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Reproductive Biology of the Tiger Shark in the Western Atlantic Ocean

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Reproductive Biology of the Tiger Shark in the Western Atlantic Ocean

By Chelsea Shields

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CERTIFICATE OF APPROVAL

The thesis "Reproductive Biology of the Tiger Shark in the Western Atlantic Ocean" submitted by Chelsea Anne Shields

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Abstract

Although tiger sharks are an important apex predator in many ecosystems, little is known about their reproduction. The goal of this study was to determine the size-at-maturity and the reproductive seasonality of tiger sharks in the western Atlantic Ocean. This was achieved using a combination of ultrasonography and measurements of plasma hormone concentrations; in particular, testosterone for males and estradiol and progesterone for females. Steroid hormone concentrations were measured using chemiluminescent assays (CLIA). Maturity was also examined through histology of reproductive organs in females and clasper calcification in males. Females were found to mature between 270 and 310 cm total length and males were found to mature between 260 and 300 cm total length. Mating was determined to occur in October/November, based on the presence of mating wounds on females and increased concentrations of testosterone in males. Some females were shown to exhibit increased plasma estradiol concentrations also during October/November; however, we do not believe that ovulation takes place until May or June based on ultrasonography data. This suggests a period of sperm storage although histological examination of the oviducal gland was not able to confirm this. Ultrasonography data, showing increasing embryo size over the course of a year, and data on minimum size of tiger sharks caught in longline surveys suggested that parturition occurs between June and September with pups being born as small as 56 cm fork length. The findings from this study show that some tiger sharks reach reproductive maturity at sizes smaller than what has been previously suggested. Additionally, the possibility of tiger sharks storing sperm suggests that their reproductive cycle is a minimum of two years long and could be up to three years in duration. This information is important for management of the species in the future. Additionally, this study adds to the limited knowledge about reproduction of elasmobranchs and how patterns of reproductive steroids can correlate with different reproductive events.

<u>Introduction</u>

The tiger shark (*Galeocerdo cuvier*) is an apex predator in tropical and temperate waters throughout the world (Castro, 2009). In the western Atlantic Ocean, they can be found in coastal and shelf waters from as far north as Nova Scotia, Canada, and as far south as Uruguay (Kohler et al., 1996; Domingo et al., 2016). In regions where the water is consistently warm such as Florida, they can be found year-round, but often migrate north to cooler water in the summer months (Kohler et al., 1996). Neonates tend to use shallow water, close to the continental shelf, but as they grow, they move into deeper and colder water (Afonso et al., 2015). Previous studies have shown that spatial ecology of tiger sharks could be regulated by factors such as use of seasonal foraging grounds, site fidelity, and the reproductive cycle (Afonso et al., 2015).

Tiger sharks can reach 4 to 4.5 m in total length (TL) and weigh over 800 kg (Castro et al., 2016). They have one of the most diverse diets of any shark species, with prey that includes bony fish, rays, other sharks, turtles, crustaceans, marine mammals, and even sea birds (Randall, 1992). Tiger sharks have also been found to ingest human garbage, including plastic and metal (Randall, 1992; Simpfendorfer, 1992). These predators are believed to have significant roles in maintaining the health of the ecosystems that they inhabit through top-down effects (Jaquemet et al., 2012; Heithaus et al., 2007).

The size and highly migratory nature of tiger sharks have made it difficult to study their general biology; this is also true about their reproduction (Sulikowski et al., 2016). Studies that have been conducted on reproduction to date have been hampered by small sample sizes and limited sampling periods, especially since most have relied on fishery-dependent sampling (Whitney and Crow, 2007).

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It is known that tiger sharks are the only member of the Family Carcharhinidae that do not nourish their young through a placental connection. They instead use a method of reproduction called aplacental viviparity (Castro et al., 2016). Castro et al. (2016) published a study documenting the development of tiger shark embryos *in utero*. Tiger shark embryos are initially nourished by yolk until their individual yolk supply is exhausted. At this point, they appear to ingest a yellowish fluid, which has been termed "embryotroph." Castro et al. tested the hypothesis that this uterine fluid nourishes the embryos. This was done by measuring the increase in dry weight from fertilized egg to embryo and determining the total energy content of the fluid by chemical oxygen demand. Based on low levels of protein, they concluded that protein was not the main source of nutrition for the embryos and suggested that lipids could be responsible. This method of nutrition allows them to increase their size by over 2000%. Castro et al. hypothesized that this type of embryonic nutrition would allow them to produce sizeable numbers of large pups.

Reproductive characteristics such as size at birth, length of gestation, and reproductive seasonality have been more difficult to study and document. They have been presented by several authors, but there is a large amount of discrepancy in the findings.

Whitney and Crow (2007) published a detailed study of the reproduction of tiger shark populations off of Hawaii. They used clasper calcification to determine that males mature at approximately 292 cm total length. They also determined that females begin to mature at, based on expansion of the uterus, approximately 290-300 cm and most are fully mature between 330 and 345 cm. There was a large amount of variation in litter size, but the mean was found to be 32.6 pups per litter. Pups were found to be born at between 76 and 89 cm, which was consistent

with the size-at-birth reported by Simpfendorfer (1992). With data from 23 pregnant females, they concluded that the gestation period of female tiger sharks is approximately 15 to 16 months, with mating occurring in January/ February. Whitney and Crow suggested that sperm storage may occur until ovulation, which was found to occur between May and July. However, it is unknown whether the gestation period and female reproductive cycle proposed by Whitney and Crow (2007) are true for tiger shark populations from other areas in their range.

Jaquemet et al. (2012) observed a pregnant tiger shark in the western Indian Ocean in the month of November. Between the two uteri, there were 42 embryos without external yolk sacs. The embryos were between 75 and 84.6 cm long and their teeth had not yet formed. They concluded that mating in this region occurs in the late winter based on a personal observation of mating wounds and parturition in the early summer. This was consistent with reports from northern Australia (Simpfendorfer, 1992). This also agreed with a gestation period of 15 to 16 months, as well as 4-5 months of sperm storage as was suggested by Whitney and Crow (2007).

Data in the western Atlantic Ocean does not support the hypothesis of a 15 to 16-month gestation period. Tiger sharks have been found with newly fertilized eggs in mid-May and term young in June, which suggests a gestation period of just over 12 months (Castro, 2009). Castro suggested that reproduction of the tiger shark in the western Atlantic Ocean is biennial. Females do not have active ovaries at the time of parturition, making it clear that the cycle is not annual. Clark and Von Schmidt (1965) also concluded that gestation in tiger sharks is slightly over a year based on the presence of both early and late term embryos at the same time of year. Similar discrepancies were found in other reproductive characteristics. It is possible that there is variability of these characteristics within the species and that regional studies will be necessary to, as Castro (2009) suggested, "fill in the gaps in our knowledge."

Sulikowski et al. (2016) recently conducted a study at Tiger Beach, in the Bahamas, in which 65 females were sampled between 2011 and 2014. This study used ultrasonography and blood hormone analysis to examine the reproductive cycle of tiger sharks. They found that ultrasonography was an effective tool in discerning pregnancy for tiger sharks. An unexpected result was a difficulty in linking steroid hormone concentrations to reproductive maturity. Specifically, juvenile female tiger sharks were found not to significantly differ in levels of estradiol or progesterone from gravid mature females. Furthermore, tiger sharks were found to have hormonal values that are among the lowest recorded compared to other yolk sac viviparous species. The patterns of estradiol and testosterone were still found to be comparable to those found in other elasmobranchs in that they showed decreased estradiol during pregnancy and increased range of estradiol, testosterone, and progesterone levels in mature animals. Given this, the authors concluded that the low estradiol and testosterone concentrations could be used to predict pregnancy in mature sharks greater than 300 cm total length. This finding made the tiger shark an ideal species to study to learn more about reproductive seasonality using plasma steroid concentrations. Based on the observation of mating scars at Tiger Beach, the authors suggested that mating occurs in the Bahamas during the winter. The lack of neonate tigers suggested that parturition occurs elsewhere.

Based on past studies, there are still aspects of tiger shark reproduction that remain poorly understood such as regional reproductive seasonality and size-of-maturity in females. Therefore, in this study, I examined the seasonality of tiger shark reproduction in the western Atlantic Ocean and how it is reflected in plasma concentrations of gonadal sex hormones. I expanded on the work of Sulikowski et al. (2016) by increasing sample size of females and put added focus on the reproduction of males. Sampling males allowed us to examine changes in

male testosterone concentrations in relation to evidence of copulation (i.e., mating wounds found on females), which was used to be more certain as to the timing of reproductive events. This study also investigated if analysis of the yolk precursor, vitellogenin, could be used to help distinguish between pregnant versus non-pregnant and, presumably, vitellogenic individuals

Methods

Animal collection

Data and samples from adult and subadult tiger sharks were obtained from a variety of locations through collaboration with several institutions (Table 1) between 2014 and 2017. Most adult and subadult tiger sharks used in this study (n=64) were caught in Port Royal Sound or Saint Helena Sound, South Carolina using circle hook drumlines. This passive fishing technique uses an anchor that is attached to monofilament gangion line with a swivel. The monofilament line was attached to a 20/0 baited circle hook. This allowed the shark to swim up to 25 m around the anchor when caught. The anchor was also attached to a line that led to an inflatable buoy. Bait used was typically Atlantic sharpnose shark or other types of small or large coastal sharks. Each drumline was checked 2 hours after deployment, in the order they were set.

Additional maturity data (and plasma samples, when possible) were obtained from adult and subadult tiger sharks collected from Bimini, Bahamas, 3 km to the SE of South Bimini. Animals were caught using bottom longlines of 500 m in length with gangions containing 16/0 circle hooks baited with barracuda spaced every 30 m. Fishing depth was 2-3 m. Lines were soaked for 24 hours and checked every 2.5 hours. Samples from Bimini were included because acoustic tracking data has documented that tiger sharks move between the two areas (B.

Table 1. Collection locations of tiger sharks above 150 cm TL.

Location	Females	Males Sampled	Sharks Sampled	Partnering Institution
North Carolina	5	0	5	OCEARCH
Port Royal Sound, South Carolina	45	15	60	South Carolina Department of Natural Resources
Saint Helena Sound, South Carolina	2	0	2	South Carolina Department of Natural Resources
Jupiter, Florida	3	0	3	Bimini Shark Lab
Sarasota, Florida	2	2	4	Mote Marine Lab
Fort Pierce Inlet	1	0	1	Florida Atlantic University at Harbor Branch
Florida Keys	1	0	1	Florida State University
Bimini	19	66	85	Bimini Shark Lab
Total	78	83	161	

Frazier, personal communication, May 16, 2018). Additional plasma samples were also provided from tiger sharks collected from other areas along the southeastern U.S., including: North Carolina (n = 5), Sarasota, Florida (n = 4), Jupiter, Florida (n=3), South Ft. Pierce Inlet, Florida (n = 1), and the Florida Keys (n = 1).

Once hooked, sharks were reeled in and secured along the side of the boat using a rope looped around the tail and a rope looped around the mid-section of the animal. The midline rope allowed the animal to be rotated while still in the water, so ultrasonography could be performed, and a blood sample could be collected. This method was selected to reduce stress to the shark. Sharks collected on the M/V OCEARCH were brought onto a platform out of the water, with a hose in the animal's mouth to allow respiration. Sex and stretched total length were recorded for each animal. Maturity was determined for males based on clasper size and calcification. Clasper measurements were also recorded for males in Bimini when possible (n=73). Many animals also had acoustic receivers implanted in them as part of a research project being conducted by the South Carolina Department of Natural Resources (SCDNR).

When possible samples (~2-10 mL) of blood were collected from the caudal vein using 16-gauge needles attached to a 5-mL syringe. The blood was transferred to 10-mL vials containing acid citrate dextrose anticoagulant modified for use with elasmobranchs (South Carolina animals) or coated with heparin (animals from other sites) and kept on ice until it could be processed. The majority of samples were processed within 10 hours, but Bimini samples were processed within a few hours and OCEARCH samples were processed immediately after collection. At the laboratory, blood was centrifuged for 5 minutes at 1500g. Plasma was removed and transferred to a cryovial, before being frozen at -20°C.

When possible, ultrasonography was performed on mature or near mature females (n=18)

to determine pregnancy status and litter size using the IBEX Pro (E.I Medical Imaging), a water resistant portable ultrasound unit, equipped with a 5-2.5 MHz 60 mm curved linear transducer with a 24-cm scan depth. Five of these individuals were caught off of North Carolina with OCEARCH. Two were caught at the Bimini sampling sites. Eleven were caught at sampling sites in South Carolina.

Although they were not used for reproductive assessments, data from tiger sharks below 150 cm TL were also used to estimate time of pupping from the various sampling locations. This included individuals caught off of South Carolina (Port Royal and St. Helena Sound) (n=116). Bimini, Bahamas (n = 46).

Plasma hormone analysis

Chemiluminescent immunoassays (CLIA) (Monobind Inc., Lake Forest, CA) were used to measure plasma hormone concentration following the manufacturer's instructions. Hormones that were measured include testosterone (T) in males and progesterone (P4), 17β estradiol (E2), and testosterone (T) in females. CLIA was be used because small volumes of plasma could be used to obtain hormone concentration. Percent recovery and parallelism was used to validate the assay. Three replicates were run for each sample to ensure consistency. The average concentration from the replicates for each animal was used. Samples were run at a dilution of 1/5 for estradiol and testosterone and 1/2 for progesterone. Recovery for estradiol, testosterone, and progesterone were determined to be 146%, 148%, and 73%, respectively. Recovery over 100% is not unusual in that hormones can separate from binding proteins during the assay.

Vitellogenin analysis

Presence of vitellogenin, the precursor to egg yolk, was determined using immunoblotting to evaluate if this approach could be used to provide evidence of follicular development in mature females. Three new antibodies were produced by Raybiotech, Inc. (Norcross, GA) against highly conserved portions of vitellogenin amino acid sequences in bonnethead *Sphyrna tiburo*; protein sequences were determined using gene sequence data obtained following induction of vitellogenin gene expression in male bonnetheads treated with estradiol (Gelsleichter, unpublished data). Tiger shark samples previously determined to exhibit high estradiol concentrations were initially used to examine presence of immunoreactive vitellogenin using all three antibodies at a dilution of 1/1000. This was done to determine which antibody, if any, would work best in this species. The antibody that was found to show to detect vitellogenin-like proteins in tiger shark was antibody 3, raised in rabbit, using the following peptide sequence KTDSRSERRILSKLINC (J. Gelsleichter, Unpublished data). Preliminary results suggested possible breakdown of the vitellogenin molecule, which is common in samples that are obtained without presence of protease inhibitors; immunoreactive bands were observed at molecular weights of ~60 kD and ~37 kD.

The presence of immunoreactive vitellogenin were tested in additional samples using immunoblot analysis, following the protocol of Tyminski et al. (2015).

Plasma samples were diluted to a 1/5 ratio in Laemmli buffer and heated at 95ºC for five minutes to denature proteins. A kaleidoscope protein standard was prepared by heating at 37ºC for one minute.

Separation of proteins was achieved using SDS electrophoresis, under reducing and denaturing conditions using Mini PROTEAN TGX precast gels (4-20%) (Bio-Rad, Hercules, CA). A kaleidoscope standard, with proteins of known sizes, was run on each gel to allow for

determination of the sizes of proteins in the sample. Proteins were transferred from the gel to an Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA), which was then incubated overnight, at 4ºC, in a 10% nonfat dry milk in Tris buffered saline (TBS) (TBS, 0.05 M Tris base, 0.15 M NaCl, pH 7.6) to block nonspecific binding.

After blocking, the membrane was briefly rinsed with TBS. It was then incubated overnight in a 1/1000 dilution of the primary antibody (rabbit polyclonal anti-*S tiburo* Vtg antibody 3) in 1% nonfat dry milk in T-TBS (TBS with 0.05% Tween 20) at 4ºC.

Following incubation in the primary antibody, the membrane was rinsed five times for five minutes each on a rocker in T-TBS. The membrane was then incubated in a 1/30,000 dilution of the secondary antibody (goat anti-rabbit Immunoglobulin-G conjugated with alkaline phosphatase) in 1% nonfat dry milk in T-TBS for 1 h at room temperature with light rocking. Afterwards, 5 more rinses were performed using the same procedure as after the primary antibody. The membrane was flipped between rinses.

BCIP/NBT (Vector Laboratories, Burlingame, CA) was used as chromogen to detect antigen-antibody complexes. The membrane was placed in the chromogen solution on the rocker for 5-10 minutes to react with the substrate conjugate. The membrane was then rinsed in distilled water to end the color reaction.

Analyzing ultrasonography data

Presence and number of embryos observed was counted. Level of detail observable was used to determine how developed the embryos were.

Analysis of data

　　　Changes in clasper size/calcification and plasma hormone concentrations relative to animal size were used to examine size at maturity using logistic regression and the Mann-Whitney U Test. Plasma hormone concentrations in animals believed to be mature were grouped by reproductive stage (confirmed by ultrasound or presence of mating wounds) and month of collection in order to determine the events of the reproductive cycle. A Mann-Whitney U test was used to determine in differences in concentrations pregnant and not pregnant animals existed. Spearman's rank was used to determine if a correlation existed between the E2 and P4 values. Additionally, data on small tiger shark catch from South Carolina and Bimini sites were examined to predict when pupping is likely occurring based on size at catch and time of year.

<u>Histology</u>

Although no animals were euthanatized specifically for the purposes of this project, samples of reproductive tract were obtained from archived collections from the University of Florida for histological examinations of reproductive organs. The initial purpose of this work was to examine if the oviducal gland functions in sperm storage; however, other portions of the female reproductive tract such as the ovary and uterus were also examined to gain additional information on size-at-maturity. Most of the animals examined were obtained as part of recreational fishing tournaments that took place between August 3rd 2003 and June 22nd 2005. Although fixation methods varied, most samples were fixed for a minimum of 48 hours in 10% formalin in seawater. Organs were eventually stored in 70% ethanol, which is the form in which we received the samples. A total of 7 females were examined, ranging in size from 182 and 380 cm (Table 2). Reproductive organs were subsampled and processed for routine paraffin histology following the methods of Tyminski et al. (2015). Histological sections (5 µm) were prepared using a rotary microtome and stained used Harris hematoxylin and eosin for observing general cell architecture. Slides were examined to identify structures using a light microscope.

Table 2. Collection time, animal size and sample type of histology samples.

ID#	TL	Date	Ovary	Oviducal	Uterus
06505952.03	272	6/22/05	Yes	Yes	Yes
010503301.34	271	1/1/2005	Yes	No	Yes
070405403.34	380	7/05/04	Yes	Yes	Yes
040505905.01	162	4/13/05	Yes	No	No
120301202.22	240	1/1/04	Yes	No	No
080304404.08	252	8/3/2003	Yes	Yes	Yes
100301928.007	182	10/18/2003	Yes	Yes	No

<u>Results</u>

Reproductive maturity and seasonality were determined using a combination of lethal and nonlethal methods, which are presented below separately for the sake of organization.

Nonlethal methods

Males

<u>Clasper Length/Calcification</u>

Clasper calcification was determined for 90 male tiger sharks with total lengths ranging from 90 to 381 cm. The smallest tiger shark with calcified claspers was 260 cm total length and the largest animal with uncalcified claspers was 299 cm total length. Using logistic regression, it was determined that animal size could be used to predict maturity correctly 95.6% of the time with a statistical significance of .003.   Clasper size was reported for 73 males caught off of Bimini. The average size of mature males sampled was 325.55 cm TL.   58 immature tiger sharks had inner clasper sizes of between 3 and 31 cm and outer clasper sizes of between 2 and 25 cm. 15 mature animals had inner clasper sizes of between 24 and 35 cm and outer clasper sizes of between 17.5 cm and 29 cm. The difference between the groups was found to be extremely significant, with a p value of less than 0.0001 according to a Mann-Whitney-U test (Fig. 1).
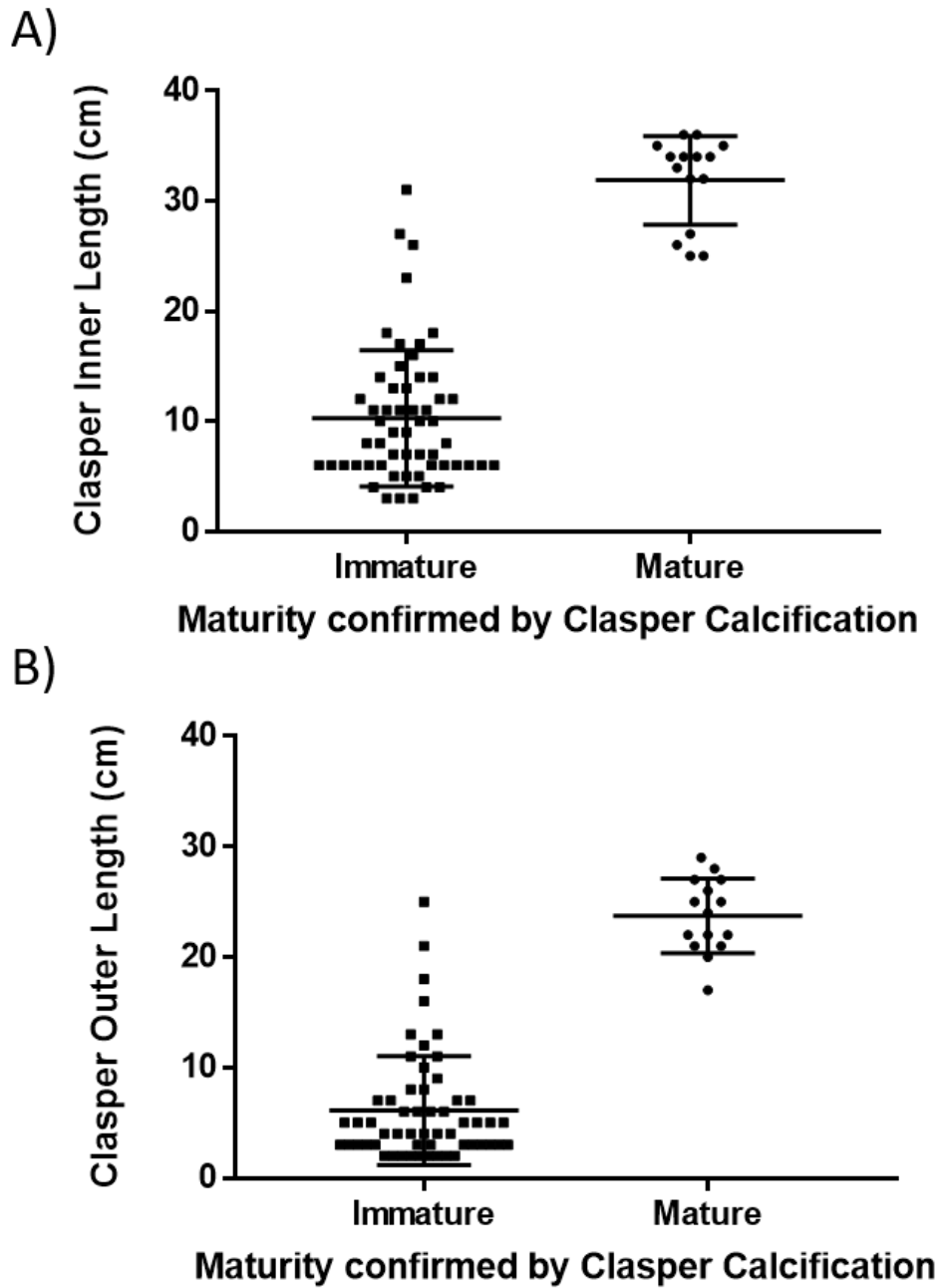
Figure 1. Mean clasper length of tiger sharks caught off of Bimini by maturity with outer bars representing standard deviation and individual data points plotted: A) Inner Clasper Length; B) Outer Clasper Length.

Testosterone was measured in 25 male tiger sharks ranging in size from 177 to 381 cm. Testosterone concentrations ranged from 0 to 17.716 ng/mL. Notable increases in testosterone concentration were observed at ~260 cm total length, a length at which some animals were observed to have a concentration of testosterone greater than ~2.5 ng/ml (Fig. 2).

Figure 2. Testosterone concentrations of male tiger sharks by total length with maturity indicated, as based on clasper calcification (n=21).

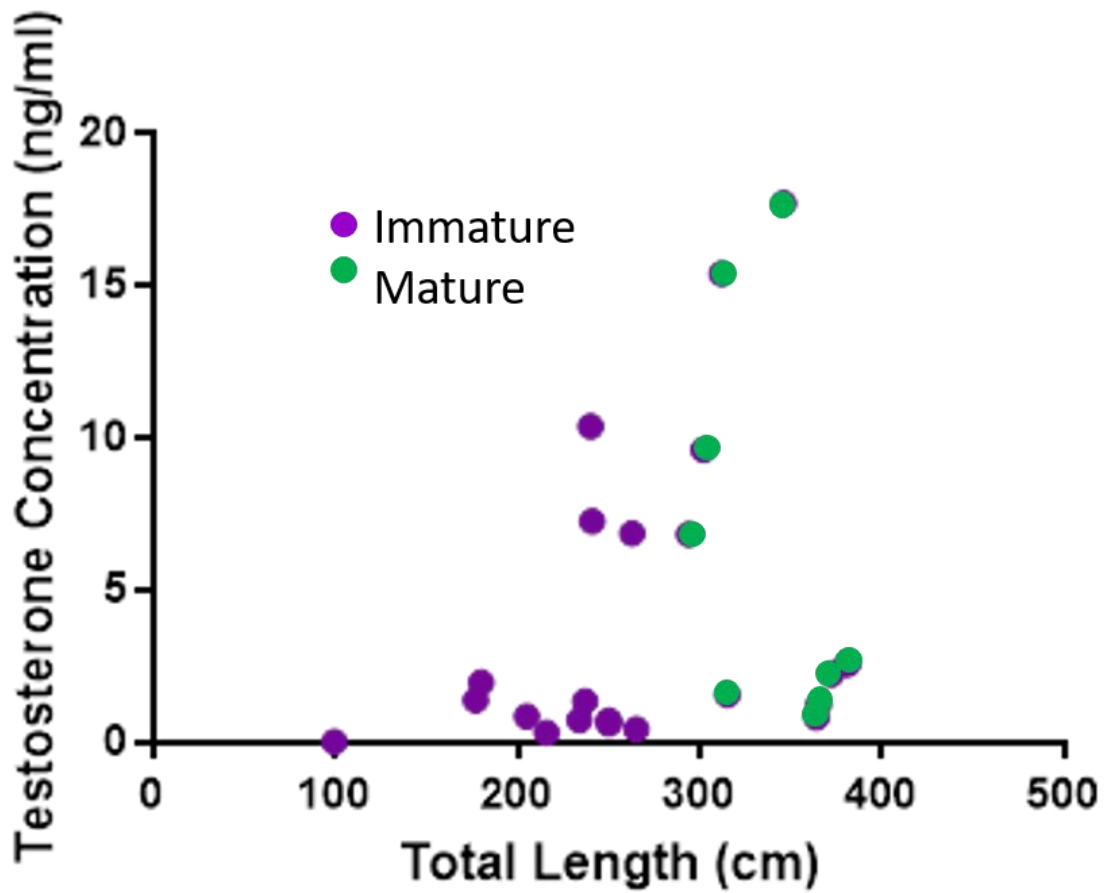Plasma testosterone concentrations were examined in a total of 9 mature males (based on clasper calcification) in relation to time of collection. A large increase in testosterone concentrations was observed in males collected in October/November, with values as high as 15.395 ng/ml and 17.716 ng/ml, compared to 1.34 ng/ml to 6.865 ng/ml earlier in the year (Fig. 3).

Females

Mating Wounds

Mating wounds (Fig. 4) were observed on 4 females, all caught in October and November, and over 330 cm in total length.

Ultrasonography

Ultrasonography was performed on 18 female tiger sharks between 240 and 396 cm TL. 6 were confirmed pregnant through ultrasonography, with the smallest confirmed pregnant animal measuring 272 cm TL. An additional animal, captured in September, was suspected to be pregnant based on visual observation of fetal movement (Frazier, pers. comm.). Embryos observed *in utero* in October were smaller and less developed than those observed in May and June (Fig. 5). Additionally, a large amount of liver displacement was visible in all gravid stages observed.

Hormone Concentrations

Estradiol (E2) was measured in 75 female tiger sharks ranging in size from 93 to 416 cm. Concentrations ranged from 43.4 to 4,125.72 pg/mL. Notable increases in estradiol

concentrations were observed in females at ~270 cm total length (Fig. 6). Progesterone (P4) was measured in 54 female tiger sharks ranging in size from 188 to 416 cm TL. Progesterone concentrations ranged from 0.38 to 9.064 pg/mL. Increased P4 concentrations were observed in a small number of animals above 270 cm in total length, with all concentrations less than 2 pg/mL below that size (Fig. 7). The animal with the highest progesterone concentration observed was only 274 cm TL.

When examining only animals above ~270 cm TL based on month of capture, a small peak in estradiol concentration was observed in May, with concentrations reaching as high as 1,902 pg/mL, and a larger peak was present in October/November, with concentrations reaching as high as 4,126 pg/mL. Estradiol for animals with probable mating wounds ranged from 81.305 pg/mL to 324.555 pg/ml. Estradiol values of confirmed pregnant animals ranged from 161 pg/mL, for an animal sampled in November, to 1,760 pg/mL, for an animal sampled in May. The other two confirmed pregnant animals sampled in May had estradiol values in the high 200 pg/mL range (Fig. 8). When examining only animals above ~270cm TL based on month of capture, no clear pattern was identified between progesterone and month (Fig. 9). There were a few animals with elevated progesterone in October (Fig. 10). Spearman's rank was used to determine degree of association between the progesterone and estradiol concentrations. It showed that there is a strong positive correlation between the variables (r_s=.498) that is statistically significant (p=.000).
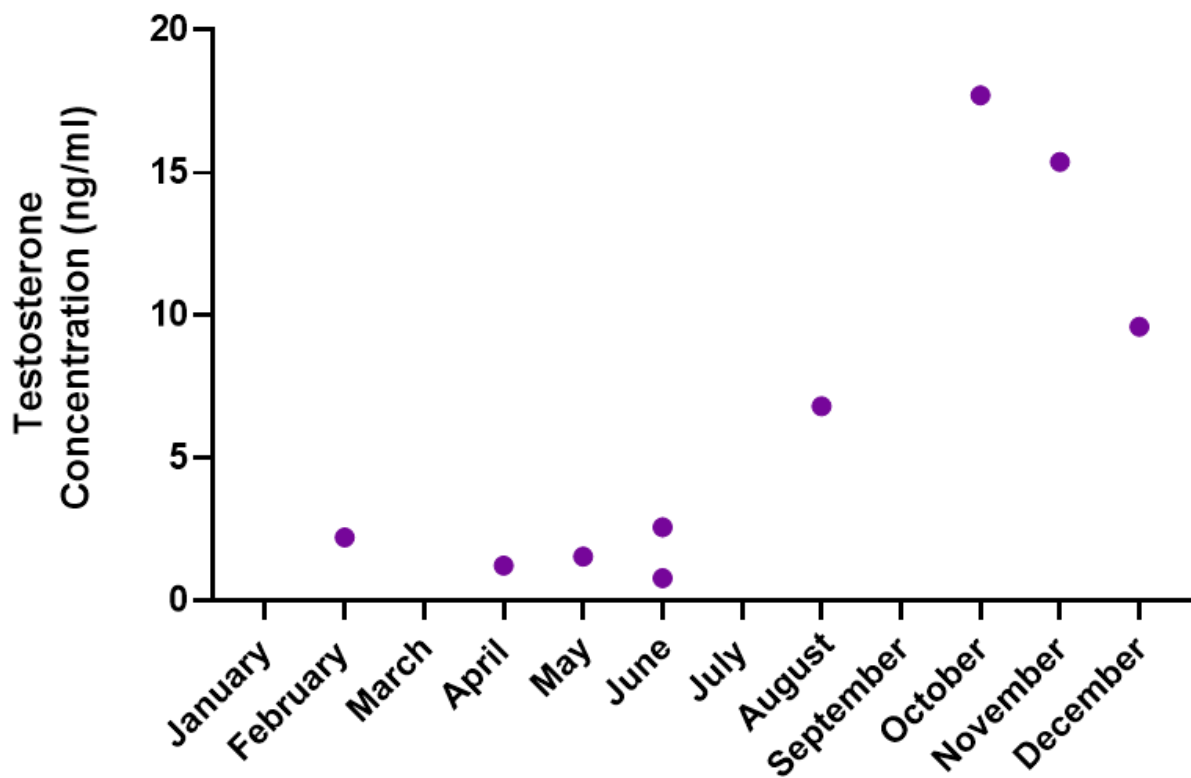
Figure 3. Testosterone concentrations of mature male tiger sharks by month (n=9).

Figure 4. Photographs of putative mating wounds on tiger sharks collected in
October and November.
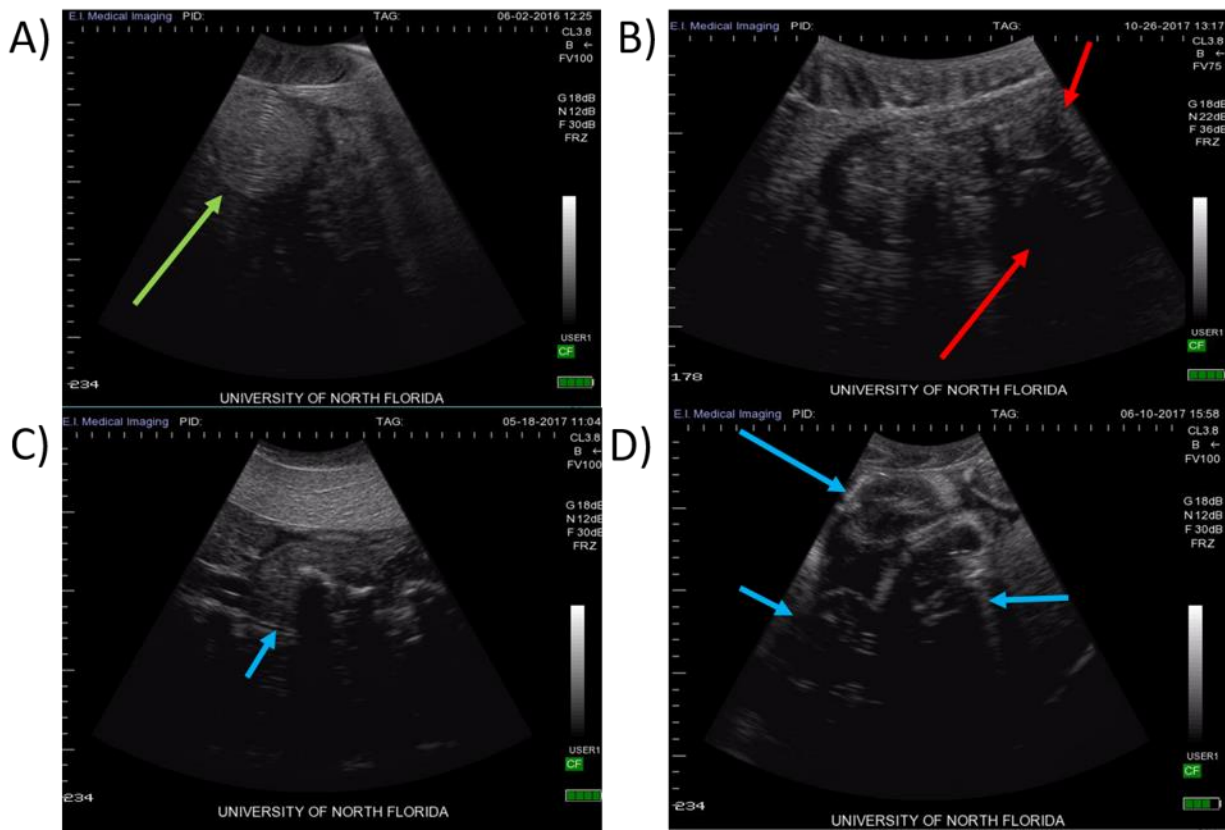
Figure 5. Ultrasonography images of tiger sharks at different reproductive stages A) non-gravid tiger shark in June, showing spiral valve (green arrow); B) mid pregnancy tiger shark in October with early pregnancy uterus (red arrows); C) late-pregnancy tiger shark in May with embryos (blue arrow); D) Near term tiger shark with embryos (blue arrows).
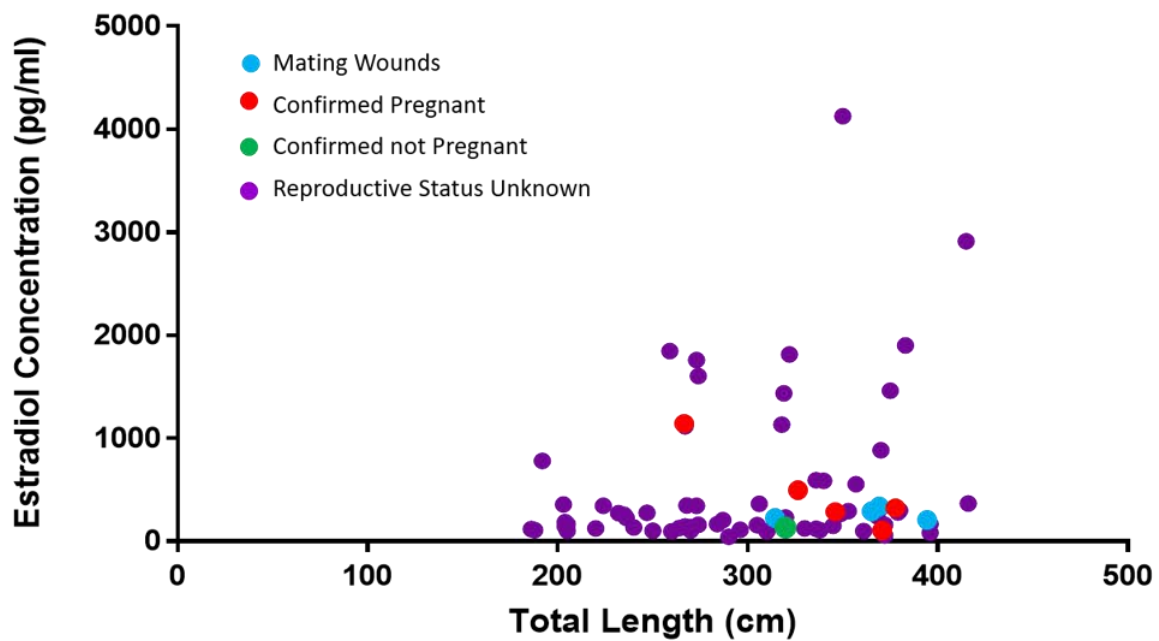
Figure 6. Plasma estradiol concentrations by total length of female tiger sharks with reproductive status indicated when possible, based on presence of external mating wounds and/or ultrasonography (n=75). All animals observed with probable mating wounds were confirmed to be not pregnant.
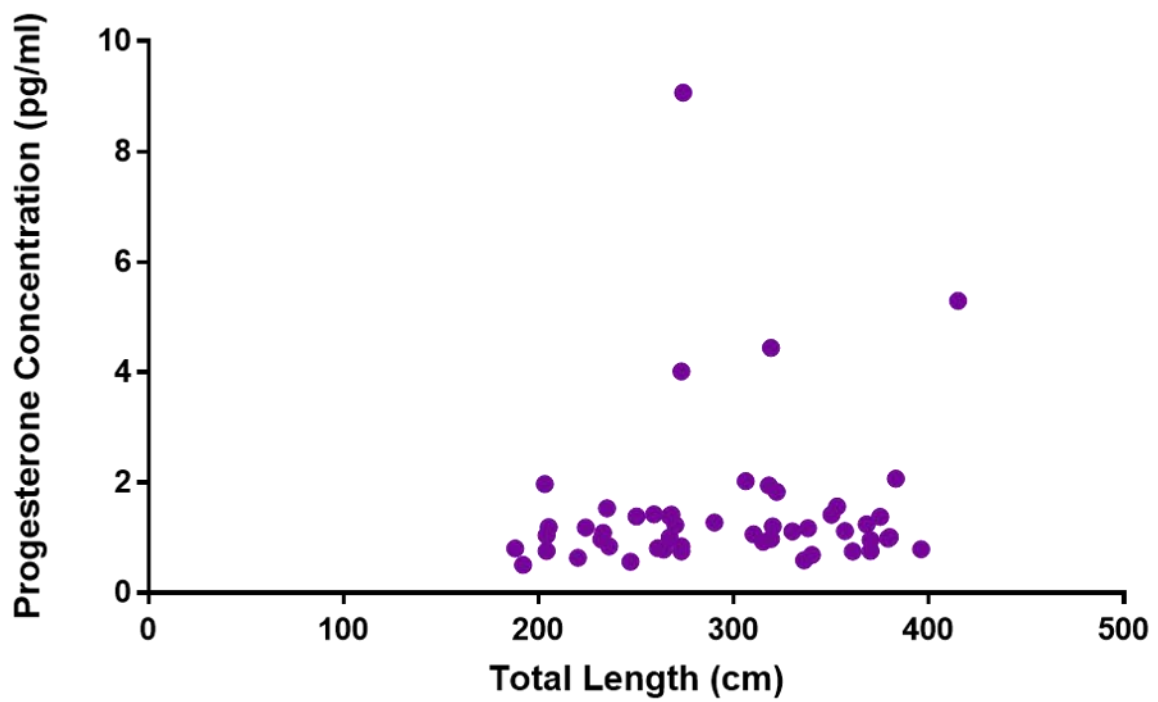
Figure 7. Plasma progesterone concentration by total length of female tiger sharks (n=54).
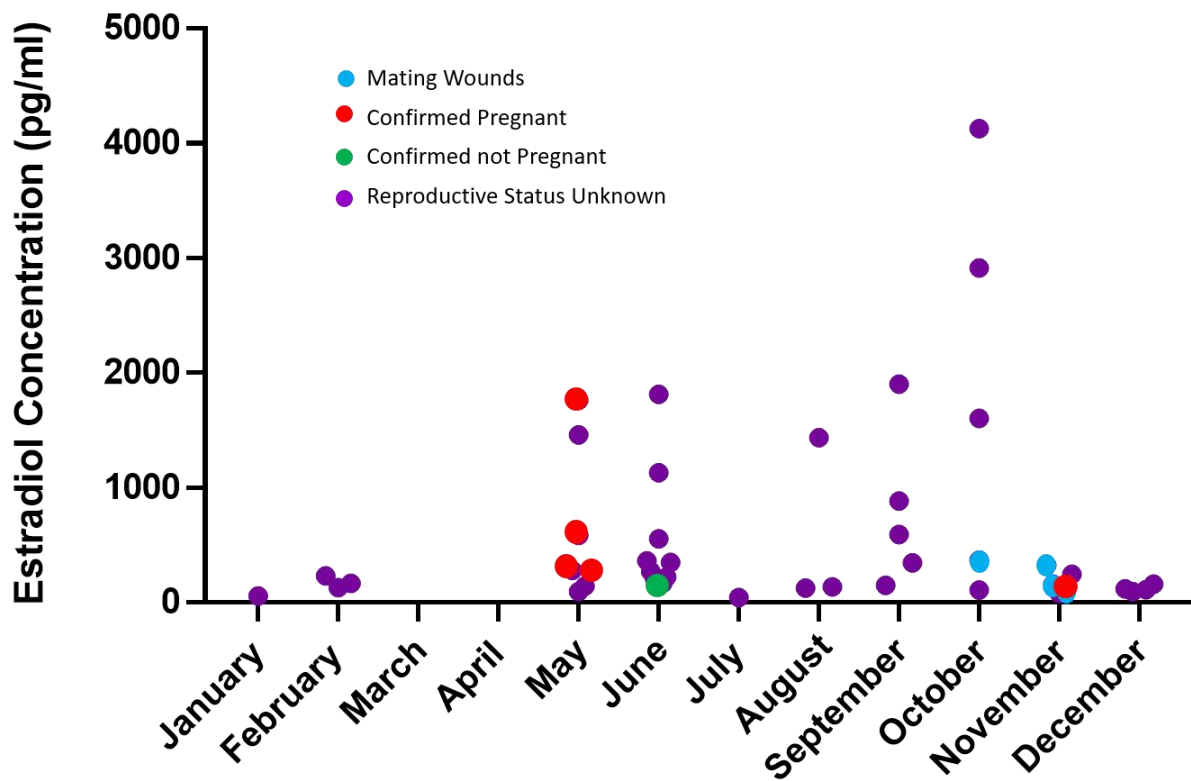
Figure 8. Plasma estradiol concentration by month for female tiger sharks (n=45) above 270 cm TL.
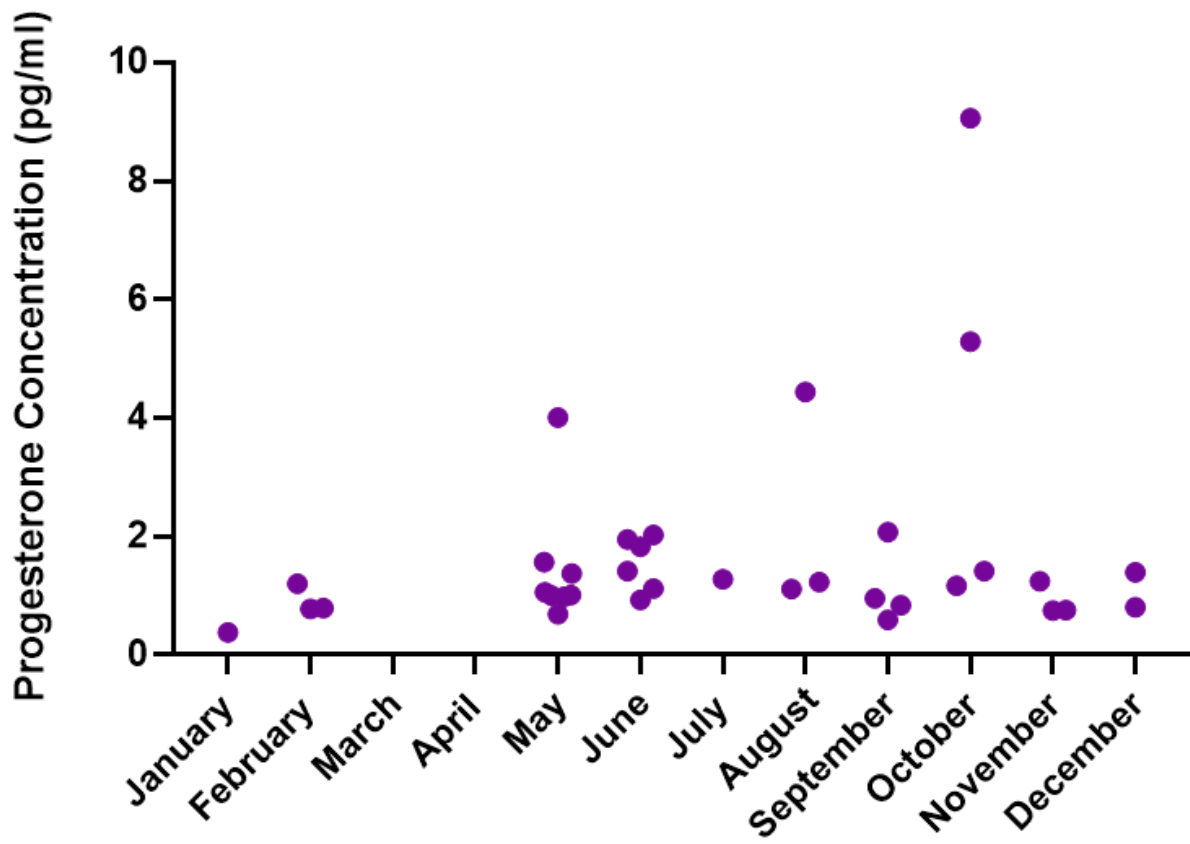
Figure 9. Plasma progesterone concentration by month for mature female tiger sharks (n=35) above 270 cm TL.
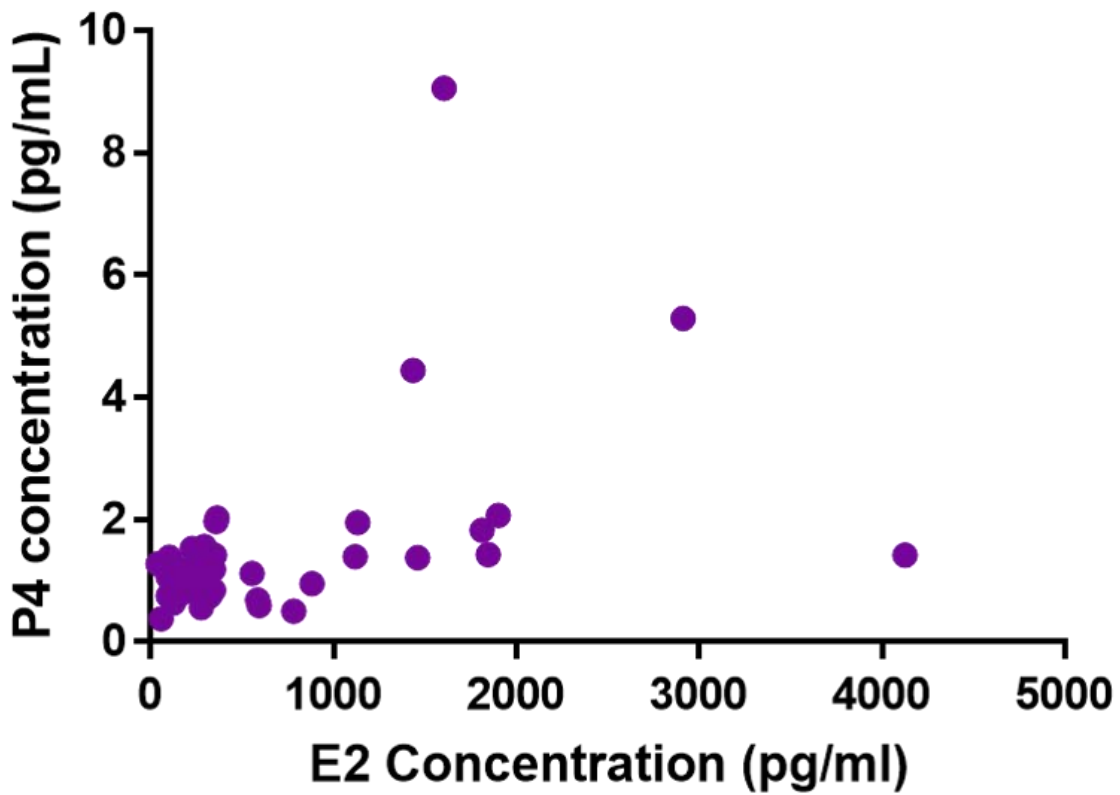
Figure 10. Concentration of estradiol compared to concentration of progesterone in female tiger sharks (n=54).

<u>Vitellogenin Analysis</u>

Western blot analysis for vitellogenin was performed on plasma of 62 female tiger sharks. Figure 11 shows a sample of the Western Blot with immunoreactive bands visible for 5 animals at ~65 kD. Immunoreactive proteins at a molecular weight consistent with that expected for vitellogenin breakdown proteins (lipovitellin, in particular) were observed in 13 animals, or ~20.9% of samples tested. 5 of these individuals were below 270 cm TL; 8 of these animals were above this size.

When examining those below 270 cm TL, immunoreactive bands were observed from animals caught in February, April, and May (Table 3). All animals who showed a band at this site were over 200 cm TL. The presence of vitellogenin in these individuals was presumed to reflect ovarian maturation.

When examining females above 270 cm TL, immunoreactive bands were observed from animals caught in January, February, April, May, June, and August (Table 4). The presence of vitellogenin in these individuals was presumed to reflect active folliculogenesis. The estradiol levels of those positive for these bands was compared to those negative for these bands and difference in means of these groups was not found to be statistically significant using the Mann-Whitney U test, with a p value of 0.78. Both pregnant and non-pregnant animals were found to be positive at this location, however no animals with mating wounds were found to be positive.

Size		B-2	B-3	B-4	B-5	B-7	B-8	B-9	B-10	B-11	B 13	B-14	B-16	B-17
~65 kD														
Sample ID	Std.	B-2	B-3	B-4	B-5	B-7	B-8	B-9	B-10	B-11	B 13	B-14	B-16	B-17
Immunoreactivity		+	+	+	+	+	-	-	-	-	-	-	-	-

Figure 11. Western blot for vitellogenin showing faint Immunoreactive bands of putative vitellogenin break-down proteins in 5 of 13 animals. Bands were also observed at other sizes that are not consistent with the size of vitellogenin breakdown proteins.

Table 3. Data for animals below 270 cm TL with positive immunoblot bands at ~60 kD using rabbit polyclonal anti-*S tiburo* Vtg antibody 3.

ID	Total Length (cm)	Month
BGC5	236	April
BGC2	203	February
BGC27	204	May
BGC18	267	May
GC50	268	May

Table 4. Data for animals above 270 cm TL with positive immunoblot bands at ~60 kD using rabbit polyclonal anti-*S tiburo* Vtg antibody 3. Pregnancy status determined using ultrasonography. N/A = no ultrasound data available.

ID	Total Length (cm)	Month	Reproductive Stage
BGC7	372	January	N/A
BGC21	320	February	N/A
BGC5	236	April	N/A
BGC4	297	April	N/A
BGC24	355	May	Pregnant
GC47	315	June	Not Pregnant
GC60	315	June	N/A
BGC3	270	August	N/A
BGC26	330	August	Pregnant

163 tiger shark pups were collected during this study. Fork length (FL) was used because total length was not recorded for all animals. Figure 12 shows the size of small tiger sharks caught compared to time of year. The smallest tiger shark (56 cm FL) was caught in July. The smallest tiger sharks caught in months following that were increasingly larger. For example, the smallest tiger shark caught in October measured 60 cm FL and the smallest tiger shark caught in January was 80 cm FL

Figure 13 shows the predicted timeline of one reproductive cycle.
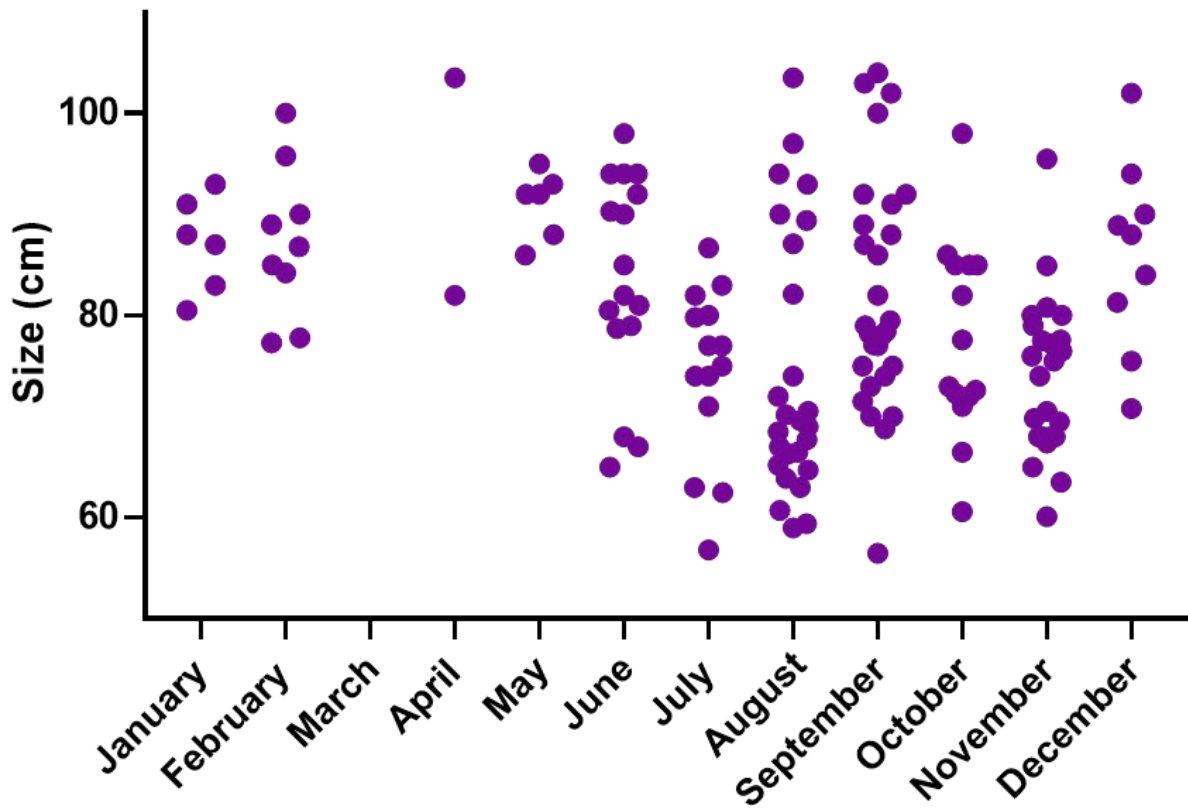
Figure 12. Fork length of tiger sharks below 110 cm fork length by month of catch.

Mating	Sperm Storage						Ovulation	Pregnancy			
Oct	Nov	Dec	Jan	Feb	March	April	May	June	July	August	Sept

Pregnancy Continued							Parturition				
Oct	Nov	Dec	Jan	Feb	March	April	May	June	July	August	Sept

Figure 13. Predicted timeline of one reproductive cycle.

Ovary

Macroscopically, ovaries from 2 animals measuring less than 220 cm total length were much smaller and had no visible developing follicles (Fig. 14). They were approximately 8 cm long, 3 cm wide, and 0.5 cm thick. The ovary from an animal with a total length of 272 cm measured 9 cm long, 7 cm wide, and 3 cm thick. It also had considerably more visible follicles. The ovary from an animal with a total length of 380 cm measured 12 cm long, 7 cm wide, and 4 cm thick, and also contained visible follicles.

Oocytes from multiple stages of development were observed in each animal examined using histology (Fig. 15). Immature and maturing animals had early stage oocytes, primordial follicles, primary follicles, and pre-vitellogenic follicles. Early stage oocytes were all less than 50 μm across. Some had clear nuclei with nucleoli present and none had a defined layer of pre-granulosa cells sur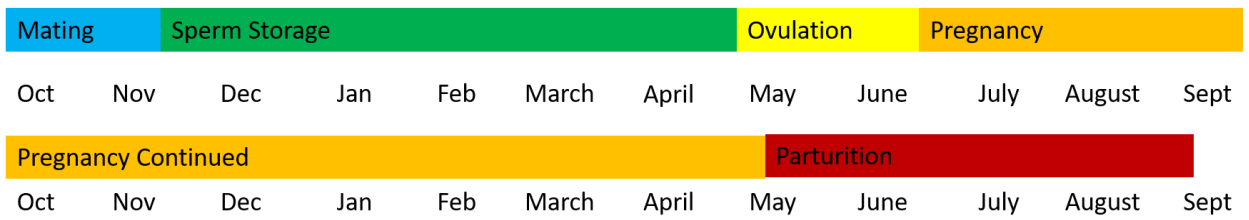rounding them. More developed follicles were about twice the size and had a single layer of flat, pre-granulosa cells surrounding the oocyte, which still had a visible nucleus. Follicles further in development were even larger (~250 μm) and had cuboidal granulosa cells surrounding the oocyte. The nucleus was no longer visible and a thin zona pellucida was present between the granulosa cells and the oocyte. Additionally, a few "globe-shaped" cells (Diaz Andrade et al., 2011) were visible between the zona pellucida and the oocyte. Larger follicles had a much thicker zona pellucida and more globe-shaped cells between the zona pellucida and the oocyte. Epithelial cells were columnar and pseudostratified. In the final stages observed,

Immature-186cm TL	Maturing- 272cm TL	Mature- 380cm TL
A) E	B)	C) E

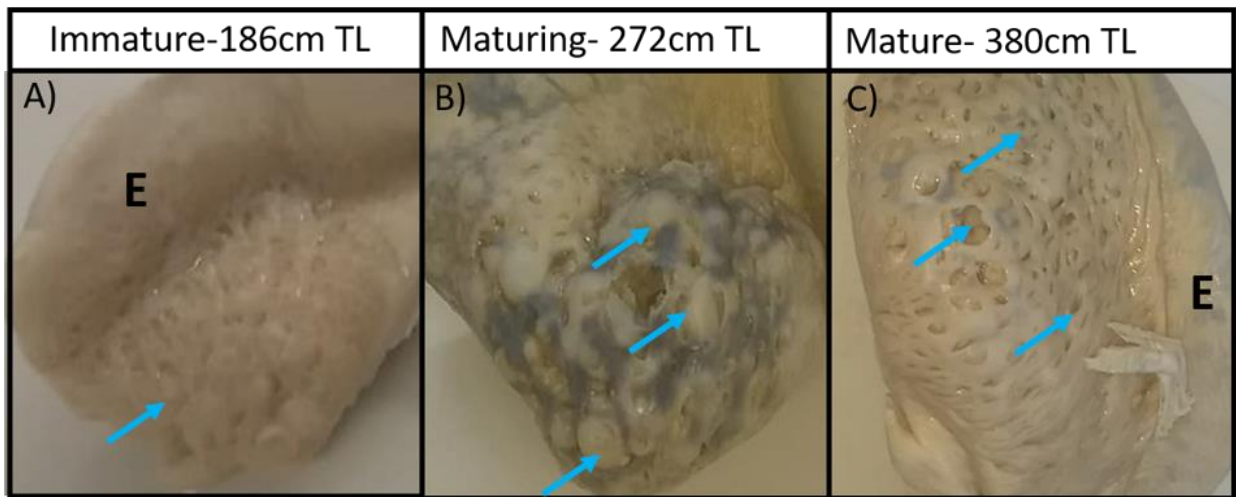Figure 14. Ovary photographs: A) Smallest ovary: 8cm long x 3cm wide x .5cm thick with small follicles (blue arrows) and ovary covering small portion of the epigonal organ (E); B) Midrange ovary: 9cm long x 7cm wide x 3cm thick with much larger follicles (blue arrows); C) Largest ovary: 12cm long x 7cm wide x 4cm thick with large follicles (blue arrows) and more of the epigonal organ (E) covered by ovary.
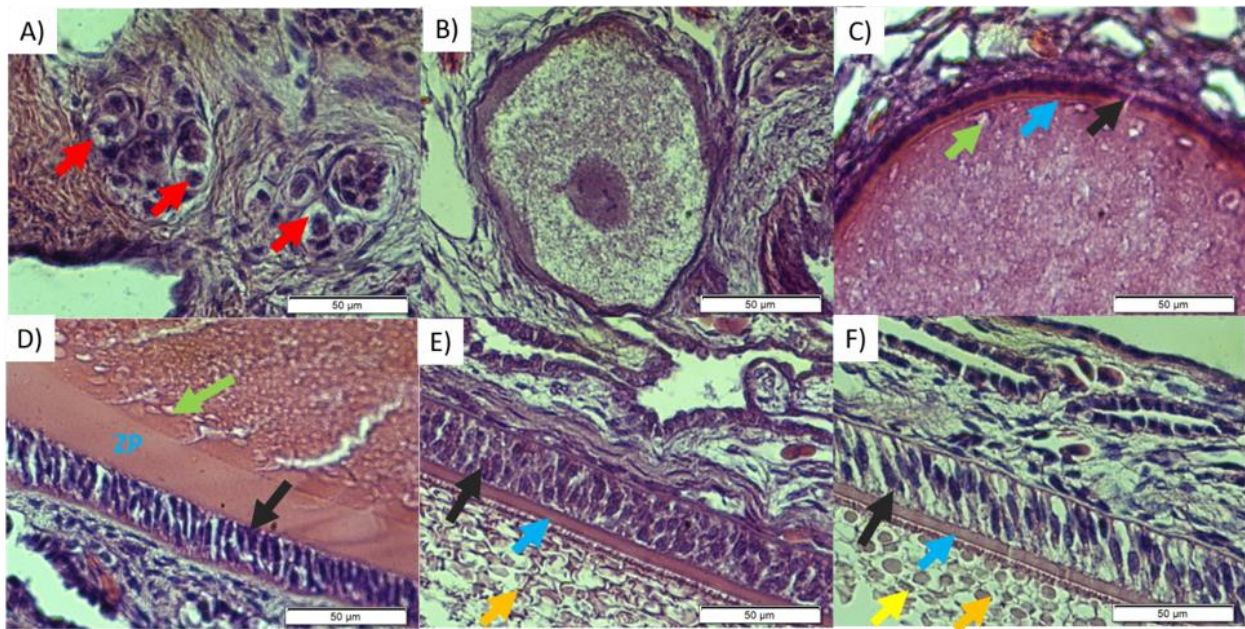
Figure 15: Histological architecture of female tiger shark ovaries demonstrating stages of ovarian development. Follicles are arranged from least to most developed: A) early stage oocytes with nuclei present (red arrows); B) primordial follicle with layer of flat epithelial cells, nucleus still present; C) primary follicle with cuboidal epithelial cells (black arrow) and zona pellucida (blue arrow) and globe-shaped cells (green arrow); D) pre-vitellogenic follicle with simple columnar epithelial cells (black arrow), thicker zona pellucida (ZP) and globe shaped cells (green arrow); E) pre-vitellogenic follicle showing pseudostratified columnar epithelial cells (black arrow), zona pellucida (blue arrow), and ooplasmic vacuoles (orange arrow); F) vitellogenic follicle showing pseudostratified columnar granulosa cells (black arrow), zona pellucida (blue arrow), and ooplasmic vacuoles (orange arrow) containing growing yolk platelets (yellow arrow). Scale bar = 50 μm.

vacuoles can be seen in the ooplasm, especially along the zona pellucida, which was thinner than it was in the previous stage. In the largest animal examined, these vacuoles show the beginning of yolk platelet development.

Follicles were also observed in some immature animals that were undergoing atresia. In these follicles, the epithelial cells appear to fold into the follicle (Fig. 16). The epithelial layer can be seen degenerating. Additionally, cells in the atrum and epithelium have clearly developed pyknotic nuclei.

Oviducal gland

Oviducal glands were obtained from 4 animals, ranging in size from 186 to 380 cm TL (Fig 17). Oviducal glands from animals below 220 cm TL were barely differentiated from the uterus and measured ~2 cm in width. Histological examination showed loose connective tissue with no evidence of secretory tubules. The luminal epithelium was straight and consisted of simple columnar epithelial cells approximately 40 μm in height. The oviducal gland from the animal measuring 272 cm TL was more differentiated from the uterus and measured ~5 cm in width. Histological examination showed some secretory tubules in the connective tissue. Additionally, invaginations were seen in the epithelium lining the lumen. The epithelial cells were pseudostratified columnar and the epithelial layer was thinner than the smaller animals, at ~20 μm thick. The largest animal had an oviducal gland that was significantly differentiated from the uterus and measured ~12 cm in width. Histological examination showed that considerably more of the loose connective tissue had been filled by secretory tubules and invaginations were more pronounced. The epithelial cells were pseudostratified columnar and the epithelial layer was about the thickness of the smaller animal, but considerably thicker than the
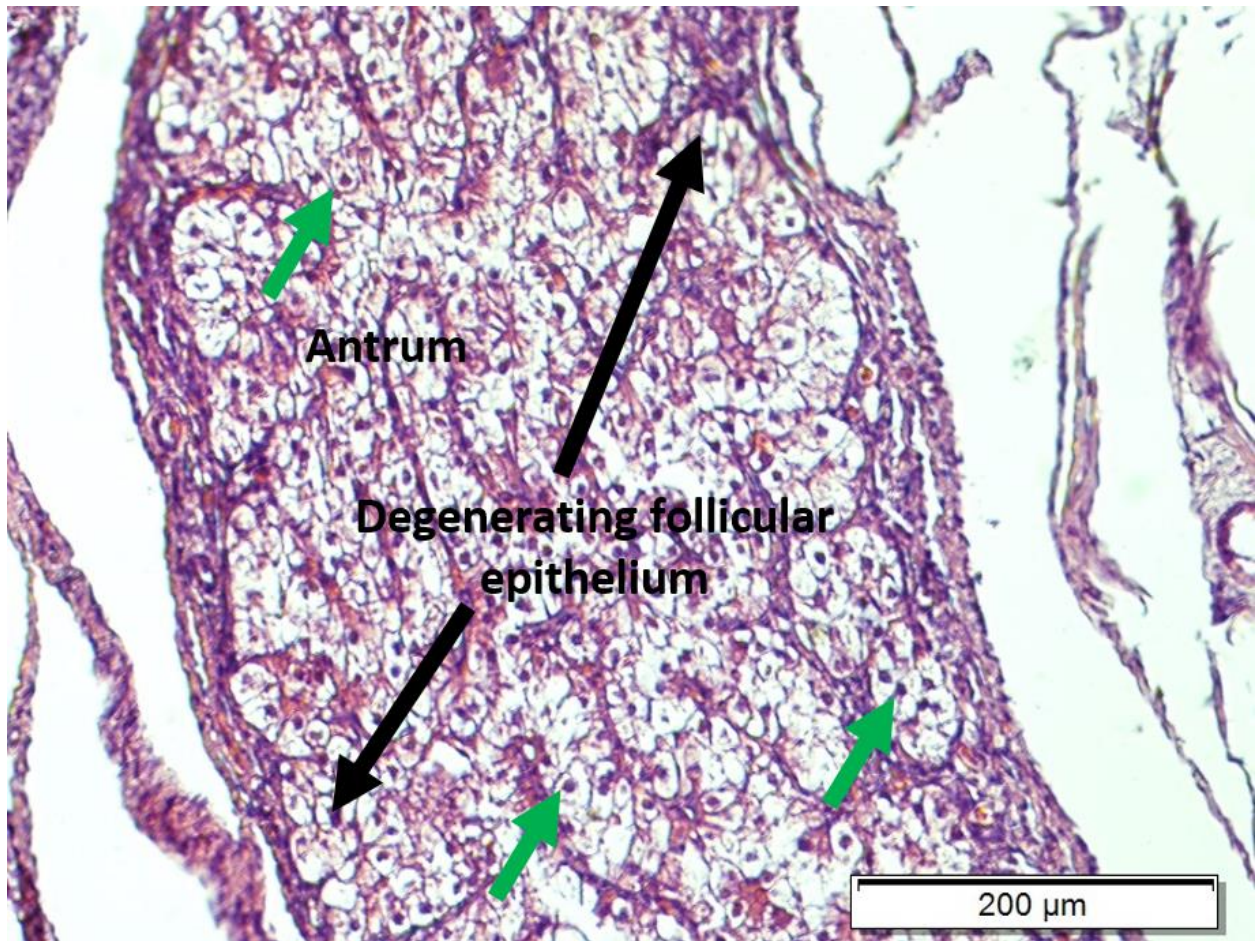
Figure 16. Atretic follicle with follicle lining folding in on follicle and antral cells with pyknotic nuclei (green arrows)
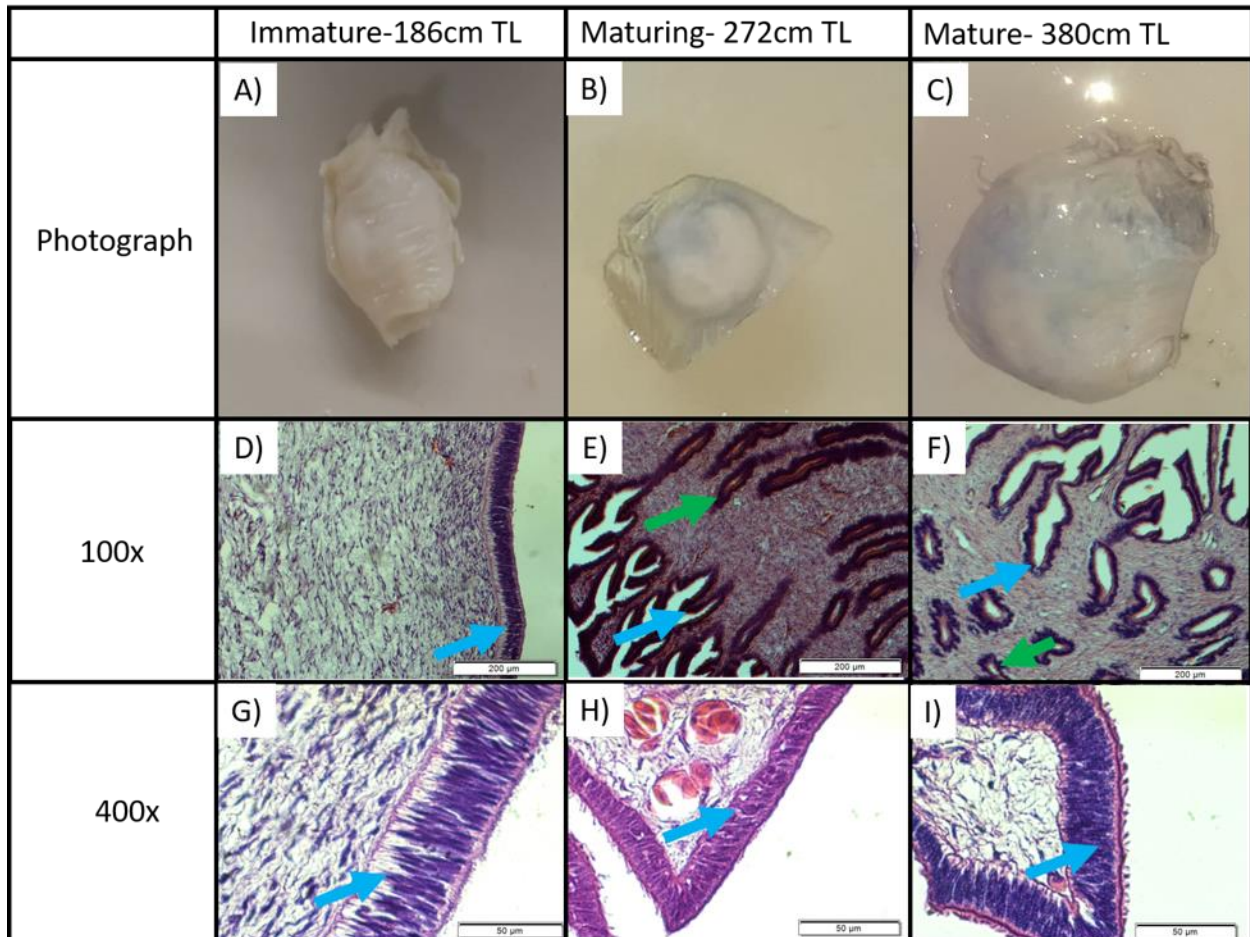
Figure 17. Morphology and histology of oviducal glands from females tiger sharks at different stages of maturity: A) Macroscopic photograph of an immature oviducal gland ~2 cm wide; B) Macroscopic photograph of a maturing oviducal gland ~5 cm wide; C) Macroscopic photograph of a mature oviducal gland ~12 cm wide; D) 100x magnification of immature oviducal gland demonstrating lack of secretory tubules and epithelial folding, Scale bar =200 µm; E) 100x magnification of maturing oviducal gland showing epithelial folding (blue arrow) and secretory tubules formation (green arrows), Scale bar =200 µm; F) 100x magnification of mature oviducal gland showing epithelial folding (blue arrow) and presence of secretory tubules (green arrows), Scale bar = 200 µm; G) 400x magnification of an immature oviducal gland with unfolded epithelium (blue arrow) composed of simple columnar epithelial cells, Scale bar =50 µm; H) 400x magnification of a maturing oviducal gland with branched epithelium (blue arrow) composed of pseudostratified columnar epithelial cells, Scale bar =50 µm; I) 400x magnification of a mature oviducal gland with branched epithelium (blue arrow) composed of pseudostratified columnar epithelial cells, Scale bar = 50 µm.

animal with a total length of 272 cm, at ~40 µm. The presence of spermatozoa was not observed in any of the oviducal glands examined; however, none of the samples obtained were collected from the proposed period of sperm storage.

Uterus

The uteri of 3 tiger sharks measuring between 270 and 380 cm total length were examined (Fig 18). The uterus of the smallest animal was approximately 3 cm wide and 2 cm thick. The uterus of the largest animal was approximately 5 cm wide and 7 cm thick.

Microscopically, distinct layers of cells were observed lining the lumen of the uterus (Fig 19). In the smallest animal, directly lining the uterus, was a layer of secretory cells, approximately 200 µm thick. This was followed by a layer of pseudostratified columnar epithelial cells, approximately 20 µm thick. Below that was a thin layer of dense connective tissue, followed by a layer of loose and highly vascularized connective tissue.

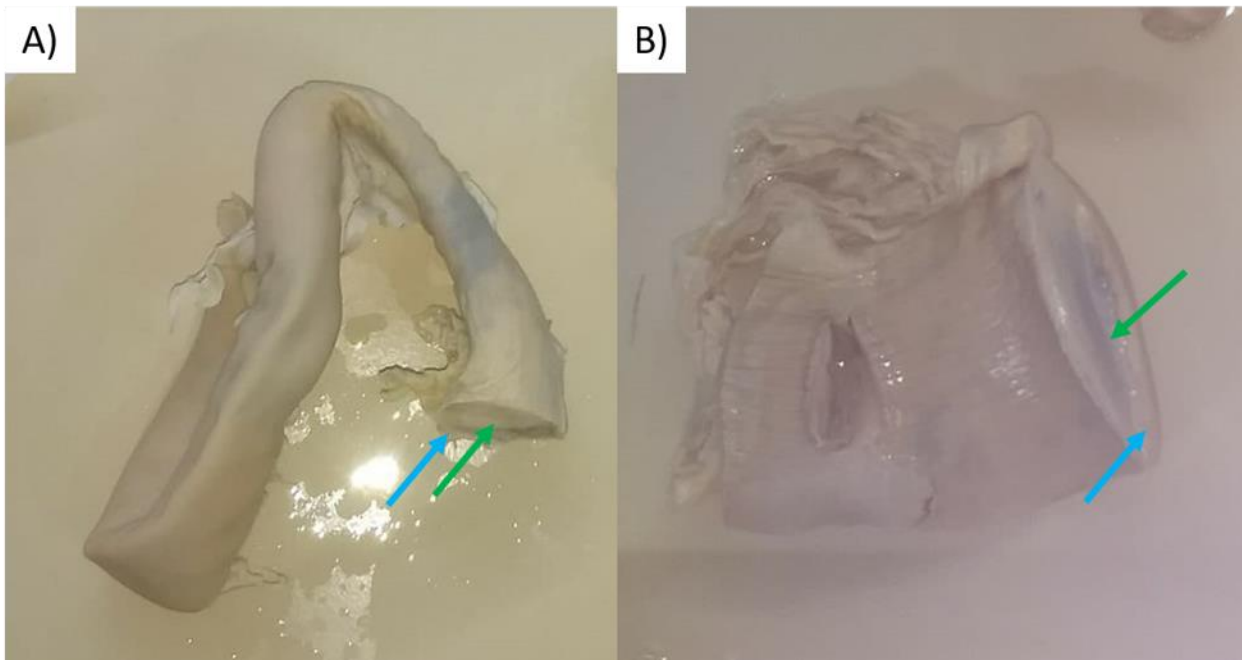Figure 18. Uterus photos: A) uterus (~3 cm wide) of animal with total length of 372 cm, showing thin perimetrium (blue arrow) and myometrium (green arrow); B) segment of uterus (~5 cm wide) of animal with a total length of 380 cm, showing thin perimetrium (blue arrow) and myometrium (green arrow).
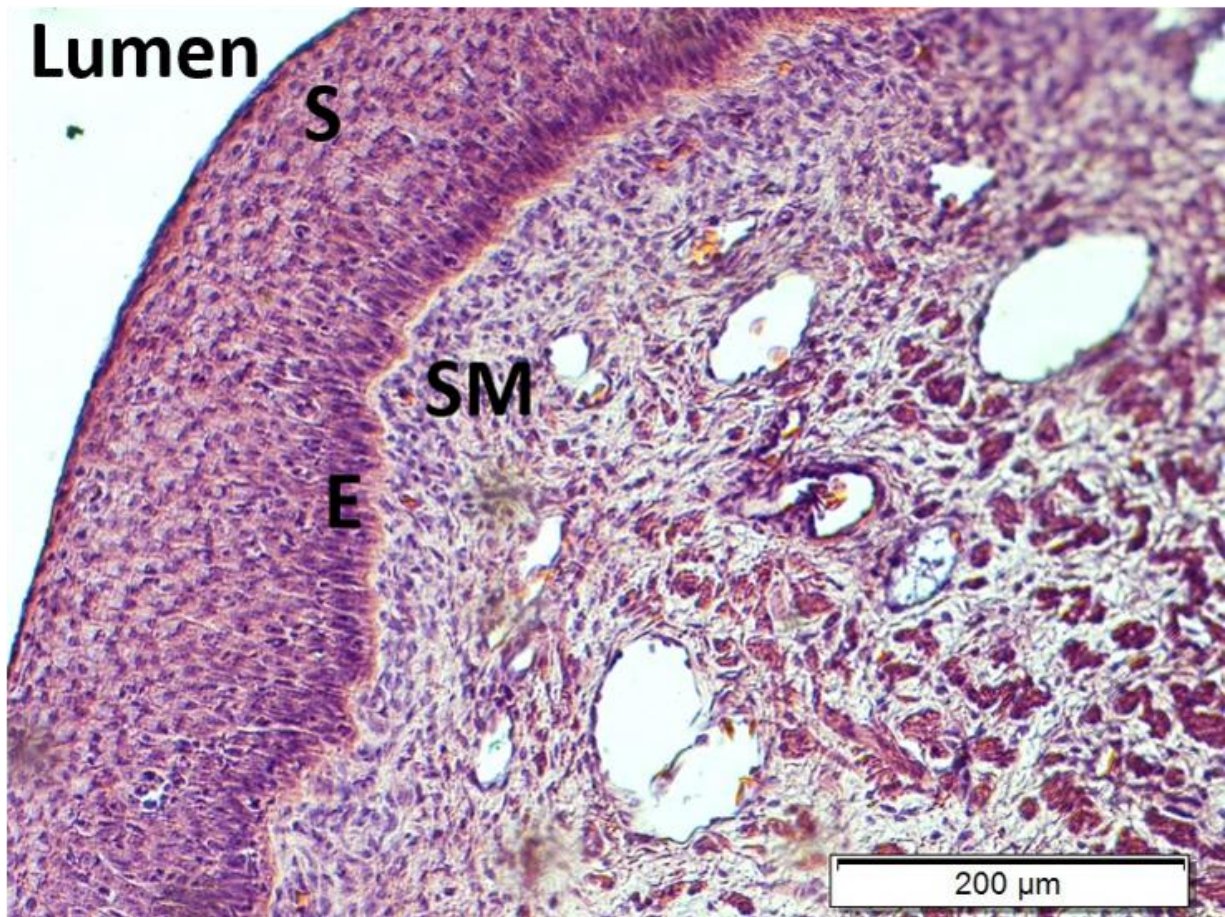
Figure 19. Uterus Histology: A) uterine lining of animal with TL of 272 cm. S: secretory layer, E: endometrial layer, SM: Submucosa.

Discussion

Maturity

Clasper length and calcification are the accepted standards for determining maturity in male elasmobranchs (Whitney and Crow, 2007). The smallest tiger shark observed with calcified claspers was 260 cm TL and the largest with uncalcified claspers was 299 cm TL, suggesting that maturity occurs between these sizes. Examination of 73 male tiger sharks for clasper size showed that maturity occurs between inner clasper size of 24 and 31 cm and outer clasper size of 17.5 to 25 cm. All animals above this range were found to have calcified claspers. This is similar to the findings of Whitney and Crow (2007), who found that claspers less than 21 cm are typically uncalcified and those greater than 25 cm are typically calcified. Estimates for size-at-maturity are supported by the increase in plasma testosterone concentrations beginning at that size. Testosterone is an androgen and increased androgen concentrations have been found to correlate with increased clasper length (Callard et al., 1991). This size at maturity for males is also consistent with what was proposed by Whitney and Crow (2007) for tiger sharks off of Hawaii. The smallest male with calcified claspers sampled in their study was also 260 cm TL, and the largest with uncalcified claspers was 314 cm TL. Clark and Von Schmidt (1965) sampled tiger sharks in the Gulf Coast. They reported that maturity occurs in males at around 290 cm TL. However, it is likely that maturity could occur in some smaller tiger sharks and this was not detected with their small sample size of only 6 mature males.

Female maturity is more difficult to determine in sharks than male maturity because of the lack of external reproductive traits. Results of this study suggest that maturity can occur in female tiger sharks as small as 270 cm TL. This is based on the determination of pregnancy in

sharks of that size. It is also supported by increases in the range of estradiol and progesterone beginning at that size, as well as observed development in reproductive organs.

A tiger shark examined in this study with a total length of only 272 cm was confirmed to be pregnant via ultrasonography. A pregnant tiger shark of ~270 cm TL was also observed by Sulikowski at Tiger Beach, Bahamas (J. Sulikowski, personal communication, Jun 19, 2017). Clark and Von Schmidt (1965) examined 41 females off the Gulf Coast and the smallest mature animal they found was 297 cm TL. Off Australia, Simpfendorfer (1992) found a pregnant tiger shark of 282 cm TL. However, Whitney and Crow (2007) suggested that maturity in females occurs between 330 and 340 cm TL off Hawaii based on widths of the oviducal gland and uterus, with the smallest pregnant animal sampled measuring at 340 cm TL. These differences could be caused by a number of factors. Sample size and sampling method, as well as the method of assigning maturity stage are known to modify the estimate of maturity (Porcu et al., 2014). It is also likely that regional differences exist between the population off the east coast of the United States mainland and those off of Hawaii.

A clear increase in the range of plasma estradiol concentrations was observed in tiger sharks beginning at approximately 270 cm TL. Estradiol is known to increase at sexual maturation in females (Rasmussen and Gruber, 1993). Additionally, the highest recorded value for progesterone was measured in an animal with a total length of only 274 cm. Rasmussen and Gruber (1993) suggest that these high levels of progesterone in maturing animals could indicate a role of progesterone in the maturity process. However, the low number of individuals exhibiting measurable progesterone levels in our study does not support this premise.

Although no tiger sharks were sacrificed for this project, samples of reproductive tract

were obtained from archives at the University of Florida and examined histologically in order to gain information that could not be obtained with other methods. Histological analysis of gonads has been shown to be very useful in many elasmobranchs and teleosts in which the gonads change appearance considerably between immaturity and maturity and throughout the reproductive cycle (Henderson and Arkhipkin, 2010; Porcu et al., 2014). We were also hopeful that we could use these samples to determine if sperm storage occurs in female tiger sharks; however, we were unable to obtain samples from individuals collected during the probably period of sperm storage.

The reproductive system of the female elasmobranch consists of a pair of ovaries, along with paired reproductive tracts consisting of oviduct, the oviducal glands, and uteri. The uteri are attached to the urogenital sinus (McMillan, 2007).

The ovary of non-lamniform sharks is on the flat surface of the epigonal organ (Rego et al., 2013). Ovaries are responsible for generating germ cells, accumulating yolk, and synthesizing and secreting hormones (Rego et al., 2013). Studying ovarian tissue at a microscopic level has been shown to reveal differences between developmental stages determined using macroscopic data (Diaz Andrade et al., 2011). The ovary is also the most common structure used for maturity determination in female elasmobranchs (Ebert, 2005).

Although length of the ovary did not seem to change much as the animals matured (8-12 cm), width increased by a higher proportion (3-7 cm) and thickness even more so (0.5-4 cm). The increase in length and width is presumably due to the growth of the animal, but the increase of thickness is likely due to the development and growth of follicles. Follicles could not be distinguished at all macroscopically in the smallest and, presumably, immature specimens. The

lack of ability to distinguish follicles macroscopically in immature animals has also been shown by Henderson and Arkhipkin (2010), for the white spotted skate (*Dentiraja cerva*)*,* and Porcu et al. (2014) for the velvet belly lantern shark (*Etmopterus spinax*).

The ovary of the next largest animal (272 cm TL) was larger than that of the immature animals, but only slightly smaller than that of the largest and confirmed mature animal. Large, well developed follicles were visible macroscopically. The increase in ovary size and presence of well-developed oocytes in maturing animals has been demonstrated by Henderson and Arkhipkin (2010) for the white spotted skate and Rego et al. (2013) for the silky shark (*Carcharhinus falciformis*).

Follicular stages observed in the ovaries of tiger sharks in this study ranged from early-stage oocytes to vitellogenic follicles. Multiple stages of development were often observed within a single animal. This has been shown by a variety of authors (Diaz Andrade et al., 2009; Diaz Andrade et al., 2011; Serra Pereira et al., 2011; Porcu et al., 2014) for the big-nose fan skate *(Sympterygia acuta)*, the smallnose fanskate *(Sympterygia bonapartii)*, the thornback ray (*Raja clavata*), and the velvet belly lantern shark, respectively.

Early stage oocytes exhibited a visible nucleus with nucleoli, without a layer of pre-granulosa cells surrounding it. Uribe and Guillette (2000) also determined that a layer of follicular or pre-granulosa cells did not surround such early-stage oocytes in the American alligator (*Alligator mississippiensis*).

More developed follicles were larger and consisted of a primary oocyte with a layer of flattened pre-granulosa cells surrounding it. This is consistent with observations by Porcu et al.

(2014) of primordial oocytes in the velvet belly lantern shak and Uribe and Guillette (2000) for the American alligator. These authors described primordial follicles as being surrounded by a single layer of flattened follicular cells.

In more developed follicles, pre-granulosa cells appeared to be cuboidal in shape. Additionally, the zona pellucida was visible between granulosa cells and the oocyte. A few globed cells were also observed between the oocyte and the zona pellucida. Uribe and Guillete (2000) found that granulosa cells in the American alligator (*Alligator mississippiensis*) became thicker as the follicle got closer to vitellogenesis, Uribe and Guillette (2000) also showed that the zona pellucida forms at this time in the American alligator. The appearance of globed cells between the zona pellucida and the oocyte was reported by Diaz Andrade et al. (2011) as characteristic of primary follicles in the smallnose fanskate *(Sympterygia bonapartii)*.

More developed follicles had pseudostratified columnar epithelial cells, a much thicker zona pellucida, and more globed cells. Porcu et al. (2014) found that the follicular epithelium thickened into a columnar double layer of cells in the velvet belly lantern shark. Other sources (Diaz Andrade et al., 2009; Diaz Andrade et al., 2011; Serra Pereira et al., 2011; Porcu et al., 2014) reported that no follicles were found past this stage of development in immature animals, for the bignose fanskate, the smallnose fanskate, the thornback ray, and the velvet belly lantern shark, respectively.

In the animals examined in this study, pre-vitellogenic follicles were observed in immature, maturing and mature animals. These follicles had a pseudostratified columnar layer of granulosa cells and a zona pellucida. The globe shaped cell were no longer visible. Additionally, vacuoles appeared to be forming from the center of the oocyte and expanding out to the zona

pellucida. The formation of ooplasmic vacuoles was demonstrated by Uribe and Guillette (2000) to be a sign of late pre-vitellogenesis in the American alligator.

The most advanced stage of follicle development observed in this paper was only observed in the largest animal examine, with a total length of 380 cm. We have identified this stage as early vitellogenesis; granulosa cells were pseudostratified and columnar. Additionally, ooplasmic vacuoles observed in the previous stage were showing formation of yolk platelets. This is indicative as the beginning of vitellogenesis and has been demonstrated as such by Uribe and Guillette (2000), and Porcu et al. (2014).

The oviducal gland is located between the oviduct and the uterus of elasmobranchs and has several important functions (Porcu et al., 2014). It is the site of fertilization and is responsible for the production of egg investments and coverings, the transport of fertilized eggs, and, in some species, the storage of sperm (Hamlett et al., 1999). The structure and development of the oviducal gland are related to the stage of maturity of the animal and the main morphology of the oviducal gland is comparable across most chondricthyans (Porcu et al., 2014).

Oviducal glands (~2 cm wide) were examined from two tiger sharks with total lengths of less than 220 cm. Both had very little differentiation from the rest of the reproductive tract, macroscopically. This was determined to be illustrative of an immature oviducal gland by Ebert (2005) for assorted skates, Henderson and Arkhipkin (2010) for the white spotted skate, Serra Pereira et al. (2011) for the thornback ray, and Porcu et al. (2014) for the velvet belly lantern shark. Histological examination showed that the lumen was lined with simple, columnar epithelial cells and the epithelium was straight and lacking invaginations. The structure also lacked secretory tubules. Lack of secretory tubules in the oviducal gland of immature

chondricthyans has been demonstrated by Porcu et al. (2014) for the velvet belly lantern shark, and Henderson and Arkhipkin (2010) for the white spotted skate. Serra Pereira et al. (2011) determined that the lumen was lined with simple columnar epithelial cells for the thornback ray.

The animal with a total length of 272 cm had an oviducal gland that was more developed, suggesting that the animal was undergoing maturity. It measured about twice the size of the immature oviducal glands and was more clearly differentiated from the uterus. Histological examinations showed the occurrence of secretory glands interspersed with connective tissue. More invaginations were present in the epithelial layer, but the thinner endometrial layer suggested that the animal was not yet fully mature. The beginning of secretory tubule formation in maturing elasmobranchs has been demonstrated by Serra Pereira et al. (2011) for the thornback ray, and Porcu et al. (2014) for the velvet belly lantern shark.

The animal measuring 380 cm TL had an oviducal gland that was extremely developed and measured ~10 cm across and clearly came from a mature animal. More numerous and complex invaginations were present in the epithelium, which consisted of pseudostratified columnar cells. Additionally, more secretory tubules were present, filling up much more of the space that was occupied by connective tissue in the maturing animal. Henderson and Arkhipkin (2010) demonstrated that the presence of secretory tubules is characteristic of mature oviducal glands.

The main role of the uterus is to protect the eggs and embryos. Other roles, such as providing nutrition, are seen in some groups such as histotroph-producing stingrays. The uterus is made up of four layers, which become increasingly distinct as the animal matures. The layer closest to the lumen is the mucosa, which consists of the epithelium and the lamina propria. The

next layer is the submucosa, which is made up of connective tissue. These are followed by the muscular and the serosa (Diaz Andrade et al., 2013). The structure of the uterus varies between elasmobranchs, based on the mode of reproduction (McMillan, 2007). Despite being a major part of the reproductive system, very few studies have examined the uterus in elasmobranchs.

The uteri of all three animals were composed primarily of tough, muscular tissue. They ranged in size from 3 cm wide by 2 cm thick for the smallest animal to 5 cm wide and 7 cm thick in the largest animal. This is consistent with findings by Diaz Andrade et al. (2013), and Porcu et al. (2014) in species of skates and the velvet belly lantern shark, respectively, who found that the uterus increased in thickness and width as animals mature. All uteri examined were determined to have come from mature or maturing animals based on examination of other reproductive structures from these animals.

Microscopically, distinct layers were visible lining the lumen of the uterus. In the smallest animal (271 cm TL), which was sampled in January, the layer directly lining the lumen was about 200 µm thick and was composed of secretory tissue. This layer was not observed in *S. acuta* or *S. bonapartii* (Diaz Andrade et al., 2013), both of which are oviparous species. It is possible that this layer is related to the production of embryotroph that Castro et al. (2016) proposed nourished tiger shark embryos after yolk supplies were used up. The second layer was the endometrial layer, which was straight and approximately 25 µm thick and composed of pseudostratified columnar cells. The next layer was the lamina propria, which is composed of dense connective tissue, and the submucosa, which was composed of vascularized loose connective tissue. The makeup of the endometrial layer and slight differentiation of the lamina propria and the submucosa is consistent with findings by Diaz Andrade et al. (2013) for

maturing *S. acuta* or *S. bonapartii.* However, small villi and ciliated endometrial cells were observed in the uterus of maturing skates*,* but not in the tiger shark. This is could be a difference relating to different reproductive modes.

Furthermore, although this study provides a minimum expected size at maturity, there is a large amount of variation between animals of the same population and it should not be expected that all animals of that size are mature. We suggest a range of maturity between 270 cm and 310 cm, before which, all animals are likely immature and after which, all animals are likely mature.

The Reproductive cycle

We suggest that mating occurs in October/ November based on the presence of mating wounds at this time. This is also supported by an increase in testosterone in males and an increase in estradiol in females during these months. Mating wounds were observed on several mature female tiger sharks in October and November. Mating wounds have been shown to be indicators of when mating is taking place in elasmobranchs (Kajiura et al., 2000). No other observations of mating wounds on tiger sharks have been reported in the western Atlantic Ocean, but Whitney and Crow (2007) reported mating wounds on tiger sharks in January and early February in Hawaii. This is likely just a regional difference between two populations that are unlikely to interact.

Additionally, there was a large spike in testosterone observed in males collected at this time. It has been thoroughly demonstrated in other species of elasmobranchs that androgens, such as testosterone, play a role in the maturation of spermatocysts during late stages of spermatogenesis (Gelsleichter and Evans, 2012). Increased androgens are also associated with

increased transport of semen through the reproductive tract (Snelson et al., 1997; Gelsleichter et al., 2002). Rasmussen and Gruber (1993) and Tricas et al. (2000) have also suggested that higher testosterone during breeding season could indicate a role of the hormone in aggressive sexual behavior.

Some female tiger sharks demonstrated a spike in estradiol just prior to this time. As it is believed that tiger sharks are not an annual species (Rivera Lopez, 1970), it is not surprising that only a portion of the animals would exhibit this change. In many species, a spike in estradiol at the time of mating is indicative of follicular development just prior to ovulation (Rasmussen and Gruber, 1993; Manire et al., 1995; Snelson et al., 1997). However, in the tiger shark, data observed by Springer (1938) and Clark and Von Schmidt (1965) suggested that newly fertilized eggs occur in the spring. Previous studies have assumed that mating had occurred recently because of this. Our data suggests that mating more likely occurs in October and November, with the possibility of delayed ovulation and pregnancy, perhaps due to sperm storage until May or June.

It is possible that increases in estradiol observed in just October and November are related to the development of the oviducal gland to prepare for sperm storage, if it occurs. Estradiol is believed to regulate the development of the oviducal gland and reproductive tract (Callard et al., 2005; Gelsleichter et al., 2012; Awruch, 2013). Additionally, the bonnethead, which has been shown to store sperm in the oviducal gland for a period of up to 6 months before ovulation, also shows an increase in estradiol at this time, parallel with oviducal gland development (Manire et al., 1995). Gonzalez De Acevedo (2014) also showed increased levels of estradiol in the bonnethead during sperm storage, as well as receptors for estradiol in sperm

and sperm storage tubules in the bonnethead oviducal gland, suggesting a role of the hormone in oviducal development and sperm storage.

Although this study was unable to find direct evidence of sperm storage in the single mature tiger shark oviducal gland examined, it is important to note that this individual was collected during July, a period that is not predicted to have sperm storage. Pratt (1993) found sperm in the oviducal gland of a mature female tiger shark. Additionally, sperm storage was hypothesized by Whitney and Crow (2007) to explain the break between mating and ovulation of tiger sharks off Hawaii. Also, it is noteworthy to mention that a recent study by Holmes et al. (2018) determined that tiger sharks exhibit a surprisingly low rate of multiple paternity. They proposed that it could indicate that sperm is stored from a single male at a time. The bonnethead which also stores sperm, has also been observed to have low multiple paternity (Chapman et al., 2004).

Based on past data and the present study, we suggest that ovulation and fertilization occurs in female tiger sharks in May/June and parturition occurs approximately 13 months later. Ovulation occurring in May/June is supported by the increase in estradiol observed in some females at that time, although this peak is not as high as that observed in October. Estradiol is known to increase when the oocyte is reaching its final maturation and ovulation (Snelson et al., 1997). Manire (1995) and Snelson (1997) suggested for the Atlantic ray, *Dasyatis sabina*, and the bonnethead, *Sphyrna tiburo*, respectively, that the elevated estradiol could be connected to the fertilized eggs moving to the uterus. Ultrasonography data shows small pups in October with much larger pups in May and June. Data on small tiger shark catch supports the premise that parturition occurs around July. Additionally, as stated earlier, the literature is clear that early

tiger shark embryos are found in the spring, based on reports by Springer (1938), Clark and Von Schmidt (1965), and Castro (2009).

Sulikowski et al. (2016) found that levels of estradiol could be used to determine pregnancy in mature tiger sharks. They found that non-pregnant tiger sharks had high estradiol concentrations, likely reflecting follicular development, while pregnant animals had lower levels of progesterone and estradiol. While the majority of the confirmed pregnant animals examined in this study had low estradiol values (200-300 pg/mL), the values of the confirmed not pregnant animals were also observed to be quite low, sometimes even lower. It is possible that this is a result of when sampling occurred and what reproductive stage the animal was in at the time. It is also likely that the low sample size of animals where ultrasonography was performed in this study influenced our findings.

Unlike estradiol, which generally a clear correlation with reproductive stages, progesterone patterns are typically less consistent in mature elasmobranchs. Rasmussen and Gruber (1993) reported low and unchanging progesterone levels in most adult sharks, particularly compared to mammals, which have elevated progesterone during pregnancy. However, Sulikowski et al. (2016) found that progesterone was highly tied to estradiol in tiger sharks. In this study, Spearman's rank showed a strong positive correlation between estradiol and progesterone values. However, the limited number of high progesterone values made trends difficult to exam.

This study also attempted to examine the presence of the yolk precursor vitellogenin, in order to determine more clearly when specific reproductive events occur. Vitellogenin, which is generally synthesized in response to rising estradiol concentrations, would be expected to be

found in shark plasma during the period before ovulation (Denslow et al., 1999; Hiramatsu et al., 2006; Ho et al., 1982). It is responsible for the growth of follicles prior to ovulation (Gracan et al., 2013). Vitellogenin produced in the liver is transferred to the oocytes in the ovaries, allowing them to accumulate yolk and grow rapidly (Castro, 2009). In the ovary, vitellogenin is cleaved into yolk proteins, which provide nutrition for developing embryos (Romano et al., 2004). Vitellogenin is prone to degradation; bands observed in immunoblots were consistent with what would be expected of vitellogenin breakdown proteins, specifically, lipovitellin, which has reported sizes of 105, 91, and 67 kD.

This study showed a number of immature and maturing tiger sharks tested positive for proteins at an appropriate size for vitellogenin component proteins. This is not surprising when combined with the results of the ovarian histology, in which pre-vitellogenic follicles were found in animals that were clearly not mature. In immature and maturing animals, the process of vitellogenesis may start, causing the follicles to begin to develop, but eventually they undergo atresia and die (Hamlett and Koob, 1999).

Of the 8 animals above 270 cm TL who were positive to the band at ~65 kD, 6 were sampled during the months leading up to or during ovulation. However, the mean estradiol value for animals which were positive for vitellogenin bands, was not significantly different from than those that were negative. Additionally, some animals that were confirmed pregnant and not pregnant were both found to exhibit immunoreactive bands at this molecular weight. It is therefore concluded that although vitellogenin may be detected in circulation, its presence may not be informative about the reproductive cycle for this species.

Ultrasonography data from this study show an increase in size and development of tiger

shark pups *in utero* from October to June. Additionally, confirmed late pregnancy animals were

caught in May and June. In June, the smallest tiger shark pups begin to be caught off of South

Carolina and Bimini. The size of the smallest pups caught at this time begins at 56 to 60 cm fork

length, and a total length of 86 to 90 cm. This is consistent with the findings of Schwartz (1989)

who reported late term embryos at 80 cm TL and free-swimming pups as small as 85 cm TL off

of North Carolina. Simpfendorfer (1992) also found that tiger pups were born between 80 and 90

cm TL. In Hawaii, Whitney and Crow (2007) determined that pups were born between 80 and 90

cm TL. However, Springer (1940) reported late term embryos in March with a total length of 62

cm and Clark and Von Schmidt (1965) found 69 cm late term embryos in May. Whitney and

Crow (2007) reported that tiger shark embryos absorb their yolk sac fairly early in gestation and

that could have caused authors to underestimate the size at birth for these pups and therefore the

gestation. We believe that this is part of the cause of the discrepancy in reported late term

embryo and pup size.

Determining the size of tiger sharks at birth, and therefore the time of parturition, has

been especially difficult due to the lack of an umbilical scar because the species is aplacental

(Whitney and Crow, 2007). Therefore, this study examined the catch size of tiger sharks below

110 cm FL to determine when parturition occurs. A clear increase in size of the neonate cohort

can be observed as more time passes after June. However, a few smaller pups are caught in

August and September. This information, combined with the observation of fetal movement in a

tiger shark in September might indicate that the pupping period for tiger sharks could be

variable, rather than highly synchronized, and could extend from May to late September. Clark

and Von Schmidt (1965) examined a tiger shark that had recently given birth in July off

Sarasota. Alves (1977) concluded that late term embryos were found from May to August off Brazil. Schwartz (1989) reported pupping from July to September off North Carolina.

Whitney and Crow (2007) captured several adult females between September and November, in Hawaii, with large vacant uteri and determined that pupping occurs in September and October based on captured neonates. The data for the western Atlantic Ocean does not suggest that pupping continues that late into the autumn. This is likely another regional difference between the two populations and a result of mating occurring at different times.

Based on the timing of reproductive events described in this paper, we suggest a reproductive timeline during which a group of tiger sharks mates in October/November, stores sperm until ovulation in May or June, and gives birth between 12 and 15 months later. Rivera Lopez (1970), Clark and Von Schmidt (1965), and Castro (2009) all suggested that gestation lasts approximately a year in tiger sharks.

Hormone data during each reproductive event could indicate a triennial reproductive cycle. Although a few animals show increases in estradiol before mating and ovulation, the majority do not. However, data from this paper does not strongly indicate whether the cycle is biennial or triennial. Several other papers have suggested evidence for a triennial cycle for tiger sharks. Rivera Lopez (1970) and Alves (1977) reported that 35% and 39% of tiger sharks were pregnant at a given time. Whitney and Crow (2007) reported that 33% or less where pregnant at a given time, except in the months where gestation periods from different reproductive groups would be expected to overlap. Even during the overlap, the percentage never went above 60%, which is much less than what would be expected for a biennial cycle.

Conclusions

As tiger sharks are one of the species that has been observed to recover in recent years, it is essential to learn about their reproduction in order to better understand how this is happening. This study adds to what is known about tiger sharks in this region by determining that the size at which some females are able to mature is lower than what has been reported in this region and considerably lower than what was found off Hawaii. This study also identified several differences in reproductive timing between these two populations, including when mating and pupping take place.

Furthermore, this study added to what is known by examining the reproduction of males, which have been mostly overlooked in all past studies. Evidence presented suggests that female tiger sharks may be storing sperm. This period of sperm storage, combined with the low incidence of multiple paternity suggests that mating either occurs with a single male, or some type of post-copulatory selection occurs. Further studies should investigate whether these processes are occurring to continue to clarify still unclear aspects of tiger shark reproduction.

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