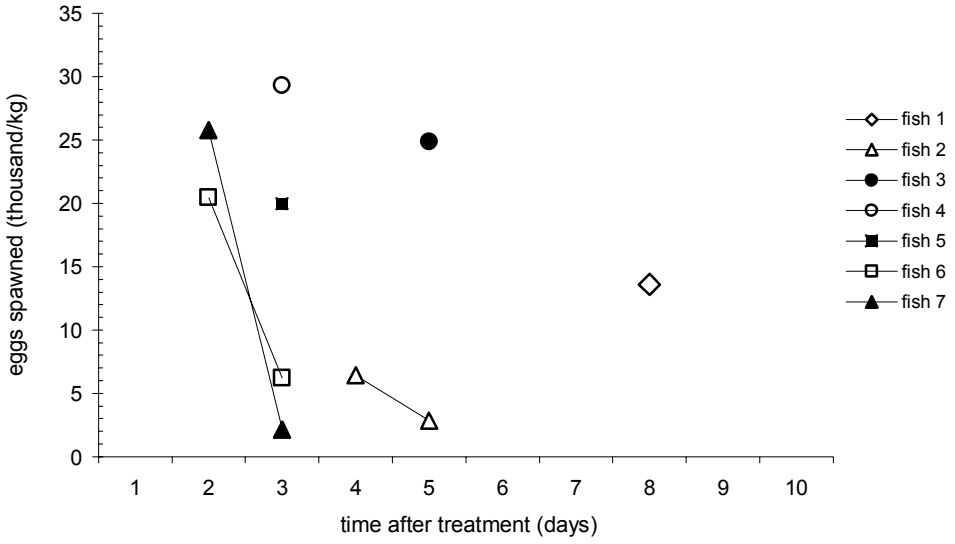


Figure 17

Fecundity of GnRHa-implanted single shad females placed in individual tanks with two males. Spawning was assessed daily for 12 days at the Manning hatchery.

III.D. Discussion

III.D.1 Spawning induction

The induction of spawning in captive fish that do not spontaniously spawn in captivity has been successfully achieved in several teleost species through the use of GnRHa administration (Zohar et al, 1986; Zohar et al., 1990a; Zohar and Mylonas., 2001; Mylonas et al., 1998; Greenwood et al., 2001). One goal of the hatchery induction experiments was to assess the potential for GnRHa treatment to induce spawning in captive female shad. As explained in the introduction, achieving the goal of hatchery-based reproduction is an integral part of establishing a viable restoration or restocking program. Results of the initial treating of shad with GnRHa in a hatchery setting were examined by Mylonas et al., (1995b). This initial study found that GnRHa implants do induce substantial spawning, whereas the control group females achieved very low fecundity. The average fecundity of females treated with GnRHa implants in the Mylonas et al (1995b) study was 53,000 eggs/kg body weight during a time period of 16 days. However, in later years it was reported by hatchery staff that despite using similar GnRHa implants the shad fecundity had been substantially decreasing (Federal aid report #NA66FA0208, 1997). Our hatchery experiments were aimed at re-evaluating the efficacy of the GnRHa implants when used in shad females, and verifying whether broodstock fecundity is indeed decreasing. In addition, our studies investigated the effects of the GnRH treatment on the profile of several key reproductive hormones. Our results show that females treated with GnRHa implants have a 3-fold higher fecundity over the control group.

The actual average fecundity of the treated group was 32,000-eggs/kg body weight in a time period of 12 days. Comparing this result with the 1995b Mylonas data, we see that there is approximately a 40% decrease in fecundity of GnRHa-treated females. Although 40% is a substantial decrease, a variation in fecundity of this magnitude can be the result of many factor, among which is natural fecundity variation. Such natural variations might be associated with the average age of the shad population, water temperature and conditions, overall fish health, etc. This difference is not, however, close to the approximately 92% decrease reported by the hatchery staff (S. Minkknnen, personal communication).

Given our hypothesis, that hatchery shad spawn only one large spawn (III.D.4), our finding of an average hatchery fecundity of 32,000 eggs/kg body weight is in agreement with the assessment of Olney et al. (2001) that the shad's batch fecundity, based on ovarian morphology, is between 25,000 and 80,000 eggs/kg body weight.

Both the spawning data and the fact that there was a significant difference in gonadal stage between the GnRHa group and the sham group strengthen the premise that GnRHa implants remain a reliable and successful tool for inducing shad to spawn in captivity.

Nevertheless, when comparing our data to Mylonas et al. (1995b) data regarding fertilization percentage, a larger difference is apparent. In the Mylonas study, the average fertilization rate was 62%. In our experiments, the fertilization percentage varied substantially, (0%-54%) and averaged approximately 15%. This difference in fertilization has a large impact on the total number of viable larvae produced. This may partially be the reason for the dramatic decrease in the total number of larvae obtained in the hatchery

over the past few years. The reason for this decline in fertilization rate is unknown, it might be a result of diminished sperm quality, water quality problems, etc. Further research is needed to elucidate this issue.

The implanted shad exhibited batch-spawning characteristics, with three distinct spawning peaks observed in females treated with GnRHa implants. The observed spawning cycle of three days (peaks at days 3, 6 and 9), is in agreement with Mylonas et al. (1995b) and Olney et al., (2001), who made empirical assessments that the shad spawning cycle is one of 2 and 4 days respectively.

III.D.2 Implants vs. Microspheres

To assess whether GnRHa implants or GnRH microspheres were a superior delivery system for inducing shad spawning, trials utilizing each delivery system were conducted.

The microspheres and implant GnRHa delivery methods have the same purpose, to deliver a sustained level of GnRH to the pituitary through the blood thereby triggering LH release and normal ovary maturation. Both of these delivery systems are based on a polymer that is combined with the GnRHa, however while the implants release GnRHa for a duration of two to four weeks, the microspheres release GnRHa for a duration of approximately 8 weeks. As a result, the rate of GnRHa release from the implants is greater than from the microspheres. Previous work has shown that both GnRHa microspheres and GnRHa implants cause an elevation of plasma LH levels (Mylonas et al., 1998). However, overall LH levels in implanted fish during the first weeks post-injection were significantly higher than in fish treated with microspheres (Mylonas et al.,

1996), due to the higher GnRHa plasma levels. In our hatchery experiments, the microsphere-treated group spawned poorly, their fecundity was significantly lower than the implanted group and was actually lower than the sham group, despite the fact that the GnRHa dosage/kg was similar in both groups. We hypothesize that the higher levels of plasma GnRHa in the implanted females relative to the microsphere-treated groups caused this difference in fecundity. It seems that the levels of GnRHa in the microsphere-treated group were not sufficient to trigger ovarian maturation and spawning. As GnRHa implants exhibited a superior ability to induce spawning in female shad, this method of GnRHa delivery is recommended for this species.

III.D.3 Hormone levels

In the context of a hatchery setting and especially with regard to fish that are being treated with GnRHa, any analysis of the reproduction hormone profile should be viewed as specific to that setting and not as an accurate reference to hormonal profiles as they occur in the wild. However, this is an important study in assessing the reproductive events and spawning success that are a result of treating captive shad with GnRHa.
The results obtained regarding mean GnRH levels in all hatchery fish prove that hrGnRH pituitary levels are much higher than those of sGnRH or cGnRH II. This indicates that hrGnRH is indeed the hypophysiotropic form of GnRH in shad. In all teleost species known to have three GnRH forms, the "species-specific" form, which is dominant in the pituitary, is also the hypophysiotropic form (Powell et al., 1994; Zohar et al., 1995; Gothilf et al., 1996; Dubois et al., 2001; Okubo et al., 2000). This "rule of thumb" appears to also apply to shad.

Knowing which of the GnRH forms is the hypophysiotropic form is a crucial step in studying the shad's reproduction system and is another step in understanding GnRH evolution and function.

Another interesting finding was that both E_2 and DHP were significantly higher in the GnRHa-implanted group than in the sham group. Both E_2 and DHP are markers of ovary stage progression and oocyte FOM. The fact that both were elevated in the treated group corroborates the efficacy of the GnRHa treatment in regard to hormonal levels. Our assumption is that untreated hatchery shad suffer from capture-induced stress, which in turn causes subnormal hypophysiotropic GnRH release to the pituitary. The low GnRH levels are not sufficient to induce normal LH release into the plasma, the result being underdeveloped oocytes, which do not produce E_2 and DHP. The fact that these two hormones are elevated in GnRHa treated fish strengthens this hypothesis. As we did not have the tools to measure shad LH directly, this hypothesis cannot be verified or disproved. However, based on studies in other fish and our E_2 and DHP results, it can be reasonably inferred that low LH levels are indeed the cause of subnormal fecundity and that administering exogenous GnRH to the HPG system circumvents this problem.

The comparison between hormonal levels and gonadal developmental stage showed that both E_2 and DHP plasma levels are significantly elevated during the later stages of ovarian development. The E_2 plasma levels were significantly higher during the stages of GVBD and atresia, while the DHP plasma levels were significantly higher only at the stage of atresia. When considering this data we must first bear in mind that plasma hormone levels do not directly mirror gonadal hormone level. Levels of gonadal hormones lag behind plasma levels of these hormones.

In many fish species, high E_2 levels are found during vitellogenesis. During late FOM, the E_2 levels drop and a serge in MIS levels occur, which induces resumption of meiosis and GV migration. In our study, elevated E_2 levels were found during the FOM and atresia stages and low levels were found at earlier stages, while DHP levels were elevated only at the atresia stage. There are several possible explanations for this result. The shad is a batch spawner, therefore the dynamics of E_2 and DHP levels differ from fish that are single batch spawners. This hypothesis is corroborated by findings in the asynchronous seabream (Gothilf et al., 1997). In the seabream, cohorts of oocytes at different developmental stages exist simultaneously in the ovary. When following the development of the most advanced cohort of oocytes, a distinct hormonal profile becomes apparent. Both DHP and E_2 levels rise during the later maturation stages (GVM and GVBD) of this distinct cohort. The hypothesis to explain this hormonal profile is that the cohorts of oocytes undergoing the last stages of FOM produce DHP, while cohorts undergoing vitellogenesis produce E_2 at higher levels than normal, thus overcoming the inhibitory effect caused by DHP. This hormonal pattern is distinctly different than the pattern observed in most group synchronous fish. A similar phenomenon seems to take place in female hatchery shad.

An additional factor is that 50% of the fish sampled were treated with GnRHa implants. In some synchronous (salmon) and group synchronous (sea bass) fish, prolonged administration of GnRHa induces a reduction in E_2 levels. This phenomenon is attributed to the fact that as soon as all oocytes reach a certain stage, they switch from E_2 production to DHP production. However in asynchronous species, we would expect to see elevation of both E_2 and DHP in response to prolonged GnRHa stimulation similar to

that seen in the American shad. This occurs because both vitellogenic and FOM oocytes are found in the ovary and produce E_2 and DHP in response to elevated GnRH levels. It is interesting to note that the T levels in female fish were not elevated at any time point. T is typically, briefly elevated in response to rising LH levels and is then aromatized into E_2. This result will be discussed further in chapter IV.

In addition to higher fecundity and elevated hormonal levels, the average gonad stage was significantly higher in the GnRHa treated group. This finding strengthens the assumption that GnRHa implant treatment alleviates the fecundity problem of captive shad, by restoring a normal hormonal profile. This is consistent with the hormonal profile as found in GnRHa treated females.

Finally, a significant correlation was found between gonadal stage and GSI. This connection verifies that both GSI and gonad stage, based on the most advanced cohort parameter, are good indications of ovarian stage and development. This finding corroborates the Olney et al., (2001) findings that both these criteria are in good agreement.

III.D.4 Single female experiment

The natural interval between spawning episodes in shad females has yet to be experimentally established. Based on ovary histology, Olney suggested that the interval is approximately four days; Mylonas et al., (1995b) on the other hand suggested a cycle of two days based on observations of group spawning dynamics in the hatchery. When examining the spawning cycle in the group setting a cycle of three days was observed, in which a spawning peak occurred on days 3, 6 and 9, but substantial spawning activity

took place also during other days, suggesting that shad do not spawn synchronously at the population level. To get a better idea of what the spawning interval actually is, we separated seven female shad and placed them in individual tanks each with two males. The spawning results, during a duration of 12 day trial were surprising, of the seven fish, four spawned only once and three spawned twice, the large spawning episodes occurred at different days post-implantation (2, 3, 5, 8). The second spawn in the three fish that spawned twice was very small, averaging 3700 eggs/kg body weight, and occurred one day after the large spawning episode. The single spawns produced during this trial were on the average of 23,730 eggs/kg body weight, which is 74% of the total average fecundity of shad in the implanted group. This suggests that the spawning peaks in the group experiment are actually not repetitive spawning by the same fish, but single fish spawning at different time points. This would mean that group batch spawning is not actually taking place, and that the three-day cycle of spawning peaks that we observed is not a batch spawning cycle but individual females spawning once, therefore is not actually a cycle. However, this hypothesis, that the shad release only one major spawn and have maybe one or several additional small spawning episodes, is not corroborated by the gonad physiology. There are three plausible explanations for these findings. One is that stress and physical deterioration (resulting in ovarian atresia) prevents the shad from spawning more than one large spawn. The second is that this abnormal spawning dynamic is due to the administration of exogenous GnRHa. It is however important to note that GnRHa treatment in other asynchronous species (sea bream, sea Bass), induced multiple spawning episodes (Zohar and Mylonas, 2001). The last is that the spawning frequency and volume per spawn differ between the single female setting and the group

setting. Social dynamics and pheromones have been shown to be very important in fish spawning behavior. Moreover the lack of these elements in a single female environment might cause abnormal spawning behavior and ovary development (Carolsfeld et al., 1997).

Our hypothesis is that the spawning behavior observed in the single female experiment, does not represent the spawning cycle as it occurs in nature. Our results may indicate that we can expect only one large spawn from shad females captured on the spawning grounds and brought to a hatchery setting, even when treated with GnRHa. However, the spawning dynamics of domesticated captive shad treated with GnRHa, that are not caught and transferred to the hatchery immediately prior to spawning, could very well be natural and comprised of multiple spawning episodes.

Chapter IV. Evaluating the wild population

IV.A Introduction

Our second major goal in this study, in addition to studying the feasibility of 60 GnRHa-induced, hatchery based shad spawning, was to gain some basic insight into shad female reproduction as it occurs in nature. In the hatchery setting, the hormonal profile and the gonad development is most probably altered due to stress and/or GnRHa treatment. Therefore we used shad sampled in the riverine setting to conduct this part of the study. There are only two studies to date that focus on shad reproduction, one from our group (Mylonas et al., 1995b) that described some basic work regarding the use of GnRHa in shad in the hatchery setting, and the other by Olney et al. (2001) that conducted extensive work on wild shad reproduction focusing on the ovarian morphology and development. Neither study examined shad reproductive endocrinology, hence no knowledge of the shad HPG axis or its action is available. However, such data is central to efforts to maximize the reproduction capabilities in captivity, for understanding shad reproduction in the wild, and for obtaining a better understanding of the action of the HPG axis in this species.

It is important to note that the shad is both an anadromous fish and has an asynchronous ovarian morphology. In general, the reproductive endocrinology of species that have these traits is poorly understood. Thus, continued study of the shad model may pave the way for a better understanding of other species with similar characteristics.

We attempted to compile reproduction-related data from wild shad caught at the Conowingo Dam in view of analyzing the HPG axis of females conducting the spawning migration. To achieve this goal, we analyzed several levels of the HPG axis and

attempted to integrate this data so as to gain some insight into the shad's reproductive endocrinology and reproductive physiology.

IV.B. Materials and methods

IV.B.1 Experimental design

We obtained adult American shad females from the Susquehanna River, Hartford County, Maryland. These females were collected at the Conowingo Dam during the spawning migration at two time-points, at the beginning of the spawning migration (4/27/02) and at its end (6/8/02). The first sampling consisted of 20 females and the second of 10 females. Several parameters and tissue samples were collected from each fish including weight, length, gonad sample, pituitary and blood for hormonal analysis.

IV.B.2 Animal sampling

The shad used for analysis were in the midst of the spawning migration when collected. They were caught the day prior to sampling in the dam's fish lift, and kept over night in a flow through holding tank supplied with water from the river. On the morning of the sampling, fish were netted and anesthetized using 2-phenoxyethanol at a concentration of 1ml/l, followed by collection of a 3ml blood sample from the caudal vasculature using a heparinized syringe. The blood was placed on ice in vials containing 100 µl of a 3mg/ml solution of aprotinin (Sigma Chemicals Co., St. Louis, MO) to prevent hormone degradation. Plasma was separated by centrifuging the blood in the lab for 15 min at 4000 x g at 4°C, the plasma was then put in 200µl aliquots at –80°C.

The fish were weighed and fork length was measured, followed by a prompt decapitation. We removed the brain and the pituitary, placed each in an Eppendorf tube, and immediately froze the samples in liquid nitrogen. The brain and pituitary samples were stored at –80°C. The whole ovary was removed, weighed, and a sample of ovarian tissue from both lobes was collected and placed in a 4% formaldehyde, 1% gluteraldehyde fixative (4F:1G) (McDowell and Trump, 1976) for later histological examination.

IV.B.3 Histology

The ovarian fragments were dehydrated in a 75-90% ethanol series over a period of 3 days and then embedded in glycol methacrylate plastic (JB-4 Mini Kit, Polysciences, Inc., Warrington PA, USA). The embedded tissues were cut into 3-4µm serial sections on a microtome (Microm, HM 340, Portsmouth, NH). The sections were stained with methylene blue/basic fuchsin (Bennett et al., 1976), and inspected using a compound microscope at 4-10x magnifications. These sections were used to assess oocyte diameter and oocyte morphology.

IV.B.4 Hormone measurements

The hormones measured were GnRH peptide levels in the pituitary, and testosterone, E_2, and DHP in the plasma. The measurements of the GnRHs and the DHP were carried out according to the assay procedures specified in chapter II.C. E_2 and T were measured using commercial E_2 and T kits for plasma hormone mesurments (Coat-

A-Count Total Testosterone/Estradiol, Diagnostic Products Corporation, Los Angeles, CA).

IV.B.5 Statistics

GnRH levels were compared using a one-way ANOVA for independent samples, followed by a Tukey's HSD post hoc test. For correlation tests, the Pearson product-moment correlation coefficient was applied. Within this test we used log transformtion to normalize E_2 and T results. All two-way comparisons were analyzed using a t-test with the exception of the DHP two-way comparison, for which we used an ordinal Mann-Whitney test due to data variation. Letters above bars represent statistically significant differences between means.

IV.C. Results

IV.C.1 Gonado-somatic index and ovarian development stages

The ovary development during the spawning migration can be broken down to several stages based on macroscopic evaluation of the size, shape and color of the ovary, as well as the presence of hydrated oocytes (which can be detected by the naked eye). Our evaluation was broken down into the three distinct stages (Fig 18) noted below:

Maturing ovary: Ovary contains oocytes at different stages of maturation (based on microscopic examination), but no fully hydrated oocytes. The external color of the ovary is orange.

Hydrated ovary: Ovary contains oocytes at different stages of maturation, among them multiple hydrated oocytes that are large and translucent. These oocytes can be seen throughout the ovary

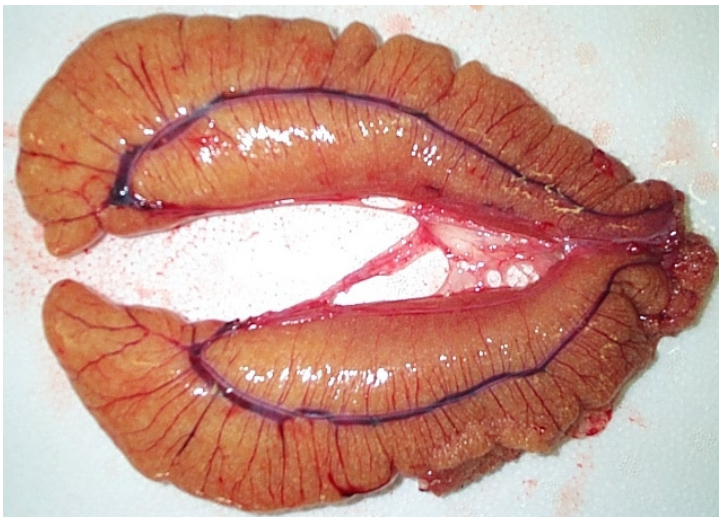
and are surrounded by oocytes of less developed stages. The external color of the ovary is reddish.

Ovulating ovary: Ovary contains oocytes at different stages of maturation. All the fully hydrated oocytes have been ovulated and are concentrated in the lateral area of the ovary in proximity to the oviduct and in the oviduct itself. The external color of the ovary is dark red.

These three distinct stages were mirrored by differences in GSI (Fig 19). The GSI of hydrated ovaries was significantly higher than the GSI of maturing ovaries. We did not have a sufficient number of ovulating ovaries to show a statistically significant difference in GSI, however we observed that the GSI of these ovaries was lower than that of the hydrated ovaries.

An additional method of assessing ovarian developmental stage is cytological based assessment of the oocytes in the ovary as discusses in chapter III. When comparing this staging to GSI there was a significant correlation between GSI and cytological-based gonad staging (Fig 20). The trend observed in this comparison was identical to the results obtained form the hatchery shad. The GSI rises with the developmental stage of the ovary, peaking at the GVBD stage, and then declining at the atretic stage. There was a statistically significant difference between the means of group 2 (Vg I) and group 4 (GVBD).

Figure 18 A.



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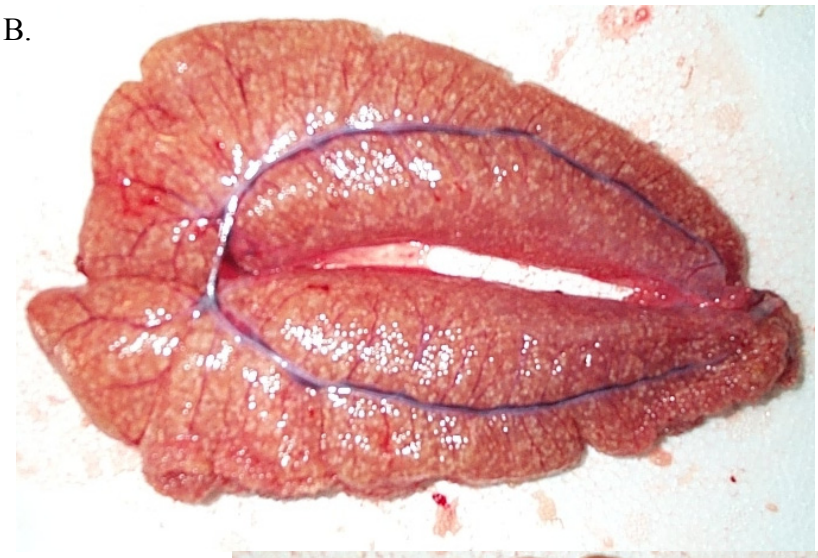

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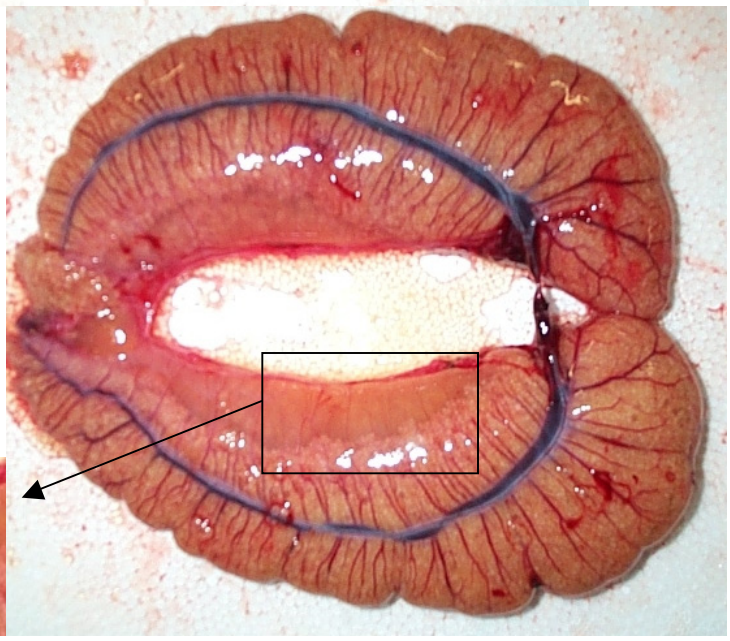

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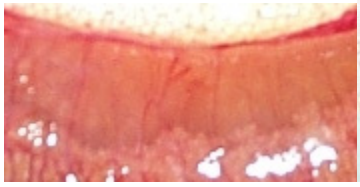


Figure 19

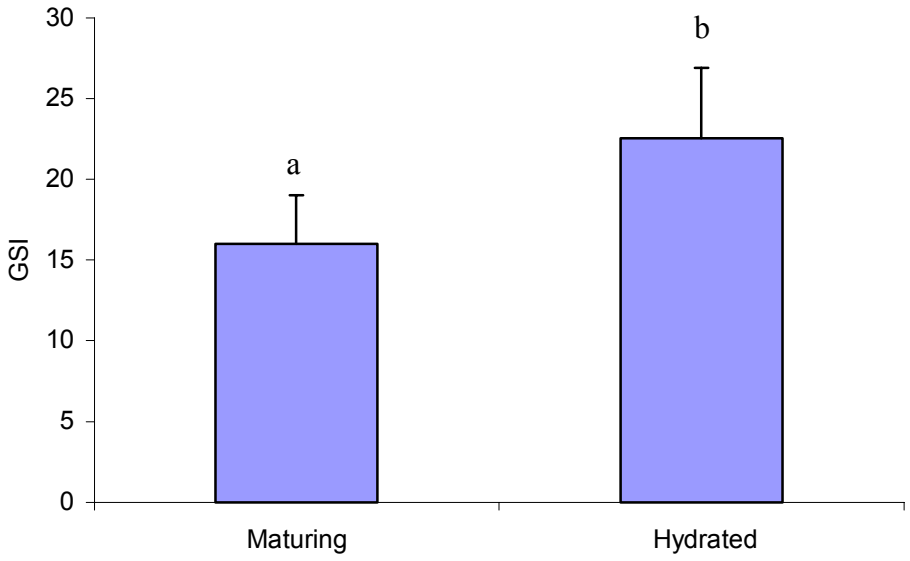


Figure 18

Three distinct stages of ovary maturation during the spawning migration: maturing ovary with orange color and no hydrated /ovulated oocytes (A), hydrated ovary with reddish color and interspersed with hydrated oocytes (B), ovulating ovary, with reddish color, all hydrated oocytes have ovulated and are concentrated in the lateral part of each ovary in proximity to the oviduct (C), enlargement of the area containing the ovulated oocytes in an ovulated ovary (D).

Figure 19

Mean (± SD) GSI of female American shad (N=18) collected at first time point. Ovaries are divided into two categories based on macroscopic evaluation (maturing/hydrated). A significant difference in GSI was found between maturing ovaries and hydrated ovaries. (t-test, $p<0.05$).

Figure 20

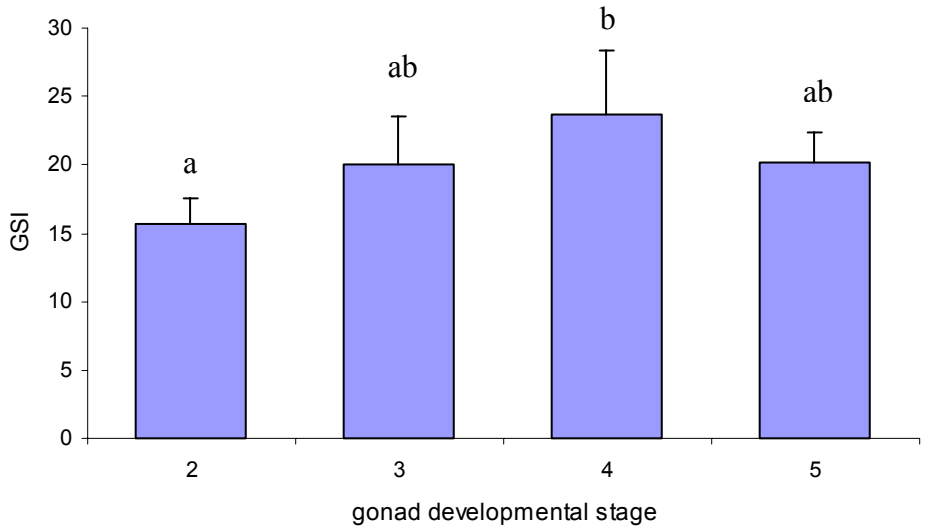


Figure 19

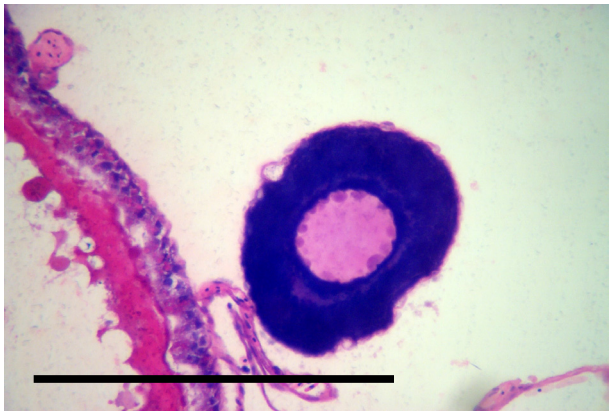
Gonad stage based on the developmental stage of the most advanced cohort of oocytes in the ovary vs. GSI. Females (N=20) collected at Conowingo Dam. Significant difference in means was found between stage 2 and stage 4. ($p<0.01$, ANOVA).

IV.C.2 Oocyte development

Another method of assessing ovarian developmental stage is by microscopically determining the developmental stage of the oocytes present in the ovary. By examining the oocyte cytology and diameter after microtome sectioning, we defined the oocyte developmental stages based on the criteria established by Mylonas et al. (1995b). This definition consists of five distinct stages of oocyte development, as discussed in I.B.2, which can be distinguished by cytological differences and diameter. We expanded this definition into six stages. The six stages are: primary growth (PG) and pre-vitellogenesis (pre-Vg), early vitellogenesis (Vg-I), late vitellogenesis (Vg-II), germinal vesicle migration (GVM), GV breakdown and hydration (GVBD), and atresia (AT) (Fig 21). Each of these stages of oocyte development was found to have distinct cytological characteristics and oocyte diameters (Table 3, Fig 21).

Figure 21

A.



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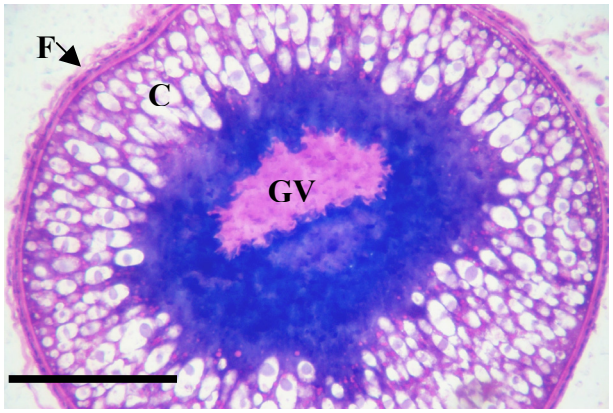

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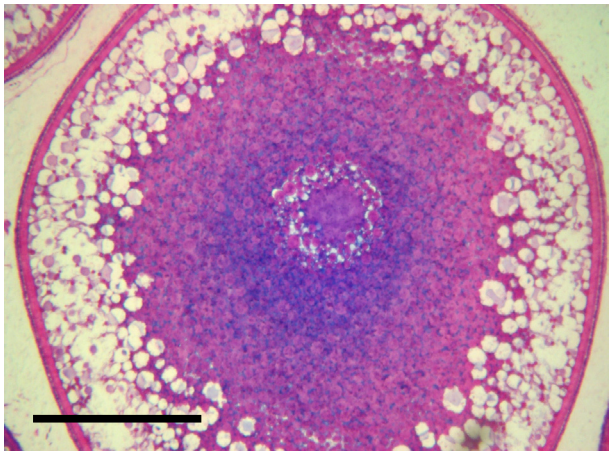

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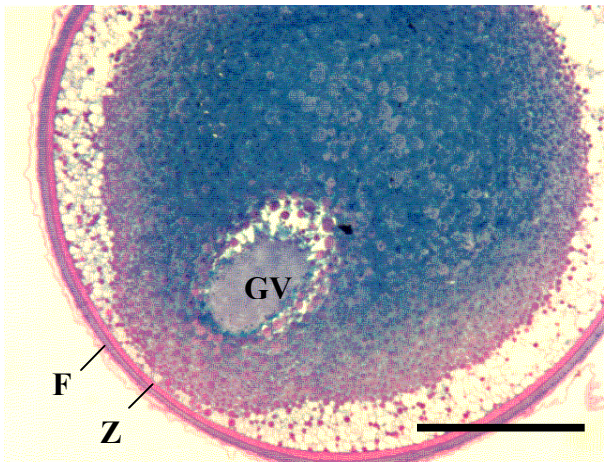

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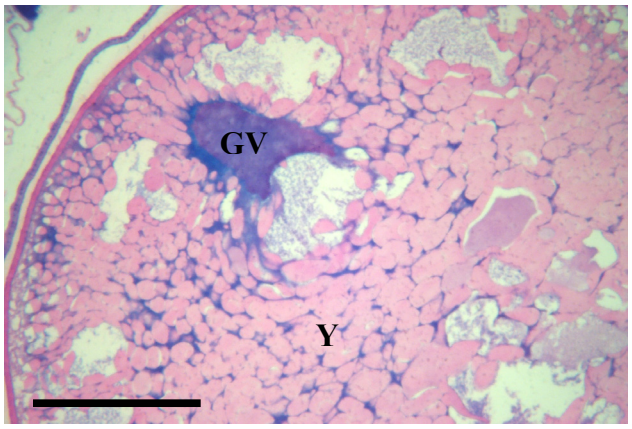

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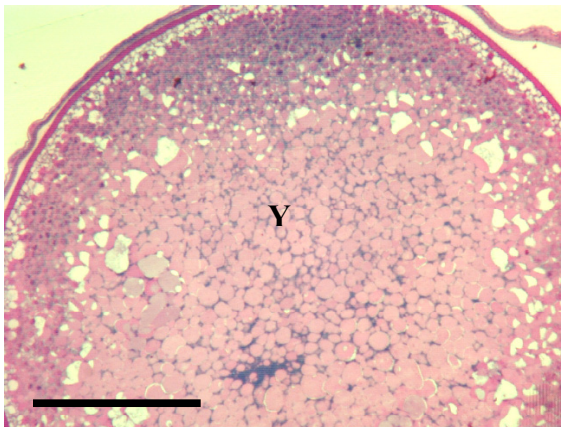

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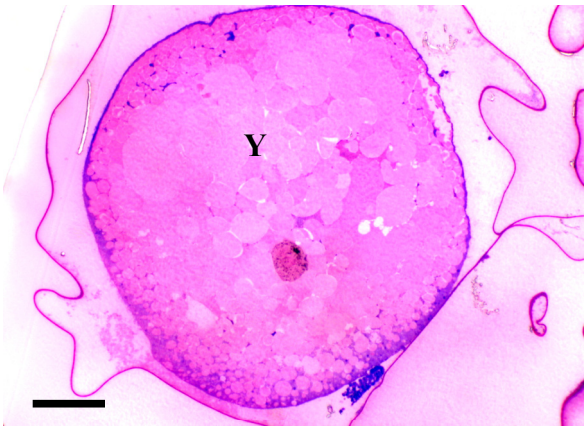

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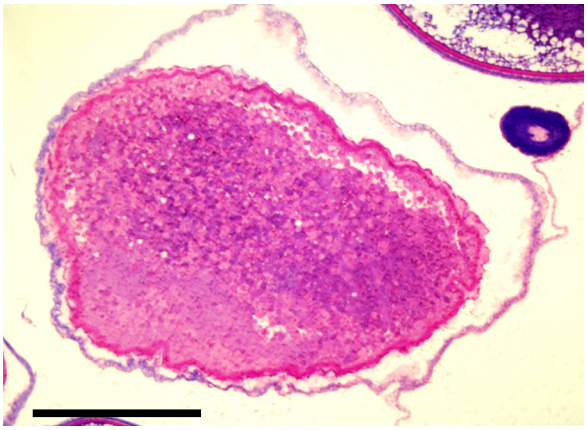

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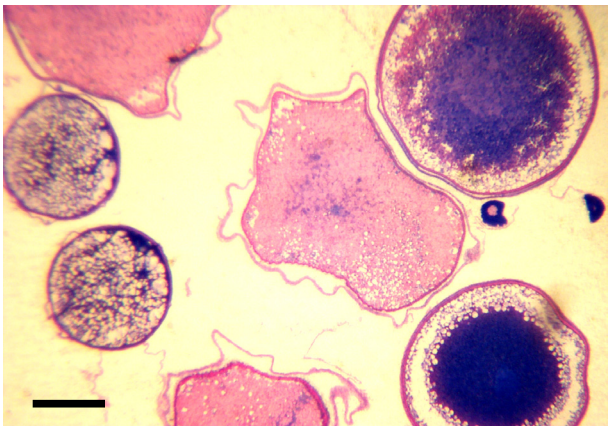

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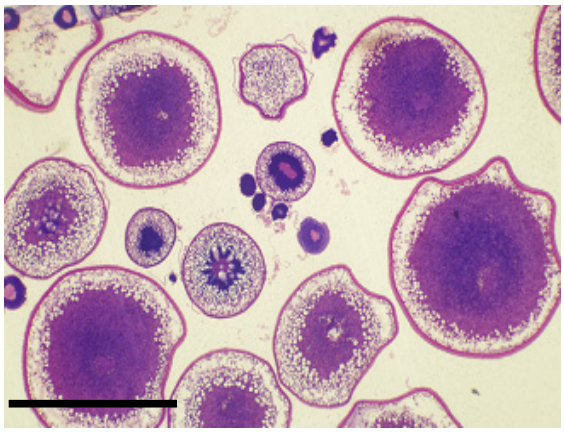

Figure 21

Light photomicrograph of oocyte sections from American shad. The photos show oocytes at various stages of development, including primary growth (PG) (A), vitellogenesis I (B), vitellogenesis II (C), germinal vesicle migration (D), coalescence of lipid droplets and GV breakdown (E, F), Fully hydrated and coalesced oocyte with no GV (G), atretic oocyte (H), and lower magnification photomicrograph of different oocyte developmental stages found simultaneously in an ovary section of two specimens (H, I). Cytological structures within the oocyte are marked as, follicular layer (F), zona radiata (Z), cortical

alveoli (C), germinal vesicle (GV), yolk globules (Y). Oocytes were embedded in glycol methacrylate and 5µm sections were stained using methylene blue/basic fuchsin. The bar at the bottom of each photograph represents 500µm.

These six stages can be defined both by different cytology and by substantial differences in diameter (Table 3). As in the hatchery females, when examining oocytes in a given specimen it is clear that the shad has an asynchronous ovary, as multiple oocyte developmental stages are present in the same ovary. The percentage of the oocytes at specific stages is significantly different between females, as exemplified in Figure 22. Nearly all females haves all stages of oocyte development present in the ovary. However, the percentage of oocytes of each given stage varies considerably, from a situation in which the majority of oocytes are at the vitellogenesis-I stage, to a situation in which the majority of oocytes at the atretic stage. These differences mirror a cycle of development in which advanced oocytes are spawned and then the next cohort of oocytes progresses to hydration and ovulation.

Table 3

Cytological differences between oocyte stages:

	Oocyte stage					
	Primary growth and Pre Vg[a]	Vg I	Vg II	GV[b] migration[c]	GV breakdown and hydration	Artesia
Diameter µm	<800	800-1,000	1,000-1,350	1,350-1,600	>1600	800 - 2400
GV visibility	Yes	Yes	Yes	Yes	Partial or None	No
GV position	Central	Central	Central	Migrating or peripheral	Disappeared or breaking down	None
Ooplasm	Dark	Cortical alveoli	Yolk Droplets	Yolk droplets	Yolk globules	Yolk globules
Zona radiata	Thin (<8µm)	Thick (>8 µm)	Thick	Thick	Thick	Disintegrating
Follicular layer	Thin (<15µm)	Thin	Thin	Thick (>20µm)	Thick	Disintegrating

[a] Vg – Vitellogenesis
[b] GV–germinal vesicle
[c] "Migration" indicates GV position between center of the oocyte and its periphery

Figure 22

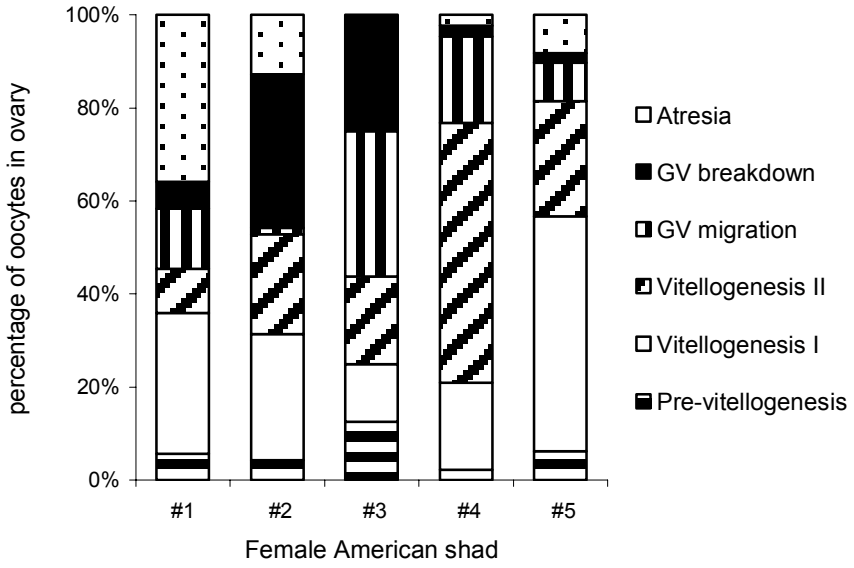

Figure 22

Quantitative analysis of oocyte stage, in ovary samples taken from five different American shad females. The relative percentage of oocytes of various stages is shown in these five ovaries.

IV.C.3 Gonadotropin releasing hormone

Pituitary GnRH peptide levels were measured in the female wild shad caught at both time points. The mean hrGnRH level (±SD) of all females (N=30) was 6.77 (±3.7) ng/pit, whereas the mean sGnRH level was 0.86 (±0.53) ng/pit and the mean cGnRH-II level was 0.153 (±0.05) ng/pit. This represents a statistically significant difference between the hrGnRH and both sGnRH and cGnRH-II (fig 23). There was no statistically significant difference between the level of the sGnRH and cGnRH-II in the pituitary. When examining a possible correlation between the hrGnRH and GSI or ovarian stage, no significant correlation was found.

Figure 23

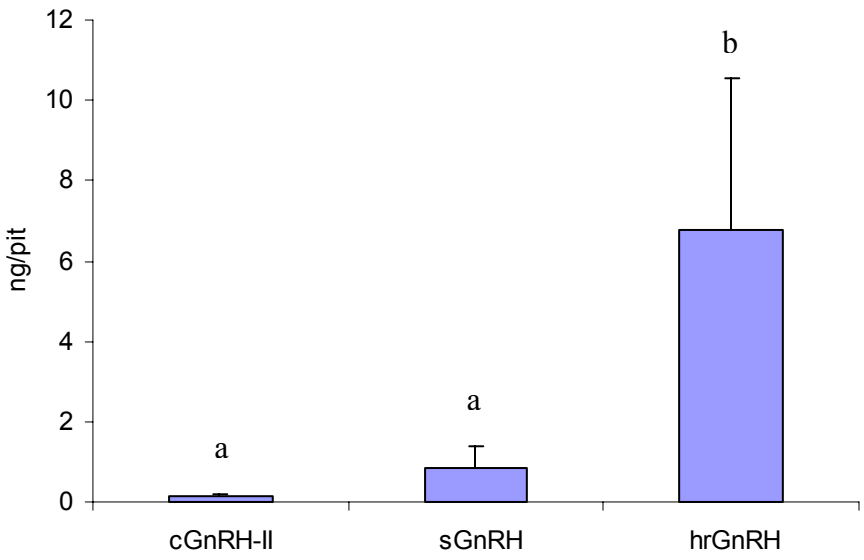

Figure 23

Mean (± SD) of GnRH peptide levels in wild female shad pituitaries (N=30) sampled at Conowingo Dam at two time points. For each GnRH, significant difference in mean from the hrGnRH mean is indicated.

(ANOVA, $P<0.01$).

IV.C.4 Estradiol, testosterone and maturation inducing steroid

As we have shown, GSI is correlated to ovarian stage, in addition, it is well established that GSI is a good indicator of ovarian development. Therefore, we used this parameter to examine variations in hormonal levels as a function of ovarian development. As stated earlier, the correlation between ovary stage and GSI is not a linear one. As can be seen in figures 16 and 20, both in hatchery and wild shad this correlation is linear up

to the stage of GVBD at which point GSI declines (atretic stage). In view of this fact, and as we were using Person's linear correlation, we excluded females that were found to be at the atretic stage from the following statistical analysis.

When measuring E_2 levels, we found that they were statistically significantly correlated to the GSI of the females. This correlation had strong negative relationship, The higher the GSI, the lower the E_2 levels in the female shad plasma (Fig 24).

An opposite correlation was found in the relationship between GSI and DHP. Here we also found a significant correlation, however, this correlation was a strong positive one, the higher the GSI the higher the DHP levels (Fig 25).

As in the captive shad, T levels were below detection level in all samples.

Figure 24

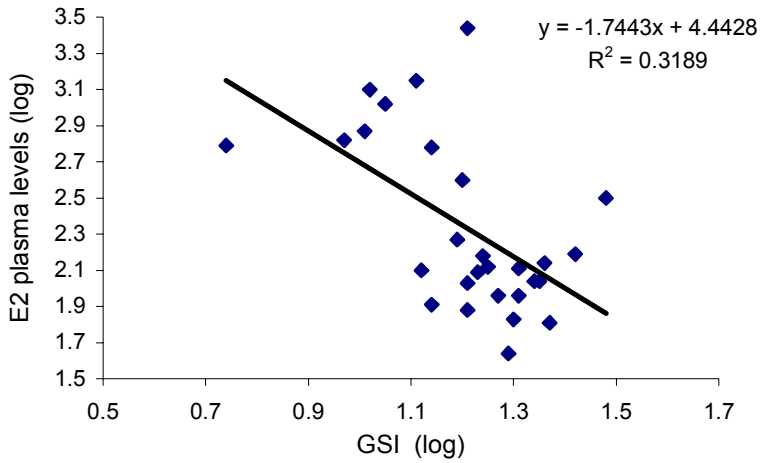

Figure 24

A statistically significant, strong negative correlation exists between GSI and E_2 in wild shad females. Females were collected at Conowingo Dam at two time points (N=29). (r = 0.565, p=0.01, Pearson's r).

Figure 25

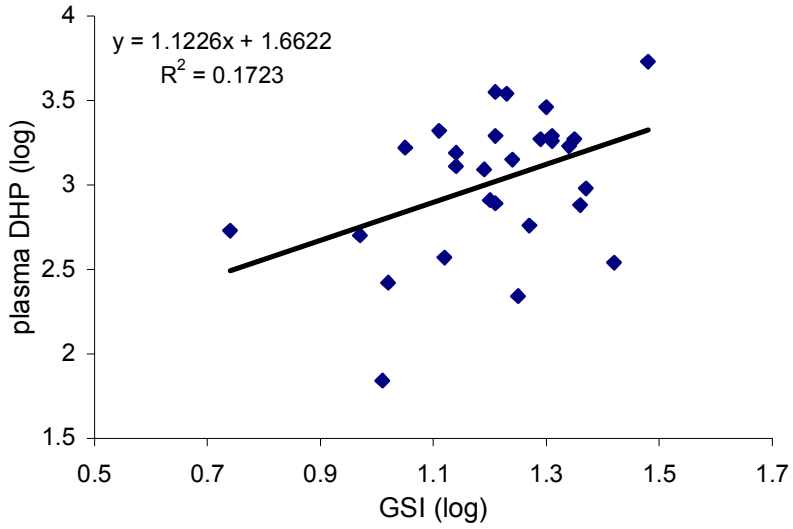

Figure 25

A statistically significant, strong positive correlation exists between GSI and DHP in wild shad females. Females were collected at Conowingo Dam at two time points (N=29). (r = 0.415, p=0.05, Pearson's r).

IV.C.5 Comparing early and late season

An additional aspect of our study of the female shad reproductive cycle was a comparison of physiological and hormonal profiles of shad caught at the beginning and the end of the spawning migration season. We conducted this comparison by sampling 20 female shad at the beginning of the spawning season (4/27/02) and 10 female shad at the end of the season (6/8/02). Mean fork length and weight (± standard deviation) for beginning of the season females was 49.3 (± 2.8) cm and 1.82 (± 0.37) kg, respectively, and for end of the season females 48.8 (± 2) cm and 1.24 (± 0.25) kg, respectively. No

statistical significance between the mean lengths of the two groups was found. However a significant difference was found between the mean weights of the two groups, the earlier group was substantially heavier 1.82 (± 0.37) vs. 1.24 (± 0.25) (Fig 26).

We also found a significant difference between the mean GSI of the two collection dates. The average GSI for beginning of season females was 19.6 ± 4 and for the end of season females was 10.4 ± 3.7 (Fig 26). The difference in GSI did not account for the total difference in body weight.

Figure 26

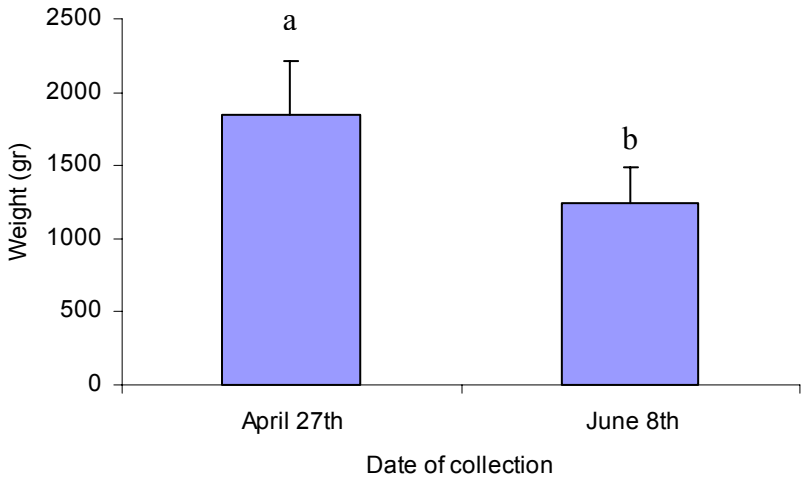

Figure 27

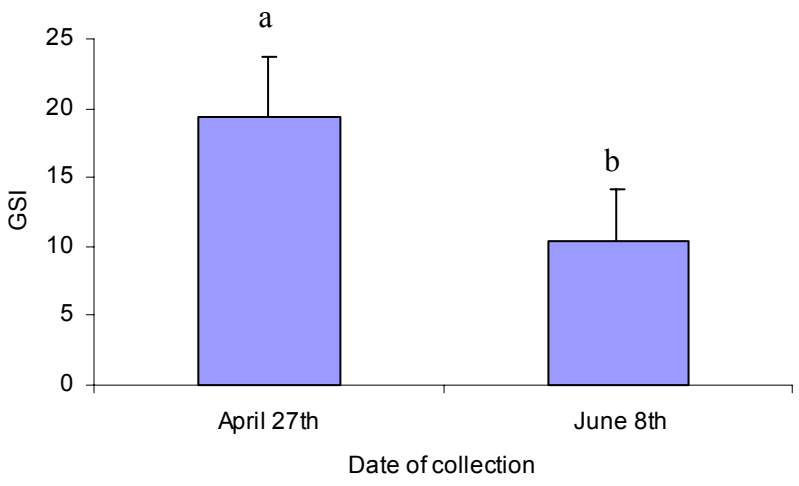

Figure 26

Mean (± SD) weight of wild shad females collected during early spawning migration (n=20) or late in the season (n=10). Sampling took place at Conowingo Dam. A significant difference in mean weight was found between the two groups. (t-test, $P<0.01$).

Figure 27

Mean (± SD) GSI of wild shad females collected during early spawning migration (n=20) or late in the season (n=10). Sampling took place at Conowingo Dam. A significant difference in mean weight was found between the two groups (t-test, $P<0.01$).

Examining the hormonal levels in the female plasma at both collection dates, several differences were apparent. The average E_2 level of the females collected at the end of the spawning season was substantially higher (9875 ± 702 pg/ml) than in the plasma of females collected at the beginning of the season (2902 ± 122 pg/ml) (Fig 28). The average DHP levels were found to be higher in females from the beginning of the season (1745 ng/ml) than in females caught at the end of the season (890 ng/ml). No significant difference in pituitary GnRH levels was found between groups.

Figure 28

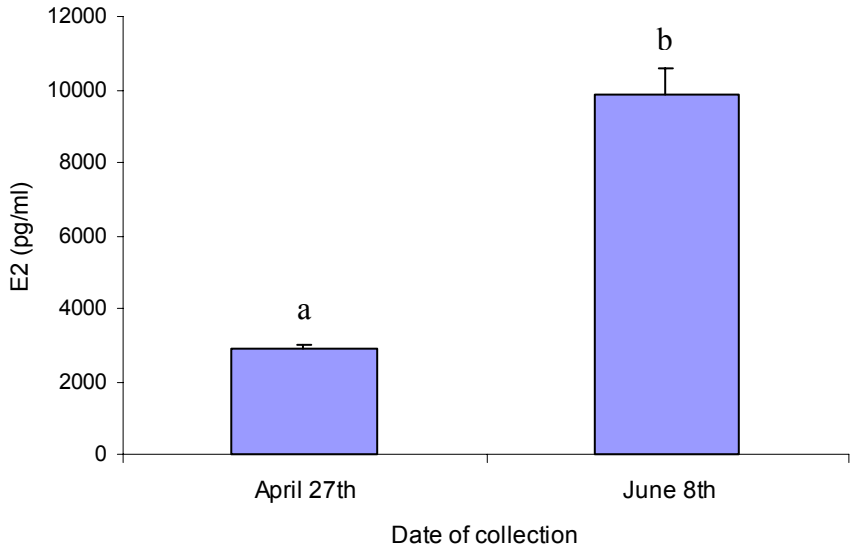

Figure 29

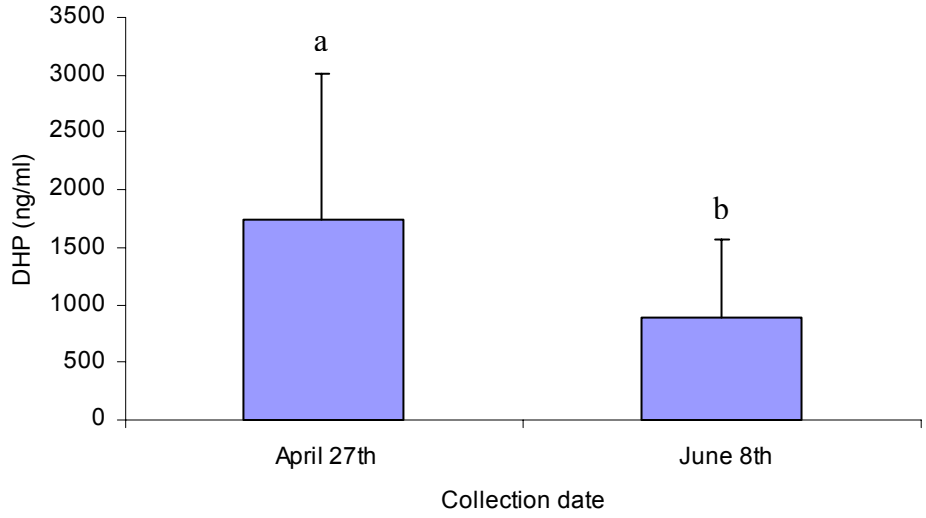

Figure 28

Mean (± SD) E_2 plasma levels of wild shad females collected at Conowingo Dam. Shad were collected during early spawning migration (n=20) or late in the season (n=10). The mean E_2 plasma level in the beginning of the season was significantly lower than mean E_2 plasma level in the end of the season (t-test, $P<0.01$).

Figure 29

Mean (± SD) DHP plasma levels of wild shad females collected at Conowingo Dam. Shad were collected during early spawning migration (n=20) or late in the season (n=10). A significant difference in mean DHP levels was found between the two groups. The mean DHP plasma level in the beginning of the season was significantly higher than mean DHP plasma level in the end of the season (Mann-Whitney, $p = 0.05$).

IV.D. Discussion

IV.D.1 Ovary stages

In this study, we focused on the American shad ovary during the spawning migration. Within this time frame, the ovary is relatively well developed. The migrating shad female enters the estuary with the ovary already at advanced stages of early vitellogenesis. All ovaries examined contained oocytes at multiple stages of development. As such, differentiating between the ovaries and assigning each ovary a specific developmental stage is extremely difficult.

A gross assessment of ovarian developmental stage can be done by macroscopic evaluation. Using this method, three distinct stages were observed during the spawning migration. These stages are characterized, by maturing, hydrated and ovulated ovaries, as described in IV.C.1. This finding is in agreement with the gonadal stages during the spawning migration as defined by Olney et al., (2001).

However, these relatively crude characterizations of ovarian stage do not reflect the subtleties of gonadal development. The developmental physiology of the

asynchronous ovary is known to be driven by the endocrine influences of the ever-changing oocyte populations. Thus, a more precise evaluation of ovarian development (i.e., oocyte population structure) is important in order to better define changes in the ovarian physiology and corresponding endocrine changes.

Another, more precise, method of classifying ovary stage is using GSI. This method seemed to work better in regard to classifying the wild shad ovarian developmental stage, but is still insufficient as a tool to precisely correlate hormonal levels to ovarian status.

A third method is using a random oocyte biopsy for a microscopic assessment of oocyte stages, based on cytology. By using this method, we are able to determine precisely which stage of development the most advanced oocytes have reached, thus we can create a numerical scale that defines a specific stage of ovarian development. When attempting to correlate this scale to hormonal levels, we attained significant results in the hatchery population but insignificant results in the wild groups.

In summary, shad ovarian developmental stages can be assessed both by macroscopic and microscopic methods. Each of these methods have some merit and, for the most part, have been shown to be in agreement with each other, as shown by Olney et al., (2001). However, as we will discuss in IV.D.3, correlating the ovary stage, based on the above techniques, to a hormonal profile is a difficult endeavor. Our findings do corroborate the hypothesis that the American shad is a batch spawner based on it's asynchronous ovarian development, and also that the shad ovaries are well developed throughout the spawning migration.

IV.D.2 Oocyte development

Oocyte development in shad is parallel to documented oocyte development in many other oviparous teleost species. When examining histological slices of individual oocytes, we can clearly categorize the oocyte stage based of cytology and size. The oocytes exhibit seven distinct developmental stages as described. The development can be grossly categorized as dormant stages (primary growth and pre-vitellogenesis), stages of vitellogenin accumulation (Vitellogenesis I and II), final FOM in which the GV migrates and breaks down, lipid droplets coalesce and the oocyte is hydrated and ovulated and lastly spawning and atresia.

In every specimen examined several of these stages, if not all of them, were simultaneously present in the ovary, hence the term asynchronous ovarian development is appropriate for the shad. The available data leaves little doubt that *Alosa sapidissima* is a batch spawner. The relative percentage of different oocyte developmental stages within each ovary varied widely between individuals. This variability is attributed to the dynamic in which batches of oocytes are developing and being spawned/reabsorbed. It also points to the conclusion that the shad, at the population level, do not spawn synchronously at given intervals, but that each female has an independent cycle of spawning, progression of the next cohort of oocytes to hydration and ovulation, and spawning once again.

IV.D.3 Hormone levels

As in the hatchery shad, the wild shad pituitary was also found to contain markedly higher levels of hrGnRH than cGnRH II or sGnRH. This is again a clear indication that the hypophysiotropic form of GnRH in shad is the hrGnRH. As mentioned, this is in agreement with the finding that in teleost species, which have a third "species-specific" GnRH form, this distinct form is the hypophysiotropic GnRH (Powell et al., 1994, Gothilf et al., 1995; Zohar et al., 1995; Dubois et al., 2001, Okubo et al., 2000).

As the GSI increases, the E2 levels decrease and the DHP levels increase, this is a known phenomenon in many teleost species (Fitzpatrick et al., 1986). The surge of E2 that causes the production and transfer of vitellogenin to the ovary is replaced, as oocytes reach FOM, with a rise in DHP levels that cause the resumption of meiosis and GVBD to occur.

Finding that this general trend occurs also in shad reinforces the basic mechanism of hormonal regulation of oocyte development. However, this finding is at odds with the finding in the hatchery that both E2 and DHP are elevated as ovarian development progresses, and with the hormonal profile observed in the asynchronous seabream (Gothilf et al., 1997). This difference can be explained in several ways. As for the hatchery experiments, the most notable explanation is that the continuous high levels of plasma GnRHa cause an unnatural elevation of both E_2 production from vitellogenic oocytes and DHP production from post-vitellogenic oocytes.

Analyzing the E_2 and DHP results given that the shad possesses an asynchronous ovary, and given the finding in sea bream (Gothilf et al., 1997) is more of a challenge.

In a synchronous, single batch spawning fish, the E2, DHP and hypophysiotropic GnRH levels correspond to distinct developmental stages of the oocytes, which all develop at the same rate and are therefore all at the same stage of development. As the oocytes develop synchronously, there is a clear cascade of events, starting with a rise in GnRH levels, continuing with a surge of E2 which enhances the production of vitellogenin and ending with a decline in E_2 levels and the production of MIS that causes the final maturation of the oocytes and resumption of meiosis (Mylonas et al., 1997; Mylonas et al., 1998,). In asynchronous fish such as shad, a clear hormonal profile is hard to detect. We believe that this is the case due to the fact that the ovary contains, at each given time point, a multitude of oocytes at various developmental stages that require both E2 and DHP.

Indeed, a somewhat different hormonal profile was found in asynchronous fish. Gothilf et al., (1997) found in the asynchronous seabream that the hormonal cycle was well correlated with characteristic reproductive events. Specifically, a surge in plasma DHP occurs approximately 8 hours before spawning, when the most advanced cohort of oocytes is at the final stage of FOM (coalesced yolk granules and peripheral GV). E2 plasma levels also begin to rise 8 hours before spawning and continue to increase until the spawning even occurs. These finding were based on a time line leading up to spawning. As seabream is a group synchronized asynchronous spawner this timeline corresponds to specific developmental stages that the most advanced cohort of oocytes undergo in all females. As discussed above, this hormonal profile is different from the one found in most synchronous fish, in that E2 levels stay elevated up to spawning, instead of declining. The suggested explanation for this is that in batch spawning fish, a

continuous supply of vitellogenin to oocytes that are at earlier stages of development is needed, hence the elevated E2 levels are needed to counter the inhibitory effect of DHP. Despite using this timeline method, the question remained as to whether the hormonal cycle observed was accurate, or if it was a result of the frequency at which blood samples were taken (every four hours) rather than an accurate hormonal cycle. As discussed, based on our findings, a similar phenomenon does not seem to occur in shad, although it also possesses asynchronous ovary development.

We suggest that the disagreement between the findings in the seabream and the findings in the wild shad are due to a mechanism by which the shad does not require elevated E_2 levels to counter DHP effects. Rather, an alternative mechanism seems to be in place that ensures a continuous supply of vitellogenin to vitellogenic oocytes in the presence of elevated DHP levels.

The effect of E2 on liver VTG production and secretion has been studied in several species. Mosconi et al (2002) showed in red seabream, which has an asynchronous ovary as well, that E2 receptor levels in the liver were substantially higher during the pre-spawning period, compared to the spawning and the post-spawning periods. While VTG receptors are known to mediate VTG absorption by oocytes, the question remains as to how VTG receptor expression is specifically elevated in some oocytes, but not others. We hypothesize that the exact hormonal profile of E2 and DHP is more complex in asynchronous than in synchronous fish species and that both E2 and E2 receptor levels are tightly regulated. We also believe that, similar to mammals, multiple additional factors are involved in the process of the asynchronous ovary development.

Some idea of how complex the regulation of asynchronous ovary development is was shown in a recent study on the vasa gene in red seabream. Vasa levels has been correlated to primordial germ cell development, in *Drosophila* vasa has been identified as one of 8 genes that are necessary maternal factors for PGC formation and its enzymatic activity shares the function of a germline specific translational regulator. Although the precise role of vasa in PGC determination is yet to be elucidated, multiple experiments have shown that there is interplay between oogenesis and the activity of the vasa protein. Therefore, changes in vasa mRNA levels as a result of hormonal manipulation can shed light on the regulation of PGC development and hence on the regulation of gonad maturation (Cardinali et al., 2003). Given these facts, Cardinali et al. (2003) examined the correlation between mature sea bream vasa gonadal mRNA levels and various hormones involved in oocyte maturation. Vasa mRNA levels were shown to be affected by several reproductive hormones, specifically E_2, GH and GnRH. A combination of GnRH and GH was shown to up-regulate vasa mRNA, yet GnRH or E2+GH down-regulated it. Thus it appears that, as occurs in mammals, factors produced by advanced oocytes and other endocrine organs, can either retard or stimulate the development of oocytes and PGCs. These factors are, in turn, regulated by a complex combination of changes in hormonal levels.

Another explanation for the lack of elevated E_2 levels during the period of time at which the advanced cohort of oocytes is undergoing FOM and producing DHP is that GSI, which was the parameter used in the wild shad experiments is not a good indicator of gonadal stage. However, given the fact that both in the hatchery and the wild shad experiments we found correlation between GSI and histology based ovary staging, and

the fact that Olney et al., (2001) found these two parameters to be in good agreement, suggest against such a conclusion.

To elucidate the exact hormonal profile and factors that regulate the shad asynchronous ovarian development, additional work will be needed. The first step will be to develop an accurate method by which to precisely determine ovarian stage. This is a goal that has not been attained to date in any teleost with asynchronous ovarian development.

IV.D.4 Comparing shad females conducting early vs. late spawning migration

Using fork length measurements to estimate age is a widely accepted method for most fish species, including shad. Shad grow approximately 100 mm per year and the fork length is correlated to the age of the fish (Mackenzie et al., 1985). As the mean lengths of the shad females caught at the beginning and the end of the spawning season were not significantly different, we assume that the average age of these two groups is similar. However, the mean weight was significantly different. It seems that shad, which are heavier, i.e., have accumulated more weight over the year, mature faster and enter the estuary earlier in the spawning season. We hypothesize that the females that are not as heavy do not have the necessary energy reserves to accommodate the onset and completion of spawning migration and therefore enter the estuary at a later date, after accumulating as much mass as possible. However, the timeframe for commencing the spawning migration is limited, as river temperatures rise rapidly.

In addition, the average GSI of the early group was higher than the later group. These two groups were caught at the same river mile, so theoretically their average

developmental stage should be similar. This discrepancy can be explained again by the hypothesis that the later fish did not, for some reason, have sufficient food during the past year or did not develop as fast, therefore both their total body weight and their GSI are decreased in comparison with the early group.

The females captured at the earlier date had a higher average GSI and a higher DHP plasma level than the later group. Whereas the females captured at the later date had a lower average GSI, and a higher average E2 plasma level that the females caught at the earlier date. This finding is in agreement with our general finding from the wild female shad, that there is a positive correlation between DHP and gonadal i.e. the more advanced the ovary (up to atresia) the higher the DHP plasma levels. But a negative correlation between E2 and gonadal stage i.e. the more advanced the ovary (up to atresia) the lower the E_2 levels.

Chapter V

Summary and future directions

Our study of shad reproductive physiology had several underlying goals. As shad are a species on the brink of endangerment, and a species for which little information characterizing basic reproductive mechanisms exists, we attempted to achieve two main goals. One goal was to initiate a study of basic female reproduction physiology and endocrinology. The other goal was to validate a method of inducing female spawning in captivity, in view of facilitating a successful restoration program.

We have shown that by using GnRHa implants, successful hatchery spawning is achievable, and we have elucidated some of the endocrine effects caused by GnRHa treatment. The main obstacle presented in this study, in the hatchery context, is the low fertilization rate, and the fact that the shad produced only one large spawn. Apparently, when transferring wild shad to the hatchery only one large spawn is achievable, for this reason using a combination of GnRHa treatment and domesticated shad, as broodstock, should be considered. Further studies are needed to elucidate the cause of the poor fertilization rates.

The shad is both an anadromous batch spawner and a primitive teleost, the most primitive teleost known to have developed a three GnRH system. Therefore it is a species of great interest in the context of teleost reproduction and basic reproductive endocrinology. In this study, we have developed some of the essential tools needed to study these aspects of shad biology. We have also expanded the knowledge of both shad reproductive physiology during the crucial timeframe of the spawning migration and the basic reproduction endocrinology of alosa species.

The major obstacles faced in this project, regarding basic shad reproductive endocrinology, was the need for a more precise method to stage ovarian development, in view of correlating such data to a hormonal profile. As we learned, achieving this in a species that has asynchronous ovarian physiology is especially difficult. There is no doubt that to achieve a better understanding of the regulatory mechanisms of asynchronous ovary development, additional research is warranted. We have however laid the groundwork for such future study.

The American shad is of historical, ecological and commercial importance to the Chesapeake Bay. Continued study of this species is recommended not only for its scientific value, but also in anticipation of future efforts to restore indigenous stocks to past levels of abundance.

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