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Non-Lethal Methods for Assessing Reproductive Status in Bonnethead Sharks (Sphyrna tiburo)

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NON-LETHAL METHODS FOR ASSESSING REPRODUCTIVE STATUS IN

BONNETHEAD SHARKS (*SPHYRNA TIBURO*)

by

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in partial fulfillment of the requirements for the degree of

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CERTIFICATE OF APPROVAL

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ABSTRACT

Reproductive biology is a necessary element for the management of elasmobranch fisheries. Traditionally, characterization of elasmobranch reproduction has involved lethal sampling to examine gross reproductive structures and development of embryos. However, this method is counterproductive to the conservation of shark populations. One non-lethal alternative is the measurement of serum hormones, which often vary according to reproductive events. Radioimmunoassay (RIA) has been used to measure hormone concentrations in reproductive endocrinology, but can be problematic for researchers. Alternatively, chemiluminescence immunoassays (CLIA) are routinely used for measuring circulating hormone concentrations in low-volume, non-extracted human serum samples. However these assays have not been previously examined for use with elasmobranch blood. In the first component of this study, I examined whether CLIA was a suitable alternative for detecting seasonal profiles of these hormones in the bonnethead, *Sphyrna tiburo*. This was accomplished by collecting serum from sexually mature male (n = 35) and female (n = 32) bonnetheads , measuring reproductive organs for maturity and reproductive stage, and measuring concentrations of testosterone (T) in males, and 17β-estradiol (E_2) and progesterone (P_4) in females using RIA and CLIA. CLIA was successfully validated for use with shark serum by assessing parallelism and spike recovery. CLIA-derived measurements were significantly correlated with those obtained with RIA (r = 0.809, 0.773, and 0.908 for T, E_2, and P_4, respectively; $p<0.0001$). However, CLIA–obtained measurements of T and E_2 concentrations were generally greater than RIA-derived values, particularly at higher concentrations. CLIA-obtained values exhibited similar patterns consistent with those of RIA and gross reproductive morphology. Overall, CLIA has been shown to be a

sensitive, reliable, and effective method for determining sex steroid concentrations and assessing overall reproductive status in *S. tiburo*.

In the second component of this study, I examined whether portable ultrasonography could be used as a non-lethal method for detecting pregnancy and determining fecundity in bonnetheads. Female bonnetheads (n = 66) were collected weekly throughout their gestation period (April to October). Reproductive status and fecundity were assessed using an $8 - 5$ MHz linear and $5 - 2.5$ MHz curvilinear array transducer. The shark was then dissected to confirm the results. Overall, detection of reproductive status using ultrasound methods demonstrated good agreement with dissection (90.9%). There was moderate and substantial agreement between the ultrasound methods and dissection method (Kappa coefficient for linear versus dissection = 61.3%; curvilinear versus dissection = 88.9%). Overall mean of fecundity assessed with the linear transducer (5.7 ± 2.4) was not significantly different from overall mean of dissection (6.6 ± 2.5), as was the curvilinear transducer (5.7 ± 2.2). In overall detection of reproductive status, ultrasound methods demonstrated good agreement with the "gold standard" method of dissection.

Evaluation of gonadal steroid chemiluminescence immunoassays (CLIA) for non-lethal

characterization of reproductive status in the bonnethead (*Sphyrna tiburo*)

INTRODUCTION

Information regarding reproductive biology is an important element needed in the management of

elasmobranch fisheries. Sharks are generally vulnerable to overfishing due to life history

characteristics such as slow growth rates, late sexual maturity, and low reproductive output

(Musick *et al.*, 2000). Therefore, information on age at maturity, reproductive periodicity, and

fecundity are strongly considered when fishery managers determine recruitment and population

status for these fishes. Traditionally, characterization of elasmobranch reproduction has involved

lethal sampling of numerous individuals to examine gross morphology of the gonads and

gonaducts, as well as the presence and size/stage of development of embryos. However, this

method is often viewed as counterproductive to the conservation of overfished shark populations,

or may be prohibited in the case of certain protected species. This emphasizes the importance of

developing and refining non-lethal approaches for examining elasmobranch reproduction

(Hammerschlag and Sulikowski, 2011).

One non-lethal alternative for determining elasmobranch reproductive status is the

measurement of circulating concentrations of gonadal steroid hormones, which often vary

according to reproductive events. For example, increases in circulating concentrations of the

gonadal sex steroid hormones testosterone (T) and 17β-estradiol (E$_2$) have been shown to increase

significantly during gametogenesis in male and female sharks, respectively, making them useful

indicators of this process (Maruska and Gelsleichter, 2011). Typically, radioimmunoassay (RIA) has been used to measure circulating gonadal steroid concentrations in most studies on elasmobranch reproductive endocrinology (Manire *et al.*, 1999; Sulikowski *et al.*, 2004; Awruch *et al.*, 2008; Lutton and Callard 2008; Henningsen *et al*., 2008). However, performing RIA can often be problematic for researchers due to the use of radioactive isotopes and the need for large sample volumes and pre-extraction of samples (Elmlinger 2011; Neupert *et al.*, 1996). Alternatively, commercially available chemiluminescence immunoassays (CLIA) are routinely used for measuring circulating hormone concentrations in low-volume, non-extracted human plasma or serum samples. However these assays have not been previously examined for use with elasmobranch blood. CLIAs were first used in the 1970s and have been shown to have greater sensitivity than RIAs or other immunoassays (Neupert *et al.*, 1996). Human steroid concentrations measured with CLIA have been compared with those measured with RIA, demonstrating good results (Rojanasakul *et al*., 1994). For example, Tummon *et al.*, (1999) demonstrated a strong correlation between 17β-estradiol (E_2) concentrations measured with CLIA and RIA overall, although correlations declined at lower concentrations. Given this, CLIA may be a suitable tool to evaluate elasmobranch sex hormones that is not as labor-intensive as RIA. However, it must be validated for use with elasmobranchs, preferably using a species with a well characterized reproductive cycle.

Therefore, the purpose of this study was to examine the use of CLIA as a tool for measuring circulating sex steroid hormone concentrations in elasmobranchs, focusing on one of the most extensively researched shark species with regards to reproductive physiology: the bonnethead (*Sphyrna tiburo*). *S. tiburo* is a small species of hammerhead that ranges from North Carolina, USA to southern Brazil (Compagno 1984). Its reproductive biology has been extensively studied

using gonad morphology/histology and sex steroid profiles, the latter of which were measured using RIA. Manire *et al.*, (1995), Manire and Rasmussen (1997) and Gelsleichter *et al.*, (2002) reported cyclical changes in serum sex steroid levels in this species that corresponded to patterns of gametogenesis, mating, and pregnancy, indicating a strong relationship between hormone profiles and reproductive status. For example, male *S. tiburo* experience an increase in circulating testosterone (T) concentrations in late summer that coincides with increases in testis size (Manire and Rasmussen, 1997). Similarly, following mating and an up to 6-month period of sperm storage, female *S. tiburo* generally experience an increase in circulating E_2 concentrations in mid-Spring that co-occurs with folliculogenesis (Manire *et al.*, 1995). Circulating progesterone (P_4) concentrations also vary cyclically in female *S. tiburo*, increasing significantly at ovulation (Manire *et al.*, 1995). In this study, we examined whether CLIA was a suitable alternative for detecting comparable seasonal profiles of these hormones in *S. tiburo* and characterizing shark reproduction in a conservation-minded, non-lethal manner.

METHODOLOGY

Biological data and sample collections

Mature male (n = 35) and female (n = 32) *S. tiburo* were collected from areas adjacent to Tampa Bay, FL using set gill nets, as part of an Environmental Protection Agency-funded study on shark ecotoxicology conducted between 1997 and 2001 (Gelsleichter *et al.*, 2005). No abnormal effects were observed in the animals used in this study. Blood samples were obtained from each shark after capture via caudal venipuncture and were immediately placed on ice, where they were allowed to clot for 3–6 h. Blood was later centrifuged (1300g) and serum samples were frozen at -80°C until thawed for hormone analysis. Sharks were measured, weighed, and transported to the

laboratory on ice for dissection and evaluation of reproductive organs, which were used to characterize maturity and reproductive stage as described in previous studies (Manire *et al.*, 1995; Manire and Rasmussen, 1997; Gelsleichter *et al.*, 2002; 2003). A variety of morphological indices of reproductive condition including testis width and the presence of semen in males, and maximum follicle diameter and pregnancy status in females were compared to CLIA-derived hormone profiles in the present study to validate the use of this method as a non-lethal alternative for characterizing reproduction.

Hormone analyses

Circulating concentrations of T, E_2, and P_4 were determined by RIA after purification by chromatography on Sephadex LH-20 microcolumns following methods described in previous studies, which have been validated for use with numerous elasmobranchs including *S. tiburo* (Manire *et al.*, 1995; Manire and Rasmussen, 1997; Gelsleichter *et al.*, 2002). Serum aliquots of 500 µL were extracted with 5 mL of freshly opened diethyl ether. The organic phase was decanted after freezing the aqueous phase in an ethanol/dry ice bath and the ether dried under a stream of air. Dried extracts were sequentially chromatographed on two different Sephadex LH-20 columns. On the first column (1.0 g LH-20 with an elution mixture containing hexane, benzene and methanol at 62:20:13 v/v), E_2 was separated from estrone and all neutral steroids. The neutral fraction from the first column was applied to the second column (2.5 g LH-20 with an elution mixture containing hexane, benzene and methanol at 85:15:5 v/v) and appropriate fractions for P_4 and T were collected. Purified steroids were then estimated using the RIA procedure described in Manire *et al.*, (1995).

CLIA-based analysis of circulating shark gonadal steroids was performed using AccuLite CLIA kits (Monobind, Lake Forest, CA) for T, E_2, and P_4 following the manufacturer's instructions. Sensitivity of each assay was reported as follows: T = 0.371 ng/mL, E_2 = 4.162 pg/mL, and P_4 = 0.105 ng/mL. Briefly, methods included pipetting standards or diluted samples in duplicate into a streptadivin-coated microplate, followed by sequential addition of a HRP-conjugated tracer reagent and an anti-hormone specific biotinylated purified rabbit IgG conjugate. Afterwards, microplates were incubated at 4C overnight for maximum binding. The following day, microplate contents were removed and washed three times with wash buffer. Then signal reagent (luminol plus hydrogen peroxide) was added to all wells and incubated for 5 minutes in the dark until the assay was read by a microplate reader (Synergy HT Multi-Mode Microplate Reader; BioTek Instruments, Winooski, VT) in luminescence mode. Each kit was evaluated for use with elasmobranch serum using parallelism and cold spike recovery (Elmlinger 2011). Parallelism was tested between the standard curve and serial dilutions of shark serum (1/1 to 1/20), which were prepared using HSA Calibrator Matrix (Monobind, Lake Forest, CA). Spike recovery was determined by adding a known amount of steroid standard to each dilution (1/1 − 1/20 for T and P_4 assays, 1/1 − 1/50 in E_2 assays) and calculating the percentage obtained in the results. Assessments of spike recovery were also used to determine the ideal dilution of sample serum to use in an assay to avoid "matrix effects," or spurious results resulting from proteins in the serum that might have interfered with antibody binding.

Data analysis

Parallelism between the standard curve and serial dilutions of shark serum were evaluated by conducting the Homogeneity of Linear Regression test on slope data using Costat software (Cohort

Software, Monterey, CA). Correlations between CLIA-derived measurements of E_2, P_4, and T, and those measured via RIA were tested separately for each steroid using Pearson's correlation coefficient. Circulating hormone concentrations determined using CLIA and RIA were grouped separately for each method by month of capture or stage and tested for significant temporal variations using ANOVA followed by Tukey-Kramer's multiple comparisons test. Differences between CLIA- and RIA-measured hormone concentrations were compared by month of capture (in males) or reproductive stage (in females) using Student's t-test. Data sets that failed tests of normality and/or equal variance were log-transformed or analyzed using appropriate nonparametric tests, i.e., Kruskal–Wallis one-way ANOVA by ranks followed by Dunn's post-test or the Mann-Whitney U test.

RESULTS

The use of commercially available CLIA kits for measuring circulating concentrations of T, E_2, and P_4 in *S. tiburo* was validated for use with shark serum. Serial dilutions of *S. tiburo* serum exhibited good parallelism, and slopes were not statistically different from standard curves ($p = 0.2951$, 0.1011, and 0.2047 for T, E_2, and P_4, respectively, Fig. 1.1).

In addition, there was good spike recovery in diluted plasma samples, particularly at dilutions of 1/10 or greater (Table 1.1). Because of some evidence for matrix effects at dilution levels below 1/10 based on lower spike recovery, samples were subsequently diluted at levels greater than this for conducting hormone measurements (1/50, 1/25, and 1/10 for T, E_2, and P_4, respectively).
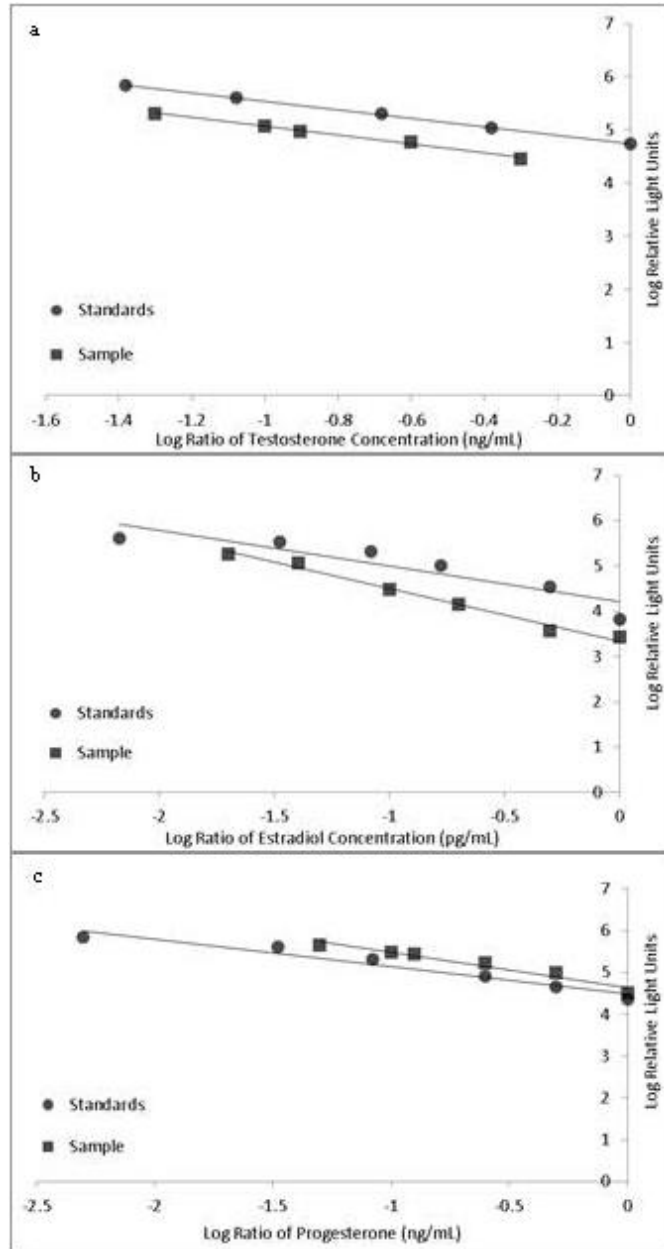
Figure 1.1. Parallelism validation between a) testosterone b), estradiol, and c) progesterone standard curve and sample dilutions. Data were log transformed. Lines represent the best fit line (testosterone: $y = -0.590x + 4.873$ for standards and $y = -0.9005x + 4.1667$ for samples, estradiol: $y = -0.791x + 4.201$ for standards and $y = -1.170x + 3.333$ for samples, progesterone: $y = -0.659x + 4.481$ for standards and $y = -0.847x + 4.632$ for samples).

Table 1.1. Spike recovery (%) in CLIA testosterone (ng/mL), estradiol (pg/mL), and progesterone (ng/mL). Measured concentrations of testosterone dilutions 1/2, 1/4, and 1/8 exceeded the standard curve and true spike recovery could not be calculated.

Sample (cold spike)	Dilution	Expected conc.	Measured conc.	Spike recovery (%)
Testosterone	1/2	18.60	12.60	-
(6 ng/mL)	1/4	18.17	12.60	-
	1/8	12.04	12.54	-
	1/10	10.44	9.19	79.10
	1/20	8.31	7.14	80.43
Estradiol	1/2	148.36	76.36	40.00
(120 pg/mL)	1/5	142.66	97.70	62.54
	1/10	141.46	114.34	77.39
	1/25	137.88	158.17	116.91
	1/50	135.59	173.98	131.99
Progesterone	1/2	26.06	32.63	154.68
(12 ng/mL)	1/4	19.41	24.06	138.76
	1/8	15.58	17.75	118.11
	1/10	15.24	18.30	125.44
	1/20	13.51	18.64	142.77

CLIA-derived measurements of serum T, E_2, and P_4 concentrations were significantly correlated with those obtained using RIA (r = 0.809, 0.773, and 0.908 for T, E_2, and P_4, respectively; $p<0.0001$; Fig. 1.2). However, CLIA–obtained measurements of T and E_2 concentrations were generally greater than RIA-derived values, particularly at higher concentrations. CLIA-obtained measurements of serum P_4 levels were occasionally higher than those measured using RIA, but results obtained using the two methods were generally more comparable than those for T and E_2.

When grouped by time of collection or reproductive stage, CLIA-obtained values of all three steroids exhibited similar patterns consistent with those of RIA-obtained concentrations. These patterns were reflective of reproductive activity (Fig. 1.3). Serum T concentrations increased markedly in males during late summer (August-September), a period during which these individuals were shown to be undergoing spermatogenesis based on morphological evaluation of the testis and reproductive ducts. Serum T concentrations remained slightly elevated in males captured during October, when copulatory activity was observed based on the presence of fresh mating scars on females. Serum E_2 concentrations increased considerably in females during the copulatory period and peaked prior to ovulation, a period characterized by extensive growth of ovarian follicles. Last, serum P_4 concentrations peaked during ovulation in females, when ovulated and presumably fertilized ova were found to be present in the lower oviducts en route to the uteri. Peak concentrations of T during late spermatogenesis, E_2 during the copulatory and pre-ovulatory periods, and P_4 during ovulation were significantly greater than those observed during several other stages for both CLIA- and RIA-measured values (Fig. 1.3).
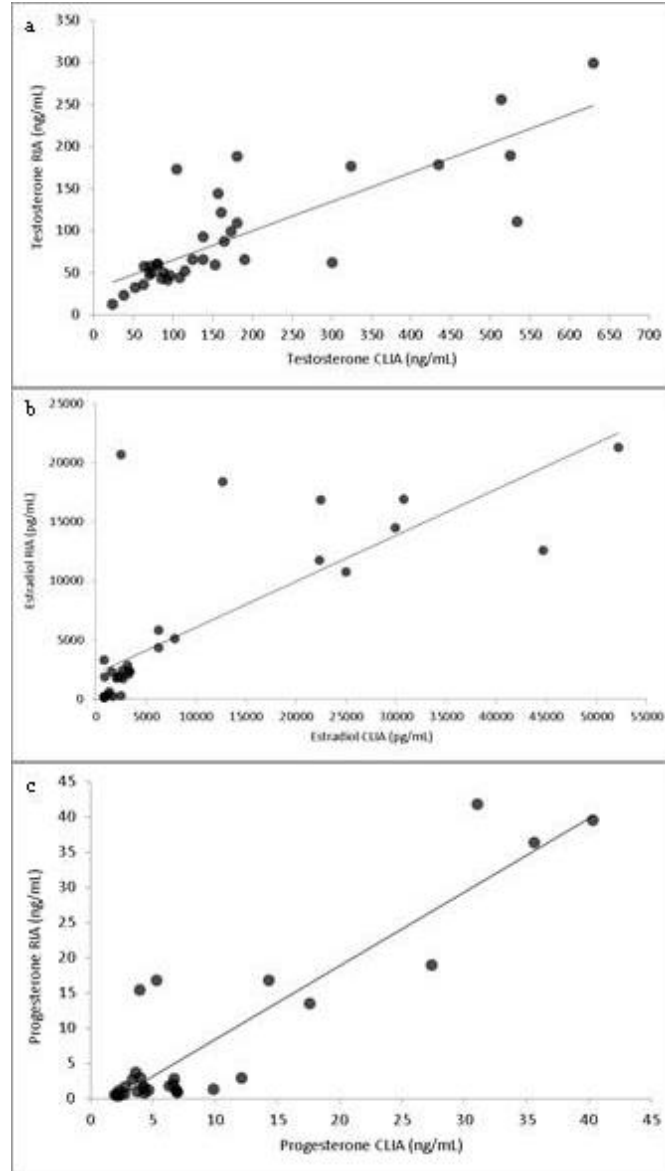
Figure 1.2. *Sphyrna tiburo* male a) testosterone concentrations (n = 35) and female b) estradiol and c) progesterone (n = 32) measured with chemiluminescence immunoassay (CLIA) compared to radioimmunoassay (RIA). Line represents a simple linear regression (testosterone: y = 0.3456x + 30.671, r = 0.809, p < 0.0001; estradiol: y = 0.3901x + 2132.7, r = 0.773, p < 0.0001; progesterone: y = 1.0471x - 2.126, r = 0.908; p < 0.0001).
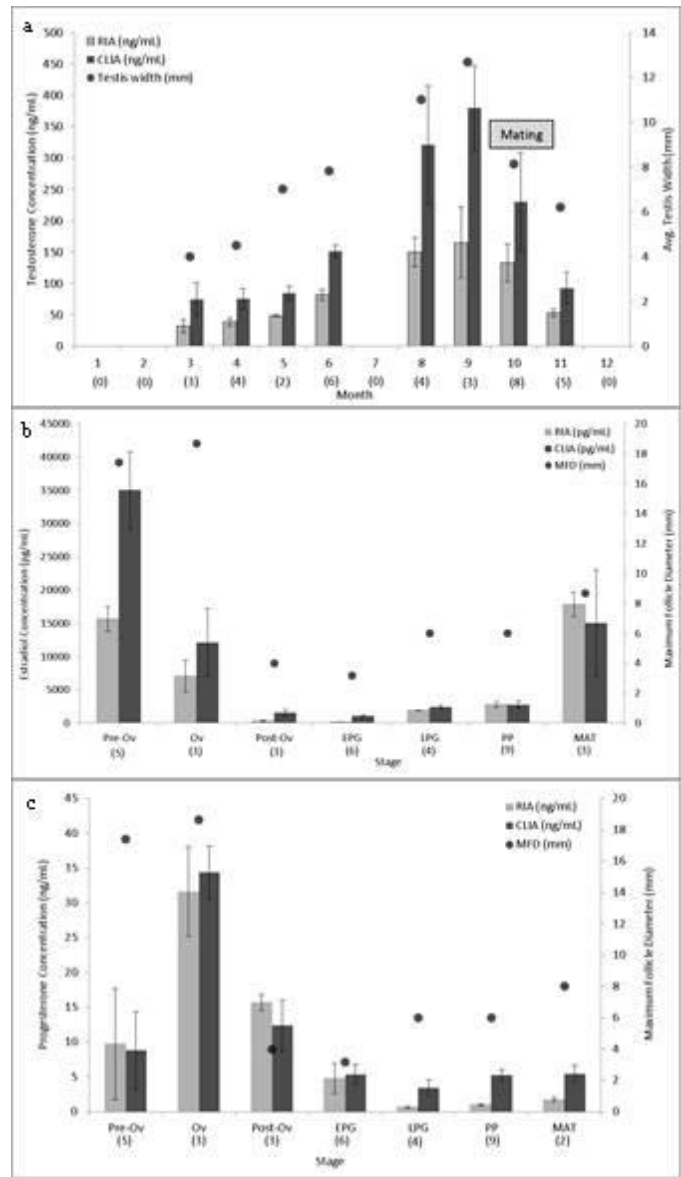
Figure 1.3. Mean *Sphyrna tiburo* a) male testosterone concentrations (n = 35) and female b) estradiol and c) progesterone concentrations (n = 32) measured with chemiluminescence immunoassay (CLIA) and radioimmunoassay (RIA) plotted by date of capture or stage. Numbers in parenthesis represent sample size. Mating occurs October. MFD: maximum follicle diameter, Pre-ov: pre-ovulatory stage, Ov: ovulatory stage, Post-ov: post-ovulatory stage, EPG: early pregnancy stage, LPG: late pregnancy stage, PP: post-partum stage, MAT: mating stage.

DISCUSSION

Overall, CLIA was shown to be a valid method for measuring serum sex steroid concentrations in *S. tiburo*. Parallelism between the standard curves and diluted shark serum demonstrated that sample analytes interacted with antibodies in a manner similar to that of the assay standards. Spike recovery was very high in only moderately diluted samples and comparable to if not greater than levels reported in previous RIA studies, which have often been below 70% for extracted samples (Gelsleichter *et al.*, 2005). Most importantly, CLIA-derived values were significantly correlated with those obtained via RIA and, although they were occasionally higher, they varied in relation to the reproductive cycle in a manner identical to that for RIA-derived values in both this and previous studies. Therefore, CLIA appears to be a suitable tool for the non-lethal characterization of shark reproduction, one that may provide unique benefits over the use of RIAs due to the limited need for pre-extraction of samples, non-use of radioisotopes, and the limited sample volume needed for conducting assays.

As demonstrated in prior studies conducted using RIA (Manire and Rasmussen 1997; Gelsleichter *et al.*, 2002), serum T concentrations measured in male bonnetheads using CLIA increased significantly during late spermatogenesis and the mating period. This presumably reflects a role for androgens in regulating some aspects of sperm production, gonaduct function, and/or copulatory behavior during these stages, a premise well supported by evidence for comparable patterns of circulating T levels in a number of other seasonally-reproducing male elasmobranchs (Maruska and Gelsleichter 2011). Serum E_2 concentrations measured in female bonnetheads using CLIA also peaked during gametogenesis, likely reflecting a role for E_2 in regulating vitellogenin production as it does in most other non-mammalian vertebrates. This, too, is supported by earlier research on circulating E_2 profiles in female *S. tiburo* as well as by studies

on both E_2 and vitellogenin levels in certain elasmobranch species (Manire *et al*., 1995; Callard *et al*., 1991). Last, like those measured by RIA in the current study and prior investigations, CLIA-measured concentrations of P_4 increased specifically during ovulation, suggesting a role for this hormone in regulating ova release and/or transport to the uterus. Prior studies have also suggested that increases in P_4 concentrations during this stage may play a direct role in inhibiting vitellogenesis and bringing the period of follicular development to a close (Maruska and Gelsleichter, 2011). The ability to detect these well-characterized hormone patterns using CLIA supports the use of this procedure in future studies.

Although they were significantly correlated with RIA-derived values, serum T, E_2, and P_4 concentrations determined using CLIA often were significantly greater, suggesting that data obtained using the two methods cannot be directly compared. This is similar to what has been observed in prior studies, e.g., Russell *et al.*, (2007) and Tummon *et al.*, (1999) found CLIA-measured concentrations of canine plasma/serum cortisol and human serum E_2, respectively, to be greater than those obtained using RIA despite strong correlations between the two procedures. The reasons for these differences as well as the ones observed in the present study remain unclear, but may be associated with a variety of factors such as differences in antibody specificity, cross-reactivity with other steroids, and dissimilar effects of sample condition (e.g., hemolysis, lipaemia) on assay performance. Nonetheless, the strong concordance between the hormone patterns determined using CLIA with those obtained via RIA still support the use of CLIA as a method for characterizing the reproductive endocrinology of *S. tiburo* and perhaps other elasmobranchs.

In conclusion, CLIA has been shown to be a sensitive, reliable, and effective method for determining circulating sex steroid concentrations and assessing overall reproductive status in *S. tiburo*. Future work includes validating and using this approach as a non-lethal alterative for

characterizing the reproductive biology of other species of sharks and rays, especially those that are threatened or endangered by human activities and may not be suitable for harvest for traditional reproductive analyses. An additional benefit of using CLIA for assessing circulating steroid concentrations in sharks is that the low sample volume needed for conducting these assays (0.5-2 µL, depending on the assay) may facilitate new studies on hormonal patterns during embryogenesis, which generally require the use of pooled blood samples or whole-body homogenates to assess (White and Thomas 1992, Lovern and Wade 2003) and have been largely unstudied in elasmobranchs.

COMPONENT II

Use of portable ultrasonography to detect pregnancy and fecundity in bonnethead females

(*Sphyrna tiburo*)

INTRODUCTION

Ultrasonography has become a widely used diagnostic tool in the veterinary field, following its introduction to obstetrics in humans in the 1960s. Ultrasound methods are non-invasive, non-lethal, and quickly yield results with a live scan (Rajamehendran *et al.*, 1994; Whitamoore *et al.*, 2010). Lindahl (1966) reported the first veterinary study using this method for ovine reproduction. Over the years, numerous bovine, equine, porcine, and other domestic livestock have become popular subjects in ultrasonography studies (e.g., Buckrell 1988; Rajamehendran *et al.*, 1994; Evans 2003; Medan and Abd El-Aty 2010). However, ultrasound machines used in these studies were generally large and fragile systems adapted from the medical profession. They were not suitable for working in the field or on non-human subjects that required specialized equipment to overcome problems with size and positioning (Rajamahendran *et al.*, 1994; Hildebrandt *et al.*, 2000). Portable, field-ready units became available at the turn of the century, designed for medical emergency applications in the battlefield (Nelson and Chason 2008; Nelson and Li 2011; Nations and Browning 2011). Only in the last two decades did versatile, affordable units become accessible for general use by wildlife biologists (see review by Broderick 2014).

More than 20 fish studies using portable and console-style ultrasound units have emerged since the late 1980's, mostly focusing on fecundity and sex determination of captive specimens

in aquaculture, aquaria, or commercially important species (see review by Novelo & Tiersch 2012). More recently, studies on ultrasonography in captive elasmobranchs have been published, primarily focusing on determination of pregnancy status and general anatomy. These include studies on the bonnethead (*Sphrna tiburo*), nurse shark (*Ginglymostoma cirratum*), and lemon shark (*Negaprion brevirostris*; Walsh *et al.*, 1993), nurse shark (*G. cirratum*; Carrier *et al.*, 2003), large-spotted dogfish (*Scyliorhinus stellaris*) and small-spotted catshark (*Scyliorhinus canicula*; Gridelli *et al.*, 2003), whitetip reef shark (*Triaenodon obesus*; Schaller 2006), sevengill shark (*Notorynchus cepedianus*; Daly *et al.*, 2007), thornback ray (*Raja clavata*) and small-spotted catshark (*Scyliorhinus canicula*; Whittamore *et al.*, 2010), dwarf ornate wobbegong (*Orectolobus ornatus*; Otway and Ellis 2012), and southern stingray (*Dasyatis americana*; Grant *et al.*, 2013).

Currently, no study has focused on using ultrasound to determine reproductive fecundity of elasmobranchs and verified results with dissection. The first ultrasound study of elasmobranchs compared ultrasonic anatomy to gross anatomy using a frozen, sectioned lemon shark (*N. brevirostris*) and determined ultrasound techniques to be a good diagnostic and research tool in the bonnethead (*S. tiburo*), nurse shark (*G. cirratum*), and lemon shark (*N. brevirostris*; Walsh *et al.*, 1993). Whittamore *et al.*, (2010) evaluated maturity status of two oviparous elasmobranchs by comparing ultrasound measurements of ovary, shell gland, egg capsules, and ovarian follicles to gross measurements with good success. Grant *et al.*, (2013) assessed health of captive and wild-caught southern stingrays (*D. americana*) by establishing liver-to-coelom ratios using ultrasound and dissection techniques to test for differences between the two groups. While anatomy and health assessments are valuable to the scope of ultrasound

and elasmobranch research, assessing pregnancy status and fecundity are important for determining life history parameters for a population (Hammerschlag and Sulikowski 2011).

The goal of this study was to determine if ultrasonography is a reliable method for determining both pregnancy status and fecundity in sharks. Historically, shark reproduction studies required lethal dissection. In protected or endangered species, lethal sampling is often not an option. Subsequently, information on their reproductive cycles and fecundity remain unavailable for conducting analyses of population growth and sustainability where it is most crucial (Dulvy *et al.*, 2014). In order to test the feasibility of a non-lethal method for determining pregnancy status and fecundity, this study examined the bonnethead (*Sphyrna tiburo*), an abundant species of shark that has previously been well-characterized in reproductive studies (Manire *et al*., 1995; Manire and Rasmussen 1997; Gelsleichter *et al*., 2003). Bonnetheads are commonly found in the coastal waters of the southeastern United States. Bonnetheads reproduce by placental viviparity and the population exhibits a synchronous annual reproductive cycle, the timing of which varies slightly depending on the population in question (Manire *et al*., 1995; Frazier *et al.*, 2013). Following male sperm production and mating in the late summer to early fall, sperm is stored in females for 3-6 months, delaying vitellogenesis, ovulation and fertilization until the following spring (Manire *et al*., 1995). Gestation lasts 4-5 months beginning with a yolk-dependent stage (lasting approximately eight to nine weeks) and followed by a placental stage that persists until parturition in late summer to early fall (Manire *et al*., 1995). This study examined female bonnetheads each week during their gestation period (April – October) with a field-ready ultrasound machine and subsequent dissection to verify results. Objectives were to detect reproductive status, gestational stage, and to determine fecundity using

two types of ultrasound transducers. Each transducer was compared to the "gold standard" dissection method by determining their levels of agreement.

METHODOLOGY

Biological data and sample collections

Female bonnetheads were collected as part of the Georgia Department of Natural Resources' bottom longline survey funded by the National Atmospheric and Oceanographic Administration Cooperative Atlantic States Shark Pupping and Nursing Survey (NOAA COASTSPAN) and Southeast Area Monitoring and Assessment Program (SEAMAP; Belcher and Greer 2015). A total of 66 females were sampled. When possible, 5 females per week were sampled during the months of gestation and parturition for this species (April to October). Sharks were euthanized using ice immersion and stored on ice until necropsy could occur, usually within 24 hours. Sharks were weighed, measured, and blood sampled from the ventral caudal vein as part of a larger study on reproduction.

Ultrasound methodology

The Ibex Pro portable ultrasound (E.I. Medical Imaging, Loveland, CO) was used to determine pregnancy status and to capture ultrasound images and video. Two transducers were used throughout the study: an 8 – 5 MHz linear array transducer which scanned up to 12 cm depth and a 5 – 2.5 MHz curved linear array transducer which scanned up to a 24 cm depth. Latex covers and a generous amount of coupling gel (used both inside and outside the cover) were used to protect the transducer from the damaging shark skin and to provide adequate sound wave transmission.

Each shark was scanned systematically by dividing the animal into four zones, scanning each zone, and recording its results (Fig. 2.1). All zones were scanned in the transverse and sagittal transducer planes on the lateral and ventral surfaces of the body. Ova were counted based on their hyperechoic properties relative to the fluid-filled space within the uterus. Embryos were counted once the vertebral spine became calcified and hyperechoic (determined to occur at a mean pup length greater than 85 mm STL). Reproductive status was determined by the presence of ova or embryos within the uterus. The term "gravid" was given to those individuals that possessed ova and/or embryos, and the term "pregnant" was avoided during early stages of gestation because fertilized ova were not easily identified at dissection until the embryo was detectable by ultrasound or visible to the naked eye.

Dissection methodology

After both transducers were used to determine number of ova or embryos, the shark was carefully dissected using a left lateral incision. Reproductive tissues were measured and sampled as part of a larger study. Ova or embryos were counted, measured, and sexed. Embryos were measured if visible to the naked eye (because of sample timing, smallest observed dissected embryo size was 12 mm STL).

Data analyses

Three methods were used to determine reproductive status: linear ultrasound transducer, curvilinear ultrasound transducer, and dissection. Reproductive status was categorized as: (1) immature; (2) mature but non-gravid; (3) gravid with ova; and (4) gravid with embryos.
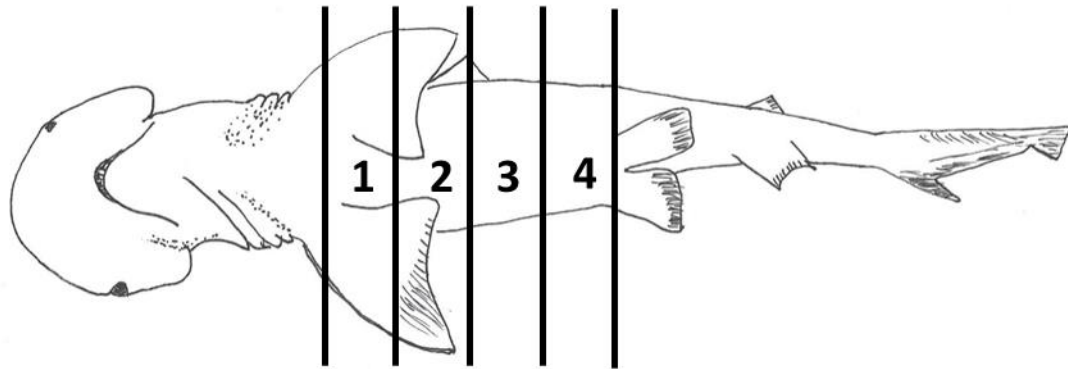
Figure 2.1. Diagram of bonnethead shark with labeled scanning zones (1 through 4).

Because of missing ultrasound scans associated with the curvilinear probe, the number of reproductive categories was reduced to three for the two analyses assessing the curvilinear. In the dissection versus curvilinear analysis, no gravid with ova females were represented. In the linear versus curvilinear, immature females were not represented. Cohen's Kappa coefficient (SAS Institute Inc. Version 9.3, Cary, NC) was used to compare the level of agreement between each method: linear transducer with dissection, curvilinear transducer with dissection, and linear with curvilinear transducers.

Bland-Altman difference plots, commonly used in method comparison studies, were used to compare quantitative data on embryo counts (fecundity) obtained using ultrasound and dissection. Statistics for the plot were calculated using the procedure outlined in Giavarina (2015). One requirement for Bland-Altman difference plot is that data are normally distributed. The Shapiro-Wilks test (Microsoft® Excel 2000/XLSTAT© Version 2015.4.01.21159, Add-insoft Inc., Brooklyn, NY) was used to test for normal distribution of the differences. The difference in embryo counts obtained using the linear transducer and dissection was not normally distributed ($W_{(0.05)} = 0.902$, $p = 0.033$) so the non-parametric Wilcoxon signed-rank test was used to determined significance. Finally, a two-sample student's t-test (Microsoft® Excel 2010) was used to determine whether the overall mean embryo counts of the ultrasound methods (linear or curvilinear ultrasound transducers) varied significantly from the overall dissection mean. This test was used to determine that when all samples are independently counted within a population of sharks, whether ultrasound method results significantly differed from the dissection method results. All graphs were constructed using Microsoft® Excel 2010 Professional Plus.

RESULTS

Ultrasound scans and dissections were conducted on 66 female bonnetheads. Ultrasound scans determined that 53 were gravid and 13 were non-reproductive. Of the 53 gravid females, 25 presented with ova and 28 presented with embryos when scanned with the ultrasound. Of the 13 non-reproductive females, 2 were categorized as immature and 11 were categorized as mature and non-gravid using the ultrasound. Of the 50 gravid females detected with dissection, 15 possessed both ova and embryos where the ultrasound only detected ova or embryos, not both. A comparison of overall results showed good agreement (90.9%) between ultrasound methods to dissection methods (Table 2.1). In all incorrectly assessed individuals, the ultrasound methods overestimated reproductive status (i.e., ultrasound methods detected ova or embryos but the individual was immature or mature but not gravid). After each zone was examined for ova, embryos, or other anatomical landmarks, the trend throughout the study indicated that the best areas to scan were zones three and four of the mother. This was in part due to other organs that scattered ultrasound waves and impaired imaging of the uteri (e.g., gut contents).

Ova were detected within the uteri via ultrasound methods during the entire yolk-dependent stage (Fig. 2.2a). Ova presented as slightly hyperechoic vacuoles within the uterus and during active scan hypoechoic fluid-filled spaces between ova were counted while moving the transducer on a transverse plane down the lateral side of the uteri. Early-gestation embryos were not detected via ultrasound methods until the spine calcified and became hyperechoic, around 85 mm STL (Fig. 2.2b). They were best scanned on the lateral side of the mother, in the sagittal plane. This size corresponded with the time embryos start developing villi on the umbilicus and were just beginning yolk-sac placental implantation yet still yolk-dependent. Yolk-dependent and placental stages were not discernable via ultrasound because once embryo spines were visible, ova were difficult to assign to a specific embryo.

Table 2.1. Overall results of reproductive status detected by ultrasound methods compared to dissection methods in female bonnethead (n = 66).

Result	Ultrasound	Dissection
Non-gravid	11	12
Immature	2	4
Gravid:	53	50
- Gravid with Ova	25	8
- Gravid with Embryos	28	42
- Both Ova and Embryos	0	15
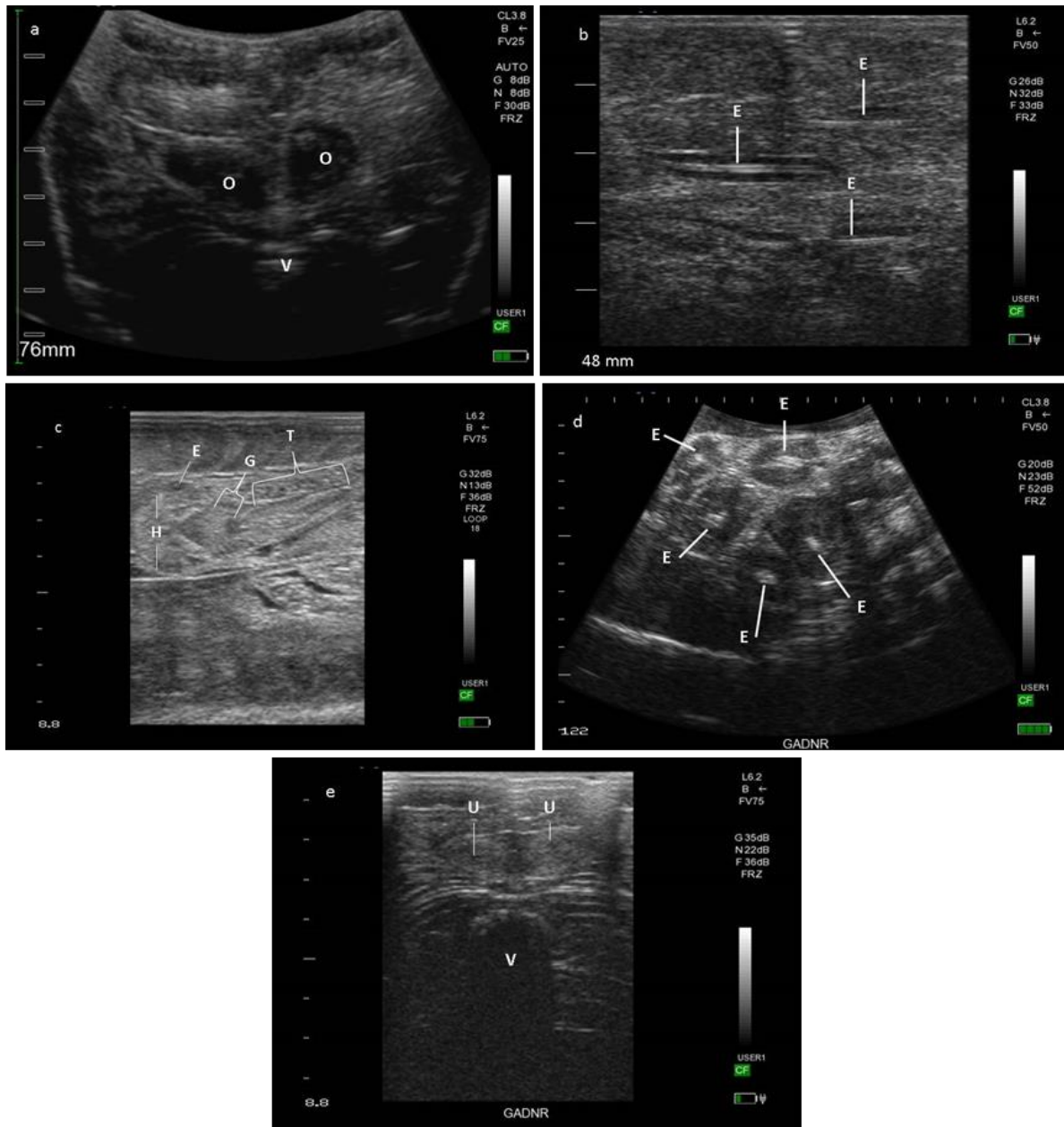
Figure 2.2 (a - e). Ultrasound scans of female bonnethead. (a) Ventral transverse scan using curvilinear ultrasound transducer of female bonnethead with one ovum (O) in each uterus. Vertebra (V) of the mother is noted for reference. Scale is 76 mm. (b) Lateral sagittal scan using linear ultrasound transducer of female bonnethead with 3 embryos (E). Average embryo size is 86.2 mm STL. Scale is 48 mm. (c) Lateral sagittal scan using linear ultrasound transducer of female bonnethead with one embryo in the uterus. Embryo anatomy is labeled: ventral side of head (H), eye (E), gills (G), trunk of body (T). Average embryo size is 120.5 mm STL. Scale is 8.8 cm. (d) Lateral transverse scan using curvilinear ultrasound transducer of female bonnethead with five embryos in utero (E). Average embryo size is 274.1 mm STL. Scale is 122 mm. (e) Ventral transverse scan using linear ultrasound transducer of female bonnethead with two post-partum uteri (U). Vertebra (V) of the mother is noted for reference. Scale is 8.8 cm.

Mid-gestation embryos were best scanned and counted on the lateral side of the mother in a transverse plane, but embryos could also be scanned and measured in the sagittal plane at this size (Fig. 2.2c). Late-gestation embryos were best scanned and counted on the lateral side of the mother in the transverse plane (Fig. 2.2d). Uteri of post-partum individuals were observed with a small hypoechoic (fluid-filled) space in the center of an enlarged uterus (up to two weeks post-partum; Fig. 2.2e). Immature individuals demonstrated a small, indiscernible uterus (data not shown). Non-gravid but mature individuals possessing a well-developed uterus from previous pregnancies demonstrated a clear hyperechoic outer shape of the organ as the ultrasound waves will reflect the surface of the uterus on the lateral side of the mother in the sagittal plane (data not shown).

There was moderate to substantial agreement between ultrasound methods and dissection in detecting reproductive status (Table 2.2). The curvilinear ultrasound transducer was better at assessing reproductive status than the linear transducer (88.9% versus 61.3%, respectively). As one would expect, the transducers agreed almost perfectly as their approach was identical (97.3%).

A scatter plot of embryo counts measured with dissection versus linear ultrasound transducer shows variability around the line of equality, as differences were highly variable yet relatively equally above and below the line of equality (Fig. 2.3a). In comparing measures of fecundity, the Wilcoxon signed-rank test showed no significant difference between the linear ultrasound transducer and dissection (T_{crit} = 30, test statistic = 55.5, p = 0.5268).

A scatter plot of embryo counts measured with dissection versus curvilinear ultrasound transducer also shows variability around the line of equality, as differences were highly variable yet relatively equal above and below the line of equality (Fig. 3b).

Table 2.2. Kappa statistic results comparing reproductive status measured by linear or curvilinear ultrasound transducers and dissection in female bonnethead.

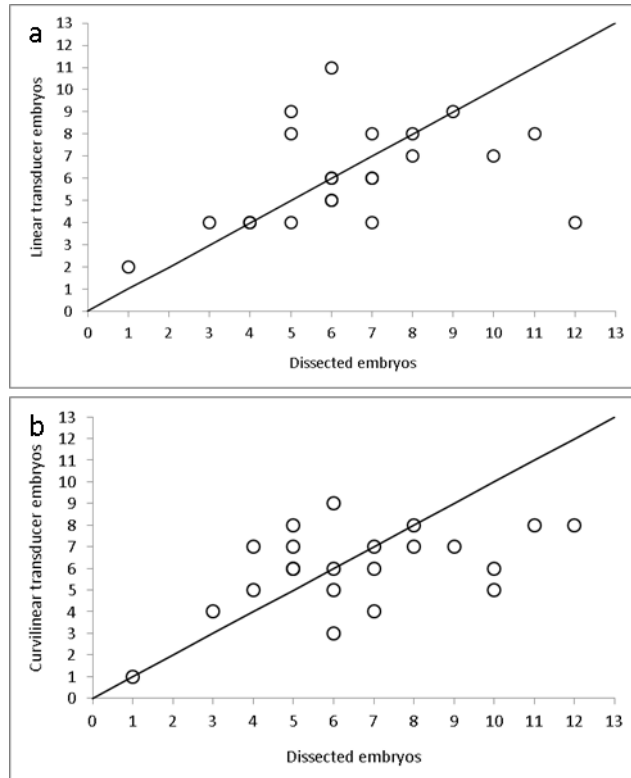| Method	n	% agreement	Kappa statistic	Result
Linear vs. Dissection	62	61.3	0.4192	Moderate agreement
Curvilinear vs. Dissection	36	88.9	0.7798	Substantial agreement
Linear vs. Curvilinear	37	97.3	0.9510	Almost perfect agreement

Figure 2.3 (a-b). Scatter plot of number of embryos counted using (a) dissection versus linear ultrasound transducer (n = 22) and (b) dissection versus curvilinear ultrasound transducer (n = 22). Line represents line of equality.

The Bland-Altman difference plot between the curvilinear ultrasound transducer and dissection resulted in the mean difference (bias) calculated at 0.55 ± 1.06 (Fig. 2.4). The bias fell within the 95% confidence intervals and was close to zero. However, there was a high degree of variability, which is to be expected in a small sample volume (Bland & Altman 1986; Giavarina 2015).

The variation of linear ultrasound transducer embryo counts did not differ significantly from the dissected embryo counts ($F_{(24, 25)} = 1.110$, $p = 0.398$). Curvilinear ultrasound transducer embryo counts also did not show significantly different variance from dissected embryo counts ($F_{(24, 22)} = 1.293$, $p = 0.274$). Mean linear transducer embryo counts (5.7 ± 2.4) compared to dissected counts (6.6 ± 2.5) were not significantly different ($T_{(51)} = 1.400$, $p = 0.168$). In addition, mean curvilinear transducer embryo counts (5.7 ± 2.2) compared to dissected embryo counts were not significantly different ($T_{(48)} = 1.404$, $p = 0.167$).
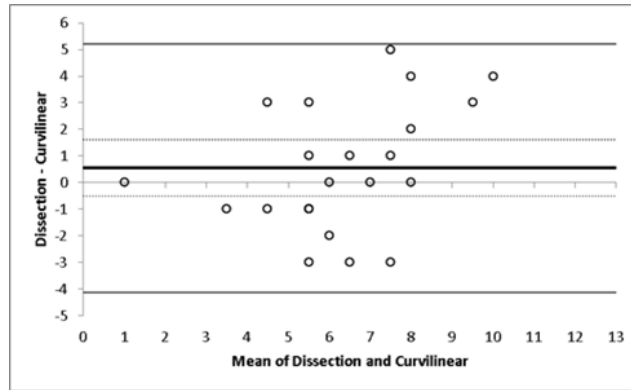
Figure 2.4. Bland-Altman difference plot of mean dissected and curvilinear ultrasound transducer embryos versus the difference of dissected and curvilinear embryos (n = 22, dark solid line indicates bias = 0.55; dotted lines indicate 95% CI = -1.60 and 0.51; solid line indicates lower LoA = -4.1 and upper LoA = 5.2)

DISCUSSION

In overall detection of reproductive status, ultrasound methods demonstrated good agreement with the "gold standard" method of dissection. At the time of publication, there are no studies assessing fecundity by combining ultrasound and dissection methods like the current study. Whittamore *et al.*, (2010) and Grant *et al.*, (2013) evaluated the use of ultrasonography for reproductive structures and shark anatomy combined with dissection but not pregnancy. Daly *et al.*, (2007) followed the ovarian progression of captive broadnose sevengill sharks (*N. cepedianus*), but pregnancy was not observed and dissection was not performed on the aquarium inhabitants. Walsh *et al.*, (1993) noted embryonic spines in one bonnethead shark, but it was an isolated observation for anatomical ultrasound reference and the dissected reference was of another shark species. Other studies used the ultrasound to confirm pregnancy for other aspects of the study and no other analyses were performed (Carrier *et al.*, 2003; Otway & Ellis 2012; Schaller 2006). The current study is the first to show a statistical comparison of pregnancy status, gestational stage, and fecundity with verification by dissection.

In the three incorrectly assessed individuals, pregnancy was overestimated by the ultrasound and likely was the result of artifact. For example, in one case of misdiagnosis early on in the study, the ultrasound methods indicated the presence of ova during early gestation where upon dissection the individual was mature but non-gravid. The gut of this female was filled with crab carapaces and other remains, obstructing the view of the uteri. In the remaining three cases, one or both transducers would indicate one or two pups in zone four of the mother, but dissection would show that the reader was imaging the rectal gland in the transverse plane.

Several aspects of ultrasound imaging can contribute to a misdiagnosis. Artifacts can have an impact on image interpretation (Nyland & Mattoon 2002). Two main categories of

artifacts include ultrasound interactions with different tissues and artifacts resulting related to operational settings and preparation (Blond & Buczinski 2009). Throughout the study, gut content became one of the most common ultrasound and dissection observations. A portion of the ultrasound waves are reflected off the surface of hard structured (hyperechoic) gut content and an anechoic area is cast behind the stomach, impairing the view of the uterus (Blond & Buczinski 2009). Other issues exist with specimen preparation (Blond & Buczinski 2009). Post-mortem individuals desiccate and the resulting concavity in the abdomen causes the transducer to have poor contact with the skin. This issue, however, would not be a factor if the ultimate purpose of a study is to scan live animals in the field.

The two transducers used in this study had varying levels of success and benefits. The linear transducer (8 – 5 MHz, 12 cm scanning depth) possessed a smaller field of view, shallow sounding, and observations showed it was appropriate for scanning ova and pups if sounded in a lateral plane in smaller species (under 2 m TL). The curvilinear transducer (5 – 2.5 MHz, 24 cm scanning depth) possessed a larger field of view, deeper sounding, and observations showed it was more appropriate for larger species (2m TL or larger; Anderson unpublished data), and appropriate for scanning embryos but better with smaller litter sizes. One should expect overestimation in larger litter sizes with both transducers depending on the species examined. Many embryos will stack upon each other and the caudal tail will double back along the trunk of the body, resembling two embryos. Other studies in fish, wildlife, and domestic animal biology have reported a margin of error in fecundity using the ultrasound (*e.g.*, Toal *et al.*, 1986; Griffin *et al.*, 2003; Zuffi *et al.*, 2005; Macbeth *et al.*, 2011). Over- and underestimation are equally reported among studies, and almost all report no statistical significance between measures of fecundity using ultrasound and dissection.

While ultrasound methods could be used to correctly identify and count ova within the uterus, they could not discriminate a fertilized egg versus a non-fertilized egg until embryo spines became hardened (around 85 mm STL). Ultrasound methods can be used to correctly identify and count embryos just beginning their transition to yolk-sac implantation in the bonnethead, and McComb *et al.*, (2005) state that embryos are still yolk-dependent until 100 mm STL. However, the ultrasound machine used in this study did not possess a high enough resolution to allow readers to identify gestational stage (yolk-dependent embryos versus yolk-sac placental attachment).

Conrath (2005) presents the argument that fecundity should be measured during late gestation to avoid overestimations that include unfertilized eggs or inviable embryos. Because fecundity could not be measured by ultrasound methods until after embryos matured to an average of 85 mm STL, around mid-gestation, this study supports that argument with several cases. Throughout the study, observations during dissection presented several unfertilized eggs that were in various states of decay and resorption. These became evident only after other embryos in the litter had progressed in development. They would not have been identifiable during early gestation using either ultrasound or dissection methods. In one particular case, the pregnant female had a decaying ovum, two decaying embryos of different sizes, and normal-sized healthy embryos all in the same litter. In this instance, at late gestation, both methods would have detected the aborted pups and not counted the unfertilized ovum.

There is only one previous study on bonnethead reproduction and fecundity in Southeast Atlantic waters (Frazier *et al*., 2013). Variation in mean litter size in this study was not significantly different than the current study ($p > 0.407$). However, the previously reported mean litter size of 8.8 ± 2.4 was significantly different than mean litter sizes found in the current study

(linear: 5.7 ± 2.4; curvilinear: 5.7 ± 2.2; dissection: 6.6 ± 2.5; $p < 0.0001$). Sample size was likely a factor in this difference, as the previous study's sample size was over three times greater than in the current study.

Ultrasound methods are a good proxy for lethal sampling and dissection. Portable ultrasound machines are beneficial to field studies, as they are compact, portable, non-invasive, and provide real-time results (Ortiz *et al.*, 2012). By simply examining pregnancy in sharks, fisheries managers can infer reproductive periodicity, cycles, and potential mating and pupping grounds. Ultrasound methods can also be used in measures of fecundity, size of maturity, or reproductive mode. Combined with other reproductive assessments, such as hormone analysis, an entire suite of reproductive measures can be determined non-lethally (Hammerschlag and Sulikowski 2011).

The potential of non-lethally detecting pregnancy in endangered and protected species yields a qualitative measure on pregnancy that is more definitive than other forms of pregnancy assessment (i.e. visual assessment and palpation; Toal *et al.*,1986; Otway and Ellis 2012). It was previously reported that visual assessment of pregnancy in sharks is likely erroneous and personal observations in this study support this argument (Otway and Ellis 2012).

There are challenges with field-ready ultrasounds such as low resolution, poor image quality, and limited penetration that can be overcome with developing technology. More work is needed to scan uteri and ovaries in species of shark that have excessive hyperechoic structures (such as hard structures in the gut), excessive size or girth (larger than 4 m STL), and in scanning large specimens in the field where boat-side scanning is the safest option. Future work includes

scanning protected species such as the oceanic whitetip (*Carcharhinus longimanus*), white shark

(*Carcharodon carcharias*), and the endangered smalltooth sawfish (*Pristis pectinata*).

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