DEVELOPING A NONINVASIVE METHOD FOR ASSESSING REPRODUCTIVE STATUS AND CHARACTERIZING GENDER- SPECIFIC PLASMA PROTEINS IN THE AMERICAN ALLIGATOR (*Alligator mississippiensis*)

By

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I dedicate this work to my family for their love and support, without which I could not have been successful in achieving my goals.

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Organochlorine pesticides (OCPs) in Florida lakes have been associated with decreased egg hatchability and increased developmental mortality in American Alligators (*Alligator mississippiensis*). Although concentrations of specific OCPs in yolk do not correlate with egg hatchability and hatchling survivability, a complex mixture of OCPs may decrease egg and embryo quality by altering maternal reproductive function. Vitellogenin (Vtg), a follicular precursor protein has long been described as a biomarker of endocrine disruption in other oviparous species. However, there is no documentation in the literature of a definitive test for identifying and measuring Vtg in this species. This is because of the inter-species variability in the amino acid sequence of this protein and thus the low cross reactivity of commercially available antibodies. To aid in the development of such an assay, Vtg proteins in plasma were identified and characterized from 10 adult female alligators collected during the peak follicular period (from Lake Griffin, FL and Rockefeller State Wildlife Refuge, LA). Two sites were chosen in an

effort to reduce site-specific bias. In addition, these sites were chosen for the ecological significance and environmental concerns associated with alligators in these geographical locations.

Our study was designed to develop a qualitative method for identifying follicular females and for assessing their reproductive status; and to identify and characterize specific plasma proteins likely to be Vtg.

Sodium dodecal sulfate-polyacrylimide gel electrophoresis (SDS-Page) analysis of plasma revealed three prominent protein bands unique to follicular females at ~250 to 450 kilo-daltons (similar to published molecular weights for Vtg or Vtg metabolites from other oviparous species). Further characterization of these proteins revealed that they were highly glycosylated and contained several phosphoserine amino acids. These proteins were isolated by SDS-Page and then confirmed by protein sequencing to have substantial homology with published Vtg sequences from other species. These data are critical for future development of an alligator specific Vtg assay. Such an assay could be used to further identify a possible mechanism for reproductive failures experienced by alligator populations in contaminant-impacted environments.

Anatomical evaluations of reproductive status validated the plasma protein screening protocol. There was a 1:1 correlation for vitellogenic females exhibiting plasma proteins in the 250 to 450 kDa range. This correlation provided significant evidence that this is an acceptable method for discerning Vtg animals from non-Vtg animals. Each animal that had the three highly expressed plasma proteins also had a larger number of follicles in the 21 mm to 25 mm or > 26 mm size classifications.

CHAPTER 1
INTRODUCTION

Heightened awareness of endocrine disruption (ED) in wildlife is expanding to a concern for humans as well. Many studies have helped to elucidate factors that may lead to the current state of environmental ED. These studies have focused on several environmental contaminants and their potential effects on many species of fish, birds, amphibians, and reptiles. While these studies have been useful in identifying several potential mechanisms of action for ED in these species, they have not completely fulfilled the purpose of representing the long-term impact on the health of the entire ecosystem. An animal model that could serve such a purpose needs to have a reasonable longevity to age-of-sexual-maturity ratio, and an upper-level predatory status in the food chain. Most bird, fish, amphibian, and reptilian species do not meet these criteria because they are sexually mature relatively soon after birth; and while they may be predators, they do not hold a very high place in the food chain. American alligators (*Alligator mississippiensis*) on the other hand, are upper-level predators, have an average life span of 50 years, and reach sexual maturity at 10 to 12 years. Their oviparous nature coupled with relatively long egg incubations make them an excellent model for studying reptilian embryonic development. Comprehensive embryonic studies have been conducted in normal alligator populations (including palatogenesis and hemoglobin amino-acid sequence development) (Densmore 1981, Le Clercq et al. 1981, Perutz et al. 1981, Ferguson 1985). For these and other reasons, the alligator is becoming a popular model

for studying ED in the environment; and subsequently for making some preliminary predictions for potential concerns for human exposure. Because of all of these attributes, we chose the American alligator as our model for the development of biomarkers to assess ED. The following work describes the development of some of these techniques.

Alligator Reproductive Anatomy and Physiology

Much of the initial information obtained on the American alligator reproductive cycle in the southeastern United States comes from studies conducted on both wild and captive populations by various investigators. Their compiled findings are summarized, beginning with sexual maturity and ending with female reproductive anatomy. Our primary focus was on Florida and Louisiana studies in order to compliment this study. These data serve as a general overview of what is known about alligator reproduction.

Age/Size of Sexual Maturity and Sex Ratios

There is some discrepancy as to when a wild alligator reaches sexual maturity. The general consensus is that sexual maturity depends on size, which depends on environmental factors (e.g., temperature and food availability) that affect growth rates. However, other factors may influence sexual maturity (such as age, genetic differences between populations, and population densities) (Ferguson 1985). While there are published methods using bone annual growth zones for aging (Peabody 1961, Hutton 1986), they are not applicable to females because the bone remodeling females undergo during egg shell formation (Wink and Elsey 1986). Some general guidelines: Louisiana males and females both reach sexual maturity at 1.8 mo or 9 to 10 y (Joanen and McNease 1980); North Carolina males at 1.8 mo or 15 y, and females at 1.8 mo or 18 y (Ferguson 1985); and in southern Florida at 12 to 14 y (Dalrymple 1996). Some believe that age/size is not the only determining factor; that social order is also important. For

example, larger, dominant males (longer than 2.7 m) are more likely to breed (Joanen and McNease 1980). These guidelines have been determined through survey analyses and may have intrinsic errors due to sampling and/or difficulties assessing age. Captive animals would seem to be an excellent control for these errors. However, animals reared in captivity develop variable growth patterns due to differences in environmental factors such as food quality and availability. This coupled with variations in mating behaviors, which develop from being in a constrained environment, leave these animals outside the norm and therefore unsuitable for calculating an average age/size for sexual maturity.

Sex ratios in wild populations have received some attention. They are calculated from survey data which again can have intrinsic errors due to sampling error and/or animal movement during the breeding season. Ferguson and Joanen (1982, 1983) tried to approach this issue from the hatchling perspective in a 4-year study; and found approximately five females to every male. However, open-water surveys show very different ratios, depending on the month of sampling. These variations are probably due to adult females remaining in the marsh, which is their preferred habitat (Ferguson 1985).

Reproductive Behavior

Courtship and mating. Typical mating rituals involve bellowing, head slapping, and snout and head rubbing (Garrick and Lang 1977, Joanen and McNease 1989, Vliet 1989), culminating with copulation and subsequent nest building and egg deposition. Specific timing of the breeding season can vary slightly depending on the geographical location. However, the following data summarized by Ferguson (1985) serve as a suitable model (similar time frames were confirmed by Guillette et al. 1997 in their Florida study). These time points are based on air temperature (which was believed to be the driving force); not on the length of day (note temperatures in parentheses at each time

point) for this particular study. However, there is no conclusive proof that temperature is the only trigger involved. Many investigators believe it is a combination of factors.

In Louisiana, both females and males begin moving to deep open-water courtship areas in the early part of March (13°C) (Ferguson 1985). Light bellowing by males (a guttural noise made to attract a mate) is heard throughout the month of April and elevates to mild bellowing by the end of April (21°C) which lasts until the middle of May (24°C) when intense bellowing begins. During this entire time, females develop ova in the ovaries; while male spermatogenesis begins in the middle of May and lasts approximately 4 weeks. Copulation begins around the third week of May and continues through the second week of June when spermatogenesis is at its peak. Females then move to shallower waters, and nest construction begins. Nest construction (see next section for a description) is complete and eggs are deposited by the end of June (27 to 28°C). The female remains to tend and protect the nest, until the next spring, when hatchlings are ready to leave the nest. Males and non breeding females on the other hand, move to deep open water for the remainder of the season, returning to their winter habitat by the middle of October (22°C).

Nest construction. The female is solely responsible for building the nest. It consists of twigs, mud, and other debris that is indigenous to the area. For example, Florida nests consist mainly of saw grass, mud, and cotton tail grass. Typically an experienced female will build a mounded den-type nest that has a tunnel-like entrance. She will guard this entrance until she hears the hatchlings chirping (still inside the egg) approximately 65 days past laying (at which time she will uncover the nest and begin tending to the hatchlings). The average number of eggs per nest is 38 in Louisiana

(Joanen and McNease 1989) and 42 to 45 in Florida (Woodward et al. 1993, Masson 1995, Guillette et al. 1997). However, there are some areas in Florida that have less eggs per nest such as Orange Lake = 33 eggs; Paynes Prairie = 34 eggs (Woodward et al. 1992) and Everglades National Park = 30 eggs (Kushlan and Jacobson 1990). Nests are usually located near the edge of a marshy area just above the water line. This can be a problem in times of draught that are followed by heavy rains because many nests can be flooded out; as was the case in Orange Lake, FL the year this study was conducted (unpublished data).

Reproductive Endocrinology

The male reproductive season begins in early spring after an increase in circulating testosterone (T) concentrations (which peak at approximately 90 ng/mL in April/May) (Lance 1983, 1984). This is concurrent with the production of mature sperm that are then stored in the seminiferous tubes.

Females also have a T surge that occurs simultaneous to an increase in 17β-estradiol (E_2); however the peak is less than $1/10^{th}$ that of mature males (Lance, 1983). This surge of a predominantly male hormone in females is not surprising, since T is the precursor for E_2.

There is some discrepancy between the Louisiana and the Florida studies as to the onset of the reproductive season in females. In Louisiana, Joanen and McNease (1980) and Lance (1989) found that the alligator reproductive season begins in early spring with an increase in circulating E_2 concentrations, which peak in April at approximately 700 pg/mL. Guillette et al. (1997) determined that Florida females appear to have a bi-phasic cycle beginning in the fall. This fall phase of increased E_2 concentrations return to summer concentrations (200 pg/mL) sometime between November and February,

however, there was no sampling during this time frame. Subsequently there is a second increase in E_2 concentrations beginning in February peaking at approximately 600 pg/mL in April/May (Guillette et al. 1997). This second rise in E_2 causes the follicles to increase in diameter from 5 mm to 40–45 mm by late May to early June. It is unclear whether these differences in E_2 cycling between the Louisiana and Florida studies is due to geographical variations or if it is just due to the Florida study including more time points (Guillette and Milnes 2001). Vitellogenesis is actively going on during this time of elevated E_2 concentrations, and Guillette et al. (1997) discussed the possibility that the fall increase in E_2 concentration served to produce an initial wave of large follicles that would in turn provide more circulating E_2 which is needed for rapid oviductal growth. The spring E_2 concentrations decrease rapidly following ovulation in June.

Subsequent to the decline in plasma E_2 concentrations, there is a rise in plasma progesterone (P) concentrations beginning in April and peaking at 5–6 ng/mL in June. This elevated concentration of plasma P continues circulating until oviposition and the beginning of luteolysis in June/July when the P concentrations decrease to 1–2 ng/mL (Guillette et al. 1997). Lance (1989) found that corpora lutea granulosa cells stained positively for 3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD) which is the enzyme responsible for the synthesis of P. It is possible that plasma P produced in the corpora lutea aids in maintaining gravidity as it does in other species (Guillette and Milnes 2001).

Female Reproductive Histology and Anatomy

There is a right and left side to the reproductive tract; the right being the larger of the two. However both sides are simultaneously involved during each breeding season.

Follicles are formed and nurtured in both the right and left ovaries and passed through the oviducts, the conduits (with various functional zones) which extend to the exterior of the body through a single vaginal opening. Folliculogenesis has been described by Uribe and Guillette (2000) as being a series of stages which are summarized in Table 1-1. Uribe and Guillette (2000) concluded that based on their histological findings, stages I–VI compared to those of other reptiles, while stages VII–IX more closely resembled birds. Other features which were similar to birds include ovarian lacunae and smooth muscle bundles surrounding the follicles. However, there were some characteristics which were unlike birds or other reptiles such as: yolk morphology (animal and vegetal pole differences); yolk platelet structure; and theca morphology. While this staging system has proved invaluable in evaluating the progress of folliculogenesis, late-stage variations are difficult to interpret. This is mainly due to the awkwardness of sectioning a 40 mm follicle for histological evaluations; they are very large with very little support tissue (Guillette and Milnes 2001).

The anatomy, and functionality of the oviduct in the American alligator is more similar to birds rather than to other reptilians. However, in contrast to birds which completely finish one egg before ovulating the next, alligators exhibit a more simultaneous ovulation and shelling of the entire clutch which is similar to reptilians (Guillette and Milnes 2001).

Table 1-1: Stages of folliculogenesis.

Stage	Oocyte Diameter (mean " SE)	Characteristics
Stage I: Previtellogenesis	42.8 " 6.6 :m	Nucleus contains chromatin in diplotene stage of meiotic prophase I. Thick chromosomes visible. One nucleolus. Squamous cells begin to surround oocyte.
Stage II: Previtellogenesis	73.8 " 6.9 :m	Nucleus contains lampbrush chromosomes and one nucleolus.
Stage III: Previtellogenesis	267.3 " 43.3 :m	Nucleus contains lampbrush chromosomes and multiple nucleoli. Squamous cells completely surround oocyte; monolayer is referred to as granulosa.
Stage IV: Previtellogenesis	486.7 " 70.1 :m	Zona pellucida at periphery of oocyte. Granulosa cells are cuboidal containing a nucleus. Theca has developed, comprised of fibroblasts.
Stage V: Previtellogenesis	1.2 " 0.9 mm	Zona pellucida is considerably thicker, consisting of two layers; an inner striated layer and an outer hyaline band.
Stage VI: Vitellogenesis	3.1 " 0.9 mm	Peripheral granules and centralized vacuoles in ooplasm. Theca has sinuses.
Stage VII: Vitellogenesis	4.5 " 1.6 mm	Granules and vacuoles have increased greatly in numbers. Vacuoles are much larger (up to 25 :m), some containing yolk platelets.
Stage VIII: Vitellogenesis	6.8 " 3.4 mm	Regional animal and vegetal poles clearly visable. Zona pellucida 18-20 :m and have well defined radiata and hyaline layers. Theca contains blood vessels, collagen fibers, and flattened lacunae.
Stage IX: Vitellogenesis	19.4 " 5.9 mm	Ooplasm is filled with large (90 :m) yolk platelets.
Stage X: Vitellogenesis	38.8 " 2.4 mm	Yolk platelets continue to grow (160 :m). Theca thickens to 180-200 :m), containing muscle cells as well.

Source: Summarized from Uribe and Guillette (2000).

The oviduct of the American alligator has been described in some detail (Palmer and Guillette 1992, Buhi et al. 1999). It has been divided into seven distinct regions each serving different purposes in preparing the mature follicle for deposition as an egg:

- The uppermost section, the anterior infundibulum, functions to receive the mature follicle.
- The posterior infundibullum and the uterine tube are muscular with mucosal folds and believed to function in albumen secretion.
- The utero-tubal junction is a transparent non-muscular, non-glandular section which connects the uterine tube to the anterior uterus.
- The anterior and posterior uterus is the site of eggshell membrane formation and eggshell calcification, respectively.
- Finally, the posterior uterus connects to the vagina where the egg exits the body.

Vitellogenin as a Reproductive Biomarker of Endocrine Disruption

Vitellogenin (Vtg) has been classified as a hormonally controlled precursor protein to several of the yolk proteins found in oviparous eggs (Ryffel 1978). Once liver Vtg production is stimulated by circulating E_2, it is post-translationally modified and circulated to the blood capillaries surrounding the follicular theca and transferred to the developing oocytes by diffusion from the follicular theca and subsequent pinocytosis by the oocytes (Wahli et al. 1981). Once in the oocytes, Vtg is proteolytically cleaved into lipovitellin and phosvitin; however the number of cleavage products is not known for most species (Ryffel 1978, Wahli et al. 1981). Characteristically, it is a highly glycosylated phospho-lipoprotein. The molecular weight ranges from ~150 to 600 kilo-daltons (Kd) depending on the species (Heppel et al. 1995, Brown et al. 1997, Allner et al. 1999, Brion et al. 2000). For example, in the African clawed-frog (*Xenopus laevis*), it occurs in the form of a dimer consisting of two 200 Kd polypeptides (Wahli et al. 1981), whereas in the Kemp's Ridley sea turtle (*Lepidochelys kempi*) the predominant Vtg protein appears at 200 Kd (Heck et al. 1997). Similarly, the isoelectric point (pI), the pH

at which the net charge of the protein is zero, ranges from ~ 6 to 7 depending on the species (Kawahara et al. 1983, James and Oliver 1997, Roubel et al. 1997). These characteristics were used collectively in the design of this study to optimize the chances of correctly identifying and characterizing Vtg in the American alligator.

Since Vtg is a maternally derived protein that is utilized by the embryo as a nutritional source, it is possible that any deviation or disruption of the pathway may alter embryo development. The full extent to which the developing embryo uses Vtg is not clearly understood, but if it could act as a carrier protein for xenobiotic chemicals, then it would stand to reason that enzymes used by the embryo to metabolize those chemicals could be turned on and the activity up-regulated. Metabolism is a complex process in that enzymes are developed to control more than one event. For example cytochrome P450 enzymes are instrumental in xenobiotics metabolism as well as steroid metabolism (Ertl et al. 1999, Sierra-Santoyo et al. 2000). With this in mind it is possible that other events in the developing embryo could be affected by this exposure. There are several potential pathways and functions to explore but first there must be a definitive method for identifying and characterizing Vtg in the species being studied. This has been done for fish and birds, but there is limited information in amphibians other than *Xenopus* and reptiles.

Vtg has been proposed as a biomarker of exposure to endocrine disrupting chemicals (EDC) in oviparous species (Sumpter and Jobling 1995). The rationale behind using Vtg as a biomarker stems from extensive research using the African clawed frog and the chicken (*Gallus domesticus*) as models for investigating E_2 induced Vtg gene activation (Ryffel 1978). Studies on the Japenese medaka (*Oryzias latipes*) revealed that

Vtg may be induced in males by E_2 and EDC to produce Vtg at a level previously determined to be indicative of a reproductive female (Gronen et al. 1999). More recently, Vtg has been investigated in Florida as a biomarker of potential endocrine disrupting effects in largemouth bass (*Micropterus salmoides*) (Bowman et al. 2002, Sepdlveda et al. 2002). Numerous studies have been conducted in other species to identify and characterize this class of proteins (Wang and Williams 1982, Wahli et al. 1989, Hartling et al. 1997) and while there has been some work done in reptilian species such as lizards and turtles (Baerga-Santini and Morales 1991, Brown et al. 1997, Morales et al. 2002, Romano et al. 2002), there is very little reported for the crocodilians (Guillette et al. 1997). The reptilians which have been investigated most conclusively in this respect are turtles and lizards. The following is a brief summary of the most recent studies published.

In the past 3 y there have been three comprehensive studies which have been essential in advancing turtle Vtg research to the point where quantitative assays are now possible. Duggan et al. (2001) analyzed plasma from the freshwater painted turtle (*Chrysemys picta*) in a seasonal study to fully characterize seasonal lipid transport in this species. They concluded that in this species, lipids and proteins control seasonal ovarian growth probably under hormonal control. These authors provide a detailed protocol for monitoring plasma lipids in turtles which utilized several techniques including the well known gravimetric method for total lipids as well as enzyme-linked immunosorbant assay (ELISA) methods for individual lipid components. Irwin et al. (2001) designed a study to analyze the potential effects of xenoestrogens present in cattle farm pond water on Vtg induction in the painted turtle. The rationale behind this study was that the

manure runoff into the ponds could be carrying metabolized (glucoronide-conjugated) hormones which bacteria in the water could subsequently cleave into active steroids. These in turn could potentially induce the turtles and fish (male and female) that inhabited the ponds to increase hepatic Vtg production, therefore altering their reproductive cycles. They used an ELISA method designed to measure Vtg in both males and females from the affected ponds and compared them to a control site. They found that water concentrations of xenoestrogens in the water were sufficient to induce Vtg production in females but not in males. Herbst et al. (2003) recently published a comprehensive study designed to analyze the Vtg protein sequence in green turtles (*Chelonia mydas*) and compared it to published sequences from other species (tuatara [*Sphenodon punctatus*], chicken, and frog). They found that the n-terminal sequence obtained from 15 cycles of Edman degradation protein sequencing was not an exact match to anything in the National Center for Biotechnology Information (NCBI) or the Expressed Sequence Tags (EST) databases. The sequence however, had 73% homology with that of the tuatara (Brown et al. 1997). They then purified plasma Vtg to produce polyclonal and monoclonal antibodies to egg yolk granules which was reactive to green turtle Vtg in both ELISA and Western blot analyses.

Lizard Vtg research has advanced from mere identification and MW determination (Carnevali et al. 1991, Baerga-Santini and Hernandez de Morales 1991) to time course analysis beginning with hepatic induction and ending with deposition in the developing follicle (Morales et al. 1996). Another interesting study conducted by Morales and Sanchez (1996) continued on their time course studies and investigated the effects of captivity on anole (*Anolis pulchellus*) Vtg production and subsequent follicular

deposition. They found that long term captivity stress induced cessation of Vtg production and circulation could be alleviated by low level E_2 hormone replacement therapy within 72 – 96 h.

Talent et al. (2002) and Brasfield et al. (2002) both published studies advocating lizards as a potential reptilian model for ecotoxicological risk assessments. Talent et al. (2002) designed an egg injection study which revealed that 17α-ethinylestradiol (an estrogenic chemical) caused male embryo feminization by impeding the development of secondary sex characteristics. Brasfield et al. (2002) designed a study to aid in the development of a protocol which could potentially be used as a quantitave tool for monitoring Vtg in western fence lizards (*Sceloporus occidentalis*). This study utilized a direct Vtg ELISA method and compared it to an indirect plasma alkaline-labile phosphate (ALP) method previously used in invertebrates (Kernaghan et al. 2002) and fish (Gagné et al. 1998, 2000, 2001) as an indirect measure of Vtg. They concluded that there was a high correlation between the two methods and that the ALP method could be a suitable measure of plasma Vtg in fence lizards.

Rosanova et al. (2002) contributed invaluable data to the Vtg field by identifying the MW and location in two liver subcellular fractions of several Vtg precursor proteins in the oviparous lizard (*Podarcis sicula*). This study utilized Western blot analysis to identify two proteins (84 and 70 kDa) located in the rough endoplasmic reticulum (RER) and four proteins (180, 150, 60, and 50 kDa) located in the smooth microsomal fraction.

Romano et al. (2002) conducted a time course study on the oviparous lizard (*Podarcis sicula*) which followed the fate of lipovitellins and phosvitins previously identified in egg yolks over a course of 44 days from ovoposition. There were two

lipovitellins at 110 and 116 kDa that remained constant in the yolk throughout the 44 day incubation. The phosvitin profile underwent various changes throughout the 44 day incubation periods; on day one there were four proteins detected at 50, 45, 29, and 14 kDa; on day 10 post ovoposition, the 29 kDa phosvitin was missing but a new one was detected at 6.5 kDa; on day 18, only two phosvitins were detected at 14 and 6.5 kDa; and finally at day 44, only the 6.5 kDa phosvitin was detected. This suggested that there was a continuous degredation of the phosphorylated proteins in the egg yolk over the course of incubation. The interpretation of this degradation was that the embryo needed to be supplied with amino acids and smaller proteins during its embryonic growth. This study confirmed a need for assays that are capable of tracking these specific phosphorylated proteins or protein fractions throughout the time course which extends from egg production in the adult female liver through the developmental period of the embryo if we are to begin to elucidate the effects that contaminants (which may cause oxidative damage and subsequent dephosphorylation) may have on the reproductive success of these and other reptilian species.

Heppel et al. (1995) attempted to develop a universal Vtg ELISA that would be reactive with plasma Vtg from several species including a snake and tuatara. The authors found that Vtg was only two to three times higher in vitellogenic females when compared to males (lizards and snakes), while the fish female reactivity was three to 10 times higher than the males depending on the species.

Previous efforts to examine Vtg proteins in crocodilians have been qualitative or semi-quantitative. Matter et al. (1998) attempted to modify the method developed by Palmer and Palmer (1995) to quantify Vtg in hatchling alligators. Briefly, they

performed Western blot analyses of hatchling plasma utilizing a rabbit anti-Vtg antibody which was raised against red-eared turtle Vtg. They were unable to detect an induction of plasma Vtg in hatchlings; however this was probably due to their young age coupled with continued lipovitellin and phosvitin contribution from their yolk sac. Brown et al. (1997) utilized an antibody raised against tuatara (*Sphenodon punctatus*) in a western blot analysis of adult female alligator plasma successfully recognizing a specific protein at ~220 kDa which they presumed to be Vtg. However this was not expanded upon, since the subject of their study was the tuatara. There has not been a quantitative assay published to date that is sensitive and specific for crocodilians. The current study was designed to characterize and isolate Vtg in the American alligator as a critical step toward the development of a quantitative assay for this species.

Background and Significance

The American alligator was placed on the United States endangered species list in 1967 (Groonbridge 1987). At that time, it was an acceptable practice to allow unlimited harvesting of animals for the sale of meat, skins, and trinkets such as teeth, claws, and skutes. It was even acceptable to harvest hatchlings and sell them as "pets". Alligator populations appeared to be diminishing, therefore monitoring of the species was begun to determine the extent of the threat for extinction. Now after years of monitoring, experts agree the species has made an important recovery and is no longer in danger of extinction (Wood et al. 1985, Woodruff et al. 1989). However, the monitoring program that was established opened a new venue for environmental research and alligators became a popular model for contaminant studies in the southeastern United States due to their place in the food chain, their longevity, and therefore their potential for bioaccumulation of xenobiotics (Hall and Henry 1992, Crain and Guilette 1998). In fact, it has been

proposed that many contaminants alligators are exposed to may be EDs (Gross et al. 1994, Guillette et al. 1994, Crain et al. 1997, Guillette and Gunderson 2001, Guillette et al. 2002). Alligator research in this area was originally conducted on eggs to determine a potential relationship between contaminants and their effects on reproductive success. However, to date no clear relationship has been established between the level of contaminants found in the eggs and embryo survival (Heinz et al. 1991). Therefore, an increasing number of researchers have begun looking at the adult female for a better understanding of mechanism(s) behind altered reproductive success.

Due to the many factors that contribute to growth and maturity in this species such as temperature, population density, and food availability and quality (Hutton 1987*)*, it is nearly impossible to determine if an adult female is reproductively active and will lay eggs in a particular year based on anatomical size alone. This coupled with permit limitations has led to the need for developing a non-invasive tool for evaluating the reproductive status of adult female alligators. The development of such a tool was the primary goal of this work. A secondary goal was to begin to isolate and characterize plasma Vtg from this species. This is of importance because it will aid future studies in elucidating a potential mode(s) of action of EDC.

Study Objectives

The objectives of the present study were to

1. Develop a qualitative method for identifying follicular females, and to
2. Identify and characterize female specific plasma proteins likely to be Vtg.

CHAPTER 2
ASSESSMENT OF REPRODUCTIVE STATUS IN FEMALE AMERICAN ALLIGATORS

Alligators are a popular model for reptilian contaminant studies due to their predatory place in the food chain, their longevity, and therefore their potential for bioaccumulation of contaminants (Hall and Henry 1992, Crain and Guilette 1998). It has been proposed that many of the contaminants that alligators are exposed to may be ED's (Gross et al. 1994, Guillette et al. 1994, Crain et al. 1997, Guillette and Gunderson 2001, Guillette et al. 2002). Contaminant research in this species was originally conducted on alligator eggs to determine a potential relationship between contaminants and their effects on reproductive success. So far, no clear relationship has been established between the level of contaminants found in the eggs and embryo survival (Heinz et al. 1991). Therefore, an increasing number of researchers have begun looking at the adult female for a better understanding of the mechanism(s) behind altered reproductive success.

Due to the many factors that contribute to growth and maturity in this species, such as temperature, population density, and food availability and quality (Hutton 1987*)*, it is nearly impossible to determine if an adult female is reproductively active and will lay eggs in a particular year based on anatomical size alone. This, coupled with permit limitations (as in Florida) has led to the need for developing a non-invasive tool for evaluating the reproductive status of adult female alligators. To date there is no such protocol published for alligators. This study was designed to develop a novel plasma

protein assay which could be utilized for the prediction of reproductive status in adult female alligators.

Vitellogenin (Vtg) protein is produced in the livers of reproductive female alligators, circulated through the blood, and subsequently deposited in the developing follicles. Along with being a reliable predictor of gravid females, it has also been proposed as a biomarker of exposure to EDC in oviparous species (Sumpter and Jobling 1995). However, to date, there has not been a quantitative assay published that is sensitive and specific for crocodilians. Heppel et al. (1995) attempted to develop a universal Vtg ELISA that would be reactive with plasma Vtg from several species including reptiles, but found that it was not sensitive enough (for reptiles) to be considered a reliable assay. Matter et al. (1998) attempted to modify the Western blot developed by Palmer and Palmer (1995) to be used as a quantitative Vtg assay in hatchling alligators. They were unable to detect an induction of plasma Vtg in the hatchlings, however this was probably due to their young age coupled with continued lipovitellin and phosvitin contribution from the yolk sac. Another factor to consider is the potential non-specific reactivity of the antibody with non-Vtg proteins. The current study was therefore designed with the intent that the data obtained herein could be used to further the efforts in developing such an assay that would be sensitive and specific for alligators.

The primary objective of this study was to screen several free-ranging alligator females and develop a reproducible method for evaluating reproductive status. Animals were screened initially for the presence of highly expressed plasma proteins specific to adult females in the 250 to 350 kDa. This is the predicted MW range for Vtg in other

oviparous species (Heppel et al. 1995, Brown et al. 1997, Allner et al. 1999, Brion et al. 2000). This is a relatively non-invasive procedure which should decrease the incidents of sacrificing animals that don't fit the study's criteria. A secondary objective of this study was to develop a standardized necropsy protocol which could be used to quantitatively assess the reproductive tract and thus be a tool for use in comparative studies.

Materials and Methods

Study Sites

Two sites were chosen in an effort to reduce site specific bias from being introduced into the individual experiments. Each site was chosen for its significance to the ecological and environmental concerns surrounding alligators in their respective geographical locations (see following sections for relevance of chosen sites).

Lake Griffin, Florida. Florida Lakes in the Ocklawaha River Basin have been the subject of environmental concern for the past few decades (Benton and Douglas 1994, Marburger et al. 2002). In the 1980's, Lake Apopka's alligator population declined noticeably suggesting a potential association with organochlorine pesticides (OCP) (Guilette et al. 1995). There were several point sources responsible for OCP contamination in Lake Apopka and subsequently the entire basin (Benton and Douglas 1994, Marburger et al. 2002). Lake Griffin, located downstream of Lake Apopka (Figure. 2-1), has moderate to elevated OCP concentrations in alligator egg yolks and decreased egg viability (Rauschenberger et al. 2003).

Rockefeller State Wildlife Refuge, Louisiana. Rockefeller Wildlife refuge was donated to the State of Louisiana in 1920, and it is comprised of 76,042 acres (this is down from the original 80,000 acres due to erosion) which border the Gulf of Mexico (Figure. 2-2). The Deed of Donation mandated that the land be maintained as a wildlife

refuge, and that there would be no public or commercial fishing or trapping. In 1983 there was an amendment to allow sport fishing and commercial trapping for the purpose of generating revenue for education and public health. This was amended again in 1987 ceasing the surplus revenue (Louisiana Department of Wildlife and Fisheries). Since then, it has been maintained as a refuge and it is staffed by a team of scientists, conservation officers, and of course a maintenance crew. The research conducted at Rockefeller has been instrumental in many of the advancements made in alligator ranching and physiology. There is limited access allowed to the public with regulations that are strictly enforced. This refuge has become popular as a reference site for many studies due to its low levels of soil contaminant concentrations (Elsey et al. 1999, Davis et al. 2001, Cobb et al. 2002) and the reduced level of stress to wildlife.

Animals

Adult female alligators (1.8 – 2.1 m) were captured by noose according to IACUC guidelines. Captures were coordinated such that animals from each site were at equivalent points in their reproductive seasons: Rockefeller animals were captured in mid April and Lake Griffin animals were captured in early May (these dates were chosen to target animals which would be in the late vitellogenic (V) stage of their reproductive cycle). These time points were confirmed to be similar when eggs were collected and staged later in the season: Rockefeller embryos were collected June 14th and staged at day 7–12 on June 29th, and Lake Griffin embryos were collected and staged at day 12 on July 1st. Animals were held in a moist cool enclosure until they were screened for the study criteria described below. Those meeting the criteria were held for sacrifice and those which did not meet the criteria were returned to their place of capture and released.

Plasma Samples

Blood (10 mL) was drawn from the occipital sinus into a heparinized syringe and transferred to heparinized tubes. The blood was set on ice until it could be centrifuged at 1000 rpm for 20 min in a Beckman J6-HC centrifuge to separate plasma from the cellular fraction. Once separated the plasma was snap-frozen in 1 mL aliquots and stored at -80°C.

Female specific protein determination

Sodium dodecal sulfate polyacrylamide gel elctrophoresis analysis was performed according to the method described by Laemmeli (1970) to screen plasma for the presence of female specific proteins in the predicted MW range (~250 to 450 kDa) for Vtg. A predetermined criteria was set to categorize the plasma profiles in the 250 to 450 kDa range as being (1) "highly vitellogenic" if the female specific protein bands were at least two to three times more intense than that of a positive control female or (2) "weak to non-vitellogenic" if the female specific proteins were less intense than those of the control female or not present at all. These intensities were measured utilizing one individual's gross visual judgment due to the nature of the field set-up and lack of availability of a scanning densitometer. The positive control female used for these and subsequent experiments had been implanted with a 180 day time release pellet containing 20 mg of E_2 in September 2001. Subsequently, plasma was drawn in December 2001 and preserved according to the protocol described previously (unpublished data, Gross et al. 2001).

Protein extractions. All chemicals utilized in this section were purchased from Sigma-Aldrich Company Corp., St Louis. MO, USA. Plasma samples (100 μL) were clarified by spinning at 10,000 rpm for 5 min in an Eppendorf microcentrifuge (to

remove cellular components). A surfactant extraction buffer (containing a protease inhibitor cocktail made up of 4-(2-aminoethyl)benzenesulfonyl fluoride [AEBSF]; ethylenediaminetetraacetate [EDTA]; Bestatin, L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine [E-64]; Leupeptin; and Aprotinin) was applied to plasma samples to liberate and denature proteins. This was prepared from a 10x extraction buffer which consisted of 500 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 5% Triton-X 100, 2% sodium dodecylsulfate (SDS), 5% sodium deoxycholate (DOC), and 20 ：L/mL protease inhibitor cocktail. The 10x buffer was added to 90 ：L of clarified plasma to give a 1x final concentration. Samples were kept on ice during extraction to minimize degradation.

Protein assay. Chemicals, pre-cast gels, protein standards, and equipment utilized in this and subsequent electrophoresis sections were purchased from Bio-Rad Laboratories, Hercules, CA, USA. Extracted protein samples were quantified according to Bradford (1976) using the Bio-Rad Protein Assay kit. A 1:100 dilution of each plasma sample was quantified by measurement against a bovine serum albumin (BSA, supplied in kit) standard curve ranging from 0 to 40 ：g/mL total protein. The micro protein assay was performed by pipetting 160 ：L of each sample dilution and standard into a 96 well plate in triplicate. Subsequently 40 ：L of G250 protein dye reagent (supplied in kit) was added to each well. Plates were incubated at room temperature for 5 min and subsequently read on a Dynex MRX Microtiter Plate Reader at 595 nm. Dynex Revelation software was used to develop a standard curve and extrapolate sample protein concentrations.

Sample preparation. For each animal, 20 ：g total plasma protein, was prepared by adding sample reducing buffer containing 12.5% 0.5 M Tris-HCL pH 6.8; 25%

glycerol; 2% SDS; 5% β-mercaptoethanol; and 1.25% bromophenol at 2 - 3 times the sample volume and boiling for 1 min to denature the protein.

Electrophoresis. The denatured protein samples were loaded onto 7.5% acrylamide denaturing gels for maximum high MW separation and reasonable band definition. A 1x running buffer (25 mM Tris base, 250 mM glycine, and 0.1% SDS) was used to perform electrophoresis on a Bio-Rad Mini-gel II apparatus powered by a Bio-Rad Power Pac 200. Molecular weights were confirmed by comparison to denatured MW standards which were run simultaneously with samples on each gel. Subsequent to electrophoresis, gels were fixed and stained with coomassie brilliant blue (CBB) protein staining solution composed of 40% methanol, 10% acetic acid, and 1% CBB overnight at room temperature with gentle agitation. The next day they were washed in several changes of de-stain (40% methanol and 10% acetic acid) to remove unbound stain and equilibrated in double deionized water (ddH_2O). The gels were then dried between two pieces of cellophane sheets in a Bio-Rad Air Dryer for preservation and subsequent documentation on a Bio-Rad GS-800 densitometer.

Data analysis. SDS-Page gels were analyzed and qualitatively graded for intensity of female specific bands in the 250 to 450 kDa MW range. These intensities were measured utilizing one individual's gross visual judgment due to the nature of the field set-up and lack of availability of a scanning densitometer. Only those animals that presented intensity in all female specific bands (when compared to a male plasma pool and the positive control female plasma [described previously]) were considered to be highly folliculogenic and subsequently chosen for sacrifice (see Figure. 2-5).

Circulating Hormone Concentrations

Plasma samples were analyzed by a standard radioimmunoassay (RIA) procedure

to determine circulating E_2 and T. This is a competitive binding assay set up to allow competition between the animal's plasma hormone and a radiolabeled standard hormone for the binding site of a protein antibody. The following is a summary of the method from Giroux (1998).

Sample extractions. For each hormone assay, plasma (50 μl) was extracted twice with 4 mL of ethyl ether in duplicate (two tubes, each containing 50 μl of plasma from each animal) for each hormone assay. Tubes were then vortexed for 1 min and then incubated in a methanol/dry ice bath for 3 to 4 min to precipitate (freeze) the aqueous fraction of the plasma. The ether/lipophilic plasma fraction was poured into a 100 mm glass tube and placed on a Labconco evaporator for 10 to 15 min. This procedure was repeated using the same tubes, thereby concentrating the two extractions together.

Hormone assays. Standard curves and samples were prepared as follows. Total count tube (TC): 350 μL phosphate buffered saline with 1% gelatin and 0.01% sodium azide (PBSGA) was added to 100 μL radioactive label to determine the upper limit of the radioactivity in the assay; non-specific binding tube (NSB): 350 μL phosphate buffer was added to 100 μL radioactive label to measure its reaction with the antibody; zero binding tube (BO), 250 μL phosphate buffer was combined with 100 μL radioactive label and 100 μL antibody to determine maximum binding of the unlabeled Ab-Ag complex; standard curve tubes, 200 μL phosphate buffer was combined with 100 μL radioactive label and 100 μL antibody and 50 μL known steroid in eight tubes of increasing concentrations from 0 pg/mL to 20,000 pg/mL; extracted sample pellets, 250 μL phosphate buffer was combined with 100 μL radioactive label and 100 μL antibody specific for either E2 or T. All tubes were incubated for 24 hours at 4°C. The next day

250 μL charcoal dextran was added to all tubes except the TC tubes and subsequently centrifuged for 10 min at 3000 rpm and 4°C in a Beckman J6-HC centrifuge to remove the unbound antibody. For each tube, 0.4 mL of the supernatant was taken off and added to 4 mL of Scintiverse scintillation fluid (Fisher Scientific, Fairlawn, NJ, USA) in scintillation vials (United Laboratory Plastics, St. Louis, MO, USA). Samples were counted on a Packard Tri-Carb scintillation counter (model 1600CA). Unknown samples were quantified against the standard curve using the Beckman EIA/RIA Immunofit.

Necropsies

Twenty adult female alligators were screened at each site. Of the 20 animals from each site, 10 highly V and 3-5 weak to non-vitellogenic (NV) animals (for contrast) were sacrificed and necropsied. Anatomical reproductive tract evaluations were performed according to a standardized protocol (Table 2-1 and 2-2). Linear measurements (total length: tip of nose to tip of tail; snout-vent length; head length; and tail girth which was measured just behind the vent) were performed using a centimeter tape. Weight was determined by suspending the animal from a kilogram scale which was attached to a fork lift. Animals were sacrificed by cervical dislocation and double pithing. Subsequently, necropsies were performed according to the following protocol saving appropriate tissues for further analysis. The abdominal cavity was exposed by making two transverse cuts: one at the vent and one just below the chest cavity. Subsequently a longitudinal cut was made on one side at the transition between the dorsal and ventral side. The outer skin and fat layer was then filleted away from the abdominal membrane and the flap retracted. The abdominal cavity was further exposed by cutting away the rib cage and through the tough outer membrane. Once inside the cavity, organs were dissected out, weighed, and

measured. The liver was weighed on a gram scale, and color and condition noted. The entire reproductive tract was removed from both the right and left sides. Photo-documentation was performed using a centimeter ruler for scale. The oviduct and ovaries were separated, weighed and measured (oviductal diameters were taken in the center of each anatomical section [defined in Chapter 1], lengths were not recorded due to expected inaccuracies subsequent to stretching and straightening). All follicles greater than 5 mm were counted, weighed, and measured using a gram scale and digital calipers.

Health and reproductive parameters were evaluated using the following formulas:

- Condition factor; K = 100 x (weight (g)/length $(cm)^3$)
- Hepatic Somatic index; HSI = 100 x (liver wt/body wt – liver wt)
- Gonadal somatic index; GSI = 100 x (gonad wt/body wt – gonad wt)

Statistics were run for mean, SEM, equality of variance, and ANOVA (for multiple groups with sites) or T-tests (for individual means between sites) when appropriate using the Minitab statistical package.

Results

SDS-Page analyses revealed three bands in the Vtg MW range (~250, 350, and 450 kDa) that were present in higher concentrations in follicular animals (indicated by brackets in Figure. 2-3). These results correlated well (10 animals out of 10) with anatomical evaluations (Figure 2-4 panels A & B). Each animal from both sites that presented intense plasma protein bands in the above mentioned MW range (Fig 2-3 panel A) also presented a highly follicular (a greater number of large [>20 mm] follicles) reproductive tract (Fig 2-4 panel A & B). Conversely, each animal from both sites that presented weak to non-existent plasma protein bands in the above mentioned MW range (Fig 2-3 panel B) also presented a weakly follicular (a greater number of small [<20 mm]

follicles) reproductive tract (Fig 2-4 panel C & D).

Table 2-1 summarizes the average anatomical evaluations of all animals from both sites that were necropsied. Overall, Lake Griffin (LG) V and NV females were significantly larger (snout-vent length, head length, tail girth, and weight) when compared to Rockefeller (R) animals. Lake Griffin V females had a significantly higher condition factor when compared to R V animals but this was not true for the NV females when sites were compared. While there were significant differences noted between sites for the previously mentioned lengths (indicated in parenthesis), the total lengths were not significant. However, there was an overall trend for the LG animals to be longer than the R animals. The Rockefeller V animals had a significantly higher hepatosomatic index (HSI) when compared to LG V animals but this was not true for the NV females. There were no significant differences for any of the previously mentioned parameters noted when the V animals were compared to the NV animals within sites.

Table 2-2 summarizes the average reproductive evaluations of all animals from both sites that were necropsied. Lake Griffin V animals had significantly larger oviductal weights and diameters when compared to R V animals. However, there were no significant differences noted for the oviductal measurements or ovarian weights for the NV females. The LG V females however had a significantly higher GSI compared to the R V animals while there was no significant difference between sites for the NV animals.

The average numbers of follicles (overall totals and size class totals) were summarized in Table 2-2. There were no significant differences in the overall number of follicles when LG V animals are compared to R V animals. However, there were differences in the distribution of these follicles in the different size classes. For instance,

LG V animals had significantly more 5–10 mm follicles in the right ovary and also contained follicles in the > 26 mm category, which was absent in the R V females, and R V animals had significantly more 16–20 mm follicles in both ovaries when compared to the LG animals. The distribution and frequency of the follicular size classes for each of the V animals are summarized for the two sites separately in Fig 2-5. These graphs reiterate the results obtained from the averages determined in Table 2-2. Overall, the LG V animals had a large number of predominantly > 26 mm follicles (Fig 2-5 panels C & D); while the R V animals had a larger number 16–22 mm and 21–25 mm follicles (Fig 2-5, panels A and B). In summary, when comparing reproductive tract measurements of V animals across sites, LG animals had significantly larger tracts containing a greater number of large follicles (> 26 mm).

However, when comparing NV animals across sites (for all of the above reproductive measurements), there were only two significant differences noted: LG NV animals had significantly more 5–10 mm follicles, whereas R NV animals had significantly more 16–20 mm follicles.

When the V animals were compared to the NV animals within sites (Table 2-2), the following significant differences were noted for the previously described reproductive measurements: LG V animals had significantly larger and heavier oviducts and ovaries than the LG NV animals, and for both sites V animals had higher GSI compared to NV animals. In addition, the R V animals had significantly more follicles overall. Conversely, there was no significant difference noted for the LG animals due to high variability in the NV animals, however, the trend indicated that there were more total follicles in the LG V animals. When the follicle size classes were compared, R V

animals had significantly more 5–10 mm, 11–15 mm and 21–25 mm follicles than the R NV animals. The LG V animals had significantly more follicles 21–25 and > 26 mm follicles, while the LG NV animals did not have any follicles of these size classes.

The average plasma E_2 concentrations were 432 ± 39 ng/mL and 571 ± 73 ng/mL for R and LG V animals, respectively. The circulating T concentrations were 219 ± 119 ng/mL and 279 ± 66 ng/mL for R and LG V animals, respectively. There was no significant difference between these values for either hormone across sites. There was no hormone analysis performed on the NV animals. This was due to technical difficulties that arose after the V animals had been analyzed.

As stated previously, there was a direct correlation (10 out 10 animals for each site) between the three female specific bands noted on the SDS-Page analysis of the plasma and the physical appearance of the reproductive tract (Figures 2-3 and 2-4).

Table 2-1. Mean ± standard error of body measurements.

	Rockefeller		Lake Griffin	
	V	NV	V	NV
Total length (cm)	232 ± 7	224 ± 10	245 ± 8	247 ± 4
Snout-vent length (cm)	120 ± 4	118 ± 5	133 ± 3[a]	154 ± 26
Head length (cm)	37 ± 1	36 ± 1	41 ± 1[a]	40 ± 0.4[a]
Tail girth (cm)	56 ± 2	53 ± 3	66 ± 3[a]	66 ± 1[a]
Weight (kg)	38 ± 4	36 ± 7	59 ± 6[a]	56 ± 4
Condition factor	0.3 ± 0.01	0.3 ± 0.02	0.4 ± 0.02[a]	0.4 ± 0.01
Hepatosomatic Index	1.3 ± 0.01[a]	1.1 ± 0.2	0.9 ± 0.04	0.9 ± 0.1

[a] Indicates a significant difference between sites ($p < 0.05$).
(vitellogenic (V) sample size: n = 10, for each site; non-vitellogenic (NV) sample size: n = 3, for each site)

Table 2-2. Mean ± standard error of the mean for reproductive measurements.

	Rockefeller				Lake Griffin			
	V		NV		V		NV	
	Left	Right	Left	Right	Left	Right	Left	Right
Oviduct diameter (mm)								
Upper	13±1	12±1	8± *	8±1	21±2[a]	19±1[a b]	11±4	10±2
Middle	17±1	15±1	14± *	11±2	19±1	18±1[b]	12±3	9±2
Lower	25±2	25±1	24± *	24±2	38±2[a b]	39±4[a b]	25±4	28±3
Oviduct weight (g/kg body weight)	4.8±0.5[b]	5.0±0.5[b]	1.7±0.5	1.8±0.9	6.9±0.7[a b]	7.3±0.8[a b]	2.1±0.7	2.2±0.7
Ovary weight (g/kg body weight)	4.4±0.5	4.9±0.5	3.0±0.8	3.2±0.9	9.2±1.2[a b]	8.7±0.9[a b]	1.0±0.2	1.1±0.1
Gonadal-somatic index:	0.9±0.1[b]		0.4±0.1		1.8±0.2[a b]		0.9±0.1	
Total number of follicles	29±4[b]	31±2[b]	6±5	5±8	34±3	34±3	13±11	20±13
5-10 mm	7±2[b]	7±1[b]	1±1	0	4±1	5±2	8±4	15±4[a]
11-15 mm	3±1[b]	4±1[b]	0	1±1	2±1	3±1	5±3	5±5
16-20 mm	10±2[a]	10±2[a]	3±2	4±4[a]	2±1	1±0.5	0	0
21-25 mm	9±3[b]	11±3[b]	1±1	0	6±3[b]	6±3[b]	0	0
> 26 mm	0	0	1±1	0	20±3[a b]	19±3[a b]	0	0
	432±4		N/A		571±73		N/A	
Plasma Estrogen (pg/mL)	219±119		N/A		279±66		N/A	
Plasma Testosterone (pg/mL)								

[a] Indicates a significant difference between sites ($p < 0.05$). [b] Indicates a significant difference between V and NV within sites ($p < 0.05$. * Missing samples from two animals, therefore no SEM. N/A: there was no hormone data available for NV animals. (vitellogenic (V) sample size: n=10, for each site; non-vitellogenic (NV) sample size: n=3, for each site)

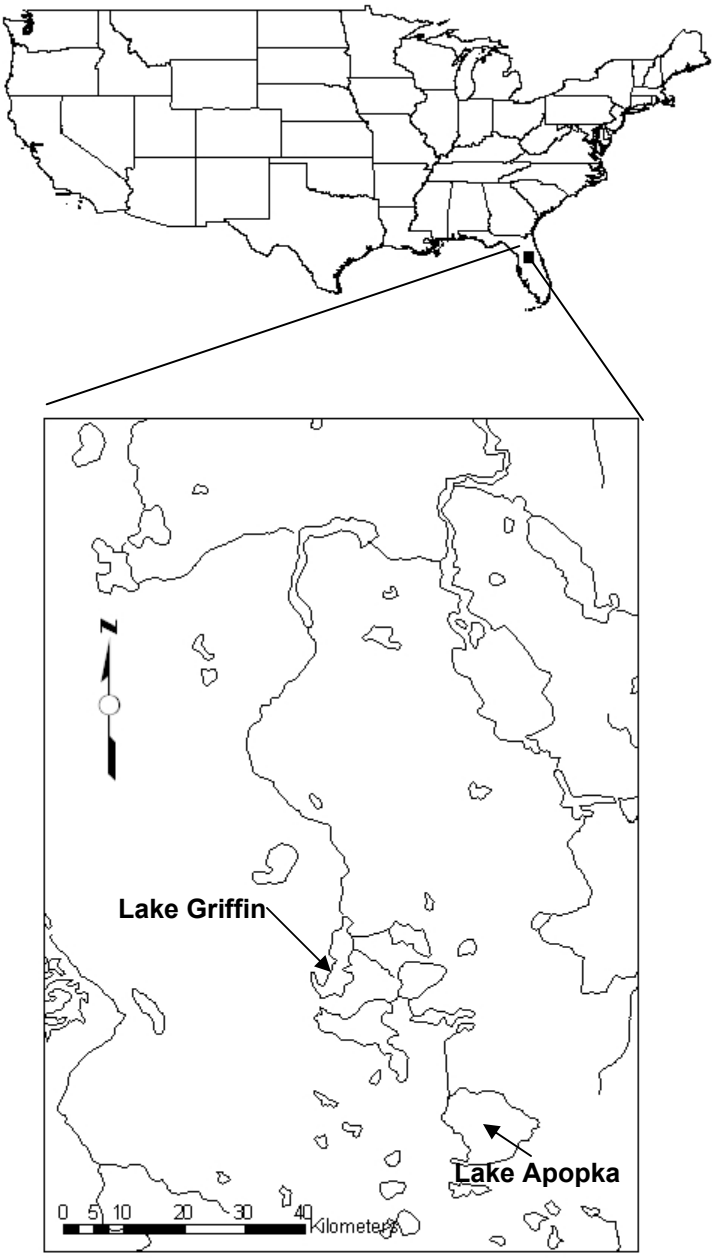

Figure 2-1. Map of the Oklawaha River Basin, Florida. Arrow indicates Lake Griifin.

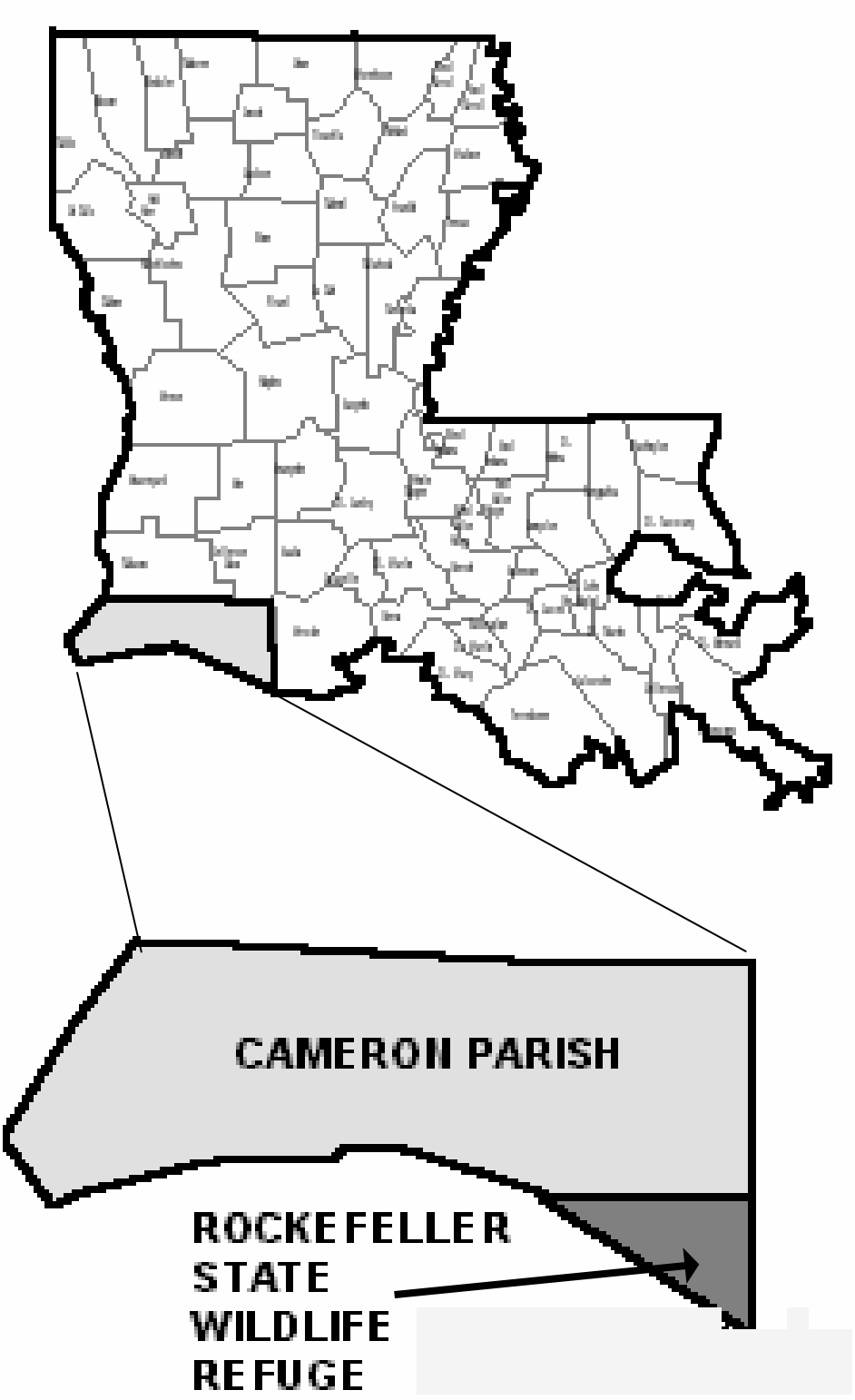

Figure 2-2. Map showing location of Rockefeller State Wildlife Refuge. Only part of the refuge is shown where the study took place (indicated by arrow).

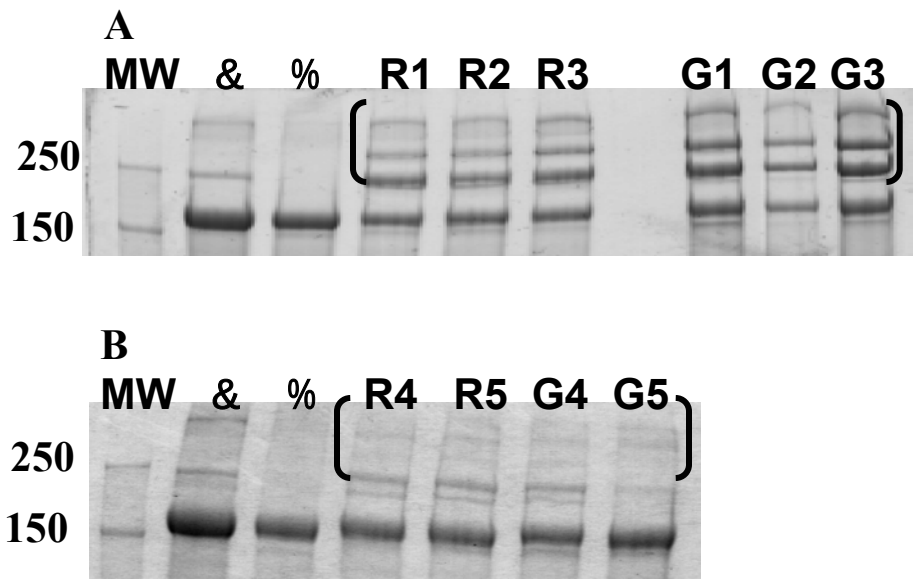

Figure 2-3. SDS-PAGE analysis of plasma samples from adult female alligator plasma. Brackets indicate expected molecular weight (MW) range for V proteins. ♀ lane contains plasma from an E2 induced control female. ♂ lane contains plasma from a control adult male alligator pool. Analysis normalized to total protein loaded. A) Vitellogenic (V) (n =3 from Lake Griffin [G] and Rockefeller [R]). B) Non-vitellogenic (NV) (n = 2 from Lake Griffin [G] and Rockefeller [R]).

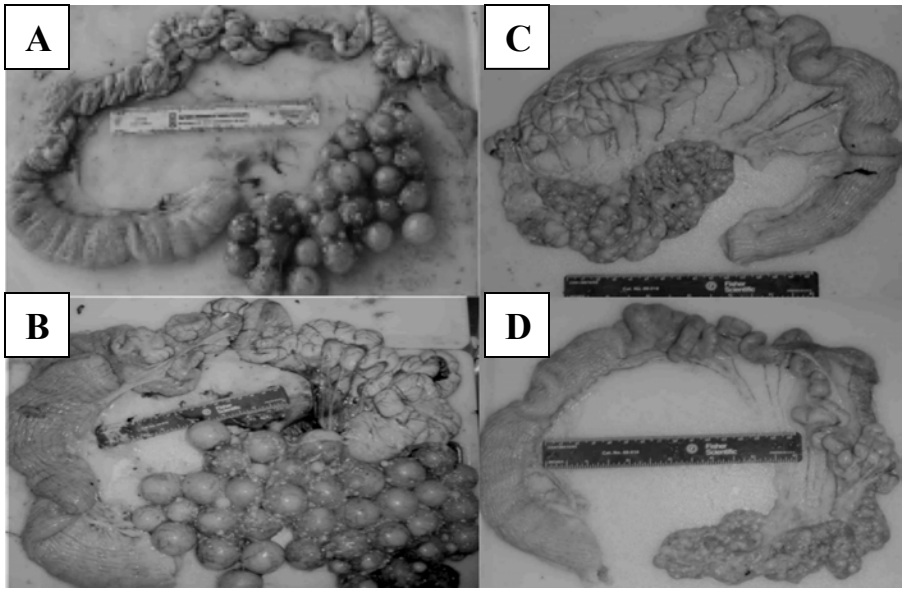

Figure 2-4. Photographic documentation of reproductive tracts of representative animals from each site. A) V Rockefeller female. B) V Lake Griffin female. C) NV pre-ovulatory Rockefeller female. D) NV pre-ovulatory Lake Griffin female.

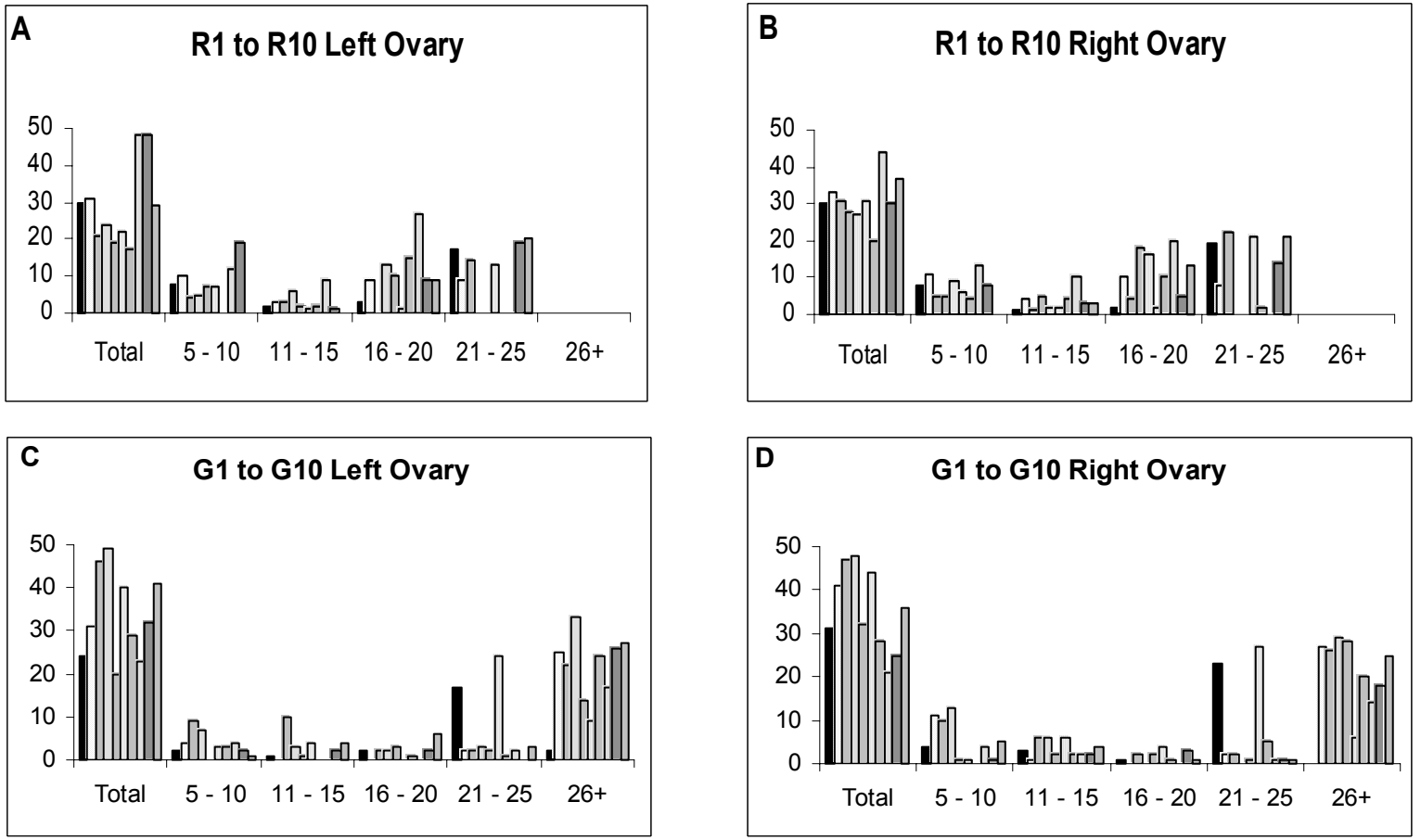

Figure 2-5: Follicular frequency distribution in right and left ovaries. Each bar represents one female alligator.
Y axis: The number of follicles in each size classification.
X axis: Total (total number of follicles for each animal), followed by each size classification (measured in mm).

Discussion

The three female specific proteins that were identified by this study have provided a good starting place for investigating plasma Vtg in alligators. They are within the predicted MW range based on information that has been published for other species, but it must be confirmed that one or all of these proteins are truly Vtg (see Chapter 3). Secondly, a full molecular characterization must be done to determine their origination and subsequent fate such as follicular deposition.

The anatomical and reproductive evaluation section of this study was designed to serve three purposes: (1) to validate the results of the plasma protein screening; (2) to provide a comprehensive data base of anatomical and reproductive parameters of both Vtg and non-Vtg adult females; and (3) to use the data generated to develop a standardized protocol to be used for future evaluations of female alligators' health and reproductive status.

The evaluation of reproductive status validated the plasma protein screening protocol. There was a 1:1 correlation for V females exhibiting plasma proteins in the 250 to 450 kDa range. This correlation provided significant evidence that this is an acceptable method for discerning Vtg animals from non-Vtg animals. Each animal that had the three highly expressed plasma proteins also had a larger number of follicles in the 21 to 25 mm or > 26mm size classifications. There was also very little inter-animal variability in the oviductal diameters. This coupled with the low variability in their sizes (within sites) confers the likelihood that they were equivalent in age and gravida (number of times that they had reproduced).

Alligator reproductive anatomy has been described comprehensively in the literature dating back as far as the 19th century. While there was no new information

acquired from this study as far as reproductive anatomy, it did provide an essential teaching tool for this student's education in reptilian anatomy. Subsequently, another opportunity arose in that a second investigator (Dr. Dave Rostal, Georgia Southern University) was simultaneously performing abdominal ultrasonography on the same animals used for this study in an effort to validate his method for use in alligators. There was a 1:1 correlation between Dr. Rostal's results (predetermined criteria for a positive ultrasound was detection of follicles >15 mm) and the anatomical evaluations performed in this study. This collaboration proved to be beneficial to both groups while limiting the needless sacrifice of additional animals.

The E_2 hormone values were close to the expected average of 700 pg/mL for adult female alligators during the latter part of the reproductive cycle for both of these geographical locations. Testosterone values however, were well above the published average of 90 ng/mL. A possible explanation for the discrepancy in the average T values obtained is that the T hormone assay was lacking in sensitivity and/or specificity for alligators. Comparing this study's E_2 analysis with others in the literature showed it to be useful in providing another parameter to evaluate the point the animals were at in their reproductive status, however it may have been improved by also including P in the hormone profile.

This study demonstrated that a qualitative analysis of female specific plasma protein in alligators was a useful and predictive measure of folliculogenesis. There was an increase in the number of follicles in LG animals which did not coincide with a higher level of plasma E_2 confirming that E_2 had already peaked for the season and that these animals were in late vitellogenesis. This also suggests that there may be a different, non-

hormonally induced pathway at work in LG animals driving them to produce a greater number of larger follicles. Another possibility is that the hormone assay performed in this study lacked sensitivity and/or specificity for alligator plasma. However, since the values were comparable to those in the literature, this is not a very likely explanation.

The anatomical evaluations demonstrated that overall LG animals were significantly larger than R animals with a higher condition factor indicating that the LG animals probably had more body fat as well. This could be due to seasonal variations between the two sites as females begin to mobilize fat deposits while they progress through their reproductive season. This could be an explanation for the larger follicles present in the LG animals. It seems to be a plausible possibility that the R animals were going to go through the same process later in the season thereby placing them slightly behind the LG animals in their reproductive status, however the egg staging data suggests the opposite. The R animals were captured and subsequently eggs were collected 2–3 weeks earlier than LG animals. The equivalency which is suggested in the materials and methods section is probably skewed because the R eggs had a longer period of time between when they were collected and when they were set in the incubator due to transportation. Two possible explanations for the reproductive differences between these two sites are: the animals were at equivalent stages when they were sacrificed with R animals having begun their season earlier; or, perhaps it is due to the fact that R females lay smaller eggs. Rockefeller animals had a higher HSI than the LG animals suggesting that they were in the process of increased hepatic protein production. Since Vtg is a hepatic protein, then perhaps they were just behind the LG animals in their Vtg production which would explain why they had smaller follicles overall. A

comprehensive time course following the production of Vtg coupled with plasma hormones would need to be done at both of these sites to fully understand the variations between these two sites.

Having a comparison of non-Vtg and Vtg animals within these sites provided another piece of information vital to the study of reproductive biology. This study reflected that 50% of the screened population at each site would go on to reproduce that year. This compares to other studies which have found that 63 to 68% of the adult female population reproduce in a given year. It is difficult to determine the accuracy of this study's data since all 20 animals from each site were not sacrificed. However, based on the three non-Vtg animals from each site that were sacrificed, it is likely that the "non-Vtg" animals would not have gone onto reproduce that year. It is a widely accepted supposition that crocodilian reproduction is temperature driven coupled with water level of the nesting areas. These two factors are inherently seasonal for each geographical location. This makes the theory of a second wave of reproductive females very unlikely.

CHAPTER 3
IDENTIFICATION AND CHARACTERIZATION OF HIGH MOLECULAR WEIGHT FEMALE SPECIFIC PLASMA PROTEIN BANDS

Vitellogenin (Vtg) has been classified as a hormonally controlled precursor protein to several of the yolk proteins found in oviparous eggs (Ryffel 1978). Once Vtg production is stimulated by circulating estradiol (E_2) in the liver, it is post-translationally modified and circulated to the blood capillaries surrounding the follicular theca and transferred to the developing oocytes by diffusion from the follicular theca and subsequent pinocytosis by the oocytes (Wahli et al. 1981). Once in the oocytes, Vtg is proteolytically cleaved into lipovitellin and phosvitin, however the number of cleavage products is not definitively known and varies between species (Ryffel 1978, Wahli et al. 1981). Characteristically, it is a highly glycosylated phospho-lipoprotein. The precursor protein (circulated through the plasma) MW ranges from ~150 to 600 kilo-daltons (kDa) depending on the species (Heppel et al. 1995, Brown et al. 1997, Allner et al. 1999, Brion et al. 2000). For example, in the African clawed-frog (*Xenopus laevis*), it occurs in the form of a dimer consisting of two 200 kDa polypeptides (Wahli et al. 1981), whereas in the Kemp's Ridley sea turtle (*Lepidochelys kempi*) the predominant Vtg protein appears at 200 kDa (Heck et al. 1997). Similarly, the isoelectric focusing point (pI) ranges from ~ 6 to 7 depending on the species (Kawahara et al. 1983, James and Oliver 1997, Roubel et al. 1997). These characteristics were used collectively in the design of this study to optimize the chances of correctly identifying and characterizing Vtg in the American alligator.

The most extensive characterization of Vtg is in fish, birds (mainly chickens and quail), and amphibians (mainly the African clawed frog). Although little is known about this protein in reptiles, this research is rapidly growing and it is gaining popularity as a model for environmental endocrine disruption.

Since Vtg is a maternally derived protein that is utilized by the embryo as a nutritional source, it is possible that any deviation or disruption of the pathway may alter embryo development. Subsequently it has been proposed as a biomarker of exposure to endocrine disrupting chemicals in oviparous species (Sumpter and Jobling 1995). The rationale behind using Vtg as a biomarker stems from extensive research using the African clawed frog and the chicken(*Gallus domesticus*) as models for investigating estrogen induced Vtg gene activation (Ryffel 1978). Studies on the Japenese medaka (*Oryzias latipes*) revealed that Vtg may be induced in males by E_2 and endocrine disrupting chemicals to produce Vtg at a level previously determined to be indicative of a reproductive female (Gronen et al. 1999). More recently, Vtg has been investigated in Florida as a biomarker of potential endocrine disrupting effects in largemouth bass (*Micropterus salmoides*) (Bowman et al. 2002, Sepdlveda et al. 2002). Numerous studies have been done in other species to identify and characterize this class of proteins (Wang and Williams 1982, Wahli et al. 1989, Hartling et al. 1997); and while there has been some work done in reptilian species (described above) such as lizards and turtles (Baerga-Santini and Hernandez de Morales 1991, Brown et al. 1997, Morales et al. 2002, Romano et al. 2002), there is very little reported for the crocodilians (Guillette et al. 1997). There has not been a quantitative assay published to date that is sensitive and specific for crocodilians. The current study was designed to characterize and isolate Vtg

in the American alligator as a critical step toward the development of a quantitative assay for this species.

The previous chapter identified three female specific plasma proteins in the 250 to 500 kDa MW range which were present in higher concentrations in follicular genic animals. Therefore the objectives of this study were to identify and characterize those high MW plasma proteins. We tested the hypothesis that Vtg was represented by one of these three bands.

Materials and Methods

Study Sites

Two sites (Rockefeller State Wildlife Refuge, Louisiana and Lake Griffin, Florida), were chosen in an effort to reduce site specific bias from being introduced into the individual experiments. Each site was chosen for its significance to the ecological and environmental concerns surrounding alligators in their respective geographical locations (see previous chapter for a description of sites).

Animals

Adult female alligators (1.8–2.1 m) were captured, euthanized, and necropsied according to IACUC guidelines as described in Chapter 2. Plasma samples were obtained and preserved as described in Chapter 2.

Female Specific Protein Determination

The following methods were performed as described in Chapter 2 with the following modifications. Chemicals, pre-cast gels, protein standards, and equipment utilized in this and subsequent electrophoresis sections were purchased from Bio-Rad Laboratories, Hercules, CA, USA, or from Sigma-Aldrich Company Corp., St Louis. MO, USA except where indicated otherwise.

Protein extractions. Plasma samples (100 μL) were clarified by spinning at 10,000 rpm for 5 min in an Eppendorf microcentrifuge (to remove RBC's and WBC's). A non-ionic surfactant extraction buffer (without inhibitor cocktail to allow for enzyme digestions) was applied to plasma samples to liberate and denature proteins. This was prepared from a 10x extraction buffer composed of 500 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 5% Triton-X 100 (SDS and DOC (due to their ionic nature) were not included in this buffer to allow for proper isoelectric focusing (IEF) described in the following section). The 10x buffer was added to 90 μl of clarified plasma to give a 1x final concentration. Samples were kept on ice during extraction and alliquotted prior to snap freezing and subsequent storage at -80°C to minimize degradation.

Protein assay. Extracted protein samples were quantified according to Bradford (1976) using the Bio-Rad Protein Assay kit previously described in Chapter 2.

Sample preparation. For each animal, 20 μg total plasma protein, was prepared by the method previously described in Chapter 2.

Electrophoresis. The denatured protein samples were then loaded onto 4 to 15% gradient acrylamide denaturing gels for maximum high MW separation while allowing for the capture of the entire protein profile from 250 kDa down to 25 kDa. Electrophoresis was then performed according to the method described previously in Chapter 2. Subsequently, gels were stained with coomassie brilliant blue for MW determination and dried between cellophane for documentation. A second set of gels were run simultaneously and stained for glycosylated proteins using a modified Periodic Acid-Schiff (PAS) method.

Isoelectric focusing analysis. Semi-purified samples (prepared as follows) were utilized to determine the pI of the three female specific proteins to allow for a cleaner more focused analysis in lieu of conventional 2D-SDS Page analysis which can be very complex therefore limiting the ability to discern the protein of interest. The denatured protein samples (one animal chosen randomly from each site, extractions described previously) were loaded onto 7.5% acrylamide denaturing gels for maximum high MW separation (7 wells for each animal). Electrophoresis was then performed according to the method described in Chapter 2. The three female specific bands of interest were then excised and eluted in SDS-Page running buffer (7 slices for each band from each animal were combined in a separate elution tube) using the Bio-Rad model 422 Electro-eluter. This yielded 6 individual semi-purified samples; 1-250 kDa, 1-350 kDa, and 1-450 kDa protein for each of the Rockefeller and Lake Griffin animals. These samples were then concentrated using Centricon YM-100 spin columns (Millipore Corporation, Billerica, MA, USA) by centrifugation at 1000 rpm for 30–60 min. Subsequently, the samples were diluted to 2 mL in PBS and re-concentrated three times to exchange the buffer and remove the SDS. Samples (10 ng quantified by the protein assay described previously in Chapter 2) were combined with rehydration buffer (8 M urea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate [CHAPS]; 40 mM dithiothreitol [DTT]; 0.2% Bio-Lyte^{TM} 3/10 ampholyte: Bio-Rad Ready/Prep 2-D starter kit), loaded onto pre-cast 7 cm immobilized pH gradient (IPG) strips (pH 5 to 8; Bio-Rad), and allowed to hydrate overnight. The strips were transferred to a clean, dry PROTEAN IEF focusing tray which was subsequently placed in the Bio-Rad PROTEAN IEF focusing cell and allowed to focus (covered with mineral oil) using the pre-set

protocol (outlined below) determined by the length and number of strips. The strips were then stained with IEF stain from Bio-Rad (27% Isopropanol; 10% acetic acid; 0.04% coomassie blue R-250; 0.05% crocein scarlet) and subsequently destained with 50% methanol and 10% acetic acid until bands were discernable. The pI was noted and recorded.

Pre-set protocol utilized to focus the proteins in the IEF focusing cell:

- Start voltage = 0 V
- End voltage = 8,000 V
- Volt-hours = 8-10,000 V-hr
- Ramp = rapid
- Temperature = 20°C

Enzyme Digests

Plasma protein extractions described above were utilized to perform the following enzyme digests in an effort to confirm which (if not all) of the three female specific proteins contained phospho-lipid and sugar moieties. The digests were then analyzed by SDS-Page according to the method described above to determine any changes in MW of the three female specific proteins created by removing their covalently bonded groups.

Deglycosylation. The E-DEGLY kit from Sigma was utilized to completely remove all N-linked and simple O-linked carbohydrates from the alligator plasma proteins. The kit contains the following enzymes; PNGase F (*Chryseobacterium* [*Flavobacterium*] *meningosepticum*) which cleaves all asparagine-linked complex, hybrid, or high mannose oligosaccharides (Tarentino et al. 1994) unless α-core fucosylated (Szkudinski et al. 1995); α-2(3,6,8,9) Neuraminidase (recombinant from *Arthrobacter ureafaciens*) which cleaves all non-reducing terminal branched and unbranched sialic acids (Uchida et al. 1979); O-Glycosidase (recombinant from

Streptococcus pneumonia) which cleaves serine or threonine-linked unsubstituted Gal-β(1-3)-GalNAc-α- (Glasgow et al. 1977, Iwase et al. 1993); β(1-4)-Galactosidase (recombinant from *Streptococcus pneumonia*) which releases only β(1-4)-linked, non-reducing terminal galactose (Glasgow et al. 1977); and β-N-Acetylglucosaminidase (recombinant from *Streptococcus pneumonia*) which cleaves all non-reducing terminal β-linked N-acetylglucosamine residues (Glasgow et al. 1977). For purposes of this study the following protocol was followed under denaturing conditions. Total plasma protein (100 :g) was diluted to 30 :l with deionized water (ddH_20) in an Eppindorf tube, 10 :l of 5x reaction buffer, 2.5 :l of denaturation solution (both supplied in kit – proprietary ingredients), 2.5 :l of Triton X-100 solution, and 1 :l of each enzyme (all in one tube to achieve complete deglycosylation) was added, mixed gently and incubated overnight at 37°C. Subsequently $1/5^{th}$ of this reaction was analyzed by SDS-Page on a 4-15% gradient gel, stained with CBB, dried, and scanned for photodocumentation (all described in chapter 2).

Phospholipase digestion. Lipoprotein lipase (LPL) is found in vivo associated with heparin sulfate proteoglycans (HSPG) at the luminal surface of vascular endothelium (Olivecrona et al. 1993). It is essentially responsible for hydrolyzing triglycerides (TG) from very low density lipoprotein (VLDL) particles (Nilsson et al. 1980, Eckel 1989). Pruneta et al. (2001) isolated plasma VLDL and added exogenous bovine LPL to monitor the TG hydrolysis. The experiment described below was a modification of that study in that after the digestion, SDS-Page analysis was performed instead of monitoring the kinetics of the assay. Lipoprotein lipase (Sigma) was added (6 to 7 units/10 :l) to 100 :g total plasma protein (diluted to 10 :l with ddH_20) and 90 :l

1x reaction buffer (100 mM sodium phosphate, 150 mM sodium chloride, and 0.5% (v/v) Triton X-100; pH 7.2). Subsequently 1/5th of this reaction was analyzed by SDS-Page on a 4 - 15% gradient gel, stained with CBB, dried, and scanned for photo-documentation (all described in Chapter 2).

Anti-Phospho-serine, -tyrosine, -threonine Western Blot Analysis

Plasma protein extractions described above were utilized to perform the following Western blot analysis in an effort to confirm which (if not all) of the three female specific proteins were phosphorylated and to identify which of the three most likely phosphorylated amino acids they contained. Electrophoresis was performed as described previously. Subsequently the protein was transferred to a 0.45 : m nitrocellulose membrane (Bio-Rad) for Western blot analysis. The protein transfer was accomplished by the following protocol optimized for the Bio-Rad mini trans-blot apparatus (Bio-Rad); gels, nitrocellulose membranes, whatman filter paper, and sponges were equilibrated for 15 minutes in transfer buffer (20% methanol in 25 mM Tris base, 250 mM glycine, and 0.01% SDS). Transfer sandwiches were then assembled in the following sequence; sponge on black side of holder, filter paper, gel, nitrocellulose, filter paper, and sponge. The holder was then locked and placed into the transfer module with the black side facing the black side of transfer module. The module was placed in the electrophoresis tank equipped with an ice block and filled with transfer buffer. Transfer proceeded at 90 volts constant for 2.5 hrs surrounded by ice to reduce chances of protein degradation due to overheating. Once the transfer was complete, the following immunoblot protocol was followed; membrane was rinsed quickly with ddH_20 and subsequently blocked for 1 h in blocking buffer (5% BSA in PBS with 0.05% Tween-20 [PBS-T]); it was then incubated

with constant agitation in a UVP HB-2000 Hybrilinker hybridation oven (Tango Technologies, Ltd., Boulder, CO, USA) in monoclonal primary antibody diluted in wash buffer (PBS-T) overnight at room temperature (RT) at the following dilutions: mouse anti-phosphoserine (Sigma) at 1:1000; mouse anti-phosphothreonine (Sigma) at 1:50; and mouse anti-phosphotyrosine (Sigma) at 1:2000. The next day the membrane was washed 3 x 5 minutes in wash buffer and subsequently incubated 1 hour at RT in goat anti-mouse IGG (alkaline phosphate conjugated) secondary antibody (Sigma) diluted to 1:30,000 in wash buffer. A final wash of 3 x 10 minutes with was buffer and 1x with ddH_20 was performed prior to color development. Detection was performed by incubating the membrane in Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega,) until bands of interest appear at desired intensity. This is a nitro blue tetazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate which turns a purple color when acted upon by alkaline phosphatase.

Alkaline Labile Phospholipid (ALP) Analysis

ALP analysis is an assay which has been used in some fish and invertebrate studies as an indirect assay for plasma or hemolypmph (respectively) Vtg. It was utilized in the current study as a secondary method to confirm the increased concentration of phospholipid proteins in the plasma of the "highly vitellogenic" females when compared to the "weak to non-vitellogenic" females. The method is described as follows:

Plasma (350 ：L) was transfered to 16 x 125 mm glass tube containing 350 ：L tert-butyl methyl ether, mixed well, and incubated at room temp for 30 min (mixing every 10 min). This step extracted the lipophillic components (lipids and lipoproteins). The tubes were centrifuged in a J6-HC centrifuge for 2min at 3,000 rpm to ensure separation.

The organic phase was transferred to a new tube and mixed with 100 ：L of 1 M NaOH and incubated at RT for 60 to 90 min (mixing well every 15 min). This step freed the alkali-labile phosphates. The tubes were centrifuged in a J6-HC centrifuge for 2 min at 3,000 rpm to ensure separation. The aqueous phase (containing the free phosphates) was transferred to a new tube for subsequent analysis by a modified (scaled down 1:5 to be performed in a 96 well format) phosphomolybdenum method (Phosphate Assay Kit; Sigma) which assays for inorganic phosphorous. Each sample (15 ：L in triplicate) was combined with development reagent consisting of TCA, molybdenum reagent, and Fiske-SubbaRow reducer and was quantified by measurement against an aqueous inorganic phosphorus standard curve (also in triplicate) ranging from 0 to 1.8 ：g/mL. A micro-protein assay was performed (as described previously) on the original plasma sample and results were utilized to report the ALP values in ：g of organic phosphate / mg protein.

Amino-acid Sequencing

Once the female specific proteins had been analyzed to determine that they had characteristics of Vtg from other species (correct MW, pI, phosphorylation, glycosylation, and phospholipid moieties), amino acid sequencing needed to be conducted to definitively identify them as Vtg. While there are publications that have utilized direct n-terminal sequencing by Edman degredation to identify Vtg, they have been marginally successful in obtaining full sequences. Therefore, for the purposes of this study internal polypeptide sequencing subsequent to enzyme digestion (of the whole protein) was chosen. The enzyme digestions reduced the protein to smaller polypeptide fragments thereby avoiding the covalently bonded groups that normally interfere with n-terminal sequencing. Plasma (25 ：g per well) from one animal from each site chosen

randomly was electrophoresed as described previously in seven wells of a 7.5% polyacrylamide gel (a separate gel for each animal). The gels were stained with coomassie brilliant blue (as described previously), the three female specific bands in the 250 to 450 kDa range in each lane were excised along with a blank gel slice, same size bands combined as one sample (keeping bands from the two animals separate; yielding 6 samples – three different size bands for each animal and 2 blank slices), and subsequently sent to the Interdisciplinary Center for Biotechnology Research (ICBR) Protein Sequencing Core for amino acid sequencing. The protocol performed by the Core is summarized below.

In-gel digestion of proteins in polyacrylamide gel pieces. Each gel slice was cut into ~1 x 2 mm sections, placed into a 1.5 mL micro centrifuge tube with 150 ΅L of 50% acetonitrile in 0.2 M ammonium bicarbonate (pH 8.9), and incubated for 30 min at 37°C. This wash buffer was removed and the wash step repeated. The gel slices were then dried completely in a speed vacuum. Endoproteinase Asp-N enzyme (Roche Laboratyories,) solution was added to each sample using a 1:20 w/w ratio with 50 ΅L 0.2 M ammonium bicarbonate (pH 8.9) and incubated for 24 h at 37°C. The total volume of the sample (gel & buffer) was estimated and 45 mM DTT was added to give a final DTT concentration of 1 mM and subsequently incubated for 20 min at 50°C. The samples were cooled to RT and an equal volume (to DTT volume) of 100 mM iodoacetic acid (IAA) was added and subsequently incubated for 20 min at RT in the dark. The supernatant was transferred to a new tube. The gel pieces were crushed and incubated for 30 min at RT with 100 ΅L of 0.1% TFA / 60% acetonitrile. This extraction buffer was transferred to a filter tube and extraction was repeated combining the 2nd extraction with

the first in the filter tube. Gel pieces were then discarded and the filter tube was centrifuged for 10 -15 min at maximum speed. The speed vacuum was used to decrease the final volume to < 150 ΅L. This filtrate was then applied to an equilibrated Vydac C18 (= 2.1 x 150 mm, 300 Å pore size, and a 5 ΅m particle size) reversed-phase HPLC column at 0.15 mL/min in 95% buffer A / 15% buffer B. Elution from the column was performed with buffer A / buffer B mixture according to the following gradient: 0-110 min (5% to 75% buffer B), and 110-120 min (75% to 85% buffer B). Elute was collected in 1.5 ml tubes which were capped immediately and stored at 4°C until sequencing was performed.

Protein sequencing. Repeated cycles of Edman degredation chemistry was utilized for n-terminal sequencing (on an Applied Biosystems model 494 HT Sequencer) of the polypeptides resulting from the enzyme digestions. Briefly, this entails the reaction of phenylisithiocyanate (PITC) with the n-terminal amino group of the polypeptide under mildly alkaline conditions to form an n-terminal PITC adduct. This was subsequently cleaved by anhydrous trifluoroacetic acid (TFA) yielding a thiazolinone derivative leaving the rest of the polypeptide intact. The thiazoline-amino acid was extracted into an organic solvent and subsequently treated with an aqueous acid to form a more stable phenylthiohydantoin (PTH) which was later identified by gas chromatography. Sixteen cycles were acquired with a sampling rate of 4.0 hz and detector scale of 1.0 AUFS.

Results

Three female specific bands were again detected by SDS-Page at ~250, 350, and 450 kDa (Figure 3-1). Upon isolation of the three bands (one Vtg plasma sample was

chosen randomly from each site for this procedure), the pI was found to be ~7.2 for all three bands in both samples (data not shown).

Glycosylation of the three female specific protein bands was determined by two methods: staining of an SDS-Page gel by a modified Periodic Acid-Schiff (PAS) method (Figure 3-2) and enzyme deglycosylation and subsequent analysis by SDS-Page to detect a shift in the electro-mobility (Figure 3-3). The PAS staining method was successful in identifying all three bands as being glycosylated in all 10 Vtg females from both sites (Figure 3-2 is a representative gel showing three animals from each site). This was further confirmed by enzyme deglycosylation of one Vtg plasma sample chosen randomly from each site and subsequent analysis SDS-Page (Figure 3-3). However the enzyme deglycosylation only showed an electrophoretic shift in the 250 kDa protein.

An indirect method for the quantification of phospholipids was used initially to establish that there was a higher concentration of these lipophilic molecules in the plasma of Vtg females when compared to non-Vtg females. There was a significantly higher amount of phospholipid protein in the plasma samples of the Vtg females when compared to a male plasma pool, however there was no significant difference noted when sites were compared (Figure 3-4). These results were not strengthened by digesting one Vtg plasma sample from each site with phospholipase and subsequent analysis by SDS-Page (Figure 3-5). There was no significant electrophoretic shift noted in the 250 to 450 kDa proteins. This enzyme only digests phospholipid moieties, it will not digest a phosphorylated amino acid.

Western blot analysis was used to identify which of the three bands (if not all) were phosphorylated and which of the three most commonly phosphphorylated amino

acids did these proteins contain. The anti-phosphoserine blots revealed that the 250, 350 and the 450 kDa protein bands contained a high concentration of phosphorylated serines (Figure 3-6 Panel A). The anti-phosphotyrosine blots revealed that only the 250 kDa band contained phosphorylated tyrosine amino acids while the anti-phosphothreonine blots did not reveal any degree of phosphorylation in any of the three bands (Figure 3-6 Panel B and Panel C respectively). This was a third method confirming that the three proteins in the 250 to 450 kDa range are phosphorylated.

Finally, while the sequencing project is still ongoing, preliminary results for the 250 kDa protein have revealed a 75 to 88 % homology when compared to published chicken, frog and fish Vtg sequences (see sequence alignments in Table 3-1). This is a small fragment resulting from the reconstruction of two out of five enzyme digest fractions of the 250 kDa protein from the Lake Griffin animal. There are 17 residues in this sequence with the highest confidence on residues 4-13. The sequence of this fragment is as follows; E (Glutamine) V (Valine) G (Glycine) I (Isoleucine) R (Argenine) A (Alanine) E (Glutamine) G (Glycine) L (Leucine) G (Glycine) X (unidentified). A sequence homology search was performed utilizing the Basic Local Alignment Search Tool (BLAST) which is provided through the National Center for Biotechnology Information (NCBI) server. Of the 100 sequences that were returned in the query, 11 of them were Vtg sequences from various species of chickens, frogs, and fish.

Discussion

This study was designed to meet the following objectives: (1) isolate and characterize the three female specific bands that had been identified in the previous screening study and (2) use what little is known in the literature about alligator Vtg to prove that one or all of those three bands are or are not Vtg.

Characteristically Vtg has been proven to be a highly glycosylated phospholipid protein in other species. The published MW weight ranges from 150 to 600 kDa depending on the species being investigated. There have also been some lower MW products which have been referred to as "Vtg-like". Vtg is a complex protein that originates in the liver of oviparous vertebrates. Much of the confusion in regards to the actual size of the protein is probably due to the fact that it undergoes extensive post-translational processing upon transfer out of the liver as well as after it begins it's journey through the bloodstream and then again prior to being taken up by the oocytes. The form that shows up in an assay is dependant on many factors including the reproductive status of the animal being tested. Vtg production and modification can be affected by hormonal influences, diet, and other environmental factors including seasonal changes. Taking all of this into account, the present study was designed to target the most likely candidates and analyze them for characteristics specific to Vtg or Vtg-like proteins. Secondly, utilize amino acid sequencing and submit this data to the available sequence banks as a method to identify Vtg.

The initial characterization of the three female specific proteins can be summarized as follows: (1) The 250 kDa protein is highly glycosylated and contains several phosphorylated serine amino acids as well as some phosphorylated tyrosines. The phospholipase digestion showed no electrophoretic mobility shift indicating that there was very little (or no) phospholipid present. The pI is ~7.2 which is in the predicted range for Vtg. (2) The 350 kDa protein is highly glycosylated and contains several phosphorylated serine amino acids as well as some phosphorylated tyrosines. The phospholipase digestion did not show an electrophoretic mobility shift indicating that

there were very little (or no) phospholipid moieties present. The pI is ~7.2 which is in the predicted range for Vtg. (3) The 450 kDa protein is glycosylated but to a lesser degree than the other two proteins. It contains several phosphorylated serine amino acids. The phospholipase digestion showed minimal electrophoretic mobility shift indicating that there was very little (or no) phospholipid present. The pI is ~7.2 which is in the predicted range for Vtg. Another explanation for the failed phospholipase digestion could be that the reaction is just not sensitive enough to discern the phospholipid moieties due to the complex nature of the Vtg protein. It is possible that these moieties are protected in the folding of the protein which would not allow for proper digestion by the phospholipase enzyme.

The preliminary amino acid sequencing revealed that the nine residues obtained from the 250 kDa protein have 75 to 88 % homology with published sequences from chickens, frogs, and fish. This data coupled with the characterization described previously infers that the 250 kDa female specific protein identified in this study is probably Vtg. Conclusion of the sequencing project should provide sufficient evidence to confirm this and to identify the other two female specific proteins as well.

Table 3-1: Amino acid sequence alignment resulting from BLAST search. Query represents the nine residues obtained from the 250 kDa female specific protein isolated from American alligator (Alligator mississippiensis) plasma.

Species	Start	Sequence	End	% homology to query sequence	Reference
QUERY	1	E V G I R A E G L	9		
Chicken (Gallus gallus)	679	E V G I R V E G L	687	88 %	Walker et al., 1983
Chicken (Gallus gallus)	679	E V G I A A E G L	687	88 %	Yamamura et al., 1995
African clawed frog (Xenopus laevis)	681	E I G I R G E G -	688	75 %	Walker et al., 1984
African clawed frog (Xenopus laevis)	681	E V A L R A E G L	689	77 %	Yoshitome, 2003
Japanese whiting (Sillago japonica)	679	E V G V R A E G -	686	87 %	Yoon, 2002
Blue tilapia (Oreochromis aureus)	679	E V G V R T E G -	686	75 %	Lim et al., 1997
Rainbow Trout (Oncorhynchus mykiss)	679	E V G V R T E G -	686	75 %	Le Guellec et al., 1988
Rainbow trout (Oncorhynchus mykiss)	679	E V G V R T E G -	686	75 %	Mouchel et al., 1996
Zebrafish (Danio rerio)	678	- - G I R A E G L	684	85 %	Wang et al., 2000
Japenese medaka (Oryzias latipes)	680	E V G V R T E G -	687	75 %	Murakami & Nakai, 2001
CONCENSUS		E V G * R * E G L			

(-) Denotes missing amino acid.
(*) Denotes lack of concensus.

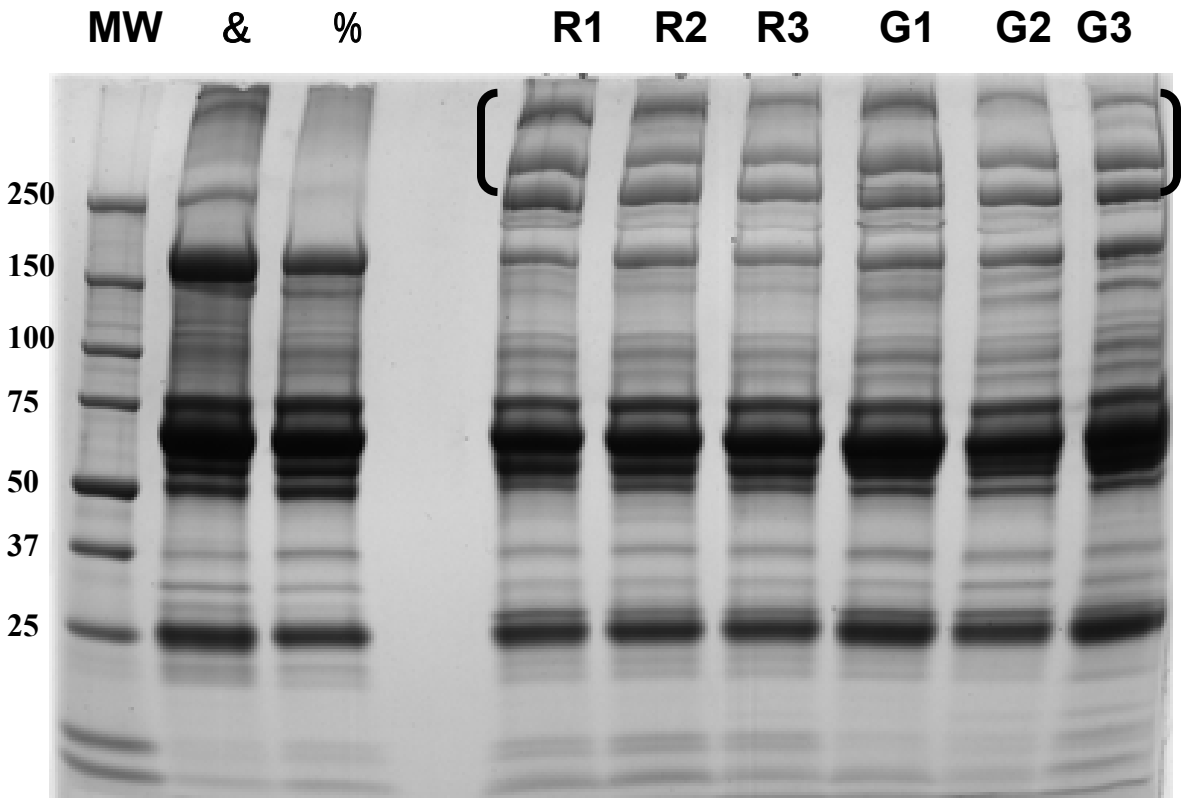

Figure 3-1. SDS-PAGE analysis of plasma samples from three Vtg adult female alligators. Three were from Lake Griffin [G] and three from Rockefeller [R]). Gel was stained with coomassie brilliant blue. Brackets surround expected molecular weight (MW) range for Vtg proteins. ♀ lane contains plasma from an E2 induced control female. ♂ lane contains plasma from a control male pool. Analysis normalized to total protein loaded. As was noted in Chapter 2 (Figure 2-3 panel A), there are three prominent bands in the 250–450 kDa MW range for both sites.

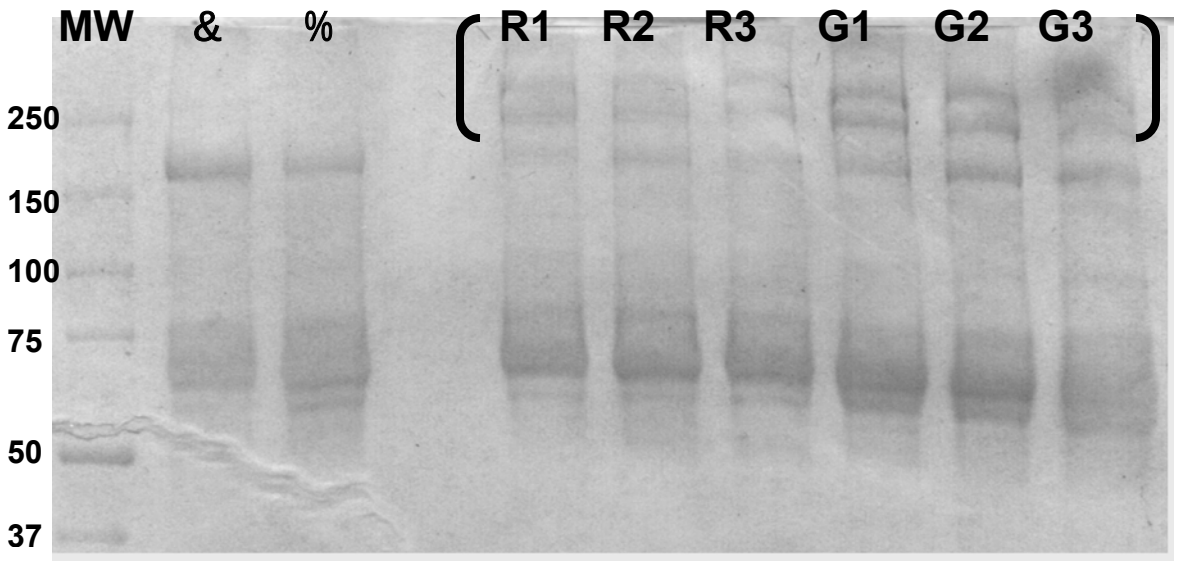

Figure 3-2. Glycosylation analysis of plasma samples. SDS-PAGE analysis of plasma samples from three Vtg adult female alligators (three from Lake Griffin [G] and Rockefeller [R]) stained for glycosylation using a modified Periodic Acid-Schiff (PAS) method. Brackets surround expected molecular weight (MW) range for Vtg proteins. ♀ lane contains plasma from an E2 induced control female. ♂ lane contains plasma from a control male pool. Analysis normalized to total protein loaded. PAS stain only stains proteins that are glycosylated. It is clear that the three bands in the 250–450 kDa range are highly glycosylated in animals from both sites.

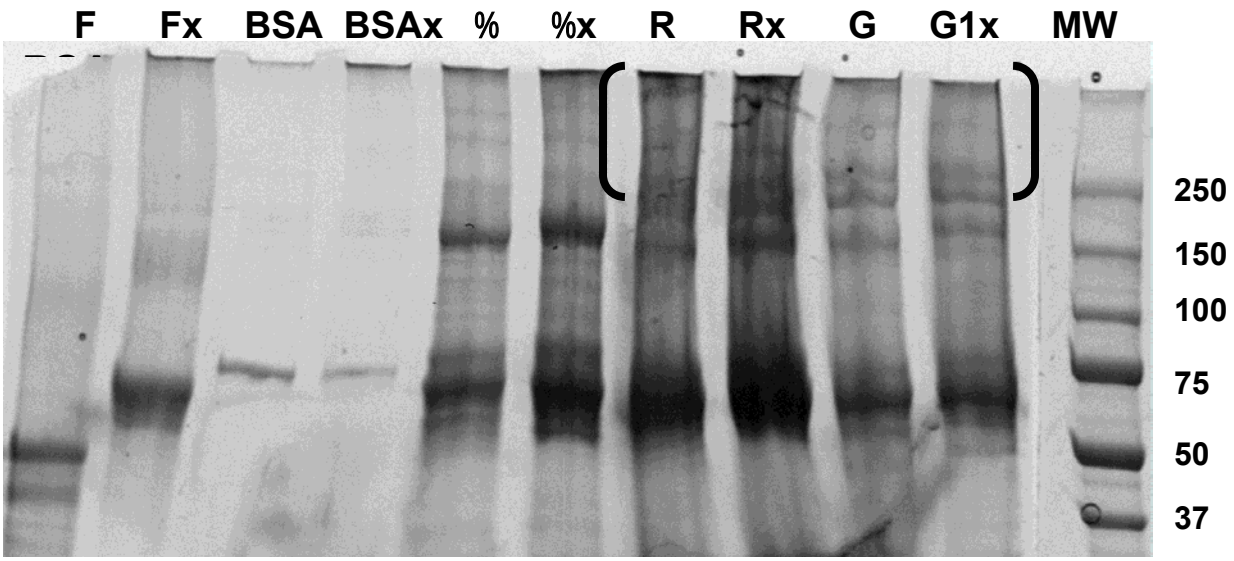

Figure 3-3. Deglycosylation analysis of alligator plasma. SDS-PAGE analysis of plasma samples from two Vtg adult female alligators (one from each site) stained with coomassie brilliant blue. One of each sample was deglycosylated by enzyme digestion prior to being electrophoresed. Samples without enzyme are indicated by X. F lanes contain Feutin; protein positive for glycosylation. BSA was included as a negative control for glycosylation. Brackets surrounds expected molecular weight (MW) range for Vtg proteins. Successful deglycosylation is identified by the electrophoretic mobility of the protein shifting down indicating a lower MW. There was only slight deglycosylation noted in the 250 kDa protein for both the Lake Griffin and the Rockefeller animals.

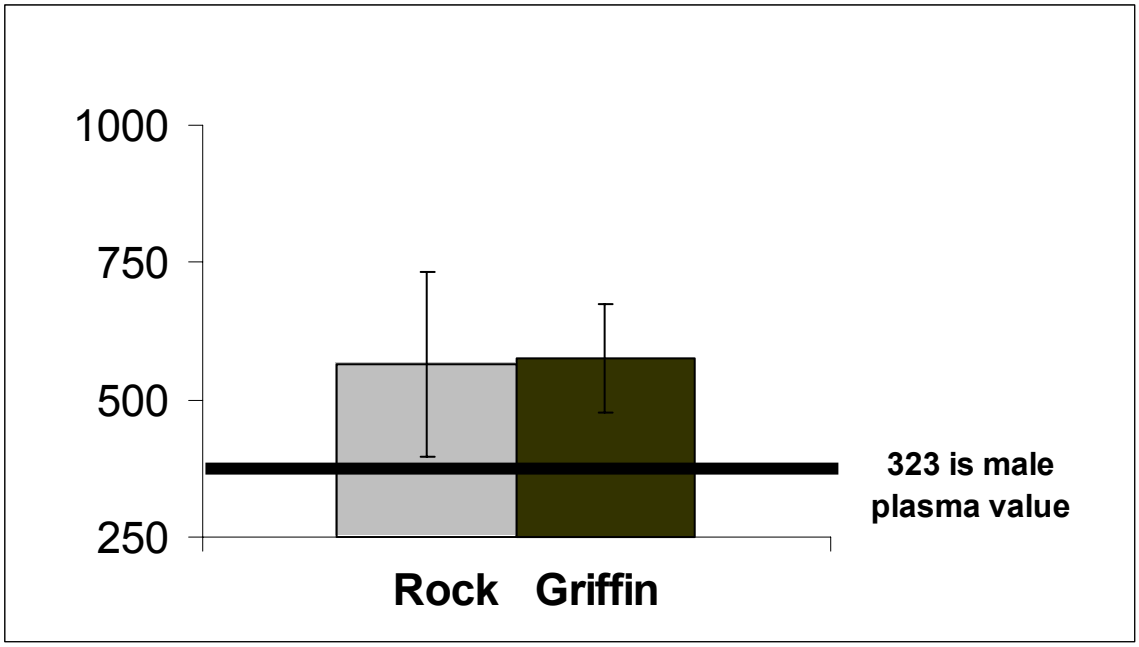

Figure 3-4. ALP analysis of plasma proteins. ALP analysis confirming the increased concentration of phospholipid proteins in the plasma of the vitellogenic females when compared to the male pool (indicated by horizontal line).

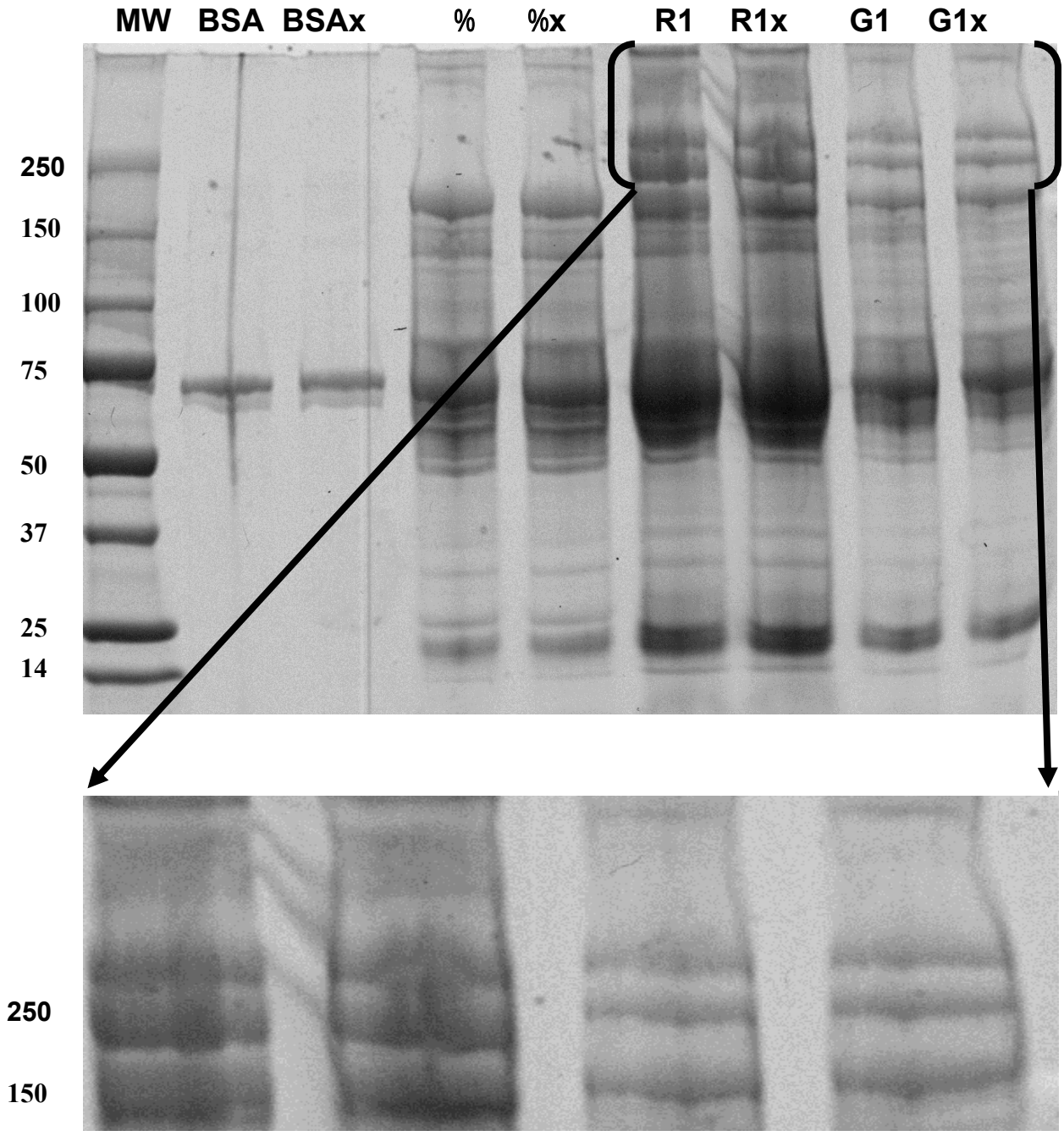

Figure 3-5. Phospholipase digestion of alligator plasma. SDS-PAGE analysis of plasma samples from two Vtg adult female alligators. One from Lake Griffin [G] and one from Rockefeller[R]) stained with coomassie brilliant blue. One of each sample was treated with phospholipase prior to being electrophoresed. Samples without enzyme are indicated by X. ♂ lane contains plasma from a control male pool. BSA was included as a negative control. Brackets surround expected molecular weight (MW) range for Vtg proteins. Successful dephosphorylation would be identified by the electrophoretic mobility of the protein shifting down indicating a lower MW. However, there was no dephosphorylation noted for either the Lake Griffin or the Rockefeller animal.

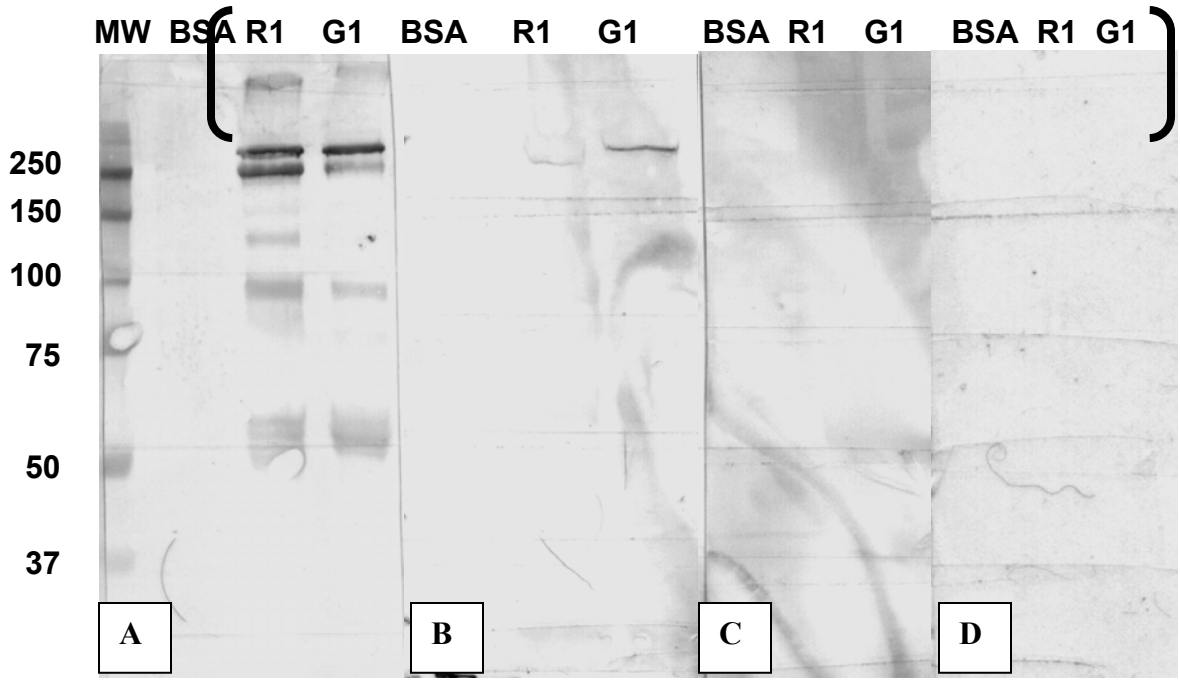

Figure 3-6. Western blot analysis of phosphorylated proteins in alligator plasma. Western blot analysis analysis of plasma samples from two Vtg adult female alligators (one from Lake Griffin [G] and one from Rockefeller [R]). BSA was included as a negative control. Brackets surround expected molecular weight (MW) range for Vtg proteins. (A) Blot was incubated in "-phospho-serine primary antibody. (B) Blot was incubated in "-phospho-tyrosine primary antibody. (C) Blot was incubated in α-phospho-threonine primary antibody. (D) Blot was incubated without primary antibody. α-phospho-serine primary antibody reacted the strongest with all three proteins in the 250–450 kDa MW range while the α-phospho-tyrosine primary antibody only reacted with the 250 kDa protein and the α-phospho-threonine primary antibody did not react with any of the three proteins.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

This is the first study that has attempted to identify Vtg in adult female American alligators through the utilization of sequence analysis coupled with limited biochemical characterization. It is therefore essential that additional sequencing be completed. These data will then be useful in developing a sensitive and specific quantitative assay for alligator Vtg. Such an assay could then be utilized throughout the entire reproductive cycle for several sites to establish a seasonal monitoring protocol. This could be expanded to a time course study designed to follow the production of Vtg and its subsequent modifications and eventual deposition in the growing follicles.

There are several possible explanations which would support that the three female specific proteins analyzed in this study are or are not Vtg or Vtg metabolites. Based on the information gathered the most likely explanation is that they are Vtg metabolites (sequencing data confirms this to be true for the 250 kDa protein) that are at different stages of post-translational modification. It is likely that their inevitable fate will be deposition in the growing oocyte to be used by the embryo as a nutritional source. However, another possibility is that one or more of them are polypeptides which have been cleaved into one or more products upon analysis by denaturing SDS-Page.

The phospholipase digestion analysis provided data that is in direct conflict with what has been previously described in the literature. As already discussed in Chapter 3, there are plausible explanations as to why this assay may have yielded negative results; complexity of the sample or lack of sensitivity of the assay. However there is another

possibility; perhaps Vtg is constructed differently than we have all assumed. It is published many times over that Vtg is a phospholipoprotein. This implies that there are phosphorylated lipid moieties attached to the protein backbone. If this were true, and the negative results were not due to interference, then the phospholipase would have cleaved these moieties from the backbone leaving a smaller phospholipid product and the remainder of the protein construct as a second product. Perhaps Vtg is more complex than was previously assumed. The western blot analysis identifying phosphorylated amino acids confirms that there are phosphate groups attached directly to the protein backbone. Further investigation of the structure and subsequent folding of the protein is warranted. This type of research would help to elucidate potential structure activity relationships between Vtg and other proteins as well as EDCs such as OCPs.

During the initial analysis of plasma protein profiles there were some subtle differences in lower MW proteins which did not fall within the target MW range for this study thereby suggesting qualitative differences in the post-translational processing of other female specific proteins in animals from Lake Griffin compared to Rockefeller. These results warrant further investigations of these plasma protein profiles from female animals from these sites as well as others to determine whether the differences may be contaminant related or whether they are just an artifact of regional genetic variations. Once this question is answered, it would be beneficial to examine the livers from the same animals looking specifically at Vtg precursors and other reproductive proteins (including metabolic enzymes) to begin to elucidate a potential mechanism(s) for metabolic alterations which may affect reproductive success in animals from OPC contaminated sites. Therefore future directions should include the same types of analysis

on the liver and ovidutcal tissues to further enhance knowledge of the alligator reproductive system at the molecular level. To date this is an underdeveloped area which could help to elucidate the mechanism(s) behind altered reproductive success in these animals. Eventually there needs to be a binding assay developed in alligators which would be able to explore the interactions of Vtg and various tissues and subsequent involvement with other proteins such as potential carrier or chaperone proteins. This could be expanded to explore possible interaction of these proteins with OCPs and other EDCs.

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BIOGRAPHICAL SKETCH

Eileen K. Monck was born June 6, 1962 in Bronx, New York. After graduating from New Britain High School in 1980, she explored different career possibilities before enrolling at Central Connecticut State University. She graduated in 1989, with a Bachelor of Science degree in biology and secondary education (with minors in chemistry and general science).

In 1989 she began working at the University of Florida as a research technician, where she developed a desire to further her education. In 1999 she began her graduate work in environmental toxicology, which she expanded to reproductive endocrinology of the American alligator. She will graduate in December 2003 with a Master of Science degree. Eileen will continue her work with alligators, under the continued supervision of Dr. Timothy Gross at the United States Geological Survey in Gainesville, Florida.

Through her college career, Eileen has been a wife, and a mother to three children; and has enjoyed exposing her children to all of the fascinating educational opportunities her career has to offer. She has often been involved in bringing science into classrooms at many age levels, and looks forward to many more opportunities to do so.