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Reproductive Biology of the Bonnethead (Sphyrna tiburo) from the Southeastern U.S. Atlantic Coast

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REPRODUCTIVE BIOLOGY OF THE BONNETHEAD (*SPHYRNA TIBURO*) FROM THE SOUTHEASTERN U.S. ATLANTIC COAST

by

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CERTIFICATE OF APPROVAL

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ABSTRACT

Understanding the life history of marine wildlife is essential for the management of both commercial and recreational fisheries. Bonnetheads (*Sphyrna tiburo*) are a component of the small coastal shark (SCS) fishery complex, and are caught regularly in both types of fisheries. Despite being well studied in the Gulf of Mexico, little is known about bonnetheads from the U.S. Atlantic coast. The goal of the first component of this study was to provide new, key information on their life history to improve management of U.S. Atlantic populations, particularly by identifying reproductive seasonality, periodicity and fecundity. This was accomplished by examining sexually mature male and female bonnetheads, collected monthly (2012-2014) from South Carolina, Georgia, and Florida waters, and monitoring changes in reproductive tract morphology. Changes reflected a seasonal reproductive cycle with an annual breeding periodicity. Histology was used to confirm events and identify other important periods in the reproductive cycle, such as sperm storage in females. Overall, Atlantic coast bonnetheads were found to exhibit reproductive patterns similar to those reported in the Gulf with slight temporal shifts in the time of mating and ovulation and slightly lower fecundity ranging between 1 and 12 with an average (±SE) of 7±3.8. Additionally, the second component of this study aimed to understand gonadal sex hormone regulation in *S. tiburo* reproduction with a particular focus on female sperm storage. Circulating plasma sex hormones increased in association with specific reproductive events. Plasma 17β-estradiol and testosterone concentrations increased during sperm storage, whereas progesterone levels increased near the end of this stage. Immunocytochemical analysis of androgen, estrogen, and progesterone receptors in the oviducal

gland, the organ that stores sperm in female bonnetheads, demonstrated that epithelial cells of sperm storage tubules and spermatozoa itself are direct targets for these hormones, playing a role in regulating this poorly understood process.

COMPONENT I:

Reproductive biology of the bonnethead (*Sphyrna tiburo*) from the southeastern U.S. Atlantic Coast

INTRODUCTION

The bonnethead is the smallest living member of the hammerhead family, Sphyrnidae, generally ranging from about 0.91-1.5 m (Chapman *et al.*, 2004; Lombardi-Carlson, 2007). Bonnetheads commonly reside in the tropical and subtropical coasts of the Western Atlantic and Eastern Pacific oceans and undergo short seasonal migrations typically in groups of about 5 to 15 individuals (Driggers *et al.*, 2014). Despite their movements towards warmer waters during autumn and winter and to cooler waters during spring and summer, bonnetheads exhibit both intra and inter-annual philopatry, resulting in long-term residency of coastal estuarine systems (Heupel *et al*., 2006; Driggers *et al*., 2014). Their diet primarily consists of crustaceans, especially portunid crabs, although cephalopods have also been shown to be important prey in certain regions (Cortes *et al.*, 1996; Chapman *et al.*, 2004; Heupel *et al.*, 2006, Bethea *et al.*, 2007).

The bonnethead is one of the most extensively studied shark species on the U.S. east coast, especially in the eastern Gulf of Mexico. Studies on reproduction in the Gulf of Mexico population show that male spermatogenesis occurs from late spring to early autumn annually and copulation occurs from November through December (Parsons, 1993; Marine *et al.*, 1995;

Manire and Rasmussen, 1997; Gelsleichter *et al.*, 2003; Nichols *et al.*, 2003; Lombardi- Carlson, 2007; Castro, 2009). Females are believed to store sperm for several months afterwards (Manire *et al.,* 1995). Ovulation and fertilization take place from mid-March to early April and parturition occurs in late summer-early autumn after a gestation period of about 4-5 months. *S. tiburo* has the shortest known gestation period in sharks and are placental viviparous (Parsons, 1993; Marine *et al.*, 1995; Manire and Rasmussen, 1997; Gelsleichter *et al.*, 2003; Nichols *et al.*, 2003; Lombardi-Carlson *et al.*, 2007; Castro, 2009).

Although Gulf of Mexico bonnetheads have been well studied, much less is known about U.S. Atlantic populations of this species. This raises concern as the U.S. bonnethead fishery stock has long been defined as occurring from North Carolina through the Straits of Florida and to the Gulf of Mexico as a single population yet it is managed solely based on life history data from the Gulf. *S. tiburo* are a component of the small coastal shark (SCS) fisheries complex and this species has high capture rates in gillnets and trawls, placing members of this group at risk of overexploitation. Therefore, it is necessary to understand their reproductive periodicity and seasonality as well as their fecundity to understand how quickly the population can grow. To address lack of knowledge regarding these populations, Frazier *et al* (2014) recently conducted a study of age, growth, and maturity of Atlantic bonnethead populations. They found that Atlantic bonnetheads mature at twice the age of their Gulf of Mexico counterparts and may live twice as long. Considerable differences in life history may result in differences in population growth between these two locales. Given age, growth and maturity differences between bonnetheads in the Atlantic and the Gulf of Mexico, it is critical to also examine whether differences occur in

reproduction between the bonnethead populations in these areas to ensure that the species is managed using the best available data.

The purpose of this study was to characterize reproductive patterns of bonnetheads from the southeastern U.S. Atlantic and compare these data to Gulf of Mexico populations. In particular, the main goals of this study were to identify the timing of reproductive events, as well as determine reproductive periodicity and fecundity. To accomplish this, we conducted morphological and histological assessments of reproductive activity in mature male and female bonnetheads collected through a large portion of their southeastern U.S. Atlantic range. The goal is to provide this life history data to fisheries managers in an effort to improve management of the species.

METHODOLOGY

Collections

Mature male and female bonnetheads were collected from April 2012 to October 2014 from estuarine, nearshore, and offshore locations in South Carolina (SC), Georgia (GA), and east Florida (FL) waters (Fig.1.1). This area makes up a large portion of the species' U.S. southeast Atlantic range. Fish were sampled using bottom longline fishing and set gill nets in fishery-independent surveys and commercial fishing trips.

Biological sampling

Following capture, sharks were sexed, weighed (kg), and measured (precaudal length (PCL), fork length (FL), total length (TL) and stretched total length (STL) in mm). Afterwards, blood (~3 mL) was sampled via caudal venipuncture using sterile syringes and needles, transferred to vacuum tubes containing anticoagulant, and kept on ice until returned to the laboratory and centrifuged to separate plasma, which was stored at -80°C until used in a companion study on reproductive endocrinology. Male maturity was determined based on the degree of clasper calcification and rotation, whereas female maturity was gauged using published data from Frazier *et al*. (2014) and later confirmed via dissection. External characteristics indicative of reproductive activity, such as the presence of mating wounds in females, were also examined.

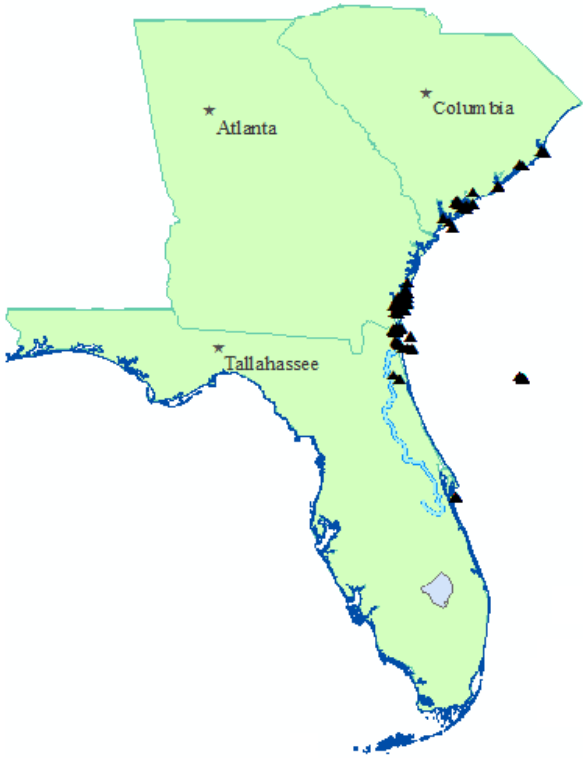

Figure 1.1. Map of South Carolina, Georgia and Florida demonstrating sites where animals were collected in the present study.

Sharks were dissected to determine reproductive stage. Length, width, and weight of each testis and width of the head epididymis were measured in males. The seminal vesicles were also examined for the presence of semen. Female ovarian follicles were categorized by color and texture with regards to egg development or reabsorption (i.e., pre-vitellogenic, vitellogenic, atretic) and enumerated, and maximum follicle diameter (MFD) was measured. Width of the oviducal glands and the uteri were also measured. If present, uterine ova or embryos were counted and embryo stretched total length (mm) and mode of nourishment (yolk-dependent or placenta-dependent) were recorded.

After obtaining morphological measurements of gonads and other reproductive structures, sections (~2-3 mm) of components of the reproductive tract were fixed in 10% formalin in elasmobranch-modified saline for 48 hours, rinsed in water and transferred to 70% ethanol for long-term storage until used in histology.

Histology

Fixed tissue samples were embedded in paraffin for routine histology as described in Gelsleichter *et al*. (2003). Following this, they were sectioned (5 μm) using a rotary microtome, mounted on poly-L-lysine coated slides, and stained with Harris hematoxylin and eosin to examine cellular architecture. In particular, histological sections from the male testis and female oviducal gland were examined for the presence of mature spermatozoa to confirm when individuals were undergoing spermatogenesis or sperm storage, respectively.

Data analysis

Morphological data on testis width and head epididymis width in males, and maximum follicle diameter, oviducal gland width, and embryo stretched total length in females were grouped by time of collection to characterize temporal patterns. To determine if significant differences were present, mean values for these measures were compared using one-way ANOVA followed by Tukey Post-Hoc multiple comparisons test if data passed tests for normality and equal variance. Normality assumptions were tested by reviewing descriptive statistics, histograms and skewness and kurotosis for the given data. Data that did not fulfill these criteria were analyzed using Kruskal-Wallis analysis of variance followed by Dunn-Bonferroni posthoc test based on data homogeneity at a significance level of 0.05. Data such as histological observations were analyzed using microscopy, following procedures described in previous studies (Parsons and Grier, 1992; Maruska *et al*., 1996; Gelsleichter *et al*., 2003). Patterns of Atlantic bonnethead reproduction were qualitatively compared with published data on *S. tiburo* from the Gulf coast to determine if patterns were similar.

RESULTS

Males

Morphological and histological analyses of reproductive condition were conducted on a total of 67 and 32 males, respectively. Testis width and head epididymis width varied seasonally in male *S. tiburo,* in which testes were significantly larger (Kruskal-Wallis and Dunn-Bonferroni, $F=46.3$, $df=8$, $p=0.000$) in late summer (August to September), then declined steadily from October to June (Fig. 1.2). Histological assessments of testis architecture demonstrated that sperm production occurred between July and September, as demonstrated by progressive increases in the abundance of late-stage germ cells such as secondary spermatocytes, elongating spermatids and mature spermatozoa (Fig. 1.3). Spermiation appeared to occur between August and September, based on increased presence of evacuated spermatocysts in the testis (Fig. 1.3). Semen was also present in the reproductive tract of all males captured during August and September, confirming that it was the period of peak sperm production and expression (Fig. 1.4). Testes were largely composed of early-stage germ cells in animals collected between February and June, suggesting that males are reproductively inactive during this time frame (Fig. 1.3).

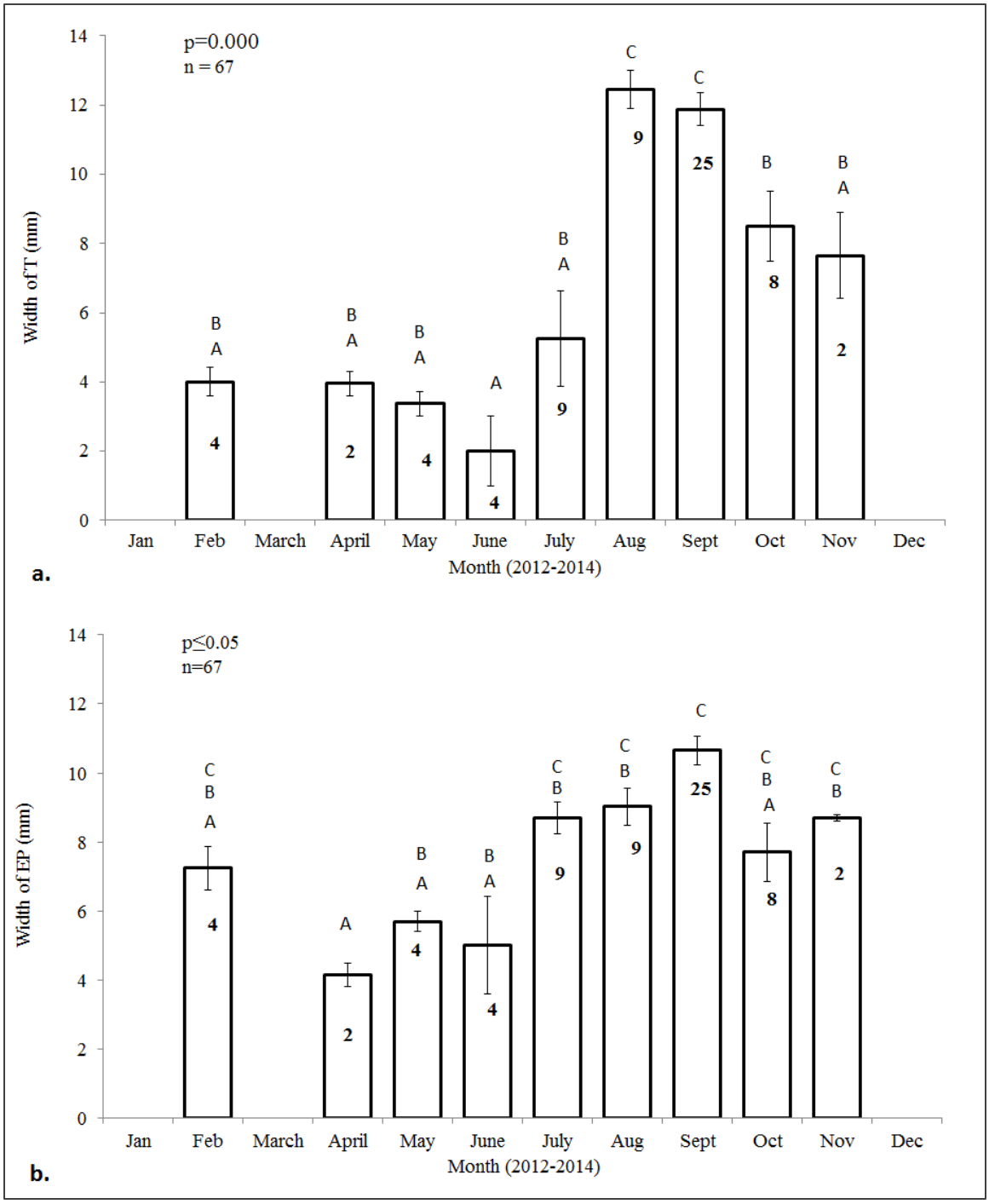

Figure 1.2. Width of a) testis and b) head epididymis in mature male bonnetheads from the U.S. Atlantic coast (n=67). Values are means (±SE). Sample size per month is shown in bars. Significance was determined using a) Kruskal-Wallis test of variance with Dunn-Bonferroni stepwise comparison (p=0.000) and b) one-way ANOVA with Tukey's post-test (p=0.085) where letters above the bars represent Homogeneous subgroups.

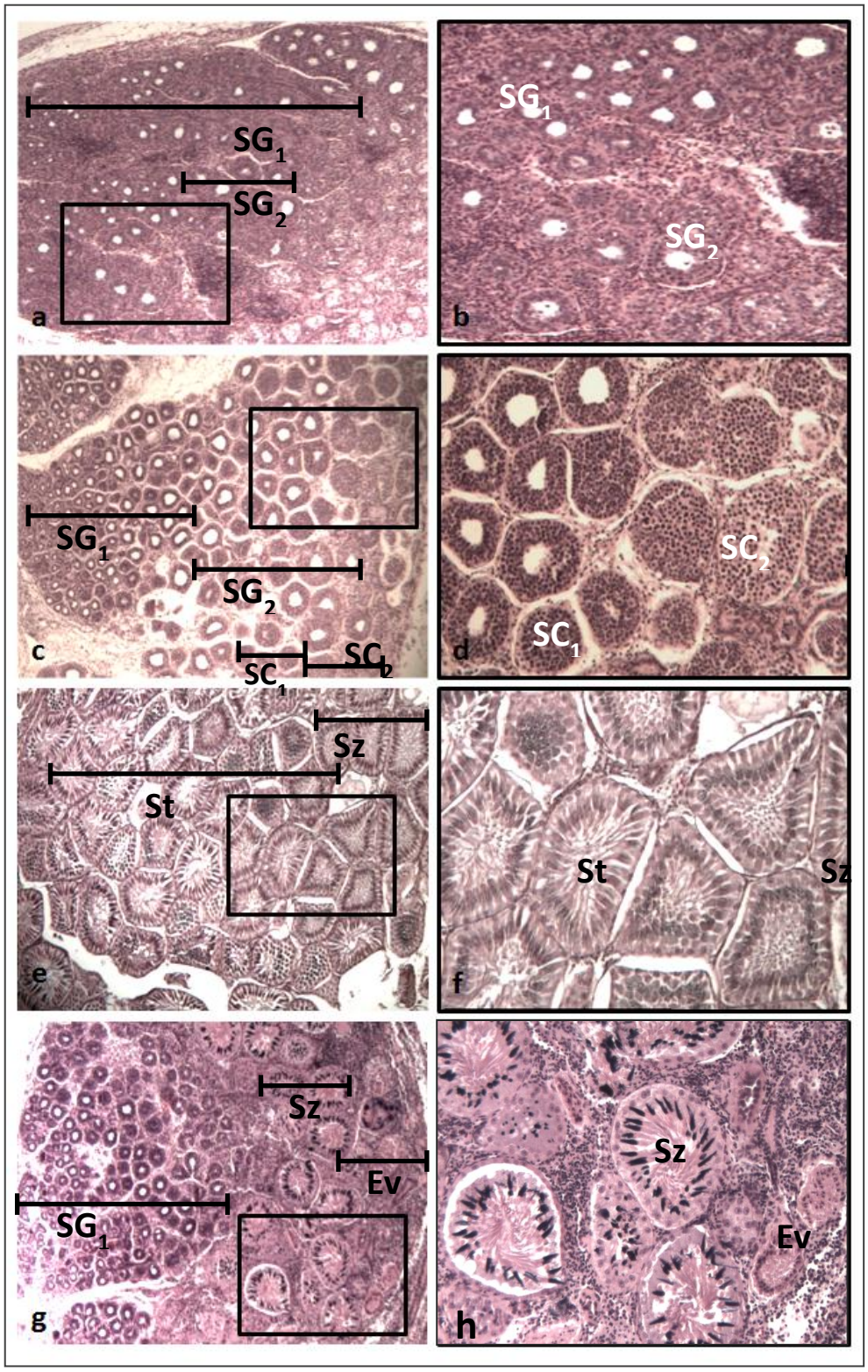

Figure 1.3. Histological transverse sections of testes from male bonnetheads collected throughout the different stages of the reproductive cycle. Images include a) 40x magnification view of a testis section of an individual from April exhibiting the presence of primary (SG_1) and

secondary spermatogonia (SG_2); b) 100x magnification of the box in a; c) 40x magnification of testis of animal collected in June additionally containing primary (SC_1) and secondary spermatocytes (SC_2); d) 100x magnification of the box in c; e) 40x magnification of testis examined in September demonstrating spermatid (St) elongation and some spermatocysts containing mature spermatozoa (Sz); f) 100x magnification of the box in e; g) 40x magnification view of testis of a November individual containing several spermatocysts with mature spermatozoa (Sz), evacuated (Ev) spermatocysts and primary spermatogonia; h) 100x magnification view of the box in g.

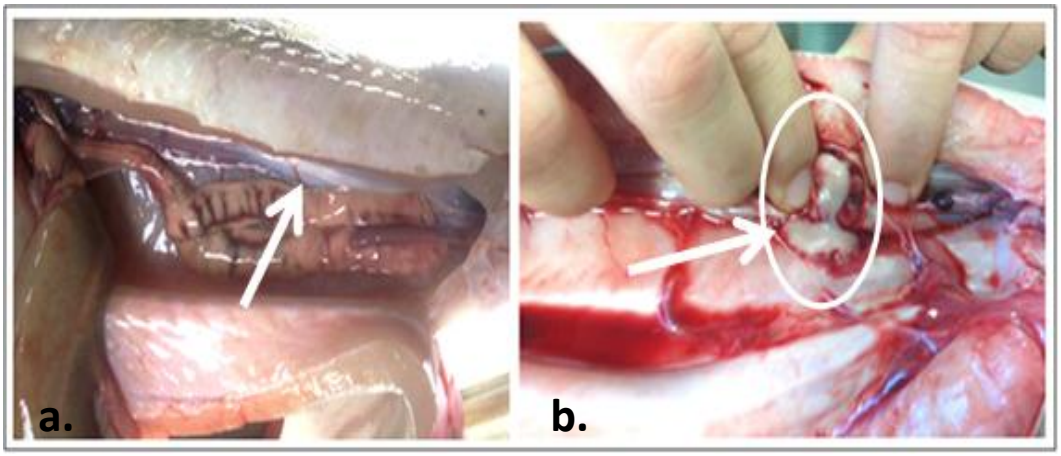

Figure 1.4. Reproductive tract of a male bonnethead caught in early October demonstrating a) the location of the seminal vesicle (arrow), and b) expression of semen from the seminal vesicle (arrow, circle).

Females

Morphological and histological assessments of reproductive condition were conducted on a total of 177 and 84 females, respectively. Mating wounds were observed in female bonnetheads collected during late September, demonstrating the occurrence of copulatory activity (Fig. 1.5). Sizeable amounts of well-defined spermatozoa were observed in the oviducal glands of females collected between September and April using histology (Fig. 1.6). Sperm were also present in oviducal glands of females collected between April and August, but were less abundant and appeared non-viable (Fig. 1.6). Maximum follicle diameter and oviducal gland width varied seasonally, both significantly peaking in April-May (Kruskal-Wallis and Dunn-Bonferroni, $F=82.9$, $df=9$, $p=0.000$ and $F=103.6$, $df=9$, $p=0.000$, respectively) and then progressively declining to their significantly lowest sizes in July-August (Fig. 1.7). Ovulated ova were present in the oviducts of some female *S. tiburo* (n=5) collected in mid-April, suggesting that the presence of spermatozoa in the oviducal gland between September and April reflected a sperm storage period of approximately 6-7 months (Fig. 1.8). Ova or embryos were present in uteri from late April to early September, indicating that gestation requires approximately 4.5-5 months (Fig. 1.9). Embryos grew rapidly during pregnancy from an average size (STL ±SE) of 51±1.7 in June to 302±5.2 in early September (n=596, Fig. 1.10). Litter size (number of embryos ±SE) ranged from 1-12 with an average of 7±0.1 (of n = 81 pregnant females examined). Non-fertile ova (n = 16) and embryos that underwent early death (n = 6) were observed in a total of 12 of the 81 pregnant females, and the overall rate of ova fertilization failure, including nonviable or partially developed embryos was 3.56%. Only 16 of the 133 mature females collected between May and September were non-pregnant, indicating that reproduction is largely annual in these populations. Of the 16, nine were noted as postpartum based on uterine scarring and one was noted to be maturing. It is possible that the remaining six non-pregnant females may have newly

matured and were captured before having the opportunity to mate or may have undergone parturition without experiencing any noticeable internal scarring of the uteri.

Figure 1.5. Presence of mating wounds in a female bonnethead (arrows).

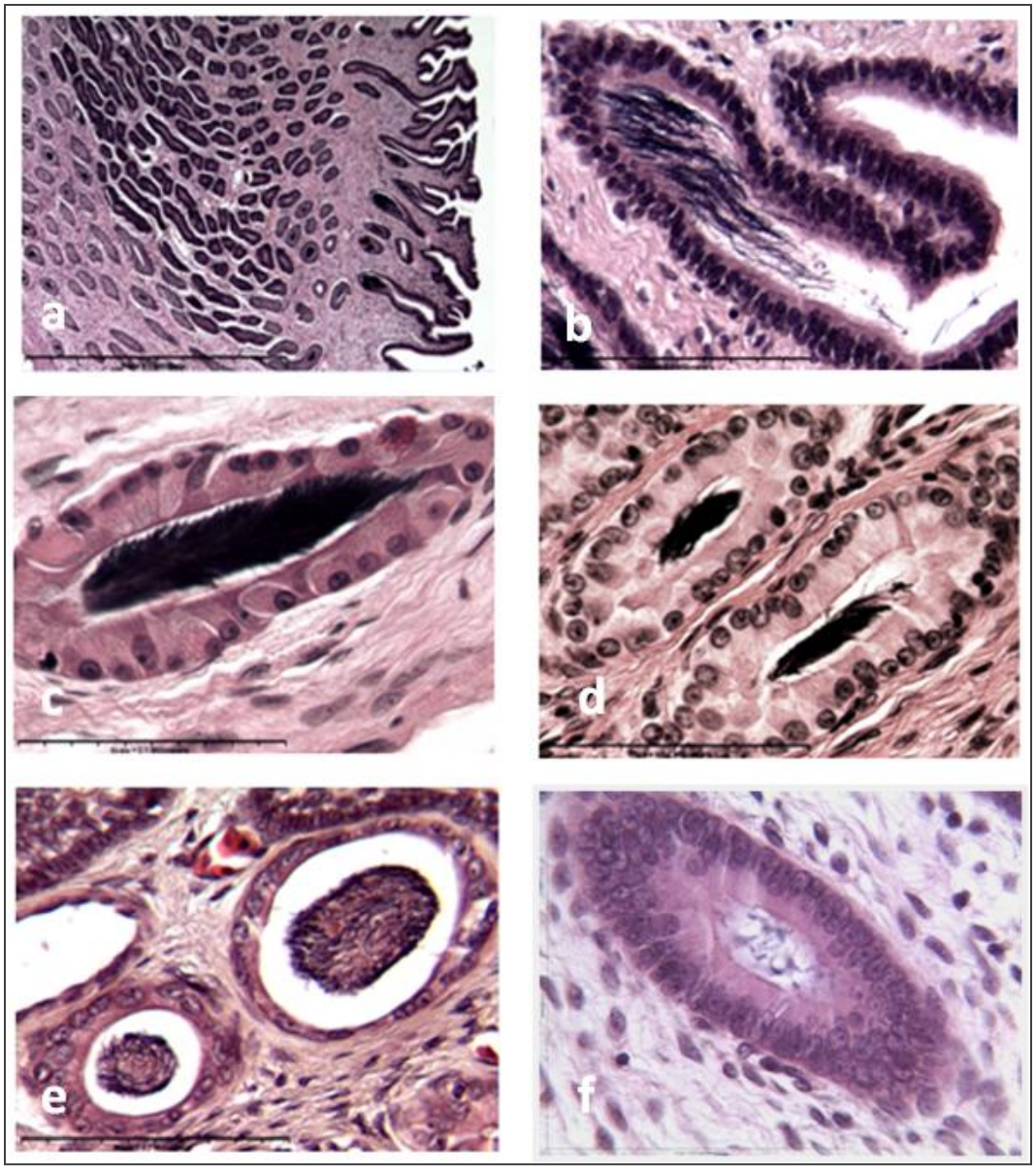

Figure 1.6. Histological architecture of oviducal gland (OG) from female bonnetheads collected during different reproductive stages. a) Transverse section of OG from a recently mated individual collected in early September, observed at 40x. At 400x the following were observed: b) close-up of sperm becoming integrated into invaginations of cells from the lumen in a recently mated individual; c) OG of individual exhibiting sperm storage in the periphery of the organ with packed sperm enclosed in tubules; d) OG of an individual collected during late winter/early spring demonstrating continuation of sperm storage; e) OG of gestating individual with residual sperm in fluid-filled matrix that appear not to be viable and clearly disassociated from vesicle epithelial cells; f) image of a non-storing tubule within the OG of a female collected in August.

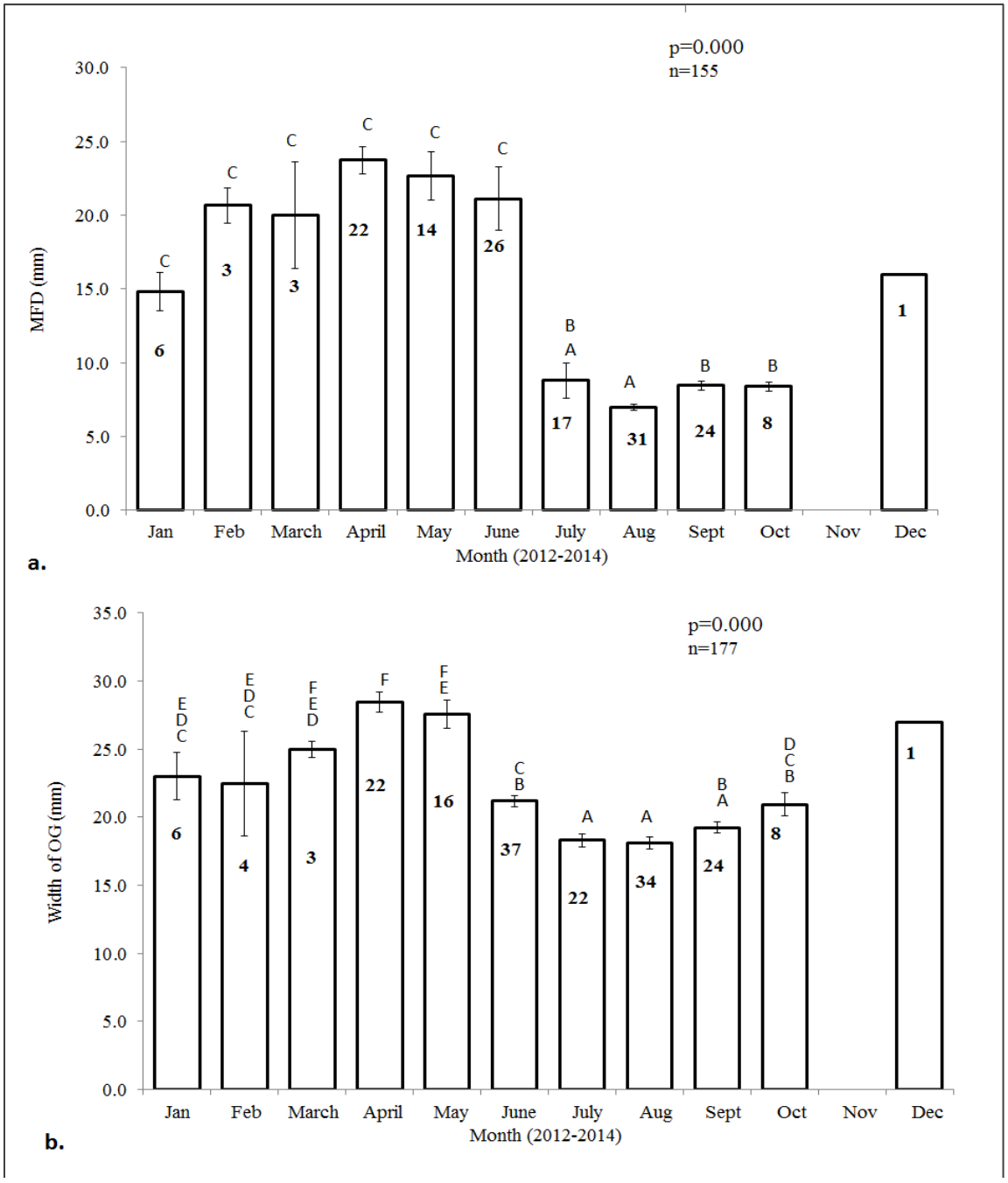

Figure 1.7. a) Maximum follicle diameter (MFD) in mature female bonnetheads from the U.S. Atlantic coast (n=155) and b) oviducal gland (OG) width (n=177). Values are means (±SE). Sample size per month is shown in bars. Significance was determined using Kruskal-Wallis analysis of variance with Dunn-Bonferroni stepwise comparisons (each with p=0.000). Homogeneous subgroups are shown using letters above the bars.

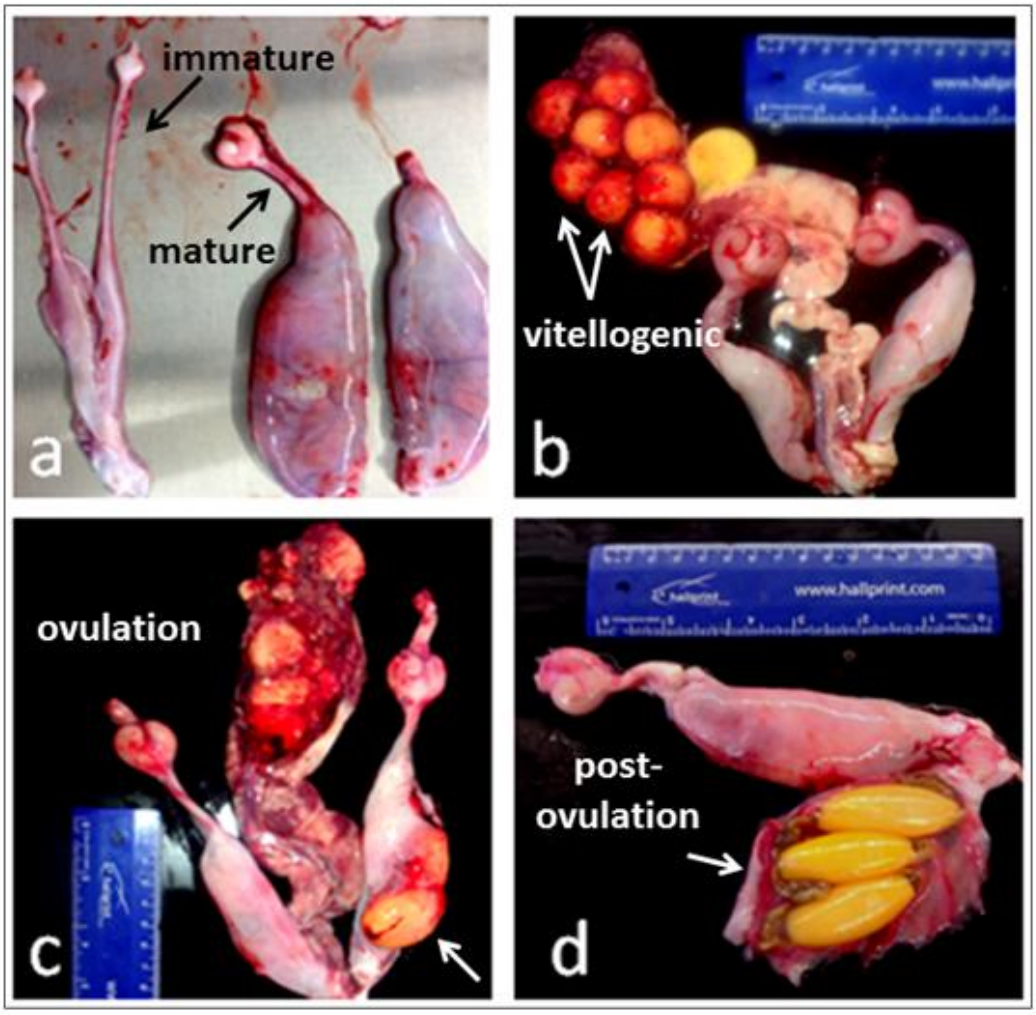

Figure 1.8. Reproductive tract of female bonnetheads caught prior to and during ovulation: a) reproductive tract of an immature female and a mature, ovulatory female including the oviducal gland, and paired uteri; b) reproductive tract of a pre-ovulatory female with enlarged, vitellogenic ova still in the ovary; c) reproductive tract of a female that is undergoing ovulation in which vitellogenic ova are present inside the uteri and some ova in the ovary appear as a mixture of vitellogenic and atretic eggs; d) reproductive tract of a postovulatory female with elongated, vitellogenic ova in the uteri.

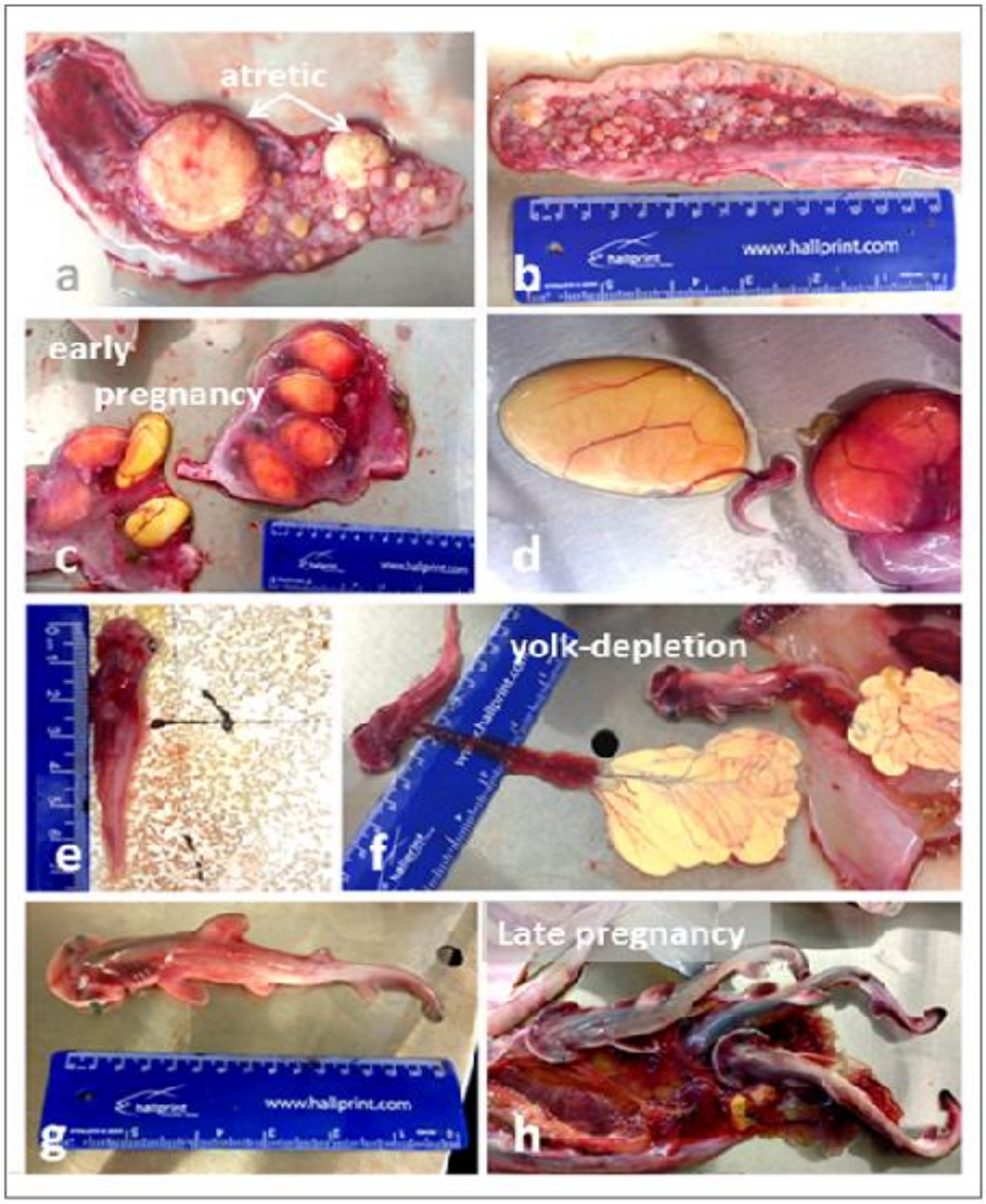

Figure 1.9. Ovarian status and transitional stages of embryonic development following fertilization within the reproductive tract of pregnant female bonnetheads. Images include: a) ovary of a female that is newly pregnant, containing atretic ova; b) ovary of a female bonnethead during late pregnancy just prior to parturition, containing small, non-vitellogenic ova; c) uteri containing ova that were recently ovulated and fertilized; d) small embryo attached to its yolk sac; e) a larger, yolk-dependent embryo; f) embryos that have depleted their yolk sac reserves while formation of the placenta has begun during implantation; g) embryo that is further in development that is larger in size and relies on a maternal placenta for nutrition uptake; h) placental embryos in condition just prior to parturition.

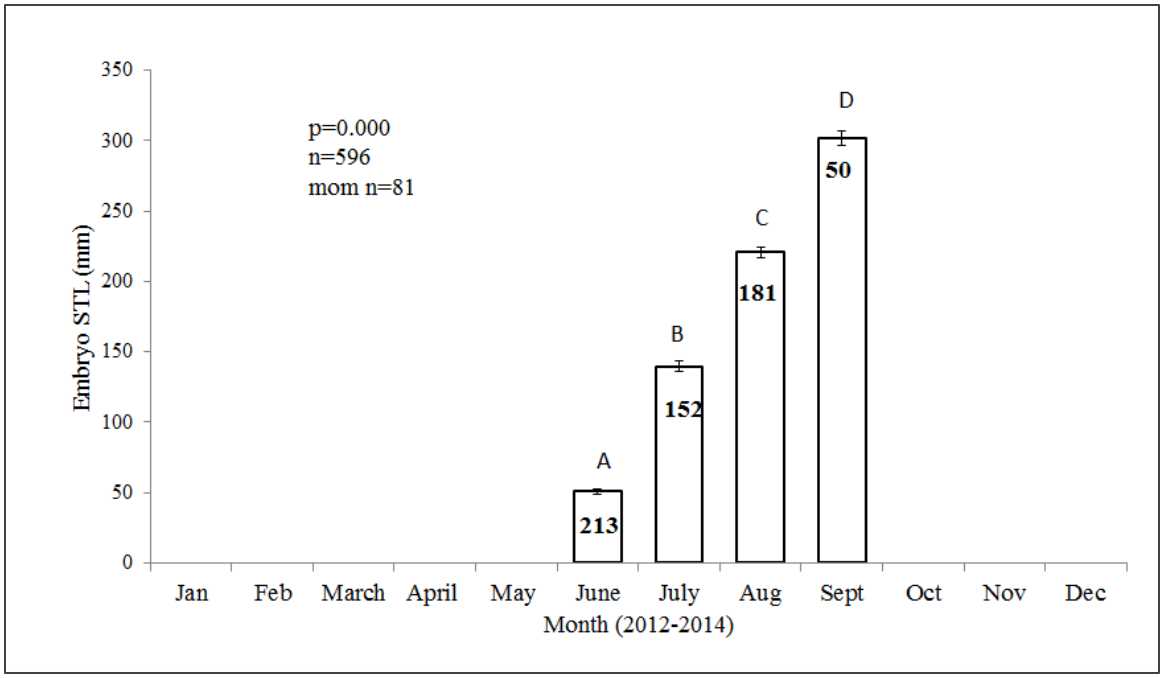

Figure 1.10. Stretched total length (STL) of embryonic bonnetheads (n=596) in the uteri of southeastern U.S. Atlantic female bonnetheads collected during pregnancy (n=81). Data includes sizes of both placental and yolk-dependent pups. Bars are means (±SE). n per month are shown in bars. Significance determined using Kruskal-Wallis followed by Dunn-Benferroni (p=0.000). Homogeneous subgroups are shown using letters above the bars.

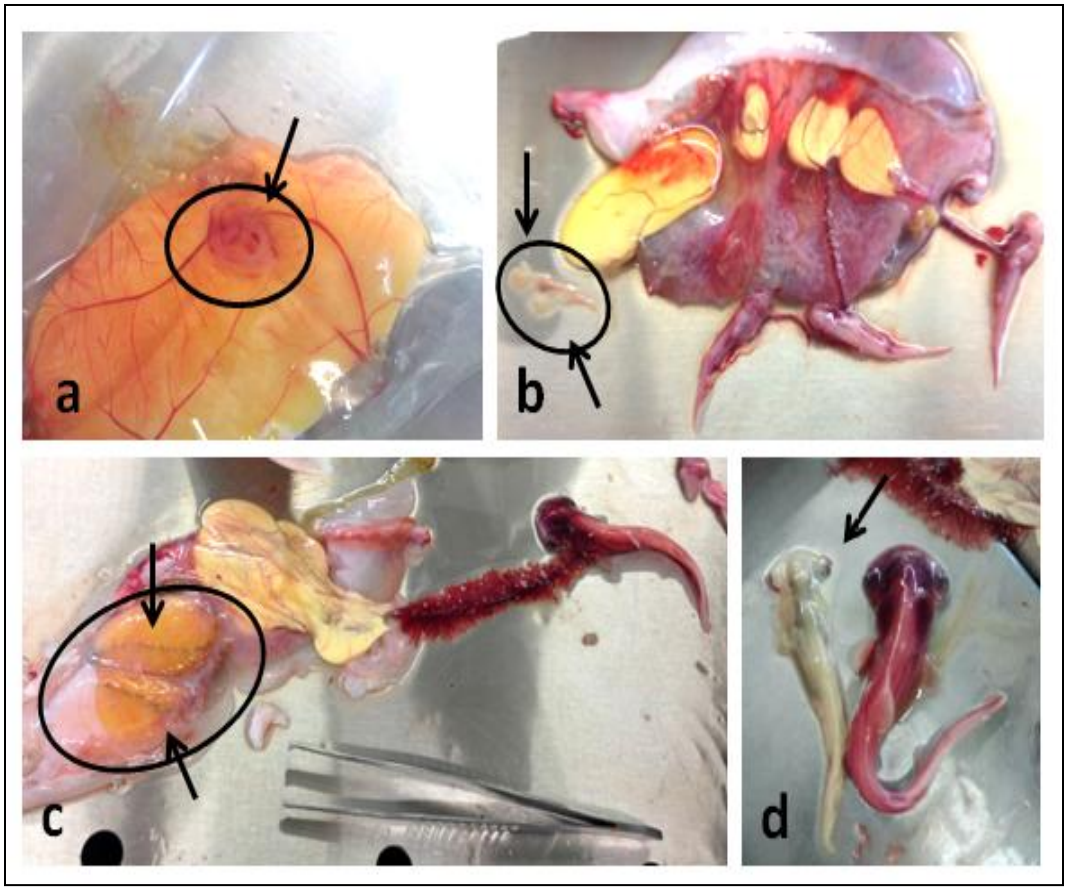

Figure 1.11. Examples of reproductive failure observed in pregnant female bonnetheads. Images include: a) early embryonic death; b) two embryos attached to a single egg; c) two ova that were never fertilized or experienced early embryo death during blastocyst formation; d) embryo that partially underwent development.

DISCUSSION

Reproductive patterns of bonnetheads from the southeastern U.S. Atlantic demonstrated a seasonal cycle and annual periodicity (Fig. 1.12). Changes in the morphology of the reproductive tract clearly reflected temporal trends in both the male and female cycles. Spermatogenesis was shown to occur from late spring to late summer using morphological and histological analysis of the male reproductive tract. Synchronized copulation in September/October was shown to be followed by storage of viable spermatozoa inside the female oviducal gland until the following spring via morphological and histological evaluation of this organ. Follicle growth occurred at the same in preparation for ovulation around late April/May. Gravid females were found to gestate for about 4-4.5 months with an average fecundity of approximately 7±3.84 pups per litter. Following sperm transfer to female bonnetheads, the male reproductive tract gradually regresses and early-stage cells dominate the testes. Sexual inactivity is believed to occur in males during this time until the next annual mating season takes place.

The current assessment of southeast Atlantic bonnethead populations showed very similar reproductive patterns to those described in their Florida Gulf coast counterparts with slight temporal shifts. Parsons (1993) reported that males in Florida Bay and Tampa Bay underwent sperm production from May through August, which peaked during the autumn and then decreased in December. These events occur in Atlantic males from mid-summer through early autumn, peaking earlier in the year than the Florida Bay and

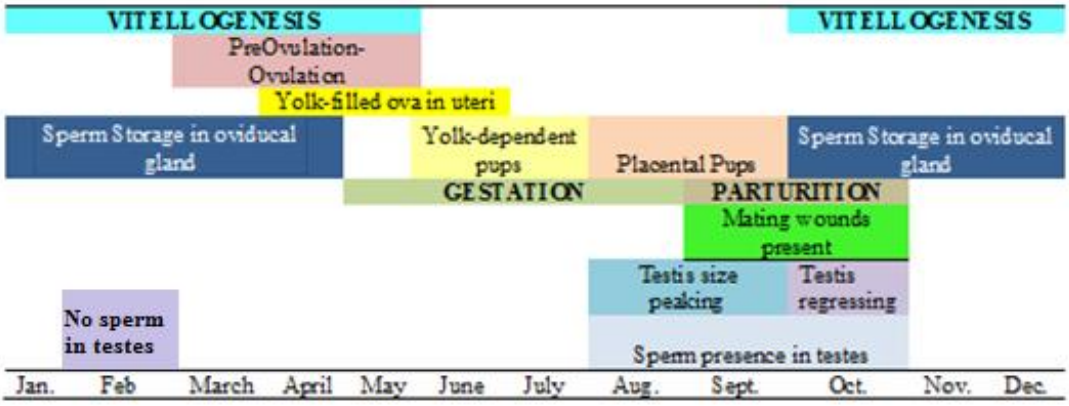

Figure 1.12. Outline of the monthly reproductive events observed to be occurring in both male and female southeastern U.S. Atlantic bonnethead sharks examined between 2012 and 2014.

Tampa Bay populations and showing a reduction in testis size as early as October. Parsons (1993) and Manire *et al*. (1995) found that females from Florida Bay, Tampa Bay and Pine Island Sound ovulate from March to April, are pregnant throughout the summer, and give birth in August through September, although recently postpartum females are noted as late as October. Female bonnetheads from the Atlantic ovulate later from April through June, experiencing parturition before the end of September. Additionally, both Gulf studies found that mating occurred from October to November where Atlantic female bonnetheads contained mating wounds beginning in September and throughout October.

The present study is unique in that southeast Atlantic male *S. tiburo* were captured in the month of February where earlier published studies contained little to no samples from the winter months. The low levels of spermatozoa observed in males during this time and the underdeveloped status of testes confirms that sexual activity is not occurring during this period. Therefore, female sperm storage rather than "sperm presence" is confirmed in this species as there is little likelihood that females would be capable of mating with males during the winter. Although this event clearly occurs, more research is needed to better understanding the process of sperm storage in this species.

In addition to understanding the mechanisms and regulation of sperm storage and its advantages for successful reproduction, it is important to also address the topic of reproductive failure. Previous studies conducted on bonnethead populations from the Gulf of Mexico noted differences in reproductive success, even among bonnethead sharks from geographically close ranges. Specifically, Parsons (1993) reported a rate of unfertilized ova and/or early embryonic

death of 27% in female *S. tiburo* in Tampa Bay while fertilization/embryonic failure occurred as low as 4.3% in Florida Bay. The present study determined that Atlantic female bonnetheads undergo unsuccessful reproduction at a rate of about 3.6% which include unsuccessful fertilization of ova and early embryonic death. Varying degrees of unsuccessful fertilization can reduce fecundity and lower population growth rates. A demographic study conducted by Cortes and Parsons (1996) calculated population growth rates of *S. tiburo* assuming complete successful fertilization and offspring in both Florida Bay and Tampa Bay and noted that such rates may impact population growth from 3.5% to as high as 22%. More studies, however, are necessary to better understand the overall implications for this on population replenishment in order to properly improve the species management in an effort to prevent species overexploitation.

In conclusion, important life history information was acquired for bonnethead populations in the U.S. Atlantic. Using quantitative and qualitative morphological and histological assessments of animals collected throughout a large portion of their Atlantic distribution, the current study determines that Atlantic bonnetheads are mating annually, have distinct seasonal reproductive patterns and bear about 7 offspring per mature female on average. The reproductive cycles described for both male and females are very similar to Gulf of Mexico bonnetheads previously studied. The time of certain reproductive events between the two locations are slightly shifted, specifically in mating and ovulation. Mating occurs during winter in the Gulf and occurs in late autumn in the Atlantic while females undergo ovulation in the spring versus in the Gulf (with a fecundity of about 10±3) and in early summer in the Atlantic (with a fecundity of about 7±4). This information will be provided to fisheries managers and will contribute to the assessment of *S. tiburo* stocks and current management regimes.

COMPONENT II:

Reproductive endocrinology of the bonnethead (*Sphyrna tiburo*) with a focus on sperm storage regulations in females

INTRODUCTION

Female sperm storage is an evolutionary adaptation that exists across several disparate taxonomic groups as the ability of storing spermatozoa within the female reproductive tract for an extended period of time following copulation (Holt and Lloyd, 2010). The independent evolution of this phenomenon throughout different animal lineages include insects, fish, amphibians, reptiles, birds and mammals, suggesting that its benefits are considerable (Calsbeek, *et al.* 2007; Holt and Lloyd, 2010; Moura *et al.*, 2011).

There are several hypotheses concerning the evolutionary benefits of this modified form of reproductive biology. It is proposed that female sperm storage may have evolved as a means for maximizing reproductive success in species whose social systems require males and females to be largely solitary, and/or for those with asynchronous reproductive cycles (Holt and Lloyd, 2010). Sperm storage may also benefit species in which males and females lack sufficient spatial coexistence and for those for which energy costs must be optimized due to low or irregular food availability. Additionally, female sperm storage may have developed due to genetic advantages from long-term access to spermatozoa (Bretman *et al.*, 2009). For instance, studies investigating paternity bias in female field crickets suggested that females prevent inbreeding by selectively

storing sperm that will optimize genes of the offspring (Bretman *et al.*, 2009). Such studies would indicate that an advantage to female sperm storage includes maximizing the genetic quality of offspring as well as ensuring maximum number of offspring and possibly may be coupled with sexual selection, such as cryptic female choice. Conversely, female sperm storage in *Anolis* lizards is believed to have evolved as a means for increasing genetic diversity of progeny through polyandry (Calsbeek *et al.*, 2007). Although the process of storing sperm in females may be similar across different animal groups, mechanistic variations in the duration of sperm storage, sperm survival and overall benefits exist for different organisms. For example, the duration of sperm storage may range from decades in female insects to as low as up to 11 days in uterine glands of dogs after copulation (Holt and Lloyd, 2010).

In addition to studies about its evolutionary benefits, a number of recent publications have addressed the physiological factors that may play an important role in regulating various aspects of sperm storage within the female reproductive tract. In particular, it has been proposed that gonadal steroid hormones (i.e. androgens, estrogens, and progesterone) are involved in the regulation of long-term survival of sperm and their release near the end of the storage period (Holt and Lloyd, 2010; Awruch, 2013). Work conducted by Yoshimura *et al.* (2000) shows that estrogen receptors (ER) and progesterone receptors (PR) were present in the cells of the sperm storage tubules in the utero-vaginal junction of mature hens, as well as in immature hens treated with diethylstilbestrol, a synthetic form of estrogen. However, ERs were not present in the reproductive tract of untreated immature individuals. These results suggest that estrogens induce expression of these receptors, which may be important for the development and maintenance of sperm storage tubules (SST) as well as the regulation of sperm storage (Yoshimura *et al.*, 2000;

Das *et al.*, 2005). More recently, progesterone has been specifically implicated in the cessation of sperm storage in birds. Ito *et al.* (2011) demonstrated that progesterone stimulates morphological changes in the SST of Japanese quail that result in the release of stored spermatozoa. Recent studies have demonstrated a potential role for androgens in regulating sperm storage in some mammals, particularly in the greater Asiatic yellow bat *Scotophilus heathii* (Roy and Krishna, 2010; 2011). These studies have demonstrated that higher testosterone levels occur during the storage period in *S. heathii*, and decrease with sperm release. In addition, androgen receptors (AR) have been localized within the epithelial cells of SST within *S. heathii*, and treatment with anti-androgens has been shown to reduce sperm survival, likely by influencing expression of pro-survival and pro-apoptotic genes (Roy and Krishna, 2010; 2011).

Many shark species have been shown to be capable of female sperm storage including the dogfish shark (*Scyliorhinus canicula*), the Oman shark (*Iago omanensis*), the Portuguese dogfish shark (*Centroscymnus coelolepis*), and 9 of 11 North Atlantic shark species, including the bonnethead (S*phyrna tiburo*) (Parsons *et al.*, 2007; Moura *et al.*, 2011). Furthermore, tangential evidence for associations between plasma sex steroid hormone concentrations and female sperm storage has been observed in some of these species; for example, increases in both estradiol and testosterone have been observed to occur during sperm storage in *S. tiburo* (Manire *et al*., 1995). However, to date, no published studies have investigated the hormone regulation of sperm storage in the female elasmobranch reproductive tract. Therefore, as a companion to research on the reproductive biology of the U.S. Atlantic bonnethead population, the main focus of this study was to examine the reproductive endocrinology of Atlantic bonnethead populations with a

particular focus on hormone regulation of female sperm storage. To accomplish this, the author validated previously described seasonal patterns in reproductive endocrinology for *S. tiburo* by examining changes in plasma testosterone (T) in males and T, 17β-estradiol (E_2), and progesterone (P_4) in females throughout the reproductive cycles. This second component of the study also examined the presence of intracellular receptors for these hormones in the oviducal gland (OG), as well as in the male testis. The purpose of the current study was to increase the understanding of the regulation of the poorly understood process of sperm storage in *S. tiburo*.

METHODOLOGY

Biological data and sample collections

Sharks were collected from South Carolina to south Florida using longlines and gillnets. All sharks captured were sexed, weighed (kg), and measured (precaudal length (PCL), fork length (FL), total length (TL) and stretched total length (STL) in millimeters). Blood (~3 mL) was collected via caudal venipuncture using sterile syringes and needles, transferred to vacuum tubes containing anticoagulant, and temporarily kept on ice until centrifuged to separate plasma, which was stored at -80°C until use for hormone analysis of testosterone (T) in males, and T, 17β-estradiol (E_2) and progesterone (P_4) in females. A sub-sample of animals were dissected to obtain morphological measurements of reproductive structures to assess reproductive stage at time of capture for a comparative reproductive study. Organ samples of the reproductive tract (including the testes from the males and the oviducal glands from the females) were collected from euthanatized individuals and fixed in 10% formalin in elasmobranch-modified saline for 48h, rinsed in water and transferred to 70% ethanol. Bonnethead organ sections (~2-3 mm) were embedded in paraffin for routine histology as described in Gelsleichter *et al*. (2003). Immunocytochemistry was used to examine presence of AR, PR, and estrogen receptor α (ERα) in histological sections.

Hormone analysis

Chemiluminescence immunoassays (CLIA) were used to measure plasma sex steroid hormone concentrations. Plasma concentrations of T in males and T, E_2 and P_4 in females were analyzed using AccuLite CLIA kits (Monobind, Lake Forest, CA) following the manufacturer's

instructions as performed by Anderson and Gelsleichter (unpublished). Luminescence in assay wells was measured using the Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). Shark plasma was diluted in HAS Calibrator Matrix (Monobind, Lake Forest, CA) prior to conducting CLIA, following validated dilution levels by Anderson and Gelsleichter (unpublished): 1/50 for measuring T in males, and 1/25 and 1/10 for measuring E_2 and P_4/T in females, respectively.

Immunocytochemistry

Immunocytochemistry was performed on the oviducal gland of female and the testis of male bonnetheads using polyclonal rabbit anti-human AR (Millipore, AR-21), polyclonal rabbit anti-human ERα (Millipore, C1355), and monoclonal mouse anti-human PR (Thermo Scientific, alpha PR-22) as primary antibodies. Tissue sections were incubated in a limonene-based solvent for deparaffinization, re-hydrated via incubation in a descending series of graded alcohols (100-95%), and rinsed in tap water. Sections were incubated at 95˚C in antigen retrieval solution (10 mM sodium citrate, pH 6.0) for a duration of 20 minutes with the goal of exposing any epitopes of the specialized target receptors that may have been masked by the fixation process. After sections were brought to room temperature, they were rinsed in reverse osmosis water and phosphate buffered saline (PBS), then blocked for nonspecific reactivity with primary antibodies via incubation in 2% normal goat serum in PBS (Vector) in PBS overnight at 4°C.

Following blocking, slides were rinsed in PBS and endogenous peroxidase activity was quenched by incubation in 1:1 hydrogen peroxide:methanol for 15 min. After rinsing twice in

PBS, sections were incubated overnight at 4°C in primary antibody diluted 1/100 in PBS containing 0.1% gelatin and 0.1% sodium azide (G-PBS).

On the final day of the procedure, sections were rinsed with PBS containing 0.05% Tween-20 (PBS-T), rinsed twice with PBS, and incubated for 30 min with secondary antibody: Anti-Rabbit Ig for AR and ER or Anti-Mouse Ig for PR (ImmPRESS HRP Anti-Rabbit or Anti-Mouse Ig (Peroxidase) Polymer Detection Kits, Vector). Afterwards, slides were rinsed with PBS three times and then incubated with the chromogen 3,3'-diaminobenzidine (ImmPACT DAB Peroxidase (HRP) Substrate, Vector) following the manufacturer's instructions. Following color development, slides were rinsed with tap water, then counter-stained with 2% methyl green (Vector) for 15-60 min at 37°C. Sections were then rinsed in tap water, dehydrated in an ascending series of graded alcohols (95-100%), cleared in a limonene-based solvent, and coverslipped using Cytoseal 60.

Immunocytochemical controls were performed via: 1) stepwise deletion of all stages of the immunocytochemical procedure; 2) replacement of the primary antibody with nonimmune rabbit IgG or diluent; and, for AR, 3) pre-absorption of the primary antibody with a 10-fold excess of the AR21 protein (the antigen used to make the PG-21 antibody).

Data analysis

Hormone concentrations in males and females were grouped by month of capture or reproductive stage, respectively, to examine temporal, stage-associated changes. Mean hormone concentrations per month or stage were compared using Kruskal-Wallis nonparametric statistics followed by Dunn-Bonferroni multiple comparisons test at a significance level of 0.05 to determine if significant differences were present between time periods, as data generally did not pass tests for normality and/or equal variance. Normality assumptions were tested by reviewing descriptive statistics, histograms and skewness and kurotosis for the given data. Data that did not fulfill these criteria were analyzed using Kruskal-Wallis analysis of variance followed by Dunn-Bonferroni posthoc test based on data homogeneity at a significance level of 0.05. Immunocytochemistry was examined using light microscopy and analyzed qualitatively**.** To assess changes in hormone receptor localization patterns, females were separated into 3 major stages based on the reproductive stage of animals at time of capture: a) mating and early sperm storage; b) mid-late sperm storage through ovulation (OV); c) post-ovulation (Post-OV) to late pregnancy (LP) and through post-parturition (PP).

RESULTS

Plasma hormone concentrations

Plasma testosterone concentrations in the mature male bonnetheads (n=57) varied seasonally (Fig. 2.1). The highest concentrations occurred from July through September with levels significantly peaking in August (Kruskal-Wallis and Dunn-Bonferroni, F=24.5, df=8, p=0.002), a period that coincided with increases in testes and epididymis size and the presence of mature spermatozoa in the testes in the same individuals, reflecting peak spermatogenesis. Plasma T concentrations were significantly lower in males collected from late autumn through the winter (Kruskal-Wallis and Dunn-Bonferroni, F=24.5, df=8, p=0.002), a time of reproductive inactivity based on reductions in testis size and the lack of mature spermatozoa in the same animals.

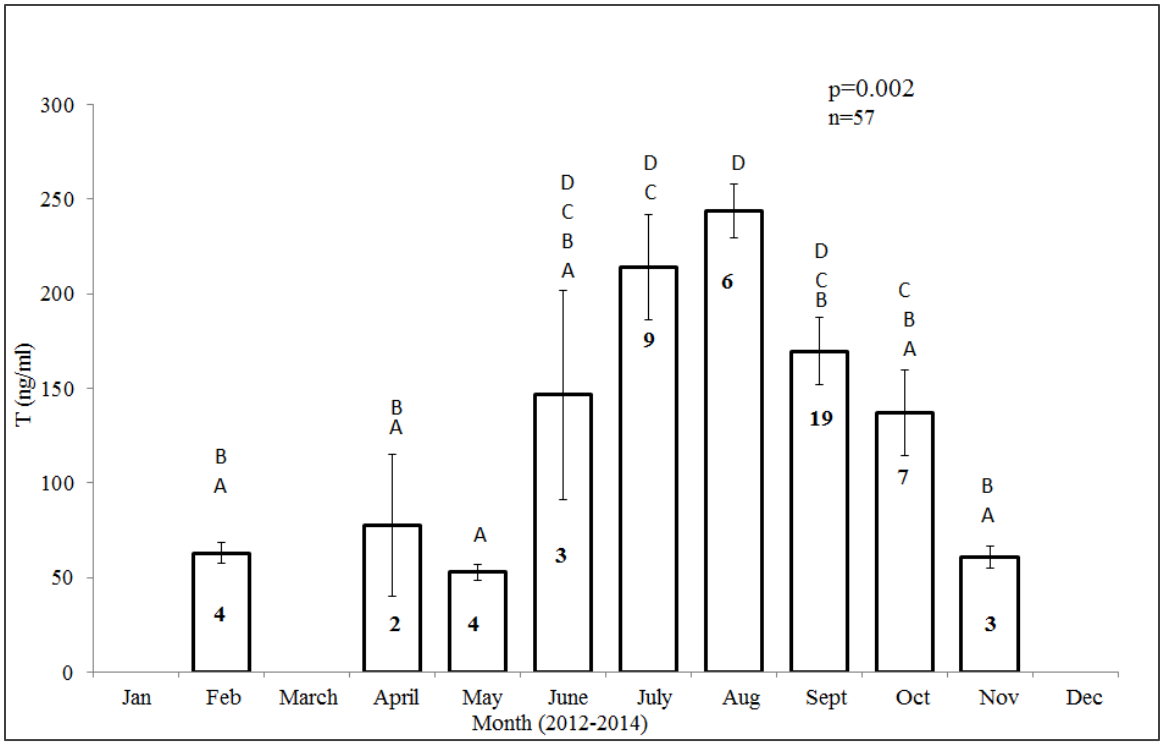

Figure 2.1. Plasma testosterone concentrations in southeastern U.S. Atlantic male bonnethead sharks (n = 57). Values are means (±SE). Sample size per month is shown in bars. Significance was determined using Kruskal-Wallis test of variance with Dunn-Bonferroni stepwise comparison (p=0.002). Homogeneous subgroups are shown using letters above the bars.

Plasma E_2 concentrations were also found to vary temporally in female bonnetheads (n=82) in relation to the reproductive cycle. Circulating E_2 concentrations (Fig. 2.2) were low in females collected just prior to and during the mating period, but exhibited significantly elevated levels in individuals collected during the sperm storage and pre-ovulatory periods (Kruskal-Wallis and Dunn-Bonferroni, F=48.2, df=7, p=0.000). Lower levels of E_2 were observed during ovulation and throughout much of the gestation with the slight exception of the post-ovulatory period, during which 1 of the 2 females collected during this stage exhibited high concentrations.

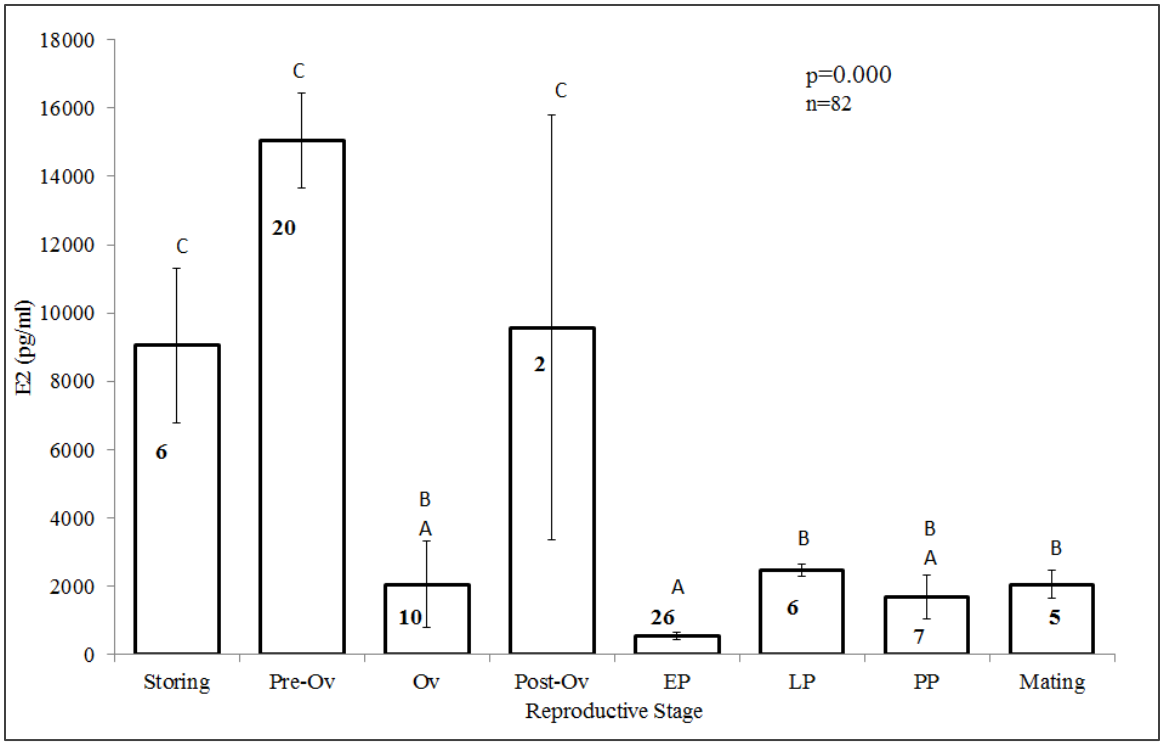

Figure 2.2. Plasma E_2 concentrations in southeastern U.S. Atlantic female bonnethead sharks (n=82). Values are means (±SE). Sample size per reproductive stage is shown in or above bars. Reproductive stages include: storing, the period after mating when females are inseminated and retain viable sperm in the oviducal glands until fertilization can take place during ovulation; pre-ovulation (Pre-OV); ovulation (OV); post-ovulation (Post-Ov); early pregnancy (EP), when embryos are yolk dependent; late pregnancy (LP), when embryos are placental; post-partum (PP); and mating. Significance was determined using Kruskal-Wallis test of variance with Dunn-Bonferroni stepwise comparison (p=0.000). Homogeneous subgroups are shown using letters above the bars.

Plasma P_4 concentrations in female bonnetheads (n=77) were also associated with different reproductive stages (Fig. 2.3). Circulating P_4 levels were low prior to and after copulation and remained low in females collected throughout much of the sperm storage period. Significant increases in plasma P_4 levels were observed in females collected during the late stages of sperm storage and follicular development, and ovulation (Kruskal-Wallis and Dunn-Bonferroni, F=28.8, df=7, p=0.000). With the possible exception of the immediate post-ovulatory period when a single female with high plasma P_4 concentrations was observed, circulating P_4 levels remained low throughout most of gestation.

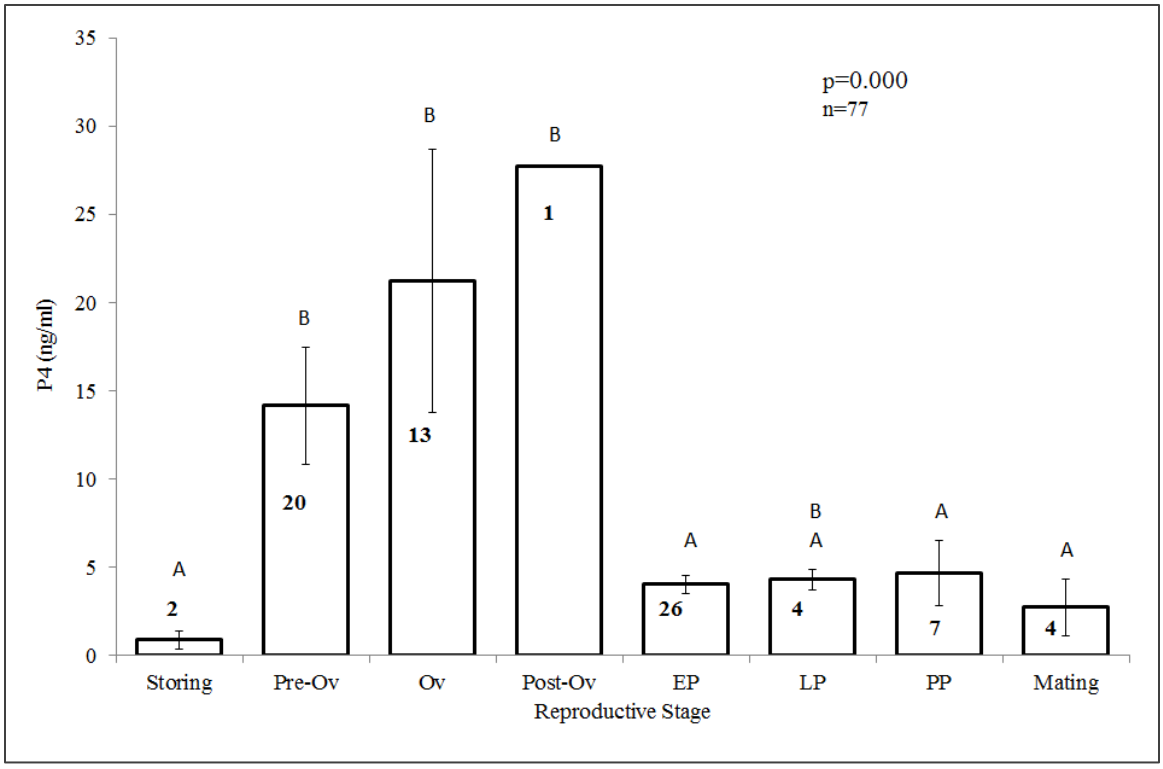

Figure 2.3. Plasma P_4 concentrations in southeastern U.S. Atlantic female bonnethead sharks (n=77). Values are means (±SE). Sample size per reproductive stage is shown in or above bars. Reproductive stages include: storing, the period after mating when females are inseminated and retain viable sperm in the oviducal glands until fertilization can take place during ovulation; pre-ovulation (Pre-OV); ovulation (OV); post-ovulation (Post-Ov); early pregnancy (EP), when embryos are yolk dependent; late pregnancy (LP), when embryos are placental; post-partum (PP); and mating**.** Significance was determined using Kruskal-Wallis test of variance with Dunn-Bonferroni stepwise comparison (p=0.000). Homogeneous subgroups are shown using letters above the bars.

Last, plasma T concentrations in mature females (n=37) also showed clear patterns throughout different reproductive stages (Fig. 2.4). Concentrations were slightly elevated in females collected during the mating period and reached significantly higher levels (Kruskal-Wallis and Dunn-Bonferroni, $F=25.4$, $df=7$, $p=0.001$) in individuals collected throughout the period of sperm storage and folliculogenesis. Hormone concentrations were markedly lower in females collected during ovulation and throughout gestation, reaching the lowest concentrations post partum.

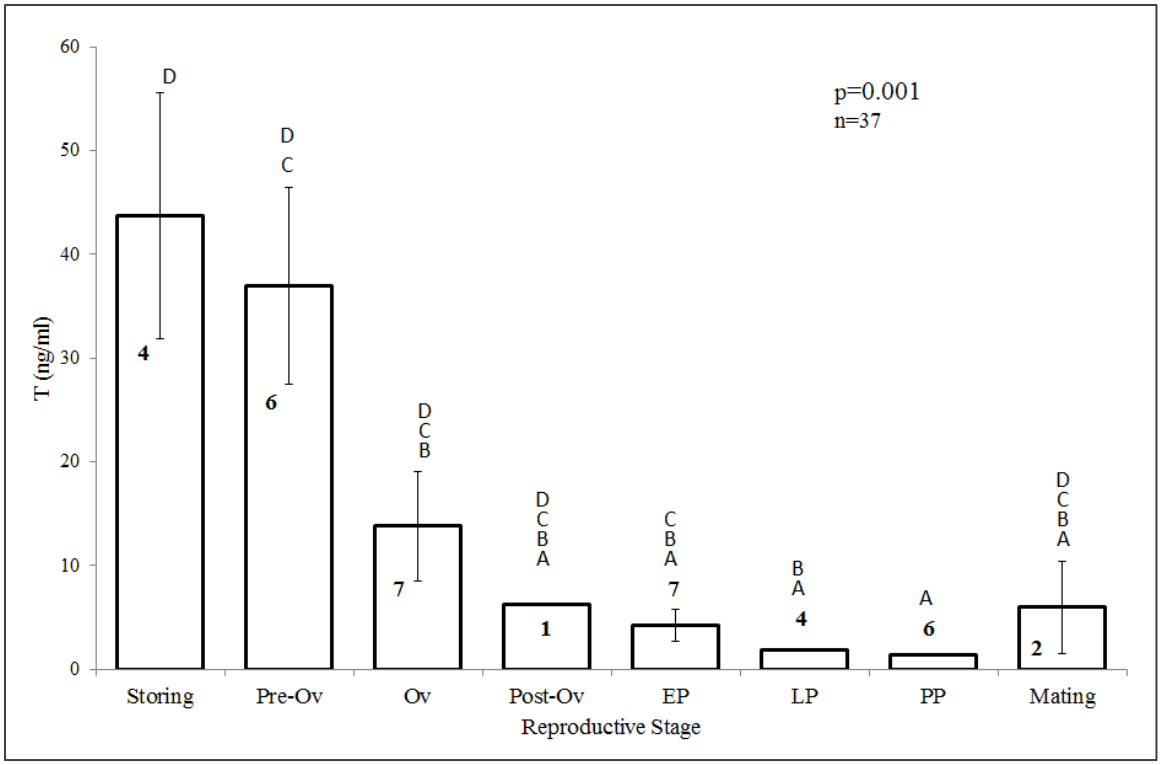

Figure 2.4. Plasma T concentrations in southeastern U.S. Atlantic female bonnethead sharks (n=37). Values are means (±SE). Sample size per reproductive stage is shown in or above bars. Reproductive stages include: storing, the period after mating when females are inseminated and retain viable sperm in the oviducal glands until fertilization can take place during ovulation; pre-ovulation (Pre-OV); ovulation (OV); post-ovulation (Post-Ov); early pregnancy (EP), when embryos are yolk dependent; late pregnancy (LP), when embryos are placental; post-partum (PP); and mating. Significance was determined using Kruskal-Wallis test of variance with Dunn-Bonferroni stepwise comparison (p=0.001). Homogeneous subgroups are shown using letters above the bars.

Immunocytochemistry

Immunocytochemistry was conducted on oviducal glands of 49 female bonnetheads representing 3 stages: mating/early sperm storage (n=19, September-January), middle to late sperm storage (n=16, February-May), and the post-storage period (n = 14, June-August). Presence of AR was most prominent in the oviducal gland during the early to late stages of sperm storage, exhibiting strong immunoreactivity in both the epithelial cells of sperm storage tubules, as well as the secretory tubules that are involved in producing eggshell membranes and various components of the post-ovulated egg. During post-ovulation, AR was strongly present in the apical and basolateral portions of the secretory tubules.

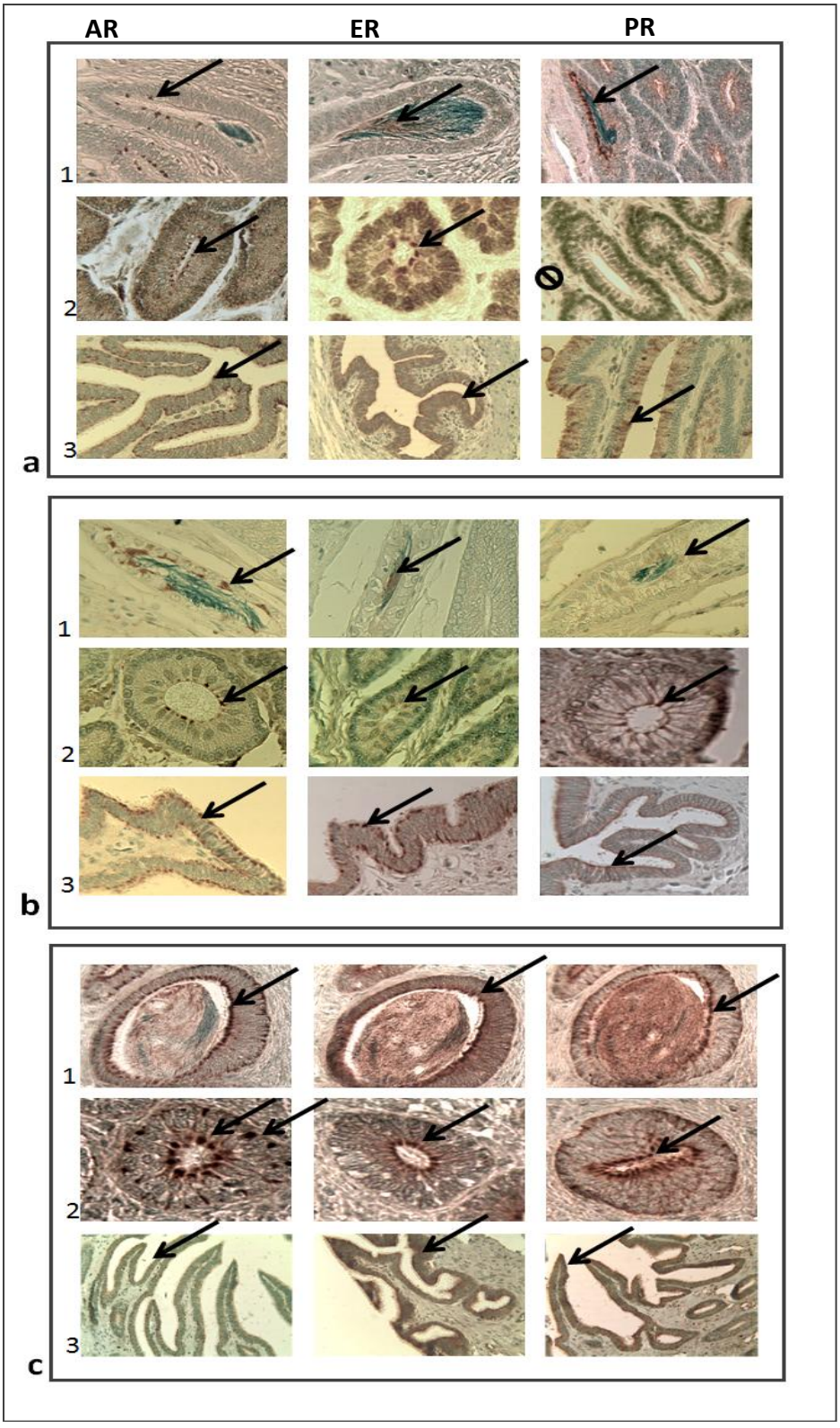

Figure 2.5. Immunocytochemical analyses of oviducal gland from female bonnethead sharks collected during various stages of sperm storage. Arrows point to sites of positive

immunolocalization. All sections were observed at 400x. Hormone receptor types are organized by column from left to right (AR, ERα, PR) and oviducal gland components are organized by row (1: storage tubules/sperm, 2: secretory tubules, 3: luminal epithelium). a) Animals collected between the stages of PP and Mating (from September to January) and undergoing initial stages of sperm storage; b) oviducal gland of animals caught in February through May, demonstrating sperm being stored until OV; c) animals collected between June and August that were classified Post-OV through LP, which exhibit residual, seemingly nonviable sperm shortly after fertilization.

Immunoreactivity of ERα was also present throughout all reproductive stages in the epithelial cells of sperm storage and secretory tubules, as well as in the luminal epithelium although immunostaining was faint in females that were undergoing ovulation and gestation (Fig. 2.5). Most notably, ERα was consistently observed within the spermatozoa itself during all stages of sperm storage.

Presence of PR was observed primarily in the secretory tubules and the luminal epithelium of females undergoing ovulation (Fig. 2.5). Localized PR was strong in sperm storage tubules in females collected during gestation. During mating, PR was not detected in the secretory tubules and only showed light staining in SST during much of the sperm storage period.

Because of positive detections of ERα in stored spermatozoa, immunocytochemistry was also conducted on testes from 43 male bonnetheads to determine if ERα receptor localization patterns in pre-spermiated cells were consistent with those observed in the female oviducal glands. Androgen, progesterone and estrogen receptors were all present in the testis of all mature males examined. Testes exhibited positive immunostaining for AR in Leydig-like interstitital cells of pre-meiotic and peri-meiotic spermatocysts, as well as in the Sertoli cells of spermatocysts containing spermatids and mature sperm. PR immunoreactivity was most prominent in males undergoing spermiation, particularly in Sertoli cells of spermatocysts containing mature spermatozoa (Fig. 2.6). ERα was present in spermatocysts containing mature spermatozoa (Fig. 2.6). Although ER was present in Sertoli cells lining the spermatocyst epithelium, localization was strongest in the tail end of spermatozoa.

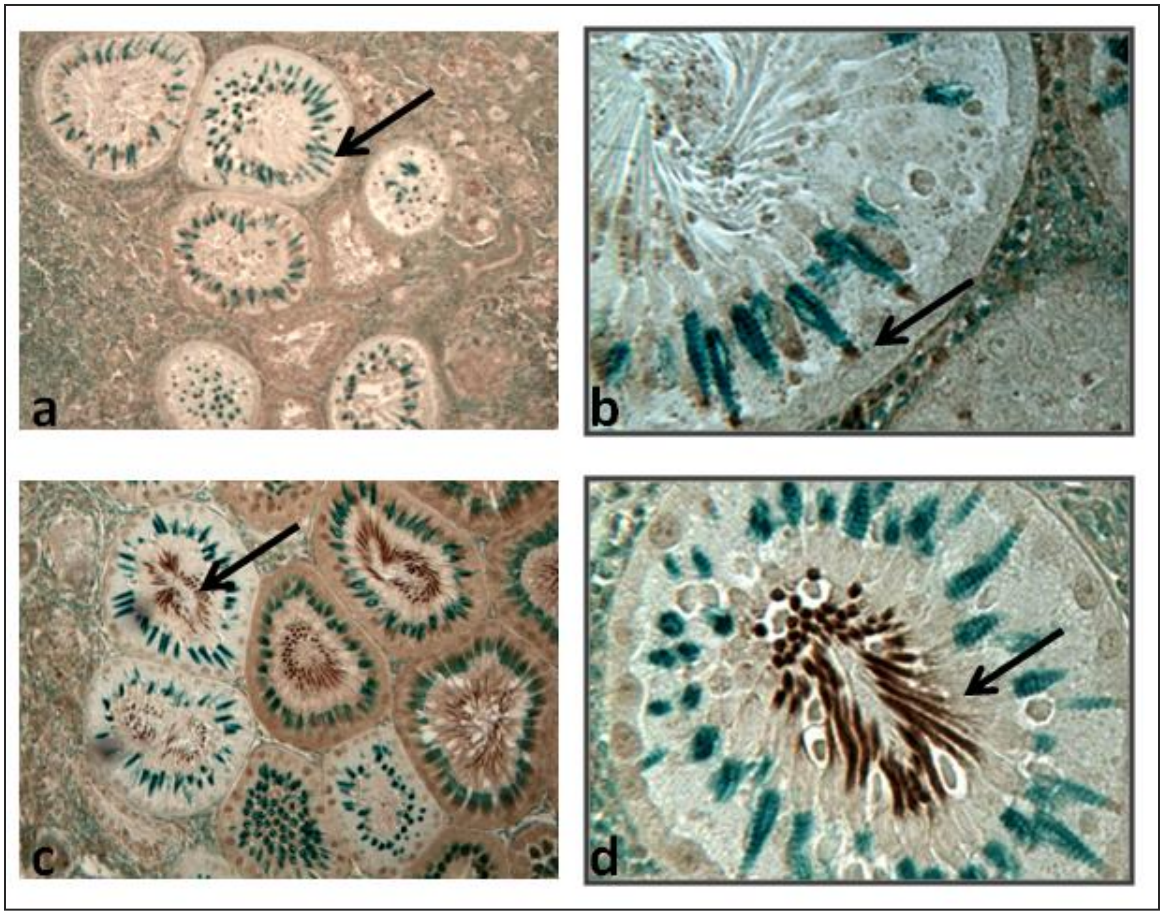

Figure 2.6. Immunocytochemical analyses of testis transverse sections from a male bonnethead collected in November 2013. Arrows point to sites of positive immunolocalization. a) Several spermatocysts containing mature sperm and exhibiting PR; observed at 100x; b) immunoreactive PR present in Sertoli cells in spermatocysts containing mature spermatozoa; observed at 400x; c) several spermatocysts containing immunoreactive ERα in the mitochondrial portion and sperm tails as well as Sertoli cells; observed at 100x; d) densely packed sperm exhibits deep immunoreactivity for ERα in the sperm tails; observed at 400x.

DISCUSSION

The results of this study show associations between temporal patterns in circulating sex steroid hormone concentrations and reproductive stages in both male and female bonnetheads from the southeast Atlantic Ocean, confirming earlier suggestions of stage-specific roles for these hormones in regulating reproduction. Plasma T concentrations in males peaked during late spermatogenesis, validating earlier work by Manire and Rasmussen (1997) and confirming a role for this hormone in regulating male gametogenesis. In females, E_2 levels peaked during the pre-ovulatory period, also corroborating previous observations (Manire et al. 1995) and reflecting a well-established role for this hormone in regulating vitellogenesis. Plasma progesterone concentrations in females increased during the late stages of follicular development and peaked during ovulation, suggesting a long-hypothesized role for P_4 in regulating this process. Most importantly for this study, both testosterone and estradiol were elevated in females during sperm storage, suggesting roles for both of these hormones in regulating this process. In contrast, progesterone levels only increased during late stages of sperm storage. Receptors for all three sex hormones were detected in the oviducal gland of female bonnetheads, suggesting that epithelial cells of sperm storage tubules and in the case of estradiol, stored sperm itself are target cells for direct actions of these hormones.

Previous research by Manire *et al*. (1995) demonstrates an increase in circulating T levels in female bonnetheads between copulation and ovulation, leading the authors to hypothesize that androgens may be directly involved in regulating certain aspects of sperm storage. The results of

this study have both corroborated these findings and provided evidence for direct effects of androgens on epithelial cells of oviducal gland sperm storage tubules, which likely play a role in maintaining sperm vitality. This hypothesis is also supported by comparable work on some sperm-storing mammals, particularly *S. heathii*, which have also been shown to exhibit increased circulating androgen levels during sperm storage as well as AR immunoreactivity in epithelial cells of sperm storage tubules in the female reproductive tract. Furthermore, Roy and Krishna (2010; 2011) demonstrated that treatment of female *S. heathii* with the anti-androgen flutamide can result in a loss of viability in stored spermatozoa, possibly due to AR-mediated changes in the expression of pro-apoptotic versus pro-survival genes by sperm storage tubule epithelial cells, which may release these factors into the tubular lumen. A similar mechanism may function to maintain sperm vitality in the bonnethead oviducal gland, warranting future studies on expression of mediators of cell death and survival in sperm storage tubules of this organ.

Although it has not been directly addressed in past studies, increases in plasma estradiol levels during sperm storage have been reported to occur in female bonnetheads, suggesting that this hormone may play a regulatory role in this process along with its well-known role in stimulating follicular development (Manire *et al.*, 1995). Like androgens, this function may be mediated through effects on epithelial cells of sperm storage tubules based on patterns of ERα immunoreactivity in the oviducal gland. However, it is also possible that E_2 may directly affect stored spermatozoa, perhaps by influencing cell metabolism, motility, and/or other traits that may contribute to its fertilizing capacity. In fact, estrogens have been found to affect sperm capacitation and acrosomal reactions in mammals (Aquila and De Amicis, 2014).

Unlike T and E_2, P_4 did not appear to be greatly involved in regulating sperm survival in female bonnetheads, as circulating levels of this hormone are low during the early to middle stages of sperm storage. However, based on increases in plasma P_4 concentrations during the late sperm storage period and ovulation, as well as PR immunoreactivity in epithelial cells of the sperm storage tubules, it is logical to suggest that P_4 may be involved in the release of stored spermatozoa. Progesterone has been proposed to affect sperm motility in some vertebrates as well as be capable of inducing the expression of sperm releasing factors in oviducal sperm storage tubules of birds (Ito *et al.*, 2011; Aquila and De Amicis, 2014).

In conclusion, hormone receptor localization suggests that T, E_2 and P_4 all appear to contribute to the regulation of sperm storage in female *S. tiburo*. All 3 hormones likely regulate the function of sperm storage tubule epithelial cells where E_2 also exhibits direct effects on stored sperm. All 3 hormones appear to also regulate secretory activity and luminal function in the oviducal gland. This study provides a better understanding of hormone regulation in reproduction of the bonnethead (*Sphyrna tiburo*). Moreover, it provides the first thorough investigation of receptor localization in female sharks to include T, P_4, and E_2. Understanding the regulation of sperm storage in females can lead to a greater understanding of how these populations benefit from this unique physiological ability, it can shed light on the overall advantages from an evolutionary perspective, and more importantly, more reliable data in this matter can ultimately provide great implications for future conservation. It is possible that sperm storage in female bonnetheads has evolved as an adaptation for cryptic female choice, possibly consisting of attempts to assuage sexual conflict by producing near equal fit offspring of both sexes. Studies have suggested other possible techniques for sustaining sperm, such as decreasing body temperatures to optimize the

environment for sperm survival, or self-sperm sustenance from mitochondria and cytoplasm while encased in a cytoplasmic storage bag within the epithelial follicle or ova. With this wide array of processes that manifest evolution at its finest, there is much need for further research on the subject (Holt and Lloyd, 2010).

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