ABSTRACT

Kennedy, Alanna Marie. Reproduction of Striped Bass *Morone saxatilis;* a structural, biochemical and functional characterization of atresia. (Under the direction of Craig V. Sullivan.)

Ovarian atresia was examined in striped bass in order to thoroughly characterize this physiological process. If the proper hormonal and temperature cues are not received, gravid female striped bass will initiate ovarian atresia. Although domestic broodfish used in aquaculture routinely complete ovarian growth, injections of gonadotropin or gonadotropin-releasing hormone are required to induce final oocyte maturation. Those fish not treated with hormones in a timely fashion will, instead, initiate ovarian atresia, thereby failing to achieve their reproductive potential for the year.

In progressively atretic striped bass, hormonal profiles showed a steady decline in circulating estradiol-17β and testosterone levels where as levels of the final maturation-inducing steroids, 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-P) and 17α, 20β-21-trihydroxy-4-pregnen-3-one (20β-S) remained basal. Testosterone levels declined significantly across the last three stages of atresia, suggesting that they maybe a useful tool in detecting the onset of atresia. In addition, *in vitro* investigations showed a general decline in steroidogenesis and follicle responsiveness to gonadotropin during atresia. A steady decline in circulating vitellogenin (Vg) levels was found as the follicles proceeded from post-vitellogenic to late atretic stages. There was also evidence of a bimodal distribution of Vg concentrations at atresia Stage 2 (mid-atresia), which suggested that absolute values of Vg would not be a useful indicator of atresia status or spawning eligibility. Contrary to prior findings in fishes, the Vg-derived yolk proteins, lipovitellin (Lv) and β'-component were not found intact or partially intact in the blood plasma of atretic animals. Results of SDS-PAGE

and Western blotting of plasma and ovarian extracts from atretic and non-atretic fish indicated that these yolk proteins are completely hydrolyzed before export from the atretic follicle. This correlated with light microscopy evaluations of atretic follicles that portrayed a general decline in the intensity of staining of the yolk granules with metanil yellow. Vg was detected via SDS-PAGE, Western blotting as well as ELISA in plasma samples from vitellogenic, post-vitellogenic and atretic animals; however, it was only detected in ovarian extracts from vitellogenic fish. Thus, absence of newly endocytosed Vg detected by Western blotting may be a useful marker for post-vitellogenesis in striped bass.

Using transmission electron microscopy, it was found that edema in the granulosa cell layer is an unambiguous characteristic of early atresia. Similar to what has been found in mammalian systems, the granulosa cells of atretic follicles appeared to lose their steroidogenic capabilities as they went through drastic morphological changes. During this time, the basement membrane, which separates the theca and granulosa cell layers of the follicle, appeared to remain intact. Therefore, evidence of phagosomes within the oocyte suggested that phagocytes of granulosa cell origin invaded the oocyte through a disintegrated zona radiata to engulf and recycle lipids, proteins and other components of the ooplasm.

REPRODUCTION OF STRIPED BASS *MORONE SAXATILIS*; A STRUCTURAL, BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF ATRESIA

by
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BIOGRAPHY

Alanna Marie Kennedy was born on October 10, 1977 and was raised in Williston Park, NY along with an older sister, Christina, and a younger brother, Edward Joseph. Her parents, Jo-Ellen and Edward, were the primary force behind her success. They gave Alanna the "roots and wings" that enabled her to tenaciously pursue her goals. She was graduated from Herricks High School in 1995 and pursued a Bachelor of Arts with a concentration in Zoology at Connecticut College in New London, CT. Prior to graduation she was accepted to NCSU as a M.S. candidate in the Department of Zoology. There Dr. Craig Sullivan, along with Drs. Naoshi Hiramatsu and Gregory Weber, provided her with the tools needed to develop and execute critical research and contribute this acquired knowledge to the scientific community. Upon completion of her degree at NCSU she will attend SUNY Stony Brook, where she has been accepted as a Ph.D. candidate in the Department of Genetics, and will investigate genetic regulators of the cell cycle.

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CHAPTER 1

INTRODUCTION

The term atresia is used to describe the breakdown of gametes and the resorption of nutrients used in their construction. In female vertebrates, atresia is a widespread process in which ovarian follicles are eliminated as a regulatory control to prevent ovulation of damaged or inferior oocytes (Van Der Kraak *et al*. 1998). In fact, in mammalian systems, the vast majority of follicles undergo atresia. Most studies of mammalian atresia have focused on granulosa cells in a preovulatory condition. A variety of biochemical markers, such as a decline in DNA synthesis in the granulosa cells, a decrease in estrogen synthesis and secretion, and a decline in gonadotropin (GtH) receptor mRNA or GtH binding to its receptors, have been used as indicators of the onset and progress of atresia. In mammals, it was found that testosterone induces and estrogen inhibits atretic processes and that atretic follicles express a higher androgen to estrogen ratio as compared to the ratio determined for non-atretic follicles (Billig *et al.* 1993). In addition, apoptosis (programmed cell death) has been identified as an important process leading to the death of ovarian follicles during mammalian atresia (Tilly *et al*. 1991). Studies conducted on preovulatory follicles suggest that, without supportive hormones such as human chorionic gonadotropin (hCG), insulin-like growth factor-I (IGF-I), and human follicle stimulating hormone (hFSH), cultured follicle cells will undergo apoptosis (Chun *et al.* 1994). Growth hormone (GH) also has been identified as an oocyte survival factor acting secondarily to the IGF-I pathway (Eisenhauer *et al.* 1995). In some cases, there is evidence that the various gonadotropins or growth hormones may act to promote follicle survival, in part, by altering follicular steroidogenesis. For example, human preovulatory follicles cultured in the presence of IGF-I produced 70-

90% more estrogen and 52-65% less progesterone (Chun *et al.* 1994). The various functional changes in follicles undergoing atresia occur before any obvious morphological change. Also preceding overt structural changes in follicles undergoing apoptosis is fragmentation of the nuclear DNA into 180-200 base pair units and multiples thereof. The DNA fragmentation is a late stage indicator of apoptosis (Huppertz *et al.* 1999).

With respect to structural changes in atretic mammalian follicles, apoptotic granulosa cells may show evidence of chromatin condensation and migration to the periphery of the nucleus, vacuolization of the cytoplasm, and cytoplasmic blebbing (Devine *et al.* 2000). Also observed in granulosa cells of atretic and apoptotic follicles is retraction of microvillar processes, a general decline in evidence of rough endoplasmic reticulum (RER), increased electron density of the cristae-depleted mitochondria, and detachment of granulosa cells from the basement membrane (Devine *et al.* 2000; Hughes and Gorospe 1991).

Ovarian atresia in non-mammalian vertebrates has received far less attention than it has in mammals. Atresia has received only basic characterization in a variety of fish species; the four stages of atresia that comprised this morphological characterization in non-mammalian vertebrates were summarized by Saidapur (1978). Typically, the "α stage" begins with degradation of the follicle and phagocytosis of the hypertrophied granulosa cells. The "β stage" is characterized by increased phagocytosis of the yolk by granulosa cells with increased hyperemia and "ingrowth" of the thecal cell layer. In the third stage (γ stage) an "intensely" orange pigment appears and the final stage (δ stage) exhibits the complete collapse of cellular structure.

There is only limited information on biochemical and morphological characteristics of atresia in non-mammalian vertebrates. The focus of that information is mainly on

conditions known to induce massive ovarian atresia in farmed fishes, including handling, nutritional, and temperature or other environmental stresses (Tyler and Sumpter 1996). This restriction of investigations on atresia in cultured fish is not surprising because, although atresia of a limited proportion of follicles early in the gametogenic cycle may help determine fish fecundity (Janz and Van Der Kraak 1997), massive ovarian atresia may be an anomaly in nature. In contrast, if captive-bred teleosts such as striped bass (*Morone saxatilis*) are not treated with exogenous hormones shortly after their oocytes are fully-grown, they fail to complete maturation and ovulate or spawn (Sullivan *et al.* 1997; Zohar and Mylonas 2001). Instead, their follicles initiate atresia, the fully-grown oocytes are degraded and their contents are eventually resorbed (Mylonas *et al.* 1997). A more thorough characterization of atresia in species such as the striped bass could identify morphological or biochemical markers for the onset of atresia that would enable biologists to test individual females for potential gamete quality prior to commitment of expensive hormone injections and limited hatchery resources. In addition, the striped bass and its relatives in the genus *Morone* could serve as models for basic research on atresia in lower vertebrates. They are well suited to this purpose because these fish routinely undergo massive ovarian atresia if they are not provided with exogenous hormone support and because the details of their reproductive physiology are well known (Sullivan *et al.* 1997).

The primary goal of the present investigation was to describe in detail the process of ovarian atresia in striped bass with attention to its hormonal, biochemical, functional, and morphological features. The reproductive biology of striped bass and its relatives has been the subject of intense investigation in recent years (Sullivan *et al.* 1997), because they are an increasingly important farmed fish in the United States (Striped Bass Growers Association

1993, 1998). The endocrinology of atresia was evaluated with respect to changing levels of circulating sex steroids and GtH-II (luteinizing hormone, LH), which are known to regulate specific reproductive processes in female striped bass. Changes during atresia in the innate steroidogenic activity and response to GtH of cultured ovarian follicles also were measured. It was anticipated that these investigations would yield insights into hormonal regulation of atresia in striped bass as well as elucidate endocrine correlates of atresia in this species.

The biochemistry of striped bass atresia and specifically the breakdown and recycling of egg yolk proteins derived from their circulating precursor, vitellogenin (Vg) also was investigated in this study. This focus is based on findings in rainbow trout (*Salmo gairdneri*), that yolk proteins derived from Vg may be recycled from the atretic oocyte to the blood plasma intact and can serve as markers of ovarian atresia (Babin 1987). Finally, structural changes in ovarian follicles undergoing atresia were described in detail with respect to gross morphology of intact follicles, tissue characteristics at the light microscope level, and ultrastructural changes elucidated via transmission electron microscopy. In summary, ovarian atresia in striped bass was described through the evaluation of functional and structural changes at multiple levels in several physiological systems. It was expected that this approach would enhance the biological understanding of atresia, provide a useful description of stages for identifying atresia, generate practical chemical and morphological markers of atresia, and suggest fruitful avenues for future basic and applied research.

LITERATURE REVIEW

The striped bass is an oviparous teleost exhibiting the group-synchronous pattern of ovarian maturation in which a single clutch of oocytes is recruited through secondary growth,

final maturation, and ovulation for a single annual spawning event (Nagahama 1983). These animals spawn only once during the spring of each year, and thus there is a distinct cyclic annual pattern of oogenesis and endocrine correlates. As is the case for other teleosts reproducing in the temperate zone, the timing of the endogenous reproductive cycle of striped bass is entrained by photothermal cues such as diurnal and seasonal changes in water temperature and day length (Blythe *et al.* 1994ab). Primary growth oocytes initiate lipid deposition in the early secondary growth phase that is during or just after the natural spawning season in April-May. This lipidation occurs without elevation of circulating sex steroids and has been shown to occur in the absence of seasonal photothermal cues (Berlinsky and Specker 1991; Clark 1998). Deposition of yolk granules in the growing oocytes is triggered by decreasing day length and water temperature around the autumnal equinox (Blythe *et al.* 1994a; Clark 1998) and is associated with a distinct elevation of circulating levels of estradiol-17β (E2) and testosterone, as well as Vg. Exogenous E2 can induce hepatic vitellogenesis in striped bass and is likely the endogenous hormone controlling this process *in vivo* (Kishida *et al.* 1992; Tao *et al.* 1993; Sullivan *et al*. 1997). Presumably, E2 is produced in the granulosa cells by the action of an aromatase enzyme that converts testosterone to E2 (Kagawa *et al*. 1982). In striped bass and congeneric species, oocyte growth ensues via deposition of lipid droplets and yolk granules in the ooplasm (Berlinsky and Specker 1991; Tao *et al*. 1993; Jackson and Sullivan 1995; Sullivan *et al.* 1997). In salmonids and other freshwater species spawning demersal eggs, oocyte growth is largely attributable to yolk protein deposition in the yolk granules (DeVlaming *et al*. 1984; Tyler 1991). However, simple examination of histological sections of fully grown oocytes of the striped bass, which spawn pelagic eggs, reveals that lipid deposition must account for at

least half of the increase in oocyte size (Berlinsky and Specker 1991; Tao *et al*. 1993). Recent research indicates that the lipid droplets are composed primarily of wax esters, which are entirely synthesized by striped bass oocytes from circulating triglyceride precursors (Lund *et al*. 2000).

The circulating egg yolk precursor, Vg, is a phosphoglycolipoprotein typically present in the blood as a dimer before being taken up by growing oocytes (Specker and Sullivan 1994). Because an entire gene family is involved in the synthesis of Vg proteins, there is ample room for both diversity and redundancy. Found in both vertebrates and invertebrates, Vg is highly conserved and there is evidence of multiple forms of this yolk protein precursor in teleosts (Folmar *et al*. 1995, Specker and Sullivan 1994, La Fleur *et al*. 1995, Kishida and Specker 1993, Matsubara *et al*. 1999). For example, three distinct Vg proteins have been detected in white perch, *Morone americana.* (Hiramatsu *et al. in press* a; Hiramatsu *et al. in press* b).

In teleosts, the yolk granules within the ooplasm contain a suite of yolk proteins derived from Vg. After Vg binds to the oocyte surface and is internalized via receptor-mediated endocytosis (Tao *et al*. 1996), it is broken down into the following components: lipovitellin (Lv), phosvitin (Pv) and β'-component (Hiramatsu *et al. in press* b). The largest Vg product is Lv, which contains approximately 20% lipid (mainly phosphatidyl choline) by weight; Pv consists largely of serine residues to which phosphate is covalently linked and to which calcium, in turn, is ionically bound. The β'-component contains neither lipid nor phosphorous and is of unknown function with respect to embryonic nutrition or physiology (Hiramatsu *et al. in press* a). The hormones and enzymes regulating binding of Vg to its receptor, internalizing of Vg, and processing of Vg into yolk proteins are unknown in striped

bass. Results of recent research on teleosts suggest that gonadotropin-I (FSH) or IgF-I may regulate Vg uptake and processing into yolk proteins in other teleosts (Tyler and Sumpter 1996).

Vitellogenin is transported from the follicular epithelium through pore canals to the zona radiata (ZR) and then into the oocyte where it forms yolk granules or spheres (Selman and Wallace 1982). In naturally vitellogenic female teleosts, Vg passes through the follicle layers intact, binds to a specific receptor on the oocyte surface and is then engulfed by the oocyte via receptor-mediated micropinocytosis (Specker and Sullivan 1994). In males, E2 introduced via injection or by proximity to vitellogenic females can trigger an otherwise latent gene response in the male liver, which then results in the synthesis of Vg (Selman and Wallace 1983). However, males possess no target for Vg (*i.e.* oocytes), so the protein returns to its hepatic origin and is broken down and recycled along with other excess or damaged proteins.

Ovarian follicles undergo vast changes during vitellogenesis. The theca is the outermost layer derived from stromal connective tissue and is characterized by collagen fibers, fibroblasts, steroidogenic cells, and capillaries or other vascular components (Nagahama 1983). Germinal epithelium-derived follicle cells flatten to form a singular granulosa cell layer below the basal lamina and above the acellular chorion or ZR. Junctional complexes between the follicle cells allow for the extracellular passage of Vg through the theca, basement membrane, granulosa cell layer, and ultimately to the oocyte surface. The ZR becomes perforated by microvillar processes, which emanate from both the oocyte and granulosa cells (Grier 2000). Endocytotic pits become evident on the surface of the oocyte as Vg is taken into the growing oocyte. Cortical granules in the apical ooplasm,

whose contents are discharged during the water hardening response of ova to fertilization, become more closely associated with Golgi bodies and developing ER while microvillar processes extending across the ZR increase in length (Kayaba *et al*. 2001). In mammalian systems, atretic follicles are characterized by a loss of these microvillar processes, an event that is also a characteristic of final follicular maturation and ovulation in teleosts (Devine *et al.* 2000; Kayaba *et al*. 2001). Additionally, previous investigations indicated the presence of hypertrophied granulosa cells in atretic follicles of striped bass and other fishes (Saidapur 1978, Jackson and Sullivan 1995; Mylonas *et al.* 1997).

In striped bass, sustained low levels of E2 and testosterone characterize most of the oocyte growth. However, these levels are consistently greater than the basal to non-detectable levels seen in reproductively quiescent animals just prior to gonadal recrudescence or in immature fish (Tao *et al.* 1993; Sullivan *et al.* 1997). At the same time, plasma Vg levels rise abruptly to high concentrations (~1 mg/ml), which are sustained for several months before spawning in maturing females. It has been suggested that in striped bass and other temperate basses in the genus *Morone*, a relatively constant and moderate E2 stimulus can provide an increased vitellogenic response, because E2 up-regulates its own hepatic receptor (Tao *et al*. 1993; Berlinsky *et al*. 1995; Jackson and Sullivan 1995; Sullivan *et al.* 1997). In all *Morone* species examined to date, there is an abrupt and sustained rise in circulating E2 and testosterone levels in the month prior to spawning that is not temporally associated with any change in circulating Vg levels or any enhanced oocyte growth. It has been postulated that the late-cycle surge of plasma E2 and testosterone levels may be involved in feedback to the brain and pituitary gland, inducing synthesis and secretion of LH at the expense of FSH production (Sullivan *et al*. 1997). However, because we still lack an

assay to measure circulating FSH in *Morone* species, and there is therefore no direct evidence to support this hypothesis. It also is possible that the late-cycle E2 and testosterone surge is involved in maintaining post-vitellogenic (fully-grown) follicles in a non-atretic state until they can enter final maturation (Patino *et al*. 2001). As in mammalian systems, the follicle cells of the teleost ovary and the hormones they produce may be oocyte survival factors that act in autocrine or paracrine fashion to delay the onset of follicular and oocyte atresia or apoptosis.

After completion of oocyte growth, ovarian follicles of striped bass and other teleost fish will initiate either final maturation or atresia. Entrance of female striped bass into these processes appears to be associated with a rise in water temperature to, or above, the optimum for fry survival (Sullivan *et al.* 1997). In fact, white bass (*Morone chrysops*) can be maintained in a non-atretic state for months. This can be accomplished by holding females with fully-grown oocytes at low temperatures (Smith *et al*. 1996). The process of final ovarian maturation in teleosts was recently reviewed in detail (Patino *et al.* 2001). Ovarian maturation also has been intensively investigated in striped bass and its relatives (Sullivan *et al.* 1997; Weber *et al.* 2000; Weber and Sullivan 2000; Weber and Sullivan 2001). Ovarian follicles of striped bass and other advanced perciform fish must acquire "maturational competence" prior to final maturation, which can be judged by their ability to complete maturation *in vitro* in response to GtH preparations. Maturation of "competent" follicles involves a shift in steroidogenic endpoints, from production of E2 and testosterone to production of C_{21} steroids, which include the final maturation-inducing steroid (MIS) hormones, 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-P) and 17α, 20β, 21,trihydroxy-4-pregnen-3-one (20β-S). The MIS, in turn, binds to specific receptors on the oocyte surface

and induces final maturation via the induction of a ubiquitous maturation-promoting factor, the keystone molecule in a final signaling pathway common to oocyte maturation in most vertebrates examined to date (Nagahama *et al.* 1994). A compelling body of evidence indicates that the MIS in *Morone* species is 20β-S (King *et al*. 1994ab, 1995, 1997).

Follicular acquisition of maturational competence appears to involve up-regulation of receptors for LH, development of gap junctions between the granulosa cells and oocytes (heterologous gap junctions, possibly portals for MIS) or between granulosa cells (homologous gap junctions), and synthesis of MIS receptors by the oocyte (Patino *et al*. 2001). In *Morone* species, maturational competence can be induced *in vivo* by injection of the fish with gonadotropin preparations (hCG) or *in vitro* by incubation of follicles in the presence of hCG or IGF-I (King *et al.* 1994a; Weber and Sullivan 2000; Weber and Sullivan 2001). The degree to which these hormones can delay onset of preovulatory atresia has not been investigated.

The specific stage of final maturation of striped bass oocytes can be judged using several cytological landmarks. These include migration of the oocyte germinal vesicle to the oocyte periphery (GVM) and its eventual breakdown (GVBD), as well as the degree of cytoplasmic maturation. Cytoplasmic maturation includes partial degradation of the Vg-derived yolk proteins (Hiramatsu *et al*. 2002), increased oocyte size due to hydration (Sullivan *et al*. 1997), and coalescence of the numerous lipid droplets in the ooplasm into one or a few large droplets (Rees and Harrell 1990). Observations from research conducted on striped bass shows that, although atresia can be initiated at any stage of final maturation, conditions leading to maturation are generally exclusive of follicular atresia (Mylonas *et al*. 1997). Consequently, it is comparatively rare to encounter females with atretic oocytes in an

advanced stage of final maturation (C.V. Sullivan, personal communication). In general, atretic oocytes appear to have undergone nuclear dissolution, which is distinct from maturational GVBD, while the germinal vesicle is in a central position and before cytoplasmic maturation is much advanced (Mylonas *et al*. 1997). These observations suggest that the endocrine and cytological factors regulating final maturation of *Morone* follicles also may be involved in the maintenance of fully-grown, preovulatory follicles in a non-atretic condition.

In wild striped bass, circulating E2 and testosterone levels increase early in final maturation, while the oocyte germinal vesicle is still migrating; peak testosterone levels lag slightly behind peak E2 levels. Androgen and estrogen levels both fall coincident with the peak in circulating 17α, β20-P and 20β-S that is concurrent with GVBD (King *et al.* 1994a) and can be induced by treatment of captive fish with a synthetic analogue of gonadotropin-releasing hormone (GnRHa). Progression of oocytes through final maturation and the changes in circulating steroid hormones described above coincide with the pronounced elevation of endogenous LH in the blood plasma induced by GnRHa (Mylonas *et al*. 1998). These observations further suggest that elevated androgen and estrogen levels might serve a dual purpose, to delay onset of atresia and to "prime" the neuroendocrine system for completion of final maturation.

Prior investigations have characterized the gross morphology and histology of atretic *Morone* follicles with reference to the annual gametogenic cycle, initiation of final maturation, and identification of candidate spawners (Jackson and Sullivan 1995; Mylonas *et al.* 1997). However, the present study is the first in which morphological, cytological, and ultrastructural changes in atretic follicles were evaluated within the context of endocrine

profiles. Results of this study illustrate the progressive loss of gonadotropin stimulation and follicular steroidogenic capacity during ovarian atresia in striped bass. Likewise, the mechanism(s) involved in recycling of Vg-derived yolk proteins, which were intensively investigated in this study, have never before been subjected to detailed examination in any vertebrate. Preliminary investigations of yolk protein recycling during atresia in primitive fishes (Babin 1987; Hiramatsu *et al*. 2002) suggested that the yolk proteins might re-enter the circulation intact. The present study shows that, in an advanced perciform teleost, which is representative of the largest category of fishes, the yolk proteins are completely degraded *in situ* before they are mobilized from the ooplasm. With respect to the developing finfish aquaculture industry, this study has generated practical morphological markers to select candidate spawners that can be identified using only a dissecting stereomicroscope. Closer examination and evaluation of broodstock with respect to the sex hormone and protein and cytological markers developed in this study could provide a useful "atretic profile" which might provide a basis for genetic selection of fish less susceptible to atresia induced by high temperatures or other factors. A greater understanding of oocyte development and subsequent demise is vital to gaining better control of reproduction and improving spawning performance of these animals. Perhaps most importantly, this study has provided the intellectual and technical framework upon which striped bass can be utilized as a general model for studying ovarian atresia in non-mammalian vertebrates.

MATERIALS AND METHODS

Experimental Animals, Blood and Tissue Samples

Adult female striped bass originating from various geographic lineages were held at the North Carolina State University (NCSU) Pamilco Aquaculture Field laboratory (PAFL) in outdoor circular pools (7.3 m in diameter X 0.92 m deep) as described previously (Hodson and Sullivan 1993; Hodson *et al*. 2000), except as otherwise noted below. All experiments involving these animals were conducted in accordance with the 1996 Guide for Care and Use of Laboratory Animals published by the National Research Council and covered under a protocol approved by the NCSU Institutional Animal Care and Use Committee. Fish were marked for individual identification with passive integrated transponders (PIT) (Destron IDI, Boulder, CO), which were injected into their lateral musculature.

Stocking rates ranged from 12-20 fish per pool, for a total biomass of 91-182 kg per pool. Fresh well water at a constant 19˚C from the Castle Hayne aquifer was mixed with brackish creek water at a flow rate of 23-38 lpm from each source and supplied to the pools. The brackish water was drawn from the surface of South Creek adjacent to PAFL or from a well point drilled into the creek bed. Salinity in the rearing tanks ranged from 2 to 10 ppt throughout the year with variation due to change in the salinity of the creek coupled with alterations in relative flow rates of creek versus well water supplied to the tanks. Water temperature in the pools showed seasonal fluctuation ranging from 12˚C to 25˚C in the winter and summer, respectively. Dissolved oxygen was maintained at greater than 70% saturation by delivery of compressed air to each pool through 2-3, 0.6 m-long, tube-style flexible membrane air diffusers (Aquatic Ecosystems; Item D324). Broodstock were fed three times each week at a rate of 6% body weight/week or 2% body weight/day. This diet

consisted of Bass Brood feed 1.27 cm diameter (nominally "0.5 inch") sinking pellets that consisted of 45% protein and 15% fat (Zeigler Bros., Inc., Gardners, PA).

In mid-April 2001, inventory was taken of the striped bass to identify their status of maturation. The fish were anesthetized in a solution (15 mg/l) of quinaldine sulfate (B.L. Mitchell, Inc., Leland, MS) and subjected to ovarian biopsy using a plastic catheter inserted into the ovary through the urogenital pore (Rees and Harrell 1990). The oocytes were held in ice-cold Cortland's Balanced Salt solution (Wolf 1963) adjusted to iso-osmotic concentration (320 mOs). Biopsy samples were viewed at 40X magnification using an Olympus SZ40 stereomicroscope fitted with an ocular micrometer. The average diameter of 3-4 of the largest follicles in the field of view was recorded. Also, the stage of maturation or atresia was assessed through the appearance of oocytes in the biopsy samples. Female maturity was staged using a standard scale for striped bass based on the degree of lipid droplet coalescence and ooplasm clarity of the oocytes (Rees and Harrell 1990). This scale was previously calibrated to specific stages of oocyte germinal vesicle migration and breakdown (Hodson and Sullivan 1993; King *et al.* 1994a). Female atresia was staged by analysis of several characteristics of the oocytes in the biopsy sample that include the degree of development of a vacuolated appearance of the ooplasm, the intensity of development of an orange ooplasm coloration, and the degree to which the oocytes exhibited an irregular (versus spherical) shape with a concurrent decrease in oocyte size.

Twelve females were selected for further analysis and placed into one of four categories:

Stage 1. "Post-vitellogenic" No oocytes in the field were considered atretic based on the criteria discussed above. Oocytes were uniformly opaque and generally ≥ 850 µm in diameter with regular spherical shape and without any orange coloration.

Stage 2. "Early Atretic" Less than 5% of the oocytes within the field were considered atretic. A few clearly atretic oocytes were apparent within the field but the rest expressed no atretic characteristics. The degree of vacuolization and orange coloration of the ooplasm of atretic oocytes was limited and the oocytes appeared uniformly spherical or only slightly irregular in shape. They were similar in size to oocytes from post-vitellogenic females.

Stage 3. "Mid-Atretic" Between 5% and 95% of the oocytes within the field were considered atretic. There was clear development of a vacuolated or granular appearance of ooplasm of atretic oocytes, often accompanied by a distinct orange coloration. Atretic oocytes were irregular in shape and exhibited a decrease in size relative to oocytes from Stage 1 or Stage 2 females. The two subcategories of this stage were:

a) Mixed – oocytes were at varying stages of atresia
b) Uniform – all oocytes were at the same stage of atresia

Stage 4. "Late Atretic" 95% or more of the oocytes within the field were considered atretic. The ooplasm of atretic oocytes appeared highly vacuolated and distinctly orange in color. All atretic oocytes were highly irregular in shape and were distinctly smaller in size than Stage 1 or Stage 2 oocytes. The two subcategories of this stage were:

a) Mixed – oocytes were at varying stages of atresia.
b) Uniform – all oocytes were at the same stage of atresia

These 12 females were moved into 6.1 m diameter X 1.2 m deep circular tanks in an insulated building outfitted with special water biofiltration, air, and water temperature control systems. Water sources and quality characteristics for subsequent indoor rearing of the fish were identical to those described above for the outdoor pools; however, water temperature was controlled to allow the fish to gradually undergo atresia over a prolonged period and allow for sampling. Prior research at PAFL conducted on the spawning of striped bass showed that sudden and sustained increases in spring water temperatures in excess of 18˚C

induced the rapid onset and progression of atresia in most female striped bass broodstock (Sullivan *et al*. 1997). Water temperature was 21˚C when the fish were introduced to the cold-bank and the temperature was incrementally decreased over four days to 15.2˚C. The temperature was gradually raised to 18˚C 10 days later and was maintained at 16-18˚C for the remainder of the experiment.

On three occasions during April and May 2001, ovarian tissue and blood samples were taken as the fish became progressively more atretic. Ovarian biopsy samples were placed in 4F:1G fixative, a 4% formaldehyde/1% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.2-7.4 (McDowell and Trump 1976). Additional biopsy samples were frozen in liquid nitrogen ($N_{(L)}$) and then stored at –80˚C until biochemical analysis. After ovarian biopsy, blood (5-10 ml) was sampled from the caudal artery by ventral insertion of a 21 gauge, 1.5-inch needle behind the anal fin using heparinized syringes. Blood samples were treated with the protease inhibitor, Aprotinin (Sigma), and the plasma was obtained by centrifugation at 10,000 X g for 5 min. The plasma was then snap frozen in $N_{(L)}$ and stored at –80˚C until use (Tao *et al.* 1993). At the time of each sampling, the identity of each fish was verified by reading its PIT tag, so that the atretic process could be followed in an individual fish.

Additional striped bass provided control samples for biochemical and histological analyses of atresia and still other striped bass were ovary donors for evaluation of steroidogenic changes *in vitro*. In early May 2001, a wild female striped bass caught in a commercial pound net near the mouth of the Roanoke River while on her spawning migration was sampled for ovarian tissue and blood to provide a control sample representative of naturally post-vitellogenic females. Also in early May, several three year-old female striped

bass were obtained from an outdoor pool and sampled as described above to provide ovarian tissue and blood for biochemical analyses. Most of these fish were at various stages of atresia at the time of sampling. However, one female that was at the13 hr stage according to Rees and Harrell (1990), indicating that she was undergoing final oocyte maturation and not atresia, had oocytes with a migrating germinal vesicle that showed clear coalescence of the lipid droplets (King *et al.* 1994ab). In early June 2001, several male striped bass from the outdoor pools were sampled as described above to obtain blood plasma as negative control samples for analyses of the presence of Vg and its yolk protein products in blood plasma of atretic females. Also as negative controls for the atresia studies, eight vitellogenic female striped bass were sampled in January 2002 from the outdoor pools as described above. For the purposes of this study, the vitellogenic females were designated as being at atresia Stage 0.

Ovary donors for *in vitro* cultures were approximately three years of age when they were obtained from an outdoor pool in mid-April 2001. The fish were subjected to ovarian biopsy and the biopsies were viewed under the stereomicroscope to evaluate their stage of maturity or atresia as described above. Aliquots of the biopsies were placed in a clearing fixative (ethanol:formalin:acetic acid, 6:3:1), followed by 100% glycerol to evaluate the status of oocyte germinal vesicle migration and breakdown associated with final maturation (King *et al*. 1994a). Ovary tissue for culture was obtained from a non-atretic, post-vitellogenic female that had not yet initiated final maturation (Stage 1), a female in the middle stages of atresia (Stage 3), and a female in late stage atresia (Stage 4). The fish were sampled for blood, their ovaries were removed, aliquots of ovarian tissue were fixed in 4F:1G and other samples were snap frozen in $N_{(L)}$ and stored at –80˚C. These selected

ovaries were then split longitudinally, placed individually in plastic bags containing 300 ml of sterile Cortland's balanced salt solution, and transported to the NCSU campus in Raleigh, NC, for *in vitro* challenge.

Steroid Hormone Radioimmunoassay

Steroid hormones were measured using highly sensitive and specific radioimmunoassays (RIAs) for E2, testosterone, 17α, β20-P, and 20β-S in striped bass blood plasma (Woods and Sullivan 1993; King *et al.* 1994a). Duplicate 25 µl aliquots of plasma were assayed for E2 and testosterone levels and 100 µl aliquots of plasma were assayed in duplicate for levels of 17α, β20-P and 20β-S. Aliquots of pooled plasma samples from male and female striped bass were run in each assay and used to correct the data for any inter-assay variation. Culture media from the *in vitro* incubations of ovarian tissue also were evaluated for E2 and testosterone levels. Duplicate 50 µl aliquots of media from each culture well were analyzed after it was determined that this volume of media would generate values falling on the linear portion of the standard curve for each assay. For each hormone, all samples from the *in vitro* cultures were analyzed in a single RIA.

Gonadotropin-II Assay

Striped bass GtH-II (sbGtH-II) was measured using a sensitive homologous RIA involving a specific antiserum directed against the β-subunit of the hormone with purified, intact sbGtH-II as the standard and radioiodinated sbGtH-II as the tracer (Blaise *et al*. 1998). Duplicate 100 µl aliquots of each plasma sample were subjected to GtH-II RIA.

Vitellogenin ELISA

Vitellogenin (Vg) levels in duplicate aliquots of plasma samples were evaluated using a homologous enzyme-linked immunosorbent assay (ELISA) as described previously (Heppell *et al.* 1999).

Electrophoresis and Immunological Procedures

Sodium dodecyl sulfate 4-15% or 8-25% polyacrylamide gradient gel electrophoresis (SDS-PAGE) of blood plasma and ovary extracts was performed under reducing conditions using a PhastSystem^{TM} automated electrophoresis unit (Amersham Pharmacia Biotech, Uppsala, Sweden) as described previously (Tao *et al*. 1993). Western blotting was accomplished using the manufacturer's companion PhastTransfer Semi-dry Transfer Kit and previously published protocols (Tao *et al.* 1993; Heppell *et al*. 1995). Antisera used for Western blotting to detect striped bass Vg or yolk proteins derived from Vg were as follows: anti-striped bass Vg (aVg), anti-white perch lipovitellin (aLv), and anti-white perch β'-component (aβ'). Details on the biochemical and immunological nature of the *Morone* egg yolk proteins and evidence for the complete specificity of these antisera have been presented previously (Tao *et al.* 1993; Hiramatsu *et al. in press* a).

Samples of blood plasma from several vitellogenic, post-vitellogenic and atretic fish were diluted in 1:10 Tris-HCl buffer (0.02 M Tris- HCl, 2% NaCl, 0.1% NaN_3, 40TIU/ml Aprotinin, pH 8.0) and further diluted 1:1 with β-mercaptoethanol and boiled for 5 min. Due to the high concentration of Vg in plasma obtained from males injected with E2, plasma from these fish was first diluted 1:30 in Tris-HCl buffer and then 1:1 with β-mercaptoethanol

before boiling for 5 min. The various plasma samples were then applied to a 4-15% SDS-PAGE gel at 4 µl/well.

Frozen samples of ovarian tissue were homogenized in Tris-HCl buffer with a Polytron PT10/35 tissue homogenizer (Brinkman, Westbury, NY) using 2 bursts at setting 5 for 30 sec and 2 bursts at setting 7 for 20 sec and then sonicated on ice using a Sonic Dismembrator 60 (Fisher Scientific LLC, Suwanee, GA) at setting 4 for 20 sec. The homogenate was then centrifuged at 1500 X g for 10 min at 4˚C. Supernatants were removed and placed in a separate vial. The pellets were resuspended in Tris-HCl buffer and additional protease inhibitor was added (Aprotinin, 40 T.I.U./ml supernatant). The homogenization and centrifugation procedures described above were then repeated and the new supernatant was added to the original one. The pooled supernatants were then progressively filtered through a 45 µm syringe filter and then a 22 µm syringe filter (Millex GV; Millipore, Bedford, MA). The resulting samples, designated as aqueous ovarian extracts, were kept in the dark on ice or snap frozen in $N_{(L)}$until use.

Ovarian extracts were diluted to a protein concentration of 1 mg/ml as assessed by a BCA Protein assay kit (Pierce, Rockford, IL) in Tris-HCL buffer and then further diluted 1:1 with β-mercaptoethanol and boiled for 5 min. The extracts were then analyzed for content of vitellogenin-derived yolk proteins and their breakdown products via 8-25% gradient SDS-PAGE and Western blotting using the PhastSystem^{TM} components and the various antisera mentioned above. Samples were analyzed on paired gels, one subjected to SDS-PAGE only and the other to SDS-PAGE followed by Western blotting, which were run simultaneously. One gel was stained with 0.1% Coomassie brilliant blue R250 (CBB) in a solution of ethanol:acetic acid:distilled water (4:1:5 v/v; CBB stain) for one hr and then destained in a

solution of methanol:acetic acid:distilled water (3:1:6 v/v). The other gel was electro-blotted onto a primed polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore) and used for Western blotting of the banded proteins as described previously (Heppell et al. 1995; Hiramatsu *et al. in press* a). Approximate molecular weights were determined by comparison of prestained SDS-PAGE Standards: (Broad Range #161-0318; BioRad, Hercules, CA) with calibrated molecular weights, to sample bands measured with electronic digital calipers (Digimatic 500-301; Mitutoyo Corporation, Kanagawa, Japan).

To verify the results of Western blotting using an orthogonal biochemical procedure, immunoelectrophoresis (IEP) of native proteins present in blood plasma and ovary extracts were conducted in 1% agarose gels using 50 mM barbital buffer and 0.9% NaCl containing 0.1% NaN_3, respectively, using routine procedures (Hiramatsu *et al. in press* a). Plasma samples were subjected to IEP without prior dilution and ovary extracts were first diluted to 1mg protein/ml before 5 µl aliquots of each sample type were applied to the gels. Antisera were absorbed with plasma from male fish (Parks *et al*. 1999) and then either used directly for IEP (aVg and aβ') or diluted 1:4 in Tris-HCl before use (aLv).

In addition, commercially available polyclonal antibodies to *bax* (PC103; Oncogene Research Products, La Jolla, CA) and *bcl-*X_L (PC104; Oncogene Research Products, La Jolla, CA), although never before used in piscine systems, were applied to uniquely prepared ovarian extracts. Previous studies have elucidated the highly conserved nature of members of the bcl-2 family of proteins, including *bax* and *bcl-*X_L, which regulate apoptosis in various tissues. After comparing aligned peptide sequences for *bax* and *bcl-*X_L obtained from GenBank for a variety of species (e.g. *Homo sapien, Mus musculus, Xenopus laevis* and *Danio rerio*), it was surmised that antibodies directed at highly conserved regions (amino

acid residues 98-117 and 150-169, respectively) of these genes would be plausible identifiers of these apoptotic genes in the striped bass. These antibodies were utilized as the primary antibody for Western blotting following SDS-PAGE of ovarian extract samples. Because homodimerization, heterodimerization and multimerization of all the bcl-2 family members are common obstacles when attempting to isolate these proteins, a non-ionic detergent was used in the homogenization buffer for ovary extracts. The homogenization buffer: 10 mM Tris-HCL, pH. 7.5, 0.5% Nonidet P-40, 10 mM EDTA, 150 mM NaCl and 1% NaN_3, used in this preparation was modified from that described by Ray and Jena (2000). This preliminary investigation is discussed in Chapter 2.

In vitro Bioassay

Incubations of ovarian follicles in 24-well culture plates and subsequent evaluation of *in vitro* follicular steroidogenesis followed routine procedures developed for striped bass (Weber and Sullivan 2000). Briefly, ovarian fragments (0.1 g) were cultured in 1.0 ml of Cortland's balanced physiological saline buffered with 15 mM Hepes, adjusted to pH 7.5, and supplemented with 1 g/L D-glucose, 0.1% BSA, 100,000 units/L sodium penicillin G, and 100 mg/L streptomycin S (King et al. 1994a). After a minimum 1 hr of pre-incubation in this (control) culture medium, the tissues were transferred to plates containing experimental media, which consisted of control media with or without addition of 25 I.U./ml human chorionic gonadotropin (hCG)(Chorulon^R, Intervet Inc., Millsboro, DE). A series of wells incubated without addition of ovarian fragments served as an additional control for background reactivity of the culture media in the various RIAs. After 24 hr of incubation in a shaking incubator at room temperature, the ovarian tissue was harvested from each well

and the incubation medium was collected. The tissue and media samples were snap-frozen in $N_{(L)}$ and held at –80˚C until use. Individual treatments (control, hCG) were replicated in quadruplicate cultures on each of two different plates.

Histological Analyses

Histological procedures and analyses were performed in the Laboratory for Advanced Electron and Light Optical Methods at the NCSU College of Veterinary Medicine under the direction of Dr. Michael Dykstra. Samples fixed in 4F:1G were processed into two embedding media. Samples for light microscopy (LM) were embedded in JB-4 (glycol methacrylate) and samples for transmission electron microscopy (EM) were embedded in Spurr (1969) resin.

For LM the fixed samples were rinsed in 0.1M Sorenson's sodium phosphate buffer (pH 7.2-7.4) twice for 10 min each. Sequential dehydration was accomplished in a graded ethanol series (50%, 75%, 95%, and 95%) for 15 min at each grade followed by two 30-min washes in 100% ethanol. To infiltrate and embed the samples, a JB-4 kit (Electron Microscopy Sciences; Fort Washington, PA) was used and product directions were followed. Embedded samples were dessicated overnight. Thin sections (3.5 μm) were cut with glass knives at 5˚. Sections were placed onto drops of distilled water (dH_2O) on glass slides and heated at approximately 60^{o}C on a hotplate to adhere the sections to the slides. The metanil yellow and periodic acid-Schiff's (PAS) reagent staining protocol used in the present study was adapted from Quintero-Hunter *et al*. (1991) and Jackson and Sullivan (1995). Durations of incubation for the present samples were longer than what was described previously. True PAS reactivity is questionable with this technique because residual glutaraldehyde bound to

the tissue can react positively with Schiff's reagent; however, a useful counterstain still results. Briefly, the slides were incubated in 1% aqueous periodic acid, rinsed with three changes of dH_2O, and then exposed to Schiff's reagent. Sections were rinsed with tap water and then incubated in Weigert's hematoxylin. Acidic 70% ethanol (pH 2.5) was used for two brief rinses and the slides were subsequently rinsed in three changes of acidic dH_2O (pH 2.5). After rinsing in tap water, the slides were incubated in 0.02% metanil yellow in acidified dH_2O. The sections were then rinsed with three changes of dH_2O followed by two rinses with 95% EtOH, two rinses of 100% EtOH and two rinses of acetone. Coverslips were applied with Polymount (Polysciences Inc., Warrington, PA). LM images were captured through the use of an Olympus Vanox AHBS3 photomicroscope equipped with a SPOT RT Slider cooled CCD camera and accompanying digital image acquisition software.

The EM samples fixed with 4F:1G fixative were rinsed twice in 0.1M Sorenson's sodium phosphate buffer (pH 7.2-7.4) two times for 15 min each. The samples were then osmicated at room temperature with 1% osmium tetroxide/0.1M phosphate buffer, pH 7.2-7.4, for 1 hr. After two rinses in dH_2O, the samples were sequentially dehydrated in an ethanolic series (50%, 75%, 95%, and 95%) for 15 min at each step, followed by two 30-min washes in 100% ethanol. After two 10-min washes in 100% acetone, the samples were infiltrated with Spurr resin as described previously (Dykstra 1993). Samples were placed in a 1:1 mixture of Spurr resin and 100% acetone for 30 min at room temperature, followed by two 1-hr steps in 100% Spurr resin. Finally, the samples were transferred to fresh 100% Spurr resin in molds with paper labels. The resin containing samples was polymerized for 8-72 hr at 70^{o}C and then semithin (0.5 μm thick) sections were cut. The semithin sections were stained with 1% Toluidine Blue O in 1% sodium borate for 15-30 sec, coverslipped, and

examined with a light microscope to determine areas of interest in the block face. After appropriate block trimming, ultrathin (80-90 nm thick) sections were cut with a diamond knife, placed onto 200 mesh grids, and stained with saturated methanolic uranyl acetate and lead citrate (Dykstra 1993). The stained ultrathin sections were examined and photographed with a Philips/FEI EM208S transmission electron microscope.

Digital Photography

Digital images of the ovarian biopsy samples were captured using a Nikon Coolpix 990 digital camera mounted on a Nikon SMZ800 binocular dissecting microscope. Ovarian follicles were photographed on glass slides in drops of ice cold, iso-osmotic (320 mOs) Cortland's balanced physiological saline. All digital images of follicles were processed so as to have a stage micrometer image inlay for accurate assessment of oocyte diameters.

Statistical Analyses

Data on blood plasma levels of Vg, E2, testosterone, 17α, β20-P and 20β-S were analyzed by category to determine statistical significance of atresia stage designations. Statistical software (SAS 8.0; SAS Institute, Cary, NC) was used to determine whether there were significant differences in Vg or hormone levels between atresia stages via use of a mixed model analysis of variance (ANOVA) procedure. Prior to analyses, variance of the data was normalized via log transformation and a variance/covariance matrix was used to compensate for repeated measures. For each parameter, the significance of the effect of repeated measures (fish effect) was determined. In statistical evaluations of the E2, testosterone, and 20β-S data, the fish effect was not significant and was dropped from the

ANOVA model. Evaluation of the 17α, β20-P data revealed a significant fish effect and repeated measures was left as a factor in the ANOVA model. Data on media levels of E2 and testosterone obtained in the *in vitro* culture experiments were first analyzed using Student's t-test to independently verify for each atresia stage whether or not addition of hCG to the culture media resulted in an increase in media steroid hormone levels. The combined data for all atresia stages was then analyzed by one-way ANOVA and contrast procedure (SAS 8.0). This was independently applied to the data for control cultures and then applied to data from the cultures incubated in the presence of hCG. The level of statistical significance established for all tests was $p \leq 0.05$.

RESULTS

In vitro Steroidogenesis

Follicular production of E2 and testosterone were compared across cultures of fish at non-atretic (Stage 1), early atretic (Stage 3) and late atretic (Stage 4) female striped bass (Figure 1). Follicles of each stage were subjected to a 24 hr challenge with hCG and compared to oocytes of the same stage held in control media. Media levels of E2 and testosterone were greatest in the media of non-atretic follicles challenged with hCG (0.8422 ng/ml and 5.184 ng/ml, respectively) and these hCG challenged follicles showed significantly higher levels of E2 and testosterone than their control counterparts. There was a notable decline in hormone secretion from follicles in the Stage 3 and Stage 4 cultures and addition of hCG to the culture media did not significantly enhance E2 or testosterone production. In fact, the Stage 4 culture yielded concentrations below detectable levels (<

minimum standard, ~0.04 ng/ml) when 50 µl was applied to the assay. Due to sample volume constraints larger volumes could not be evaluated in triplicate.

A comparison of follicular steroidogenesis across the three *in vitro* cultures indicated that the decline of E2 and testosterone production between non-atretic and early atretic follicles was significant for both control and hCG-challenged cultures. However, the decline of these parameters between early and late atretic follicles was not shown to be significant. With the exception of the late atretic group, the cultured follicles produced significantly more testosterone than E2 independent of the inclusion of hCG in the culture media.

Steroid Hormones in Blood Plasma

Plasma samples from vitellogenic (Stage 0) fish were taken at a different time of year and under distinct environmental conditions as those taken from the post-vitellogenic and atretic fish sampled during this study. For this reason plasma steroid hormone levels in the vitellogenic females were not assessed for statistical significance against the linear study, although absolute values were compared (Table 1). Plasma E2 levels increased more than twice when comparing Stage 0 to Stage 1 plasma samples. A steady decline was detected in circulating E2 from Stage 1 through Stage 4. In fact, ~39% of Stage 4 samples had plasma E2 levels that were below detection limits for the RIA (< minimum standard, ~0.08 ng/ml) when 25 µl sample volumes were assayed. Plasma levels of testosterone exhibited a pattern of change similar to E2 levels with respect to the progress of ovarian atresia. Testosterone levels in post-vitellogenic females were similar to those assayed in vitellogenic fish but they declined steadily with advancing atresia, becoming non-detectable (< minimum standard, ~0.08 ng/ml), in ~50% of females at atresia Stage 3 and ~83% of females at atresia Stage 4.

Plasma levels of 17α, β20-P and 20β-S did not vary with atresia stage, remaining basal but still within detection limits (< minimum standard, ~0.02 ng/ml), in females at all stages of atresia. Plasma from vitellogenic females was not assayed for 17α, β20-P or 20β-S levels because prior research established that these steroids are not detectable in vitellogenic female striped bass (King *et al.* 1994a).

Plasma Vitellogenin Levels

Plasma Vg levels in vitellogenic females were not compared statistically to those of the post-vitellogenic and atretic fish for the reasons stated in the analysis of plasma E2 and testosterone levels. Although the sampling schedule and experimental design makes such a comparison invalid, absolute values of plasma Vg for vitellogenic females are reported and graphically compared to values for the other fish in Figure 2. Vitellogenic (Stage 0) females were found to have an average Vg concentration of 1.068 mg/ml, which is slightly more than half of the mean value determined for post-vitellogenic (Stage 1) fish. Figure 2 shows an overall decline in plasma Vg levels as ovarian atresia progresses. It was found that the decline that occurs between Stage 1 and Stage 2 is not significant. Uniquely, the variability found in Stage 2 is greater than that found in any other stage and the distribution of these concentrations appears to be bimodal. Plasma Vg levels in Stage 3 females were significantly lower than in Stage 1 fish, and a further significant decrease in circulating Vg was evident as females progressed from atresia Stage 3 to Stage 4. It is important to note that plasma Vg levels always remained within detection limits of the ELISA (> minimum standard, 5.86 ng/ml).

SDS-PAGE and Western Blotting

Results of SDS-PAGE and Western blotting of blood plasma samples run on 4-15% gradient gels and ovarian extracts run on 8-25% gradient gels are shown in Figure 3. As noted, Western blotting was accomplished using specific, polyclonal antisera raised in rabbits against *Morone* Vg (Heppell *et al.* 1999) or Lv, and β'-component (Hiramatsu *et al. in press* a). Vitellogenin from *Morone* species and other teleosts is highly prone to physical degradation (Tao *et al*. 1993, 1996). Accordingly, plasma and ovarian tissue from the female striped bass were divided into smaller aliquots when first obtained and these aliquots, utilized for SDS-PAGE and Western blotting, underwent a single freeze-thaw cycle during analyses. Western blots of plasma samples from both vitellogenic, post-vitellogenic, and atretic females, as well as E2-induced males were conducted using aVg, aLV, and aβ' and all of these antibodies detected a main band with an apparent mass of ~170 kDa. This band possesses a molecular weight identical to the major band induced in males by via injection with E2. In addition, Western blotting of plasma from E2-induced males revealed minor bands at the ~100 kDa and ~90 kDa positions. No other bands were detected using the aforementioned antisera.

Electrophoresis of extracts of ovary samples from vitellogenic females on an 8-25% gradient gel indicated a faint high molecular weight band corresponding to ~140 kDa which was detected by all antisera (aVg, aLv, and aβ') in Western blotting. This band was not seen in Western blots of ovary extracts from post-vitellogenic or atretic females.

In Western blots of ovary extracts from vitellogenic, post-vitellogenic and atretic females, the aVg antiserum also detected major bands corresponding to the ~102 kDa, ~94 kDa, and ~75 kDa positions on the blot, although immunostaining intensities varied between

the different categories of females. Specifically, samples from vitellogenic and post-vitellogenic females generated a bold ~102 kDa band and faint bands at the other two positions, whereas the ~94 kDa and ~75 kDa bands were more intensely stained when samples from atretic females were used. The aVg antiserum also detected minor bands at ~116 kDa and ~84 kDa positions on blots of ovary extract samples from vitellogenic females.

When the antiserum against *Morone* Lv was used for Western blotting, banding patterns of immunostained ovarian proteins were similar to those detected by aVg. Major bands were detected at the ~102 kDa, ~94 kDa and ~75 kDa positions on the blots when ovary extract samples from vitellogenic, post-vitellogenic and atretic females were used. However, several minor bands of low apparent mass (*i.e.* ~23 kDa, ~18 kDa, ~14 kDa) were additionally revealed that were not apparent on blots developed using aVg.

Atretic ovarian extracts showed a relatively higher staining intensity of the ~18 kDa and ~14 kDa bands. Western blots of ovary extracts from vitellogenic, post-vitellogenic, and atretic females detected a solitary major band corresponding to a mass of ~7 kDa when probed with aβ' antiserum. Blots immunostained with aVg or aLv revealed minor bands of high molecular weight when analyzing ovarian extracts from vitellogenic fish.

Findings regarding the specificity and reactivity of the three antisera used in Western blotting were verified via IEP (Figure 4). None of the antisera reacted with male plasma. Native Vg in the plasma of vitellogenic fish was strongly detected by aVg and aLv and appeared as a single clear precipitin line migrating near the sample well. These same antisera showed faint immunoreactivity with plasma from atretic animals, each generating a single diffuse precipitin that electrophoresed farther down the gel than the lines generated when plasma from vitellogenic females was used. Two distinct precipitin lines were created by

aVg reacted against electrophoresed proteins from extracts of post-vitellogenic and atretic ovaries. The band closest to the sample well represents Lv because an identical line was elucidated when the samples precipitated with aLv. The more distant precipitin line represents β'-component and this is known because an identical band was present when the same samples were reacted with aβ' (data not shown). Of note is the lack of a precipitin line representing either Lv or β'-component when extracts of atretic ovaries were subjected to IEP using aVg.

Ovarian Morphology

Stereomicroscopy of Biopsy Samples

Digital images of ovarian follicles at each of the stages of atresia (Stage 1-4) are shown in Figure 5. As noted, freshly biopsied samples were placed in ice-cold, iso-osmotic balanced physiological saline and images were captured using a digital camera mounted on a dissecting stereomicroscope.

The maximum diameter of follicles obtained from vitellogenic (Stage 0) females in January was 600 μm with the majority of oocytes in the field ~500 μm and the oocytes were uniformly spherical in shape and coloration was uniformly opaque with a light green hue (data not shown). Based on results of prior research (Sullivan *et al*. 1997), these females would have been in mid-vitellogenesis at this time. Ultimately, their follicles would have reached ≥900 μm diameter by the time they became post-vitellogenic and eligible for induced spawning.

Typical post-vitellogenic (Stage 1) follicles were 900-1000 μm diameter and contained follicles that were uniformly spherical and opaque (Figure 5A). Early atretic

(Stage 2) follicles appear slightly vacuolated, especially at their periphery, and begin to take on an irregular or oval shape that is evident in samples examined in cross section (Figure 5B). These same follicles maintain an opaque coloration with a light green hue. Mixed in shape (spherical to irregular), opacity (opaque to highly translucent), and color (light green to orange), mid-atretic follicles (Stage 3) appear increasingly vacuolated and this characteristic increases through late atresia (Figure 5C). Also characteristic of Stage 3 is a general thickening of the peripheral band surrounding the oocyte. By late stage atresia (Stage 4), the oocytes are highly vacuolated, become increasingly transparent and somewhat decrease in diameter (~700-800 μm). Follicles obtained from females in the later atresia stages have generally lost their pale green coloration and range from yellow to dark orange in color. Even with this drastic change in appearance, the follicles appear intact (Figure 5D).

In biopsy samples obtained from females in the later stages of atresia (Stages 3 and 4), there is an increasing occurrence of follicles at early developmental stages; *i.e.* follicles containing primary and early secondary growth oocytes (Figure 5C and 5D). In addition, the ovaries of females at the later atresia stages had follicles that were uniformly atretic, all being at the same atresia stage (uniform), or their individual follicles were at various stages of atresia ranging from early to late (mixed).

These general observations on the physical appearance of post-vitellogenic and atretic follicles were, in part, stated earlier in the Materials and Methods section, because morphological characteristics of sampled follicles were used to sort females into atresia stages prior to biochemical and immunological analyses.

Light Microscopy

Histological sections of ovarian follicles at Stage 1-4 of atresia viewed by light microscopy (LM) are illustrated in Figure 6A, B (Stages 1 and 2) and Figure 7A, B (Stages 3 and 4). Stage 0 follicles are not illustrated in this investigation because the histology of vitellogenic follicles has been repeatedly described (Specker *et al.* 1987; Berlinsky and Specker 1991; Berlinsky *et al*. 1995; Tao *et al.* 1993) and is generally similar to that of other teleosts (Selman and Wallace 1989). In addition, the histological appearance of vitellogenic striped bass follicles is quite similar to that of post-vitellogenic follicles. However, the small and presumably newly formed yolk granules may be absent from the peripheral ooplasm in post-vitellogenic follicles. Follicles at both stages contain an oocyte with yolk granules that stain intensely with metanil yellow as well as lipid droplet remnants, which are indicated by circular voids dispersed throughout the ooplasm (Figure 6A). Also shown are PAS-positive cortical granules restricted to the ooplasm periphery (Figure 6A, inset). In mid-sagittal sections of follicles, an intact and basophilic germinal vesicle with multiple peripheral nucleoli is centrally located in the ooplasm (not shown in Figure 6A; Mylonas *et al.* 1997). The oocyte is bounded by a thick ZR that stains strongly with both metanil yellow and PAS and is overlain by layers of follicle cells (Figure 6A, inset). Similar to other teleosts (Selman and Wallace 1989), the follicle cell layers overlying the ZR consist of a monolayer of basophilic granulosa cells, a strongly PAS-positive basal lamina, and the multi-cellular thecal layer, which includes steroidogenic cells, fibroblasts, vascular tissue, and surface epithelium (Figure 6).

As illustrated in Figure 6, histological preparations of biopsy samples of Stage 1 and Stage 2 follicles have shown that the theca cell layer often becomes detached from the

follicle during sampling and sample processing. The granulosa layer was variable in appearance among samples taken from females at Stages 1 and 2. This layer begins to appear vacuolated. This vacuolization is not due to intracellular inclusions or vacuoles developing within the granulosa cells but rather is attributable to increasing extracellular edema in the granulosa cell layer. The degree of edema may appear slight (Figure 6B, inset) or extreme (Figure 6A, inset) in follicles from Stage 1 or 2 females, depending on the individual follicles examined.

In post-vitellogenic, non-atretic follicles (Figure 6A), the lipid droplet remnants are typically uniform, circular and evenly dispersed throughout the ooplasm. However, in Stage 2 follicles the lipid droplets begin to coalesce in a manner quite different than what is observed during final oocyte maturation. In early atretic oocytes, the coalescence occurs among groups of lipid droplets immediately adjacent to one another, and it does not proceed to generate one or a few major lipid droplets as seen in maturing oocytes (Mylonas *et al*. 1997). Thus, the limited coalescence generates oocytes whose ooplasm is filled with numerous remnants of coalesced lipid droplets, and these have highly irregular margins, especially in the peripheral ooplasm. Also beginning at Stage 2 is the dissolution of the germinal vesicle, which was most often observed breaking down in a central position or just as it was beginning to migrate to the oocyte periphery in association with onset of final oocyte maturation (Figure 6B). These characteristics of lipid droplet coalescence and nuclear dissolution during early atresia of striped bass follicles have been reported previously (Mylonas *et al*. 1997).

As follicles progress to Stage 3, there is a pronounced thickening of the ZR followed by its differentiation into the acidophilic ZR *interna* (ZRI), which stains intensely with

metanil yellow, and the basophilic ZR *externa* (Figure 7A, see smaller follicle on the left). The ZRI and the ZR *externa* (ZRE) later begin to loose contact with one another and, by the time the oocyte becomes highly irregular (non-spherical) in shape, integrity of both ZR layers is breached in several places (Figure 7A, inset). Also at this time, edema in the granulosa layer, first noticeable at atresia Stages 1 and 2, becomes extreme. In the space formerly occupied by ooplasm, the yolk granules become more diffuse in appearance and stain less intensely with metanil yellow. While the ZR components, granulosa cell layer, and ooplasm are undergoing tremendous change, the basement membrane separating the granulosa cells and theca remains intact as shown in the following section. By the time follicles have progressed to atresia Stage 4, the ZR components have largely disintegrated, a clear granulosa cell layer cannot be identified, and inclusions (lipid droplets, yolk granules) originally present in the ooplasm extend all the way out to the basement membrane. The follicle appears highly vacuolated due to the prevalence of lipid droplets. However, the few yolk granules remaining at this stage no longer stain well with metanil yellow or PAS and are, instead, weakly basophilic. The theca also thickens considerably, apparently due, in part, to hyperplasia of its connective tissue elements. There is a clustering of spindle shaped cells resembling fibroblasts around and within the thecal layer, extending from the basement membrane to the follicle periphery. These cells express variable staining characteristics in that most are strongly basophilic throughout, but the cytoplasm of small clusters of these cells stains intensely with metanil yellow. Primary growth and early secondary growth (lipid stage) follicles (Sullivan *et al.* 1997) are frequently encountered within the same field as Stage 3 and Stage 4 follicles, likely as a result of a biopsy sampling artifact based on decreased size and abundance of remaining atretic follicles.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to further illustrate the structural changes occurring in striped bass follicles undergoing atresia and to elucidate the cellular processes underlying the morphological changes observed at the LM level as discussed. As noted, Stage 1 follicles resemble vitellogenic follicles in structure. The ooplasm is bounded by the ZR, which is overlain by a monolayer of mitochondria-rich granulosa cells. Granulosa cells are filled with smooth and rough ER and Golgi bodies. Surrounding the granulosa cells is an acellular and fibrillar basement membrane, which is ultimately enclosed by the multi-cellular theca (Figure 8A). At this stage, the granulosa cells fill the space between the basement membrane and the ZR and are closely associated with the ZR as well as neighboring granulosa cells. Microvillar processes emanating from both the ooplasm and the granulosa cells penetrate as finger-like projections through pores in the ZR and make contact with one another. The acellular ZR is highly stratified but consists of four distinct major layers of different electron density, all of which are perforated by the microvillar cell processes (Figure 8A). The ooplasm contains electron-dense yolk granules (not shown) and highly electron-dense lipid droplets as well as mitochondria and some fine granular areas that suggest the presence of proteinaceous material.

As previously noted, during atresia Stages 1 and 2, the granulosa cell layer begins to become fluid filled due to intracellular edema (Figure 8B). The microvillar processes appear to begin to recede in conjunction with a general decline in intracellular contact across the ZR. However, this latter observation requires conformation via more intensive sampling of follicles at this stage and more detailed examination of junctional complexes between the granulosa cells and oocyte. The granulosa cells become increasingly electron dense and

continue to exhibit intact mitochondria coupled with plentiful RER and Golgi bodies. Again it is evident that the basal lamina between the granulosa cells and theca remains intact but the overlying theca cells and the surface epithelium appear to become disorganized (not shown, discussed below).

Atresia Stage 3 shows that the basal lamina is still intact but becomes increasingly more serpentine, the theca is very disorganized and increasingly hyperemic, and the ZR assumes a digested, disintegrating appearance (not shown; see discussion of atresia Stage 4 and Figure 8). Evidence of multiple nuclei is found within the ooplasm (Figure 9A), and the elongated, pyramidal shape of these nuclei is similar to that of granulosa cells in follicle layers exhibiting edema during atresia Stages 1 and 2. The cells bearing these pyramidal nuclei often contain membranous whorls, reminiscent of those seen in macrophages, which suggests that they are engaged in phagocytosis (Figure 9A). In addition, these nuclei are often closely associated with lipid droplets (Figure 9A).

Follicles in a late stage of atresia (Stage 4) exhibit an increasingly tortuous, although intact, basal lamina with increased vascularization of the highly disorganized theca (Figure 9). Nests of fibroblasts are frequently encountered in the theca (not shown; see Figure 7b). Ooplasmic disorder increases, membranous whorls are evident throughout the ooplasm (Figure 10A), and it seems that intracellular debris that was not phagocytized by invading granulosa cells begins to degrade, eventually disappearing (Figure 10B). Eventually the ooplasmic space is largely devoid of organelles or other inclusions, although some lipid droplets are still present at this late stage of atresia (Figure 10B).

Induced Spawning

One female (PIT tag #791A), of the group that was repetitively sampled while undergoing ovarian atresia, did not progress to mid- to late atresia stages (Stages 3 and 4) during the experiment. This individual underwent an increase in circulating Vg levels between sampling periods one to two and accounted for the two highest Vg concentrations measured during the study, including those measured in vitellogenic (Stage 0) animals. On 27 May 2001, after sampling of the fish was terminated and all of the other females were determined to have highly atretic oocytes, this female was still at atresia Stage 2 and the maximum diameter of her ovarian follicles was still 940 μm. She was then implanted with a cholesterol/cellulose pellet containing 300 μg (~36 μg/kg body weight) of a synthetic analogue of human gonadotropin-releasing hormone (GnRHa), in order to induce maturation and spawning (Hodson and Sullivan 1993). Two days later, she was subjected to ovarian biopsy and upon examination of her follicles it was found that they were at an advanced stage of final maturation (11 hr Stage of Rees and Harrell 1990; corresponding to a stage just preceding breakdown of the peripheral germinal vesicle). She was then injected with 330 I.U. hCG per kg body weight and was spawned the next day, producing 703,799 eggs of which 40.3% (N=283,631) were fertile as judged by the presence of a well formed embryo several hours after fertilization. It was noted that these ova did not water harden as well as those of fish spawned during the normal reproductive season (April).

DISCUSSION

The present study involves fundamental research conducted to elucidate the process of ovarian atresia in striped bass and its regulation by reproductive hormones. Within the

scope of this investigation was the examination of yolk proteins derived from Vg and how they are recycled during ovarian atresia. Also central to this research were the more detailed descriptions of the morphology, histology, and ultrastructure of non-mammalian atretic follicles than exists in the present literature (Mylonas *et al*. 1997). Although previous studies have generally described atretic follicles of striped bass and other teleosts (Berlinsky and Specker 1991, Mylonas *et al.* 1997), this is the first investigation to simultaneously examine their morphological, biochemical, and functional properties.

Prior research on reproduction of striped bass demonstrated the value of measuring reproductive hormones and proteins as markers for specific stages of maturation and to identify suitable candidates for induced spawning (Sullivan *et al*. 1997). These methods were first calibrated to morphological and histological characteristics of the maturing gonads. In addition, *in vitro* tests of follicular steroidogenesis and oocyte maturation have been developed to judge the maturational competency of female striped bass (Weber *et al.* 2000). The results of the present research were anticipated to generate similar morphological, biochemical, or functional markers of atresia. Such markers could then be utilized in hatchery propagation of striped bass by providing the means to recognize when atresia is imminent in individual females. Those fish could then be promptly induced to spawn before their ovaries become dysfunctional and their reproductive output is lost for the year. Also, practical markers of atresia would benefit applied research conducted to discover ways to delay or block onset of atresia in striped bass. For example, techniques for delaying atresia based on holding females at low temperature (Smith *et al*. 1996), which have already been developed for white bass, could be more rapidly optimized for striped bass if physiological processes leading to atresia could be conveniently monitored.

Mylonas *et al*. (1998) suggested that, as teleosts progress through atresia, there is a significant decline in their ability to respond to GtH due to a general decline in the steroidogenic capabilities of the ovarian follicle cells. In the present study, cultured follicles from three different stages of atresia were challenged with hCG and compared to follicles that were held in serum-free conditions for relative measurement of E2 and testosterone production. These hormones were chosen for evaluation primarily because their reproductive functions in striped bass have been thoroughly characterized (Sullivan *et al*. 1997). Secondarily, *in vitro* production of these hormones by follicles from post-vitellogenic, non-atretic *Morone* females has been evaluated in several prior studies (King *et al.* 1994b, 1995; Weber and Sullivan 2000). Also, striking elevations in circulating E2 and testosterone are characteristic of post-vitellogenic striped bass both immediately prior to, and during final maturation (King *et al.* 1994a; Jackson and Sullivan 1995; Mylonas *et al.* 1997; Weber *et al*. 2000). Finally, high levels of production of both hormones in response to hCG characterizes the early stages of final maturation of Morone follicles *in vitro* (King *et al.* 1994a; 1995). Therefore, it was logical to suspect that these sex hormones might be involved in regulating atresia in striped bass, as they are in controlling atresia and apoptosis of follicles of other species (Hughes and Gorospe 1991; Nahum *et al*. 1996; Janz and Van Der Kraak 1997; Choi *et al.* 2000).

It was found that fully-grown follicles from non-atretic striped bass produced both E2 and testosterone *in vitro* and production of these hormones was strongly stimulated by hCG. As shown in prior reports on culture of *Morone* follicles (King *et al.* 1994a; 1995), *in vitro* testosterone production by non-atretic striped bass follicles was substantial, being several-fold greater than E2 production independent of whether or not the follicles were exposed to

hCG. The reverse was true with regard to circulating levels of E2 in non-atretic (Stage 1) females; E2 levels were over three times greater than testosterone levels. This disparity between results could be due to several factors, including differential metabolism and/or clearance of the two steroids *in vivo*. Alternatively, post-vitellogenic follicles may require hormonal support in addition to GtH in order to maintain high levels of aromatase activity *in vitro*. Current models for follicular steroidogenesis in teleosts indicate that E2 production is localized to the granulosa cells in which aromatase converts testosterone, produced locally or by the theca cells, to estrogen (Petrino *et al*. 1989; Patino *et al.* 2001). With the exception of GtH, the other hormones that are responsible for maintenance of high levels of aromatase activity by striped bass granulosa cells are not known. It is likely that several endocrine, paracrine, or autocrine factors are involved. For example, IGF-I is known to regulate maturational competence, responsiveness to gonadotropin, steroidogenesis, and oocyte maturation in *Morone* follicles (Weber and Sullivan 2000, 2001; Weber *et al.* 2000).

In this study, plasma levels of teleost final MIS were maintained at constant low levels in females at all stages of atresia. In prior research, compelling evidence has been amassed that the primary MIS in striped bass and other *Morone* species is 20β-S; it is suspected that 17α, β20-P is the immediate biosynthetic precursor to 20β-S (King *et al*. 1994ab, 1995, 1997; Sullivan *et al.* 1997). The only change in plasma levels of these C_{21} steroids was a significant decline in 17α, β20-P levels in females at late stages of atresia. Final maturation of striped bass oocytes is accompanied by a pronounced surge in circulating levels of 17α, β20-P and 20β-S just prior to breakdown of the germinal vesicle after their migration to the oocyte periphery (King *et al*. 1994a). The spike in 17α, β20-P and 20β-S levels, which is preceded by a precipitous decline (hours to days) in plasma E2 and

testosterone levels, is also seen when follicles are induced to undergo final maturation *in vitro* (King *et al*. 1994ab). However, the decrease in plasma E2 and testosterone associated with atresia is clearly part of a different physiological phenomenon. It extended over several weeks and was not followed by any increase in C_{21} steroids or associated with morphological evidence of follicular progress through final maturation.

Measurement of circulating levels of E2 and testosterone and evaluation of follicular steroidogenesis *in vitro* indicated that progression of atresia in striped bass is characterized by a general loss of steroidogenic ability as well as a decreased responsiveness to GtH. However, there was no evidence that selective down-regulation of aromatase activity is a component of the decline in steroid production. Steroid production and responsiveness to hCG by follicles from an early atretic (Stage 2) female were minimal, and follicles taken from a female in an advanced stage of atresia did not produce significant amounts of E2 or testosterone even when challenged with hCG. Likewise, average blood plasma levels of E2 and testosterone declined progressively with advancing atresia. In the case of E2, this decline was statistically significant only for late atretic (Stage 4) females.

As observed in prior studies of vitellogenic and maturing striped bass, changing testosterone levels were highly correlated with E2 levels in females undergoing atresia (Woods and Sullivan 1993; Blythe *et al.* 1994). However, plasma testosterone levels declined significantly between each stage of atresia after Stage 2 (early atresia), suggesting that testosterone assays could be used to verify a female's viability to spawn, despite the fact that sampled follicles would be considered partially atretic by the gross morphological standards used in this study. The one female in this study who did not progress to advanced atresia was classified as being at atresia Stage 2 at the induction of spawning. However, this

individual yielded a fecundity and fertility comparable to non-atretic striped bass spawned during the normal reproductive season (Sullivan *et al*. 1997; Hodson *et al*. 2002). Thus, testosterone levels may better indicate the viability of potential spawners than does morphological assessment of ovarian biopsies. The only difference noted in the spawning performance of this female was that her oocytes did not water harden as rapidly or fully as females induced to spawn earlier in the season. It is possible that function of the cortical alveoli, which are located in the peripheral ooplasm and contribute to water hardening by depositing osmotically active materials into the perivitelline space, may be impaired early in atresia or that the chorion itself is somehow compromised. As noted, the chorion (ZR) is delaminated and ultimately breached during the latter stages of atresia and it is possible that damage to this structure begins early in the atretic process.

The pattern of change in circulating E2 and testosterone during the gametogenic cycle of all *Morone* species examined to date is decidedly biphasic (Sullivan *et al*. 1997). Most oocyte growth occurs while circulating levels of these steroids are sustained at low levels (usually < 0.5 ng/ml) and it is only during the last few weeks before spawning that the hormone titers are strongly elevated (~1-3 ng/ml). Comparison of the absolute levels of E2 and testosterone in Stage 0 (vitellogenic) females of this study to results from previous investigations indicates that the Stage 0 fish were in early vitellogenesis. This is consistent with the time they were sampled (January). Plasma levels of E2 and testosterone would have been several-fold higher if these fish were sampled later in the year, and especially during the last few weeks before spawning. As discussed above, previous research has shown that there is a significant increase of plasma E2 and testosterone levels in the plasma of post-vitellogenic *Morone* females early during final maturation, as germinal vesicles are migrating

to the periphery of maturing oocytes. If females in this study were proceeding toward final maturation and not atresia, then their plasma E2 and testosterone levels should have increased considerably after Stage 0. It seems that levels of these sex steroids that were measured in females at Stage 1 and beyond are really endpoints of a steep decline in these hormones associated with atresia.

The results of the *in vitro* evaluations of follicular steroidogenesis clearly indicate that loss of responsiveness to GtH, in part, causes the decrease in circulating steroid hormones during atresia in striped bass. It is now generally accepted that most teleosts possess a dual GtH system. This system includes FSH (GtH-I) and LH (GtH-II) that are structurally homologous and functionally analogous to their mammalian counterparts (Kawauchi ***et al.*** 1989). Striped bass GtH-II has been biochemically purified and immunoassays (RIA and ELISA) to detect the protein in blood have been developed (Blaise *et al*. 1998; Mananos *et al.* 1997). It is known that circulating GtH-II levels peak in striped bass coincident with the peak in 17α, β20-P and 20β-S levels that accompanies germinal vesicle breakdown in maturing oocytes (Mylonas *et al*. 1997; Mylonas *et al*. 1998). Before and after this time, GtH-II is present at low or non-detectable levels in the blood plasma. Follicle-stimulating hormone appears to be a better candidate for potential regulation of follicle survival in opposition to atretic processes. This is because, in most fishes examined to date, FSH is engaged in regulation of E2 and testosterone production during the majority of the gametogenic cycle, exclusive of final maturation (Gomez ***et al.*** 1999; Moriyama *et al*. 1997). However, GtH-I has not been purified from striped bass and immunoassays are not available to detect the protein. In striped bass, as in some other teleosts, pituitary levels of the hormone are too low to accommodate available purification methods. It is likely that striped

bass maintain relatively low GtH-I concentrations in the blood and their pituitary gland may release the hormone as soon as it is produced (Sullivan *et al.* 1997). As is the case for GtH-II, the gene encoding striped bass GtH-I has been cloned as cDNA (Hassin *et al*. 1995). This result indicates that GtH-I mRNA, and potentially the encoded protein, are produced by the striped bass pituitary gland. Detection of changes in GtH-I (or GtH-II) mRNA associated with atresia was not possible in the present study, as designed, because the repeated sampling of individual females undergoing atresia precluded sampling of pituitary tissue for mRNA measurements. However, blood plasma samples were taken and sent to the laboratory of Professor Yonathan Zohar for analyses of GtH-II levels. At the time of this writing, the GtH-II data is unavailable. When available, results of these analyses will be presented in a report evaluating potential involvement of changes in circulating GtH-II levels in the decline of plasma E2 and testosterone during atresia.

The waning steroid hormone production by atretic females can be correlated to the morphological findings indicating a drastic change in the structure of follicle cells undergoing atresia. Specifically, the granulosa cells undergo a striking metamorphosis, from cuboidal, steroidogenic nurse cells arranged in a monolayer between the ZR and basement membrane into large, irregularly shaped phagocytes that breach the ZR, completely fill the space formerly occupied by ooplasm, and consume lipid droplets, yolk granules and other yolk components. We identified the cells invading the ooplasm as granulosa cells because, unlike the ZR, we found no evidence that the basement membrane of the follicle is breached, even late in atresia. Furthermore, nuclei of these cells bear a strong resemblance to those of granulosa cells in non-atretic follicles. Finally, there was no evidence of cell division in the granulosa cell layer, such as would be evident if the granulosa cells were only acting as

progenitors to the cell line producing phagocytes which invade the ooplasm. It is not surprising that this profound metamorphosis of the granulosa, from endocrine cells to phagocytes, would be accompanied by a loss of steroidogenic capacity. Considered together with the histological and ultrastructural assessments made in this study, it seems that atretic follicle cells (theca and granulosa) cease steroidogenic processes and are destined to one of three fates: apoptosis, necrosis, or phagocytosis. Although apoptosis was not specifically investigated in this study, it has become apparent that programmed cell death is a major mechanism of follicular atresia in mammals (Morita and Tilly 1999). Clear signs of cellular necrosis were observed in both the theca and granulosa cell layers of striped bass follicles undergoing atresia in this study (data not shown).

The decline in circulating E2 levels in females undergoing atresia in this study was temporally associated with a parallel decline in circulating levels of the yolk protein precursor, Vg. However, although the decline of E2 and testosterone levels with advancing atresia stage was accompanied by a similar decline in Vg levels, statistical assessment of sex steroid and Vg levels in individual females did not reveal a significant correlation. As discussed previously in the Literature Review, E2 and testosterone levels generally fluctuate in tandem in female striped bass, presumably due to their product-precursor relationship, and it is well established that E2 induces hepatic vitellogenesis in this species (Kishida *et al*. 1992; Tao *et al.* 1993). In prior research on vitellogenic female striped bass, E2 and Vg levels were significantly correlated (Tao *et al.* 1993; Blythe *et al.* 1994), albeit weakly, due to the biphasic nature of changing E2 levels during gametogenesis as discussed above, and due to the fact that Vg levels rise abruptly to a sustained plateau early during secondary oocyte growth. Nonetheless, it was found that plasma Vg levels were significantly correlated

with oocyte growth and have potential value as a test for onset of maturation and progression of gametogenesis in striped bass (Blythe *et al.* 1994). In contrast, absolute levels of Vg do not seem to have predictive value with respect to the status of atresia. Within atresia stages there was substantial variation in plasma Vg levels, and for fish at atresia Stages 2 and 3 the distribution of blood Vg concentrations was clearly bimodal. In another study of post-vitellogenic, non-atretic striped bass, blood Vg levels were evaluated with respect to their ability to predict the competency of females for hormone-induced spawning (Weber *et al.* 2000). It was found that Vg levels had little predictive value due to the high degree of variation between females. It appears that following secondary oocyte growth, during which time deposition of yolk proteins derived from Vg is a major mechanism for increasing the mass of the oocytes (Specker and Sullivan 1994), plasma Vg levels have little utility in the identification of maturation or atresia in individual fish.

The metabolic fate during atresia of egg yolk proteins derived from Vg also was investigated in this study. In previous research on more primitive fishes, such as rainbow trout and hybrid sturgeon (*Huso huso* female x *Acipenser ruthenus* male), it was shown that Lv and β'-component were recycled from the atretic oocytes to the blood plasma in largely intact form (Babin 1987; Fujii *et al.* 1991). These could then serve as markers for the onset of atresia in individual females. However, the phenomenon of yolk protein recycling has not previously been investigated in more advanced teleosts, including perciform fish like striped bass. In salmonid fishes, barfin flounder (*Verasper moseri*), and several other teleosts, Vg gives rise to three major yolk proteins, Lv, Pv, and β'-component (Specker and Sullivan 1994; Hiramatsu *et al. in press* a). These three yolk proteins were recently purified from white perch, biochemically characterized, and used as antigens to develop specific antisera

(Hiramatsu *et al. in press* a). Results of SDS-PAGE and Western blotting of proteins in blood plasma and ovarian extracts performed using these antisera revealed that the corresponding yolk proteins of striped bass are completely degraded in the follicle during atresia and not exported to the bloodstream intact or partially-intact for recycling. This finding is in contrast to observations on rainbow trout and hybrid sturgeon, in which Lv or β'-component was found circulating intact in atretic animals (Babin 1987; Fujii *et al*. 1991). Neither the β'-component nor any Lv fragment could be detected in plasma of atretic striped bass by Western blotting, which suggests that they are completely hydrolyzed within the oocyte before the amino acids are recycled back into the blood stream.

While Lv is the major lipoprotein supplying amino acids and lipids to developing embryos, and Pv transports materials for skeletal development (phosphate and calcium) bound to its extensive polyserine domain, the exact function of β'-component is unknown (Specker and Sullivan 1994). It is now recognized that teleost β'-component is derived from the C-terminus of Vg and contains five of the conserved cysteine residues which characterize this Vg domain (Hiramatsu *et al. in press* a). These observations, along with protein structure-function considerations, suggest that the tertiary structure of β'-component may be important, perhaps for retention of some signaling function of the molecule. In a study of salmonids it was found that β'-component remained intact during the later stages of embryonic and larval development (Hiramatsu *et al.* 2002). It was suggested that usage of β'-component by salmonid embryos does not require prior breakdown of this protein. Because the present study revealed contrary evidence, that β'-component is not recycled intact in striped bass, it would be interesting to investigate whether or not this breakdown only occurs in atretic follicles or if this yolk protein is broken down for use in developing

striped bass embryos as well. In the latter case, a potential signaling function for β'-component in perciform teleost embryos would not be indicated.

Evidence to support the conclusion that striped bass yolk proteins are completely degraded in atretic follicles comes from several observations. First, the ~170 kDa band that was distinguished in all of the plasma samples as well as in the ovarian extracts from vitellogenic animals undoubtedly represents the intact Vg polypeptide monomer and not a breakdown product of Vg-derived yolk proteins. This same subunit molecular weight was obtained for Vg purified from striped bass (Tao *et al.* 1993) and white perch (Tao *et al*. 1996). In addition, the E2-induced male plasma not only illustrated a very strong 170 kDa vitellogenin band but also exhibited bands corresponding to ~ 100 kDa and ~90 kDa. These latter bands are believed to arise from proteolytic degradation of Vg or mechanical damage to the molecule occurring during freeze-thaw cycles (Tao *et al.* 1993; Hiramatsu *et al. in press* b). Using the aforementioned triumvirate of antisera, Western blotting of plasma samples from vitellogenic, post-vitellogenic, and atretic females revealed no bands other than those attributable to Vg or its breakdown products. This strongly supports the notion that striped bass yolk proteins are completely degraded in atretic follicles.

Analysis of ovarian extracts from vitellogenic females by 8-25% gradient SDS-PAGE and Western blotting revealed a high molecular weight band (~145 kDa) that was verified to be the Vg monomer by comparison with results for plasma from E2-induced males run in the same electrophoresis system (data not shown) and through its recognition by the aVg, aLv, and aβ' antisera in Western blots. Generation of this band by ovarian extracts from vitellogenic females could result from contamination of the ovary samples by blood bearing high concentrations of Vg or the presence in the oocytes of newly endocytosed Vg that has

not yet been converted into yolk protein products. Only the latter scenario seems plausible because ovarian extracts from post-vitellogenic and atretic females did not generate the Vg band, even though blood levels of Vg were higher in post-vitellogenic females than in vitellogenic animals. As noted, the vitellogenic females in this study were sampled early in their gametogenic cycle when their Vg levels were not as high as maximal values achievable during the season. It also should be noted that, in the context of this study, "post-vitellogenic" refers to females whose oocytes are fully-grown and no longer incorporating yolk proteins derived from Vg, although they still had high levels of Vg in their circulation. The absence of Vg in ovarian extracts from post-vitellogenic fish supports this characterization of post-vitellogenic females and suggests that termination of vitellogenic oocyte growth involves cessation of Vg uptake and processing by the oocytes in the face of constitutively high circulating Vg levels.

In Western blots developed with aVg, bands corresponding to ~102 kDa, ~94 kDa, and ~75 kDa were generated by ovarian extracts from vitellogenic, post-vitellogenic, and atretic females. These same bands also were antigenic to the aLv antiserum and, therefore, likely represent Lv-derived proteins. On SDS-PAGE gels and Western blots developed with aLv, ovarian extracts from atretic females showed evidence of an increase in staining intensity of breakdown products at lower molecular weights (~23 kDa, ~18 kDa, and ~14 kDa bands), which is suggestive of progressive breakdown of Lv and its derivatives as atresia proceeds. Teleost Lv generally consists of a heavy chain and a light chain, which are covalently linked but derived from different domains of the parent Vg molecule (Matsubara *et al.* 1999). Results of recent detailed analyses of the biochemistry of egg yolk proteins in white perch (Hiramatsu *et al*. *in press* a) strongly suggest that the ~102 kDa band represents

the Lv heavy chain (Lvhc) of striped bass, the ~94 kDa band is a breakdown product of the Lvhc, and the ~75 kDa band represents the Lv light chain (Lvlc).

Antiserum specificity was apparent from results of IEP in that all three antisera were clearly female-specific; no precipitin lines formed when male plasma was analyzed. The precipitin lines that formed by aVg and aLv reacting against Vg in plasma from atretic females were more diffuse and formed farther down the gel than when plasma from vitellogenic females was analyzed. These results suggest that Vg is already partially degraded as it circulates in atretic females, although the epitopes reacting against aVg or aLv remain intact. This type of degradation, evident in IEP, has been observed when plasma samples from vitellogenic animals have undergone repeated freeze thaw cycles (N. Hiramatsu, personal communication). As the plasma samples used for IEP were not subjected to repeated freeze-thaw cycles, it seems likely that Vg may have been proteolytically damaged in plasma from atretic fish, perhaps as part of the process of clearing Vg from the plasma in the absence of its normal target, the vitellogenic ovary. The results of IEP also verified the lack of intact β'-component in the blood of atretic females, which corroborates the novel finding that this protein is completely hydrolyzed prior to being recycled back into the blood of atretic female striped bass.

Although there have been extensive morphological characterizations of oocyte development in fish, most of these investigations conclude at ovulation (Andrade *et al.* 2001, Mohanty and Verma 1999, Selman *et al*. 1993). The first study that examined the resorption process in teleosts only investigated the postovulatory follicle of *Astyanax bimaculatus lacustris* (Drummond *et al*. 2000). It was suggested that apoptosis plays a critical role in this process by acting to eliminate the wall of follicular cells that remain after spawning.

Apoptotic cells were identified by characteristics such as chromatin condensation and apoptotic bodies in the apical cytoplasm. Cells that proceeded toward apoptosis were phagocytized by normal follicular cells while other cells underwent a necrosis-like process. Programmed cell death is a highly conserved event found in organisms from *Caenorhabditis elegans* to humans and in a wide variety of roles. Apoptosis is a way of eliminating unhealthy cells or remodeling existing tissues. For these reasons it is likely that apoptosis plays a role in teleost ovarian atresia. Although the apoptotic process can be evaluated by labeling DNA fragmentation in histological preparations, this technique was not applied in the present investigation.

Light micrographs portrayed early vitellogenic samples with smaller oocytes that had not completed uptake of yolk proteins. Selman and Wallace (1989) suggest that Vg is taken up by pinocytotic activity and this uptake accounts for a substantial portion of oocyte growth. Stage 1 oocytes were definitively larger than those at Stage 0 and Stage 1 oocytes had equally dispersed yolk granules as opposed to Stage 0 oocytes that primarily showed yolk granules staining around the periphery. Primary and secondary oocytes appeared within the same field as Stage 3 and Stage 4 oocytes. This is consistent with previous depictions of atresia in striped bass in that, like other vertebrates, the juvenile ovary contains its lifetime complement of primary growth oocytes (Sullivan *et al.* 1997). A study conducted on rainbow trout (*Oncorhynchus mykiss*) removed one of the pair of ovaries during the beginning of vitellogenesis (Tyler *et al.* 1996). Rainbow trout, like striped bass, are single-clutch group-synchronous spawners and are found to have a second population of oocytes (at primary and secondary growth stages) that develop along with the present season's spawnable oocytes. Tyler *et al*. (1996) found that by removing one of the ovaries the

remaining ovary containing primary and secondary growth oocytes, along with the clutch of the current season, expressed a compensatory growth effect to recruit the young oocytes and preparation for spawning a year early. During this rapid growth of primary and secondary oocytes, the clutch that was intended to spawn remained on a growth curve comparable to controls with both ovaries intact.

This seems similar to what was found in histological sections of ovarian tissue in the present study. Secondary growth oocytes became visible within the same sections as the highly atretic oocytes. There are two possibilities to account for the multiple stages present: 1) Early secondary stage oocytes are developed a season before their intended maturation year or 2) The onset of atresia acts as a signal to begin recruiting the next cohort of oocytes through secondary growth. Evidence supporting the former hypothesis was provided by Berlinsky and Specker (1991) who observed early secondary growth oocytes alongside nearly fully-grown oocytes just prior to the spawning season. The latter hypothesis should be tested utilizing the knowledge of atresia and techniques developed in the present study.

Light micrographs of the various atresia stages not only illustrate the morphological changes of the ZR, granulosa cells, theca and surface epithelium, but also are suggestive of biochemical changes within the ooplasm. Yolk granules are deeply stained in the early stages (0,1,2) but, by Stage 3, there is a noticeable decline in yolk granule staining and a degeneration of the infrastructure of the oocyte. This corresponds to the yolk protein breakdown found via Western blotting. Evidently the yolk granules are broken down into component parts and are subsequently exported from the oocyte. Electron micrographs of Stage 3 oocytes illustrate granulosa cell nuclei closely associated with membrane-bound proteinaceous material as well as lipid droplets, within the cytoplasm, all enclosed within the

same plasma membrane. This, of course, suggests that both remaining lipids and proteins are engulfed and contained within vacuoles of the now phagocytic granulosa cells. In addition, membranous whorls that typify phagosomes were found within these invading cells.

At the LM level it appeared that the granulosa cells become hypertrophied. Electron microscopy provided evidence that the granulosa cells themselves do not hypertrophy but rather the space between the ZR and basement membrane enlarges as a result of edema. The cause of this edema has yet to be identified. However, during the duration of the developing edema the follicle cells themselves remain healthy as shown by abundant electron-dense mitochondria coupled with plentiful amounts of rough ER and some Golgi bodies. This means that the follicle cells still possess their metabolic machinery. Because some evidence of granulosa cell lysis is found between the basement membrane and ZR, it is likely that not all granulosa cells become phagocytic but rather some undergo a necrosis-like process (not shown). It is possible that, similar to what is found in post-ovulatory situations (Drummond *et al*. 2000), the majority of follicle cells initiate apoptosis while other follicular cells change from steroid producing cells to phagocytic cells and engulf the remaining apoptotic follicular layer as well as the contents of the degrading oocyte. Lambert's 1966 investigation of the guppy (*Poecilia reticulata*) ovary supports this idea in that he found granulosa cells associated with a morphologically healthy oocyte produce glucose -6 phosphate dehydrogenase (G-6PD). This enzyme is critical to the production of nicotinamide adenine dinucleotide phosphate in the citric acid cycle and is highly elevated in steroidogenic tissue. This researcher also found that G-6PD was not detected in the atretic follicle but there were high levels of non-specific esterases and acid phosphatases, which are indicative of a degenerating steroid-producing tissue. Finally, those follicle cells that are not phagocytized

become necrotic. In cyclostomes, histological analysis correlates atresia with the development of cells originating from follicle cells that phagocytize the contents of the oocytes (Saidapur 1978).

Changes also may occur in the microvillar processes that connect the follicle cells to the ooplasm. As vitellogenesis progresses, it was found that microvillar processes increase in number and length. During the stages of maturation in which the germinal vesicle migrates to the periphery it has been found that a retraction of the microvillar processes occurs; by the time of FOM and ovulation there is a complete lack of evidence of these processes (Kayaba *et al.* 2001). The loss of contact between follicular cells and the oocyte also is evident prior to ovulation when a substantial "void" or edema develops between the follicle layer and the ZR (Nagahama 1983). No real evidence of the retraction of microvillar processes corresponding to progressing atresia has been found in the present study. Disintegration of the ZR, however, clearly occurs around Stage 3. Perhaps an enzyme similar to that secreted by a hatching embryo is synthesized by one form of the changed follicle cell. How this acellular material is digested or what triggers the digestion is, as yet, unknown.

Throughout the stages of atresia investigated in this study and all of the associated morphological and biochemical changes, the basement membrane appears to remain intact. The only change of note is that it becomes increasingly tortuous. Because of the difficulty in obtaining biopsy samples past Stage 4 we are, as yet, unable to completely characterize the degradation of this membrane. It is possible that after the majority of yolk granules degrade and most of the lipid droplets are engulfed and digested by phagocytic granulosa cells, the basement membrane is also degraded and the breakdown products are recycled back into the blood. The disorganized thecal layer just outside the basement membrane becomes

increasingly vascularized from Stage 0 through Stage 4, which could indicate a path by which breakdown products are recycled. In addition, LM sections showed development of nucleated cells surrounding very late stage atretic follicles. These cells are probably fibroblasts that had once given structure to the ovary and are now collapsing as a result of the decline in the integrity of the oocytes within. In avian systems, fibroblasts are known to invade the scars from ovulated follicles as well as the lipoidal masses of highly atretic oocytes and phagocytize the lipid within the oocyte (Marshall and Coombs 1957). Some fibroblasts even become larger and more swollen than others and are then identified as "lipid cells." This phagocytic property of the fibroblasts may account for how the remaining lipid is removed from the highly degraded oocyte and may explain the development of these cells and their differential staining in the light micrographs of Stage 4 follicles.

Ultimately, advanced teleosts that are not provided with the proper hormonal and thermal cues will begin the undesirable process of ovarian atresia. This process can begin at a variety of stages of development and the absolute cues that initiate this process are unknown. The present study was an attempt to further our understanding through a wide array of available techniques and to suggest areas of future research. Understanding the atretic process more fully increase the likelihood that aquaculturists will be able to identify stages of this process and, potentially, to delay or prevent its onset.

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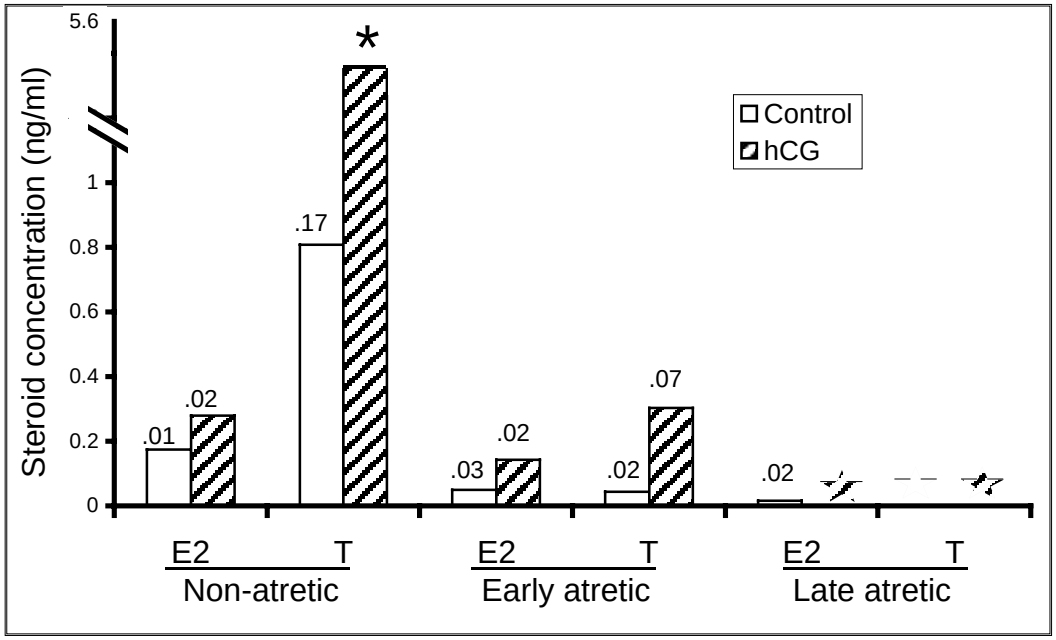

Figure 1. Estradiol-17β (E2) and testosterone (T) concentrations in culture media after incubation of ovarian follicles at three stages of atresia: Non-atretic = Post-vitellogenic or Stage 1, Early atretic = Stage 3, Late atretic = Stage 4, for 24 h in the absence (Control, open bar) or presence of 25 I.U./ml media of human chorionic gonadotropin (hCG, hatched bar). The height of the bar indicates the mean for triplicate incubations and the number above the bar indicates SEM. The asterisk indicates a mean ± SEM of 5.164 ± 0.31 ng/ml. Star indicates hormone levels below the detection limits of the assays.

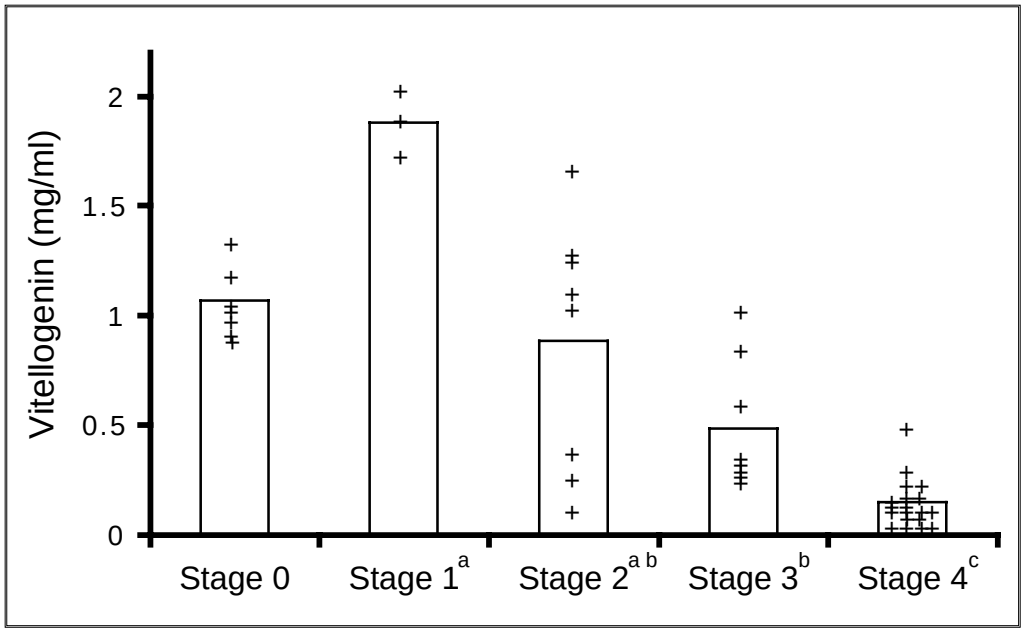

Figure 2. Vitellogenin concentrations in blood plasma of fish at different stages of ovarian atresia. (Stage 0 = Vitellogenic, N=8, Stage 1 = Post-vitellogenic and Non-atretic, N=3; Stage 2 = Early atretic, N=8; Stage 3 = Mid-atretic, N=8; Stage 4 = Late atretic, N=18). Plus signs indicate values for individual females. Stages followed by a common superscript are not significantly different. Stage 0 was not statistically assessed for significant differences from the other stages due to the distinct sampling conditions for this stage.

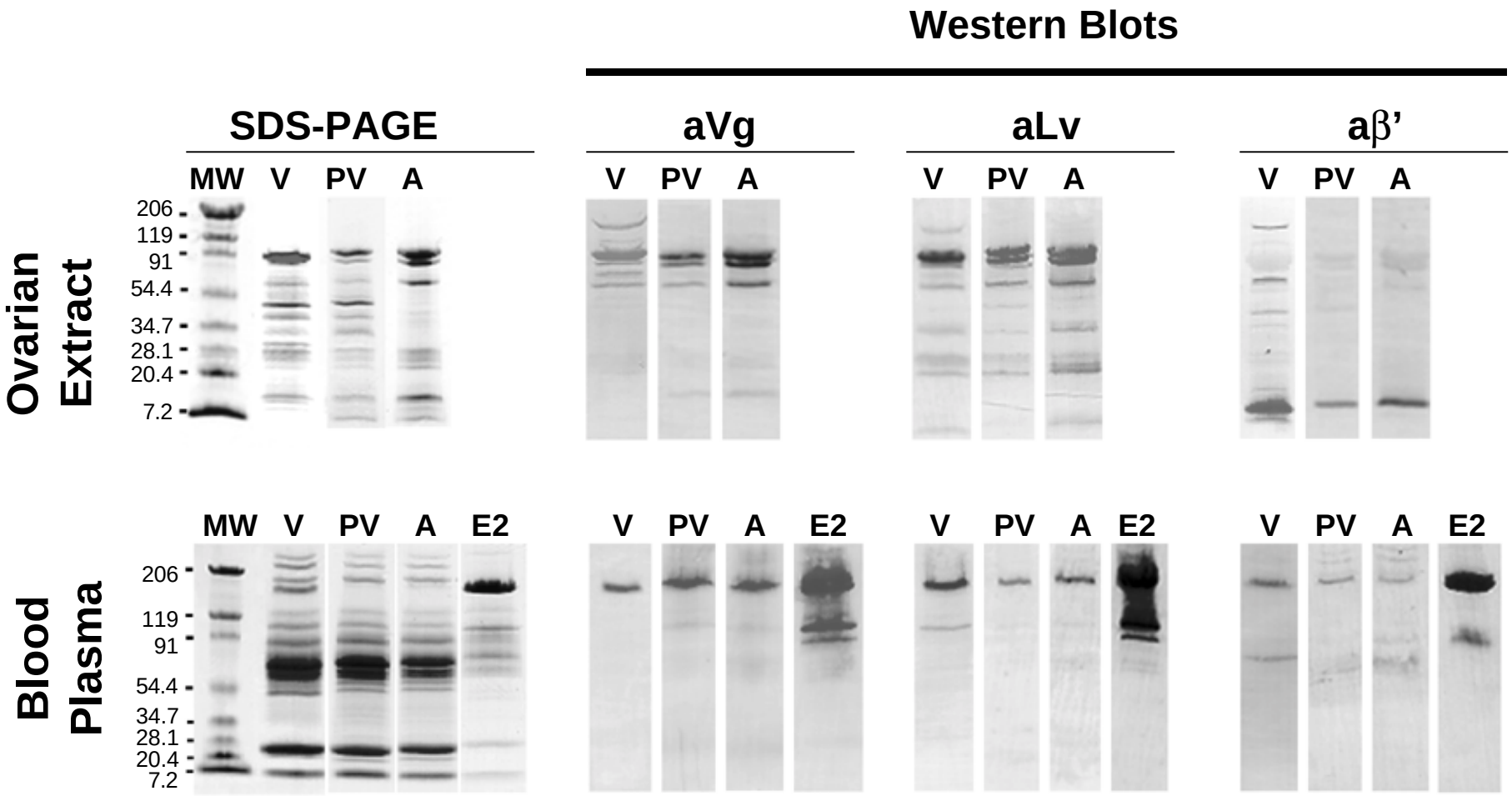

Figure 3. Results of SDS-PAGE and corresponding Western blots of ovarian extracts and blood plasma from Vitellogenic (V, Stage 0), Post-vitellogenic (PV, Stage 1), and Late atretic (A, atresia Stage 4) females and plasma from males that had been injected with estradiol-17β (E2). Numbers on the left indicate the mass (kDa) and position of the molecular weight (MW) markers in 8-25% gradient SDS-PAGE (Ovarian Extract) or 4-15% gradient SDS -PAGE (Blood Plasma). The antisera used for Western blotting were directed against *Morone* vitellogenin (aVg), lipovitellin (aLv), and β'-component (aβ').

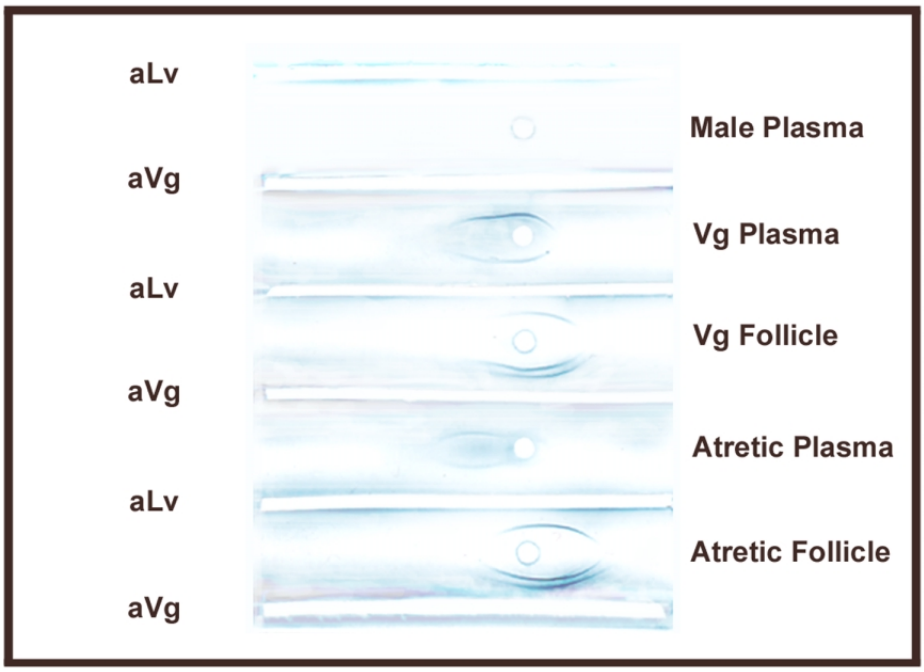

Figure 4. Results of immunoelectrophoresis of blood plasma samples from mature males (Male Plasma), Vitellogenic females (Vg Plasma), and Atretic females (Atretic Plasma) or extracts of ovarian follicles from Vitellogenic females (Vg Follicle) or Atretic females (Atretic Follicle). The antisera used were directed against *Morone* vitellogenin (aVg) or lipovitellin (aLv).

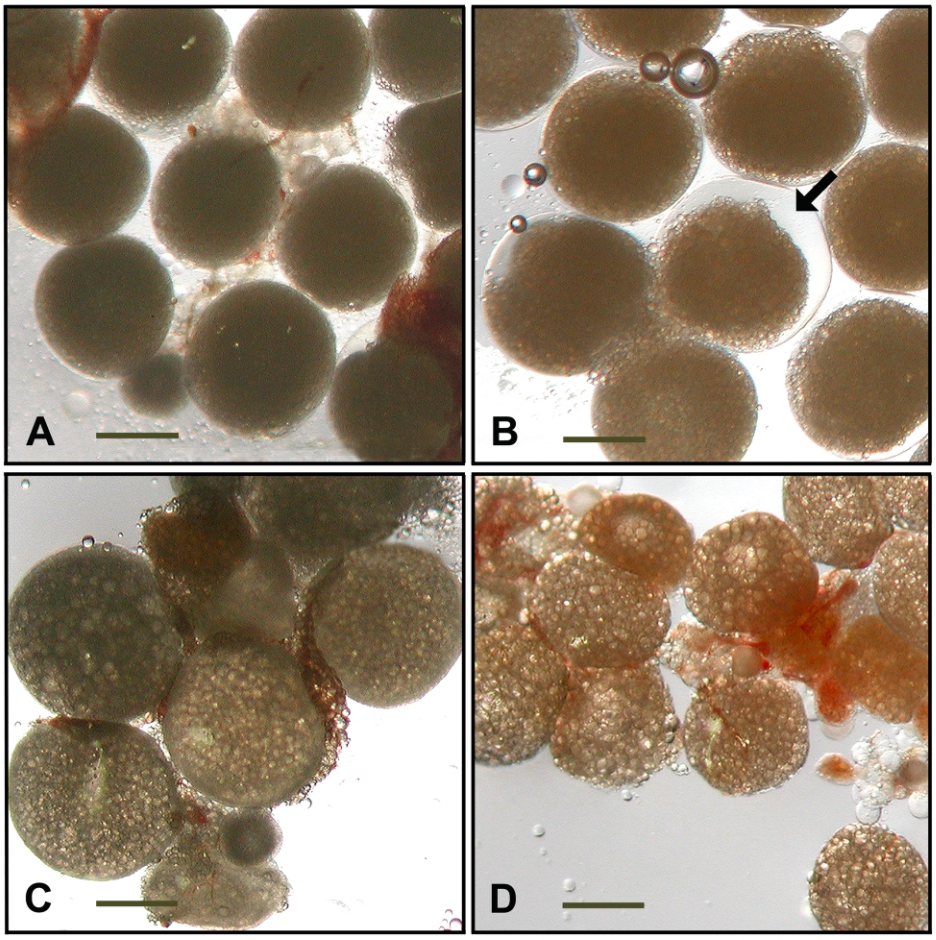

Figure 5. Ovarian follicles at different stages of atresia as viewed with the dissecting stereomicroscope. The stages are as follows: Stage 1 (Post-vitellogenic and Non-atretic, A), Stage 2 (Early atretic, B), Stage 3 (Mid-atretic, C) and Stage 4 (Late atretic, D). Horizontal bars = 500 µm. The arrow in panel B indicates a detached chorion on an osmotically compromised oocyte due to damage occurring during ovarian biopsy.

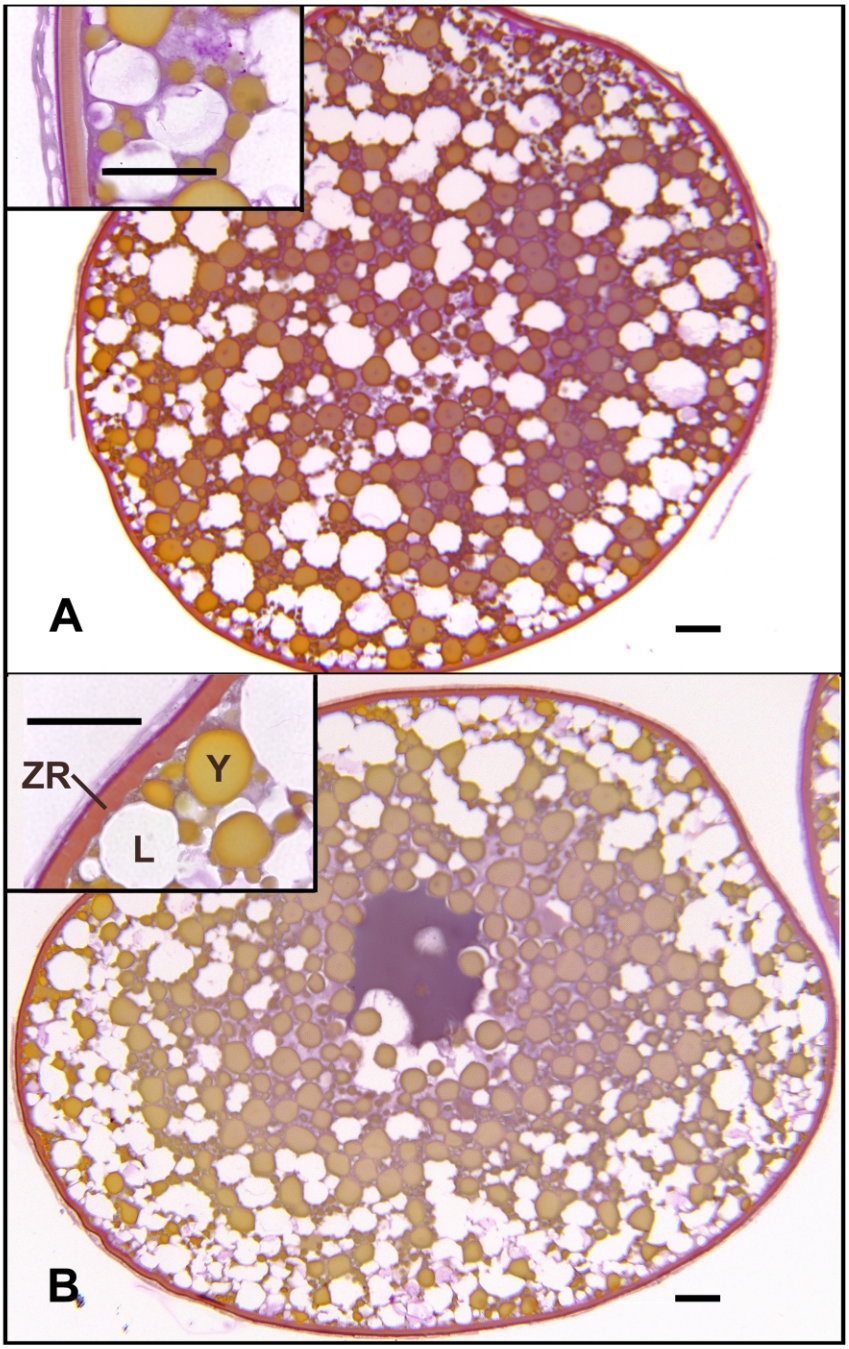

Figure 6. Light micrographs of ovarian follicles at atresia Stage 1 (Post-vitellogenic and Non-atretic, A) and Stage 2 (Early atretic, B). Insets show the peripheral ooplasm, containing lipid droplet remnants (L) and yolk granules (Y), the zona radiata (ZR), and the follicle cell layers at higher magnification (see inset, panel B). Horizontal bars = 50 µm. Sections were stained with PAS and metanil yellow. Changes in the arrangement of lipid droplet remnants and the staining properties of yolk granules are evident between stages.

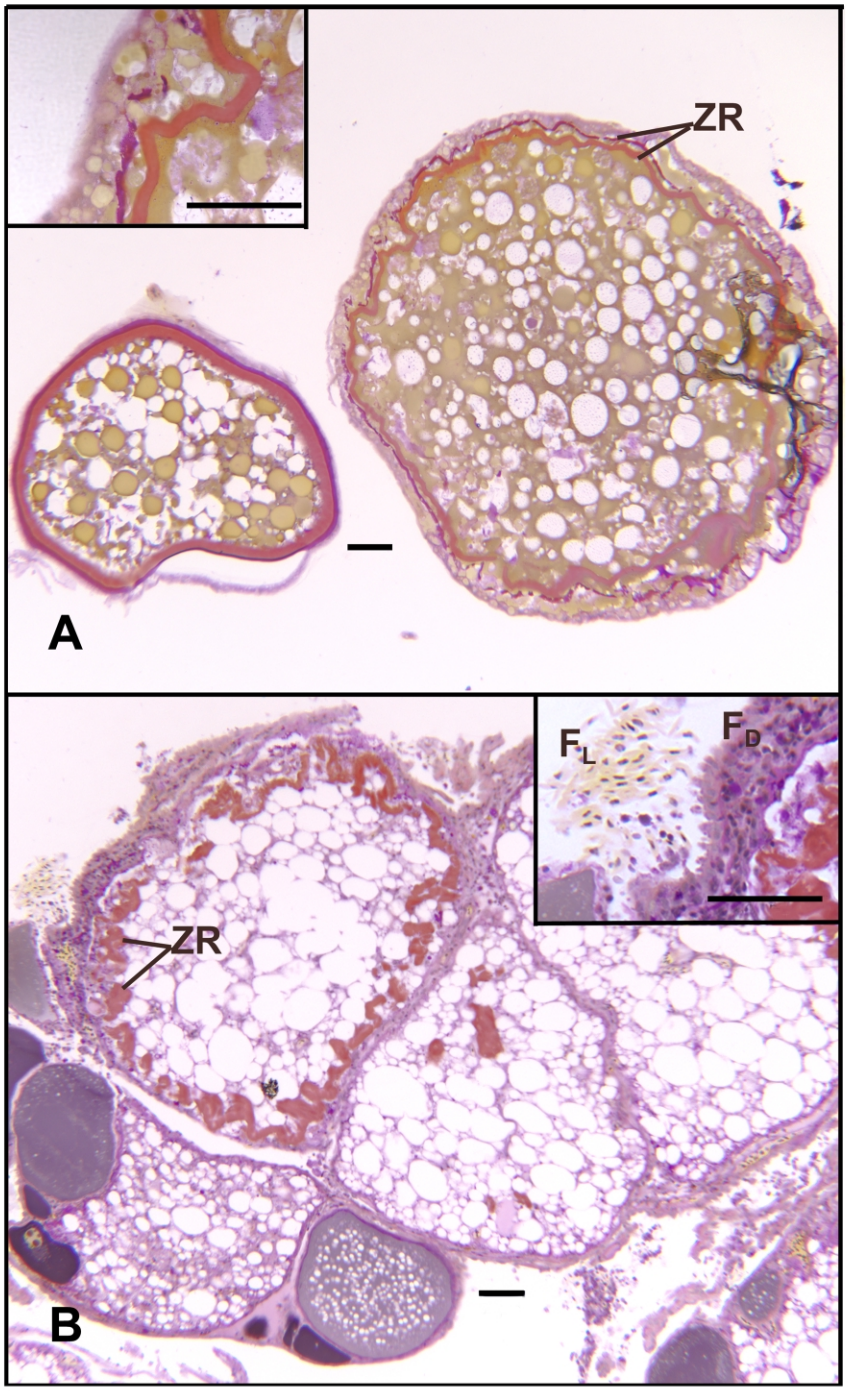

Figure 7. Light micrographs of ovarian follicles at atresia Stage 3 (Mid-atretic, A) and Stage 4 (Late atretic, B). Insets show the follicle periphery, as in Figure 6. Horizontal bars = 50 µm. Sections were stained with PAS and metanil yellow. Note that the zona radiata (ZR) becomes more sinusoidal, delaminates, and is finally breached during Stages 3 and 4 (inset, panel A; panel B). The presence at the follicle periphery of dark (FD) and lightly (FL) stained fibroblasts becomes evident by Stage 4 (Inset, panel B).

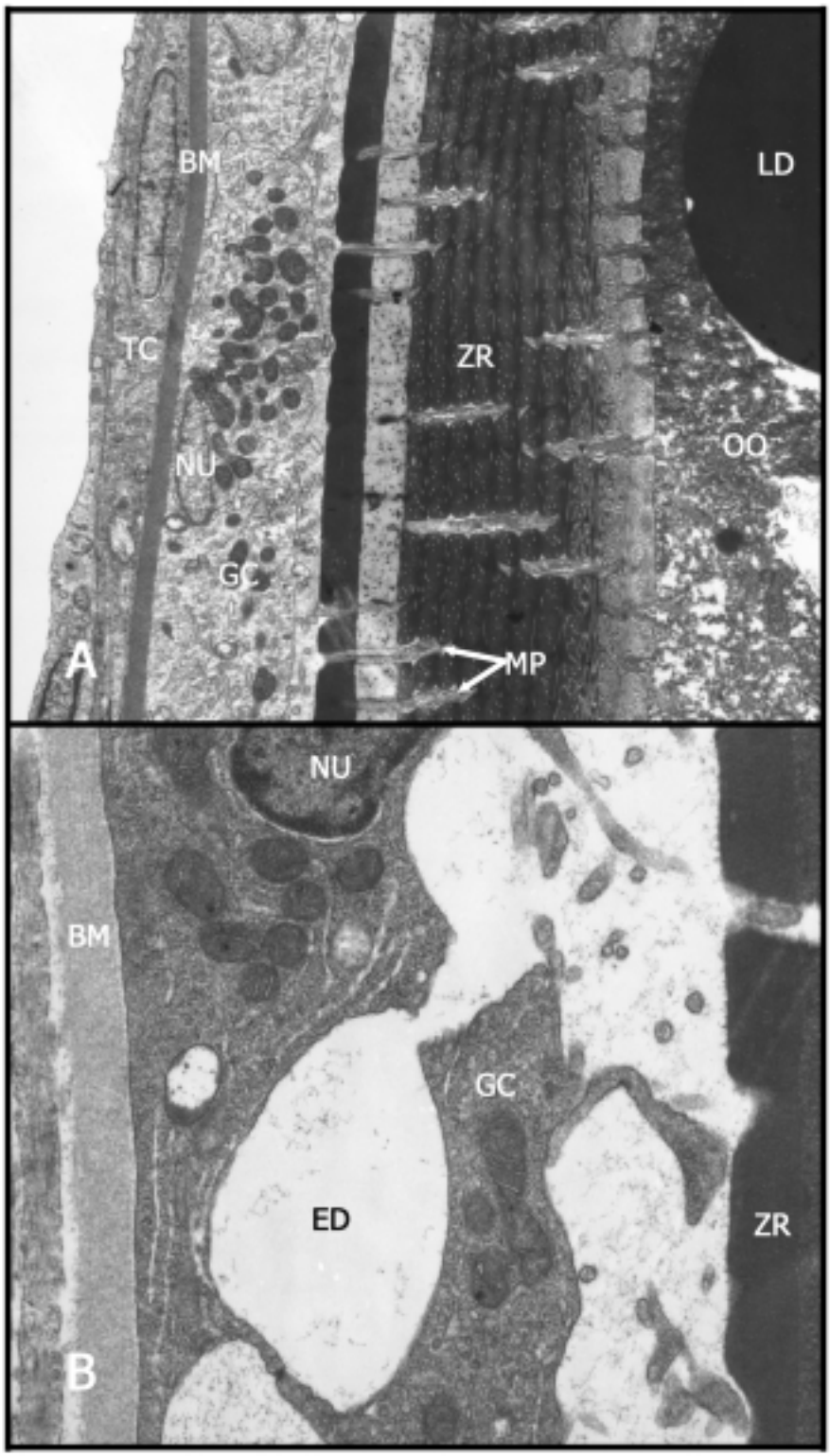

Figure 8. Transmission electron micrographs of follicles undergoing atresia. (A) 6,800X The intact outer layers of a Stage 1 follicle, extending from the apical ooplasm (right) to the follicle surface (left). The multi-cellular thecal layer (TC) overlays the basement membrane (BM), granulosa cell layer (GC), and multi-laminate zona radiata (ZR), which is extensively perforated by microvillar processes (MP) from the granulosa cells and oocyte. The ooplasma (OO), containing electron dense lipid droplets (LD) and yolk granules (not shown) is evident beneath the ZR. (B) 19,000X During atresia Stage 2 (Early atretic), the GC layer beginsto develop edema (ED) with an apparent decrease in intercellular contact between the granulosa cells and oocyte due to retraction of microvillar processes. The theca layer begins to become disorganized although the basement membrane remains intact.

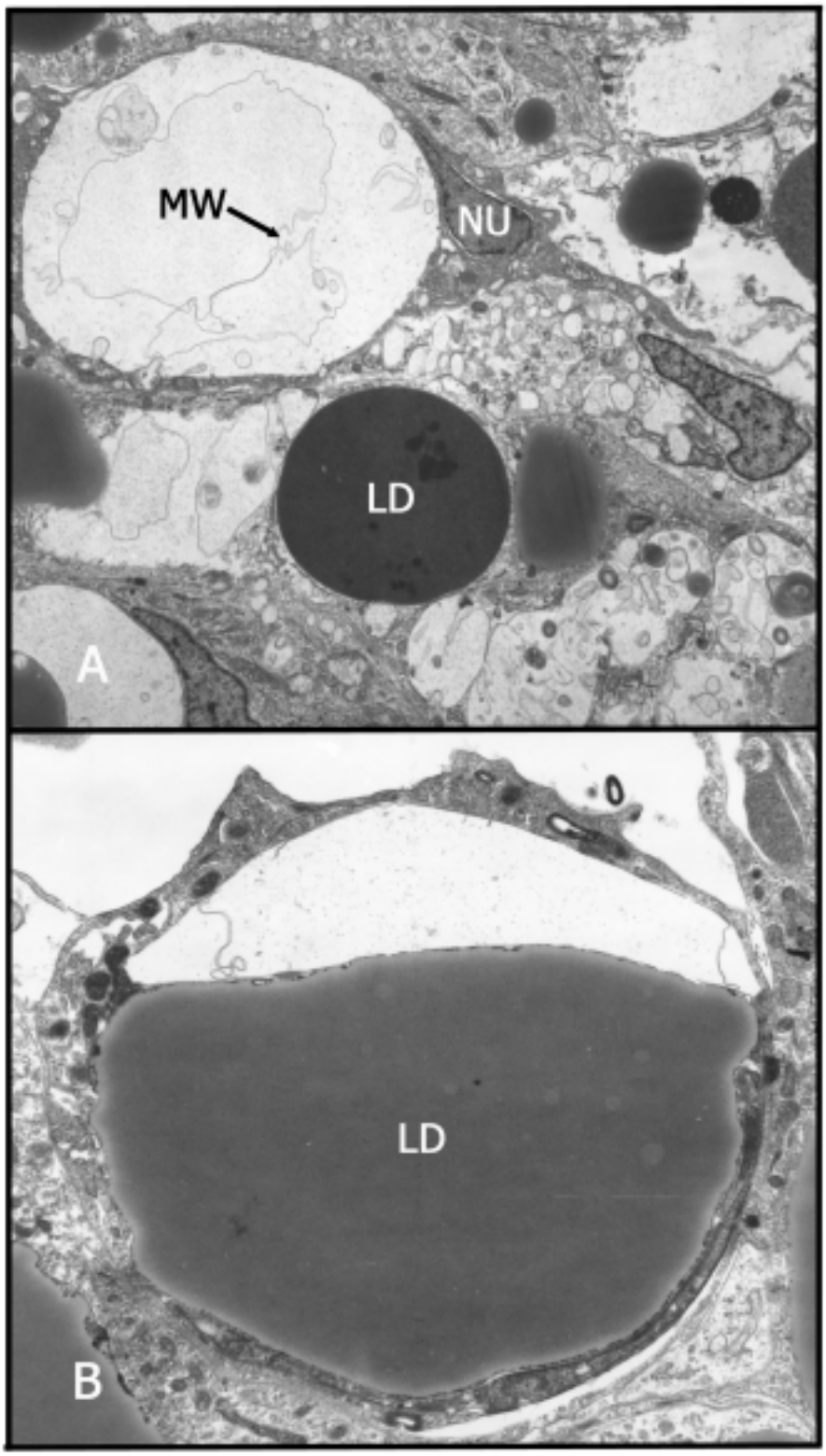

Figure 9. Transmission electron micrographs of follicles undergoing atresia. (A) 3,800X During Stage 3 (mid-atretic) and Stage 4 (Late atretic), phagocytic cells with elongated, pyramidal-shaped nuclei invade the ooplasm and are associated with vacuoles containing membranous whorls (MW). (B) 6,500X Lipid droplets (LD) and yolk granules (not shown) from the ooplasm also are engulfed by cell processes emanating from the invading phagocytic cells.

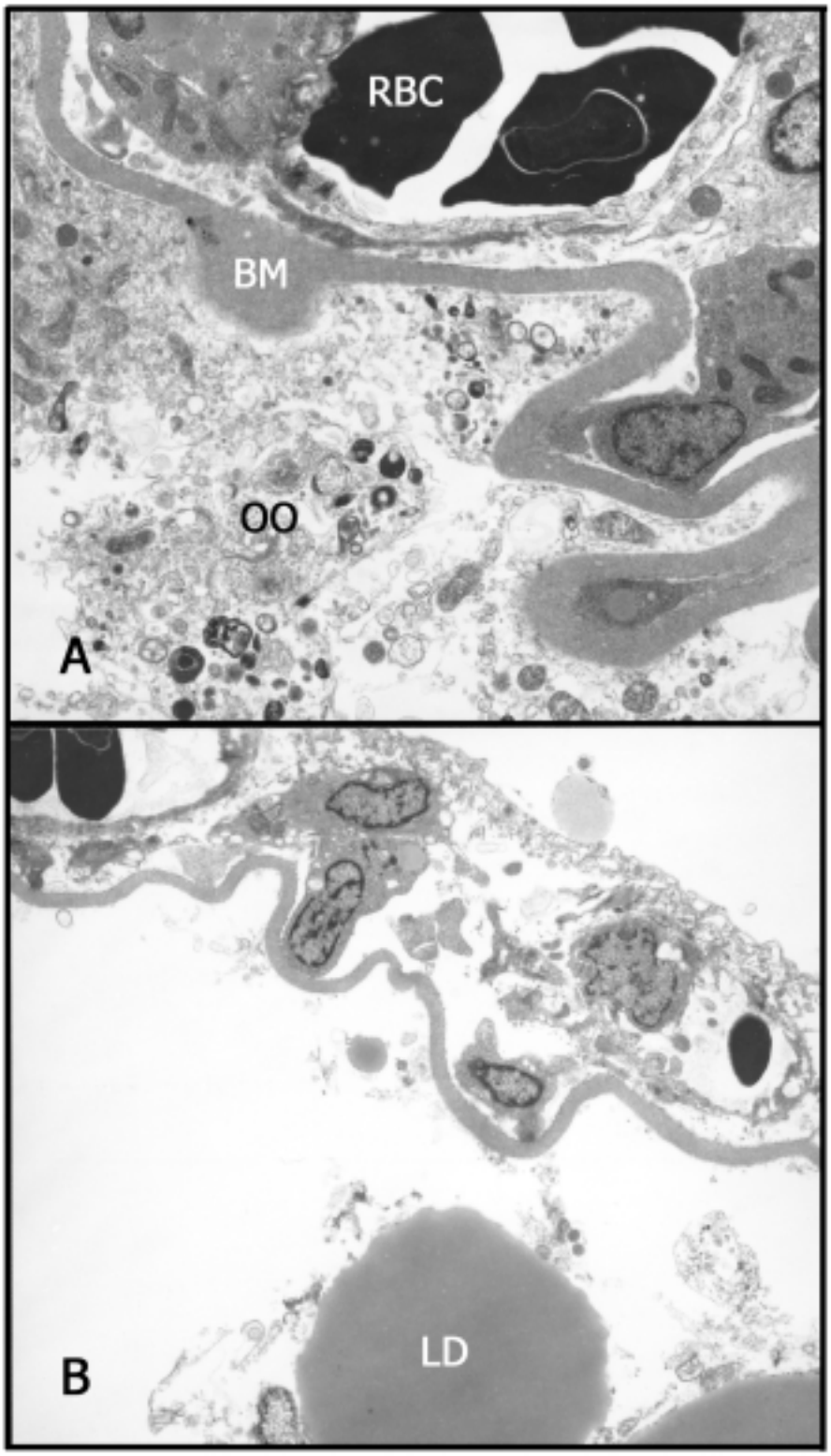

Figure 10. Transmission electron micrographs of follicles undergoing atresia. (A) 6,000X Stage 4 (Late atretic) follicle whose basement membrane has become increasingly sinusoidal but is still intact. The highly disorganized theca layer becomes increasingly well vascularized as illustrated by red blood cells (RBC) present in thecal capillaries (upper right). Ooplasm contents (OO) remain within the intact basement membrane. (B) 4,000X At this late stage of atresia, remnant of the yolk granules are very rare (not shown) and the former ooplasm appears progressively more empty, although not all lipid droplets (LD) have been completely phagocytized.

Table 1. Hormone concentrations by stage as determined by RIA are presented as the Mean ± SEM. Stage 0 values are 0.3553 ± 0.03 and 0.3072 ± 0.02 for Estradiol 17β and Testosterone, respectively. Values followed by a common superscript are not significantly different from other values of the same hormone.

Hormone Concentration (ng/ml)

Stage	*N*	Estradiol 17β	Testosterone	17α, 20β-P	20β-S
1	3	0.9588 ± 0.09^{a}	0.2852 ± 0.08^{a}	0.1756 ± 0.02^{ab}	0.1857 ± 0.02^{a}
2	8	0.5490 ± 0.09^{a}	0.2864 ± 0.04^{a}	0.1343 ± 0.02^{a}	0.1386 ± 0.02^{a}
3	8	0.1997 ± 0.03^{ab}	0.0808 ± 0.03^{b}	0.1248 ± 0.02^{a}	0.1580 ± 0.02^{a}
4	18	0.0933 ± 0.02^{b}	0.0116 ± 0.01^{c}	0.0855 ± 0.02^{b}	0.0842 ± 0.02^{a}

CHAPTER 2

APOPTOSIS: AREAS OF FUTURE RESEARCH

Reproduction and the processes supporting reproduction can be considered the foremost energy sinks in an animal's existence. Atresia is a biological attempt to regain energy that is lost when there is a failure in the development or expulsion of gametes. For humans, approximately 99% of oocytes become atretic; therefore atresia maybe considered a "normal" occurrence and actual ovulation may be considered an anomaly. In fish, although many oocytes reach maturity, there are always cells that are reabsorbed via atretic processes. In mammalian systems the process of apoptosis or programmed cell death is known to play a role in atresia, however this process has not been thoroughly explored in teleosts.

The term apoptosis was first coined in the 1970's and was described as "complimentary but opposite" to the process of mitosis and cellular division (Kerr *et al*. 1972). In healthy systems, the stringently regulated death of cells maintains a homeostatic balance with the creation and growth of new cells. Apoptosis plays a wide variety of roles including growth and remodeling of tissues, development and differentiation, and elimination of damaged cells in both plant and animal species. In ovine systems it has been suggested that apoptosis may play a key role in the disassociation of a follicle from ovarian tissue during ovulation (Murdoch 1995). However, in compromised systems homeostasis has been disrupted and either there is an increase in cell size and/or proliferation for which apoptosis cannot compensate, or there is an up-regulation of apoptosis in which cells die at a greater rate than healthy cells can be generated. An array of pathologies can develop from this disruption including cancers, ischemic stroke, and autoimmune and neurodegenerative diseases.

It is important to distinguish between two closely related mechanisms that both result in cell death: necrosis and apoptosis. Necrosis is a process in which cells degrade as a result of an exogenous insult. Primarily necrosis happens as a result of trauma or injury, whereas apoptosis is a developmental change that is programmed into the genetics of a cell. Necrosis also is a generalized trauma resulting in damage to a central cell as well as surrounding cells and it involves no mRNA or protein synthesis. In comparison, apoptosis almost always involves mRNA or protein synthesis and can occur in single cells, leaving neighboring cells untouched. An important distinction is that apoptosis is "programmed:" it is an active cell death and requires ATP; necrosis is considered passive. To distinguish these two processes under a microscope one might examine a necrotic cell and see general cell hypertrophy and lysis with accompanied random DNA fragmentation. Examination of apoptotic cells, on the other hand, would reveal cell shrinkage, chromatin condensation and apoptotic bodies (clusters of uniformly cleaved DNA fragments). Particularly characteristic of apoptotic cells is the shrinkage of individual cells and the subsequent loss of contact with cells immediately surrounding it.

The anti-apoptotic gene *bcl-2*, the pro-apoptotic gene *bax* and their analogues are said to be central to programmed cell death in a variety of species, from *Caenorhabditis elegans* to *Homo sapiens,* and have been found in many tissues. In fact, these genes have been so highly conserved over the course of evolution that human genes can act as a substitute for *C. elegans* genes and will carry out their functions completely (Vaux *et al*. 1992). Pro-apoptotic *drob-1* in *Drosophila melanogaster* has been found to act much like mammalian *bax* and *ced-3* and *ced-4* in *C. elegans* (Gaumer *et al*. 2000). The zebrafish, *Danio rerio,* has been found to contain the protein zfBLP1 which is 50% homologous to the human bcl-X_L and

functions as one of the Bcl-2 family of proteins in that it contains all four of the domains that have been found to be most highly conserved (Chen *et al*. 2001). It was suggested by Inohara and Nunez (2000) that *D. rerio* could "bridge the gap" between the human and nematode genes due to the discovery of 37 apoptotic regulators that were found to have significant homology with mammalian species. Although the majority of research being conducted in this species is focused on changes in gene expression during development in embryonic and retinal tissues (Chen *et al*. 2000; Link *et al*. 2001), any elucidations of the apoptotic mechanism could be broadly applied to a variety of tissue systems.

The Bcl-2 family of proteins is known to play an integral role in programmed cell death. Before a cell is apoptoticly induced, bax can be found in the cytosol in its monomeric form; however, the anti-apoptotic members of the Bcl-2 family such as bcl-X_L are usually membrane-bound, and located within the nucleus, endoplasmic reticulum and/or the mitochondria (Hsu *et al.* 1997). When the apoptotic cascade begins, bax moves from its inactive location in the cytosol to the mitochondrial and nuclear membranes where it homodimerizes to form a pore in mitochondrion (Schlesinger *et al*. 1997). The mitochondria is a key player in apoptotic regulation not only because this organelle is targeted by the main genetic regulators of apoptosis, but also because the pore formation leads to a release of cytochrome C, as well as other cytochromes (Kluck *et al*. 1999). Cytochrome C is known to facilitate the production of ATP via the electron transport chain. It also indirectly induces a conformational change, which cause the release of caspases. Caspase-3 triggers the cleavage of caspase-activated DNases from its inhibitor and the DNases then cause DNA fragmentation and, ultimately, cell death (Enari *et al*. 1998). Regular DNA fragmentation into 180-200 base pairs and their multiples has been widely established as a reliable

characteristic of late stage apoptosis. This DNA fragmentation is the final act of a cascade of events that begins with alteration in gene regulation. Bcl-2 acts as an inhibitor of the cascade by heterodimerizing with bax, thereby inhibiting mitochondrial pore formation and preventing cell death.

The DNases responsible for this regular fragmentation fall into three classes; cation-independent endonucleases, Mg^{2+} endonucleases, and Ca^{2+}/Mg^{2+} endonucleases. These categories and their potential mechanisms have been thoroughly described (Counis and Torriglia 2000). DNA fragmentation precedes apoptotic morphological changes within a cell; therefore, this characteristic maybe useful in the assessment of spawning. A correlation between incidences of DNA fragmentation in the granulosa cells and fish fecundity would be of practical value in aquaculture. Such predictive information could be used to prevent fish breeders from committing to expensive hormone injections for an animal that will have a lower fecundity than that of a fish with intact DNA.

The uniformly cleaved DNA fragments characteristic of apoptosis can be distinguished from the haphazard cleavage of necrotic cells through the use of gel electrophoresis and commercially available kits such as TACS 2TdT/Blue Label kit (Trevigen) and ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Intergen Company). These kits are TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) kits and have been used in studies focused on DNA fragmentation in both LR White-embedded mammalian tissues and paraffin embedded tissues from *Xenopus laevis*, *D. rerio*, *Catostomus commersoni*, and a variety of avian systems (Goping *et al*. 1999, Bever and Fekete 1999, Tilly *et al*. 1991, Gaverieli *et al*. 1992). These kits allow researchers to identify regular DNA fragmentation among and within cells and to distinguish apoptotic cells from necrotic cells

within the granulosa layer. For the current project, both *in vivo* and *in vitro* samples were fixed in 4% formaldehyde freshly prepared from paraformaldehyde and were promptly embedded in highly porous, hydrophilic LR White resin for use in immunocytochemical applications (Goping *et al*. 1999). These samples would be ideal for use in DNA fragmentation studies.

Healthy follicles from both striped bass and white bass were taken into culture and challenged with hCG for an *in vitro* competency study. These healthy follicles were exposed to three concentrations of doxorubicin (DOX), a light sensitive chemical that acts to form free radicals as well as intercalating itself into DNA and causing cellular damage (Ohtsubo *et al.* 2000). DOX is widely used as a chemotheraputant due to its role in causing DNA fragmentation (Perez *et al.* 1997, Coley *et al.* 2000, Goren *et al*. 2000). Normal unfertilized mouse oocytes that are treated with DOX undergo apoptosis; however, bax deficient mice are not affected, suggesting that DOX activates the bcl-2: bax pathway and that without bax, DOX is rendered ineffective (Perez *et al.* 1997). Other apoptosis inducers include UV irradiation, which has been successful in *Mus musculus* (Perez *et al*. 2000), etoposide and staurosporine in *D. melanogaster* (Nezis *et al*. 2000) and X-ray radiation in *D. melanogaster* (Gaumer *et al*. 2000). Although a wide variety of mechanisms have been used to induce apoptosis in a number of different species, DOX was selected for use in this study because of its broad application (Jurisicova *et al*. 2000). The *in vitro* samples were embedded in LR White resin so they are ready for DNA fragmentation detection and corresponding samples were snap frozen in $N_{(L)}$ for use in other techniques. The aforementioned method will definitively establish apoptotic mechanisms in that regular DNA fragmentation is a hallmark of an apoptotic cell (Hall 1999).

Once it is determined that apoptotic processes are occurring in the granulosa cells, it would then behoove researchers to identify the early stage genetic regulators of programmed cell death. As mentioned previously, two genes of particular interest, *bcl-X_L* and *bax*, are thought to control apoptotic processes and by determining the ratio of the expression of these two genes, it would be possible to foresee an impending cell death (Oltavi *et al*. 1993, Johnson *et al*. 1996). It is known that *bax* is up-regulated when a cell is deprived of hormonal support therefore a negative feedback loop may facilitate the rapid demise of the granulosa cells (Murdoch 1995). This application has great potential in that if the path towards cell death in a follicle can be identified early (i.e. prior to DNA fragmentation) then there is the possibility that the cascade can be stopped or reversed. This would be a colossal achievement and would contribute greatly to control follicle demise and subsequent control of reproduction.

The study conducted by Gaumer and colleagues (2000) suggests that there is an effect of mammalian bax and bcl-2 in developing *Drosophila* embryos and they function in a similar manner. In fact, recent studies used human *bcl-2* RNA to rescue *Xenopus* cells that were previously treated with UV, α-amanitin, or cycloheximide (Hensey and Gautier 1997, Hensey and Gautier 1998) to induce an apoptosis-like cascade. Human *bcl-2* RNA was not only shown to rescue the cells but, when injected prophylacticly it significantly reduced the percentage of cells undergoing programmed cell death after being exposed to the aforementioned apoptotic stimuli. In *D. rerio*, zfBLP1 has been found to have a 50% homology with the Bcl-2 family of proteins (Chen *et al.* 2000). Because this protein family is so highly conserved across species, antibodies for mammalian bcl-2 and bax have had important roles in identifying these proteins in other systems. Previous studies have

approached the identification of members of the Bcl-2 protein family by creating a cDNA library of the tissues of interest (Cruz-Reyes and Tata 1995).

Already, we have determined that these regulators do exist in the samples taken from the present study. SDS-PAGE and Western Blotting analysis with commercially available antibodies directed against bcl-X_L and bax obtained from Calbiochem (PC103, PC104) have yielded banding patterns similar to those identified in previous studies. The primary difficulty encountered is the common, widely reviewed problem of homodimerization, heterodimerization and mulitimerization in the presence of detergents (Hsu and Youle 1998). Most previous studies that have detected these family members (in mammalian systems) have not been able to separate these proteins into monomers and, thus, they do not run at the proper molecular weight. The most successful investigation utilized the zwitterionic detergent Chaps, which prevents bax/bcl-2 and bax/bcl-X_L heterodimerization formation in murine thymocytes (Hsu and Youle 1998); however, not only has this not been tested in teleosts but it also does not prevent dimerization with other Bcl-2 family members. Although a very cursory investigation, our research yielded identical results using two different homogenization buffers, one with ionic detergent and one with non-ionic detergent (Zwain and Amato 2001, Ray and Jena 2000). This obstacle does not seem as if it would be easy to overcome. Although it may not be feasible to manipulate these proteins to run at their established molecular weights, an alternate plan may involve synthesizing the amino acid sequence against which the antibody is directed. Then it can be verified that the antibody is actually identifying the target protein and nothing else and one would be able to confirm the existence of those proteins within the sample. At that point, corresponding samples that have been snap frozen in liquid nitrogen and could be used to prepare mRNA for cDNA cloning of

candidate genes such as *bcl-X_L* and *bax* through the use of a commercially available kit such as Lambda-Zap cDNA Library Kit (Stratagene). Similar to techniques applied to *X. laevis* (Cruz-Reyes and Tata 1995), by creating a cDNA library we will then be able to conduct a low stringency screening for human *bcl-2* and *bax*. Remaining frozen biopsy samples can potentially be analyzed for gene expression via semi-quantitative, reverse transcriptase-polymerase chain reaction. Ultimately there is the potential to isolate and sequence these genes in striped bass and create specific antibodies for use in a "litmus test" to determine eligibility of broodstock for spawning.

Apoptosis is an elaborate but elegant system, which has not been fully elucidated in any organism. The "program" for apoptosis is written in the DNA sequence of a cell and certain conditions need to be met in order for a cell to initiate this form of cell death. Apoptosis affects every aspect of cell life and death from the most morphologically simple to the most morphologically complex. It is involved with all facets of biology from cell growth and differentiation to reproduction and development. Thus, it is understandable why the elucidation of apoptotic mechanisms has become one of the foremost priorities of the scientific community.

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