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Characterization of Andrenocortical Tissue Morphology, Histology, and Steroid Synthesis Among Finetooth (*Carcharhinus isodon*), Blacktip (*Carcharhinus limbatus*), Atlantic Sharpnose (*Rhizoprionodon terraenovae*), and Bonnethead (*Sphyrna tiburo*) Sharks

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CHARACTERIZATION OF ANDRENOCORTICAL TISSUE MORPHOLOGY, HISTOLOGY, AND STEROID SYNTHESIS AMONG FINETOOTH (*CARCHARHINUS ISODON*), BLACKTIP (*CARCHARHINUS LIMBATUS*), ATLANTIC SHARPNOSE (*RHIZOPRIONODON TERRAENOVAE*), AND BONNETHEAD (*SPHYRNA TIBURO*) SHARKS

by

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ABSTRACT

CHARACTERIZATION OF ANDRENOCORTICAL TISSUE MORPHOLOGY, HISTOLOGY, AND STEROID SYNTHESIS AMONG FINETOOTH (*CARCHARHINUS ISODON*), BLACKTIP (*CARCHARHINUS LIMBATUS*), ATLANTIC SHARPNOSE (*RHIZOPRIONODON TERRAENOVAE*), AND BONNETHEAD (*SPHYRNA TIBURO*) SHARKS

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Adrenocortical (interrenal) tissue is composed of steroidogenic cells that produce corticosteroids involved in the stress response and hydromineral balance. Previous research characterizing the elasmobranch interrenal suggests that the number of interrenal bodies that produce the single primary corticosteroid, 1α-hydroxycorticosterone (1α-OHB), varies among species. However, potential species-specific differences in the amount of interrenal tissue and major steroid products produced have been understudied. To address this critical gap in our understanding of elasmobranch biology, this study examined interrenal morphology, cell structure, steroidogenic enzyme distribution (3βHSD), and steroid production in four shark species: Finetooth *Carcharhinus isodon*, Blacktip *Carcharhinus limbatus*, Atlantic Sharpnose *Rhizoprionodon terraenovae*, and Bonnethead *Sphyrna tiburo*. Morphological analysis revealed differences in interrenal structures among species. Finetooth and Blacktip Sharks have numerous discrete white interrenal bodies along the dorsal midline of the kidney. The Atlantic Sharpnose Shark interrenal is a single yellow-white to dark green body along the kidney midline.

Bonnethead sharks possess one thin strip of white tissue extending from the anterior portion of the kidney ending past the division of the kidney lobes. Histological assessment of cell type and 3βHSD expression confirmed putative interrenal cytology and steroidogenic potential among all species. *Ex vivo* tissue incubations confirmed steroidogenesis and validated 1α-OHB as the dominant steroid product; however, corticosterone (B) and deoxycorticosterone (DOC) were also produced. This study is the first to describe interrenal morphology in Atlantic Sharpnose and Bonnethead sharks. These results advance our understanding of elasmobranch corticosteroid production as well as potential mechanisms for species-specific responses to acute and chronic stress.

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DEDICATION

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
B	Corticosterone
CRH	Corticotropin-releasing hormone
DHA	Dehydroepiandrosterone
DOC	11-deoxycorticosterone
HPI	Hypothalamus-pituitary-interrenal axis
HPLC	High-pressure liquid chromatography
HPT	Hypothalamic-pituitary-thyroid
ISI	Interrenal-somatic index
NAD	Nicotinamide adenine dinucleotide
NBT	Nitroblue Tetrazolium
OCT	Optimal Cutting Temperature
PBS	Phosphate-buffered saline
P450scc	P450 side chain cleavage
StAR	Steroidogenic acute regulatory protein
-S negative control	Negative controls of no oil substrate
S/-NAD negative control	Negative controls of oil substrate without NAD
TMAO	Trimethylamine oxide
1α-OHB	1α-hydroxycorticosterone
3βHSD	3β-hydroxysteroid dehydrogenase
17βHSD	17β-hydroxysteroid dehydrogenase
11βHSD	11β-hydroxysteroid dehydrogenase

CHAPTER I - INTRODUCTION

Adrenocortical tissue (adrenal or interrenal tissue) is present in all vertebrates and plays a crucial role in the physiological regulation of both the stress axis and hydromineral balance. In mammals, the adrenal glands are located superficially, dorsal to the kidneys, and are composed of two cell layers: an outer cortex and an inner medulla. The adrenal medulla contains chromaffin cells, neuroendocrine cells that produce adrenaline and noradrenaline when stimulated by the stress cascade. The mammalian outer cortex is composed of three zones – zona glomerulosa, zona fasciculata, and zona reticularis – and produces the steroid hormones aldosterone and cortisol. Aldosterone is a mineralocorticoid produced in the adrenal zona glomerulosa that modulates blood pressure and electrolyte balance to regulate hydromineral balance. Cortisol, a glucocorticoid produced in the zona fasciculata, regulates metabolism and facilitates the synthesis and release of glucose, and is, therefore, a crucial component of the primary stress response. The zona reticularis produces precursors to reproductive steroids.

Unlike mammalian adrenal tissue, elasmobranch interrenal cells produce the novel corticosteroid hormone 1α-hydroxycorticosterone (1α-OHB). This steroid is believed to act as both a mineralocorticoid and glucocorticoid (reviewed by Anderson 2012). Previous studies have confirmed 1α-OHB as the predominant product of the elasmobranch interrenal but debate the presence of other products such as corticosterone (B), and 11-deoxycorticosterone (DOC) (Table 1.1). Variation in experimental results regarding steroid products and quantities could be due to the historical difficulty in quantifying steroid products, particularly 1α-OHB, and cross-reactivity of available assays for corticosteroid quantification.

The morphology of mammalian adrenal glands is well understood, however, there is a relative lack of information regarding adrenal or interrenal tissue structure in elasmobranchs as compared to non-mammalian vertebrates (Figure 1.1). The mammalian adrenal structure is located superficially and dorsal to both kidneys. A similar structure of adrenal tissue located above the kidney is seen in birds (Chester-Jones 1957). Teleosts have an intra-renal distribution, where both interrenal and chromaffin cells are dispersed throughout the anterior region of the kidney (Nandi 1962). Chondrichthyan (including the subclass elasmobranchii) interrenal tissue is composed of a varying number of distinct bodies, is believed to consist of one cell type, and is located on the dorso-medial surface of the kidney (Idler 1972). Chromaffin cells are present as separate bodies lying adjacent and on both sides of interrenal tissue.

Elasmobranch interrenal tissue was first noted by Retzius (1819) and further described by numerous scientists from the 1830s to the 1850s (Nagel 1836, Stannius 1846; Leydig 1851, 1852, and 1853). Early studies from several authors (reviewed in Chester-Jones 1957) characterized interrenal tissue into three structural groups: rod-shaped, horseshoe shaped, and concentrated type. These different morphologies have been observed in multiple species: the rod type was present in Small-Spotted Catshark *Scyliorhinus canicula* (previously *Scyllium canicula),* Nursehound Shark *Scyliorhinus stellaris* (previously *Scyllium cutulus),* Common Smoothhound *Mustelus mustelus* (previously *Mustelus laevis/M. vulgaris),* Blue Shark *Prionace glauca* (previously *Carcharias glaucus),* Tope Shark *Galeorhinus galeus* (previously *Galeus canis)*, and one holocephalan, Rabbitfish *Chimaera monstrosa* (Dittus 1941); the horseshoe type was found within the ray genus, *Raja,* including Starry Skate *R. asterias,* Common Skate

Dipturus batis (previously *R. batis),* Thornback Ray *R. clavata,* and Longnose Skate *Dipturus oxyrinchus (previously R. laeviraja oxyrhinus)* (Grynfellt 1904a); and the concentrated type was observed in the genus *Torpedo*, e.g., Common Torpedo Ray *Torpedo torpedo* (previously *T. ocellata*), and Marbled Electric Ray *T. marmorata* (Kisch 1928). Variation in interrenal mass among species has been documented by Hartman *et al.* (1943), who investigated interrenal mass among ten species of elasmobranchs and found large differences among Little Skate *Raja erinacea,* Smooth Dogfish *Mustelus canis*, and Spiny Dogfish *Squalus acanthias*, as well as distinctions in interrenal mass between genders. Observations of the general structure of interrenal tissue in multiple elasmobranch species have been described; however, unlike other taxa, considerable variations in location and quality among species have been documented.

Due to the anatomically distinct structure of interrenal bodies, lack of characterization and description of interrenal tissue in multiple species, and undefined interrenal steroid production in chondrichthyans, the goal of my study is to further describe interrenal morphology and steroid production for four species of sharks commonly found in the northern Gulf of Mexico. A greater understanding of chondrichthyan interrenal tissue may provide explanations for observed variations in the stress response among different species. Therefore, my study describes interrenal morphology, examines histological characteristics, and examines steroid production among four species of sharks: Finetooth Shark *Carcharhinus isodon*, Blacktip Shark *Carcharhinus limbatus*, Atlantic Sharpnose Shark *Rhizoprionodon terraenovae*, and Bonnethead *Sphyrna tiburo* (Figure 1.2).

To describe gross morphology, Chapter I details the structure and distribution of interrenal tissue in these four species of sharks. Chapter II validates putative interrenal tissue cell structure and the presence of steroidogenic enzymes using hematoxylin and eosin histology and histochemical localization of the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (3βHSD), respectively. Investigating steroid production, Chapter III confirms steroid synthesis and possible species-specific differences in products from *ex vivo* incubations of interrenal tissue.

1.1 Animal Models

Finetooth, Blacktip, Atlantic Sharpnose, and Bonnethead sharks were chosen as model organisms. All species are abundant in the Mississippi Sound and are in the order Carcharhiniformes.

Finetooth Sharks are coastal species reaching total lengths of 156 cm for females and 145 cm for males (Castro 2011). This species resides along the coast during the summer and migrates offshore during the winter. They are found in the Atlantic Ocean from North Carolina to Cuba (including the Gulf of Mexico) and in southern Brazil. They congregate in schools to prey on Atlantic Menhaden *Brevoortia patronus*. Identifying features include a steel-blue coloration, pointed snout, large eyes, slender teeth, and large gill slits.

The Blacktip Shark is considered a large coastal species that resides both near-shore and offshore but habitually in depths less than 30 meters (Castro 2011). Maximum total length is approximately 156 cm for females and 145 cm for males. They are a cosmopolitan species that inhabit tropical and subtropical waters, including the Gulf of

Mexico. Blacktip Sharks are identified by distinctive black tips on all fins except the anal fin, with the first dorsal fin originating over the trailing edge of the pectoral fins.

Atlantic Sharpnose Sharks are the most abundant species in the Mississippi Sound and are considered a small coastal species. This species has a maximum total length of 110 cm for both sexes (Castro 2011). They are found along the Atlantic coast of the U.S. from New Brunswick, Canada, through the Gulf of Mexico and coast of Brazil. They are characterized by a long snout, long labial furrows on the corners of the mouth, and small white spots along their sides.

Bonnethead sharks are small coastal sharks found in warm waters along the U.S. Atlantic coast, including the Gulf of Mexico and Caribbean Sea, and along the Pacific coast, from southern California to Ecuador (Castro 2011). During winter months Bonnethead sharks migrate closer to the equator. Bonnethead sharks average total lengths 100-120 cm for both sexes with a maximum length of 150 cm total length. A spade-shaped head and high first dorsal fin are distinguishing characters for these sharks.

Table 1.1

Variability of steroid products from selected elasmobranch species.

Species	Sex	1α-OHB	B	Reference
Thorny Skate	M/F	0.6 ± 0.08	0.021	Truscott and Idler 1972
Spiny Dogfish	M/F	2.3 ± 0.5	1.5 ± 1	Truscott and Idler 1972
Blue Shark	M/F	0.87 ± 0.05	0.3	Truscott and Idler 1972
Shortfin Mako	M/F	5.3	0.052	Truscott and Idler 1972
Small-Spotted Catshark	F	3.97 ± 0.28	---	Hazon and Henderson 1984

1 µg per 100mL plasma; Steroid hormones: 1α-OHB- 1α-hydroxycorticosterone, B- Corticosterone.

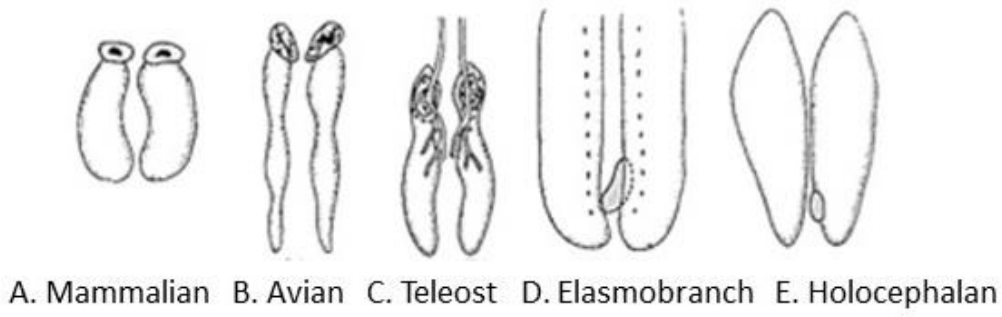


Figure 1.1 Variation in adrenal/interrenal (densely stippled) and chromaffin (black) tissue distribution among major taxa.

(Idler 1972).

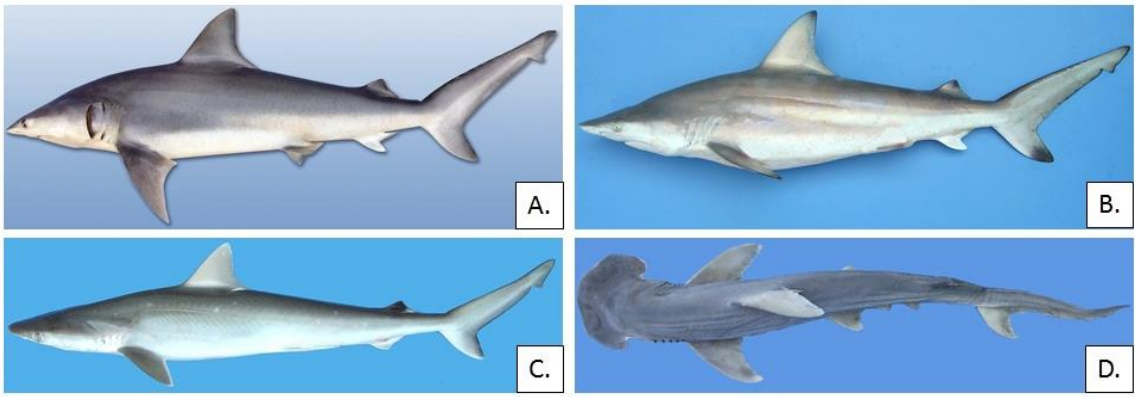


Figure 1.2 Four species of coastal sharks.

A. Finetooth Shark B. Blacktip Shark C. Atlantic Sharpnose Shark D. Bonnethead (Hoffmayer *et al.* 2007).

CHAPTER II – CHARACTERIZATION OF INTERRENAL MORPHOLOGY AND BIOMETRIC ANALYSIS OF INTERRENAL-SOMATIC INDEX AMONG SPECIES, SEX, AND ONTOGENY

2.1 Introduction

Interrenal coloration and structure vary among elasmobranch families. In most species, interrenal tissue has a yellowish-white coloration that makes discrete adrenal bodies distinguishable from kidney tissue and therefore facilitates accurate identification (Hartman *et al.* 1943). Skates contain one or two interrenal bodies lying ventro-medial to the kidneys (Hartman *et al.* 1943), while stingrays typically have a single interrenal body (Evans, personal communication). Some shark species contain individual interrenal bodies embedded medially on the dorsal side of the posterior portion of the kidney, with chromaffin (neuroendocrine) tissue present as thin masses left and right of the kidney (Figure 2.1) (Hartman *et al.* 1943; Chester-Jones 1957). Jones (1957) describes shark interrenal tissue as having three possible structure types: rod, horseshoe, and concentrated (Figure 2.2). Investigation of the Finetooth Shark interrenal morphology (Figure 2.3) reveals the expected shark morphology of a concentrated tissue type with numerous distinct interrenal bodies embedded dorso-medially to the kidney tissue. This tissue in Finetooth Sharks has been positively identified as interrenal due to its ability to synthesize corticosteroids *ex vivo* (Evans, unpublished). Blacktip, Atlantic Sharpnose, and Bonnethead sharks have interrenal morphology not previously described in the literature, which further supports the need for additional studies.

Previous studies have observed differences in interrenal size among elasmobranch families; for example, Smooth Dogfish *Mustelus canis* and Spiny Dogfish *Squalus*

acanthias possess relatively larger interrenal glands compared to the Little Skate *Leucoraja erinacea* (Hartman *et al.* 1943). Hartman *et al.* (1943) also discovered a significant positive interrenal-somatic index (ISI), a measure of interrenal mass relative to total body mass, correlations within female Little Skate, Spiny Dogfish, and Smooth Dogfish, and within the males of Little Skate and Smooth Dogfish. To compare the relative relationship of interrenal mass between individuals and species in this study, ISI was calculated.

Species-specific differences in structure and relative interrenal mass may impact the rate of synthesis and ultimately baseline and maximum circulating levels of corticosteroids, potentially affecting the stress and/or osmoregulatory physiology of each species (e.g., species-specific responses to stress). The presence of multiple interrenal bodies in sharks, variation among species, and a lack of understanding of interrenal tissue in other elasmobranch species led to my investigation of the variation in size, number, morphology, and distribution of the interrenal bodies among species. The specific goals of this chapter were to 1) describe interrenal morphology, and 2) investigate the relative relationship of interrenal mass among four shark species.

2.2 Methods

2.2.1 Sample Collection

Finetooth (n=129), Blacktip (n=91), Atlantic Sharpnose (n=69), and Bonnethead (n=53) sharks were collected in 2015 (March to November) and 2016 (March to September) in the Mississippi Sound, Mississippi and around the Chandeleur Islands, Louisiana. Samples were collected during longline and gillnet surveys conducted by The

University of Southern Mississippi's Center for Fisheries Research and Development, as well as from local and commercial fishermen in these same regions.

Upon collection, sex, length [precaudal (PCL), fork (FL), and stretched total (STL); to the nearest millimeter], and weight (kg) were determined before the animal was euthanized by pithing and vertebral severance just posterior to the skull.

All procedures were approved by The University of Southern Mississippi Institutional Animal Care and Use Committee, protocol numbers 11092217 (longline; Appendix A), and 13101704 (gillnet and longline).

2.2.2 Biometrics (Dissection and Interrenal-Somatic Index)

Animals collected for biometric analysis were placed on ice until return to the laboratory. An incision was made from the cloaca to the pectoral girdle to open the body cavity and allow for excision of the kidney tissue and interrenal bodies. For both tissues, weight (g), length (mm) and width (mm; interrenal bodies only) were determined. Kidney tissue was photographed to observe interrenal placement and morphology. A count of interrenal bodies was recorded before the tissue was removed from the kidney and weighed. Animals were classified by maturity (immature, mature), with gestating sharks within pregnant individuals classified as pups. Transitional animals that were reproductively immature but displayed characteristics of gonadal change such as suspension of uterine tissue and enlargement of testes without calcified or rotatable claspers, and rhipidion ability to splay, were not included in the ISI analysis.

Interrenal-somatic index (ISI) was calculated as the percentage of interrenal mass relative to total body mass. Calculated ISI was then multiplied by a standardization factor of 10^6 (n-values in Table 2.1).

$$ISI = \left(\frac{interrenal\ tissue\ mass\ (g)}{total\ body\ mass\ (g)}\right) * (10^6)$$

2.2.3 Statistical Analysis

To examine if interrenal mass varied throughout the life history of each species, the correlation coefficient of interrenal mass and body mass within each species was calculated for all sex-maturity stage combinations (immature-males, immature-females, mature-males and mature-females). Biometric comparisons of ISI between maturity stages and sexes were statistically assessed; no comparisons between species were made due to the high degree of variability in morphology (see Results below). The ISI data violated assumptions of normality and variance homogeneity (as indicated by results from Shapiro-Wilks and Levene tests, respectively), thus a nonparametric Kruskal-Wallis test on ranks was used to compare median estimates for sex-maturity stage combinations within species groups. A multiple comparisons Kruskal-Wallis post hoc test was used to determine which sex-maturity stage combinations were significantly different from one another within each species (at $p<0.05$).

2.3 Results

Distinct morphological differences with regards to interrenal tissue were observed among shark species. Finetooth and Blacktip Shark interrenal tissue followed the expected rod-shaped morphology of numerous discrete bodies embedded dorso-medially in kidney tissue (Figures 2.3 and 2.4, respectively). Both species have small distinct oval

to round bodies that are whitish to yellow in coloration. Interrenal bodies appear distinct from one another with a few bodies appearing rod-like or merging with adjacent bodies. In several larger individuals, interrenal bodies appeared to be larger in size and merged with adjacent interrenal bodies forming lobular structures (Figure 2.5). All interrenal bodies were located under a thin membrane encapsulating the kidney tissue. Atlantic Sharpnose Sharks have one discrete interrenal body embedded dorso-medially within the kidney that is greenish-brown or yellowish-white in coloration (Figure 2.6, A and B, respectively) and could be a modified classification as an elongated rod-shaped. This tissue has a speckled or granulated texture and is surrounded by a very thin membrane less robust than that of the Finetooth and Blacktip Sharks. The putative interrenal tissue in Bonnethead sharks is composed of one ribbon-like strip embedded dorso-medially in kidney tissue, which extends past the division of the anterior and posterior kidney lobes (Figure 2.7). The anterior of the tissue is extremely thin and ends just past the anterior separation of the kidney lobes. The posterior end of the tissue is cylindrical; the extended length, texture, and thinness of the interrenal tissue suggest a new classification is warranted. While this tissue is discrete, it is less robust than the other species and difficult to remove from the kidney. In each species, interrenal tissue is bordered by distinct rows of chromaffin tissue, which appear as discrete white bodies much smaller than that of the interrenal tissue.

When available, pups were examined for the presence of interrenal tissue. Pup interrenal tissue was variable in size and easily distinguished and identifiable in Finetooth (5 female, 3 male) at approximately 4 and 11 months gestation and in Atlantic Sharpnose Sharks (3 female, 4 male) at approximately 11 months gestation. No interrenal was

obvious in Blacktip Shark pups (4 female) at approximately 3 months gestation. The approximate gestation month was based on the estimated time of mating; July for Finetooth Sharks (Hendon *et al.* 2013), June for Blacktip Sharks (Castro 1996) and Atlantic Sharpnose Sharks (Hoffmayer *et al.* 2013). Interrenal bodies within Finetooth Shark pups were located under a thin membrane, white in coloration and very small. Tissue coloration in Atlantic Sharpnose Shark pups was like that of the kidney, appearing slightly darker red to brown than the kidney surrounding it. No yellow-white or green coloration or distinct granules were present in putative interrenal tissue from Atlantic Sharpnose pups. Small tissue masses and an insufficient number of individuals limited further analyses of pup interrenal tissue.

The relationship between interrenal mass and body mass was examined using correlation analyses (Figure 2.9). Within all species, Finetooth Sharks have the strongest linear relationship (R^2=0.8180) followed by Blacktip (R^2=0.7198), Atlantic Sharpnose (R^2=0.5839), and Bonnethead sharks (R^2=0.4966). In general, interrenal mass increased with body mass, however, the correlations were more variable when analyzed by sex-maturity stage categories.

Within species, significance differences were observed among sex-maturity stage combinations. A significant difference was found within Finetooth Sharks ($p < 0.001$); post hoc analysis revealed significant pairwise differences between immature and mature males (average ISI ± standard error respectively; 17.96 ± 1.16 and 10.62 ± 0.66), immature females and mature males (15.56 ± 0.81 and 10.62 ± 0.66), and mature females and immature males (10.45 ± 1.86 and 17.96 ± 1.16). Within Blacktip Sharks significant differences ($p = 0.007969$) were revealed between immature and mature females (10.11 ±

0.60 and 5.84 ± 0.59), and mature females and immature males (5.84 ± 0.59 and 10.78 ± 1.18). Atlantic Sharpnose Sharks had significant differences within groups (p=0.01279), and post hoc analysis revealed a difference between immature females and immature males (28.77 ± 2.91 and 16.15 ± 4.45). Bonnethead sharks also had a significant difference (p = 0.01428), and post hoc analysis revealed a difference between immature and mature males (63.68 ± 12.28 and 29.44 ± 1.87).

Due to the nonparametric nature of ISI, comparison of medians within species was visualized using box and whisker plots (Figure 2.10). Within Finetooth Sharks there was a widespread range of ISI in immature females and immature males, however, medians for both groups were similar (females 17.34, males 16.30). Mature males had a narrower range closely followed by mature females with medians of 9.86 and 7.93, respectively. Blacktip Shark immature males had the largest range, while mature females had the smallest. Medians of immature males (9.60) and females (10.69) were similar. Atlantic Sharpnose Sharks had widespread ranges, and medians varied such that no groups were similar (immature males 10.54, mature males 17.86, immature females 32.13, mature females 23.10). Bonnethead shark ranges were similar to those of Atlantic Sharpnose Sharks with increased variability in medians among groups (immature males 57.90, mature males 27.41, immature females 49.32, mature females 30.96). The widest range was observed among immature females (18.42- 161.71).

2.4 Discussion

Novel variability of interrenal tissue morphology among species was observed. This is the first description of the Atlantic Sharpnose and Bonnethead shark interrenal tissue. Finetooth and Blacktip Sharks have morphology similar to previous literature.

Distinct features of Atlantic Sharpnose Shark interrenal tissue are one interrenal body instead of numerous bodies, and the variable coloration of the tissue. Bonnethead shark interrenal tissue is distinguished by the thinness of the elongated tissue.

The notable difference in coloration and structure in Atlantic Sharpnose Shark interrenal tissue is unexplained, but may be due to evolutionary divergence within the Carcharhinidae family, which includes Atlantic Sharpnose, Blacktip and Finetooth Sharks. The Bonnethead shark is in the monophyletic family Sphyrnidae, and is phylogenetically and morphologically distinct from the Carcharhinidae (Naylor 1992). Within Carcharinidae, Atlantic Sharpnose Sharks are in the monophyletic genus *Rhizoprionodon*, forming a clade with the Tiger Shark *Galeocerdo cuvier*, as both exhibit long labial furrows and pre-anal ridges (Compagno 1984; Naylor 1992). Blacktip and Finetooth Sharks are in the genus *Carcharhinus*. The distinctions within the genus *Carcharhinus*, however, are more variable with Finetooth and Blacktip Sharks having varying degrees of relatedness, (Naylor 1992) and such distinctions are still debated. The phylogenetic relationship between genus and family morphological variation requires further elucidation of evolutionary divergence and genetic investigation within shark families to clarify if Atlantic Sharpnose Shark interrenal coloration and morphology may be due to a phylogenetic difference.

It is also possible that the distinct coloration observed in Atlantic Sharpnose Shark interrenal tissue is related to iron storage, lipid metabolism, or additional causes of pigmentation. The presence of phagocytes containing haemosiderin, a product of iron breakdown - distinguished by brownish-yellow granules filling the cytoplasm and unusual nuclei- present in haemopoietic tissue within kidneys of Nile Tilapia

Oreochromis niloticus could be one possible example (Agius 1980, 1981; Pulsford *et al.* 1982; Zuasti *et al.* 1989; Abdel-Rahman 1997; Bin-Dohish 2001, 2003; Sarmento *et al.* 2004). As interrenal tissue is highly vascularized this coloration could be due to blood cells being broken down within the tissue. A test using Prussian blue to detect iron presence via histology would help clarify this concept among species in future studies. Another explanation could be lipofuscin, a fine granular pigment containing lysosomal degradation of lipids found in liver, kidney, heart muscle, adrenals, nerve cells and ganglion, although this pigment is often present in hematoxylin and eosin staining (Young and Heath 2000). Future testing with the Armed Forces Institute of Pathology method or oil red (Luna 1968) could confirm the presence of lipofuscin. This pigment is related to aging but no differences in coloration were obvious among Atlantic Sharpnose Sharks based on sex or maturity. Another possibility could be structures similar to "melanomacrophage centers" within the head kidney of select teleosts, sometimes encapsulated but ranging in coloration from dark brown to yellow granular or heterogeneous pigments observed within interrenal cells by Gallo and Civinini (2003). In Gilt-Head Sea Bream *Sparus aurata* and Sea Bass *Dicentrarchus labrax* these "melanomacrophage centers" are thought to function in scavenging or ingesting cell debris, and melanin from melanocytes (Meseguer *et al.* 1994). If coloration is due to breakdown of interrenal cells or lipid cells the greater presence of this coloration within Atlantic Sharpnose Sharks could possibly indicate a rapid turnover rate for interrenal cells. Future histological tests to determine presence or quality of fat, phospholipids, or unsaturated lipids within cells could further elucidate differences (Luna 1968).

Within all species, the interrenal mass increased as the body mass increased. While variability is present within each species, this progression is logical as larger organisms may require more interrenal tissue to supply sufficient amounts of corticosteroids to maintain the stress response or hydromineral balance. Linear relationships may vary due to variation in reproductive and growth development among animals, as smaller species such as Atlantic Sharpnose and Bonnethead sharks do not undergo the large increases in size, characteristic of Finetooth and Blacktip Sharks, and therefore could have relatively lower changes in interrenal mass due to size.

Within-species ISI was highly variable, with differences observed among sex-maturity stage combinations. This variability could result from multiple influences (e.g. low sample sizes in certain size classes such as mature females, any interruption in the normal growth and development that limits energy availability for interrenal growth). Variation in interrenal mass (or condition) and body mass among individuals may be explained by the health of the animal, overall energy expenditure, or other influences (i.e. poor health, increase in stomach mass, reproductive status, or previous exposure to a severe stressor). Extremely low interrenal masses from immature animals or the difference in the order of magnitude of body mass (1000's of grams) in relation to decimal grams may also expand range values. Future studies could expand our understanding of such influences on interrenal condition and growth by investigating the stress response and any associated histological changes through different life stages or environmental conditions (e.g. preovulatory, gravid different seasons, young of the year still within a hypo-responsive period, etc.).

Table 2.1

N-values for biometric analysis by species, gender, and maturity.

	Female		Male		
	Immature	Mature	Immature	Mature	Total
Finetooth Shark	63	5	44	17	129
Blacktip Shark	52	12	21	6	91
Atlantic Sharpnose Shark	15	6	13	35	69
Bonnethead	8	7	6	32	53
					367

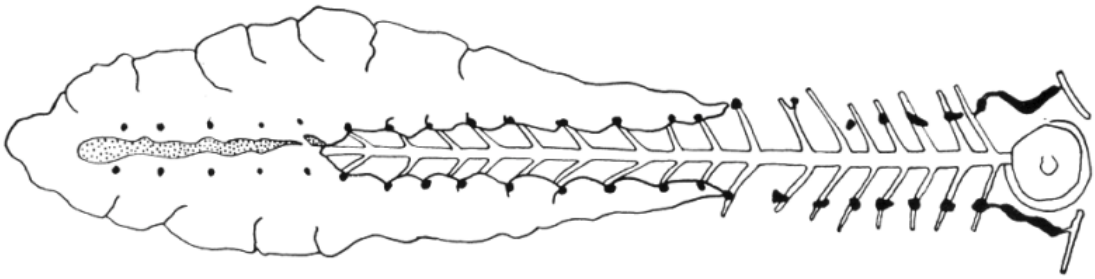


Figure 2.1 Example of kidney tissue (white), denoting interrenal (stippled) and chromaffin (black) morphology.

Greater Spotted Dogfish *Scyllium catulus* (Vincent 1897).

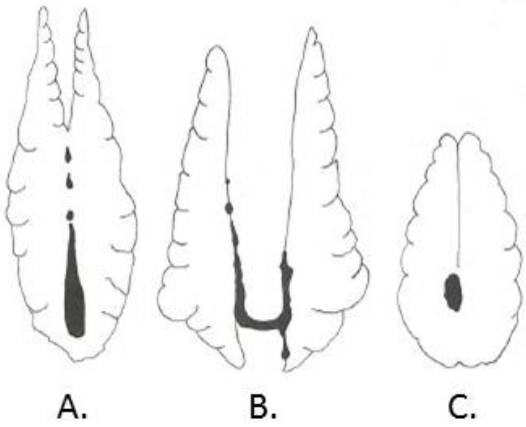


Figure 2.2 Morphological types of interrenal tissue.

A. Rod-shaped B. Horseshoe shaped C. Concentrated type (Chester-Jones 1957).

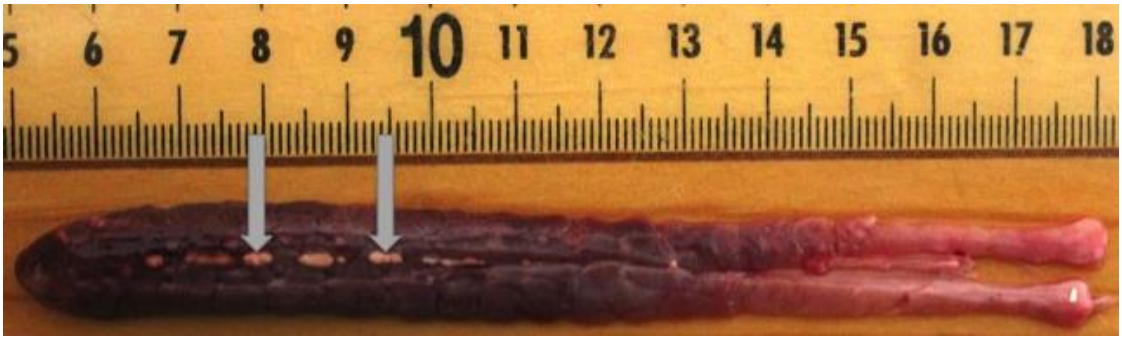


Figure 2.3 Finetooth Shark interrenal bodies (indicated by the arrows) in kidney tissue.

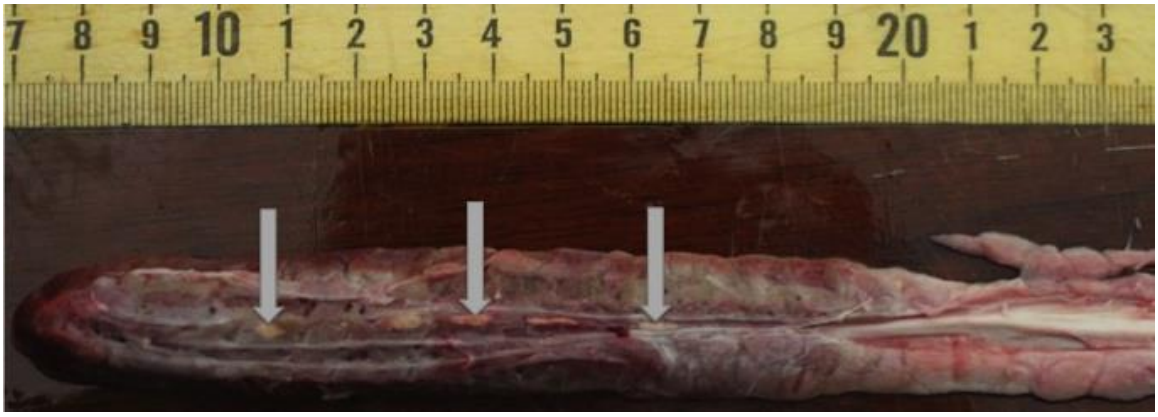


Figure 2.4 Blacktip Shark interrenal bodies (indicated by the arrows) in kidney tissue.

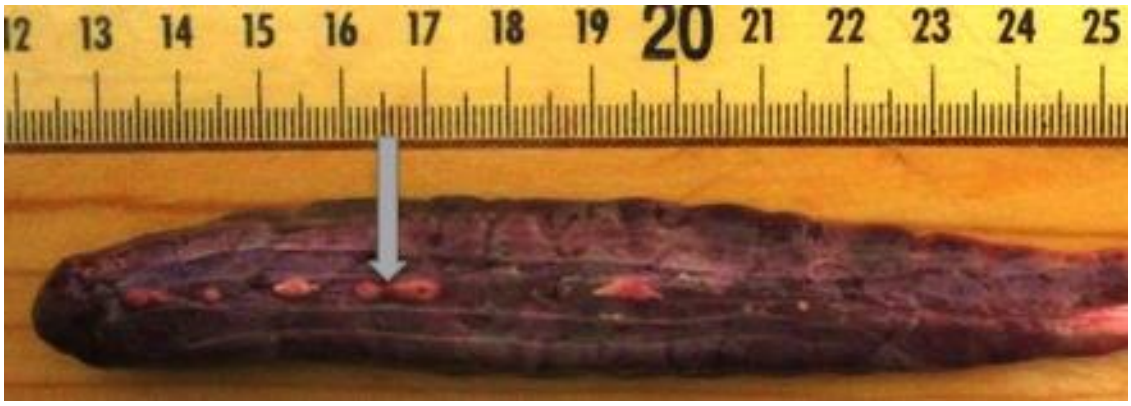


Figure 2.5 Formation of lobular structure (indicated by the arrows) when adjacent interrenal bodies increase in size and merge together.

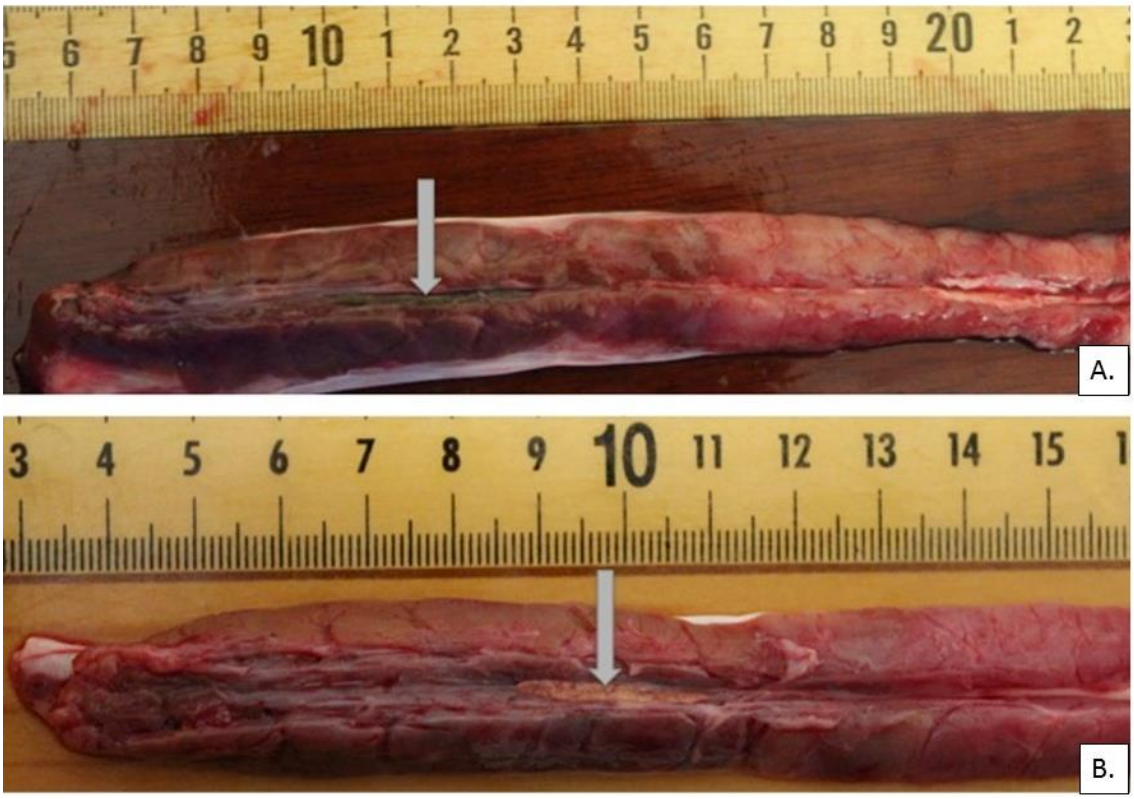


Figure 2.6 Atlantic Sharpnose Shark interrenal tissue displaying the color variation of putative interrenal bodies (indicated by the arrows) within kidney tissue.

A. Indicating dark coloration B. Indicating lighter coloration.

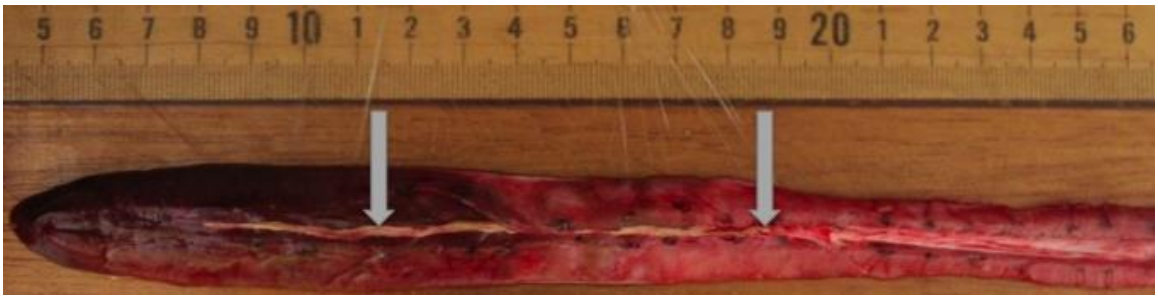


Figure 2.7 Bonnethead putative interrenal body (indicated by the arrows) in kidney tissue.

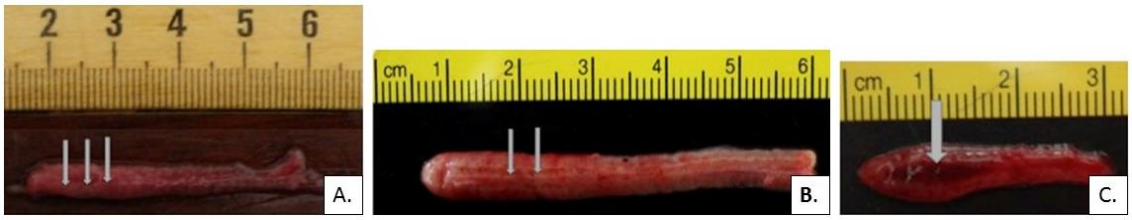


Figure 2.8 Interrenal tissue present in pups.

A. Finetooth Shark (approximately 4 months gestation) B. Blacktip shark (interrenal tissue not distinguishable at approximately 3 months gestation) C. Atlantic Sharpnose Shark (approximately 11 months gestation).

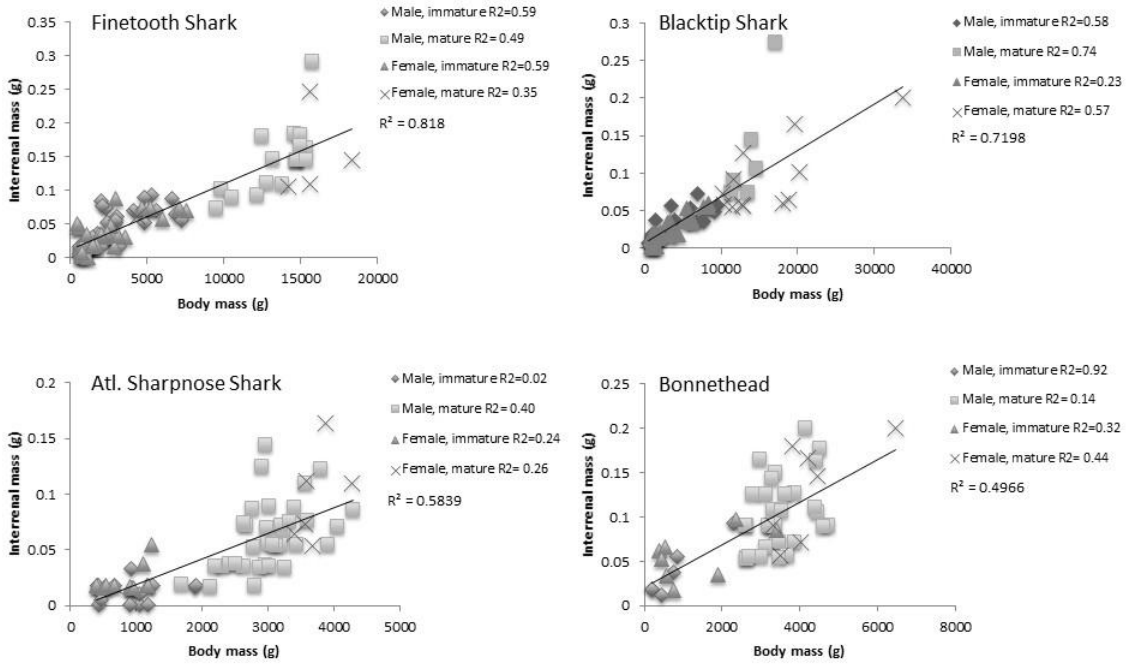


Figure 2.9 Comparison of Finetooth, Blacktip, Atlantic Sharpnose, and Bonnethead shark interrenal mass (g) to body mass (g) in each sex-maturity stage.

R^2 values are presented for all sex-maturity stages and linear trend-line with corresponding R^2 value for all individuals (regardless of sex-maturity stage) within the species.

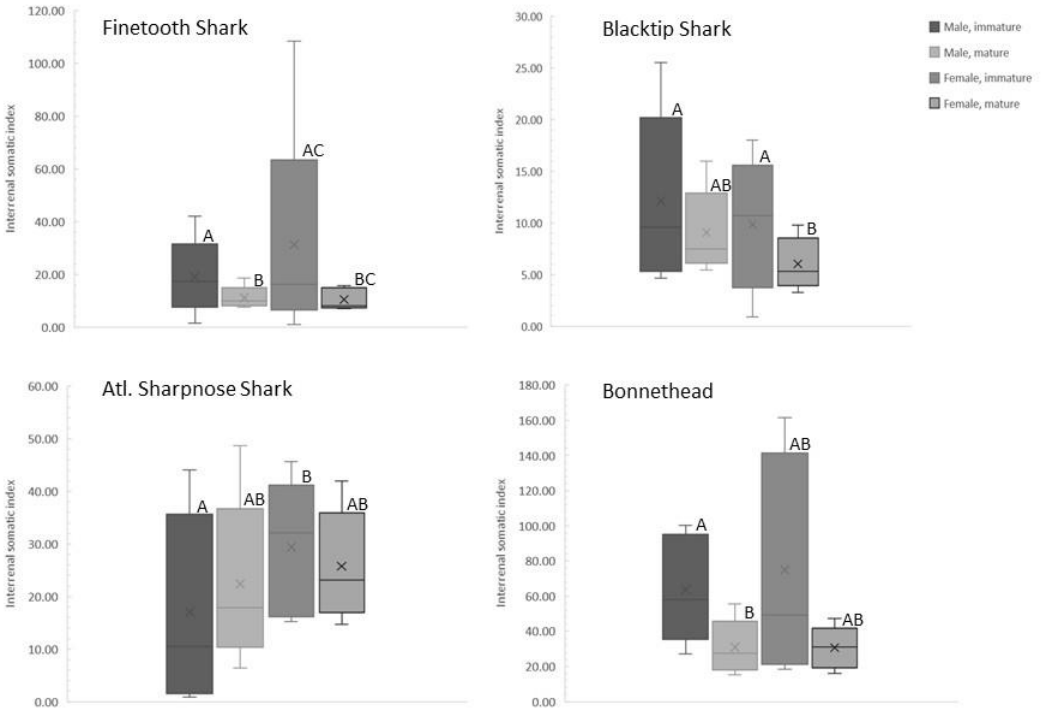


Figure 2.10 Finetooth, Blacktip, Atlantic Sharpnose, and Bonnethead shark interrenal-somatic index among sex-maturity stages.

Boxes indicate 25th and 75th percentiles with bars denoting minimum and maximum values. Median values are indicated by the line dividing the 25th and 75th percentile boxes, while X indicates the sample population mean.

CHAPTER III – HISTOLOGICAL VERIFICATION OF INTERRENAL CELL TYPE AND POTENTIAL FOR STEROID PRODUCTION VIA DETECTION OF 3βHSD

3.1 Introduction

3.1.1 Hematoxylin and Eosin Histology

Much of what is known about fish cytology is based on previous research on teleosts. Teleost interrenal cells are integrated within the head kidney among renal and chromaffin cells; in contrast, elasmobranch interrenal tissue are discrete bodies. Rocha *et al.* (2001) investigated teleost interrenal and chromaffin cells and found one type of interrenal cell and two types of chromaffin cells; each type of chromaffin cell is responsible for the production of epinephrine or norepinephrine. Although teleost and elasmobranch interrenal cells appear structurally distinct, similar cytological characteristics are expected to be present between interrenal cells of both groups: cylindrical, homogenous, and contain numerous mitochondria and smooth endoplasmic reticula (Rocha *et al.* 2001).

Interrenal cell structure and function among teleosts varies during reproductive stages. Patterns in interrenal cell cytology corresponding to the annual reproductive cycle in female and male Three-Spined Stickleback *Gasterosteus aculeatus* were observed by Civinini *et al.* (1997, 2001). Civinini *et al.* (2001) categorized interrenal cells into four reproductive phases by distinct appearance: quiescence, prereproductive, reproductive, and postreproductive. During the quiescence and prereproductive period interrenal cells appear sponge-like, with few polygonal dark cells present separated by intercellular space; these interrenal cells have abundant large oval or round mitochondria with dense

matrices. The prereproductive phase is characterized by numerous endoplasmic reticuli and secretory vesicles in conjunction with mitochondrial proliferation. Within the reproductive period, interrenal cells are more extensive and an increased number of darker cells were observed, the mitochondria appear to fuse together with a less dense matrix and the cell membrane begins to appear discontinuous. The postreproductive phase is distinguished by restoration of the cell membrane, reorganization of mitochondria and other organelles, and marked tissue degeneration; dark cells are more abundant and form cords. The characteristics of teleost interrenal cells follow the cyclic release of steroids for reproductive changes. Furthermore, there was indication that during the reproductive phase, alterations in cell appearance could be affected by stress, or either physical (e.g. handling) or osmoregulatory challenges (Gallo *et al.* 1997). Variation in cell distribution and type could indicate functional differences (e.g. greater number of cells could potential indicate great steroid production).

3.1.2 3βHSD Histology

3β-Hydroxysteroid dehydrogenase (3βHSD) is a steroidogenic enzyme that converts pregnenolone to progesterone within the corticosteroid synthesis pathway. This reaction occurs in the early stages of steroidogenesis and is a rate-limiting step in the process of corticosteroid production (Nunez *et al.* 2006). An immunohistochemical reaction, using Nitroblue Tetrazolium (NBT) as a colored indicator, is used to confirm the presence of 3βHSD (Chieffi 1965). If 3βHSD is present, cells are stained blue-dark purple; the degree of NBT color change correlates to enzymatic expression. A chosen precursor is converted by 3βHSD in the presence of a cofactor, nicotinamide adenine dinucleotide (NAD), resulting in conversion of NAD to NADH. The NADH reacts with

NBT, a redox indicator, which results in the formation of insoluble NBT diformazan that stains cells blue-purple (Park *et al.* 1968). Negative controls (reactions without DHA or NAD) impact the functionality of the reaction and resulted in no staining to very light staining.

3βHSD has been identified in the interrenal cells of Marbled Electric Ray *Torpedo marmorata*, Common Torpedo Ray *Torpedo torpedo* (previously *T. ocellata*), Nursehound Shark *Scyliorhinus stellaris*, and the Small-Spotted Catshark *Scyliorhinus canicula* by Chieffi (1965) using biochemical and histochemical methods. In gonadal tissue, distribution of 3βHSD varied by the reproductive phases of male and female Three-Spined Stickleback (Civinini *et al.* 1997, 2001). During the quiescence, prereproductive, and postreproductive phases 3βHSD was identified via histochemical staining but varied in relative abundance and distribution. Within the reproductive phase, staining demonstrated that 3βHSD was always present. 3βHSD presence and expression in steroidogenic tissues (i.e. gonadal and interrenal) varies depending on reproductive cycle, thus, differences in enzyme distribution and expression may be present among the four species of shark investigated in this study.

The lack of literature describing interrenal cells in elasmobranchs, combined with observed novel morphological differences in interrenal tissue among species and the potential for cytological differences due to reproduction cycles, facilitated an investigation of interrenal cell type and distribution to verify putative interrenal tissue and investigate potential influences on species-specific responses. Furthermore, verification that putative interrenal tissue has steroidogenic potential could clarify function and indicate differences to potentially explain species-specific responses to stress. Therefore,

the specific goals of this chapter are to 1) describe interrenal cytology to confirm putative interrenal tissue, and 2) determine the steroidogenic potential through the presence of the steroidogenic enzyme 3βHSD.

3.2 Methods

3.2.1 Hematoxylin and Eosin Histology

To observe cell type and structure, interrenal tissues from Finetooth (n=13), Blacktip (n=20), Atlantic Sharpnose (n=20), and Bonnethead (n=14) sharks were collected within 12 hours of euthanization. Due to the difficulty in obtaining fresh samples from Bonnethead and mature Finetooth Sharks, a proportion of interrenal tissue was collected opportunistically within two days of euthanization (Finetooth n=7, Bonnethead n=6); these animals remained on ice until processing. Interrenal tissues from immature and mature, male and female individuals were collected for each species (n=5 per group). For comparison, interrenal tissue was collected from one male and one female Atlantic Stingray *Dasyatis sabia*, which were rapidly euthanized. Interrenal bodies collected for histology were fixed in 10% neutral buffered formalin (J.T. Baker) for a minimum of one week. Prior to dehydration, tissues collected from Bonnethead sharks were divided into three sections: anterior, central, and posterior.

After fixation, tissue was rinsed in low-flowing tap water overnight. To dehydrate, tissue was placed in 60% ethanol for two hours, 70% ethanol for two hours, and fresh 70% ethanol for a minimum of two hours before being further dehydrated and impregnated with paraffin using Thermo Scientific™ Shandon™ Excelsior™ ES Tissue Processor (Appendix B). Within one hour of completion of processing, tissue was

embedded in paraplast using a Shandon™ Histocentre 2 Embedding Center and stored at 4°C.

Prior to sectioning, tissue was placed on ice for a minimum of 30 minutes. Tissue was sliced on an AO® Rotary Microtome with a disposable Accu-Edge Low Profile Microtome Blade at a thickness of 4 µm. Slices were placed in a S/P Brand Tissue Flotation Bath heated to 37-42°C, which contained distilled water and one cap-full of Sta-On (Surgipath). Tissue sections were carefully collected on a slide and placed on a slide warmer for a minimum of two hours to ensure complete desiccation.

Tissue was stained and counterstained with Hematoxylin (EK Industries) and Eosin (Richard Allen Scientific) following the methods of Luna (1968; Appendix B). Slides were then affixed with a cover slip using mounting medium (Richard-Allan Scientific) and allowed to completely dry before microscopic evaluation.

3.2.2 3βHSD Histology

To determine presence and location of the steroidogenic enzyme 3βHSD, three interrenal samples per species were removed from individuals immediately after euthanization and rapidly processed (<10 minutes).

Interrenal tissue was placed in Tissue-Tek® Optimal Cutting Temperature (OCT) compound (Sakura® Finetek) for a minimum of five minutes before being placed in fresh OCT compound and rapidly frozen using a dry ice and reagent grade ethanol (Fisher Scientific) slurry. Tissue was stored at -80°C until slicing. Tissues collected from Bonnethead sharks were divided into three sections prior to freezing.

Enzyme staining followed the methods of Bara *et al.* (1965). Slides were prepared with a coat of oil solution (95% ethyl ether, 5% mineral oil) and 2 mg of

dehydroepiandrosterone (DHA; Chem Impex international) per 1 mL of solvent. Following applications of the oil coating, slides were allowed to dry and then placed in a Sakura® Tissue-Tek Cryo3 Plus cryostat maintained at −25°C. Tissue was cut into 15 µm sections and carefully placed over the oil deposit before being dried for 1 to 2 minutes. Slides were then incubated at 38°C for 30 minutes in Krebs solution containing 1 mg mL^{-1} NAD (Chem Impex international) and 0.5 mg mL^{-1} Nitro-BT (Chem Impex international). Sections were then rinsed with phosphate-buffered saline (PBS, 1X), fixed for 10 minutes in l0% neutral buffered formalin, rinsed again with PBS, and then affixed with a cover slip using DAPI-flouromount-G (Southern Biotech) before imaging. Negative controls of no oil substrate (-S negative control) and oil substrate without NAD (S/-NAD negative control) were used to confirm positive staining of 3βHSD.

3.3 Results

3.3.1 Hematoxylin and Eosin Histology

Interrenal cells are characterized by cell shape (polygonal or round), homogenous cell type, the presence of dark purple nuclei and an apparent cell membrane. Intercellular granulation and cytoplasmic lipid content is variable and affects cell densities. Slight differences in staining intensity were seen within all species. Cells were often arranged in circular cord or cluster formations (Figure 3.1). Cell density was observed to increase around blood vessels (Figure 3.1). Interrenal tissue was highly vascularized; hemorrhaging, blood cells and blood vessels were often present (Figure 3.1). Blood cells (dark pink in coloration) were larger than interrenal cells, nucleated, and had non-granulated intercellular space. A majority of interrenal samples were surround by kidney tissue. Kidney tissue was distinguished by tubules, brush border epithelial cells, and

collecting ducts (Figure 3.1). The border between interrenal and kidney cells contained collagen fibers that appear wave-like with folded layers.

Atlantic Stingray interrenal cells were examined as a model for interrenal cell characteristics. At 10X magnification, the tissue appeared homogenous with clusters of cells. The interrenal body was surrounded by kidney tissue and blood vessels were present (Figure 3.2 A and B). At 40X magnification, circular bundles or clusters of cells (distinguished as a basement membrane surrounded by connective tissue) were the most distinctive characteristics. Cells were polygonal and contain granular cytoplasm and lipid vacuoles. Cell abundance varied among individuals. The density of interrenal cells within the Atlantic Stingray did not appear to vary within the tissue. Denser congregations of cells, with little to no cellular space around blood vessels (indicated by increased density of nuclei) was observed in several individuals.

Finetooth Shark interrenal cells were similar to those of the Atlantic Stingray with round cells in clusters surrounded by basement membrane (Figure 3.2 C and D). Bundles of cell clusters were less common and surrounded by less connective tissue than observed in the Atlantic Stingray. Certain sections of the tissue had more corded cell arrangements of the clustered cells. More granular cytoplasm was observed in Finetooth Shark interrenal cells with lipid vacuoles but was less abundant than observed in the Atlantic Stingray. No prominent differences in cell density, zonation, or location were observed among sexes or maturity stages. Vascularization and red blood cells were present.

Blacktip Shark interrenal cell structure was like that of Finetooth Sharks (Figure 3.2 E and F). Clusters of cells were present in bundles encapsulated by basement membranes of various amounts of surrounding connective tissue. Corded patterns existed

in some sections of the tissues but were not observed in all individuals. Clusters were smaller than those seen in Finetooth Sharks. Cells were round or polygonal and contained granular cytoplasm but more lipid vacuoles that resulted in an increase in cellular space and a decrease in cell density. Within a section of tissue, the density of cells varied. Vascularization was not as common as in Finetooth Sharks but red blood cells were present within tissues. No distinct differences in cell density and appearance between sexes or maturity was observed.

The structure of Atlantic Sharpnose Shark interrenal cells was unique when compared to other species examined (Figure 3.2 G and H). Overall, the tissue appeared to have an oily sheen or texture giving the cells a globular cluster appearance. Compared to Finetooth and Blacktip Sharks, Atlantic Sharpnose Sharks had increased vascularization with distinct blood vessels and clusters of blood cells. The majority of cells were round and clustered but cord-like structuring was present in several individuals. Connective basement membrane tissue surrounded clusters of cells in some individuals but was not consistent, as observed in other species. Granular cytoplasm was abundant with lipid vacuoles present in some cells. Cytoplasm and lipid vacuoles resulted in an apparent increase in intercellular spaces compared to Finetooth and Blacktip Sharks, and therefore, Atlantic Sharpnose Sharks had a lower interrenal cell density. Darker cells, located at the edge of the interrenal and brush border transitional area (adjacent to the kidneys), were seen in samples from several Atlantic Sharpnose Sharks. No zonation was observed in Atlantic Sharpnose Shark interrenal tissue. Additionally, no distinctions were observed between sexes and maturities. Pups appeared to have less cytoplasmic and lipid vacuoles and thus a slight increase in cell density.

Bonnethead shark interrenal tissue was examined in three sections: anterior, central, and posterior (Figure 3.2 I-N). Throughout all sections, interrenal cell density and vascularization appeared to be more abundant than observed in other species. The anterior section had the greatest cell density, with connective tissue present and indistinct clusters (Figure 3.2 I and J). Cells were arranged in columnar and bundled areas with some variability between individuals. Both granular cytoplasm and lipid vacuoles were present. Cells in the central section were slightly less dense than the anterior section with more cytoplasm and lipid vacuoles (Figure 3.2 K and L). Small, irregular clusters of cells were present but not as distinct as those seen in Finetooth or Blacktip Sharks. Small cell clusters were surrounded by connective tissue. Cells with larger sections of granular cytoplasm were present among the densely-clustered cells. Within the posterior section, cell density was similar to the central section and contained more polygonal or oval cells with cytoplasm and lipid vacuoles (Figure 3.2 M and N). No obvious section-specific difference in cell appearance was identified, only slight differences in density and staining coloration (Figure 3.3). No apparent differences among sexes and maturity were observed.

Among all species, putative interrenal tissue consisted of one cell structure and type with characteristics consistent with steroidogenic cells. Observed differences were dependent on connective tissue and density due to cytoplasm and lipid vacuoles.

3.3.2 3βHSD Histology

All species exhibited positive staining for 3βHSD enzyme expression. No substantial difference was seen in 3βHSD staining among species. Among all species and within individual slices, variation in coloration was present, however, no consistent

pattern was identified. Oily droplets were present within all interrenal tissues. All negative controls demonstrated less intense staining than experimental slides; -S control had less staining (Figure 3.3 G) and S/-NAD control had minimal coloration (Figure 3.3 H). The stain was effective for all trials. Kidney tissue, when present, contained a brush border with low levels of 3βHSD expression adjacent to layers of kidney tubules which varied in 3βHSD expression. Tubule and brush border cell types were visibly discernable from the interrenal tissue (visible in Figure 3.3 C).

Among all species, the intensity of 3βHSD stain varied slightly. Condition of the tissue was variable; this was particularly notable in Atlantic Sharpnose Sharks, in which tissue was more prone to shearing during slicing. Occasionally, slices were darker than others but did not follow an apparent pattern.

3βHSD expression was present in Finetooth Sharks with no zonation or distinct differences in 3βHSD expression within interrenal tissue (Figure 3.3 A). Cells appeared granular with consistent cell density. Overall interrenal cells did not appear corded. Kidney cells, when present, contained brush border cells with lighter coloration adjacent to tubule cells with sections of greater 3βHSD expression.

Blacktip Sharks had similar 3βHSD expression to Finetooth Sharks (Figure 3.3 B). No zonation of expression was seen. Cell density and 3βHSD expression appeared consistent throughout the interrenal body with no distinct cording of cells. Kidney tissue, when present, was similar to observations in Finetooth Sharks, however, some tubule cells at the most exterior edges showed greater 3βHSD expression than identified in Finetooth Sharks.

The interrenal tissue of Atlantic Sharpnose Sharks was less robust and more prone to shearing than other species (Figure 3.3 C). Slices, prone to tearing apart, contained space within the tissue so cell density was difficult to assess. Slight variations in staining indicate a difference in 3βHSD expression within the core of the tissue. Atlantic Sharpnose Shark kidney tissue was similar to the other species examined with lighter coloration in brush border cells and kidney tubules.

No difference in 3βHSD expression among Bonnethead sections was detected, and expression was similar among all three portions of the interrenal strip. The central section (Figure 3.3 E) appeared more granulated than the anterior (Figure 3.3 D) and posterior (Figure 3.3 F) tissue, which both had a slight granular texture. No zonation of expression was observed within all three sections. Cells near the exterior of interrenal tissue had greater expression and lipid droplets were abundant and easily distinguishable within the tissue. When present, kidney brush border cells had light staining while kidney tubule expression varied from light to dark staining.

3.4 Discussion

3.4.1 Hematoxylin and Eosin Histology

Similarities to cell structure and shape from past literature, the presence of lipid vacuoles, and 3βHSD expression support the hypothesis that putative interrenal tissue in examined species was correctly identified. Cytology of interrenal tissue was distinguished by steroidogenic cell characteristics: numerous, round or oval mitochondria containing tubular cristae that appear granular, large smooth endoplasmic reticulum, and less abundant rough endoplasmic reticulum, and Golgi complexes (Gallo and Civinini 2003). Clusters of cells were often arranged in bundles connected by collagen through

tight junctions, desmosomes, and interdigitations. Atlantic Sharpnose and Bonnethead sharks contained less connective tissue than Blacktip and Finetooth Sharks. This finding that not all species examined contain distinct collagen clusters is supported by previous studies. While teleost interrenal cells are scattered within the head kidney, Goldfish *Carassius auratus* (Yamamoto and Onozato 1965; Ogawa 1967; Kagawa and Nagahama 1980), Fathead Minnow *Pimephales promelas* (Yoakim and Grizzle 1980), and Common Rudd *Scardinius erythrophthalmus* (Mastrolia *et al.* 1981) interrenal cells are not separated from surrounding chromaffin and lymphoid cells by connective tissue.

Previously, two distinct types of interrenal cells have been described (Agrawal and Srivastava 1978; Mahon *et al.* 1962). Type one, cubic or columnar shaped cells, contain granular cytoplasm and circular nuclei and are typically arranged in compact strands or cords (Agrawal and Srivastava 1978). Type two, irregularly shaped cells, have cytoplasmic extensions (occasionally), irregularly outlined nuclei, and are heterochromatic. The percentage of the two types of interrenal cells is highly inconsistent and depends on the individual, reproductive period, and stress or other deleterious conditions (Mahon *et al.* 1962; Civinini *et al.* 1997, 2001; Gallo and Civinini 2003). The second cell type (i.e. irregularly shaped cells) has notably varied in hematoxylin and eosin stain intensity (Mahon *et al.* 1962). In the current study, observed variations in cytology (i.e. bundled clusters, or more columnar corded appearance) and staining intensity may be explained by degeneration, lipid content, stress, and reproductive status (Figure 3.4).

Changes in interrenal cytology, dark or irregular cells and deviations in staining could be due to cyclic regeneration of interrenal tissue. In teleosts, interrenal tissue has

been observed to degrade and regenerate, including periods with no detectable interrenal tissue (Gallo and Civinini 2003). Degraded interrenal cells and cellular components have also been observed in adjacent blood vessels in addition to interrenal cell phagocytosis by macrophages (Gallo and Civinini 2003). To replace degraded cells, interrenal cells must regenerate by differentiation as observed by Gallo and Civinini (2003). These regenerating or differentiating cells were classified as light or dark depending on density within the cytoplasm and nucleus from observations of interrenal cells from Three-Spined Sticklebacks (Gallo and Civinini 2003). Darker cells were hypothesized to be newly differentiated cells, which can break down and phagocytose remaining degenerating cells (Gallo and Civinini 2003). Differences in cell coloration and increases in dark cells or nuclei observed in Atlantic Sharpnose Sharks could therefore potentially be explained by degradation, macrophages, and regeneration of tissue.

In the current study, cell density was variable among all elasmobranch species. This density difference could be due to the amount of lipids present within interrenal bodies. Interrenal cells are lipophilic: during the histological process, lipid droplets are dehydrated and appear as empty vacuoles in the cells. The density of lipid vacuoles appears to vary among elasmobranch species, which manifests as differences in vacuole density among species: less lipid space allows for an increase in cell density. Lipid density may also vary due to lipid-depleting cellular processes, such as steroidogenesis. Exposure to prolonged stress prior to capture could cause depletion in stored lipids due to an increase corticosteroid synthesis. Additionally, upon capture, a delay in sampling could further exacerbate stress and thus lipid depletion, which may cause a notable

variation in cell appearance. Differences in stress levels could be due to fishing practices, handling time, and prior stressors.

Ease of tissue shredding observed in the Atlantic Sharpnose Shark interrenal tissue is potentially due to greater lipid content, which could result in a 'softer' texture that is more likely to tear. Previous studies have shown that activation of interrenal cells resulted in depletion of lipid content and increase in 3βHSD expression (Hanke and Chester-Jones 1966). In these activated tissues, lipid stores become depleted and after time have an atrophied appearance with "shrunken" cells that are dark in coloration and have smaller nuclei.

Differences in interrenal histology of the four shark species investigated could also be due reproductive status. Observed variation in density, granulation, cell space, or lipid vacuoles within cells may be due to differences in secretion stages. Four phases of interrenal cells (previously discussed) were found to correspond to breeding cycles in the Olive Barb *Systomus sarana* (Chakrabarti and Ghosh 2014). During these cycles, interrenal and chromaffin cells displayed hyper- and hypo-activity, changes in structure, and cytology that corresponded to testis activity and structural changes (Chakrabarti and Ghosh 2014). Post-spawning interrenal stimulation of gonadal tissue is less proficient and enters a chromophobic state (Chakrabarti and Ghosh 2014; Nagahama 1994). Changes in interrenal tissue are therefore closely linked to gonadal activity of Olive Barb (Chakrabarti and Ghosh 2014). Also, unique mitochondrial cytological changes between sexes due to reproductive phases throughout the year have been observed in Three-Spined Sticklebacks (Civinini *et al.* 1994, 1997, 2001). In females, mitochondria can fuse into ''supergiant'' mitochondria about the size of the nuclei or greater. While this fusion

is present in males, the mitochondria do not reach a size comparable to that seen in females. Females also frequently develop dense and tubular mitochondrial cristae and increased vacuolization (Gallo and Civinini 2003). It was not possible to observe such cellular detail in the current study, but it is possible that ‘‘supergiant’’ mitochondria are present in shark tissue given observed darker cell coloration or greater granulation within cells. Observations of Pacu *Piaractus mesopotamicus* (Gazola *et al.* 1995) and Olive Barb (Chakrabarti and Ghosh 2014) found that a slight increase in gonadosomatic index initiates the process of storing cytoplasmic granules. At the onset of maturation and spawning, interrenal and chromaffin cells contain granulated structures that denote a release of steroids. Therefore, the maturation and growth period of an individual can affect interrenal cytology and steroid production.

3.4.2 3βHSD Histology

Some individuals demonstrated a false positive stain from both negative controls. A false positive stain in both -S and S/-NAD negative controls could be due to abundant levels of endogenous NAD or precursors present in the tissue, possibly indicated by the numerous lipid droplets present within the tissue. Additionally, other precursors or steroidogenic enzymes could use the NAD and DHA triggering the NBT. However, there is no way of determining if that was the case in this study. While slight differences in coloration were observed, no true zonation or patterns of localization were observed. These results are similar to studies by Chieffi (1965) on the Small-Spotted Catshark, where areas of increased 3βHSD expression were identified by concentrations of positively stained cells but there was no evidence of zonation. Studies in Goldfish and Three-Spined Stickleback observed variation in 3βHSD staining, in which not all cells

displayed the same amount of stain (Kagawa and Nagahama 1980; Gallo and Civinini 2003).

Other enzymes vary in staining expression in *in vitro* experiments. 17β-hydroxysteroid dehydrogenase (17βHSD; responsible for the interconversion of estrone to estradiol and androstenedione to testosterone) and 11β-hydroxysteroid dehydrogenase (11βHSD; regulates oxidation of cortisol and corticosterone to cortisone and 11-dehydrocorticosterone, and the interconversion of 11β-hydroxy- and 11-ketoandrogens) have shown positive expression in interrenal cells of some species (Bara 1968, 1972; Yaron 1970; Hooli and Nadkarni 1974; Saidapur *et al.* 1976; Shanbhag and Nadkarni, 1977; Kulkarni and Sathyanesan 1978). High 17βHSD and 11βHSD expression within head kidney was observed in Rainbow Trout *Oncorhynchus mykiss* (Arai *et al.* 1969; Schulz and Blüm, 1991) and Crucian Carp *Carassius carassius L*. (Zeqiri and Hyvärinen 1997); however, African Catfish *Clarias gariepinus* (Vermeulen *et al.* 1995) had low levels of expression. No positive histochemical reaction for 17βHSD was found in Three-Spined Stickleback (Civinini *et al.* 1997, 2001). In comparison with 3βHSD, expression of 17βHSD was weaker and substrate dependent in that study.

Stress level and life history of an individual will impact enzymatic expression and therefore, 3βHSD expression. Observations of mammalian adrenal tissue have found reduced or abnormal cells between adrenal zones based on functional states (Idler 1972). In this study, interrenal tissue was harvested as quickly as possible, however, pre-capture or capture and handling stress could have impacted enzymatic expression. An animal subjected to pre-capture stress may have up-regulated enzymatic expression and thus, a

greater presence of positive staining than an animal that experienced less stress. Other changes in staining intensity, cell activity, or structure could be due to an individual's functional state (i.e. maturity, reproductive status, and age). Also, differences in staining could be due to functional differences in cell state. Cytochemical observation of interrenal tissue in Three-Spined Stickleback using etiocholanolone as a substrate revealed differences in 3βHSD expression depending on three interrenal cell phases (Gallo and Civinini 2003). During phase one, cells are negative to the stain with slight stain precipitates (found on the exterior smooth endoplasmic reticulum and mitochondria interior), whereas within phase two and three greater 3βHSD expression is present and precipitation increases.

Novel morphology was validated by interrenal cell type and steroidogenic potential. Although slight differences were observed, all species contained interrenal cells with no indication of other cell types, and all cells appeared to have steroidogenic potential. Investigation of differences in steroid potential (e.g. more quantitative methods of defining enzyme expression) by stress level or maturity would further enhance our understanding of elasmobranch stress physiology at the cellular level.

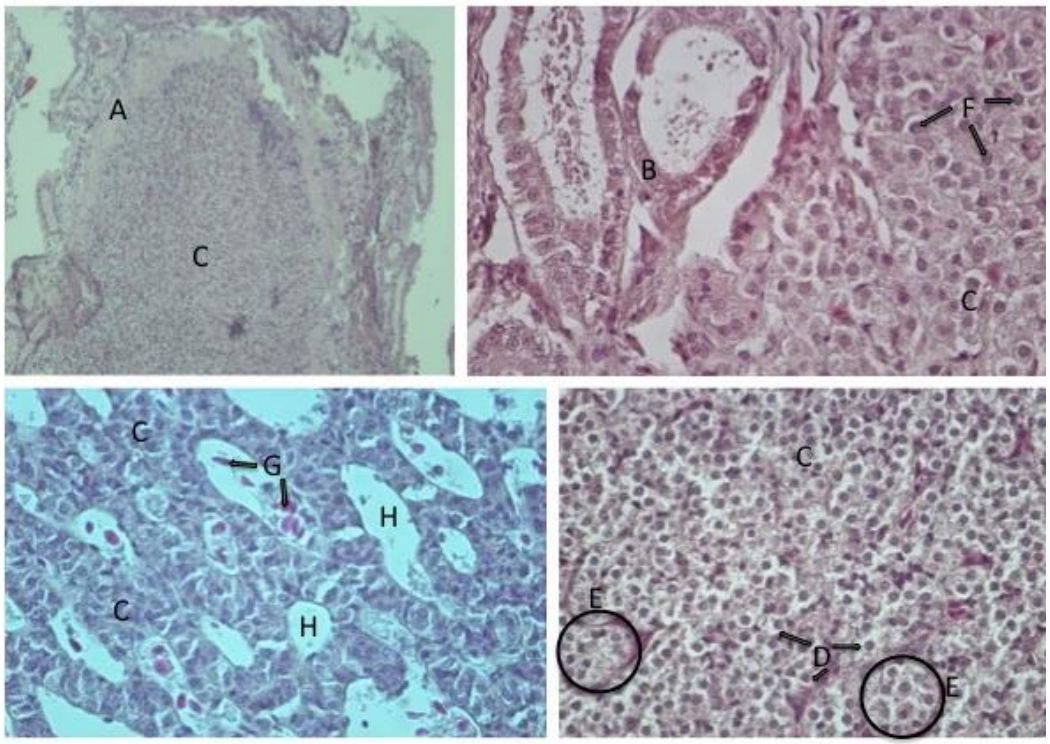
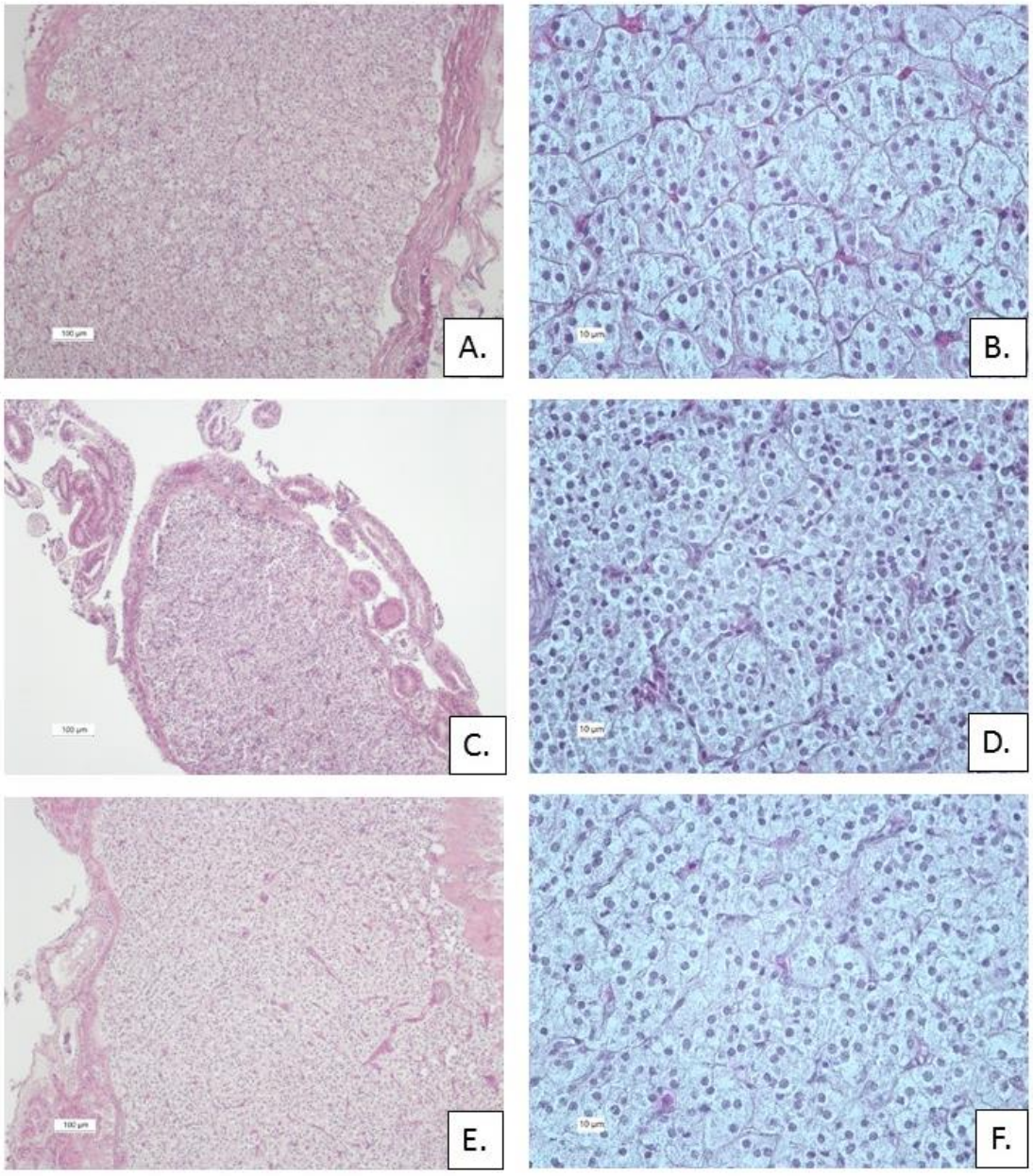


Figure 3.1 Hematoxylin and eosin histological staining of interrenal tissue indicating cytological structures.

A. Kidney border cells B. Kidney tubule cells C. Interrenal cells D. Cytoplasm space E. Cellular bundles (within circles) F. Nuclei G. Blood cells H. Blood vessels.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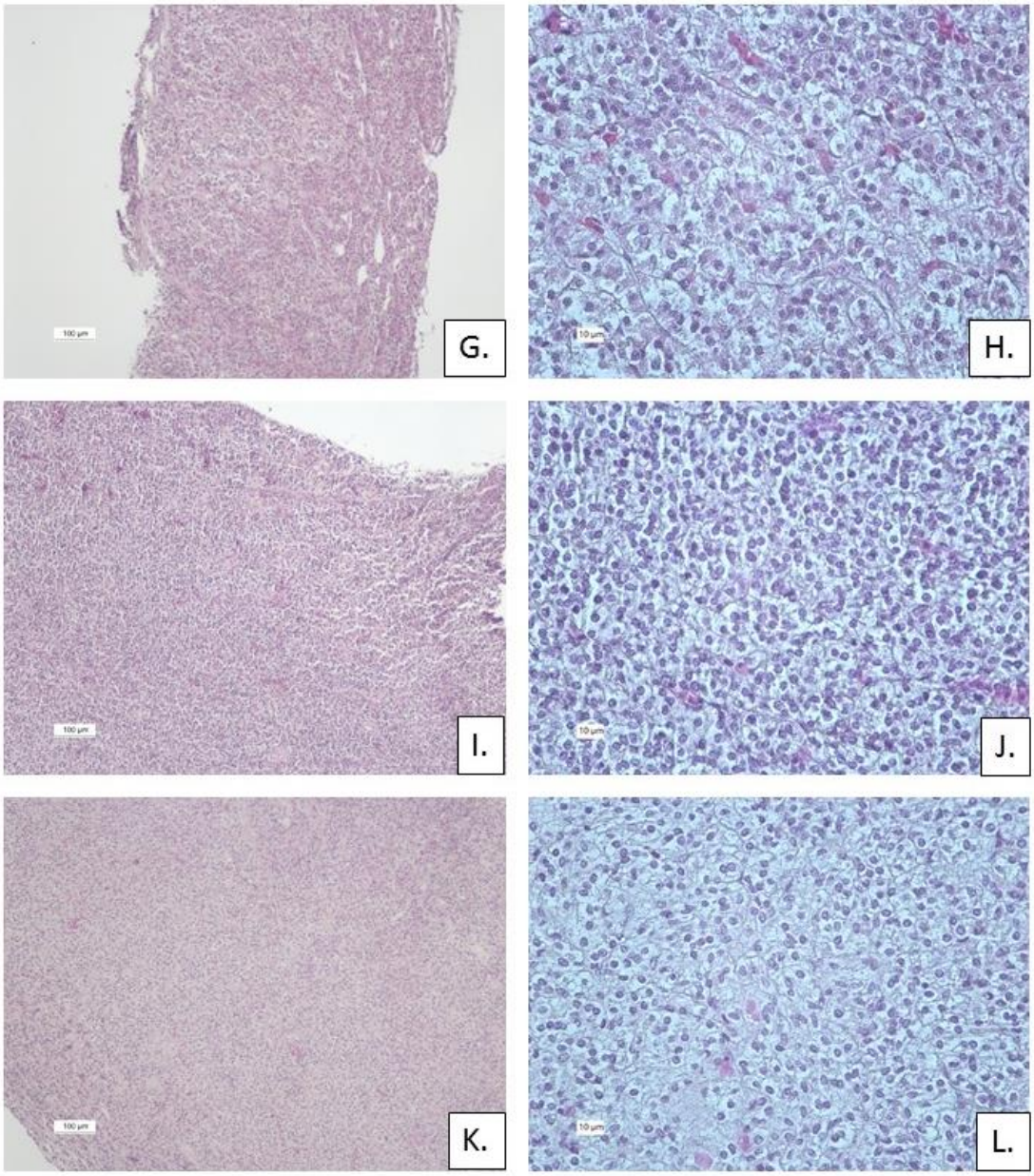
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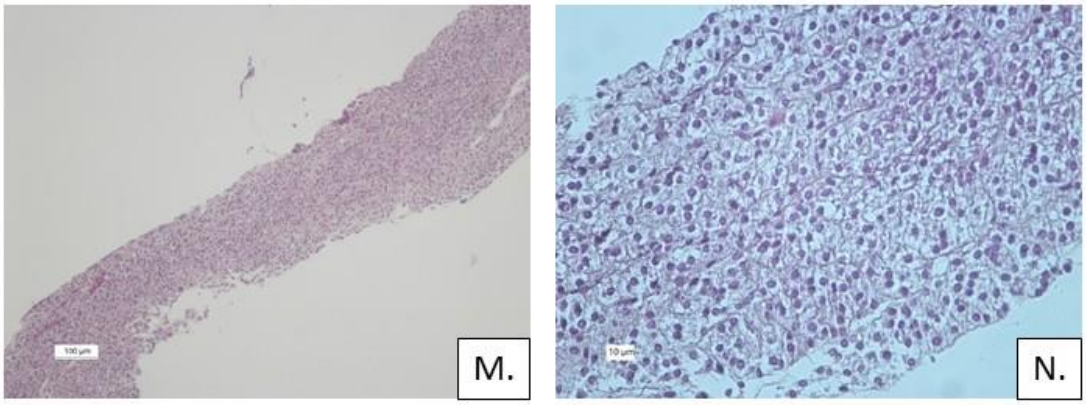
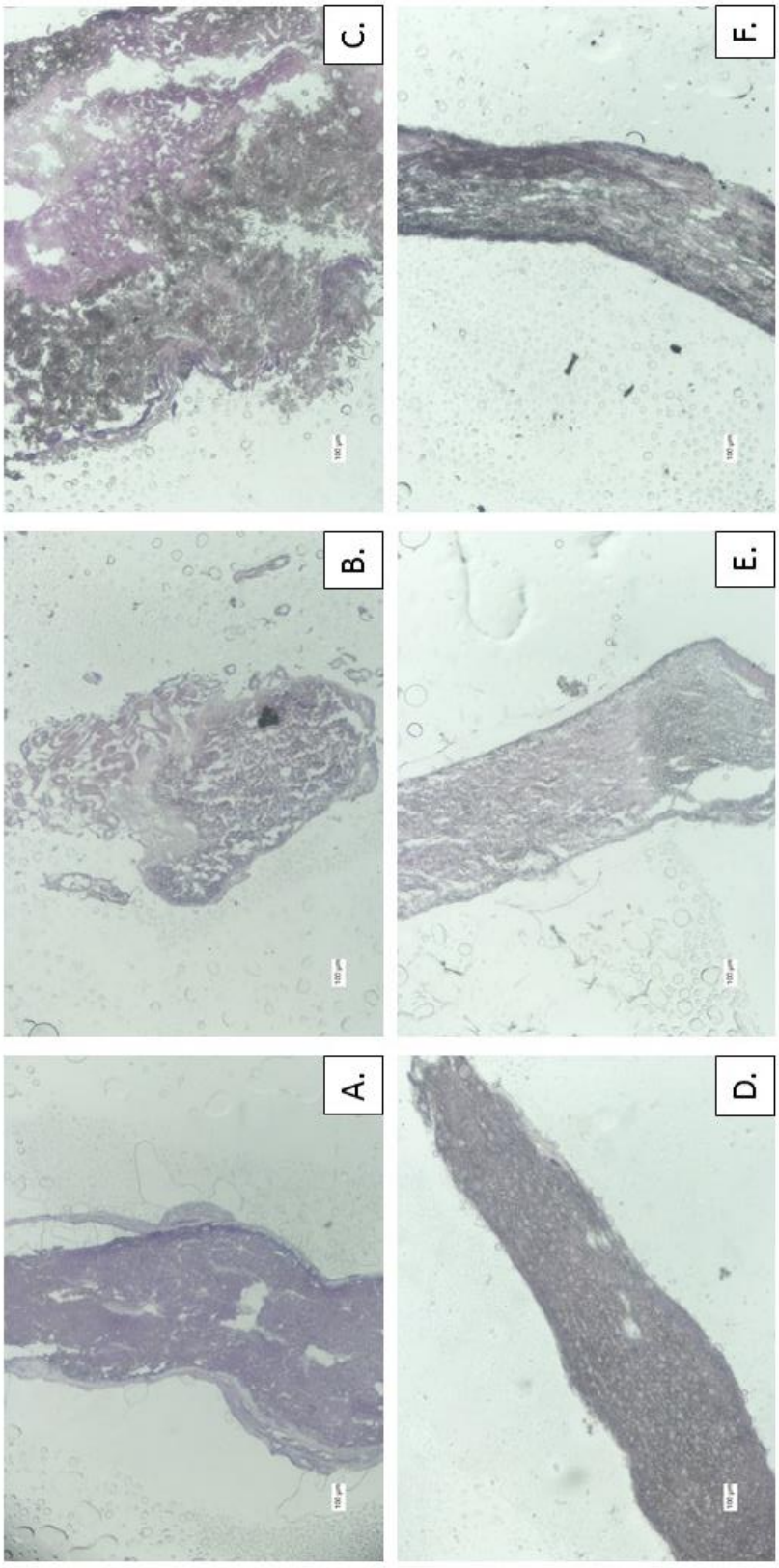


Figure 3.2 Hematoxylin and eosin histological staining of interrenal cells.

A. Atlantic Stingray (10x) B. Atlantic Stingray (40x) C. Finetooth Shark (10x) D. Finetooth Shark (40x) E. Blacktip Shark (10x) F. Blacktip Shark (40X) G. Atlantic Sharpnose Shark (10x) H. Atlantic Sharpnose Shark (40x) I. Bonnethead shark anterior section (10x) J. Bonnethead shark anterior section (40x) K. Bonnethead shark central section (10x) L. Bonnethead shark central section (40x) M. Bonnethead shark posterior section (10x) N. Bonnethead shark posterior section (40x).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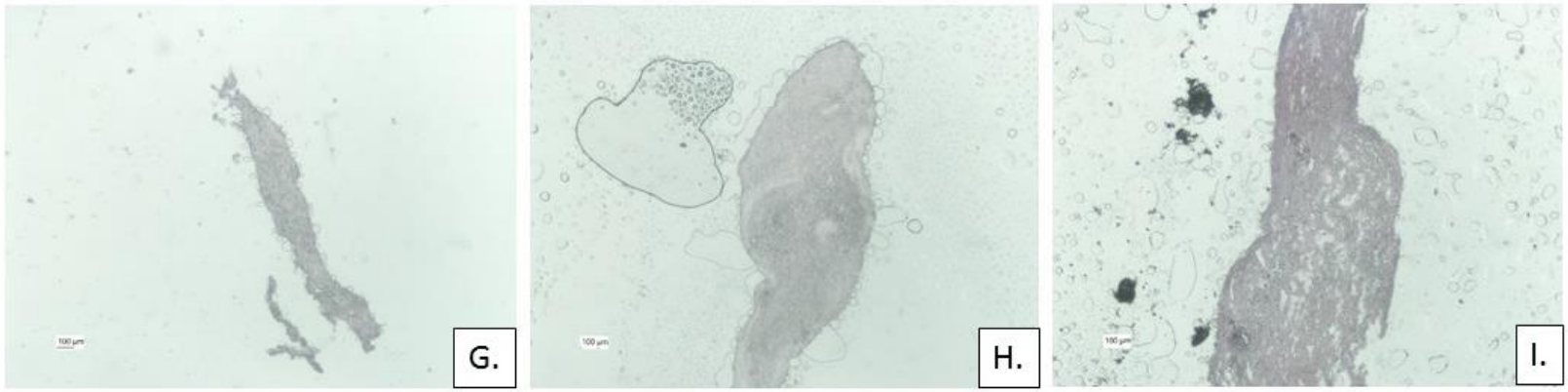

Figure 3.3 3βHSD immunohistochemical staining.

A. Finetooth Shark B. Blacktip Shark C. Atlantic Sharpnose Shark D. Bonnethead shark anterior section E. Bonnethead shark central section F. Bonnethead shark posterior section G. Bonnethead shark anterior section –S negative control H. Bonnethead shark anterior section S/-NAD negative control I. Bonnethead shark anterior section (all images are at 4X magnification).

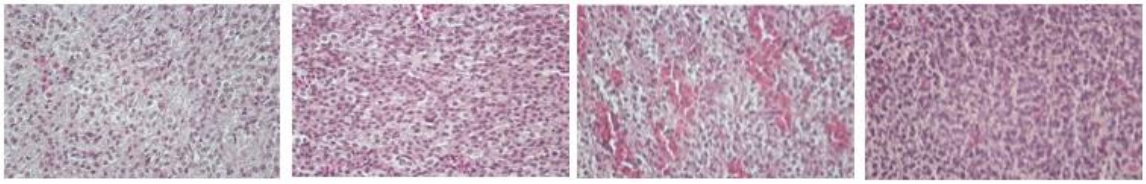

Figure 3.4 Example of the range of staining intensity of hematoxylin and eosin histological staining within a species.

Images from Bonnethead sharks, 40X magnification.

CHAPTER IV – *EX VIVO* DETERMINATION OF INTERRENAL STEROID SYNTHESIS

4.1 Introduction

The production of corticosteroids by interrenal tissue is a critical component of the primary stress response. In teleosts and elasmobranchs, steroid production is initiated in interrenal cells via a hormonal cascade activated by the hypothalamus-pituitary-interrenal axis (HPI). Analogous to the mammalian hypothalamus-pituitary-adrenal (HPA) axis, the HPI axis acts in a similar manner. Following the perception of a stressor, the hypothalamus releases corticotropin-releasing hormone (CRH), which induces the pituitary to produce adrenocorticotropic hormone (ACTH). ACTH is released into the blood stream and transported to interrenal tissue where corticosteroid production is stimulated. Corticosteroids mediate the stress response by increasing circulating levels of metabolic fuels, such as glucose. However, chronic elevation of corticosteroids can negatively impact certain physiological processes such as osmotic balance, the immune response, and reproductive function. In addition to the HPI axis, the stress response also involves the neuroendocrine release of catecholamines. Catecholamines, epinephrine (adrenaline) or norepinephrine (noradrenalin), are released from chromaffin tissue (adjacent to the interrenal bodies) in response to sympathetic nervous system stimulation, and function to further mobilize glucose, increase blood pressure, and, in fishes, adjust gill ventilation and up-regulate chloride cell activity (Grant *et al.* 1969; Metcalfe and Butler 1984; Holmgren *et al.* 1992a; Sollid and Nilsson 2006).

Within interrenal cells, the regulation of steroid production is determined by rate-limiting proteins: steroidogenic acute regulatory protein (StAR) and P450 side chain cleavage (P450scc) (Evans and Nunez 2010). The steroidogenic pathway is a series of enzymatic reactions that begins when cholesterol is transported into the inner mitochondrial membrane by the transport protein StAR, the rate-limiting step in steroid synthesis. P450scc then converts cholesterol to pregnenolone as the first committed step in the steroidogenic pathway (Figure 4.1). Pregnenolone is then converted to progesterone by 3βHSD. Production of corticosteroids requires 3βHSD, which converts hydroxyl groups to ketones and is a rate-limiting step in the process of corticosteroid production. Through multiple enzymatic reactions, progesterone is transformed into 11-deoxycorticosterone (DOC), corticosterone (B), and finally 1α-OHB. Corticosteroid production and release is regulated by the expression of StAR, P450scc, and 3βHSD, and thus measuring expression of these key enzymes via molecular and histological markers determines the presence of steroidogenic cells and tissues.

If steroids are indeed produced, determining which steroids are produced as final products will further elucidate the function of a tissue. Historical uncertainty regarding steroid products in elasmobranchs (i.e. the major and potentially biologically active products of the pathway: 1α-OHB, B, and DOC; Figure 4.1) due to the lack of efficient assays has recently been addressed as the ability to measure corticosteroids has improved. Previously, Phillips and Mulrow (1959) suggested that teleost interrenal tissue produced cortisone, aldosterone, and cortisol. Idler (1972) reviewed numerous elasmobranch species and reported the production of significant amounts of both 1α-OHB and B

depending on the species (Bull Shark *Carcharhinus leucas,* Blacktip Shark *Carcharhinus limbatus,* Dusky Shark *Carcharhinus obscurus,* Tiger Shark *Galeocerdo cuvier,* Spiny Dogfish *Squalus acanthias*). Nunez and Trant (1999) demonstrated that the Atlantic Stingray *Dasyatis sabina* interrenal tissue predominantly produces 1α-OHB *in vitro*, in addition to small amounts of 11-dehydrocorticosterone when tissue is stimulated with ACTH. However, in earlier studies (prior to the development of more advanced methods for steroid identification) methods for measuring steroid products *in vitro* may have been inefficient or misleading due to cross-reactivity. Only recently have new methods adequately measured 1α-OHB (Evans and Nunez 2010; Evans and Wheaton personal communication).

This chapter will provide evidence of interrenal steroid production and identify interrenal steroid products. Confirmation of steroid production will: 1) provide evidence that putative interrenal tissue is functioning as expected, and 2) further our understanding of specific steroids produced by interrenal tissue across species.

4.2 Methods

4.2.1 *Ex vivo* Incubation

To ascertain steroid production, interrenal tissue from Finetooth (n= 3), Blacktip (n=3), Atlantic Sharpnose (n=3), and Bonnethead (n=3) sharks was rapidly collected from euthanized sharks. Tissue collected from Bonnethead sharks was divided into three sections (anterior, central, and posterior) and incubated separately to determine steroid production throughout the full length of the tissue.

Incubation protocol followed the methods of Nunez and Trant (1999), and Idler and Truscott (1966). Tissue was removed from the kidney with forceps and minced with a scalpel. Approximately 50 mg of tissue was placed in a 12-well plate with 2 mL of elasmobranch Ringer's solution (ER; 240 mM NaCl, 7 mM KCl, 10 mM $CaCl_2$, 4.9 mM $MgCl_2$, 2.3 mM $NaHCO_3$, 0.5 mM $Na_2 HPO_4 \cdot (2H_2 O)$, 0.5 mM Na_2SO_4, 360 mM urea, 60 mM trimethylamine oxide (TMAO), 1% glucose) on an orbital shaker for 30 minutes at 26 °C. After an initial incubation to remove background steroid products, media was exchanged with 2 mL of fresh ER and 1 µL of ^{14}C-labeled progesterone in EtOH [0.1uCi/µL] (American Radiolabeled Chemicals) for 4.5 hours. Upon conclusion, all incubation media samples, and interrenal tissue samples were stored separately at -80°C until steroid extraction.

4.2.2 Steroid Extraction and High-Pressure Liquid Chromatography

Media was thawed, centrifuged (1.5 minutes at 1300 x g), and placed in borosilicate glass tubes. The organic phase was extracted using two aliquots of 2.5 mL dichloromethane (BDH). The organic phase was gently dried on an N-Evap™ Organomation analytical evaporator using nitrogen gas in a 42 °C water bath. Steroids were then dissolved and vortexed in 250 µL of a 50% methanol and 50% acetonitrile solution before being sealed with Parafilm and stored at -10 °C.

Following the methods of Evans *et al.* (2010) and Nunez and Trant (1999), two solutions were used to equilibrate the high-pressure liquid chromatography machine (HPLC): Solution A (10% methanol and 10% acetonitrile in water) and Solution B (50% methanol and 50% acetonitrile). To measure total radioactivity, media was thawed and 50

µL of each sample was added to 5 mL of Ecolum MP scintillation fluid (Aquazol) and run on a scintillation counter. Of the remaining sample, 100 µL was injected into a 250 × 4.6 mm Ultrasphere C18 reverse phase column (Beckman, Fullerton, CA) with a 5 µm particle size with a flow rate of 1 mL min^{-1}. Steroids were collected through the column at a gradient of 40% to 100% of Solution B over 28 minutes. Fractions were collected at 6-9.5 minutes (1α-OHB), 10-13 minutes (B), and 14.5-17.5 minutes (DOC). Fraction collection time was determined as the three minutes surrounding the elution peak of 25 µM standards of DOC (Sigma), B (Sigma), and 1α-OHB (1 mM in methanol, provided by John Rimoldi, University of Mississippi), similar to the methods of Evans *et al.* (2010). To quantify steroid production, 15 mL of Ecolum MP scintillation fluid (Aquazol) was added to each fraction and run on a scintillation counter.

4.2.3 Statistical Analysis

To determine statistical differences within species, steroid production of each product (i.e. DOC, B, and 1α-OHB) was statistically examined using an analysis of variance with a Tukey post hoc test. P-values less than 0.05 were considered significant.

4.3 Results

Steroid production was confirmed in interrenal tissue from all four species. All individuals produced 1α-OHB as the primary steroid product, with varying amounts of B and DOC (Table 4.1). Overall, Atlantic Sharpnose Sharks had the greatest production of all three steroid products among species with regards to percent precursor converted. Blacktip Sharks had similar, though slightly lower, production compared to Atlantic Sharpnose Sharks. Finetooth and Bonnethead sharks had lower total steroid production.

In relation to 1α-OHB production within each species, Atlantic Sharpnose and Finetooth Sharks had similarly high production of B compared to Blacktip and Bonnethead sharks (Table 4.2). A similar pattern was found for DOC production among the four species: Atlantic Sharpnose Sharks produced the greatest amount of DOC, roughly equivalent to the amount of B produced in Atlantic Sharpnose Sharks. Bonnethead sharks had almost no production of DOC.

Within species, Finetooth Sharks produced 1α-OHB as the major product followed by almost equal amounts of B and DOC (Figure 4.2). The production of 1α-OHB, B, and DOC were not significantly different in Finetooth Sharks. Among Blacktip Sharks, 1α-OHB was the dominant product followed by B and DOC (Figure 4.3). The production of 1α-OHB by Blacktip Sharks was significantly different from the production of B and DOC; production of B and DOC were not significantly different.

Atlantic Sharpnose Sharks had the greatest overall steroid production, with 1α-OHB being the major product, followed by B and then DOC (Figure 4.4). The production of all steroid products was significantly different from each other.

This trend continued within Bonnethead sharks: 1α-OHB was the dominant product, followed by B and DOC (Figure 4.5). A negligible amount of DOC was produced. The production of 1α-OHB was significantly different from B and DOC, whereas the production of B and DOC were not significantly different.

4.4 Discussion

Steroid production among all species supported my hypothesis that putative interrenal tissue was functionally consistent with previous studies of interrenal tissue in

the four shark species investigated. Production of 1α-OHB among all species indicated that putative interrenal tissue in these species functions as expected in elasmobranchs. However, species-specific differences in the production of B and DOC may elucidate physiological roles in systems other than the stress response and osmoregulation via 1α-OHB alone. There are also multiple factors including life stage, stress levels, cellular degeneration, and interaction with other physiological processes, which can potentially influence steroid production among and within individuals of a species. However, due to the remarkable consistency in steroid product ratios between individuals within a species (e.g. note the low standard error variation in Figures 4.2-4.5) the most likely reason for differences observed is indeed species-specific corticosteroid profiles.

Alterations in total 1α-OHB and other corticosteroid production can be due to life stage. To preclude negative effects of elevated corticosteroids during the early stages of development, juveniles from many vertebrate taxa have a "hypo-responsive period" with regards to stress. During the hypo-responsive period, which often occurs just after parturition, the stress axis does not fully function due to highly sensitive negative feedback mechanisms that limit ACTH secretion and the ability to produce detectable levels of corticosteroids and/or ACTH (Walker 1986; Barry *et al.* 1995). The hypo-responsive period may also be caused by a delay in HPI axis development (Feist and Schreck 2002; Barry *et al.* 1995). In mammals, this hypo-responsive period is thought to be an adaptive mechanism to counteract deleterious effects that corticosteroids can have on development of the central nervous system (Sapolsky and Meaney 1986). While

juveniles were not tested in this study, adaptive mechanisms or adjustments in energy partitioning could still affect the adult animals stress response or steroid production.

An individual's environment and perception of stressors can also impact steroidogenesis. An individual previously stressed by environmental conditions could respond with greater steroid production due to up-regulated enzyme expression (i.e. P450scc, 3βHSD), or alternatively less production because cholesterol stores have been depleted. A rapidly sacrificed individual with little exposure to stress prior to capture could have higher levels of steroid production because cholesterol stores were not depleted. Through anecdotal experience, Atlantic Sharpnose and Bonnethead sharks are not capable of managing capture-and-handling methods as well as Blacktip and Finetooth Sharks, and often succumb to the negative effects of stress (personal observation). Manire *et al.* (2001) compared multiple biochemical markers of stress in Bonnethead, Blacktip, and Bull Sharks and observed the greatest mortality in Bonnethead sharks. Species-specific responses were thought to be related to respiratory physiology, length of time spent struggling after capture, and level of net entanglement around the gills (Manire *et al.* 2001). This difference in the stress response could also suggest an increase in levels of 1α-OHB required to initiate or maintain the stress response in certain species or an excess of steroid production that overwhelmed the stress axis.

Another factor affecting total corticosteroid production is the influence of other physiological systems or endocrine interactions with the HPI stress response. Studies on teleosts identified complex endocrine relationships between the stress axis and thyroid function. Castañeda Cortés *et al.* (2014) reviewed the interaction between HPI axis and

the hypothalamic-pituitary-thyroid (HPT) axis through the influence of CRH as a neuroregulator, which interacts with thyroid and interrenal activity. Peter (2011) showed that thyroid hormones play a modulatory role in the stress response as well as osmoregulation and metabolism. Associations between the stress axis and thyroid hormones suggest complex interactions and multiple factors that could regulate steroid production. An additional perspective in teleosts suggests that complex endocrine actions such as an immune-controlled feedback mechanism, gonadotrophic stimulation of interrenal tissue, and influences of hydromineral hormones and catecholamines on interrenal sensitivity regulate the actions of the interrenal tissue (Schreck *et al.* 1989). Because these regulatory factors occur in teleost fish and the stress axis is conserved, it is plausible that similar processes occur within elasmobranch physiology.

Reproductive cycle and status could also influence interrenal production of steroids. In multiple species of teleosts, interrenal cells can produce a variety of androgens from incubated tissue, African Catfish *Clarias gariepinus* (Vermeulen *et al.* 1995) produce a variety of androgens; Mozambique Tilapia *Oreochromis mossambicus* (Balm *et al.* 1989) and Coho Salmon *Oncorhychus kisutch* produce androstenedione (Schreck *et al.* 1989); Sockeye Salmon *Oncorhynchus nerka* (Kubokawa *et al.* 2001) produce testosterone. Atlantic Salmon *Salmo salar* produce 17a,20b-dihydroxyprogesterone from precursors (Sangalang and Freeman 1988), which suggests interrenal tissue may be an alternate source of steroids which induces oocyte maturation (Nagahama 1997) and spermiation (Todo *et al.* 2000) in several fish taxa. The production of cortisol, as well as basal cortisol levels in teleosts, is influenced by multiple factors

(e.g. maturity and reproductive status, seasonal cycles, and migratory periods; Pankhurst 2011). Sex steroids, depending on season and maturity, can modify the pituitary–interrenal axis. In Rainbow Trout and Brown Trout *Salmo trutta* (Pottinger *et al.* 1996; Young *et al.* 1996), androgens have a suppressive action while 17β-estradiol enhanced corticotropic activity. Influences of synthetic cortisol (dexamethasone) in Sockeye Salmon demonstrated a sex-dependent response with a stronger response in spawning females, which also had a greater response to ACTH injections (Fagerlund 1970). Future studies are needed to explore whether the production of androgens or estrogens significantly affect corticosteroid production in elasmobranchs.

It is possible that in the current study tissue may have been exposed to excessive quantities of precursor, and therefore the tissue may be unable to continue producing 1α-OHB and thus intermediate products, such as DOC and B, accumulated. However, as noted above the consistency of results between individuals of the same species supports the validity of species-specific differences in product ratios. Regarding the low conversion rates in Bonnethead and Finetooth sharks, it is possible that steroidogenesis proceeds more slowly in interrenal tissue of these species, or conversely, that tissue from these species may not handle the media and incubation methods as robustly as tissue from Blacktip and Atlantic Sharpnose Sharks. Conversely, Atlantic Sharpnose Sharks had abundant cytoplasmic lipid stores in interrenal cells and the greatest overall steroidogenesis *ex vivo*, which could indicate that greater stores of steroid precursor result in greater steroid production.

The production of relatively large amounts of B and DOC compared to 1α-OHB was unexpected, based on the hypothesis that 1α-OHB is the dominant corticosteroid in elasmobranchs and central regulator of both the stress and osmoregulatory systems. As suggested, the levels produced may be due to overstimulation, however, B and DOC play biologically active roles in multiple vertebrate classes and therefore these results warrant further investigation. For example, B is a potent glucocorticoid in birds, reptiles, and amphibians (Jones and Henderson 1976) and DOC is a mineralocorticoid in teleosts (Bentley 1998; Sturm *et al.* 2005). Also, using the DNA-ligand binding domain of the mineralocorticoid receptor and the glucocorticoid receptors in the Little Skate *Leucoraja erinacea*, Carroll *et al.* (2008) found that B and DOC-bound to both the glucocorticoid and mineralocorticoid receptors. Both B and DOC bind with greater sensitivity to the mineralocorticoid receptor than did 1α-OHB. The presence of B and DOC and their potential to bind to receptors suggest that these steroids could play biologically active roles in elasmobranch physiology.

Future investigation of HPLC steroid peaks using an inline radioactive HPLC to further clarify peaks would distinguish other interrenal products in addition to the major products (1α-OHB, B, and DOC) targeted in this study. A recently developed Enzyme-linked immunosorbent assay accurately quantifies 1α-OHB (Evans and Wheaton personal communication), which could also more accurately quantify 1α-OHB production. Expansion of species- and sex-specific steroid production data through further *ex vivo* incubations are needed to develop a more complete understanding of steroid production in elasmobranchs.

Table 4.1

Percent of total radioactivity of steroidogenic products from *ex vivo* incubations.

	1α-OHB	B	DOC
Finetooth Shark	8.84	4.36	4.38
Blacktip Shark	21.85	7.34	4.23
Atlantic Sharpnose Shark	33.12	14.99	5.74
Bonnethead	7.52	1.50	0.06

Table 4.2

Ratio of steroidogenic products in relation to the production of 1α-OHB.

	1α-OHB	B	DOC
Finetooth Shark	1	0.49	0.50
Blacktip Shark	1	0.34	0.19
Atlantic Sharpnose Shark	1	0.45	0.17
Bonnethead	1	0.20	0.01

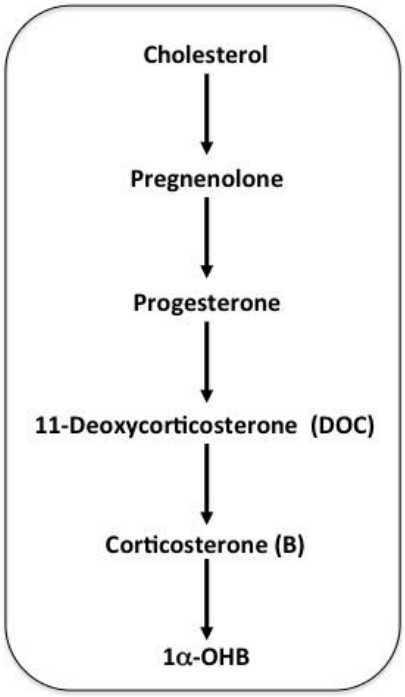

Figure 4.1 Section of the steroidogenic pathway from the conversion of cholesterol to the production of 1α-OHB.

Each arrow represents different enzymes responsible for the conversion of steroids.

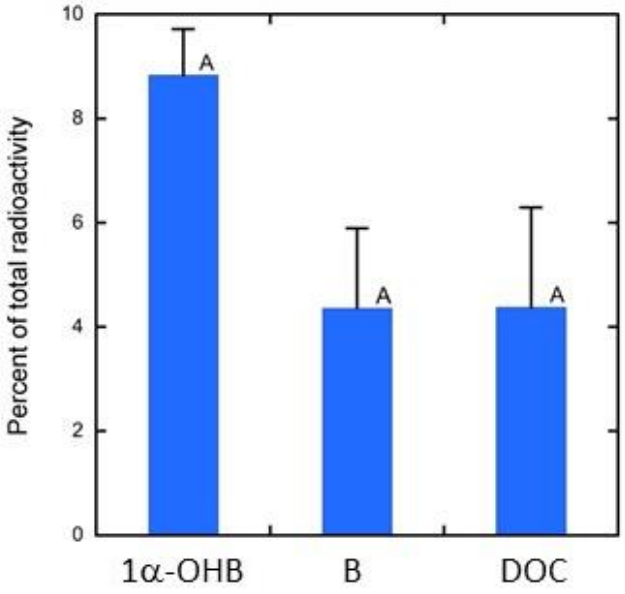

Figure 4.2 Percent of total radioactivity input and steroid products for Finetooth Shark.

(n=3) Error bars indicate standard error of the mean corticosteroid produced.

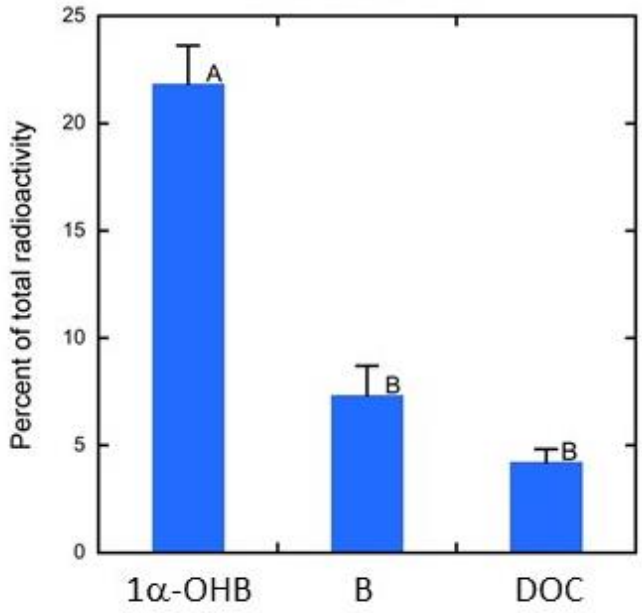

Figure 4.3 Percent of total radioactivity input and steroid products for Blacktip Shark.

(n=3) Error bars indicate standard error of the mean corticosteroid produced.

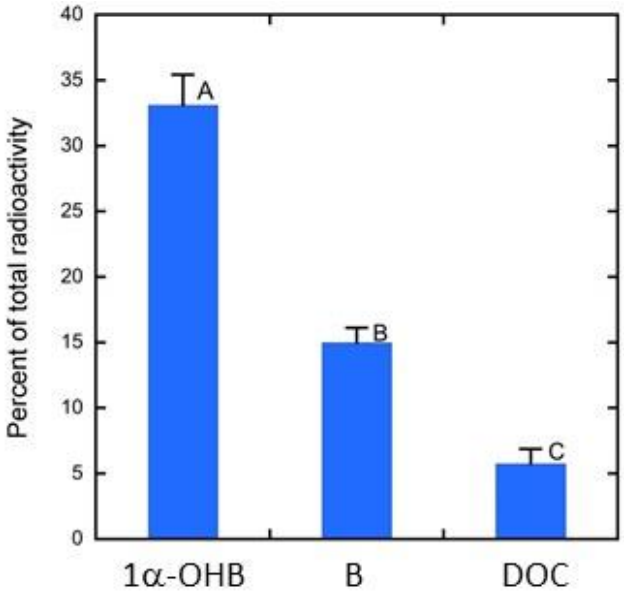

Figure 4.4 Percent of total radioactivity input and steroid products for Atlantic Sharpnose Sharks.

(n=3) Error bars indicate standard error of the mean corticosteroid produced.

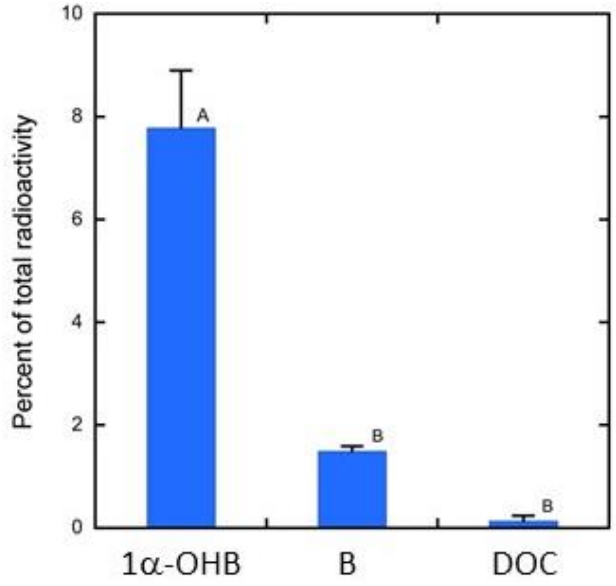

Figure 4.5 Percent of total radioactivity input and steroid products for Bonnethead sharks.

(n=3) Error bars indicate standard error of the mean corticosteroid produced.

CHAPTER V – CONCLUSION

Future research regarding interrenal morphology, cytology, and functionality would increase understanding of elasmobranch physiology, species-specific traits, and comparative biology. Among all species in the current study, cytoplasmic lipid stores were observed, 3βHSD expression was present, and steroid production was verified. There was no distinct connection between lipid presence, enzymatic expression, and steroidogenesis observed within this study.

Histological examination established that all putative tissues were indeed interrenal tissue. Future analysis of interrenal cytology through histological tests using lipid indicators (e.g. Sudan black or oil red) could help to further elucidate intra- and inter-specific differences in morphology. Further investigations could elaborate upon differences in coloration or capsule structure. The difference in coloration in Atlantic Sharpnose Shark interrenal tissue could not be determined in this study. The darker coloration was hypothesized to be the result of differences in blood flow due to increased vascularization, clotting or blood related to interrenal cell phase, however, no obvious distinctions were seen in hematoxylin and eosin staining within Atlantic Sharpnose Sharks. Further, Bonnethead sharks, which lack these distinct brown pigments, had greater vascularization than Atlantic Sharpnose Sharks. Additional tests could elucidate other pigments within the tissue to determine the cause of differences in coloration.

Investigating the capsule or membrane tissue surrounding interrenal cells could elucidate structural differences and explain variations in stress physiology among species. Finetooth and Blacktip Sharks have robust and distinct interrenal body capsules in

comparison with the softer tissue of Atlantic Sharpnose and Bonnethead sharks. While no obvious indications of membrane structure were seen during histological examination, further investigation could enhance these ideas. The thin membrane surrounding Atlantic Sharpnose Shark tissue could influence steroid transport: membrane thickness or protein structure could have unique characteristics that alter the movement of steroids, which could explain higher steroidogenic production. This may also explain the thinness and structural variability in the posterior portion of Bonnethead tissue. In a comparative view, the morphological appearance of a tissue tends to mimic the functionality, thus, these alterations in structure may play a vital role in interrenal physiology.

Further understanding of morphology and histology can help understand steroidogenic potential and functionality. Histological tests to quantify lipid presence or observations of stages of cytology under different stages of stress would provide insight into shark physiology. Differences in lipid content within cells may explain variations in steroid production. The stress level and lipid content of an individual will impact production, and it is also possible that there is a species-specific difference in quantity of lipid stored. Species with a lower rate of steroid production relative to others may possess limited ability to store lipids, variations in membrane permeability, or a decreased need to store lipids due to a lower steroid production rate.

Differences observed among species with regards to the stress response and a lack of knowledge regarding elasmobranch interrenal tissue warranted in-depth investigation of interrenal tissue among selected elasmobranch species. This study is the first to describe Atlantic Sharpnose and Bonnethead shark interrenal morphology, clarify the

production of steroid hormones B, DOC, and 1α-OHB in these species and suggest the potential for another biologically active role for B and DOC within elasmobranchs. My research elucidates differences in interrenal morphology, validates putative interrenal tissue and the presence of 3βHSD steroidogenic enzymes through histology, and verifies *ex vivo* steroid synthesis and species-specific differences. These results increase understanding of taxa-specific differences in interrenal tissue, therefore advancing comparative anatomy within elasmobranch species and augmenting research on elasmobranch stress physiology.

APPENDIX A – Institutional Animal Care and Use Committee Approval Form

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.6791 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**15101509 (Replaces 13101704)**
PROJECT TITLE:	**Mississippi Coastal Sport Fish Studies/Gulf Shark Pupping and Nursery Study**
PROPOSED PROJECT DATES:	**10/2015 - 09/2018**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Jill Hendon**
DEPARTMENT:	**Fisheries Research & Development**
FUNDING AGENCY/SPONSOR:	**N/A**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2018**

Frank Moore, PhD
IACUC Chair

10/01/2015
Date

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.4063 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**11092217**
PROJECT TITLE:	**Southeast monitoring and assessment program (SEAMAP) Bottom Longline**
PROPOSED PROJECT DATES:	**9/2014 – 9/ 2017**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Jill Hendon**
DEPARTMENT:	**CFRD**
FUNDING AGENCY/SPONSOR:	**NOAA DoC**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2017**

Frank Moore, Ph.D.
IACUC Chair

Date 10-15-14

APPENDIX B – Histological Processing

Table B.1

Dehydration and paraffin impregnation steps for the Thermo Scientific™ Shandon™ Excelsior™ ES Tissue Processor.

Step	Solution	Duration (hr.)
1	70% EtOH	Hold
2	80% EtOH	1
3	95% EtOH	0:40
4	95% EtOH	0:40
5	95% EtOH	0:40
6	100% EtOH	0:40
7	100% EtOH	1
8	100% EtOH	1
9	Xylene Substitute	1
10	Xylene Substitute	1
11	Xylene Substitute	1
12	Paraplast Plus	0:40
13	Paraplast Plus	0:40
14	Paraplast Plus	0:40

(Corey 2016).

Table B.2

Hematoxylin and eosin staining procedures.

Step	Solution	Duration
1	Xylene Sub.	3 min.
2	Xylene Sub.	3 min.
3	Xylene Sub.	3 min.
4	100% EtOH	10 dips
5	100% EtOH	10 dips
6	95% EtOH	10 dips
7	95% EtOH	10 dips
8	80% EtOH	10 dips
9	80% EtOH	10 dips
10	50% EtOH	10 dips
11	Distilled Water	1 min.
12	Hematoxylin 2	4 min.
13	Water – rinse well	------
14	Acid water	2 dips
15	Water – rinse well	------
16	Blueing water	30 sec.
17	Water – rinse well	------
18	95% EtOH	10 dips
19	Eosin Y	1 min.
20	Blot Blot Blot	------
21	95% EtOH	10 dips
22	95% EtOH	10 dips
23	95% EtOH	10 dips
24	100% EtOH	1 min.
25	100% EtOH	1 min.
26	100% EtOH	1 min.
27	Xylene Substitute	1 min.
28	Xylene Substitute	1 min.
29	Xylene Substitute	1 min.
30	Xylene Substitute	1 min.

Adapted from Corey 2016.

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