DISSERTATION

THE PROCESSES FOR DETERMINING THE RISK FACTORS INVOLVED WITH THE MORBIDITY AND MORTALITY OF THE SOUTHERN STINGRAY, *DASYATIS AMERICANA,* AT AN AQUARIUM

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ABSTRACT

THE PROCESSES FOR DETERMINING THE RISK FACTORS INVOLVED WITH THE MORBIDITY AND MORTALITY OF THE SOUTHERN STINGRAY, *DASYATIS AMERICANA,* AT AN AQUARIUM

During the first few years of opening a new touch pool exhibit, approximately 36 southern stingrays died at the Downtown Aquarium in Denver. Many of these stingrays presented with similar necropsy findings: a small, dark (described as dark, dark brown, black, or blue) liver indicative of lipid and glycogen storage depletion and many of the females were described as having oophoritis. At the time, only limited information was available regarding anatomy, clinical diagnostics (hematologic and plasma biochemical profiles and imaging), and histology for southern stingrays. As this project progressed and the need for this information became apparent, much of it was compiled and organized into a website or desktop application (Appendix 4) in order to provide baseline knowledge.

The first part of this project involved reviewing the gross necropsy reports, histopathology reports, and histology slides. The gross necropsy reports were divided among stingrays with known and unknown causes of death. Known causes of death included water quality mishaps and accidental deaths from inadvertently jumping out of the exhibit. Precautionary measures were put in place to avoid further deaths from those particular situations. Preliminary evaluation of the necropsy reports suggested that the odds of a stingray having a small liver is almost 40 times as high when the cause of death is unknown compared to a known cause of death and the odds of it being at the aquarium less than three months is 57

times as high when the cause of death is unknown compared to known. Stratifying by liver size revealed it as a confounder thereby making it the basis for the subsequent study. The goal of evaluating the stingray liver, as it relates to morbidity and mortality in this situation, was to understand conditions of lipid depletion, better manage those conditions, and develop a health monitoring system to avoid severe conditions. In reviewing the reports and slides regarding oophoritis, it was discovered that the histologic diagnosis was likely misidentified in the majority of cases due to the close association of the epigonal organ, which is a hematopoietic organ, with the ovaries. The gross diagnoses of oophoritis stemmed from an initial histopathology report and what was viewed as hemorrhagic ovaries was likely normal, reproductively mature, active folliculogenesis. In only a few cases the ovaries were necrotic making a diagnosis of oophoritis appropriate.

The next step of this project was to determine the risk factors involved with hepatic lipid depletion. Uniquely identified (by PIT tag) living stingrays in the collection as well as new arrivals were routinely examined. Examinations included physical, ultrasound, and blood parameter assessments. A quantitative diagnostic approach was validated and used to establish a liver-to-coelom ratio (or liver size percentage) by ultrasonography to assess liver size. Arbitrarily, a liver-to-coelom ratio of 70% (small liver) was selected to assess the following risk factors: wingspan, new arrivals, season, folliculogenesis, and pregnancy. A generalized linear mixed model (X^2_{LR}=72.031, df = 1, p<<0.001) was used to analyze the risk factors for the outcome, small liver (yes or no). The predictors (with odds ratio [confidence intervals]) in the final model included pregnancy (yes) (30.978 [6.803, 141.066]), time in captivity (≤ 3 months) (2.534 [0.945,6.790]), and wingspan (> 60 cm) (0.530 [0.206, 1.365]). Although time in

captivity and wingspan were not statistically significant, they improved the fit of the model. Pregnancy had the greatest effect at predicting small livers.

One time during the study period, new southern stingrays were wild caught and added to the touch pool exhibit. This provided an opportunity to compare the liver size, hematological values, and plasma biochemical values of current acclimated rays to the new arrivals. New arrivals or stingrays in captivity for less than three months showed a statistically significant difference ($p < 0.05$) compared to acclimated rays in liver size, in some hematologic values (plasma protein and PCV), and in some plasma biochemical values (bicarbonate, urea, calcium, cholesterol, chloride, globulin, and potassium). Significant differences in liver-to-coelom ratios existed between the two stingray groups when compared at introduction (median difference = 30.9%, p=0.007) and after eight months (median difference = 20.5%, p=0.008); and within the acclimated group (median difference= 20.4%, p=0.018) and wild-caught group (median difference 31%, p=0.008) when comparing livers at introduction and after eight months. The hematological and plasma biochemical values showed no differences after eight months of cohabitation suggesting that the parameters were affected by environmental and dietary changes.

Routinely examining the stingrays and collecting consistent clinical information helped to establish a system for monitoring their health. The protocol promoted stability for the stingray collection and allowed for captive breeding to take place. Captive breeding served to populate the collection without the need for wild capture, which was cost-effective and reduced the risk of outside disease. In order to maximize space at the aquarium, a gestation study was conducted to predict parturition date ranges. Eight pregnant stingrays were monitored during three gestation sessions for two years. The fetal body depth measurements were taken using

ultrasound. The first two gestation sessions were used to develop a linear regression model to predict a parturition date range and the third gestation session was used to assess the accuracy of the model. The regression model was Days Before Parturition = 139.75-31.249*Fetal Body Depth. This model was tested on three stingrays and predicted the parturition dates for two of them within 1-2 weeks and the third one within one month. There are many factors that can affect gestation length but clinically this model was helpful in determining parturition date ranges at this aquarium.

Two other findings observed while reviewing histology slides was hepatic melanomacrophages and epigonal organ edema and hemorrhage. Because melanomacrophages have been indicated as biomarkers for chronic stress in some fish, counts were taken per high power field and analyzed among stingrays for liver size and the presence of both follicles and epigonal organ edema. There were significant differences in counts between stingrays with small and large livers and between stingrays with absent to mild and severe epigonal organ edema. It was unknown as to whether or not this was an independent pathologic finding for the epigonal organ or if there was a reproductive component. After completion of this project, one study stingray that had been moved to a larger exhibit, died from a water quality mishap. Presumptively, she was in a state of follicular stasis or was not able to reproduce. She had many opportunities to mate with males but was never pregnant. Ultrasonographically, she constantly had large follicles and a large, fluid-filled, trophonemata-lined uterus. Other than the reproductive findings, she was clinically healthy and had no other issues. Upon gross necropsy, her ovary was filled with multiple, variable-sized follicles. Histologically, the liver was adequately filled with lipid, the ovary was normal, and the only abnormality was a hemorrhagic epigonal organ. Another southern stingray, not in the study, also died from the same water

quality mishap. She also had similar gross necropsy and histological findings with the addition of mild epigonal organ edema. Both of the stingrays had low melanomacrophage counts, which coincides with these cells being biomarkers for chronic stress. Although the reproductive anatomy was not structurally abnormal, the condition presented here appears abnormal. Further study is needed to evaluate the specific ovarian cycle of the southern stingray; the communication (vascular, immunologic, or hormonal) between the gonad and epigonal organ; and the possible clinical, histological, and hormonal differences between folliculogenesis and follicular stasis.

This project provided this aquarium with a protocol to assess the health of southern stingrays, which has also been used for other elasmobranchs at this facility. Collaboration with other aquariums to further investigate the reproduction condition is also underway.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PERTINENT PUBLICATIONS

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CHAPTER 1: Introduction and Literature Review

1.1 Background: The Roles of Zoos and Aquariums

Aquaria and zoos uphold many different roles including exhibition of live animals, sharing research, zoological education, advocating for animal welfare, and conservation (Packer and Ballantyne 2010). With more attention towards the declining global environment, the idea and practice of conservation is as important as ever. As human population increases, the depletion of natural resources also increases. These natural resources include, but are not limited to rainforests, woodlands, farmlands, freshwater lakes and oceans (Olney 2005). In an effort to reverse these trends, it is important to educate the public. Public zoos and aquariums accredited by the Association of Zoos and Aquariums (AZA) host 143 million visitors each year (Vehrs et al. 2006) and zoos and aquariums in general host over 700 million visitors each year (WAZA) providing this to be an excellent educational source. The World Zoo and Aquarium Conservation Strategy have spearheaded this approach of integration using these public facilities. A quote from the strategy regarding zoos and aquariums (WZACS Introduction, p. 9, Olney 2005):

> Only zoos, aquariums and botanic gardens can operate across the whole spectrum of conservation activities, from ex situ breeding of threatened species, research, public education, training and influencing and advocacy, through to in situ support of species, populations and their habitats; they uniquely have a massive 'captive audience' of visitors whose knowledge, understanding, attitude, behaviour and involvement can all be positively influenced and harnessed.

With this idea in mind, it is important for public aquaria to uphold the responsibility of public awareness and education as their part in conservation. There are large-scale, international

studies being conducted to evaluate the perception of public awareness as well as the effectiveness of *in situ* conservation efforts (Packer and Ballantyne 2010; Gusset and Dick 2010; Gusset and Dick 2011).

There are thousands of species represented at public aquaria and not only does this contribute to the public's education but also to the aquarium staff and researchers' education. It is also the responsibility of these facilities to learn as much as they can from their collections in order to provide the best care possible to these animals in captivity and possibly contribute to biological data to understand their wild counterparts.

Popular exhibits, seeing animals in their 'natural environment', and interacting with animals appear to make an impression on the public and prompt reflecting on their experience and what they have learned (Packer and Ballantyne 2010). One of the most popular exhibits at the Downtown Aquarium at Denver as well as many other aquariums is the stingray feeding touch pool. This is an interactive exhibit where visitors at the aquarium are given the opportunity to feed and touch the stingrays. Southern stingrays, *Dasyatis americana,* and Cownose stingrays, *Rhinoptera bonasus,* inhabit the exhibit. Other locations within the aquarium where larger southern stingrays are displayed include a large exhibit with the tunnel. The tunnel allows guests to walk through the tank and see the animals from other perspectives (from below). The stingrays are especially intriguing to visitors because, being benthic in nature, they tend to settle on the top of the tunnel allowing visitors to stand underneath them. Due to the attraction these exhibits have on the public, they play a vital role in the aquarium's conservation and awareness mission.

1.2 Southern stingrays in captivity

Southern stingrays, *Dasyatis americana*, are classified as Data Deficient globally by the International Union for Conservation of Nature (IUCN). The IUCN tracks species' vulnerability by their Red List of Threatened Species™. Known species of plants, fungi, and animals with Adequate Data are classified in accordance with IUCN on their risk of extinction from lowest (Least Concern) to highest risk (Extinct in the wild). In the United States, southern stingrays are classified as Least Concern whereas in other South American countries, like Brazil, they are classified as Vulnerable due to increased fishing (Grubbs et al. 2006). The Data Deficient status globally indicates that there is not sufficient information regarding distribution and population worldwide in order to assess the risk of extinction. Clinical, biological, ecological, and population studies can contribute to the information regarding this species to have a better understanding of its status and conservation. Although elasmobranchs in captivity should serve to help educate the public, they are also a resource for research to further understand the biology and contribute to information to conserve wild populations.

The southern stingray is a commonly exhibited elasmobranch in public aquaria. There are over 120 facilities that exhibit elasmobranchs worldwide (AES 2008 census). Forty-three of these facilities have Southern stingrays on display making them the second most represented stingray species to cownose stingrays, *Rhinoptera bonasus* (AES 2008 census). As mentioned, stingrays are housed in a variety of exhibits including touch pools, which allows actual visitor interaction (touching and feeding). Other exhibits that allow visitors unique perspectives of the animals include tunnels, bubbles, side inserts, and open floors. There are some studies conducted at zoos and aquariums attempting to assess the impact of the institution such as the Association of Zoos and Aquariums-sponsored project (Dierking et al. 2002) and the Monterey

Bay Aquarium's conservation mission (Yalowitz 2004) but there are also reports challenging the validity of similar studies (Marino et al. 2010).

There are very few publications focused on southern stingrays. Most of the research focus has been on other elasmobranchs or elasmobranch groups. For this review, there will be emphasis placed on the elasmobranch liver, reproductive system and other publications specific to Southern stingrays related to the objectives of this study. There have been other related studies conducted on other species of stingray, which have been referred to and include the following broad categories: anatomy, imaging, hematology and plasma biochemical references, reproduction, and histology.

1.3 Anatomy and Imaging Review

1.3.1 Gross Anatomy Review

Elasmobranchs are cartilaginous vertebrates within the class Chondrichthyes and include sharks, rays, and skates. Their endoskeleton is made of cartilage and the body of the skeleton is separated into segments: axial (skull), vertebral (spine), and appendicular (fins) (Compagno 1999). The appendicular skeleton creates the levels of the pectoral and pelvic girdles, which support the fins (or wings and fins in the stingray's case). Similar to teleosts, they are gill-breathing with no lungs and depending on the species have four to seven gill openings or slits. Unlike teleosts, their endoskeleton is composed of calcified cartilage and they contain no swim bladder or adipose tissue (Compagno 1999). Buoyancy, if needed, is assisted by fat storage in the liver.

A normal liver in marine elasmobranchs usually appears to be a large, lipid-filled, organ that occupies the majority of the ventral coelomic cavity, whose cranial and caudal margins are

outlined by the pectoral and pelvic cartilaginous girdles, respectively. In some cases the composition of the normal liver may approach 80 percent of lipids (Holmgren and Nilsson 1999). These fatty livers are important to these animals as they depend on them for energy and buoyancy (Holmgren and Nilsson 1999). It has also been documented that in some shark species, ketone bodies are a main fuel source and are present regardless of when they ate their last meal (Watson and Dickson 2001). This may imply that when food is scarce, these animals will rely on their lipid stores over their glycogen stores (Carrier, 2004). With this information, it can be speculated that the decreased liver size may be due to starvation or increases in energy demand.

Carrier (2004) describes two endocrine pathways that may also create similar results. The secretion of catecholamines from the chromaffin tissue stimulates the mobilization of lipid stores for energy and thereby will decrease the size of the liver. The second pathway works as an inhibitory process but may produce a similar outcome. Stress induces the hypothalamo-pituitary-interrenal (HPI) axis to secrete 1α-hydroxycorticosterone (1α-OHB), which inhibits lipid storage thereby disallowing the liver to be large.

Surrounded by the liver, in the cranial right ventral quadrant of the coelomic cavity, is the gall bladder. Although it appears that the gall bladder in elasmobranchs functions similarly to that of mammals in the ray, *Raja erinacea*, and dogfish, *Squalus acanthias*, the activity of its function varies (Holmgren and Nilsson 1999). For example, the process of bile production is similar to that of mammals but the rate at which it is produced is much lower, approximately 100 times that of a rodent (Boyer et al. 1976). In contrast to mammals, bile acids form alcohol sulfate esters as opposed to taurine salts (Holmgren and Nilsson 1999).

Dorsal to the liver, within the coelomic cavity, are where the majority of the other internal organs are located. The direct digestive system includes a crushing plate in mouth, esophagus, stomach (cardiac and pyloric), spiral intestine, rectum, and cloaca. Aside from the liver and gall bladder, another indirect digestive organ is the pancreas.

The heart is located in its own cavity just cranial to the coelomic cavity and pectoral cartilaginous girdle (which separates it from the coelomic cavity) and just caudal and medial to the gill arches (Tota 1999).

Reproductive anatomy is reviewed in section 1.5.1. Female southern stingrays (as well as other elasmobranchs classified as myliobatiformes) possess a unique anatomical characteristic in that one of their lymphomyeloid tissues or organs, called the epigonal organ, is adjacent to or encompasses the ovary. The epigonal organ, similar to bone marrow in mammals with its production of leukocytes, is a dynamic organ but the variation in size as well as its association with the ovary is unclear but likely due to endocrine factors (Lutton et al. 2005). Because of its close proximity to the ovary, it is thought that the immune effects of the epigonal organ may directly affect reproduction by altering ovarian processes (Lutton et al. 2005; Lutton and Callard 2008).

1.3.2 Lipid Metabolism in Elasmobranchs

A summary of lipid metabolism is shown in Figure 1.1. A gastrointestinal microanatomical difference between elasmobranchs and mammals include the presence of oxynticopeptic cells in elasmobranchs compared with separate parietal (oxyntic) and chief (peptic) cells in mammals (Rebolledo and Vial 1979). Similar secretions are noted despite the combined efforts of the cells, with the addition of chitinases secreted in elasmobranchs.

Elasmobranchs generally have a short gastrointestinal tract (GIT). The stomachs are similar to one another but the intestines are quite different. The largest portion of the intestinal tract is very short and referred to as a spiral intestine or spiral valve. The spiral intestine is comprised of several leaflets (the formation varies amongst species) to increase surface area for absorption. There are no proper glands lining the intestinal wall, instead the secreting cells are located in the folds and crypts. Despite the limited information regarding fat absorption, it is assumed to be similar to that of mammals as the colipases secreted by the pancreas are homologous to mammalian colipases (Ballantyne 1997).

General differences regarding energy and metabolism among elasmobranchs when compared to mammals includes the lack of adipose tissue, the majority of fat is stored in their liver (normal process), they have little to no albumin and therefore little to no circulating fatty acids, fatty acid oxidation (beta-oxidation) does not occur in extrahepatic tissue, and their primary fuel source is ketone bodies evident by high concentrations in circulation regardless of when their last meal occurred (Watson and Dickson 2001). The activity of ketone body formation in the liver is high and evident by increased activity in CPT-I (in the carnitine transport system), 3-hydroxyacyl CoA dehydrogenase (in beta-oxidation), thiolase (in beta-oxidation), HMG CoA synthase (in ketogenesis), and 3-hydroxybutyrate dehydrogenase (in ketogenesis in the liver) as shown in Table 1.1 (Zammit and Newsholme 1979; Treberg et al. 2006). Ketone oxidation is high in extrahepatic tissues shown by increased levels of 3-hydroxybutyrate dehydrogenase and oxoacid CoA transferase. Unlike mammals, elasmobranchs are in a chronic state of ketosis. Due to their high demand for ketones and given that the liver is the only organ that performs ketogenesis at high levels, the liver is normally composed of large

amounts of lipid (up to 80%) and therefore hepatic lipidosis is not a pathologic condition in elasmobranchs.

Table 1.1. Summary of enzyme activity, fatty acids, and ketones in elasmobranchs compared to mammals (Ballantyne 1997; Watson and Dickson 2001; Zammit 1979; Treberg et al. 2006)

Enzyme	Elasmobranchs	Mammals
CPT 1	↑↑ Liver, not detected in heart or red muscle	↑ Liver between meals, detected in other organs
HOAD	↑↑ Liver, very low in other organs, none in heart, red muscle	↑ All organs between meals
Thiolase	↑ Liver	↓ Liver
HMG Synthase	↑↑ Liver, not detected in heart or red muscle	↑ Liver during starvation
3-HBD	↑ Liver, ↑ muscle	N/A
OAT	↑↑ Heart and red muscle, low in liver	↑ Liver
Plasma NEFA	Low	High
Plasma KB	High	Low

CPT = Carnitine Palmitoyl Transferase
HOAD = 3-Hydroxyacyl CoA Dehydrogenase
HMG = 3-Hydroxy-3-Methylglutaryl CoA
HBD = β-Hydroxybutyrate Dehydrogenase
OAT = 3-Oxoacid CoA Transferase
NEFA = Non-Esterified Fatty Acid
KB = Ketone Body

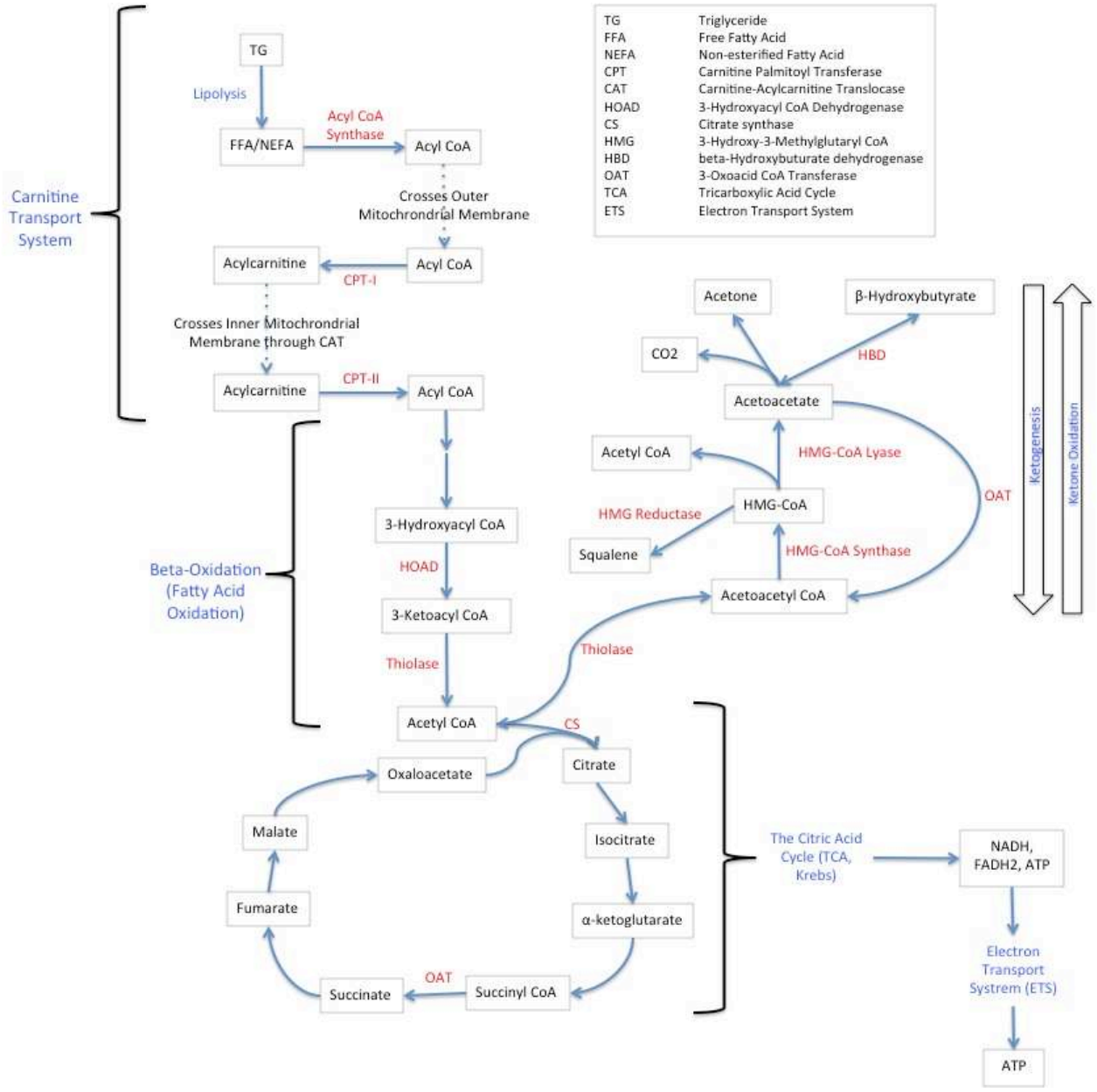

Figure 1.1. Summary of Lipid Metabolism in Elasmobranchs

1.3.3 Imaging Review

There are very few publications on imaging techniques in fish with the majority of them being on teleosts. Stetter (2002 and 2004) describes equipment, technique, and little on interpretation for radiography, ultrasonography, CT, MRI, and endoscopy in fish. Ultrasonography is an excellent diagnostic tool for fish as they can remain in the water with no need for acoustic gel. Several elasmobranch species undergo tonic immobility when placed in dorsal recumbency therefore sedation may not be needed with this imaging modality (Henningsen 1994; Stamper 2007). Walsh et al.'s (1993) ultrasonography publication shows transverse ultrasound images with actual cut sections in cross section for comparison. Although the results were accomplished using a variety of sharks, the information can be extrapolated to some degree to stingrays and is useful for determining a process for identifying structures via ultrasound. For example, the fatty liver in elasmobranchs has a similar echogenicity as mammals with hepatic lipidosis (Nyland et al. 2002; Mathiesen et al. 2002).

1.4 Hematology and Biochemistry Review

Blood collection and sample handling has been widely published (Campbell 2012; Campbell 2015; Grant 2015; Noga 2010; Walsh and Luer 2004; Southgate 2001). Once the sample has been collected, options for anticoagulants and preservation include EDTA, lithium heparin, buffered formalin, a combination of EDTA and lithium heparin, or ACD solution A (Arnold et al. 2014, Walsh and Luer 2004, Grant 2015).

Hematological and biochemical evaluations in fish are not routinely performed in the clinical setting due to cost and the difficulty of interpretation (Campbell 2012). Part of the difficulty leading to interpretation is the differences in nomenclature for hematological study.

The results for hematology include total white blood cell count, differential (neutrophil, heterophil, lymphocyte, monocyte, eosinophil and basophil counts), plasma protein and packed cell volume. The terminology for granulocytes varies in the literature. Due to the staining characteristics of the granules in elasmobranch granulocytes, many resources describe these cells by physical appearance using avian (and mammalian) terminology (Walsh and Luer 2004). For example, granulocytes with heterophilic-staining granules are termed heterophils, granulocytes with red-staining granulocytes are termed eosinophils and granulocytes with purple-staining granules are termed basophils (Walsh and Luer 2004). Another publication refers to these cells by different names but also by physical appearance in the sandbar shark; cells with a segmented nucleus and colorless cytoplasm are termed neutrophils, cells with many, fine, red, rod-shaped granules are termed fine eosinophilic granulocytes (FEG) and cells with few, pale red, round granules are termed coarse eosinophilic granulocytes (CEG) (Arnold 2005). Campbell (2015) refers to studies in the lesser dogfish for classification of granulocytes as G_1 (granulocyte type 1) which would be physically equivalent to the avian heterophil, G_2 (granulocyte type 2) which would be physically equivalent to the mammalian neutrophil, and G_3 (granulocyte type 3) which would be physically equivalent to an eosinophil. Following this scheme, basophils may also be referred to as G_4 (granulocyte type 4). Despite the discrepancy in nomenclature, hematological evaluation can be performed relatively inexpensively using a hemacytometer and stained slide for differential. Due to the nucleated red blood cells, using an automated analyzer is not possible therefore manual counts are performed in laboratories. The procedure could easily be performed at much lower costs in-house with minimal training (Grant 2015).

There are some studies reporting hematological and biochemical reference intervals in fish; however very few were conducted for elasmobranchs. Current elasmobranch hematological

and biochemical studies include common thresher (*Alopias* vulpinus), dusky shark (*Carcharhinus obscurus*), great white shark (*Carcharodon carcharias*), tiger shark (*Galeocerdo cuvieri*), Atlantic shortfin mako (*Isurus oxyrinchus*), blue shark (*Prionace glauca*), scalloped hammerhead shark (*Sphyrna lewini*), smooth dogfish (*Mustelus canis*), captive whale shark (*Rhincodon typus*), captive cownose stingrays (*Rhinoptera bonasus*), Atlantic sharpnose sharks (*Rhizoprionodon terraenovae*), spiny dogfish (*Squalus acanthias*), bonnethead sharks (*Sphyrna tiburo*), wild southern stingrays (*Dasyatis americana*), sandbar sharks (*Carcharhinus plumbeus*), Clearnose skate (*Raja eglanteria*), Atlantic stingray (*Dasyatis Sabina*), blacktip shark (*Carcharhinus limbatus*), nurse shark (*Ginglymostoma cirratum*), wild dwarf ornate wobbegong sharks (*Orectolobus ornatus*), free-living sand tiger sharks (*Carcharias taurus*), and white-spotted bamboo sharks (*Chiloscyllium plagiosum*) (Arnold 2005; Emery 1986; Ferreira et al. 2010; Haman et al. 2010; Dove et al. 2010; Cain et al. 2004; Harms et al. 2002; Walsh and Luer 2004; Otway et al. 2011; Otway 2015; Alexander et al. 2016). Few studies have evaluated factors that affect hematological and biochemical parameters in elasmobranchs and some examples for hematology include endothermic versus ectothermic sharks (Emery 1986), sex (Persky et al. 2012), venipuncture site (Mylniczenko et al. 2006), tourist sites versus non-tourist sites (Semeniuk et al. 2009), and inflammatory disease (Alexander et al. 2016). Even fewer studies have been published assessing factors affecting plasma biochemical values but some examples are shown for stress during capture and transport in juvenile dusky sharks, *Carcharhinus obscurus* (Cliff and Thurman 1984), changes in glucose with epinephrine administration in the nursehound shark, *Scyliorhinus stellaris* (DeRoos and DeRoos 1978), changes in glucose following insulin infusion in spiny dogfish, *Squalus acanthias* (DeRoos et al.

1985), and changes in electrolytes from freshwater to seawater in elasmobranchs (Hazon et al. 2003).

The article specific for biochemical reference for wild-caught southern stingrays was a study of 28 wild-caught southern stingrays from three different locations along the coasts of South Carolina, Georgia, and Northern Florida (Cain et al. 2004). The objectives of this study were to provide descriptive statistics for plasma biochemistry results and determine the existence and strength of associations between size and plasma analytes, the time on deck before sampling, determine plasma analytes, lactate and glucose values by sex and by region (north compared to south), and total solids and total protein. Because the distribution was not normal, the descriptive statistics results for plasma biochemistry analytes were displayed as the median, 10^{th} and 90^{th} percentile. The following conclusions were made regarding the other objectives: no significant difference between sex ratios and regions, no differences in disk width or body weight with respect to region, osmolality was significantly higher ($p<0.0001$) in the south than in the north, no significant differences in blood chemistry with respect to weight, disk width or sex, plasma lactate and calcium were the only values significantly but not strongly associated positively with time on deck, plasma lactate was significantly but not strongly associated with glucose, and total solids and total protein values were linearly related.

The limitations of this study are that these were wild-caught southern stingrays in three relatively proximal regions. This species can be found along the east coast of the United States, throughout the Gulf of Mexico, and in northern South America. This data represents this species in a focused location. Other limitation may include stress-related conditions, season variations (hormonal changes), and possibly repeat subjects. Regardless, this is the only study of this kind for southern stingrays and provides a reference for this research project. In this project, the

hematological and biochemical review will provide a reference range for captive southern stingrays as well as one reference for interpretation between two different conditions.

1.5 Female Reproduction Review

1.5.1 Anatomy

The reproductive anatomy and physiology of elasmobranchs is similar for males amongst species but varies slightly for females. The general female reproductive anatomy in elasmobranchs consists of ovaries, ostia, oviduct, oviducal gland (shell or nidamental gland), uterus, cervix, and urogenital sinus (Hamlett and Koob 1999; Henningsen et al. 2004; Walker 2005; Musick and Ellis 2005). The oviduct may be referred to by any of its parts: the ostium, anterior oviduct, oviducal gland, isthmus (species-specific), and dilated terminal region (Hamlett and Koob 1999). Depending on the species or group, the ovary and oviducal gland may be unilaterally or bilaterally functional and when unilaterally functional the anatomy on the opposite side may be rudimentary or nonexistent (Lutton et al. 2005; Hamlett et al. 2005; Henningsen et al. 2004; Wyffels 2009). Fertilization often occurs in proximal oviduct or oviducal gland depending on species (Hamlett et al. 2005; Lutton et al. 2005). Sperm storage within the female reproductive tract occurs in many lower vertebrates although the length of time for storage varies from several months in amphibians to several years in reptiles (Holt and Lloyd 2010). Sperm storage typically occurs in the terminal zone of the oviducal gland in elasmobranchs (Hamlett et al. 2005). Sperm storage has been identified in several elasmobranch species including the smoothhound, *Mustelus canis* (Hamlett et al. 2002b), the gummy shark, *Mustelus antarcticus* (Storrie et al. 2008), and Oman shark, *Iago omanensis* (Hamlett et al. 2002a). Sperm storage has not been specifically identified in the southern stingray.

The association of the ovary with the epigonal organ also varies amongst species. The location and association of ovaries with respect to the epigonal organ has been described as either internal or external but there have been few studies conducted and there is little information on this topic (Lutton et al. 2005). Because the epigonal organ is a dynamic lymphomyeloid structure and the size and shape of the ovary changes in response to hormonal stimuli, the relationship between the two organs may be in flux and difficult to classify at any given time (Lutton et al. 2005). In the southern stingray, the ovary is only on the left side and closely associated with the epigonal organ although appears to be concentrated along the distal margin. There is a single, left-sided, ostium, oviduct, oviducal gland and uterus in the southern stingray.

1.5.2 Maturity status

Reproductive maturity in females is denoted by the time of their first ovulation (Walker 2005; Ramirez-Mosqueda et al. 2012). It has been suggested that follicular development, oviducal gland width, and uterus width may be used to determine maturity in female southern stingrays; however, actual descriptions and measurements of these parameters have not been well documented (Henningsen and Leaf 2010; Henningsen et al. 2004). Size, more specifically disc width, at maturity with confirmation by pregnancy is more often documented. For female southern stingrays, 70-80 cm disc widths have been associated with sexual maturity and one studied confirmed maturity in southern stingrays that ranged in size from 82.5-90 cm (Henningsen and Leaf 2010).

1.5.3 Mating

Reproductive behaviors may be stimulated by visual, biochemical, or electroreceptive means (Henningsen et al. 2004; Pratt and Carrier 2005). Mating behaviors in stingrays typically begin with precopulatory following, parallel swimming, biting, female avoidance/acceptance, or clasper flexion of the male (Maruska and Gelsleichter 2011; Pratt and Carrier 2005). The male southern stingray(s) typically follow and bite the wings or tail of the female often leaving abrasions and scars. Other behavior observed specific to southern stingrays include multiple males following and grasping as well as copulation starting over the bottom and may continue into the substrate (Pratt and Carrier 2005). Females may remain upright with the male(s) either above or below her during copulation. The male stingray(s) inserts a clasper into the oviduct to deliver sperm (Pratt and Carrier 2005). Multiple males may copulate in rapid succession with a single female but paternal DNA differences in offspring is under investigation (Pratt and Carrier 2005).

1.5.4 Reproductive cycles

Extrinsic factors such as temperature, light, and population density can affect reproductive physiology in elasmobranchs. External stimuli on sensory organs relay messages to the hypothalamic-pituitary-gonadal axis, which controls oogenesis and steroidogenesis (Lutton et al. 2005). Gonadotropin-producing cells exist in the ventral lobe of the pituitary gland in elasmobranchs but differ from other animals in that there is not a direct vascular or neuronal connection to the hypothalamus, but rather a portal system (Henningsen 1999; Lutton et al. 2005). The ventral lobe is likely the main source for the gonadotropins, GTP I (similar to follicle stimulating hormone in mammals and is increased during vitellogenesis) and GTP II (similar to

luteinizing hormone in mammals and increased prior to oocyte maturation), and therefore responsible for follicular development (Lutton et al. 2005; Henningsen 1999). The ovulatory cycle process from oogonia to mature follicle appears consistent in vertebrates but the signals in which the process operates differs between animal classes. For example, final oocyte maturation (FOM) from the secondary oocyte is stimulated by a hormone called maturation inducing hormone (MIH) or maturation inducing steroid (MIS) in amphibians, reptiles, and teleosts but has yet to be identified in elasmobranchs (Henningsen 1999). Epigonal-derived substances have also been speculated as affecting the gonadal processes (Lutton et al. 2005; Lutton and Callard 2008).

Reproductive cycles have been described as three types, presumably in wild populations, based on ovarian cycle and gestation: continuous, seasonal, and punctuated (Koob and Callard 1999; Maruska and Gelsleichter 2011). Continuous cycles occur in elasmobranchs that are continuously reproductively active, that is mating or breeding, pregnant, and pupping throughout a year. The activity is synchronized with environmental factors and they are pregnant for the majority of the year. Seasonal cycles occur in elasmobranchs that are pregnant for part of the year (approximately six months) and non-pregnant the rest of the year. Although environmental factors also play a role, they are seasonal and therefore populations synchronize at the same time within a year. Punctuated cycles occur in elasmobranchs that are pregnant for approximately one year and non-pregnant for approximately one year. In some species, they are non-pregnant for up to two years; depending on the time it requires oocytes to reach ovulatory size (Koop and Callard 1999). Southern stingrays follow the seasonal cycle category; however, in captivity it has been documented for them to experience almost two pregnancies yearly (Henningsen 2000). In other ray species as well as the southern stingray, it has been noted that mating and parturition

occur at different times of the year (Henningsen et al. 2004). Although gestation has been defined as the time from copulation to parturition, a more accurate definition would be fertilization to parturition. Regardless of how gestation is defined, it is worth noting that several species have the ability to delay embryonic development, known as embryonic diapause, store sperm, and partake in parthenogenesis (Henningsen et al. 2004; Waltrick et al. 2012; Wyffels 2009; Hamlett et al. 2005). Embryonic diapause occurs during the blastodisc stage of development and can last between four and ten months (Waltrick et al. 2012). Species documented as undergoing diapause include bluntnose stingray (*Dasyatis sayi*), whiptail stingray (*Dasyatis brevis*), pelagic stingray (*Pteroplatytrygon violacea*), masked stingaree (*Trygonoptera personalis*), longheaded eagle ray *(Aetobatus flagellum*), Brazilian guitarfish *(Rhinobatos horkelii*), shovelnose guitarfish *(Rhinobatos productus),* ringstreaked guitarfish *(Rhinobatos hynnicephalus),* blackchin guitarfish *(Rhinobatos cemiculus),* common guitarfish *(Rhinobatos rhinobatos),* fiddler ray *(Trygonorrhina fasciata),* Australian sharpnose shark *(Rhizoprionodon taylori)* (Wyffels 2009; Waltrick et al. 2012). Sperm storage in the terminal zone of the oviducal gland has been documented in several elasmobranch species and may last from days to years (Hamlett et al. 2002).

Ovulatory cycles do not necessarily correspond to the reproductive cycle or mode of female elasmobranchs. In the wild, elasmobranchs that cycle continuously will also continue to develop follicles throughout their pregnancy or later in gestation. Seasonal and punctuated breeders develop follicles outside of gestation or when they are not pregnant (Koob and Callard 1999). In captivity this likely changes due to the constant environmental conditions which likely alter the reproductive endocrinology. Hormone levels with respect to ovulatory cycles have been studied but not specifically in the southern stingray. Stingrays with similar reproductive modes

(uterine viviparity) have been studied very little but hormones measured in the marbled electric ray (*Torpedo marmorata*) and Atlantic stingray (*Dasyatis sabina*) include progesterone (P_4), 17β-oestradiol (E_2), testosterone (T), and 5α-dihydrotestosterone (DHT) (Henningsen 1999; Lutton et al. 2005). In *D. sabina*, E_2 and T were elevated during the pre-ovulatory phase, P_4 peaks twice – slightly one month prior to ovulation then more profoundly at ovulation, and a second spike in E_2 occurs one month prior to parturition and with developed enlarged oocytes in the ovary (Henningsen 1999; Lutton et al. 2005). There are conflicting studies as to the outcome of the enlarged oocytes, as to whether they become solely atretic or possibly ovulate (Henningsen 1999). It is also worthy to note that this cycle in *D. sabina* is developed on wild caught stingrays over the course of one calendar year (seasonal cycling) with respect to time of year (Lutton et al. 2005; Koob and Callard 1999).

1.5.5 Parity

The reproductive modes in elasmobranchs have been classified as oviparous (egg-laying) or viviparous (live-bearing) (Maruska and Gelsleichter 2011). Viviparous elasmobranchs are further divided into reproductive modes of aplacental viviparity and placental viviparity or the means by which they receive nutrients of lecithotrophy (yolk sac viviparity) and matrotrophy (maternal supplementation) (Hamlett et al. 2005). Two modes of aplacental viviparous species receive nutrients by way of matrotrophy, specifically trophonemata or histotrophy and oophagy and uterine cannibalism or adelphotrophy. Placental viviparous species also receive nutrients by matrotrophy (Hamlett et al. 2005). Species with aplacental viviparity, which receive nutrients by yolk sac, are referred to as lecithotrophic viviparity (Figure 1.2).

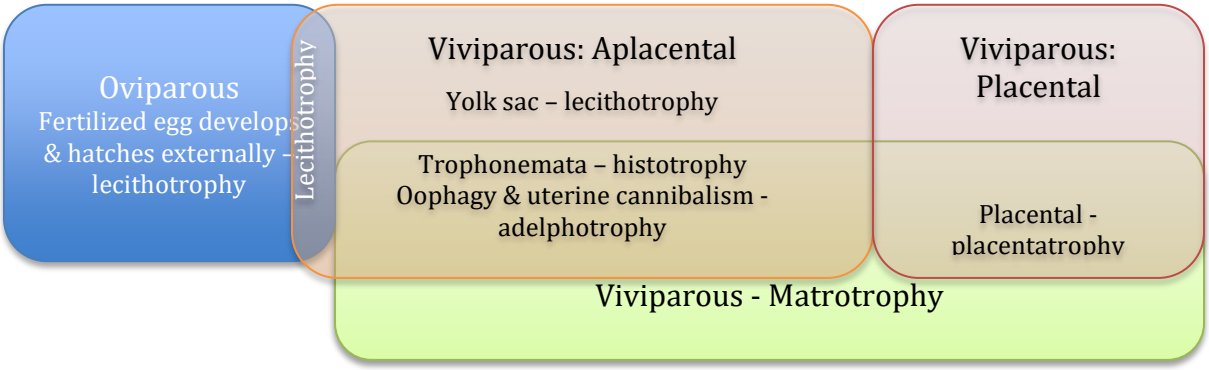

Figure 1.2. Overview of reproductive modes and nutrient delivery. Viviparous species are live-bearers compared to oviparous species which lay eggs. Lecithotrophic species produce embryos that eat yolk from a stored source (egg or yolk sac) compared to matrotrophic species where the female directly provides nutrients to the embryos. Viviparous specieds are often further classified as aplacental and placental (Maruska and Gelsleichter 2011; Hamlett et al. 2005; Henningsen 1999).

The southern stingray falls under the category of aplacental viviparity with trophonemata and histotroph, specifically the uterus functions as an internal incubator with trophonemata that supply nutrients to the embryos during gestation (Hamlett et al. 1996; Hamlett et al. 2005; Lutton et al. 2005). Initially, fertilized eggs contain an embryo with egg jelly before "hatching" from the egg capsule in utero (Wyffels 2009). The trophonemata are finger-like extensions of the uterine mucosa that increase in length during gestation. The projections deliver histotroph or uterine milk (sometimes referred to as uterolactation) to the developing embryos throughout gestation (Hamlett et al. 1996; Hamlett et al. 2005). The amounts of histotroph as well as the contents also change throughout gestation (Wyffels 2009). Histotroph contains varying amounts of lipids, proteins, and carbohydrates, which are delivered through the gill filaments of the embryos and spiracles, gills, and mouth of the developed fetuses (Wyffels 2009; Hamlett and Koob 1999).

1.5.6 Reproduction in captivity

Breeding any elasmobranch species in captivity requires planning (Henningsen et al. 2004). Southern stingrays appear to do well in a captive setting for a long period of time and breed without encouragement. Details involved with the breeding environment, health records, and any information pertaining to the reproductive event (copulation, gestation, litter size, pup parameters, etc.) should be noted. Evidence of similarities between captive breeding and wild reproduction exists and therefore any additional information may contribute to wild stingrays' behavior and reproduction (Henningsen et al. 2004).

Southern stingray reproduction in captivity likely varies with respect to environmental cues such as temperature and photoperiod, but appears to occur every 4.4-7.5 months as opposed to annually in the wild (Henningsen 2000; Henningsen et al. 2004; Ramirez-Mosqueda et al. 2012). Notes on reproductive biology in the southern stingray by Henningsen (2000) describes the statistics on neonate size and weights as well as litter size with respect to maternal size and to sizes and weights of the neonates. It concluded that the litter size is directly proportional to maternal size and indirectly proportional to size and weights of the neonates. Southern stingrays typically produce two to 10 offspring per litter with an average of four to five pups in captivity compared to two to seven pups in the wild (Henningsen 2000; Ramirez-Mosqueda et al. 2012). Reproductive abnormalities in elasmobranchs have been documented such as stillborn fetuses in sand tiger sharks (*Carcharias taurus*), southern stingray (*Dasyatis americana*), and leopard shark (*Triakis semifasciata*); dystocia or egg-binding in spotted wobbegongs (*Orectolobus maculatus*); infertile ova from sand tiger sharks; release egg capsules without yolk or embryos from nurse sharks (*Ginglymostoma cirratum*); and dystocia or failure of parturition resulting in over-

gestation in cownose stingrays (*Rhinoptera bonasus*) (Henningsen et al. 2004; Henningsen 1999).

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CHAPTER 2: Research Overview and Specific Aims

2.1 Research Overview

2.1.1 Statement of the Problem

During the first few years of opening a new touch pool exhibit, approximately 36 southern stingrays died at the Downtown Aquarium in Denver (formerly Colorado's Ocean Journey). Seventeen of these stingrays presented with similar necropsy findings: a small, dark (described as dark, dark brown, black, or blue) liver indicative of lipid and glycogen storage depletion and many of the females were described as having oophoritis (Figure 2.1). Large, lipid-filled livers are a normal structure of many marine elasmobranchs. Fats and protein are the main energy sources for these animals and the large, fatty livers also aid in buoyancy for some elasmobranchs (Hamlett 1999). This particular problem has not been specifically addressed in previous research. It is important to find the cause of death in these animals but more so to detect the risk factors involved with the disease in order to prevent mortality.

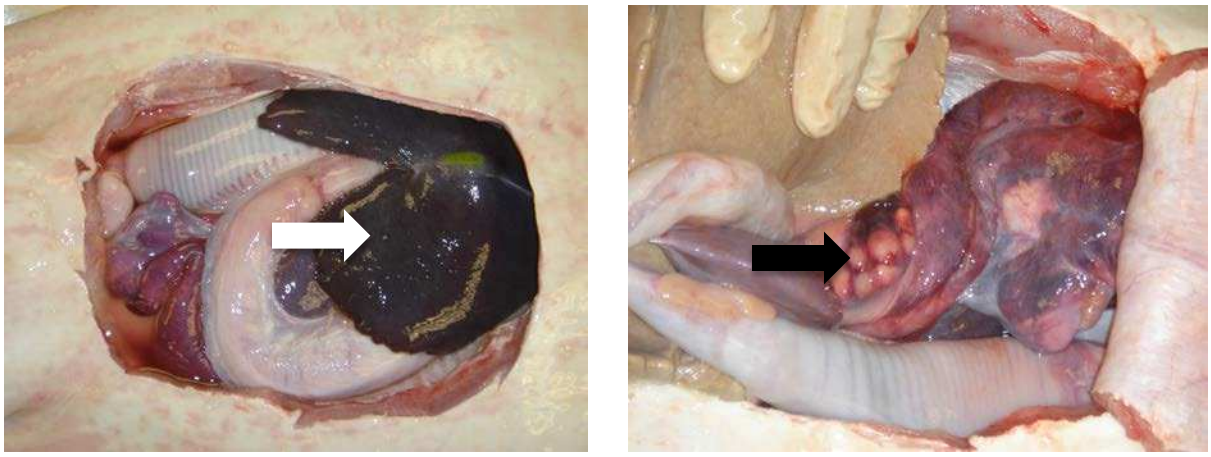

Figure 2.1. Gross necropsy findings on female southern stingrays. Left: The white arrow is pointing to a small, dark liver. Right: The black arrow is pointing to follicles on a hemorrhagic ovary.

The majority of elasmobranch research focuses on sharks. Although there are over 100 species of rays and skates represented in public aquaria (AES 2008 census), there is little research done on any one species. Most of the books regarding elasmobranchs discuss characteristics in sharks and then extrapolate or compare and contrast with rays and skates.

Research, specifically on southern stingrays, was beneficial for this particular aquarium in preventing mortality and also providing optimal care for these captive animals. This species is included in a taxon that is considered data deficient according to the World Conservation Union for wild populations. Data deficient is defined as not enough information to determine its risk of extinction (Grubbs et al. 2006). Audiences that may find this research useful include aquarists working at other aquaria, marine biologists and aquatic veterinarians.

2.1.2 Significance of the Problem

The southern stingrays at the aquarium were wild caught since the stingrays had not successfully naturally reproduced on their own in house. When stingrays from the captive population were lost, the exhibit collection was replenished with stingrays from the wild. So, the significance of this problem was two-fold: one part being that these stingrays, for some reason, were dying in captivity at this aquarium; the other part being that the collection is restocked using wild-caught stingrays, which could contribute to depleting wild populations.

The cause of these mortalities may be husbandry related; therefore, it was crucial to identify the risk factors involved. It would be unfair to these animals to continue placing them in a harmful or stressful situation. So, by investigating the environment, diet, mating behaviors, and by performing physical examinations, there was an opportunity to improve conditions or health status in order to avoid further mortalities. In maintaining the well being of these animals,

the aquarium was also more financially responsible. Although cost was another significant part of the problem, it played a much lower role. There was a large cost involved with acquiring wild-caught stingrays. The cost included, but was not limited to, a third party catching requested animals (based on species, approximate age and sex), shipping them from Florida to Denver using a mobile life support system, and the actual charge for the animal. It was not uncommon for animals to die during transport, as this process can be very stressful. The initial health of the animals during catch was unknown as they were likely not examined at the time they were caught. Other considerations when acquiring new fish were comingling populations. Without knowing the health status of the recently caught population, new arrivals were placed in quarantine for several weeks prior to placing them in the exhibit. They also typically received preventative treatment for parasites while in quarantine. Even then it was still not guaranteed that they would not transmit parasites or other diseases to the established captive population.

2.1.3 Research Questions

At the start of this project there were several questions, many of which were answered within the individual studies and many that were answered during the preliminary research or pilot studies. The following questions were initially formulated, organized (Figure 2.1), evolved into the objectives, and refined into the specific aims.

- What was the cause of death of the southern stingrays (*Dasyatis americana)* that had similar findings on gross necropsy - small, dark liver and hemorrhagic ovaries?
- Was the cause of death a result of different etiologies secondary to being immunocompromised from chronic stressful situations?
- Were the small livers a result of stress and/or starvation?

- Is the liver size of the stingrays measurable?
- Were the stingrays showing clinical signs before death?
- How are the hematology and biochemistry values interpreted for this species?
- What are normal histological findings compared to pathology for the southern stingray?
- Was there a reproductive component? Or was this secondary to the suspected stressful condition? Or was this the underlying cause of the stress?
- Were these problems associated with recent acquisitions? Were other aquariums seeing this problem? Do they wild catch or captive breed?

2.1.4 Objectives of the Project

The specific aims for this project were conducted as formal studies. As part of the studies, objectives were established and are listed here. Some objectives needed to be established in order to conduct the chapter studies (for example, identifying the gross anatomy and confirming it with histological evaluation had to be completed before organs and tissues could be identified using the ultrasound). The results of this work are represented in a website (southernstingray.businesscatalyst.com) and serve as appendix 4 for this project.

- To identify normal anatomy of the southern stingray, *Dasyatis americana* (Appendix 4-website)
- To identify normal and pathological ultrasonographic imaging of the southern stingray housed at the aquarium (Appendix 4-website)
- To identify normal and pathologic histologic findings of the southern stingray (Appendix 4-website, Chapter 3)

- Identify small livers in southern stingrays – is this a risk factor for mortality? (Chapter 3 & 4)
- Identify risk factors for small livers involved with environmental, biological, or reproductive conditions (Chapter 3).
- To understand hematological and plasma biochemical values of captive southern stingrays (Chapter 5)
- To predict a parturition date range for captive southern stingrays (Chapter 6)
- Identify a relationship between disc size and follicle size and correlate it to reproductive maturity in the southern stingray (TBD)

2.2 Specific Aim 1 (Chapter 3: Morbidity Investigation)

There have been several southern stingray deaths at the Downtown Aquarium in Denver (formerly Colorado's Ocean Journey). Gross findings on necropsy varied but a subjective increase in small livers and hemorrhagic ovaries (presumptive oophoritis) in several female stingrays prompted a closer investigation. Preliminary evaluation of the necropsy reports suggested that the odds of having a small liver or residing at the aquarium for less than three months are higher among stingrays with an unknown cause of death. An observational study was conducted to assess risk factors for small livers. **The specific aim of this chapter was to assess the influence of environmental, biological, and reproductive factors that may contribute to small livers.** *The hypothesis for specific aim 1 is that pregnancy and newly acquired stingrays would be at risk for smaller livers*. A generalized linear mixed model was used to evaluate the independent variables possibly contributing to changes in the dependent variable.

2.3 Specific Aim 2 (Chapter 4: Anatomy & Imaging for Liver Size)

To gain a better understanding of the dynamics of liver size and clinical health status, the stingrays were evaluated using ultrasound imaging. Anatomy of elasmobranchs is well documented and the current publications were reviewed so gross anatomy could be identified specifically for southern stingrays. Results of specific gross and microanatomy are illustrated in Appendix 4 (website). Once the specific anatomy was identified both grossly and sonographically, determining liver size using an ultrasound could be validated. **The specific aim of this chapter was to validate the ultrasound as a tool for determining liver length with respect to the coelomic cavity length and to assess the difference between liver lengths among two groups of stingrays – those acclimated to captivity to those recently acquired.** *The hypothesis for specific aim 2 is that there would be a difference between the two groups (rejecting the null hypothesis). The liver lengths of the recently acquired stingrays will be smaller compared to the stingrays that have been acclimated for several years.* It is expected that the recent additions will be in a negative metabolic state and therefore have smaller livers from lipid depletion.

2.4 Specific Aim 3 (Chapter 5: Hematology & Biochemistry)

There are not many hematological and biochemical reference ranges for elasmobranchs and even fewer studies evaluating interpretation of these types of diagnostics. Currently, for southern stingrays, there is one biochemical reference for wild southern stingrays (Cain et al. 2004) and no studies involving interpretation of diagnostics. This chapter will introduce hematological and plasma biochemical values for captive (formally wild) southern stingrays at different time periods during captivity from acclimated to captivity for several years to recently

introduced into captivity. **The specific aim of this chapter is to compare hematological and plasma biochemical values between established and newly captive stingray groups**. *The hypothesis for specific aim 3 is that there would be a difference in parameters that are affected by water quality and diet such as packed cell volume, protein, electrolytes, and cholesterol.*

2.5 Specific Aim 4 (Chapter 6: Captive Reproduction)

In addition to gaining a better understanding of the overall clinical health of southern stingrays in captivity, the pursuit of conservation is also important. Part of the conservation effort role in zoos and aquariums is captive breeding. Once a health monitoring protocol was in place at the aquarium, captive breeding began to occur within the exhibit naturally. This activity provided an opportunity to study reproduction in captive southern stingrays. As more pregnant stingrays were identified, it was apparent that space was a potential issue – the aquarium would not be able to separate pregnant females from the other exhibited animals for the entire gestation. **The specific aim of this chapter is to predict a parturition date range for captive southern stingrays.** *The hypothesis for specific aim 4 is that a parturition date range would be predicted within two weeks.* This will allow aquarium staff to separate the pregnant females for one month compared to the possible 4.5-7.5 months (reported gestation lengths in captive southern stingrays).

2.6 Theoretical Framework (General Methodology)

2.6.1 Research Design

This research project was designed to answer many of the questions posed: What was the cause of death of the southern stingrays at the downtown aquarium that had similar findings (small, dark livers and hemorrhagic ovaries) on gross necropsy? Were the small livers a result of stress and/or starvation? Was the cause of death a result of different etiologies secondary to being immunocompromised from chronic stressful situations? Is the liver size of these stingrays measurable? Were the stingrays showing any clinical signs before death? How are hematology and biochemical panels interpreted for this species? What are normal histological findings compared to pathology for the southern stingray? Was there a reproductive component? Or was this secondary to the suspected stressful condition? Or was this the underlying cause of the stress? Are they induced ovulators? Is the female to male ratio a factor? Was the problem related to recent acquisitions? Were other aquariums having this problem? Do they wild catch or captive breed?

Ultimately, the purpose was to gain a better understanding of the pathophysiology of the disease processes that were affecting the southern stingrays, intervene and reverse the condition, and to prevent further mortalities at this particular facility. The small, dark liver was common to all fatalities. There were three broad categories to investigate given this small amount of information: predict animals with disease, determine cause and possible treatment, and evaluate the husbandry and associated risk factors to prevent it. Many of the dead stingrays had hemorrhagic ovaries or oophoritis. This condition was also taken into consideration during these investigations (see overview of research design diagram below). In order to better understand the pathophysiology, many fundamental components must be

identified, such as anatomy, hematological and biochemical parameters, imaging, and histology. This was achieved through reviewing necropsy videos and photos, continued necropsies, ultrasonographically imaging dead specimens in conjunction with necropsies and obtaining and evaluating blood samples.

There were several studies conducted during the research period. Preliminary work involved retrospective research using necropsy records, examining living animals at every opportunity, and performing thorough necropsy examinations. Because the concentration was a particular problem at this particular facility, the group was defined. Animals of questionable health will be moved to quarantine and evaluated at a higher frequency with the option for treatments. Treatment depends on the specific clinical signs and results of husbandry investigation.

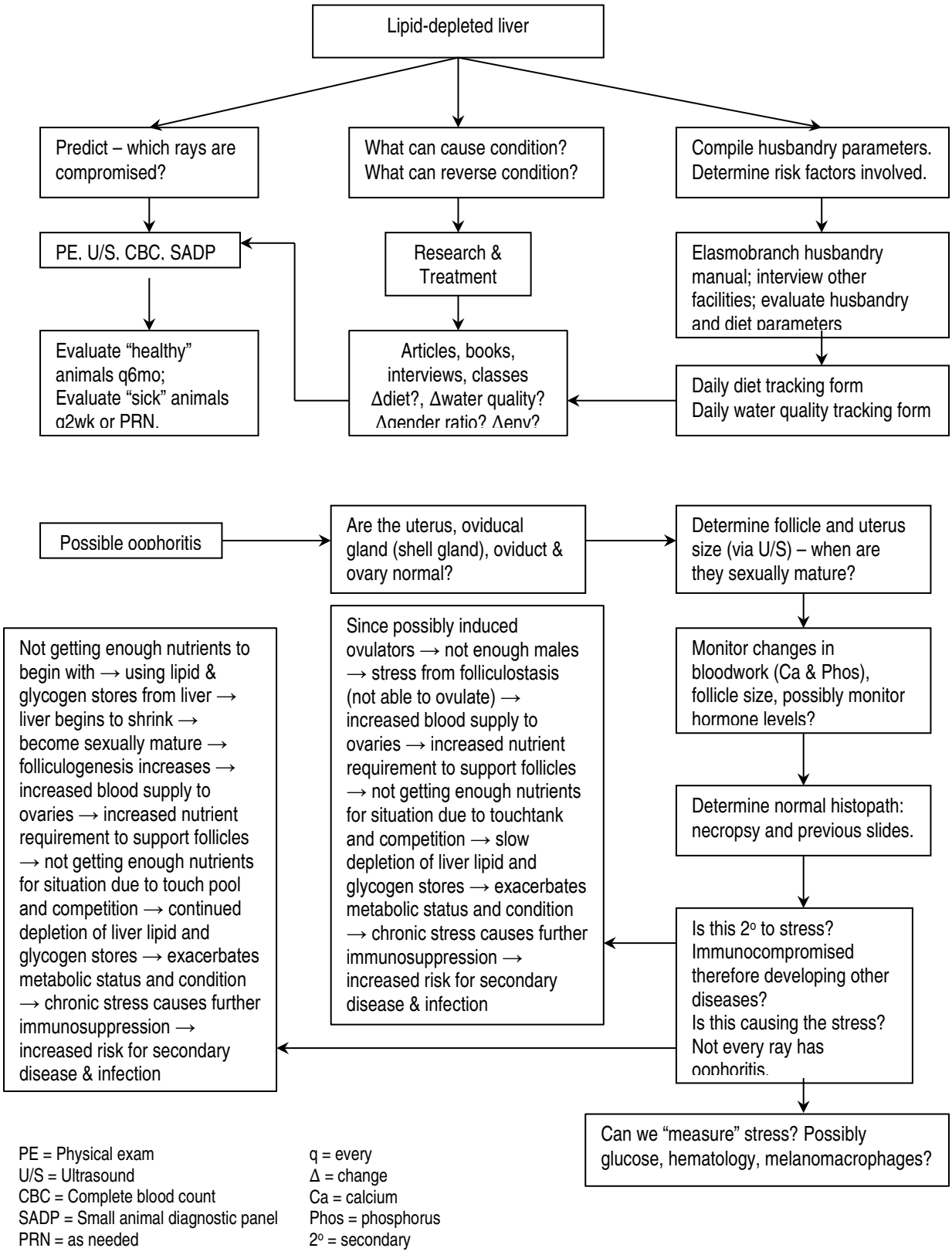

Figure 2.2. Overview of Research Design and Questions

2.6.2 Subject Selection

The subjects selected were the southern stingray, *Dasyatis americana*. The population studied was specifically the collection at the Downtown Aquarium at Denver (DAD). At the start of the project there were 43 southern stingrays, 35 females and eight males. Of the 43 stingrays, 22 were wild-caught (20 females and two males) and 21 were captive-bred (15 females and six males). There were 18 wild-caught females in their displayed exhibit, the ray touch pool. The southern stingrays in the touch pool share the tank with five cownose stingrays, *Rhinoptera bonasus* (three females and two males). During the preliminary and formal research, two groups of wild caught rays and two groups of captive rays were added at various times.

The primary focus was on the females. The majority of the adult southern stingrays were on exhibit in the touch pool. This is an approximate 12,000-gallon tank with approximate dimensions of 10 meters long by five meters wide by varying depths from 20 centimeters to one meter. The substrate is sparse sand. The southern stingrays are benthic marine animals.

This population was selected due to the mortality rate in this collection at this aquarium over several years. The focus is on the female population since they represent the majority of the collection and part of the problem appears to be reproductive.

During interviews of other facilities, information was obtained regarding other populations of the same species at their respective locations. Other facilities also had a combination of captive-bred and wild-caught southern stingrays as well as females and males but no other facility was experiencing this particular issue.

The Colorado State University animal care and use research protocol review form (A-100) was submitted and approved: IACUC Protocol #08-282A-01.

2.6.3 General examination

Sampled selections of female southern stingrays in the aquarium touch pool were examined routinely at least once every six months. During periods where specific studies were conducted, all stingrays in the collection were examined and data collection was typically completed within a three-day time frame. The physical examination, ultrasound evaluation, blood collection and unique identification of the stingrays were performed at the touch pool. Due to the hours of operation of the aquarium, the procedures were done prior to business opening which dictated completion time. The procedures required at least four people to maximize efficiency – one aquarist in the tank, one person to record findings, one phlebotomist and one sonographer (although the sonographer collected blood in short-handed situations). The sonographer, with the help of the aquarist, performed the physical examination.

One southern stingray was captured at a time using a net and brought to the side of the pool. Their body condition score (BCS) was subjectively assessed on a scale of one to five using the muscular flesh slope to the dorsal spine. The integument was evaluated and any lacerations, abrasions, contusions, ulcerations, notches, regions of discoloration, or abnormalities in tail length was recorded on the physical exam form (Appendix 5).

The stingrays were restrained using tonic immobilization (Stamper et al. 2007). The animals were placed in dorsal recumbency to perform the physical exam, the ultrasonographic evaluation and to collect blood. If tonic immobilization was unsatisfactorily achieved, then an anesthetic agent, tricaine methane sulfonate (MS-222), was used for sedation. In those cases, a separate container holding 40 gallons of marine water was used to deliver the sedative (11 grams of MS-222 with equal parts sodium bicarbonate to establish a 75 parts per million concentration). This was in preparation for sedation but was never necessary.

Venipuncture was always attempted first after placing them in dorsal recumbency to avoid stress further affecting results on the hematological and biochemical profiles. Using a three-milliliter (3-cc) syringe with a 23-gauge one-inch needle, the proximal tail vein was used as the venipuncture site. Approximately five to ten centimeters from the base of the tail, on midline of the ventral tail, was the venipuncture site. One and a half milliliters of blood was obtained and placed in lithium heparin microtainers. A blood film (without anticoagulant) was also made at the time of sample collection. The microtainers and blood smear were labeled with the appropriate PIT tag number and stored in a cooler with an ice pack for transport back to the Veterinary Teaching Hospital Clinical Pathology lab. Here, the plasma was analyzed (Roche Hitachi 917 Chemistry analyzer) for biochemical analyte values and manual cell counts were obtained (Natt-Herrick's solution and stain and hemacytometer).

Using a saltwater resistant measuring device, such as a tape measure, the following measurements were taken (in centimeters): distance from snout to vent, wingspan (or disc width), and length of coelomic cavity (distance from pectoral cartilaginous girdle to pelvic cartilaginous girdle). The results were recorded on the examination form (Appendix 5). The respiratory rate was obtained by counting gill slit movements and recorded. The heart rate was obtained using the ultrasound machine (Aloka SSD-900 with a 7.5MHz linear probe). The heart was imaged in order to count contractions.

The liver measurement also involved use of the ultrasound equipment. At first, it was thought that the ultrasound probe would need protection from the potential damaging effects of the saltwater therefore a large, plastic palpation glove was used to encase the probe. Later it was revealed that this was not necessary and for the majority of exams it was not used. Ultrasound gel was not needed on the animal as the water acted to eliminate any air artifact. The probe was

positioned at the caudal aspect of the ventrum, just cranial to the vent, in a sagittal position. The reference point of the probe was in a cranial direction with the opposite end over the pelvic girdle. The pelvic girdle should cast an acoustic shadow when viewed on the imaging monitor. Due to the large liver size in this species of stingray, the liver should also be viewed in the same image. The image can be frozen on the screen in order to measure the distance from the caudal tip of the liver to the cranial tip of the pelvic girdle. This distance was then compared to the length of the coelomic cavity and a relationship is established as a percentage. In cases of debilitated animals, it was suspected that the liver would not extend to the pelvic girdle therefore would not be captured within the same image. The distance from the caudal tip of the liver to the pelvic girdle was measured using the ultrasound and another device such as a ruler. The probe was positioned over the liver so that the caudal tip of the liver is level with the caudal tip of the probe. The distance from the caudal tip of the probe to the palpated pelvic girdle was measured. The reliability of this process was tested during necropsies. This procedure is described in more detail in chapter 4.

Ultrasonographic evaluation included identification of the following organs: heart, esophagus, liver, gall bladder, stomach, spiral intestine, pancreas, spleen, epigonal organ, ovary, oviducal gland, and uterus. Reliability of correct organ identification was confirmed during necropsies. The following measurements were obtained during examinations: stomach wall thickness, uterine wall thickness, whole uterus thickness, and follicle diameter. An image of each organ was saved in sagittal and transverse positions. As the liver decreases in size, the echogenicity will also change (Mathiesen et al. 2002); therefore liver echogenicity will subjectively be compared to the spleen as it is in mammals. On previous necropsies it had been observed that these animals can also have a substantial amount of free fluid in their coelomic

cavity. The ultrasound exam was used to detect any free fluid in the coelomic cavity and also provide guidance for coelomocentesis for fluid analysis. Imaging was also useful in the event of a pregnancy. All images were saved and measurements and findings were recorded on the evaluation form for each stingray.

The stingrays were uniquely identified with a passive integrated transponder (PIT) tag using microchips detectable by the AVID identification system. These tags provided identification without detriment to the animal (Marshall et al. 2004). PIT tags were placed in the musculature of the left wing in females and in the right wing of males. Placement was confirmed with the reader. The PIT tag number was adhered to the evaluation form.

Any animal that was found to have a small liver, a liver hypoechoic compared to the spleen, large follicles and free coelomic fluid were moved to quarantine for further evaluation. Southern stingrays that appeared emaciated (low BCS) or that had irregularities in their heart rate or respiratory rate were also considered debilitated and moved into quarantine for further evaluation, treatment or both. Evaluation of these animals took place during routine veterinary visits to the aquarium (twice monthly). Constant communication with the curator of fishes and aquarium staff was pertinent for monitoring health status of these animals. Water quality parameters and diet were closely monitored and tracked (records kept by aquarists). Additional unscheduled visits were necessary for further evaluation or treatment.

The same procedures, excluding blood collection, were applied to deceased animals during the necropsy. External, ultrasonographic and internal measurements were taken. Digital photographs, video and imaging, as well as the necropsy and pathology reports (from CSU Diagnostic Laboratory) were used for documenting the findings. A history was gathered from the aquarists and curators and recorded on the form. Any external lesions were identified and

recorded. An incision was made through the skin along the cartilaginous edges thereby making a circular opening into the coelomic cavity. The liver and gall bladder were evaluated then removed to inspect the other organs, which lie dorsal to the liver. Any coelomic effusion was collected for fluid analysis or culture. The following organs were collected for histologic evaluation in separate neutral buffered formalin (10%) containers to assure proper organ identification: liver, gall bladder, esophagus (and organ of leydig), stomach, small intestine, spiral intestine, rectocolon, rectal gland, kidney, interrenal gland, spleen, pancreas, epigonal organ, ovary (and follicles), oviduct, oviducal gland, uterus, thyroid, brain, skin, ampullae of Lorenzini, muscle, cartilage, eye, gills and heart. The samples were submitted to the Colorado State University diagnostic laboratory.

Conditions of Testing

During routine examinations, the testing and data collection were done at the aquarium touch pool between six o'clock and ten o'clock in the morning during the week. The recheck examinations were done during routine visits to the aquarium or during a special visit if required.

Animals in quarantine were transported to a separate holding container for examination. They were captured in a net, similar to the touch pool protocol, and then transferred to the holding container. The examination continued as described for the touch pool.

Treatments

As data were collected and studied, the treatment protocols were established. Examples of treatments included increasing food intake to provide more energy for metabolic demand during stressful situations, folliculogenesis, or pregnancy. Stressful situations included changes in husbandry conditions, construction, trauma, or illness. With much of this research being an observational study, in order to ultimately establish (and prevent) cause of death, after treatment was administered, retesting was done to see how treatment affected the population. Other examples of treatment included changes in the diet, changes in the water quality parameters, or medication as deemed appropriate. Medical treatments were required in the event of a suspected disease process. This may be caused by immunosuppression, which places them at risk of other diseases or conditions. For example, some parasites are normal commensals of these fish but when immunocompromised, the parasites can overgrow and debilitate the health of the fish. In this type of situation, treatment of the parasite may be necessary.

Data Analysis

Ultrasonographic, gross and histologic images of the organs were collected during the course of this study. The collection will serve as a reference for further studies and veterinary examinations.

Southern stingrays at the aquarium were considered healthy if they had a BCS of equal or greater to two and a half, had a liver that comprised at least 70 percent of the coelomic cavity, had little to no free fluid in the coelomic cavity, had no deep external lesions and were reportedly eating and behaving normally in the exhibit. The plasma biochemical and hematological results from these animals allowed for some interpretation of these values. Values for the following

analytes were obtained: glucose, blood urea nitrogen (BUN), creatinine, phosphorus, calcium, total protein, albumin, globulin, cholesterol, t-bilirubin, creatine kinase, aspartate aminotransferase, sodium, potassium, chlorine, and bicarbonate. Values were also obtained for the following hematological parameters: total white blood cells, G_1 (type I or heterophil) granulocytes, G_2 (type II or neutrophil) granulocytes, G_3 (type III or eosinophil) granulocytes, basophils, monocytes, lymphocytes, refractometer protein, and packed cell volume (Campbell and Ellis 2007). The analysis and descriptive statistics are described in detail in chapter 5.

2.6.4 Limitations of the Study

There were several limitations of this study that were taken into consideration. First, the majority of the examinations were performed at the aquarium. This was not a laboratory environment and therefore it was important to respect the operation of the facility and work with the staff in order to accomplish the task at hand. It was necessary to schedule examinations and coordinate the logistics with the staff. Since there were multiple individuals involved, it was at times difficult to coordinate schedules.

Results of these examinations required some of the animals to be moved into quarantine. The space in quarantine limited the number of animals that could be moved. This required these animals to remain in the exhibit until a quarantine tank was available. Moving these animals at a later date depended on the aquarists' schedules and took time to locate that particular animal in the touch pool. Another thing considered was quarantine environments, although set up to be similar to the exhibits, they were a different environment which could have an affect on some of the measured parameters.

Some of the animals in quarantine required treatments. The quarantine systems as well as the touch pool system required daily monitoring (record keeping was done at the aquarium by aquarium staff). Treatments and monitoring were also done by the aquarists and depended on their schedule. Other duties and tasks of the aquarium staff determined the compliance in attending to the treatments and form completion.

Some other minor limitations of this study included the physical distance from Colorado State University to the aquarium; ease (or lack there of) of weighing the animals, and that the group being studied was from one location under similar husbandry conditions. The distance to the aquarium affected the time in getting to unhealthy animals and in obtaining dead animals as early as possible for necropsy. Weighing the animals was an issue since using a hanging scale was not feasible at the touch pool. A flat scale could have been used but water presented problems with the electronics and often the animals overhung the scale thereby skewing the weights. The limitation of the population being in one location was that there was not a separate group for a control or another group, such as a wild population, for comparison.

With any research project, funding was an issue. The diagnostics were limited to the funds the aquarium was willing to spend. Other testing (like hormone assays) was not done due to lack of funding. There was no other outside funding obtained.

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CHAPTER 3: Investigation of the Morbidity and Mortalities of the Southern stingray, *Dasyatis americana*, at an Aquarium

3.1 Synopsis

Zoos and aquariums house thousands of stingrays throughout the world. Stingray exhibits are particularly popular due to their impressive wingspan seen in larger exhibits and the interactive component involved with touch pools. Stingrays are also included in ecotourism sites where guests may interact in a relatively natural environment. Regardless of the captive or semi-wild environment, stingray health is of concern. The aquarium in this study stocked their touch pool with wild-caught southern stingrays and experienced several mortalities. Necropsy records were reviewed in order to assess for any commonalities. A case-controlled study was conducted and resulted in a presumptive conclusion that liver size and time at the aquarium were more consistently seen when the cause of death was unknown with liver size being a confounder. The liver size with respect to the coelomic cavity was used as the outcome for this observational study to assess health status. A generalized linear mixed model (X^2_{LR}=72.031, df = 1, p<<0.001) was used to analyze the risk factors for decreased liver size (liver length ≤ 70% of the coelomic cavity). The predictors (with odds ratio [confidence intervals]) in the final model included pregnancy (yes) (30.978 [6.803, 141.066]), time in captivity (≤ 3 months) (2.534 [0.945,6.790]), and wingspan (> 60 cm) (0.530 [0.206, 1.365]). Although time in captivity and wingspan were not statistically significant, they improved the fit of the model. Pregnancy had the greatest effect at predicting small livers.

3.2 Introduction

Nearly 10,000 captive elasmobranchs are exhibited in hundreds of aquariums all over the world with almost half of them being rays or skates (AES Census 2008). The southern stingray, *Dasyatis americana*, is the second most displayed stingray at these facilities. They are often used to stock touch pools when they are small and then transferred to larger exhibits as they mature. They can grow to an impressive two meters in width (wingspan) with females being larger than males and have been reported to live up to twenty years or more in captivity (personal communication, Henningsen 2007). Southern stingrays are also used in wildlife tourism to provide human-wildlife interactions (Semeniuk et al. 2010). With increasing popularity to exhibit and offer interaction with these animals, monitoring their health is essential but can be difficult due to the limited information available.

During the first couple of years after opening a new touch pool exhibit, an aquarium experienced several mortalities in their southern stingray collection. Based on a preliminary analysis of stingray necropsy records at this aquarium, a small liver was associated with many of the cases with an unknown cause of death (Appendix 2). The reason for the decreased liver size was unknown, therefore this variable was the basis of investigation (outcome) for this study. Given this information, it was decided to examine and monitor stingrays in an effort to assess potential risk factors associated with small livers to improve conditions and avoid additional mortalities.

Elasmobranchs do not have adipose tissue and therefore fat is stored in the liver attributing to its change in size and color (Ballantyne 1997; Holmgren and Nilsson 1999; Rossouw 1987). The animals' condition could be based on the amount of lipid stored in the liver but there have been no studies (to the author's knowledge) that identify ideal conditions based on

liver content. It is common to associate emaciation with the depletion of hepatocyte lipid stores though (Garner 2013). Since these animals were exhibited in an interactive petting and feeding pool, the season (low/high) for feed sales was taken into consideration along with reproductive status (pregnant or not), and size (wingspan) because these variables make biological sense. Reproductive activity and size increases the need for energy and therefore the potential need to store energy. Other variables considered were follicle size and time in captivity.

The objective of this study was to determine the potential risk factors associated with a decreased liver size. The hypothesis of this research was that the probability of a southern stingray having a lipid-depleted liver would be greater if they were undergoing folliculogenesis, were pregnant, and/or recently wild caught (new to captivity) during the low season (September-February) at the aquarium. This information can be used to establish a protocol for monitoring the health of this collection to potentially avoid further mortalities. Identification of these risk factors and their influence on the health of the animal may assist aquarium staff, veterinarians, and researchers with husbandry and clinical decisions.

3.3 Materials and Methods

This study was approved by the animal care and use committee at Colorado State University.

This was an observational, longitudinal (repeated measures) study design and included the female southern stingrays (subjects) from the entire collection at the Downtown Aquarium at Denver. A sample of the stingray collection was examined on a regular basis (at least once every six months or if they received new arrivals). The routine examinations were used to identify potentially compromised animals.

Only females were examined as they represented the majority of stingrays and because changes in reproductive status can change lipid content in the liver. The routine examinations were performed as described in chapter 2 and included collecting the following information (independent variables): wingspan (cm), time in captivity (days), follicle diameter (cm), pregnant (yes or no), and season (high or low). Univariate analysis was performed by creating design variables for the continuous variables, which lead to dichotomizing follicles size (≤ or > 1 cm), time in captivity (≤ or > 3 months), and wingspan (≤ or > 60 cm).

The variables were selected based on available information and biological sense. The high season was defined from March to August and represents higher attendance at the aquarium thereby increased touch pool feed sales; Low season represents September through February and has lower attendance and feed sales. Since liver size, specifically a small liver, was the concern, it served as the outcome or dependent variable (target). The dependent variable was dichotomous and was defined as either a lipid-depleted or small liver (liver ≤ 70% the length of the coelomic cavity) or having a lipid-filled or large liver (liver > 70% the length of the coelomic cavity). The establishment of a liver-to-coelom ratio or liver percentage is described in detail in chapter 4. Any animal with a liver-to-coelom ratio less than or equal to 70% was transferred to quarantine for further evaluation and possible treatment. Information obtained on stingrays in quarantine was not included in the longitudinal study. The basis for selecting 70% as a cutoff value for potential treatment was selected arbitrarily. Subjectively, dead stingray livers were 50% or smaller, and without knowing the rate at which the liver mobilizes lipid, 70% was selected as a size that could likely recover with attention and without severe debilitation. A summary of the possible variables for use in the model is shown in Table 3.1.

Table 3.1. Summary of potential variables.

Variable	IV or DV	Number of levels	Level of measurement
Follicles > 1cm (LF)	IV	2	Dichotomous
Captive ≤ 3mo (CT3M)	IV	2	Dichotomous
Wingspan > 60cm (WS60)	IV	2	Dichotomous
Pregnant (Preg)	IV	2	Dichotomous
Season (LS)	IV	2	Dichotomous
Liver size	DV	2	Dichotomous

IV: independent variable
DV: dependent variable

The limitations of this study included the small sample size, the lack of generalizability, the lack of individual diet information, the lack of feed sales information, and the long duration between examinations. Other forms of potential bias may include information bias if the animals are incorrectly classified or measured.

Statistical Analyses

The collected data was analyzed using a statistical software package (IBM® SPSS® Statistics version 23 release 23.0.0.0, IBM corporation, Armonk, NY and Microsoft® Excel® Version 14.6.8, Redmond, WA).

A generalized linear mixed model (Mixed model – Generalized linear in SPSS) was used to analyze the data. A generalized linear mixed model was selected due to multiple observations representing repeated measurements from a single stingray (subject), the non-normal distribution (use of link function), and because the outcome was categorical (dichotomous). Univariate analysis was performed first for the categorical variables as well as for design variables created from continuous variables. For the generalized linear mixed model, the target or outcome variable was the dependent variable (Liver Size of small or large) using a binary logistic

regression model for the target distribution and relationship (logit link) with the linear model. The potential fixed effects included the intercept, pregnancy (yes/no), time in captivity (≤ or > 3m), season (low/high), follicle size (≤ or > 1cm), wingspan (≤ or > 60cm), and interaction terms. The final model only included pregnancy, time in captivity (≤ or > 3m), and wingspan (≤ or > 60cm) as fixed effects. The random effects included the intercept and pregnancy during the building process but the intercept-only model was used in the final model. The random effect covariance type defaulted to variance components. The information criteria provided by the software output included -2Log pseudo likelihood ratio (LR) and Akaike (AIC) and were used to assess the fit of the model and helped determine variable inclusion. It is recommended to use a second-order AIC (AIC_c) with a small sample size/parameters of less than 40 (Anderson and Burnham 2002; Myung et al. 2009). The AIC_c was calculated using an equation that considered the sample size and number of parameters for each model iteration (Myung et al. 2009). Models with smaller LR and AIC_c, compared to the intercept-only model (reference) were considered better-fit models. Variables were added one at a time to the model and the chi-squared distribution was calculated using the LR. Significance of the chi-squared distribution at the 0.05 level warranted inclusion of a variable as it indicated an improvement to the fit of the model. The selected model also had the lowest AIC_c.

3.4 Results

Over a five-year period, 41 stingrays were examined during 15 examination periods, which equated to 114 observations. Of the 114 observations, the livers were found be less than 70% the length of the coelom 29 times and large livers (greater than 70% the length of the coelomic cavity) were found 85 times. The small or large liver groups were stratified by

independent variable category (season, pregnancy, follicle size, time in captivity, and wingspan) (Table 3.2). There were only 76 observations for follicle size therefore it was not included in the model building process. Also, there were four observations that did not include wingspan measurements. This was due to either that step being overlooked for that stingray or there was not a measuring device available.

Table 3.2. Summary of the data from the study. Small livers are those whose lengths are ≤70% the coelomic length and large livers are those whose lengths are >70% the coelomic length.

		Small Livers (n=29)	**Large Livers (n=85)**	**Total**
Season				
	Low (Sept-Feb)	15	36	51
	High (Mar-Aug)	14	49	63
	Total	29	85	114
Pregnancy				
	Yes	10	3	13
	No	19	82	101
	Total	29	85	114
Follicle size				
	> 1 cm	9	44	53
	≤ 1 cm	11	12	23
	Total	20	56	76
Time at Aquarium				
	≤ 3 months	11	23	34
	> 3 months	18	62	80
	Total	29	85	114
Wingspan				
	> 60 cm	13	42	55
	≤ 60 cm	14	41	55
	Total	27	83	110

The mixed model was performed and the combination of variables that provided the best fit (X^2_{LR}=72.031, df = 1, p<<0.001) is shown in Table 3.3. The AIC_c was the lowest for this model (456.872) compared to the intercept-only model (499.561). Additional random effects did

not change the LR, therefore it was not beneficial to further increase the complexity of the model by including them.

Table 3.3. Generalized linear mixed model results for predicting stingrays with liver sizes less than 70% of coelomic cavity length.

Variable	Coeff. (β)	SE	Sig.	$\exp^{\beta}$	95% CI	
Intercept	-2.146	1.573	0.175	0.117	0.005	2.647
Pregnant	3.433	0.765	0.000	30.978	6.803	141.066
Cap time ≤ 3m (CT3M)	0.930	0.497	0.064	2.534	0.945	6.790
Wingspan > 60 cm (WS60)	-0.635	0.477	0.186	0.530	0.206	1.365

The mixed model coefficients are denoted by 'Coeff.' in Table 3.3. To translate the information given by the coefficients to a probability (p) of predicting whether or not a stingray has a liver size of less than 70% of the coelomic cavity, one may use the following formula (coefficients are used if that variable is true in a given scenario):

$$Logit\ (p) = Log\left(\frac{p}{1-p}\right) = -2.146 + 3.433\ (pregnant) + 0.930\ (CT3M) - 0.635\ (WS60)$$

The significant predictor variable was pregnancy (p = 0.000). The odds ratio [confidence interval] for this variable was 30.978 [6.803, 141.066]. For pregnant stingrays, this means that the odds of being pregnant are more than 30 times as high among stingrays with small livers as among stingrays with large livers. The pregnant variable's confidence intervals confirmed its

significance, as it did not include 1 in its range. The odds of a stingray having a small liver are increasingly greater if the stingray is pregnant. Using the predictor equation developed from the generalized linear mixed model analysis, pregnancy (95%) had the highest prediction percentage when it was the only variable and together with all variables (97%) only increased the probability of livers less than 70% the length of the coelom by 2%. Biologically, it makes sense that pregnant animals are most likely to have a small liver since they are most likely to be in a negative metabolic state.

3.5 Discussion

Elasmobranch livers serve the purpose of lipid storage for metabolic function, as a future fuel source, follicle development for mature females, and for buoyancy (Ballantyne 1997; Rossouw 1987). Knowing that the liver functions as the sole location for lipid storage, which fluctuates depending on the metabolic demand and caloric intake, it is considered a dynamic organ (much like adipose tissue in mammals). Similar to other animals, a decrease in fat or being severely under conditioned can compromise their health and even contribute to death, which was the suspicion in the unknown cause of death in many of the rays discussed in Appendix 2. Based on the information learned from reviewing the necropsy records, this study was conducted to confirm possible risk factors contributing to lipid depletion in southern stingray livers. The risk factors included in the final model were pregnancy, recent arrivals (a captivity time of less than three months), and a wingspan of greater than 60 cm. Risk factors that were considered but not included in the final model were follicle size and season.

Follicle size as a continuous variable, as well as dichotomized to follicles less than or greater than one centimeter, were initially considered but not included in the analysis due to too

many missing values per observation. The generalized linear mixed model function in SPSS eliminates entire subject observations from the study if values for any variable are missing in some cases. When running certain analyses, errors were encountered due to the lack of observations therefore follicle size was eliminated from the study. From a practical perspective, including follicle size does not make sense since it is not a parameter that the aquarists could take into consideration (other than guessing based on stingray size). The aquarist would not be able to look at an individual stingray and detect the size of its follicles unless they were using an ultrasound or the stingray was deceased and they were performing a necropsy. If they were using an ultrasound to measure follicles, then there would be no need to use that as a predictor since they could easily use the ultrasound to view the liver. From a biological perspective, follicle content includes lipids so, during vitellogenesis and folliculogenesis, they are mobilized from the liver. Animals undergoing folliculogenesis do not mobilize enough lipids from the liver to result in drastic depletion thereby putting the animal in a potentially compromising status (follicles alone would have little effect on liver size in a captive environment). This process occurs in other elasmobranch species over the course of several months to years and has not disrupted the metabolic need for lipid (Rossouw 1987).

Follicle size overlaps with other variables such as age, which is represented by size (the older the stingray, the larger the stingray, which is more likely reproductively mature). Also, since many of these stingrays were wild caught and transferred to captivity, time in captivity may also overlap with follicle size. Typically, smaller stingrays were harvested from the wild to increase the number for transport and display (the smaller the group of stingrays, the more that could be transported and the more that could fit into the exhibit). So, in those cases, the reciprocal of the follicle variable being a strong predictor is somewhat misleading. Certainly

immature (not yet reproductively mature) stingrays will have smaller follicles, so perhaps age (or rather size) would have been a confounder in this case.

Season (low/high) was considered but did not contribute significantly to the model. The thought behind including this variable was based on aquarium attendance and touch pool feed sales. Unfortunately neither attendance nor feed sales data were available during the time frame and therefore were not included. Daily diet was also not included. This information, although helpful in understanding the fluctuations (if any) of feed provided, would not have pertained to individuals. Daily diets were fed by total number of individuals in this exhibit and the feed was broadcasted to the exhibit (not handfed to each stingray). Public feedings were handfed to individual stingrays but tracking that information would be nearly impossible. One or two volunteers monitored groups of guests and the stingrays were not generally identifiable by unique markings. Also, daily diets were adjusted by the attending aquarist based on their observation of "slow" or "busy" days, which could have altered the results based on the season variable alone. Along these same lines, stingrays identified with small livers during exams were "treated" with an increased diet and the liver size was monitored during routine veterinary visits (approximately once every other week) although this information was not included in the study.

Shortly after the beginning of this study period, a group of wild caught stingrays were transported to the aquarium and added to the exhibit. This group made up the majority of the subjects that fell into the recently acquired category (captivity less than three months group). Analyses on this group compared to the acclimated group at the time are described in more detail in chapters 4 and 5. Because the stingrays were examined routinely and adjustments to diet were made accordingly, either to the exhibit (based on the aquarist's observations) or to individuals (based on exam findings), there was a reduced risk of compromised health. Because there were

no massive die-offs during the rest of the study period, the need to introduce additional stingrays was eliminated. At the end of this study period, female stingrays were reproducing and the pups could have also been categorized as being in captivity for less than three months; however the intent behind this variable involved adults transitioning to a new environment. Although neonates also commonly have small livers, there are other factors to consider in addition to a new environment. It is standard practice to closely monitor neonates and offer them a variety of food as soon as they are metabolically ready (once the yolk sac is depleted) at higher rates compared to adults (Janse et al. 2004; Janse and Schrama 2010). Growth curves in captive southern stingrays have been reported (Henningsen and Leaf 2010). Due to different circumstances surrounding neonates, they were not included in the routine examinations and therefore not included in this study. Examining the newly acquired adults prompted the attention requirement for adjustments in feed in these situations.

The newly acquired stingrays also represented a group with wingspans less than 60 cm overlapping small size and short captivity time. This is opposed with pregnant stingrays, which typically have a minimum wingspan of 70 cm (Henningsen and Leaf 2010; Ramirez-Mosqueda et al. 2012). Also, larger stingrays, in general, have a higher energy demand therefore would require a higher caloric intake. A wingspan over 60 cm in this study improved the model as expected; however, it is important to understand the situations of the given variables as a short captivity time also improved the model. Overall, the growth of the female stingrays used in this study followed a logarithmic curve as suggested in another study (Henningsen and Leaf 2010). All pregnant rays were over 60 cm and the majority of them expended much of their reserves during their pregnancy resulting in a small liver. Based on the size of livers that were observed during gestation (often between 50-60%), it indicated that the estimation of a liver being “small”

at less than or equal to 70% was very conservative as there were no deaths on closely monitored pregnant rays with livers less than 60%. So for pregnancy and wingspan, because the model improved with the addition of the variable wingspan greater than 60 cm, it added to the effectiveness of just using pregnancy alone or pregnancy with captivity time. Had the model had not progressively improved with the addition of the variables (CT3M and WS60) then that would have reinforced that only pregnancy was informative as a predictor.

Retrospectively, the distribution of liver sizes expressed as percentages was reviewed among the living (from this chapter's study) and deceased (from the study in appendix 2) rays to evaluate the arbitrary decision of a 70% cut off for "treatment." This distribution is represented by Figure 3.1. The deceased stingrays with liver size percentages from 70-100 were all from known causes (water quality problems or jumping out of the exhibit). The deceased rays with liver size percentages of less than 60 were all unknown at the time and were categorized to the ranges by qualitative coding. For instance, based on liver color, livers described in the necropsy reports as "blue" were assumed to be in the 30-39 range and those reported as "dark" were assumed to be in the 40-49 range. Livers outside of those two ranges, either smaller or larger, were either expressed as a percentage in the necropsy report or an image was provided in the record and the measurement was extrapolated. Based on this information, the cutoff was conservative as expected and could have been decreased to 60%.

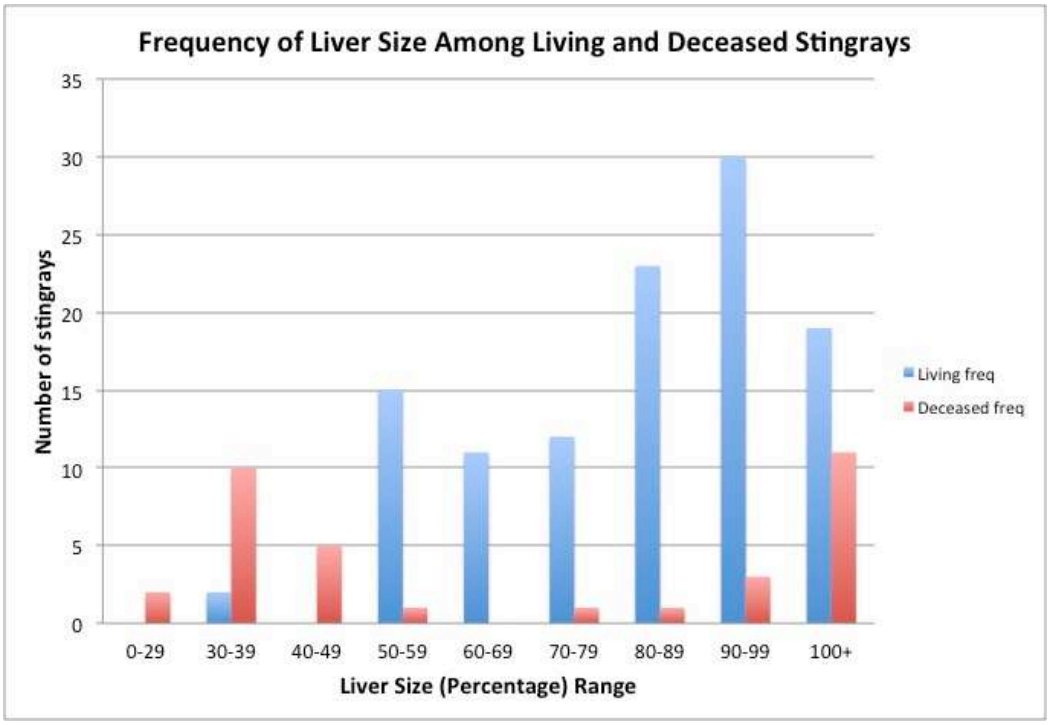

Figure 3.1 The distribution of liver size (percentage) ranges among living (from this study) and deceased (from appendix 2 study) stingrays.

For future study, things to consider might include the association of liver length with depth, liver echogenicity with respect to size, individual stingray's diet content, the amount and frequency of the broadcasted diet, attendance, and feed sales. Since this study was conducted, many of these southern stingrays outgrew the touch pool exhibit and were moved to either another location (a different aquarium) or to a larger exhibit within the aquarium. Often times larger fish are individually fed within larger exhibits to ensure they are being offered an adequate diet, to deter consumption of tank-mates, and to ensure they are receiving necessary supplements. It is exceptionally more difficult to coordinate capture to examine animals housed in the larger exhibits so examination frequency would be reduced.

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CHAPTER 4: Validation of the ultrasound-guided technique to establish a liver-to-coelom ratio and a comparative analysis of the ratios among acclimated and recently wild-caught Southern stingrays, *Dasyatis americana*[1]

4.1 Synopsis

Southern stingrays, *Dasyatis americana*, are a well-represented elasmobranch species in public aquaria and other facilities throughout the world. This study was conducted at a facility that experienced some mortality and replenished the collection with wild-caught stingrays. A common necropsy finding among the stingrays was a small, dark liver. The objectives of this study were to assess the reliability of an ultrasound-guided technique for establishing a liver-to-coelom ratio by calculating the approximate length of the liver with respect to the coelomic cavity length and then to compare ratios between acclimated captive and wild-caught stingrays. The ultrasound validation phase of the study measured the distance from the caudal margin of the liver to the pelvic cartilaginous girdle and compared it to the actual distance measured during the necropsy or surgery. There was no significant difference found between the ultrasound and actual distance measurements (p=0.945). This technique was then used to establish liver-to-coelom ratios and compare two groups of stingrays, presumably under different metabolic states at different periods. Liver-to-coelom ratios were established during initial examinations as well as eight months after cohabitation in a touch pool exhibit. Significant differences in liver-to-coelom ratios existed between the two stingray groups when compared at introduction (median difference = 30.9%, p=0.007) and after eight months (median difference = 20.5%, p=0.008); and

[1] Grant K.R., Campbell T.W., Silver T.I., Olea-Popelka F.J. (2013) Validation of an ultrasound-guided technique to establish a liver-to-coelom ratio and a comparative analysis of the ratios among acclimated and recently wild-caught southern stingrays, *Dasyatis americana*. *Zoo Biology* 32(1):104-111. Reprinted with permission from John Wiley and Sons, License number 3856201421705

within the acclimated group (median difference= 20.4%, p=0.018) and wild-caught group (median difference 31%, p=0.008) when comparing livers at introduction and after eight months.

4.2 Introduction

Elasmobranchs have been an attraction in public aquarium exhibits since the late 1800's (Koob, 2004). The Southern stingray, *Dasyatis americana*, is a well-represented stingray species in public aquariums throughout the world. The Southern stingray is exhibited in over 48 facilities worldwide and is the second most represented marine stingray species (AES census, 2008; Firchau et al., 2004). They are commonly displayed in feeding or touch pools where the public may interact with the animals contributing to their popularity (Jeffery and Wandersee, 1996). Due to the public involvement with these types of facilities, monitoring feedings and caloric intake for these animals is challenging. The study presented here will introduce a method for assessing body condition based on relative liver size and will use this method to compare stingrays in presumably different metabolic states.

The liver is a large organ in elasmobranchs and may occupy the majority of the coelomic cavity. In some shark species the liver extends to the cloaca (Walsh et al., 1993). The liver is the primary location for triacylglycerol storage (Zammit and Newsholme, 1979). The liver of benthic elasmobranch species may weigh between 1% and 6% of their body weight of which 80% may be lipid (Holmgren and Nilsson, 1999). The lipid stores provide energy between meals and, in some species, assist with buoyancy. It is suspected that under stressful situations, long periods between meals, or during times of high energy or nutritional demand, the lipid stores become depleted thereby altering the size of the liver. In addition to a decreased size, lipid

depletion will decrease the echogenicity of the liver during ultrasound examination (Mathiesen et al., 2002; Nyland and Park, 1983; d'Anjou, 2008).

Animals in this study were housed in a commercial interactive pool with Southern and Cownose stingrays, *Dasyatis americana* and *Rhinoptera bonasus*, respectively. Over a three-year period this facility experienced intermittent mortalities involving their recently wild-caught, adult, female Southern stingrays. Necropsy records noted that all of these animals had a small, dark liver upon gross necropsy, which was described as lipid or glycogen depletion in the pathology reports. Records indicated that there were no signs of illness prior to death and in many cases the animals ate until the day before they died.

Since the most evident macroscopic lesion at necropsy, in all cases, involved the size of the liver, the objective of the initial phase was to determine the reliability of accurately measuring the distance between the caudal margin of the liver and the pelvic cartilaginous girdle using an ultrasound-guided technique. This distance implies a relative liver length compared to the coelomic length and was used to establish a liver-to-coelom ratio (liver size %) to identify potentially compromised animals.

The second phase of this study compares the liver size percentages of recently wild-caught Southern stingrays to those acclimated to captivity using the previously described ultrasound technique. Since recently wild-caught stingrays are likely in a negative metabolic state due to the stress of capture and transport, it is suspected that they are more nutritionally compromised than those acclimated to captivity.

4.3 Materials and Methods

Validation of the ultrasound-guided technique for establishing the liver-to-coelom ratio

The purpose of this phase of the study was to validate an ultrasound-guided technique to measure the liver. This phase was exploratory in which available subjects, fourteen adult Southern stingrays (13 females and one male), were used. The ultrasound exams were performed and measurements were taken on 13 deceased animals and one animal under general anesthesia for surgery. The ultrasound exams and necropsies were performed within 24 hours of death.

All but one of these animals lived in the touch pool exhibit. The fourteenth stingray lived in a much larger exhibit with a variety of other animals. The exhibit and water quality parameters were consistent with recommendations for captive elasmobranchs (Mohan and Aiken, 2004). The offered diet consisted of a variety of fish, such as smelt, pollock, capelin, mackerel; as well as squid and shrimp. The stingrays were also supplemented with an elasmobranch vitamin (Vita-Zu®, Mazuri®, St. Louis, MO) once weekly.

Each stingray was placed in dorsal recumbency for sonography. Thirteen imaging exams were performed post mortem and one during surgery. The stingray undergoing surgery was placed in a shallow bath with recirculating saltwater treated with 100 parts per million (ppm) of tricaine methanesulfonate (Finquel® or MS-222®, Argent Laboratories, Redmond, WA). The length of the coelomic cavity was established by palpating and measuring the distance (in centimeters) between the pectoral and pelvic cartilaginous girdles on ventral midline (Figure 1).

Ultrasound examinations were performed using a 7.5 MHz linear array transducer with a commercial ultrasound unit (Aloka SSD-900v, Aloka, Inc. Wallingford, CT). The overall gain, time gain compensation (TGC), and depth settings were adjusted to maximize image resolution

and organ visualization. With the transducer in a sagittal position, on ventral midline, just caudal to the pectoral cartilaginous girdle, the liver was identified. The caudal margin of the liver was located with the ultrasound along the ventral midline. The pelvic cartilaginous girdle was identified by palpation just cranial to the vent (Figure 4.1).

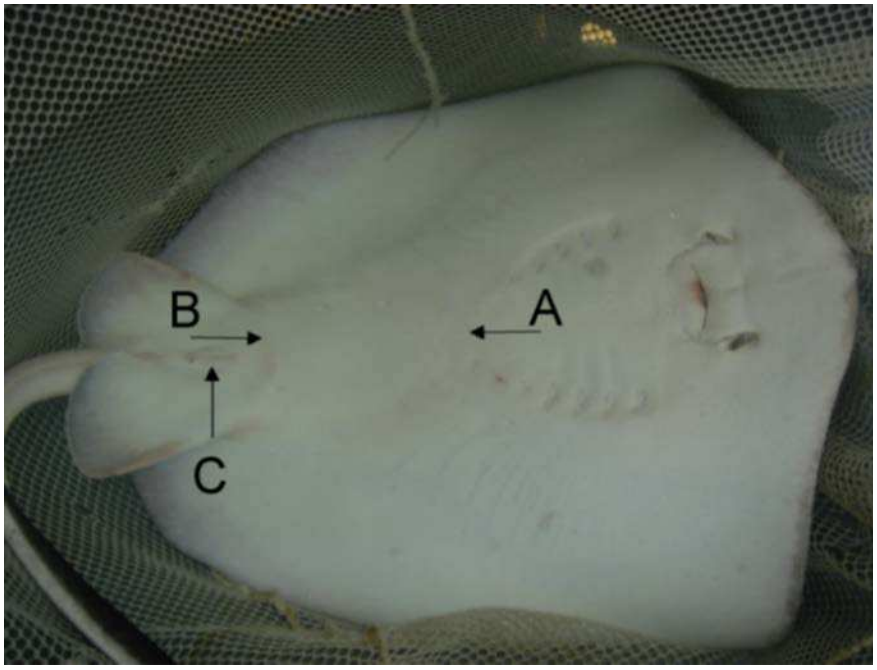

Figure 4.1. Ventral view of a female Southern stingray, *Dasyatis americana*: the pectoral cartilaginous girdle (A), the pelvic cartilaginous girdle (B), and the vent (C)

If the cartilage and the caudal liver margin were captured within the same view, then the distance between the two landmarks was measured using the ultrasound unit (Figure 4.2). If the two landmarks were not captured within the same image, then the caudal edge of the transducer was aligned with the caudal margin of the liver and the distance between the caudal edge of the transducer and the cartilage was measured with a ruler. The estimated liver length is calculated by subtracting the distance between the liver and pelvic cartilage from the coelomic cavity

length. The liver size is expressed as a percent of the coelom (or liver-to-coelom ratio) by dividing the estimated liver length by the coelomic cavity length.

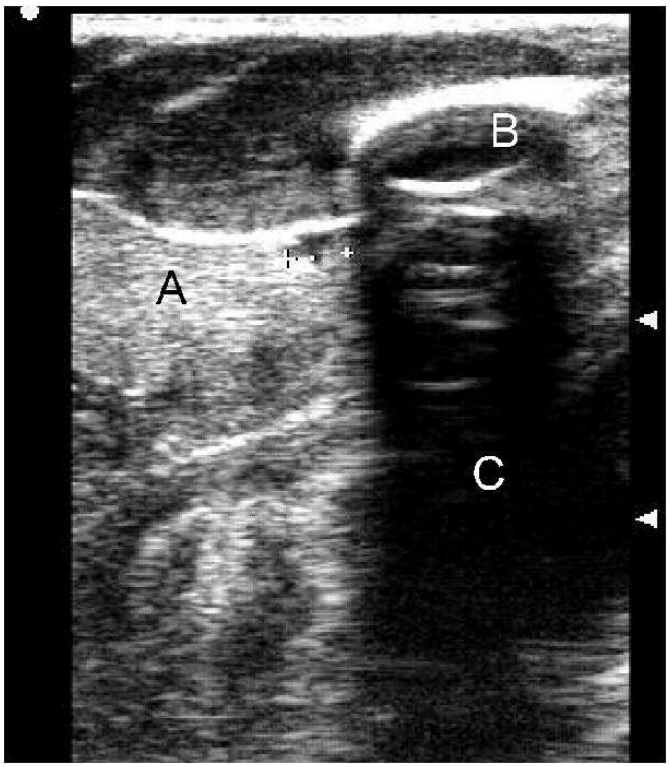

Figure 4.2. Ultrasound image of the caudal, mid coelom in a female Southern stingray, *Dasyatis americana*. Note the caudal margin of the liver (A) and the pelvic cartilaginous girdle (B). Similar to that of bone, the cartilage produces a distal acoustic shadow (C). The distance between them can be measured using the ultrasound unit as noted by the dotted line in the image. The large '+' symbol is at the caudal margin of the liver and the small '+' symbol is at the cranial edge of the pelvic girdle.

The necropsy was performed by making a circular incision along the border of the cartilage surrounding the coelomic cavity. The contents of the coelom were exposed during necropsy. The distance between the caudal margin of the liver on ventral midline and the pelvic cartilaginous girdle just cranial to the vent was measured in centimeters. This distance and the distance obtained using the ultrasound provided two measurements for each stingray (paired data).

Comparative analysis of ratios among acclimated and wild-caught stingrays

This phase was prospective using a cohort of twenty female Southern stingrays. Nine of the stingrays were acclimated to the touch pool exhibit for a minimum of two years (acclimated stingrays) and 11 of the stingrays were recently introduced to the exhibit after being captured from the wild (wild-caught stingrays). Initial examination of the acclimated stingrays occurred one month prior to the introduction of the wild-caught stingrays. Upon arrival to the aquarium, the wild-caught stingrays were treated with two ppm of praziquantel (Fishman Chemical, LLC, Ft. Pierce, FL) for five days while quarantined for two weeks. The 11 wild-caught stingrays were examined on two different occasions within one month of arrival.

Physical examinations of both groups consisted of placing the animal in dorsal recumbency for measurements and ultrasound imaging. The stingrays were captured with a large nylon net and manually turned into dorsal recumbency. Although the barbs are clipped due to public interaction, careful handling by trained personnel ensured the safety of those involved with the examinations. Measurements recorded included wingspan (largest distance from wing tip to wing tip), snout-to-vent length, length of coelomic cavity (pectoral to pelvic cartilaginous girdle measurement), and liver length (using the ultrasound-guided technique). Liver size percentages were established for each stingray. An ultrasound image comparing liver and spleen echogenicity was also recorded.

The wild-caught stingrays were introduced into the touch pool exhibit approximately two weeks after arrival. The husbandry and diet for the stingrays were identical to those described previously. In an effort to monitor the health status of the collection, physical and ultrasound examinations were performed twice yearly. Therefore, eight months after introduction, examinations were repeated.

Validation of the ultrasound-guided technique for establishing the liver-to-coelom ratio –

StatTools, Palisade Corporation, Ithaca, NY was used for statistical analysis. The Wilcoxon signed rank test was used to compare the median distances measured from the caudal liver margin to the pelvic cartilaginous girdle using the ultrasound-guided technique and the measurement taken during necropsy among each subject (paired data). We tested the null hypothesis that there will be no difference between the liver-to-cartilage distance measurements when using the ultrasound-guided technique compared to the measurement taken during necropsy or surgery.

Comparative analysis of ratios among acclimated and wild-caught stingrays –

The liver size median percentages were compared between the two stingray groups (acclimated vs. wild-caught) using the Wilcoxon rank sum test at the time of introduction and after eight months of cohabitation. The liver-to-coelom percentages were compared between time periods (introduction vs. eight months) within each stingray group (paired data) using the Wilcoxon signed rank test using SPSS 17.0 for windows, release 17.0.2. We tested the null hypotheses that liver size percentages between stingrays groups and time points were not different. Statistical significance (rejection of the null hypotheses) was considered at $p<0.05$.

The wild-caught stingray examinations were completed within one month of arrival, two-weeks apart. The liver size percentages were subjectively evaluated between the two exams and showed no difference.

4.4 Results

Validation of the ultrasound-guided technique for establishing the liver-to-coelom ratio

Table 4.1 represents the data for 14 Southern stingrays. The minimum and maximum actual liver-cartilage distances were zero and 15 cm, respectively. The minimum and maximum differences between the ultrasound-guided measurement and actual distance were zero and two centimeters, respectively. Six of the 14 stingrays had a distance measurement of zero centimeters between the ultrasound guided and actual distances. Two of the 14 stingrays had distance differences of two centimeters. The remaining six observations had a difference between measurements of 0.31, 1.37, 1.40, 1.48, and two at 1.67 cm. The median difference between the measurements of the two methods for the liver-cartilage distance was not statistically significant (median difference = 0.84 cm, $p = 0.945$, Table 4.1).

Table 4.1. Measurements from the caudal liver edge to the pelvic girdle in Southern stingrays using an ultrasound-guided technique (US) and those taken during necropsy or surgery (N/S).

Stingray	US (cm)	N/S (cm)	|Difference|
1	14.5	14.5	0
2	0	0	0
3	1.67	0	1.67
4	0.31	0	0.31
5	0	0	0
6	1.4	0	1.4
7	3.52	5	1.48
8	0	0	0
9	0	0	0
10	3.37	2	1.37
11	1.67	0	1.67
12	13	15	2
13	0	0	0
14	7	9	2
Median	1.54	0	0.84
Mean	3.32	3.25	0.85
St Dev	4.85	5.53	0.86

Comparative analysis of ratios among acclimated and wild-caught stingrays

The results from the comparisons between the two groups of stingray liver size percentages are shown in Table 4.2. The median liver size percentages of the acclimated stingrays and wild-caught stingrays at introduction were significantly different (p=0.007) at 90.9% and 60.0%, respectively. Likewise, comparing the groups after eight months of cohabitation, the median liver size percentages of the acclimated and wild-caught stingrays showed a contrasting significant difference (p=0.008) at 70.5% and 91.0%, respectively.

Liver measurements were also compared within groups. The values for the acclimated and wild-caught rays at introduction were compared to values obtained eight months later. There was a significant difference for liver size within the wild-caught stingray group between time periods (median difference = 31%, p=0.008) and for the acclimated group (median difference = 20.4%, p=0.018).

Table 4.2. Descriptive measurements among recently wild-caught Southern stingrays and acclimated Southern stingrays and comparisons of liver-to-coelom ratio (liver size percentages).

	Acclimated stingrays						Recently wild-caught stingrays						**Wilcoxon rank sum test** *p-value*
	n	Min	Max	Median	Mean	sd	n	Min	Max	Median	Mean	sd	
Liver size-Intro (%)	7	80	104	90.9	92.9	7.1	11	30	85	60.0	59.5	17.1	***0.007***
Liver size-8mo (%)*	8	53	83	70.5	69.9	9.5	11	58	100	91.0	86.9	12.6	***0.008***
Wilcoxon sign rank test p-value	--	--	--	***0.018***	--	--	--	--	--	***0.008***	--	--	--

*The second measurement taken after eight months of cohabitation. Wilcoxon sign rank tests were used to compare median liver sizes at different time points among the same stingrays. The Wilcoxon rank sum test was used to compare median liver size among different groups of stingrays (at two different time points).
p-values (bolded) indicates significance (<0.05)

4.5 Discussion

The purpose of this study was to assess the accuracy of measuring an estimated liver length relative to the coelom using an ultrasound-guided technique and to use this technique to compare stingrays acclimated to a captive environment to those recently wild-caught. The small difference between measurements in the ultrasound validation phase confirmed the accuracy of the ultrasound-guided measurements. We did not have a predefined hypothesis to test regarding the difference between the two measurements. Although small (median difference = 0.84 cm, p=0.945), there were observed differences between ultrasound-guided and actual measurements in some stingrays; however, we did not find a clinical/anatomical relevant difference between the measurements between the two methods in our study. Considering the variability in our data, a post-hoc power analysis indicated our sample (n=14) would be sufficient to detect a significant difference of one centimeter between methods if that difference existed, with a power of 80% and 95% confidence.

The accuracy of taking liver to cartilage distance measurements may vary depending on whether or not the image captures both the caudal liver margin and the pelvic girdle. If the ultrasound image captures both landmarks, then the measurement can be taken directly with the ultrasound unit and the only variability is probe position. The variability in the actual distance measured when both landmarks are captured within the image is identifying a clean border on the pelvic girdle. The cartilage is clearly defined on the ultrasound image as it produces a distal acoustic shadow (Figure 4.2). During necropsy, the soft tissue needed to be removed in order to establish a definite point of measurement on the cartilage. If the liver is small and not imaged with the cartilage, then measuring the distance requires an external measuring device and a well-positioned probe. The caudal edge of the probe must be aligned with the caudal margin of the

liver at which point the distance is measured from the caudal edge of the probe to the palpated pelvic girdle. The margin of error may involve the probe position, the variability of the point at which to measure from the probe, accurately palpating the pelvic girdle, and the variability of the point at which to measure to the cartilage. Again, if the probe is not on midline, this may alter the distance measured as well. The other factor that may add to the variability is movement by the animal.

A liver with decreased fat stores may not only decrease in size but also display a decreased echogenicity when imaged with an ultrasound unit. The liver may have a similar echogenicity or appear hypoechoic when compared to the spleen (Figure 4.3A) or epigonal organ. In cases where it is difficult to discern organs, identifying the liver to measure the distance from the caudal margin to the pelvic cartilage may be challenging. A comparison of the echogenicity, gross appearance, and corresponding histology of a liver with depleted lipid stores and a normal lipid-filled liver are shown in Figures 4.3 and 4.4, respectively. The ultrasound image of the lipid-depleted liver (Figure 4.3A) shows a small liver ventrally with similar echogenicity compared to the spleen. The corresponding gross image from necropsy shows the small, dark liver extending to the curvature of the stomach. The spleen is dorsal to the liver and therefore is not seen. The darker color of the liver during necropsy is an indication of depleted lipid stores (Rossouw, 1987). Histologically this liver showed marked depletion of fat from the hepatocytes. Although there were some fat vacuoles present, overall the liver was severely lipid-depleted which is apparent when compared to the histology of a normal liver (Figure 4.4C). The ultrasound image of the lipid-filled liver shows a large and hyperechoic liver compared to the spleen, which is dorsal to the liver (Figure 4.4A). While in elasmobranchs it represents a normal condition, increased echogenicity is consistent in other animals with abnormal fatty infiltration to

the liver (Nyland et al., 2002; Stetter, 2004). The histology of this normal stingray liver shows the majority of hepatocytes with fat vacuoles, which is similar to hepatic lipidosis in other animals (Cebra et al., 1997; Cooper, 2002). On necropsy this liver is large and a light tan color (Figure 4.4B).

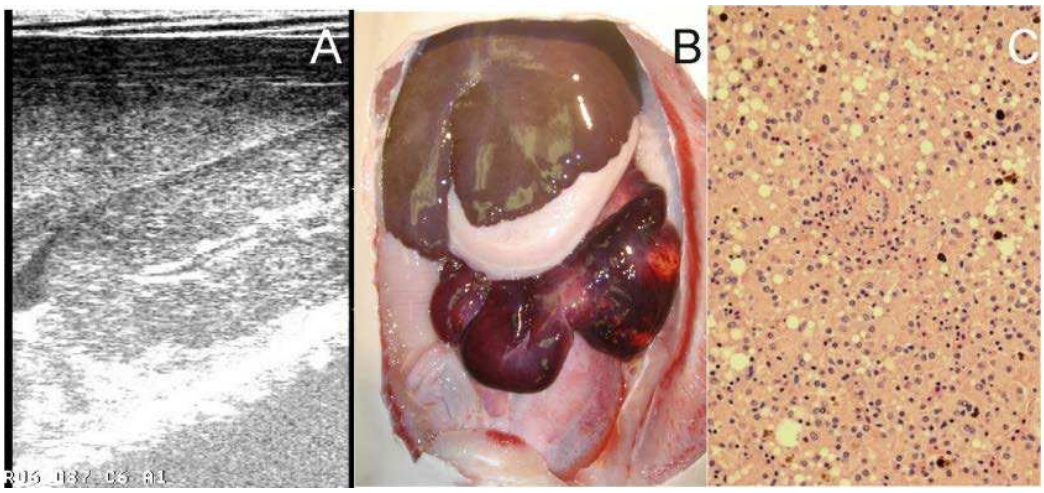

Figure 4.3. These images are from the same Southern stingray, *Dasyatis americana,* with a lipid-depleted liver. (A) This ultrasound image is captured with the linear transducer in a sagittal position, mid to cranial coelom on ventral midline. The liver is ventral (top of image) to the spleen (middle of image). Note the similar echogenicity between the liver and the spleen. (B) This image shows the open coelom during necropsy. The caudal margin of the liver does not extend beyond the curvature of the stomach. (C) Histology of the liver shows some fat vacuoles but is severely depleted overall, HE stain

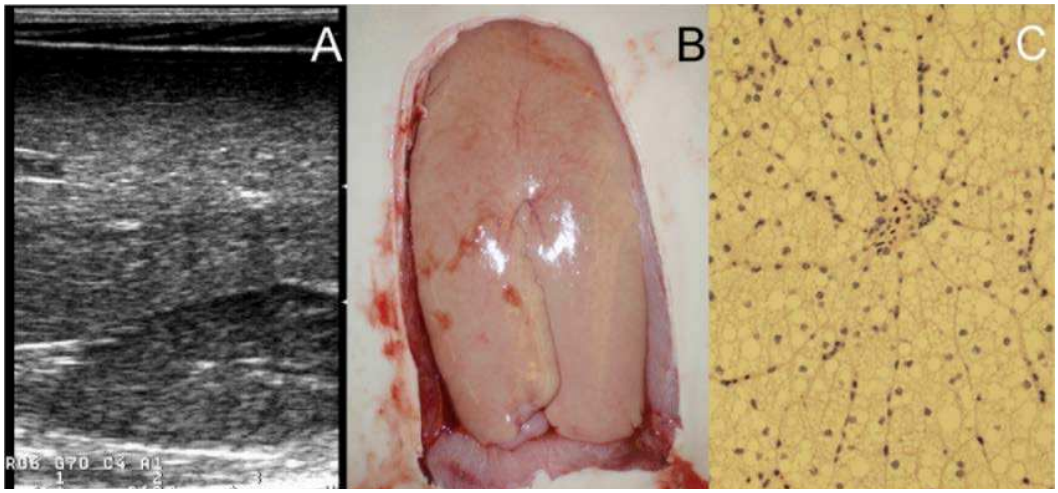

Figure 4.4. These images are from the same Southern stingray, *Dasyatis americana*, with a lipid-filled liver. (A) The ultrasound image is with the linear transducer in a sagittal, mid coelom, ventral midline position. The liver is occupying the majority of the image (top half of image A) and is hyperechoic compared to the spleen (bottom of image A). (B) The open coelom during necropsy. Only the liver can be seen, as it is large and lipid-filled. (C) Histology (HE stain) of the lipid-filled liver with the majority of the hepatocytes containing fat vacuoles.

To further evaluate the liver size using this technique under presumably different metabolic states, two groups of stingrays were compared. One group originated from the wild and had been in captivity for at least two years while the other group was recently acquired from the wild. The results provided evidence to support the hypotheses that wild-caught stingrays' liver size percentages are significantly different compared to the acclimated stingrays between the two groups at introduction. The liver size percentages were significantly different when analyzed between groups at introduction and after eight months of cohabitation as well as within both groups. The smaller liver size percentages in the wild-caught stingrays at introduction were expected due to possible stress of the capture and long transport, stress from the new and unfamiliar environment, and anorexia. Southern stingrays reside in the western Atlantic (Cain et al., 2004; Chapman et al., 2003) and Gulf of Mexico (Lytle and Lytle, 1994; Semeniuk et al., 2007) so the transport distance to this facility was over 2,000 miles. The exact time that lapsed from capture to arrival is unknown but it is likely that these animals relied on their fat stores for energy during the majority of this process. Their liver sizes were unknown at time of capture, so livers may have been depleted in the wild. Regardless of nutritional status prior capture, the transport and anorexia likely contributed to their negative metabolic states. This study only confirmed that they arrived with relatively small livers.

Within one month of introducing the recently wild-caught group to the touch pool the amount of food provided to the exhibit was six kilograms daily. This food was given in addition to the amount provided by the public. There were no stingrays lost during this transition and they were re-evaluated after eight months. Unexpectedly, there was an inverse relationship with liver size percentages between groups after eight months. The wild-caught group's median liver size percentage was significantly higher than the acclimated group's median liver percentage.

One explanation for the decreased liver size in the acclimated group is competition. The acclimated group may have become accustomed to daily feedings whereas the wild-caught group was accustomed to foraging in the wild and therefore capitalized on the opportunity. One study conducted in Grand Cayman Southern stingrays found that there were behavior changes between tourist sites and non-tourist sites (Semeniuk and Rothley, 2008). The stingrays in the tourist sites appeared to display more aggressive competitive behaviors and exhibited more injuries compared to stingrays in non-tourist sites (Semeniuk and Rothley, 2008). One year after introduction, an informal examination of ten randomly selected stingrays from the total group (six were from the wild-caught group and four from the previously acclimated group) showed no difference in liver-to-coelom ratios with all of them between 90% and 100% (data not shown).

Based on the wingspan measurements of the wild-caught group, it is suspected that these stingrays were younger. The median wingspan difference between groups at introduction and eight months later was 21 cm and 20.5 cm, respectively. These differences were significant ($p<0.001$). Both groups increased in size similarly over the eight-month cohabitation period with median wingspan differences of 9.5 cm in the acclimated group and 10 cm in the wild-caught group. The wild-caught group was significantly smaller which may imply that these rays were not yet sexually mature and therefore the nutrient demand during folliculogenesis was absent. Vitellogenic precursors originate from the liver (Hamlett et al., 2005; Hamlett and Koob, 1999). A decreased amount of lipid in the liver may possibly contribute to small follicles. This correlation between liver size and follicle size has been shown in other elasmobranchs (Walker TI, 2005). Follicle size was not recorded during the examinations due to difficultly visualizing them in many of the stingrays within the wild-caught group. This may have aided with their successful transition into captivity.

Animals that have undergone a potentially stressful event, such as transport, and that are anorexic may be in a vulnerable condition that possibly predisposes them to opportunistic pathogens or other immunosuppressive diseases. It is important to quarantine, examine, and provide additional nutritional support through the capture and transport transition. Ultrasound is a noninvasive approach for evaluating the liver-to-coelom ratio and hepatic echogenicity in recently captured elasmobranchs. This technique can be easily incorporated into the routine physical examination and will provide insight into the nutritional status of the animals. Routine examinations with established collections are also necessary to gain more accurate health assessments. Stingrays in these types of exhibits are often difficult to monitor feedings and many of the animals may appear to be eating when actually they are mouthing or playing with the food. Further investigation is necessary to evaluate the dynamics and physiology of the elasmobranch liver during different metabolic states.

Conclusions

1. The ultrasound serves as a useful tool in approximating the relative length of the liver when compared to the cartilaginous borders of the coelomic cavity.
2. The ultrasound-guided technique for establishing a liver-to-coelom percentage showed a significant difference between relative liver size percentages among stingrays recently wild-caught compared to stingrays that were acclimated to captivity.
3. Further studies are needed to determine the liver-to-coelom percentage at which intervention is necessary and to better understand the dynamics of the elasmobranch liver.

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CHAPTER 5: Hematology and Plasma Biochemistry Value Differences Between Acclimated and Recently Captive Female Southern Stingrays, *Dasyatis Americana*

5.1 Synopsis

Southern stingrays are used for interaction and education in captive and wild settings therefore it is important to monitor their health conditions. Diagnostic tools that are useful for assessing health in other animals are hematology and plasma biochemistry profiles. Certain reference intervals have been established in this species; however, interpretation of intervals in stingrays under different conditions are lacking. The primary aim was to compare hematological and plasma biochemical values between seventeen female stingrays that were acclimated to captivity (n=8 adult) to those recently collected from the wild (n=9 immature). Examinations included measuring disc width, ultrasound evaluation of the coelomic cavity, and blood collection. The examinations were performed on both test groups at two time points: prior to introduction of the recently captive rays to the aquarium exhibit and eight months after cohabitation. Hematology analysis included manual WBC counts, leukocyte differential, PCV, and plasma protein. Plasma chemistry profiles included aspartate aminotransferase, bicarbonate, urea, calcium, creatine kinase, cholesterol, chloride, globulin, glucose, phosphorus, potassium, sodium, and total protein. The two groups of stingrays' results were compared using the Wilcoxon Rank Sum test. The following parameters were found to have statistically significant differences ($p<0.05$) prior to introduction: bicarbonate, urea, calcium, cholesterol, chloride, globulin, potassium, total protein, and PCV. The recently-captive rays had higher median values of urea, chloride, and potassium. There were no significant differences after eight months of

cohabitation. Data interpretation for hematology and plasma chemistry values may be affected by the environmental changes for stingrays.

5.2 Introduction

Southern stingrays (*Dasyatis americana*) belong to the Dasyatidae family, subclass elasmobranchii, and naturally reside in the western Atlantic ocean and Gulf of Mexico (Grubbs et al. 2006). In the wild, they are used in nature-based tourism and, in captivity, they are one of the most represented marine stingray species in public aquaria (Semeniuk et al. 2009; Firchau et al. 2004; 2008 AES Census). In public aquaria they are often displayed in interactive exhibits such as feeding or touch pools, which contribute to their popularity. Maintaining a healthy collection is important both for the animals and for public education. Diagnostic tools that may be useful in assessing the health of these animals are hematological and plasma biochemical profiles (Campbell 2015; Grant 2015). There are, however, a lack of reference intervals for many species and little information exists regarding interpretation of changes outside of those intervals. There are many factors that may influence cellular or physiologic changes in elasmobranchs such as environment (water parameters and quality, temperature, season), nutrition, age, sex, species, stress, and disease (Clauss et al. 2008; Southgate 2001). Intervals and medians for selected blood values for this species have been previously reported based on 28 individuals caught in trawls (Cain et al. 2004).

The facility used in this study maintains a southern stingray collection in a touch tank for public interaction. Wild southern stingrays were acquired to add to the collection. The objective of this study was to compare hematological and plasma biochemical values between female southern stingrays that were acclimated to a captive aquarium environment to those recently

introduced to the facility from the wild. It was suspected that the recently-captive stingrays were nutritionally deprived as well as stressed from capture and environmental changes at the time of examinations and therefore differences in analytes relating to those changes would be seen.

5.3 Materials and Methods

This study was approved by the animal care and use committee at Colorado State University.

Animals

There were two groups of stingrays used in this study: the first group had been in captivity for at least two years (acclimated rays) and the second group were newly acquired from the wild and transported approximately 2,000 miles to the aquarium (recently-captive rays). There were 25 stingrays total (13 acclimated rays and 12 recently-captive rays) and the sample size for each group depended on the stingrays caught during a given examination session and successful data or blood collection from individual animals. For this study, eight acclimated and nine recently-captive rays were used. This population was used in another study (Grant et al. 2013), therefore some descriptive statistics, like disc width, may vary slightly due to the different combinations of animals used within each group.

All stingrays were uniquely identified with a passive integrated transponder (PIT) tag (Avid Identification Systems, Inc., Norco CA). Physical examinations were performed and blood was collected, prior to the arrival of the new stingrays, on the acclimated rays and within one month of arrival, during two sessions, on the recently-captive rays. Information such as

capture process, the duration from capture to arrival, the water quality during transport, and other transport conditions was unknown.

Husbandry

Upon arrival to the facility, the recently-captive rays were quarantined for two weeks and treated with two parts per million (ppm) of praziquantel (Fishman Chemical, LLC, Ft. Pierce, FL) for five days for potential parasites. Both the exhibit and quarantine systems were maintained under similar parameters. The acclimated and recently-captive rays were housed in a 45,000-liter exhibit and 11,400-liter quarantine tank, respectively. Each tank contained artificial saltwater with average water quality parameters maintained at 75 degrees Fahrenheit (24 degrees Celsius), 7.5-8.0 pH, 33‰ salinity, zero ammonia, less than 0.05 ppm nitrite, and less than 150 ppm nitrate. Their diet consisted of smelt, pollock, capelin, mackerel, squid, or shrimp daily along with an elasmobranch vitamin supplement (Vita-Zu®, Mazuri®, St. Louis, MO) provided once weekly. The acclimated rays were also presented with feed purchased from the public of varied amounts.

Blood sampling and processing

Blood sampling and physical examinations were performed prior to introduction (acclimated rays within three months of new ray introduction; recently-captive rays within one month after arrival) and eight months after cohabitation of the two groups. The stingrays were handled by manual restraint and placed in dorsal recumbency to induce tonic immobility (Henningsen 1994, Stamper 2007). Physical examinations were conducted after blood collection and included measuring the disc width (DW) of each stingray and a coelomic ultrasound

examination. During the ultrasound examination, liver lengths were measured (Grant et al. 2013) as well as follicle diameters when possible. Although follicle size was not initially recorded in medical records, review of the saved ultrasound images allowed for follicle diameter measurement.

Blood was collected from the caudal tail vein by a ventral approach using a 3-mL syringe and 23-gauge needle (Noga 2010). Blood was immediately transferred into lithium heparin containers (Microtainers® BD, Franklin Lakes, NJ) and fresh blood smears were made. The whole blood samples were maintained in a cooler and submitted to the Colorado State University Diagnostic Laboratory (Clinical Pathology Laboratory, Fort Collins, CO) within four hours of collection. Hematological and plasma biochemical diagnostic profiles were performed. Hematological profiles included manual WBC counts using the Natt-Herrick method (Natt-Pette™, Exotic Animal Solutions, Inc., Hueytown, AL), leukocyte differentials, plasma protein, and packed cell volume (PCV). Leukocyte differentials were determined using Wright's-giemsa stained blood smears and the following cell nomenclature: G_1 (granulocyte type I or heterophil-like cells), G_2 (granulocyte type II or neutrophil-like cells), G_3 (granulocyte type III or eosinophil-like cells), basophils, lymphocytes, and monocytes (Campbell 2015; Grant 2015). Plasma protein was measured by refractometer and PCV by microhematocrit centrifugation. The following plasma chemistry tests were analyzed using the Roche Hitachi 917 (Block Scientific, Nutley, NJ): aspartate aminotransferase (AST), bicarbonate, blood urea nitrogen (urea), calcium, creatine kinase (CK), cholesterol, chloride, globulins, glucose, phosphorus, potassium, sodium, and total protein (biuret method).

Statistical analysis

The data were analyzed using a commercial statistical software package (IBM® SPSS® Statistics version 23 release 23.0.0.0, IBM Corporation, Armonk, NY). Histograms of the data were used to evaluate distribution. Due to the small sample size and violations of assumptions for parametric testing, a nonparametric test, the Wilcoxon Rank Sum test, was used to compare the values for the hematological profiles, plasma biochemistry profiles, disc widths, and follicle size between the two groups. Statistical significance was considered with a probability value of less than 0.05.

The entire process was repeated eight months after the recently-captive rays were introduced into the touch tank.

In addition to the comparative analysis, the correlation between protein values from the hematological profile reports (refractometer) and the biochemistry profile reports (biuret) from both sessions was evaluated using the Spearman's rho correlation coefficient (IBM® SPSS® Statistics version 23 release 23.0.0.0, IBM Corporation, Armonk, NY).

5.4 Results

All of the stingrays were apparently healthy. Prior to introduction, the DW of the acclimated rays (median=60 cm) was significantly larger (p=0.001) compared to the recently-captive rays (median=40 cm). The diameter of the follicles in the acclimated rays (median=1.41 cm) were significantly larger (p=0.001) compared to the diameter of the follicles of the recently-captive rays (median=0.60 cm). Eight months after cohabitation, the DW of the acclimated (median=64 cm) were still significantly larger (p<0.001) compared to the recently-captive rays (median=51 cm). There was no difference in follicle diameter (acclimated ray median=1.7 cm, recently-captive ray median=1.52 cm, p=0.277).

The descriptive and comparative results from the hematological and plasma biochemistry profiles are shown (Tables 5.1-5.4). Significant differences were found between the two test groups in the first sample session for PCV and protein by refractometer, both of which were higher in the acclimated group (Table 5.1). There were no significant differences in WBC counts between the two test groups at either of the two sampling sessions. There were significant differences in bicarbonate, urea, calcium, cholesterol, chloride, globulin, potassium, and total protein between stingray groups prior to introduction (Table 5.3). The acclimated rays had higher bicarbonate, calcium, cholesterol, globulin, and total protein compared to the recently-captive rays, which had higher urea, chloride, and potassium at introduction. These results, along with the results from another study (Cain et al. 2004) that established reference intervals of wild stingrays are summarized in Table 5.5. After eight months of cohabitation in the touch tank exhibit, all hematological and plasma biochemical values showed no statistically significant differences therefore the data were combined and summarized in Tables 5.2 and 5.4, respectively.

There was a significant positive correlation (n=33, r=0.954, $p<0.0001$) between protein values when computing the results from the refractometer and biuret methods on all stingrays from both time periods (Figure 5.1).

Table 5.1. Comparative results of statistically significant different hematological values between acclimated and recently-captive southern stingrays prior to introduction. P-values < 0.05 are considered statistically significant.

	Acclimated stingrays		Recently-captive stingrays		
Plasma Biochemistry Value	n	Median (min-max)	n	Median (min-max)	*p*-value
Plasma protein (g/dL)	8	7.9 (7.1-8.6)	9	5.6 (5.4-6.2)	0.000
PCV (%)	8	29 (24-36)	9	24 (21-31)	0.015

Table 5.2. Descriptive results of hematological values combined from the two stingray groups after eight months of cohabitation.

Hematological Value	n	Median (min-max)
WBC ($x10^3$/uL)	17	28.9 (6.2-55.5)
G_1 ($x10^3$/uL)	17	5.4 (2.2-13.8)
G_2 ($x10^3$/uL)	17	0.0 (0.0-0.3)
G_3 ($x10^3$/uL)	17	1.2 (0.2-5.0)
Basophils ($x10^3$/uL)	17	0.0 (0.0-0.1)
Lymphocytes ($x10^3$/uL)	17	21.7 (1.4-46.2)
Monocytes ($x10^3$/uL)	17	0.0 (0.0-2.0)
Plasma protein (g/dL)	17	7.0 (4.5-7.8)
PCV (%)	17	31 (20-48)

Table 5.3. Comparative results of statistically significant different plasma biochemistry values between acclimated and recently-captive southern stingrays prior to introduction. P-values < 0.05 are considered statistically significant.

	Acclimated stingrays		Recently-captive stingrays		
Plasma Biochemistry Values	n	Median (min-max)	n	Median (min-max)	*p*-value
Bicarbonate (mEq/L)	8	5.3 (3.9-5.7)	9	4.1 (3.1-5.4)	0.027
Urea (mg/dL)	8	1050 (880-1075)	9	1110 (780-1330)	0.036
Calcium (mg/dL)	8	17.2 (16.3-18.3)	9	15.5 (14.6-17.2)	0.002
Cholesterol (mg/dL)	8	205 (139-291)	9	122 (23-176)	0.004
Chloride (mEq/L)	8	247 (168-269)	9	285 (259-313)	0.002
Globulin (g/dL)	7	3.4 (3.0-3.9)	9	1.5 (1.3-1.8)	0.000
Potassium (mEq/L)	8	3.1 (1.7-3.5)	9	3.7 (2.1-5.8)	0.006
Total protein (g/dL)	8	4.4 (3.7-4.9)	9	2.5 (2.3-2.8)	0.000

Table 5.4. Descriptive results of plasma biochemistry values combined from the two groups of stingrays after eight months of cohabitation.

8 months of cohabitation	n	Median (min-max)
AST (U/L)	17	11 (5-27)
Bicarbonate (mEq/L)	17	2.7 (2.1-4.0)
Urea (mg/dL)	17	1050 (870-1130)
Calcium (mg/dL)	17	16.7 (12.6-18.5)
CK (U/L)	17	218 (94-653)
Cholesterol (mg/dL)	17	263 (78-335)
Chloride (mEq/L)	17	265 (254-280)
Globulin (g/dL)	17	2.4 (1.5-3.0)
Glucose (mg/dL)	16	45 (22-66)
Phosphorus (mg/dL)	17	4.8 (3.9-6.7)
Potassium (mEq/L)	17	3.3 (2.6-6.1)
Sodium (mEq/L)	17	261 (250-274)
Total protein (g/dL)	17	3.4 (1.7-4.0)

Table 5.5. A summary of the plasma parameter medians that were significantly different in this study as well as those from a study (Cain et al. 2004) that established reference intervals for wild-caught southern stingrays.

Parameter	Acclimated rays	Recently-captive rays	Wild-caught rays*
Bicarbonate (mEq/L)	5.3	4.1	<5
Urea (mg/dL)	1050	1110	1243
Calcium (mg/dL)	17.2	15.5	16.5
Cholesterol (mg/dL)	205	122	NR
Chloride (mEq/L)	247	285	342
Globulin (g/dL)	3.4	1.5	NR
Potassium (mEq/L)	3.1	3.7	5.0
Total protein (g/dL)	4.4	2.5	2.6
PCV (%)	29	24	22
Sodium (mEq/L)[a]	274	277	315

[a]Na was not significantly different in this study but included here due to its association with Cl.
NR = not reported
* Cain et al. 2004

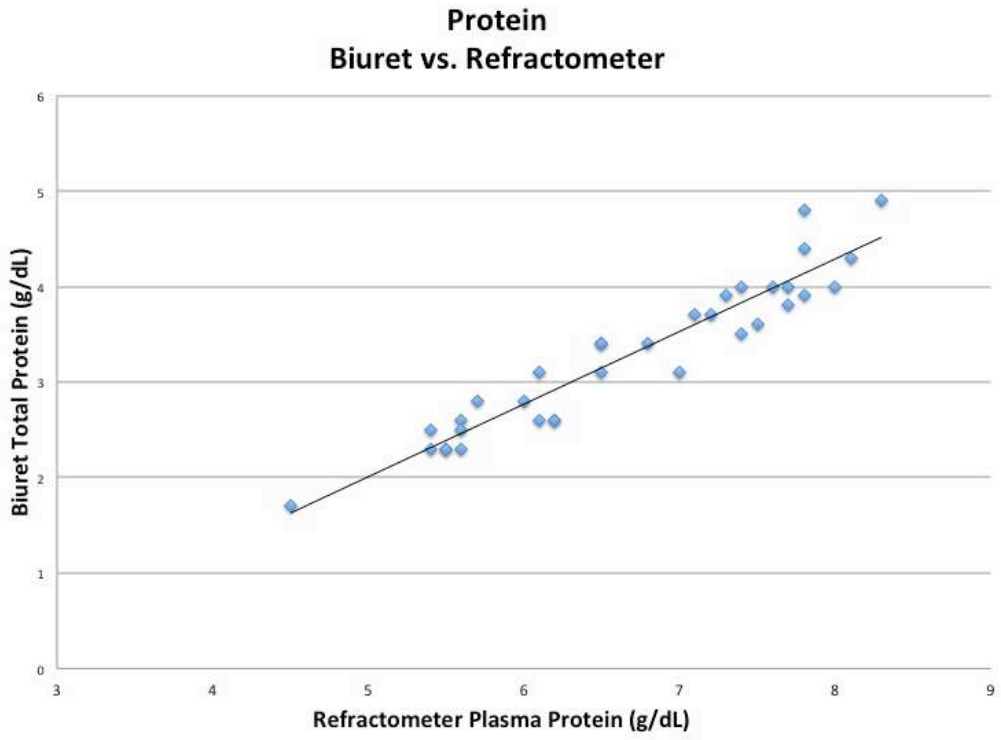

Figure 5.1. The total protein Spearman's rho correlation (n=33, r=0.954, p<0.0001) between the results from the refractometer (RP) and biuret (BP) methods in all the southern stingrays from both time periods.

5.5 Discussion

The wild-caught stingrays introduced into captivity were captured off the southern coast of Florida and transported over 2000 miles to the aquarium. Information regarding the capture technique, the duration from capture to arrival, the life support system during transport, water quality during transport, and feedings during capture and transport were unknown. Although the examinations of the recently-captive rays were completed after they were introduced into a similar environment as the acclimated rays, their previous ocean and transport environments may have played a role in the differences in the electrolytes and urea. Marine elasmobranchs readily move water and salt across the gill epithelium and osmoregulation is achieved by balancing water through renal excretion and balancing sodium and chloride levels with various organs

(Evans et al. 2004). They normally maintain their blood osmolarity slightly higher than their environment. This is accomplished by retaining high levels of solutes, such as sodium, chloride, urea, and trimethylamine oxide (TMAO) (Evans et al. 2004; Hammerschlag 2006; Anderson et al. 2007). By remaining hyperosmotic compared to the environment, they have less water loss and thus avoid dehydration (Hammerschlag 2006). Because they have the ability of regulating their electrolyte and urea plasma concentrations based on their environment, one explanation of the differences between chloride, potassium, and urea may be that the recently-captive rays were previously exposed to an environment that was higher in salinity. Although there was not a significant difference between groups when comparing sodium, the median values of sodium were higher in the recently-captive group, which would be expected if exposed to higher salinities. The primary organs involved with regulation of these solutes (sodium, chloride, potassium, and urea) are the rectal gland and kidney. The rectal gland of elasmobranchs controls the majority of salt excretion, with secretory fluid having higher concentrations of NaCl compared to the surrounding seawater, but also contains ion pumps and channels that transport potassium across the basolateral cell membranes (Evans et al. 2004). A cotransport protein (NKCC), a Na-K activated ATPase, a K^+ channel, and a Cl^- channel on the basolateral cell membrane have been shown to osmoregulate *Squalus acanthias* (Evans et al. 2004). Initially being in an environment with a higher salinity may have resulted in higher concentrations of these ions until the rectal gland could excrete adequate amounts to regulate to the new environment. The kidney is involved with sodium and chloride movement, although to a lesser extent, as well as urea reabsorption and clearance (Evans et al. 2004; Hammerschlag 2006). Some marine elasmobranchs seem to acclimate to lower salinities, not only by increasing urine flow (thus eliminating urea, sodium, and chloride), but also possibly by decreasing urea synthesis

in the liver (Tam et al. 2003; Hazon et al. 2003; Anderson et al. 2005). Table 5 shows a summary of the significantly different values in this study and in Cain's study. Although a different analyzer was used in the study by Cain et al., the results complement the trend in this study. For example, the parameters that were elevated in the recently-captive rays compared to the acclimated rays (urea, chloride, and potassium) were also shown to be higher in the wild-caught rays.

Another contributing factor explaining changes in electrolytes is stress. There are a number of factors that may influence stress in fish including water quality, environmental conditions, social environment, handling, transport, nutrition, therapeutics, and pathogens (Clauss et al 2008; Pasnik et al. 2010). The recently-captive rays were possibly experiencing chronic stress from capture, confinement, overcrowding, transport, or environmental (poor water quality) and dietary change (Skomal and Bernal 2010). The transition of wild animals into captivity is classified as chronic stress (lasting days to weeks) and may prolong the differences in blood values depending on the severity of the stressor and the time it takes to acclimate (Skomal and Bernal 2010; Manire et al. 2007). Osmoregulatory function is affected by stressful events and may not immediately respond nor quickly stabilize (Eddy 1981); however, it has also been reported that increased sodium and chloride from marine fish, undergoing capture stress, normalized within 24 hours (Eddy 1981; Wells et al. 1986; Cliff and Thurman 1984). The increase of sodium and chloride is mainly attributed to an increase of water outflow. Potassium also remained elevated in previous studies presumably from muscle (intracellular) leakage (Wells et al. 1986; Cliff and Thurman 1984). The stingrays in this study probably experienced relatively different degrees of stress during the entire process from capture to exhibition. Although the examinations were performed several weeks after their arrival, they were disrupted

during the move from quarantine to exhibit and again during their examinations. The examination process between the two groups was the same; however, the acclimated rays were much more accustomed to human interaction and routine examinations.

Other hematological or plasma biochemical values in fish shown to be affected by either chronic or acute stress include glucose, leukocyte counts, bicarbonate, PCV, protein, lactate, hemoglobin, and cortisol (in teleosts) (Clauss et al. 2008; Evans et al. 2004; Smith et al. 2004; Stoskopf 2010; Ross and Ross 2008; Roberts et al. 2010). In this study, lactate, hemoglobin, and cortisol (not applicable) were not analyzed and no significant differences were seen in glucose or with the leukocyte counts. Measuring cortisol in elasmobranchs is not applicable since it does not exist. The major stress hormone in elasmobranchs is considered 1α-hydroxycorticosterone (1α-OH-B) and is difficult to measure (Manire et al. 2007; Skomal and Mandelman 2012). Although corticosterone (also from the interrenal or adrenocorticoid gland), a 1α-OH-B precursor, has also been found in serum and feces when studying stress response, the amount of increased concentrations and cross-reactivity with 1α-OH-B, support that it is not likely a primary stress hormone (Anderson 2012; Karsten et al. 2003; Manire et al. 2007; Skomal and Mandelman 2012). The glucose results in this study may not be reliable given the duration between collection and analysis. The samples were not centrifuged to separate cellular components from plasma and therefore were vulnerable to glucose consumption (generally at a rate of 10% per hour) (Weiser 2012). The glucose results in this study were similar to those in wild southern stingrays (Cain et al. 2004) and were lower compared to captive cownose stingrays (Ferreira et al. 2010). A stress leukogram in elasmobranchs is similar to that of other fish in that it is represented by a general leukocytosis with lymphopenia and relative granulocytosis (Campbell 2015; Clauss et al. 2008; Grant 2015; Roberts et al. 2010). There was not a

significant difference in these cell counts but the recently-captive rays had a slightly higher leukocyte count with a median increase in lymphocytes and decrease in granulocytes compared to the acclimated rays. Overall, the WBC counts in the pooled data after eight months of cohabitation (Table 2) appeared subjectively higher based on the authors' experience. The values for WBC counts are similar compared to other reports from wild caught free-ranging Atlantic sharpnose sharks (*Rhizoprionodon terraenovae*), bonnethead sharks (*Sphyrna tiburo*), and spiny dogfish (*Squalus acanthias*) but higher compared to captive cownose stingrays (*Rhinoptera bonasus*) (Haman et al. 2012; Ferreira et al. 2010). When comparing these values to a study done on white-spotted bamboo sharks (*Chiloscyllium plagiosum*), the values are most similar to the pre-operative males with traumatic clasper wounds (Alexander et al. 2016). The stingrays in this study had presumed bite wounds as a result of mating behavior which may have induced an inflammatory response but further studies in hematological values for southern stingrays are needed.

The remaining analytes (bicarbonate, PCV, and protein) potentially affected by stress were significantly different between the two groups. The direction of the change in bicarbonate showed the acclimated rays having higher levels of bicarbonate compared to the recently-captive rays. Hyperactivity from stress may cause an acidosis thereby decreasing the bicarbonate (Smith et al. 2004). The acidosis may be from respiratory or metabolic mechanisms in the recently-captive rays. The type of acidosis in elasmobranchs appears to vary among species and is caused by a relative hypoxia or an increase in anaerobic activity (Skomal and Bernal 2010). Either type of acidosis is a potential cause for a decrease in bicarbonate in this study but exercising to fatigue is more probable especially upon entering quarantine and the exhibit. The decreased PCV and protein in the recently-captive rays may be a result from stress, age, diet, or disease. The median

PCV for this group was greater than 20% and therefore would not be classified as an anemia (Campbell 2015; Clauss et al. 2008). Stress from acclimating to captivity, starvation, and confinement are known to decrease the PCV in fish which certainly may have been the case here (Stoskopf 2010; Roberts et al. 2010). A study evaluating blood analytes between wild southern stingrays in a tourist site versus a non-tourist site resulted with lower PCV and protein levels in the tourist site rays which was attributed to those rays being in a poorer state (Semeniuk et al. 2009). Although being in a poorer state is subjective, this is possibly the case of the recently-captive rays in this study. It is likely that they were tightly confined during transport in suboptimal water conditions with a lack of nutritional support. They were in a negative metabolic state after arriving to the facility based on the small liver sizes (Grant et al. 2013). The lack of nutritional support may also explain the difference in plasma cholesterol.

Another cause for a lower PCV and protein in stingrays is blood loss from parasites. There was no apparent blood loss from the recently-captive rays during examination; however, mild blood loss from parasitism is possible. Wild elasmobranchs have been reported with a number of different external and internal parasites (Ruhnke 1994). Naturally, these animals may not succumb to the infestation of such parasites, but in a stressful situation, proliferation and detriment may occur. These stingrays were not specifically tested for any parasites when they arrived but as part of the aquarium's protocol for new animal arrivals and introductions, the rays were held in quarantine and treated with praziquantel. The exams were performed after treatment but if parasites contributed to the lower PCV, then perhaps not enough time lapsed for adequate red blood cell regeneration.

Younger fish of the same species also tend to have lower PCVs compared to older fish (Clauss et al. 2008; Roberts et al. 2010). The exact ages of the rays here are unknown but using

size to estimate relative age implies that the acclimated rays were older. The recently-captive rays had smaller disc widths compared to the acclimated rays. The difference in size may also contribute to the difference in protein. Presuming that the larger rays are older and thereby reproductively mature (DW>70 cm, based on the authors' experience with pregnant rays at this facility), increases in protein may be explained by increased mobilization of vitellogenin. The follicles in the acclimated rays were larger compared to the recently-captive rays' follicles which also may be an indication of reproductive maturity. A study done on wild stingrays exposed to public interaction showed a possible association between increased DW and elevated serum protein levels; however, the stingrays analyzed in that study were all larger than the largest ray in this study (Semeniuk et al. 2009). The suspected difference in reproductive maturity may also account for the difference in plasma calcium levels with the acclimated rays mobilizing more calcium (Palmeiro et al. 2007). Although size may be a contributing factor to the differences in these analytes; environment, diet, and stress are more likely contributing to the differences as size was still significantly different after eight months. After eight months, the largest of the recently-captive rays measured at 54 cm indicating that they were not reproductively mature yet.

After the eight month cohabitation period, there were no differences in plasma biochemistry values between the acclimated and recently-captive rays (Table 4). These results were similar to those reported for wild caught bonnethead sharks (*Sphyrna tiburo*) and captive smooth dogfish (*Mustelus canis*) (Harms et al. 2002; Persky et al. 2012).

The hematologic and plasma biochemistry profiles each provided values for protein. A difference in protein values existed as the plasma protein reported in the hematological report was measured using a refractometer whereas the total protein from the plasma biochemistry profile was measured using spectrophotometry, the biuret method in this case. It was expected

that the refractometer would overestimate the protein values as this method is based on the refractive index of the fluid and other solutes may contribute (Stoskopf 2010; Weiser and Allison 2012). The positive correlation between methods has been previously demonstrated in wild southern rays as well as in other species (Cain et al. 2004; Cray et al. 2008; George and O'Neill 2001; Harms et al. 2002).

In conclusion, capture, confinement, transport, and environmental and nutritional changes were probable factors involved with the differences in hematology and plasma biochemistry values found in the stingrays in this study. Although differences were noted around the time of introduction, there were no differences after eight months and it appeared that the recently-captive rays were acclimated to their new environment. The results presented in this study may serve as a hematological and plasma biochemical baseline for southern stingrays maintained in similar environmental conditions.

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CHAPTER 6: Brief Report: Predicting parturition dates ranges from ultrasonographic fetus fetal body depth measurements of southern stingrays, *Dasyatis americana*

6.1 Synopsis

Zoos and aquariums are often involved in captive breeding of certain species for a variety of reasons. Captive breeding and research programs operate in accordance with other organizations or individually with regards to the species, location of the facility, or other needs (reintroduction, sustainability, exhibition, education, or research). The aquarium in this study began breeding southern stingrays as the collection matured. One issue faced was limited space. The pregnant animals were maintained on exhibit, which put the pups at risk of being eaten or traumatized by other fish if born unsupervised. To allow the females a safer birthing environment, a better understanding of female stingray reproduction and gestation were required. The purpose of this study was to track fetal body depth measurements of pregnant southern stingrays to predict an approximate parturition date range. Obtaining a parturition date range would allow for temporary holding in quarantine for the females to pup safely. Eight pregnant stingrays were monitored during three gestation sessions for two years. The fetal body depth measurements were taken using ultrasound. The first two gestation sessions were used to develop a linear regression model to predict a parturition date range and the third gestation session was used to assess the accuracy of the model. The regression model was Days Before Parturition = 139.75-31.249*Fetal Body Depth. This model was tested on three stingrays and predicted the parturition dates for two of them within 1-2 weeks and the third one within one month. There are many factors that can affect gestation length but clinically this model was helpful in determining parturition date ranges at this aquarium.

6.2 Introduction

Southern stingrays inhabit over 120 facilities worldwide and are frequently visited in popular tourist sites (AES Census 2008, Semeniuk and Rothley 2008). Stingrays exhibited in aquariums likely arrived in those situations as a result of wild capture or captive breeding while those interacting in ecotourism reside in their relatively natural environment.

The International Union for Conservation of Nature (IUCN) Red List of Threatened Species™ compiles and reports information regarding the extinction risk of known species of plants, fungi, and animals. The evaluated species are divided into two categories: Adequate Data and Data Deficient. The species for which there is adequate data are further classified by their risk of extinction from lowest to highest risk: Least Concerned (LC), Near Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CR), Extinct in the wild (EW), and Extinct (EX) (IUCN Red List of Threatened Species™ 2015). The southern stingray's global status is Data Deficient meaning "there is inadequate information to make a direct, or indirect, assessment of its risk of extinction based on its distribution and/or population status" (IUCN Red List of Threatened Species™ 2015, Grubbs et al. 2006). In the United States it is assessed as Least Concern due to an apparent healthy population with not much threat but in Brazil it is considered Vulnerable due to increased fishing (Grubbs et al. 2006). Anecdotal reports suggest that wild populations are declining (SEZARC 2014) and information from the Mexican Official Standard suggest a decline in batoids (Ramirez-Mosqueda et al. 2012).

Zoos and aquariums are active participants in species conservation efforts in a variety of avenues. Captive breeding and research programs operate in accordance with other organizations or individually with regards to the species, location of the facility, or other needs (reintroduction, sustainability, exhibition, education, or research). For example, threatened or

endangered species may be involved in large-scale programs such as the Species Survival Plan® (SSP program, Association of Zoos & Aquariums), other locally-run programs like New England Aquarium's lobster research program (American Lobster Research Program, New England Aquarium), or Monterey Bay Aquarium's research on great white sharks (Shark Research, Monterey Bay). Animals not classified as threatened or endangered may be involved in captive breeding programs for reasons such as potential depletion of the species in the wild thus becoming threatened or endangered, research, sustainable living (farming or aquaculture), the high cost of harvesting from the wild, or to reduce the potential risk of spreading diseases to captive collections from wild populations.

Elasmobranch reproduction is complex as it may be described as two modes, which include oviparity and viviparity depending on the species. Understanding the details of each mode uncovers the complexity of reproduction in this animal group. Viviparous species are either placental (placentrophy) or aplacental which are further classified as yolk sac (lecithotrophy), with trophonemata (histotrophy), or oophagous or intrauterine cannibalism (adelphotrophy) (Hamlett et al., 2005). Southern stingrays are aplacental viviparous species with trophonemata (finger-like projections extending from the uterine mucosa) that secrete histotroph or uterine milk to supply nutrients to the fetuses. The offspring are referred to as pups and the act of giving birth is often referred to as pupping.

At this particular facility, captive breeding began as the southern stingray collection matured. Captive breeding was beneficial in the respect that it would eliminate the need to capture wild animals and transport them in order to stock the exhibit, reduce the risk of introducing potential disease from a wild population, and reduce the cost of transport. One obstacle, however, was the facility was limited with space therefore pregnant animals were

maintained on exhibit which put the pups at risk of being eaten or traumatized by other fish if born unsupervised. In order to alleviate these issues, a better understanding of the female stingray reproductive stages and gestation duration were required. The purpose of this study was to track fetal body depths of pregnant southern stingrays to predict an approximate parturition date range. The aquarium was limited in space; however, if given an estimated parturition date range, then it would be manageable to separate pregnant females temporarily thus giving them a safer place to pup and reducing the risk of pup deaths.

6.3 Materials and Methods

This study was approved by the Colorado State University animal care and use committee.

The stingrays at this facility were uniquely identified using a passive integrated transponder (PIT) tag (Avid Identification Systems, Inc., Norco CA). They were housed in a 45,000-liter touch pool exposed to filtered natural sunlight and with artificial saltwater maintained at 75 degrees Fahrenheit (24 degrees Celsius), 7.5-8.0 pH, 33‰ salinity, zero ammonia, less than 0.05 parts per million (ppm) nitrite, and less than 150 ppm nitrate. Their diet consisted of sharing approximately 4.5 kilograms of a combination of smelt, pollock, capelin, mackerel, squid, or shrimp daily along with an elasmobranch vitamin supplement (Vita-Zu®, Mazuri®, St. Louis, MO) provided once weekly. The pool was shared with cownose stingrays (*Rhinoptera bonasus*) and allowed public interaction with an option to purchase feed.

As part of routine physical examinations, the disc widths (DW, in cm) and liver-to-coelom ratios (liver %) were measured (Grant et al. 2013). From the routine exams, eight

pregnant, captive, female southern stingrays were used for this study. The time of conception was unknown and the animals were identified as pregnant during a routine physical examination.

The stingrays were monitored for up to two years and fetal body depth measurements (in cm) were recorded as often as possible, usually once every 1-4 weeks, until parturition. Examinations were performed in the exhibit by catching the animal with a nylon or rubber net and placing her in dorsal recumbency. In dorsal recumbency, this species undergoes tonic immobilization. The ultrasound measurements were taken using a 7.5-MHz linear array transducer and ultrasound unit (Aloka SSD-990v, Aloka, Inc. Wallingford, CT). The probe was positioned on each animal such that a transverse orientation of the fetus could be seen (Figure 6.1). Subjectively, the location of the greatest fetal body depth was determined and measured. If there was more than one pup, attempts were made to measure each one. For stingrays with more than one measurement per examination, the fetal body depths were averaged for use in the prediction model. Once the stingrays gave birth, the number of pups (NP) and the days from the first examination until parturition were recorded (DEP).

All of the stingrays in this study experienced their first pregnancy. There were three separate gestation periods among the eight stingrays of which fetal body depth measurements were collected. The measurements from the first two gestation sessions were used to formulate the model for predicting parturition dates and the last gestation period measurements were used to assess the model for accuracy.

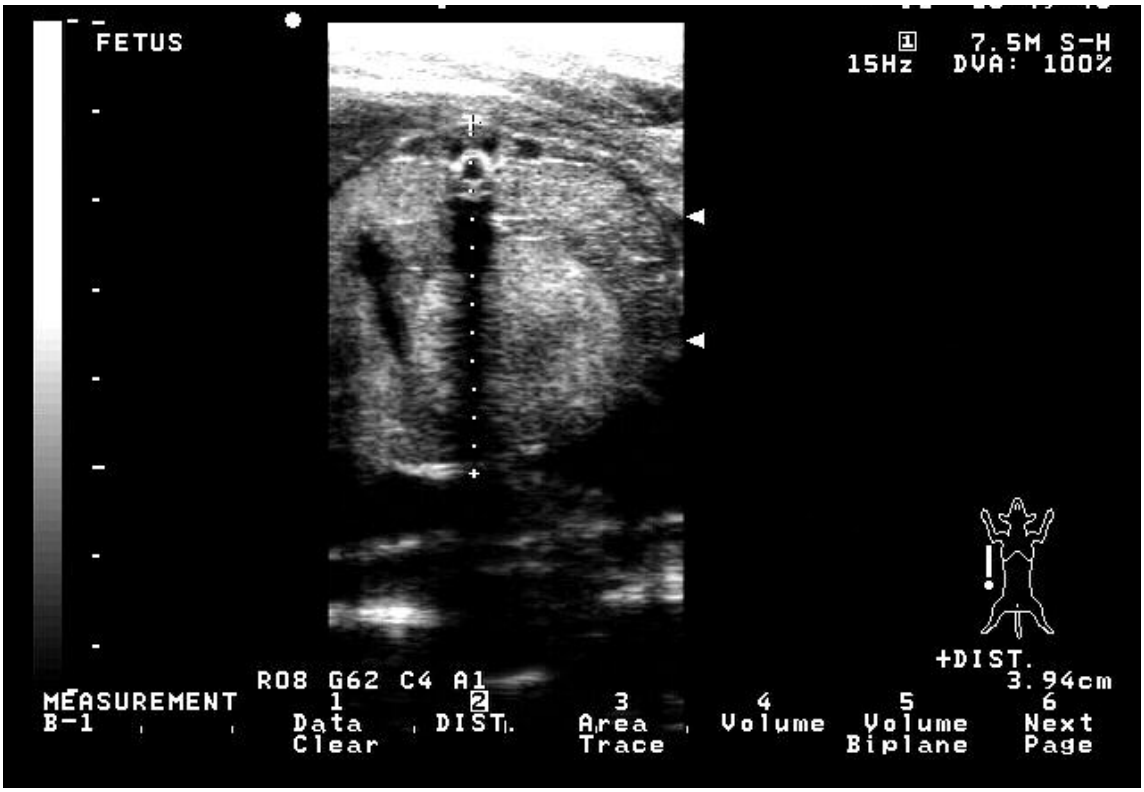

Figure 6.1. An ultrasonographic image of a transverse orientation of the coelomic cavity of a southern stingray fetus. The dotted line bookended by a large and small plus sign represent the fetal body depth measurement. The distance in this example is 3.94 cm shown in the lower right corner of the figure. The dotted line is seen against the acoustic shadow made by cartilaginous spine. The round, hyperechoic structure that partially crosses the acoustic shadow is the fetus' spiral intestine.

Statistical Analysis

In total, 48 measurements of the fetal body depth over time were used from the first two gestation sessions for development of the parturition date prediction model. All data were analyzed using a statistical software package (IBM® SPSS® Statistics version 23 release 23.0.0.0, IBM corporation, Armonk, NY). A linear mixed model was used to analyze fetal body depth as a potential predictor for days before parturition. This model accounts for the longitudinally collected data of the females (subjects) and the dependency of the fetuses to the female which were repeatedly measured. There was only one possible predictor, fetus size as a

continuous variable, therefore it was the only variable used. The outcome, days until parturition, was also continuous. To further setup the analysis, fetus size including the intercept were fixed effects and the intercept only was used for random effects. Subject groupings were the pregnant female stingrays and the covariance type was variance components. Significance was considered with a *p* value of less than 0.05.

During the third gestation session, three stingrays were pregnant and 38 measurements were taken to assess the model equation by descriptive statistics and a box and whisker plot. A bar chart was used to visualize the increase of average fetal depths over the gestation period.

6.4 Results

Eight stingrays (identified as stingrays 1-8) were monitored in this study, which included three gestation periods or sessions. Two stingrays were pregnant during all three periods, one was pregnant twice, and five were pregnant only once. The time between litters of each of the three stingrays monitored during the second and third gestation sessions were approximately 7-8 months apart (i.e., the stingrays that pupped during the second gestation session each had their next litter 7-8 months later). The parturition dates for the litters during the second and third sessions were stingray 4-June 9th and Jan 21st, stingray 6-June 9th and Dec 28th, and stingray 7-May 24th and Dec 8th, respectively. The descriptive statistics are summarized in Table 6.1 and include the disc width measurements (DW), the liver-to-coelom ratio (Liver %), the gestation sessions of which the stingrays were pregnant (GS), the total days from the first examination until parturition for a given stingray and their respective gestation session (DEP), the number of measurements recorded during the gestation session (MR), and the number of pups produced by stingray per gestation session (NP). The gestation time periods covered were: one occurred from

mid-July until the end of November, two occurred from the end of April until mid-June, and three occurred from mid-September until mid-January.

As fetal body depths (FBD) increased, there was a decrease in the days remaining before parturition (DBP) from the first two gestations sessions. There was a strong correlation between measurements and days (n=48, r=-0.852, CI [-0.915, -0.749], p<0.0001) shown on the scatter plot (Figure 6.2). The linear mixed model equation representing this relationship is: DBP = 143.80-FBD*32.90 meaning that for every one centimeter growth in fetal body depth, the pregnant female is 32.90 days closer to parturition. Approximately 35% of the variability can be explained by the pregnant females.

During the third gestation session, two stingrays (4 and 7) were examined five times and stingray 6 was examined four times. During each examination, 1-4 measurements were taken. The depths were averaged by date and charted to show increased fetal growth by stingray (Figure 6.3). Descriptive statistics were used to assess the model equation. The median (minimum, maximum) difference in days between the predicted and actual parturition dates for stingray 4 was 29 (14, 47) days, stingray 6 was 11 (-2, 20) days, and stingray 7 was 6 (-9, 10) days (Figure 6.4).

Table 6.1. A summary of descriptive statistics for each stingray.

Stingray	DW (cm)	Liver %	GS	DEP	MR	NP
1	69	56.3	1	48	1	2
2	82	59	1	109	6	3
3	72	59.5	1	116	1	2
4	85	58.1	1	107	6	2
	NR	NR	2	40	5	4
	102	58.3	3	121	13[a]	4
5	96	62.5	1	116	7	4
6	71	88.2	1	91	3	3
	NR	NR	2	91	6	4
	83	52.5	3	90	12[a]	3
7	NR	NR	2	24	4	2
	79	50	3	77	13[a]	4
8	93	NR*	2	54	7	6

DW (cm) = disc width measured in centimeters
Liver % = the liver length divided by the distance from pectoral to pelvic girdle
GS = gestation session
DEP = total days from first exam until parturition
MR = number of measurements recorded during gestation session
NP = number of pups from that gestation session
NR = measurement not recorded
NR* = measurement not recorded but note in record - extended to cranial aspect of the stomach
a = multiple measurements taken during individual exams

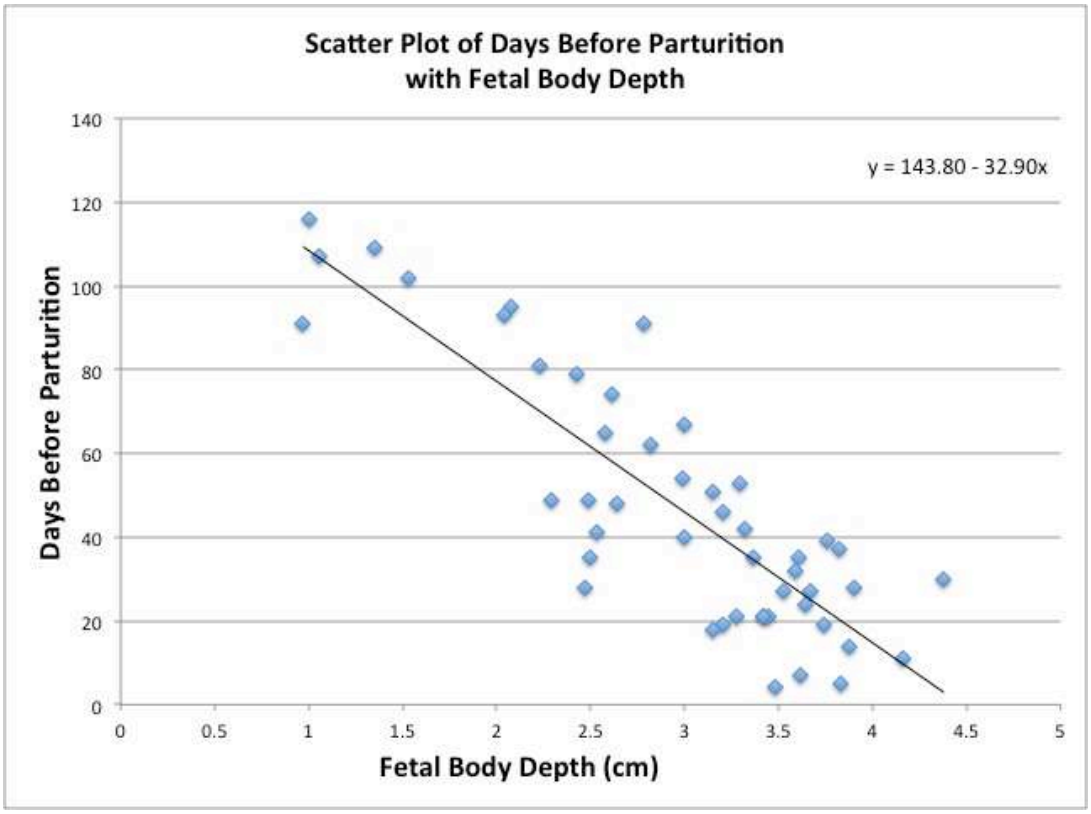

Figure 6.2. Scatter plot and best-fit line of 48 paired samples of fetal body depth (FBD) and days before parturition (DBP) (Pearson correlation coefficient, -0.852, CI [-0.915, -0.749], p<0.0001). The linear regression model equation representing this relationship is: DBP = 143.80-FBD*32.90.

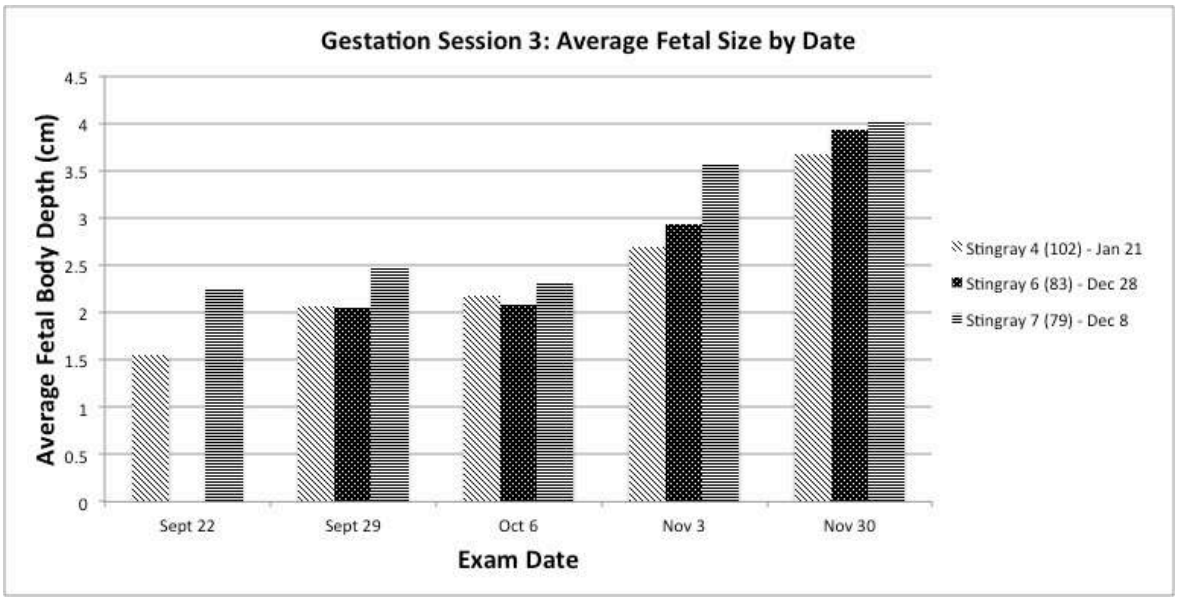

Figure 6.3. Bar chart for the average fetal body depths of the three stingrays pregnant during the third gestation period by examination date. The legend shows the stingray identification number, the disc width of the pregnant stingray in parentheses, and the actual parturition date.

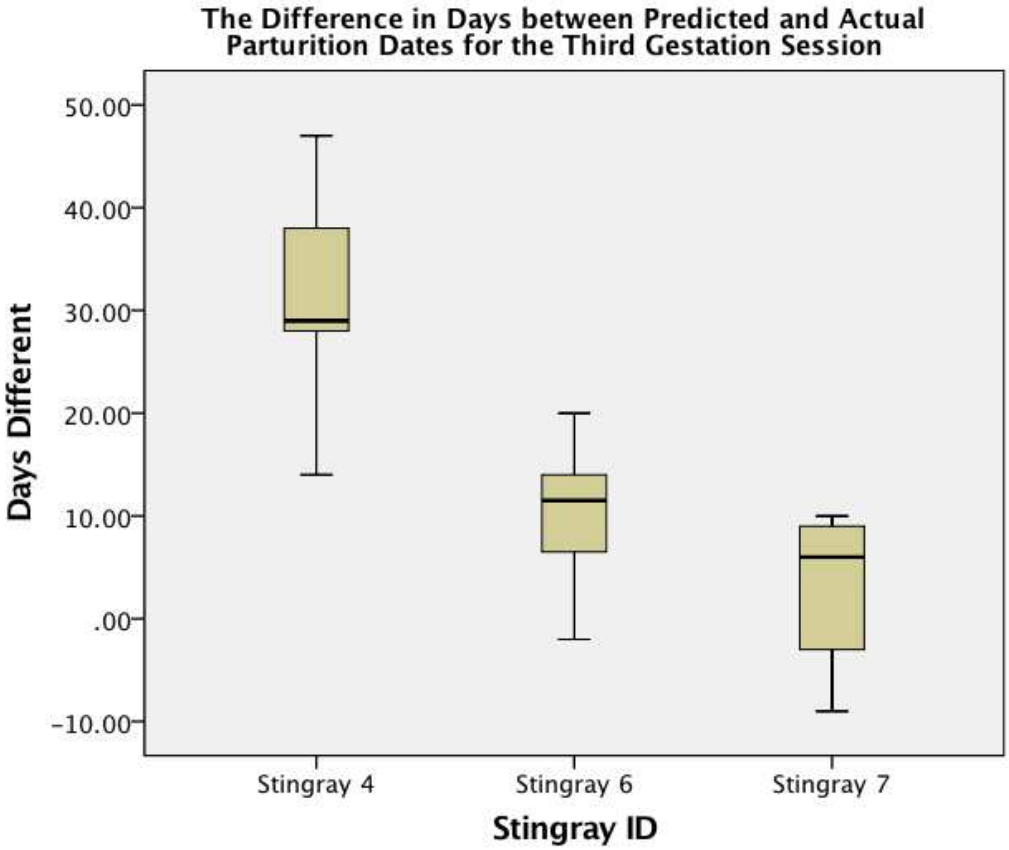

Figure 6.4. Box and whisker plot of the difference in days between the predicted and actual parturition dates for the three stingrays in the third gestation session.

6.5 Discussion

The purpose of this study was to track fetal body depths to see if this could be a predictor for parturition date. This aquarium did not have ancillary space to hold pregnant females for their entire gestation but would be able to move them temporarily if given a parturition date range. Overall, eight stingrays participated during three different gestation sessions over two years. The metabolic toll of pregnancy was confirmed by the decrease in liver lengths with respect to the lengths of the coloemic cavities, ranging from 50-88%. It was also noted that the disc width of the pregnant females ranged from 69-102 cm. This range is similar to the disc widths of reproductively mature captive females at other facilities (Henningsen and Leaf 2010) and in the wild (Ramirez-Mosqueda et al. 2012).

Gestation in southern stingrays has been perceived as the period from copulation to parturition and is reported as 4.4-7.5 months in captive rays (Henningsen 2000) and eight months in wild rays (Ramirez- Mosqueda et al. 2012). A more accurate definition of gestation is the time from fertilization until parturition (Wyffels 2009). Although copulation was not seen in this study (and time of fertilization is unknown), the length of gestation appears to coincide with the report on captive rays. Three stingrays had consecutive pregnancies and the time between litters was 7-8 months in each case therefore the length of gestation was less and fits within the previously reported range for captive stingrays. Likewise, fecundity of southern stingrays in this study (2-6 pups) was similar to that of other reports (Henningsen 2000; Hamlett et al. 1996; Ramirez- Mosqueda et al. 2012).

The linear regression model was developed from the measurements recorded from the first two gestation sessions: DBP = 143.80-32.90l*FBD, where DBP refers to the days before parturition and FBD refers to the fetal body depths. Using this equation to predict date ranges

for the third gestation session resulted in ranges within approximately 1-2 weeks for stingrays 6 and 7 but 2-6 weeks for stingray 4. Despite having a disc width of approximately 20 cm larger than stingray 6 and 7 in this group, stingray 4 gave birth to a similar number of pups. Maternal size has been reported to vary directly with litter size although may differ between wild and captive stingrays (Henningsen 2000; Ramirez- Mosqueda et al. 2012). Perhaps with stingray 4 being the largest stingray, it allowed for more physical space for pup development thereby presumably prolonging her gestation period. Likewise, stingray 7 was the smallest stingray and carried the relatively largest fetuses throughout the third gestation session. Stingray 7 pupped the earliest after the last examination, which may be explained by a relatively smaller uterine capacity. Uterine capacity in other species has been thought to limit litter size either due to morphology or size of the female (Ebert and Cowley 2009). Throughout the gestation period, stingray 4's fetuses were the smallest on average from the first examination date. Previous parturition dates for stingrays 4, 6, and 7 were June 9th, June 9th, and May 24th, respectively. The third gestation session began the same year and the first exam was not started until September 22nd. Certainly, stingray 4's gestation period could have started later compared to stingray 6 and 7 but it is still likely in that scenario that the pups were larger at the time of parturition compared to the other stingrays' pups. Although litter size has been shown to vary directly with maternal size, it has also been shown to vary indirectly with the size of the pups (Henningsen 2000), which supports the presumption that stingray 4's pups were larger. Based on the model, stingray 4's predicted parturition date was December 25th and the actual date was January 21st, almost a difference of a month compared to the 1-2 weeks of the other two stingrays. Measurements beyond the last exam date were not performed because it was suspected that the females could be within two weeks of parturition and stress from capture and restraint may influence reproduction

(Henningsen 1999). In some cases abortion has occurred in capturing wild stingrays and examining captive stingrays (Ramirez- Mosqueda et al. 2012; White et al. 2001; Mollet et al. 2002).

Factors that may affect reproduction, and therefore gestation or parturition, in elasmobranchs in general may include season, stress, hormone levels, diet, photoperiod, temperature, and social structure (Henningsen 1999; Fahy et al. 2007; Waltrick et al. 2012; Maruska and Gelsleichter 2011). In this study, any of these factors may have played a role in affecting reproductive events at any given time; however, perceived season, diet, photoperiod, temperature, and social structure remained relatively constant at this aquarium. Although some factors appear to remain constant, they cannot be ruled out of having some effect. Stress and hormone levels may contribute to changes in reproduction as well. For example, increased levels of plasma progesterone are proposed to impact follicular development, embryonic development, and parturition in the Australian sharpnose shark (*Rhizoprionodon taylori*) (Waltrick et al. 2012). Hormone levels contribute to the ovarian cycles, which can occur either concurrently or in sequence with gestation (Ramirez- Mosqueda et al. 2012). Correlations between progesterone and day length, progesterone and water temperature, and inverse correlations between progesterone and the change in day length have been shown in round stingrays (*Urobatis halleri*) (Mull et al. 2010). The coordination of ovarian cycling and gestation likely depend on hormonal levels, which likely coincide with environmental cues and vitellogenesis. With stingray 4 being the largest ray in the touch pool, perhaps the relative space seemed more confined and inadequate for pupping and therefore delayed parturition. A report of a captive cownose stingray (*Rhinoptera bonasus*) presumably failing to pup due to artificial environmental conditions from endocrine inhibition has been documented (Henningsen 1999).

In future studies, it would be advisable to measure and weigh the pups upon parturition to get a better indication of correlation between pup sizes at parturition compared to just body depths in utero. Other helpful measurements to consider would be disc width of the fetuses in utero or maternal weight changes throughout gestation. Other variables for consideration to include in the model for determining parturition dates might be maternal size and hormone levels. And although there are many factors that can affect gestation length, clinically this model was helpful in determining parturition date ranges at this aquarium.

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CHAPTER 7: Concluding Remarks

7.1 Significance of work

Animals in captivity serve a purpose for continuing education, preservation, and conservation of their species but their presence in captivity is not without risk to their health. There are many factors involved with the healthcare of these animals, which may include environment, diet, transport, reproduction, and preventative care. The information gained from captive animals can also be useful to understand the biology, physiology, and ecology of their wild counterparts. The work presented in this dissertation is intended to further the knowledge of care and reproduction for southern stingrays in captivity. The stingrays in this project were housed in a relatively new touch pool at an aquarium. Within the first couple of years of having the new touch pool exhibit, it was noticed that there were quite a few stingray deaths, which initiated this project.

7.1.1 Research at a public aquarium

The research at the aquarium followed the protocol for conducting research in a public aquarium (Smale et al. 2004). This project was coordinated between Colorado State University and the Downtown Aquarium in Denver therefore equipment and other resources were available. The appropriate approvals were sustained by Colorado State University's IACUC and the Denver Aquarium's management. Consideration for the animals' well being as well as normal operation of the aquarium was also important. This was accomplished by conducting regular physical examinations or other procedures as deemed appropriate for diagnosis or treatment (as one would conduct normally in any clinical setting) during closed business hours of the facility

when possible. The aquarium provided at least one aquarist to assist with all coordinated examinations.

Advantages to conducting research at the aquarium were availability of subjects (stingrays), having access to necropsy records, as well as being able to monitor and review history and environmental parameters. The challenges presented with conducting a study at the aquarium were discussed briefly in section 2.6.4 (Limitations) of chapter 2 but include small sample size with lack of generalizability, lack of a controlled environment (compared to a laboratory), and the distance from the university to the aquarium. The number of stingrays in the collection at the aquarium determined the sample size. Concerning the controlled environment, there are many areas and resources at the aquarium in which a controlled study can be conducted. The difference in this case was that the intent was to study exhibit animals in the environment in which they were displayed in captivity making it, as expected, an environment that was drastically different from a laboratory setting. Although this presented added challenge, it offered a realistic approach to understanding the health concerns of stingrays in these conditions and the results were representative of stingrays in their captive habitat. Unfortunately, comparing the captive stingrays to their wild conspecifics was not possible due to location and lack of funding. Comparisons to other studies were done when possible. The distance from the aquarium to the university posed a challenge because when a stingray died, it took time for them to either be delivered to the hospital or to travel to the aquarium to perform the necropsy. When communication was done relatively quickly, the animal could be placed on ice in the interim. Many times the stingrays were placed in the freezer, which compromised results.

The routine veterinary visits only occurred every other week and, during times of stingray examination, additional days were added as needed to conduct all the examinations in a reasonable time frame. There were or, at least, could have been many more variables introduced without warning or explanation which is very similar to conducting clinical studies with client-owned pets. For example, stingrays were moved to other, larger exhibits (or worse, other facilities) when they outgrew the touch pool and PIT tags were not scanned and therefore not identified. Another challenge encountered was the communication with and availability of the aquarium staff. Often times aquarists would rotate the care of exhibits or care would be shifted when the primary aquarist was off work. If a stingray experienced anorexia, trauma, illness, fighting, or death during these shift changes or between shifts, occasionally it was not communicated until the next veterinary visit. The delay in mortality awareness was particularly unfortunate due to the potential valuable information that may have been collected during that necropsy (often in these situations, the stingrays were autolytic and/or frozen). This presented an opportunity for education and communication. The elasmobranch necropsy procedure document (Appendix 6) was specifically created to help the aquarium staff perform necropsies, identify organs, and appropriately collect samples in the absence of the veterinarians. This proved to be a large learning curve and education and communication continue.

The benefit of doing a project or study like this with exhibited animals and having to work with such a variety of employees at the aquarium was that it improved communication as a whole. Direct communication was established and it was clear to everyone involved that everyone had the same goal of maintaining and improving the health of the animals. As potential morbidity occurred, the teamwork needed to make adjustments to the facility or exhibit, water quality, health monitoring, and treatment administration was apparent. Although the

specific cause of death was not determined in many of the stingrays that died initially, changes based on suspected risk factors improved the situation and decreased mortality at this facility.

Although the formal studies of this project are complete, the activity and protocol surrounding the exercise of routine examinations has not been abandoned. Many of the stingrays in these studies have moved to other facilities or larger exhibits that are not as accessible, but with the importance of regular health exams, it is routine to coordinate and execute annual examinations on major species at this facility.

Along with the importance of educating and communicating with the aquarium on organ identification and sample collection, it is also critical to communicate with the laboratory technicians and pathologists. Proper organ identification and thorough history are essential in the first steps to understanding the mechanisms and process of physiologic change and potential diagnoses.

7.1.2 Follow up and conclusions

The initial investigation began because there were more deaths than expected when stocking a relatively new exhibit. Initial necropsy reports indicated that the livers were small and dark brown, bluish, or black and often no other descriptions, details, or histories were provided. Interviews of the aquarists were not very useful, as the aquarium no longer employed many of them. One curator alluded to a possible toxicity (such as accidentally being treated with copper) but there was no record of any incident. Records from the transport company are not normally provided and were not available. The possible toxicity or some other intermittent event is possible since the discoloration of a bluish tinge of the liver was not observed once this study officially began. Another explanation for this could be related to size. It seemed that the more

severe the lipid depletion, the darker the liver presented. So, due to the close monitoring of the stingrays' livers that were in the study and the consideration for a small liver less than 70% (thereby treatment administered), the livers in these stingrays never decreased to a size that would elicit the darker discoloration described in the reports or witnessed early on. Small livers appearing dark with a bluish, brownish, or grayish tone (depending on how one sees it) have been shown elsewhere and described as emaciation (Garner 2013). What came from this project, as a process for determining the risk factors for morbidity and mortality, was a process for determining baseline anatomy, imaging, hematology, biochemistry, and histology for southern stingrays. Because much of the time was spent establishing and confirming this information, it was compiled into a desktop application or interactive website that is accessible and user-friendly in hopes of promoting stingrays' health at other facilities (Appendix 4, southernstingray.businesscatalyst.com). One thing that was not well established in this study was obtaining weights in the stingrays. Monitoring weights can be extremely helpful with regards to the health of any animal. Weights were never obtained in live animals because there was not a feasible way to do so at the exhibit.

The small livers in these studies were associated with recently acquired stingrays that were likely nutritionally deprived and pregnant stingrays with a greater energy demand, both being in a negative metabolic state. The condition of the recently acquired stingrays may or may not have existed upon capture and the process of capture, transport, crowding, trauma, and poor water quality contributed or exacerbated the condition. The discoloration of the liver likely corresponds to the degree of lipid-depletion although toxicity cannot be ruled out. It appeared in this research that the smaller livers presented as darker livers. Weighing the livers and calculating the hepatosomatic index (HSI) would support this assumption (Sherman and Gilliam

1996). Small livers were also seen during suspected feed competition and pregnancy once the stingrays had been acclimated to captivity. The animals experiencing these conditions were under more controlled circumstances and all of them recovered uneventfully.

In review of all the records and histology slides, the suspected oophoritis (or necrosis, cysts, or abscesses) on gross necropsy was likely an observation of an ovary with large follicles as seen with reproductively mature adult stingrays. The histologic diagnosis of oophoritis was misidentified due to the close proximity of the ovary to the epigonal organ. In some cases, this was likely a normal presentation of folliculogenesis but in other cases, the stingrays may have been experiencing follicular stasis or another unknown reproductive condition. Stingrays that experience follicular stasis may not be able to ovulate or extrinsic/intrinsic factors are not allowing her to ovulate. If the follicles are not reabsorbed then they are considered to be in pre-ovulatory follicular stasis, which can act like a compressive coelomic mass. In other animals this causes inappetence, anorexia, and lethargy and treatment includes an ovariectomy (Backues and Ramsay 1994; Rivera 2008).

Upon further review of the histology sections, melanomacrophages in the liver and epigonal organ edema were noted and further evaluated. The epigonal organ was edematous to varying degrees. It is unknown as to whether or not there is a correlation among cases with epigonal organ edema and follicular stasis or liver size. The epigonal organ is a lymphomyeloid or hematopoietic organ and produces leukocytes similarly to the bone marrow in higher vertebrates. The morphology of the epigonal organ is unique compared to other vertebrates due to the close association it has with the gonad. The function of this association is not well understood but in the skate there is vascular and cellular communication between the epigonal organ and ovary (Lutton and Callard 2008). Interaction of these organs includes evidence of

apoptosis of epigonal leukocytes by progesterone and testosterone (Lutton and Callard 2007) and by an epigonal organ derived substance that inhibits spermatogenesis in males (Piferrer and Callard 1995). Perhaps there is a relationship between epigonal organ edema and the potential follicular stasis presumably seen here. From this study, it can be stated that stingrays found dead with a diagnosis of hemorrhagic or necrotic ovaries (presumed follicular stasis with epigonal-ovarian complex edema) also had small livers but it is not clear as to whether the two conditions were related. An unknown disease or condition that disrupted the immune system involving the epigonal organ and subsequently affecting the ovary due to its morphological relationship (Lutton et al. 2005; Lutton and Callard 2008) to the epigonal organ cannot be ruled out. Such a condition may also affect the nutritional status thereby changing the size of the liver.

In a pilot study, the difference in melanomacrophage counts was different between liver size and degree of epigonal organ edema. Identifying follicular stasis or epigonal edema ante mortem was not done due to extreme difficulty and antiquated equipment. In one case where the liver was extremely reduced in size (23%), the epigonal organ was removed in an attempt to perform an ovariectomy. Histologically, there was no evidence of epigonal organ edema in that case. Perhaps, the epigonal organ is responding to shock as the animal is dying and therefore not present in a living animal but further study is needed. In some ante mortem cases, cystic ovaries with or without an enlarged, fluid-filled uterus were occasionally identified ultrasonographically (as was an enlarged uterus without cystic ovaries) and hematological and biochemical results were uneventful. Antibiotic treatment was administered in one case; however, the stingray's reproductive condition did not change. Further studies are needed to evaluate the possible association with stress from disease and melanomacrophage counts or epigonal organ edema as well as determining the difference between normal follicle maturation and follicular stasis in this

species. In the future, with this information, sample collection for histology should include several tissue samples from various locations of the liver, epigonal organ, gonad, and epigonal organ-gonad complex.

It is worthy to note that at the start of this project, 12 other aquariums were visited and staff, biologists, or veterinarians were interviewed inquiring about this problem. None of these other facilities, at the time, had experienced this issue. Other aquariums are now experiencing, what appears to be, reproductive issues with their female stingrays and research efforts are being conducted by the South-East Zoo Alliance for Reproductive Conservation organization (SEZARC 2014) and collaboration with this aquarium has been discussed. They suggest that the hormones, estradiol and progestin, may contribute to this condition (SEZARC). Another publication suggests that female stingrays in captivity that are housed with all-female groups develop a reproductive disorder attributed in part by extremely elevated circulating estradiol and low progesterone (Penfold et al. 2014). Thus far, there is no evidence from this study that the reproductive condition is the primary cause of death but in some (older, unmonitored) cases there were no other findings. In future evaluations, hormone assays should be evaluated, along with ultrasonographic status of the ovary (follicular size and characteristics), uterus, and epigonal organ.

Overall, a possible scenario regarding the newly arrived stingrays to the aquarium with an unknown cause of death could be that they may or may not have been nutritionally deprived when captured $\rightarrow$ chronic stress from the capture and transport process (plus possible underlying disease) $\rightarrow$ epigonal organ edema (if edema develops in association with the epigonal organ-ovarian complex condition) $\Leftrightarrow$ reproductively mature females experienced follicular stasis (environmental conditions are not met and inadequate lipid stores) $\rightarrow$ anorexia $\rightarrow$ further

depleting liver of lipid stores → emaciation → epigonal organ edema (if the epigonal organ is a shock organ) → death.

This was a very small sample size but continued assessment of these findings may help further explain the presumed follicular stasis or reproductive disease. Questions to answer for future studies might include:

1. Differentiating ovaries that have and have not ovulated (identify folliculogenesis and follicular stasis). Perhaps this may include looking at corpora atretica (or atretic follicles or follicular atresia) versus corpora lutea in post mortem samples and ultrasound antemortem (Lutton et al. 2005; Cek et al. 2009; Diaz-Andrade et al. 2011).
2. Establish ovarian cycle or status. During necropsy, use ultrasound imaging to capture images of follicles of different stages and evaluate them histologically.
3. Evaluating hormone assays with ovarian and reproductive cycles. Are the hormonal signals coordinated for ovulation and uterine pregnancy preparation? It appears that this is the case (at least in one stingray here) and, if so, then confirm hormonal peaks for ovulation (then there must be inhibitory effects that prevent it) or if no hormonal peaks for ovulation, then what promotes uterine preparedness? Plasma or muscle tissue may likely be used for progesterone, testosterone, and estradiol evaluation (Prohaska et al. 2013).
4. Further evaluation of the communication (immunologic and vascular/hormonal) between the epigonal organ and ovary. And would an ovariectomy (but leaving the epigonal organ) from a dorsal approach be beneficial in females suspected of

follicular stasis? And, perhaps reviewing male stingray epigonal organs for a relationship of edema with different disease processes.

As a result of this research, including the preliminary work as well as the formal studies, an understanding of the previous mortalities and a process for monitoring the health of the stingrays was established. Once attention was brought to this issue, the aquarium has not experienced many unknown causes of death and the aquarists are observant to potential risk factors that may compromise the stingrays' health.

7.2 Specific Aim 1 (Risk Factors for Small Livers)

Several southern stingray deaths over the course of a few years after installation of a touch pool exhibit prompted this investigation. Initial necropsy and pathology reports indicated hepatic lipid atrophy and oophoritis but it was unclear as to whether or not these findings attributed to the deaths. An analysis of contributors for the unknown causes of death indicated that liver size and the time in captivity played a role. Liver size was found to be a confounder therefore served as the dependent variable for this study. The collection was observed and examined over a five-year period and the risk factors associated with small livers included pregnancy, time in captivity, and wingspan.

Examining the rays regularly and using liver size as a guide for nutritional status appeared to resolve the issue with unknown cause of mortality in the touch pool exhibit. These rays have since been moved to other larger exhibits (some to other facilities) making routine examinations more challenging. The aquarists though are more aware of external body condition (muscle mass), signs of pregnancy, and appetite and are able to identify and quarantine questionable animals for further examination. In the larger exhibits, typically larger individual

animals are fed as opposed to broadcast feeding, which should allow closer observation by the aquarists. The adult southern stingrays reside in three exhibits meaning at least three aquarists are responsible for their care and feeding. Challenges still exist and include the rotating of exhibit care among aquarists and communication between the aquarists and veterinarians.

7.3 Specific Aim 2 (Liver measurement)

A size decrease in liver or hepatic lipid atrophy was a consistent finding upon gross necropsy in several cases prompting the start of this project. As part of routine physical examinations, evaluating the length of the liver with respect to the length of the coelomic opening, bordered by the pectoral and pelvic cartilaginous girdles, provided one point of reference to their metabolic state. Evaluation was done with ultrasound imaging therefore it was a relatively easy process and a non-invasive diagnostic technique with very little to no harm or pain afflicted on the stingray. Evaluating the liver routinely helped guide the health of the collection and condition of individual stingrays especially since obtaining weights was difficult.

The difficulty in conducting examinations arose once the stingrays outgrew the touch pool and were moved to larger exhibits or other facilities. Of course, stingrays moved to other facilities were no longer in the aquarium's collection and therefore were not being monitored any longer. Stingrays moved to other larger exhibits were also difficult to monitor because access to them was much more difficult. Generally, they were moved to one of two other exhibits, Under the Sea or Dining. Six stingrays that were moved to the dining exhibit experienced harsh feed competition and two of them were found dead shortly after the transition. The aquarists performed the necropsies and the incident and results were revealed during the next veterinary visit. The aquarists noted that the livers of both stingrays were small but measurements and

other details were unknown therefore emaciation or starvation was suspected as the primary cause of death. Because it was difficult for the aquarists to monitor feed intake for individuals in that particular exhibit and it was difficult to examine them at that exhibit, the remaining four stingrays were moved into quarantine. Upon examination of the stingrays in quarantine, all of their livers were lass than 50% and hematological and biochemical profiles were unremarkable with the exception that all but one ray had a mild leukocytosis. There were no other findings explaining the leukocytosis therefore a stress leukogram was suspected. Based on this information, the stingrays remained in quarantine where individual feeding and ingestion could be closely monitored. The stingrays were examined weekly until their liver sizes increased in approximately two months and they were moved either back to the touch pool or to the other, larger exhibit.

Measuring the liver length is an easy and quick evaluation for body condition and metabolic status. Possibly liver length along with a muscle mass evaluation (flesh slope along the spine) and weight would be an even better general indication of nutritional or metabolic status. Liver depth measurements using an ultrasound could also be considered for a more complete estimate of liver size. Liver depth measurements would likely need to be measured at multiple locations since the livers appear to taper off from the dorsal aspect first (i.e. the ventral aspect of the liver tends to extend more caudally than the dorsal aspect when the liver length shortens).

7.4 Specific Aim 3 (Hematology and Biochemistry)

This specific aim concentrated on the hematological and biochemical profiles of the captive southern stingray. This study discussed the interpretation of differing values between the

acclimated group and recently acquired group. Due to the limitation of study subjects and the fact that they are housed in the same facility under similar conditions, more studies like this are needed to gain a broader understanding of what and how extrinsic and intrinsic factors may affect these values. Further studies here may include collecting blood from this collection as well as associated water quality parameters and collecting similar data from other facilities to see if there are correlations between biochemistry values and water quality parameters. Other areas of study might also include hematological profiles of wild caught southern stingrays. Further study is always needed in this arena for any elasmobranch species and consistency in granulocyte nomenclature would also be helpful. Granulocyte nomenclature could be more specified with cytochemical studies evaluating possible function of these cells.

7.5 Specific Aim 4 (Predicting parturition date range)

This specific aim focused on captive breeding and predicting a parturition date range in order to reduce the amount of time pregnant stingrays needed to be in quarantine. With the small sample size of pregnant stingrays and the space limitation for stocking the pups, the study ran for three reproduction cycles. The first two cycles were used to develop the model for prediction and the third cycle was used to assess the accuracy of the model prediction. Of the three pregnant stingrays in the third cycle, the model predicted two parturition dates within two weeks and under predicted the third one by two more weeks (within one month total). It would always be better to have more subjects and examine them more frequently and consistently, but, again, because this study was conducted at an exhibit at the aquarium, it was important to respect the production of the facility. With the information that was obtained from this study, it proved useful for this aquarium. The pregnant stingrays were moved to quarantine when requested and

usually pupped within two weeks. Future studies could include more measurements (cranial body, caudal body, length, etc.), noting organ development, hormone assays, and measurements and weights of the female (pre- and post-parturition) and pups (post-parturition).

For this study, only reproducing females were used. During subject selection, it was noted that several females appeared prepared for pregnancy (large follicles and a large, fluid-filled uterus with long trophonemata) but never became pregnant (no evidence of egg capsules, embryos, or fetuses). One of these females died after completion of the study due to a water quality mishap. On gross necropsy, the liver was relatively large and she had hemorrhagic ovaries. This presentation is suspected to be the reproductive disease described by SEZARC (SEZARC 2014) or what is proposed here as follicular stasis (or other reproductive condition). Histologically, there were no lesions noted for the ovary. The epigonal organ was severely erythematous and mildly edematous. These females are likely in follicular stasis and not ovulating for some reason. This particular stingray was with males and therefore had the opportunity to mate but perhaps was not able to ovulate or environmental cues (or other signals) were inhibiting ovulation. Unfortunately, oviducal gland was not submitted for histological evaluation therefore sperm storage could not be evaluated. There were no sperm observed in the uterus but this female had not been with males for over two years. From this study, it appears as though this presentation is not the cause of death but in other cases it cannot be ruled out as a contributing factor.

Once the aquarium reached a limit for stocking pups, males and females were separated to halt reproduction. Approximately two years later, one stingray that had never been pregnant and had a wingspan of 62 cm at the time this study had started, became pregnant. Using the parturition prediction model established in chapter 6, it was predicted that she would pup in

14.75 days therefore she was moved to quarantine. She pupped 15 days later in quarantine. It is suspected that this stingray had stored sperm, which has been described in elasmobranchs but not for this species to the authors' knowledge (Hamlett et al. 2002; Storrie et al. 2008). Closer examination of histological sections of the oviducal glands for sperm may reinforce the presumption that this species also stores sperm.

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APPENDIX 1: Definition of Terms

Ampullae of Lorenzini	Specialized electrosensory receptors located in the skin on the head and wings.
Aplacental viviparity with uterine villi	The reproductive mode of elasmobranchs where secrete histotroph to provide nutrients to the embryos.
Aplacental yolk sac viviparity	The reproductive mode of elasmobranchs where the embryos rely heavily on the yolk for nutrients.
Benthic	Bottom-dwelling animals in a sea or lake
Coelom or Coelomic cavity	The body cavity of fish, reptiles or birds since they do not possess a diaphragm
Coelomocentesis	Tapping the coelomic cavity with a needle and aspirating fluid
Echogenicity	The ability of creating an echo relating to ultrasound imaging
Elasmobranch	Pertaining to the subclass elasmobranchii, cartilaginous fish
Epigonal organ	The lymphomyeloid structure in elasmobranchs, closely associated with the ovaries in females
Follicular stasis or folliculostasis	When the act of creating follicles and ovulating stops
Folliculogenesis	The act of creating new follicles for ovulation
Histotroph	The lipid-rich secretions or “milk” produced by trophonemata during uterolactation in preparation for or during gestation.
Husbandry	Domestic management, overall parameters for caretaking of an exhibit and its animals
Immunocompromised	The immune system of an animal is compromised by stress or disease
Immunosuppressed	The immune system of an animal is suppressed by stress or disease
Interrenal gland	A gland in elasmobranchs similar to mammalian adrenal glands located dorsally between the two kidneys.
Lecithotrophy or lecithotrophic	When the embryo only receives its nutrition from egg yolk
Matrotrophy or matrotrophic	When the embryo receives nutrients from the female

Melanomacrophage	Macrophage aggregates; pigment-containing cells within the liver of elasmobranchs
Necropsy	The equivalent of an autopsy in an animal
Oophoritis	Inflammation of the ovaries
Organ of Leydig	Hematopoietic organ attached to the esophagus
Ovoviviparity	See aplacental yolk sac viviparity
Pelagic	Used to describe the location where a fish may reside relating to the open sea or ocean
Pups	The offspring of stingrays
Trophonemata	The finger-like projection extending from the mucosal surface of the uterus, which provides the nutrients to the fetuses during gestation.
Viviparous	Live bearers

APPENDIX 2: Mortality Investigation Preliminary Research

Synopsis

An aquarium experienced several southern stingray deaths over course of several years after opening a touch pool exhibit. Similar findings during necropsy included a small, dark liver and hemorrhagic ovaries in the females. A thorough review of the necropsy records, histopathology reports, and histology slides was conducted to assess for any risk factors. From the necropsy records, consistent variables to assess as risk factors for a case-control study included age (juvenile/adult), liver size (small/large), follicles (absent/present), and time at the aquarium (</> three months). Liver size and time at aquarium were statistically significant (odds ratios > 1 and 95% confidence intervals not including 1). The odds of a stingray having a small liver is almost 40 times as high when the cause of death is unknown compared to a known cause of death and the odds of it being at the aquarium less than 3 months is 57 times as high when the cause of death is unknown compared to known. These two variables were stratified and liver appeared to be a confounder. Elasmobranch histology identification can be somewhat challenging due to their unique anatomy especially for those who do not routinely evaluate these species therefore some organ identification was incorrect on the histopathology reports. After evaluation of the histology slides, it is likely that the diagnosis of oophoritis was made due to confusion with its association with the epigonal organ. Common findings included hepatic melanomacrophages, normal ovaries, and epigonal organ edema.

Introduction

An aquarium added a touch pool exhibit and experienced several deaths within their stingray collection used to populate the pool. The deaths occurred over several years but the death rate appeared to be higher than expected. This prompted a closer look at the mortalities.

During a brief review of several necropsy records at the facility, the reports indicated no clinical signs prior to death; during gross necropsies, all of these animals had small, dark livers; for cases that were submitted to a diagnostic laboratory for histopathology, severe lipid depletion in the liver was confirmed; and one other common finding among many of the female animals was a hemorrhagic ovary, often diagnosed as oophoritis. These findings prompted a more in-depth evaluation. The purpose of this preliminary investigation is to gain a better understanding of the risk factors involved with the deaths of these stingrays by reviewing the necropsy records and the histology. The suspicion is that recent arrivals are likely more at risk of death due to the stress of transport, lack of nutrition, and suboptimal environmental conditions.

Methods

Necropsy Records Review

As a pilot case-control study, necropsy records were reviewed from 36 stingrays and limited consistent information gathered from these records included age (juvenile/adult), liver size (small/large), follicles (present/absent), time at the aquarium (<3mo/>3mo), and cause of death (unknown/known). A three-month time frame was selected for the variable 'time at the aquarium' because three months would allow for arrival, quarantine (with treatment protocol), veterinary examination, and acclimation to the exhibits for the adults. Cases were defined as those with an unknown cause of death and controls were those with a known cause of death.

Known causes of death included water quality problems and accidental (by inadvertently jumping out of the exhibit). To prevent further accidental deaths, the exhibit was modified to avoid exits from the exhibit by stingrays swimming out on the shallow end of the pool. In order to evaluate each variable independent of the unknown or known cause of death, the odds ratios (retrospective) and 95% confidence intervals were calculated using a statistical software package (Epi Info™, version 7.1.5). Significant risk factors were stratified to assess for interaction and variables that did not act independently were also checked for confounding.

Histology Review

The histology slides for cases that were submitted to the diagnostic lab were retrieved. The slides were reviewed for consistent organ identification and for any other features that may have been overlooked during the initial evaluation. Histological observations were noted.

Results

Necropsy Records Review

The data collected from the necropsy records are summarized in Table A2.1. Four necropsy reports (gross findings) stated oophoritis. Two of the reports stated that two other and three other stingrays also died from their respective groups with similar gross findings (small, dark bluish livers and necrotic or hemorrhagic ovaries). This increases the total number of deaths to 41; however, there were not individual necropsy reports for those five stingrays, therefore they were not included in the analysis.

Table A2.1. Summary of data from necropsy records

	Cases (n=19)	Controls (n=17)	OR	95% CI
Age				
Juvenile	6	0	9	[0.99, 81.93]
Adult	13	17		
Liver size				
Small	17	3	39.67	[5.79, 271.64]
Large	2	14		
Follicles				
Present	11	15	0.18	[0.03, 1.04]
Absent	8	2		
Time at Aquarium				
< 3 months	15	0	57.6	[6.07, 546.61]
> 3 months	4	17		

OR: Odds ratio
CI: Confidence interval

Liver size and time at aquarium were statistically significant (odds ratios > 1 and 95% confidence intervals not including 1). The odds of a stingray having a small liver is almost 40 times as high when the cause of death is unknown compared to a known cause of death and the odds of it being at the aquarium less than 3 months is 57 times as high when the cause of death is unknown compared to known. The sample odds ratios for these variables are not very precise as shown by the wide confidence intervals, but they do not include 1 therefore small livers and less than three months at the aquarium are statistically significant risk factors for unknown causes of death at the 0.05 level.

The association between time at the aquarium and unknown cause of death was further explored (stratified) among those with and without a small liver (Table A2.2).

Table A2.2. The association between time at the aquarium and unknown cause of death among those with small and large livers.

		Unknown cause of death			
Liver size	**Time at aquarium**	**Yes**	**No**	**OR**	**95% CI**
Small	< 3 months	14	0	112.5	[7.22,4409.14]
	> 3 months	3	3	7.5	[0.73,97.29]
Large	< 3 months	1	0	15.0	[0.58,803.98]
	> 3 months	1	14	1.0*	--

*Referent group
OR = Odds ratio
CI = Confidence interval

Effect modification (interaction) is used to assess the risk of the outcome (unknown cause of death) if exposure to two risk factors (small liver and less than three months at the aquarium) are involved. The increase in an unknown cause of death due to liver size and time at the aquarium is perfectly multiplicative (15 * 7.5 = 112.5, more than additive and no multiplicative interaction) and therefore liver size and time at the aquarium do not act independently making it necessary to check for confounding.

Table A2.3. Strata-specific odds ratios of those at the aquarium less than and greater than three months among those with a small and large liver.

	Unknown cause of death			
	Yes	**No**	**OR**	**95% CI**
Small Livers				
< 3 months	14	0	15	[1.29, 174.29]
> 3 months	3	3		
Large Livers				
< 3 months	1	0	15	[0.9, 251.07]
> 3 months	1	14		

OR = Odds ratio
CI = Confidence interval

The small liver is associated with less than three months at the aquarium, is a risk factor for an unknown cause of death, and is not a link in the direct (biological, physical, or chemical)

causal chain between stingrays at the aquarium for less than three months and an unknown cause of death. The liver size variable was found to be a confounder because the stratum-specific odds ratios (OR = 15 in Table A2.3) for those at the aquarium less than and greater than three months were the same but different from the crude odds ratio (OR = 57.6 in Table A2.1) by much more than 10%.

Histology Review

Elasmobranch histology identification can be somewhat challenging due to their unique anatomy especially for those who do not routinely evaluate these species. Inevitably, errors were made which complicated the investigation. In three reports the epigonal organ was mistaken for spleen, in one report the epigonal organ was mistaken for lymph node, in one report the epigonal organ was submitted as gonad and reported as unidentifiable, in one report the stomach was mistaken for bladder, in one report the uterus was mistaken for intestine, and two reports identified the liver as normal when it was severely lipid depleted.

For cases that diagnosed oophoritis, they described the ovary as being "infiltrated with numerous heterophils and fewer mononuclear cells." Upon further review of the histology of the ovary, it was determined that there was likely confusion regarding the diagnosis of oophoritis due to the ovary's close proximity to the epigonal organ. A more consistent finding among the epigonal-ovarian complex was edema. A consistent diagnosis for the liver was lipid atrophy and/or glycogen depletion.

Discussion

This preliminary study showed, given the limited number of variables for risk factors, that the liver was a confounder for the stingray deaths at the aquarium. The odds for being at the aquarium for less than three months were high when the cause of death was unknown, therefore the likelihood of recent arrivals having a small liver is good. It should be noted that the wide confidence intervals are likely due to a small sample size and the distribution of the animals in each group (exposure and outcome).

In order for a small liver to be the sole risk factor for death, it seems that a more severe compromise or disease process would need to be present unless the animals are truly starving to death. Considering all the variables evaluated here, and knowing that the liver is likely a confounder, it would appear that the actual cause of the outcome (unknown cause of death) is exacerbated by the variables that contribute to the confounder. In an elasmobranch pathology study, southern stingrays were one of the most commonly submitted species, which corresponds to the southern stingray being one of the most exhibited elasmobranchs (Garner 2013, AES 2008). Although nutritional disease was the diagnosis in 12% of all cases, it was diagnosed as the sole problem in only 1.5% of all cases (Garner 2013). The nutritional contribution to disease processes was based on the depletion or lack of lipid stores from hepatocytes (Garner 2013). A subjective observation in this study was the association of liver size and color. Larger livers were off-white to tan and smaller livers were dark brown to black (or gray or blue) (Figure A2.1). Similar observations in the association between liver color and lipid content were found in one study on the lesser sand shark (*Rhinbatos annulatus*) (Rossouw 1987).

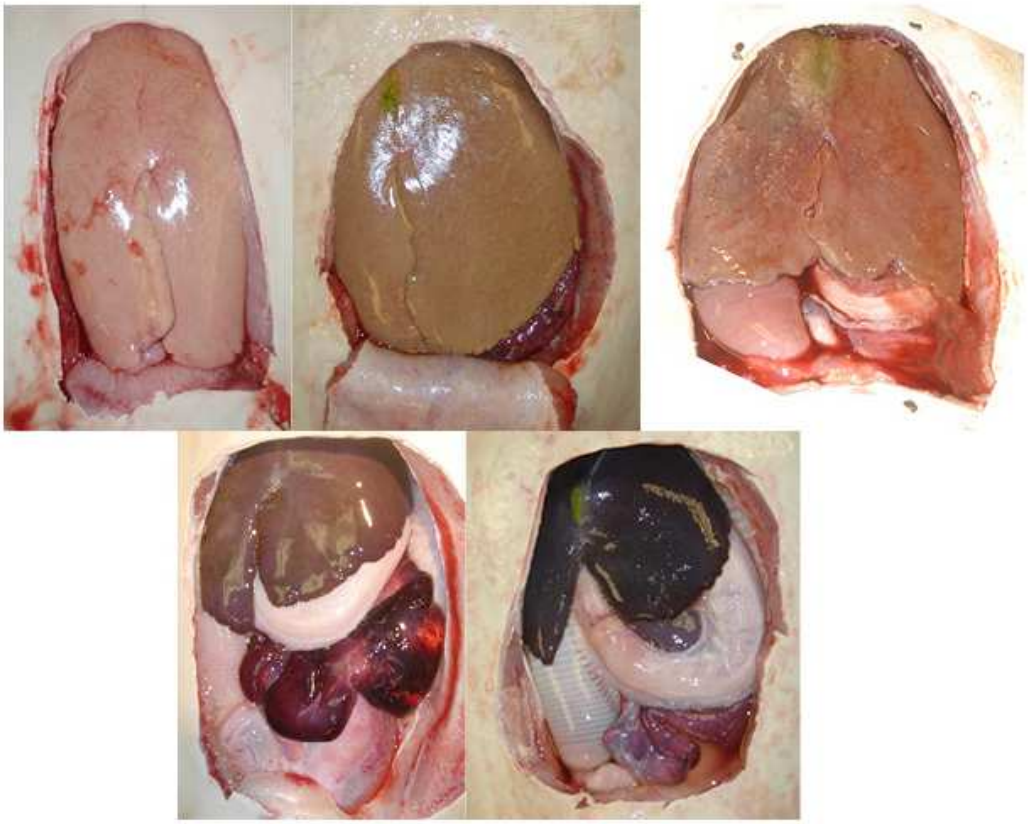

Figure A2.1. Five livers from various female southern stingrays during necropsy showing the progression from top left to bottom right of large, lipid-filled to small, lipid-depleted and varying in color from light to dark, respectively. These images are not to scale. The liver in the bottom left image is likely larger than that bottom right image as seen by the caudal margin of the liver on the smaller curvature of the stomach.

Four necropsy reports (gross findings) stated oophoritis. Two of the reports stated that two other and three other stingrays also died from their respective groups with similar gross findings (small, dark bluish livers and necrotic or hemorrhagic ovaries) but individual reports were not documented because the aquarists assumed it was the same "liver and oophoritis disease" that has been diagnosed. The issue was that the presumed diagnoses on gross necropsies came from one previous histopathology report and continued. Although oophoritis was reported in many of the necropsy and diagnostic lab results, it did not seem to play a role in

subsequent deaths probably due to the young age and reproductive status of many of the stingrays. It appeared that what was likely being described, as oophoritis grossly, was a presumably normal reproductive maturation process or follicular stasis. One histopathology report stated that the ovary was normal with adjacent edema and another one also stated a normal ovary despite the gross description of hemorrhagic ovaries in the gross findings. In the yellow stingray, *Urolophus jamaicensis*, another aplacental viviparous species, during folliculogenesis and vitellogenesis, inward follicular folding occurs to increase surface area for yolk transport (Hamlett et al. 1999; Hamlett et al. 2005). Vascularization also proliferates into these folds and persists to ovulation. In a study evaluating the epigonal organ-ovary complex of the skate, *Leucoraja erinacea*, it was noted that angiogenesis and vasculogenesis increased (developed and became more abundant) during the reproductive development period and is greater during advanced follicular development (Lutton and Callard 2008). The development of the southern stingray ovaries appears to be similar in both of the species in the same way. When they are reproductively inactive or immature, the skate ovaries are completely embedded within the epigonal organ, which is also seen with the southern stingray (Lutton and Callard 2008). Females that do not ovulate and remain in a state of folliculostasis, or continue to develop follicles without absorbing them, are likely in trouble. After reviewing the histology slides, in the majority of cases, the ovary and follicles appeared normal. Histopathology reports with oophoritis as the diagnosis had misidentified the epigonal organ as ovarian inflammation therefore the problem that contributed at the start of this project was misidentified as oophoritis. A more appropriate conclusion is that these females are not ovulating and therefore experiencing pre-ovulatory folliculostasis or follicular stasis. In these cases, a necrotic ovary may be seen but is likely dependent on the duration of the condition. This condition has been identified in

reptiles where it is commonly referred to as pre-ovulatory egg-binding or retained follicles (Sykes 2010; Chitty and Raftery 2013). Pre-ovulatory follicular stasis in reptiles is often resolved with ovariectomies (Backues and Ramsay 1994; Rivera 2008). This surgery was conducted in one stingray during this study; however, she died four days later due to dehiscence. The ovary was removed with a majority of the epigonal organ (mistaking it as part of the gonad at the time). There are no other reports of this type of surgery in elasmobranchs and it is unknown as to the potential consequences for removing the epigonal organ. Also, if this or a similar procedure is attempted, a ventral midline approach should be reconsidered, especially in benthic species.

Recent arrivals or having spent less time in captivity may definitely contribute to the small liver confounder but may also include other problems. For example, an existing disease or potential condition may exist and the stress of catching, transport, crowding, trauma, change in water quality, or new environment may immunosuppress the stingray allowing the disease (such as parasitism) an opportunity to overwhelm the animal. Parasitism has been reported in other elasmobranch deaths (Borucinska and Adams 2013; Marancik et al. 2012). There were some deaths upon arrival as well as some that died shortly after arrival. Stingrays that were dead on arrival appeared severely traumatized. Other factors in the latter scenario that may contribute to an unknown cause of death include continued or chronic stress, fighting, or falling further into a negative metabolic balance from food competition or aggression. With each stressful event and considering the duration of the stressor, physiologically any resources will be used to either combat the stressor or be used for maintenance. Depending on how the animal allocates their resources, and the degree and length of the stressful event, may determine or contribute to their probability of survival (McNamara and Buchanan 2005). After reviewing the histology slides of

the liver in some of these cases, it was noted that moderate numbers of melanomacrophages existed. Although the presence of melanomacrophages in hematopoietic tissues is normal, presence in the liver in increasing size and frequency may indicate exposures to environmental stress and therefore is considered a biomarker for that condition (Agius and Roberts 2003; Adams et al. 2015; Borucinska et al. 2009).

The next steps included a more formal approach to counting melanomacrophages and comparing those counts between different conditions (Appendix 3). The living collection was examined on a routine basis in an observational study to further evaluate the risk factors (Chapter 3). Also, due to the dynamic characteristic of the liver and the consistency of lipid depletion among cases, a study evaluating the liver size was conducted (Chapter 4).

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APPENDIX 3: Hepatic Melanomacrophage Pilot Study

Synopsis

An aquarium experienced multiple southern stingray deaths over the course of several years. Many of the deaths occurred in recently wild-caught stingrays that were captured and transported to the aquarium to stock the exhibit. Common findings on gross necropsy included small, dark livers and presumably hemorrhagic ovaries in females. Histologic findings confirmed hepatic lipid-depletion but also noted hepatic melanomacrophages. The presence of melanomacrophages in the liver and hematopoietic organs of fish may indicate starvation, stress, or other environmental change. A negative metabolic state was presumed in these cases due to the decreased size of the liver and confirmation of lipid-depletion. Stress also likely contributed to the condition of the stingrays but is difficult to assess post-mortem. The purpose of this study was to establish melanomacrophage counts in cases where liver tissue was submitted to the diagnostic lab and compare counts between cases using dichotomous variables of liver size, presence of hemorrhagic ovaries, and presence and degree of epigonal organ edema. The difference in melanomacrophage counts was significant ($p<0.05$) for liver size ($p=0.048$) and epigonal organ edema ($p=0.042$) but not significant for the presence of hemorrhagic ovaries ($p=0.222$). The increased counts may be due to starvation and/or stress, given their situations, but further study in fish, especially elasmobranchs, is needed.

Introduction

An aquarium with a touch pool exhibit experienced several deaths within their southern stingray collection used to populate the pool. The deaths occurred over several years but the

death rate seemed higher than expected. Upon initial review of the facility's records, the reports indicated no clinical signs prior to death; during gross necropsies, all of these animals had small, dark livers; for cases that were submitted to a diagnostic laboratory for histopathology, severe lipid depletion in the liver was confirmed; and one other common finding among many of the female animals was a hemorrhagic ovary and often diagnosed as oophoritis on the histopathology reports.

Of the 36 stingray deaths, 15 cases were submitted to the diagnostic laboratory for histological evaluation and diagnosis. The diagnostic lab reports and histology slides were retrieved for further evaluation. The organ tissues sampled for each case varied. In some cases, only the ovary, and associated epigonal organ, and liver were submitted. Extensive review of these cases aided in the proper identification of gross anatomy and histology for future examinations and necropsies.

During review of the liver histology and reports, many cases showed and reported melanomacrophages or infiltrates of hemosiderin (or hemosiderosis) or pigmented cells. Melanomacrophage centers in fish are typically found in hematopoietic tissue, primarily spleen or kidney, but have also been noted in the liver in some teleost fish compared to predominantly in the liver in elasmobranchs (Agius 1980; Agius 1983; Wolke 1992). The intracytoplasmic pigments usually consist of melanin, lipofuscin, or hemosiderin (Wolke 1992). Changes in morphology within a species due to age, gender, nutritional status (starvation), tissue breakdown, hemolysis, iron and hemoglobin metabolism, and inflammatory and immunological conditions have been described (Agius and Roberts 2003; Borucinska et al. 2008). When experiencing environmental stressors, such as pollution or poor water quality, the increase in size or frequency of the melanomacrophages has served as a reliable biomarker for these conditions in teleosts

(Agius and Roberts 2003). Although very little research has been conducted on elasmobranchs and hepatic melanomacrophages, one study established a baseline of cells per high power field in three shark species, the blue shark (*Prionacae glauca*), the shortfin mako (*Isurus oxyrhinchus*), and the thresher (*Alopias vulpinus*) (Borucinska et al. 2009). One case report included the presumptive increase in hepatic melanomacrophages (from a baseline of 12 cells/hpf to 62 cells/hpf) in a wild-caught blue shark with hepatic cholangiocarcinoma and testicular mesothelioma (Borucinska et al. 2003). Another study reported the size and number of melanomacrophage centers around the portal vein compared to other areas in the livers of a freshwater stingray (*Potamotrygon motoro*) (Engracia de Moraes et al. 2016). Due to their possible association with stress and the likelihood that stress was a factor in the deaths, the melanomacrophage counts will be recorded in this study.

Generally, the purpose for reviewing the slides in these cases was to gain a better understanding of organ identification both grossly and histologically. The objective for this part of the study was to determine if hepatic melanomacrophages are potential biomarkers for stress in southern stingrays. This was accomplished by evaluating the descriptive statistics of hepatic melanomacrophage counts in the 15 cases submitted to the diagnostic lab. The research questions for this portion of the study are:

1. What is the baseline melanomacrophages count in southern stingrays?
2. Do melanomacrophage counts differ between stingrays with different potential causes of death or necropsy findings (liver size, hemorrhagic ovary presence, epigonal edema presence)?

Methods

This was a retrospective pilot study comparing melanomacrophage counts. The histology reports, as well as the histology slides, were used to collect data and the information from the following variables was recorded for each case: stingray ID, the presumed primary contributor to death, gross liver appearance (small or large), adequate lipid stores (yes or no), gross hemorrhagic follicles (yes or no), histological epigonal organ edema (yes or no), and hepatic melanomacrophage count per high power field (40x). Gross necropsy reports were used to determine whether the liver was small or large (as stated in the report) as well as whether or not the ovary appeared hemorrhagic (or stated presumptive oophoritis or necrosis).

The liver and epigonal tissue were histologically evaluated for each case. Similar to the shark study (Borucinska et al. 2009) five random areas of the liver were selected and the melanomacrophages in each high power field (40x) area were counted. In this study, only one liver tissue sample was used for each stingray due to the limited number of tissue samples taken at the time of necropsy, therefore only the five random areas were used for counts (not 15 as in the shark study). The liver was also evaluated for adequate lipid stores as a dichotomous variable, either adequate or not (Figure A3.1). In order for a liver to be considered as having adequate liver stores, lipid vacuoles had to be present throughout the tissue sample. Liver tissue samples with sparse or no hepatic lipid vacuoles were considered inadequate. The epigonal organ was evaluated for the degree of edema, either absent to mild or severe. Epigonal tissue samples were considered severely edematous when fluid severely disrupted the normal reticular stroma of uniform mature granulocytes (Figure A3.2).

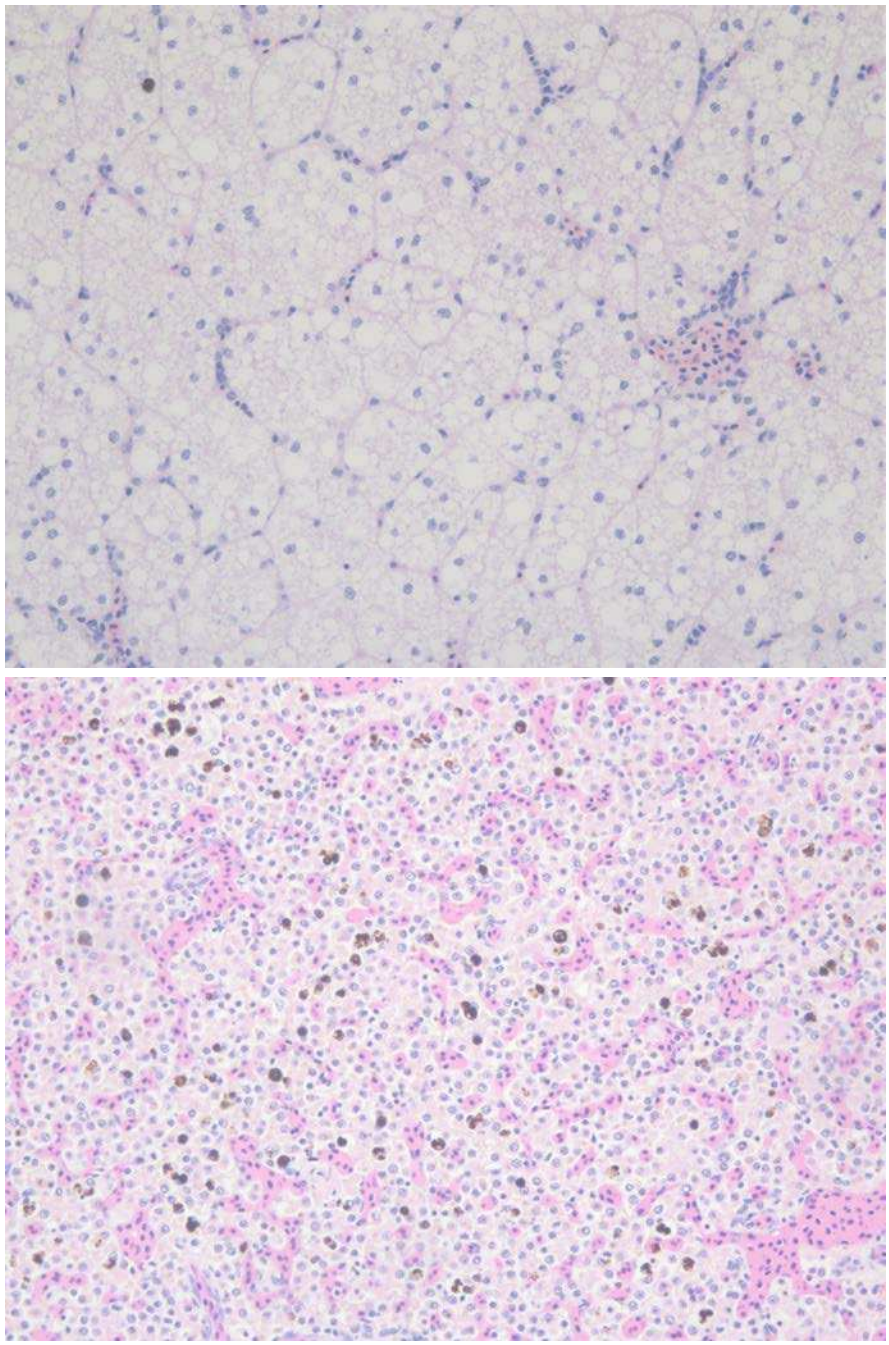

Figure A3.1. Histologic sections of southern stingray livers (H&E, x200). Top: example of a liver with adequate lipid stores. Hepatocytes are vaculoated with lipid. There is one melanomacrophage in the upper left corner. Bottom: example of liver with lipid depletion. There are several pigmented cells or melanomacrophages.

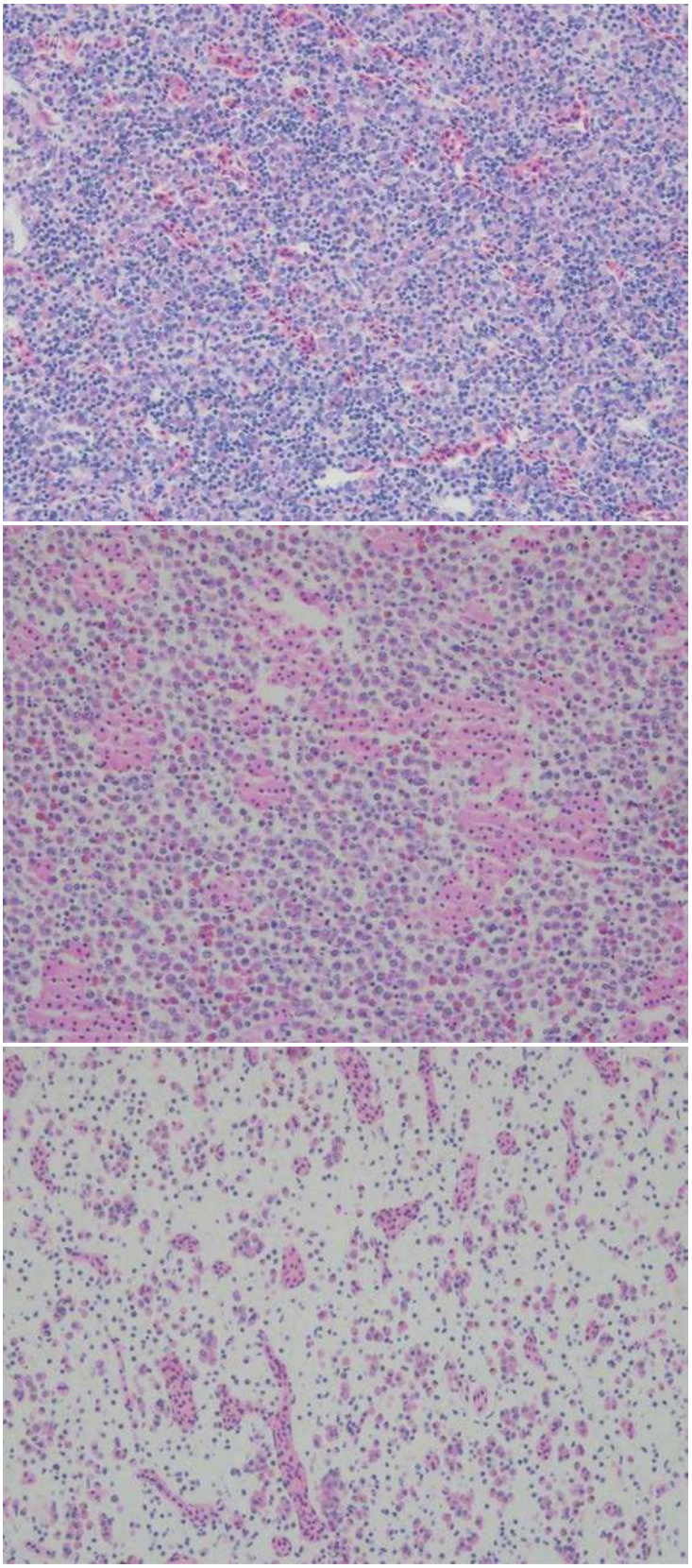

Figure A3.2. Histologic sections of southern stingray epigonal organs (H&E, x200).

Top: Normal epigonal organ

Middle: Epigonal organ with mild edema

Bottom: Epigonal organ with severe edema

Statistical Analyses

The Mann-Whitney U test was used to compare the difference in melanomacrophage counts between small and large livers, presence or absence of hemorrhagic ovaries, and the absent to mild or severe epigonal edema. A probability value of less than 0.05 was considered statistically significant.

Results

There were 12 cases in total because one slide set was missing and two of the cases were severely autolytic. Adequacy of lipid stores coincided with liver size description therefore only the liver size variable was used. Two of the stingrays were male so the ovary data did not apply but they were included to increase the sample size for the liver size and epigonal organ edema. There were three cases that had melanomacrophage counts that were too numerous to count and therefore were recorded as greater than 100. Because some counts were greater than 100 and not specifically counted, means are not presented. The remaining descriptive data are shown in Table A3.1.

As for the primary contributors to death, six were recent arrivals (in captivity for less than three months), two had anorexia and lethargy, one died from high ammonia levels, and three died from low dissolved oxygen levels. The four stingrays that died from water quality problems were all acute deaths and the necropsies were done within two hours of death. Of the four stingrays, all of them had absent to mild epigonal edema, three had large livers, and two had hemorrhagic ovaries. The two cases of anorexia or lethargy had mild and severe epigonal edema, respectively; one had a large liver; and they both had hemorrhagic ovaries. The epigonal organ in both of those cases was also erythematous or hemorrhagic. The recent arrival cases all

had small livers, two had mild epigonal edema, and all the females (four) had hemorrhagic ovaries.

The difference between melanomacrophage counts among liver size and epigonal organ edema were significantly different (p = 0.048 and 0.042, respectively) and counts among hemorrhagic ovaries was not significantly different (p = 0.222).

Table A3.1. Descriptive statistics for melanomacrophage counts (MMC) for liver size, hemorrhagic ovary, and epigonal organ edema. Numbers in parentheses represent the sample number.

	Median MMC	**Min MMC**	**Max MMC**	***p*-value**
Liver size				
Large/adequate lipid (3)	10	1	27	0.048
Small/inadequate lipid (9)	67	25	>100	
Gross hemorrhagic ovary				
Yes (8)	34	10	68	0.222
No (1)	1	1	1	
Epigonal organ edema				
Absent-mild (7)	26	1	>100	0.042
Severe (4)	68	45	>100	

Discussion

Although the presence of melanomacrophages in hematopoietic tissues is normal, presence in the liver in increasing size and frequency may indicate exposures to environmental stress and therefore is considered a biomarker for that condition (Agius and Roberts 2003; Adams et al. 2015; Borucinska et al. 2009). Another biomarker used to identify stress is follicular atresia (Adams et al. 2015), which was also identified in one report (Figure A3.3).

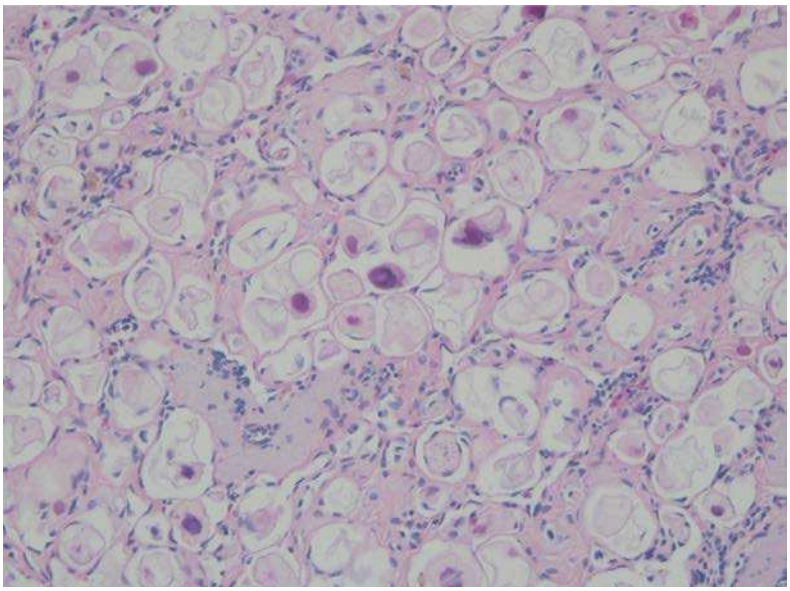

Figure A3.3. Histologic section of southern stingray ovary with many atretic follicles (H&E, x200)

A baseline count in this species was not established due to the small sample size and lack of information surrounding the deaths (like time from death to necropsy). The counts were significantly different among the small and large liver as well as in absent to mild and severe epigonal organ edema but not different among the absent or present hemorrhagic ovary. There was only one stingray that did not have a hemorrhagic ovary reported but looking back in the record, this stingray's wingspan was only 41 cm, so not reproductively mature. Unfortunately records for the other stingrays were not as complete but for the cases where wingspan was recorded, the next smallest ray was 62 cm, indicating they were all likely reproductively mature. An explanation may be that the hemorrhagic follicles are a normal process of reproductive maturity and not an indication of follicular stasis or other abnormal reproductive condition. There was also one case that was not included in the melanomacrophage count comparison because tissues samples only included the ovary and an "unidentified organ" but not the liver.

The stingray in this case had seven fetuses in utero and the report also noted “cysts on ovaries” which likely referred to large, vitellogenic (yolk-filled) follicles. It is not surprising that only “hemorrhagic” ovaries are found in reproductively mature females (since they are the ones with prominent follicles) but the two cases here (the reproductively immature ray and the pregnant ray) support the thought that it is not an abnormal finding in, at least, some cases. The question now becomes, can normal, mature follicles be differentiated from early follicular stasis?

The melanomacrophage counts were significantly different among the small and large liver, which would be expected. With lipid depletion, the hepatocytes are physically closer together therefore more cells are seen per high power field. This allows for melanomacrophages the opportunity to also increase in density per high power field and therefore it was difficult to discern whether the melanomacrophages were truly increased in animals with small livers. In one pathology study, gross and histological images of cownose livers were shown for both a lipid-filled and lipid-depleted liver (Garner 2013). In the corresponding histology images of the livers, it appeared that there are more melanomacrophages in the lipid-full liver compared to the lipid-depleted liver but the slides would be needed to confirm (Garner 2013). Certainly, more research is needed here and it would be ideal, in anticipation of looking for these features on histology, to select multiple tissue samples and make appropriate tissue cuts. The melanomacrophage counts among absent to mild and severe edema in the epigonal organ was also significantly different. This, too, is not surprising if one assumes that the stingrays with small livers are experiencing a negative metabolic balance due to a disease process or condition that may also produce edema in the epigonal organ. This is also assuming that edema in the epigonal organ is abnormal. There is one case study describing edema and hemorrhage in the

epigonal organ of a bonnethead shark, *Sphyrna tiburo*, with a bacterial infection and associated septicemia (Camus et al. 2013).

This pilot study was conducted in part to support the thought that stress was a contributor to the stingrays' condition. Typical assessment of melanomacrophages occurs on wild-caught fish to evaluate the effects of environmental change (toxin exposure, starvation, etc.) (Mizuno et al. 2002), although there are also reports aimed at determining possible function (Meseguer et al. 1994). Most of the information regarding fish is in regards to teleosts, which may not accurately apply to elasmobranchs (Agius 1983). Clinically, there would likely be other less invasive indicators (physical exam findings, ultrasonographic imaging of the liver, changes in blood parameters, etc.) of stress in a well-monitored collection therefore collecting a liver biopsy for the sole purpose of counting melanomacrophages would be unreasonable. It is still an interesting finding and should be evaluated during any histological review of the liver or hematopoietic organs.

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APPENDIX 4: Southern Stingray, *Dasyatis americana* Website/desktop application

Website: southernstingray.businesscatalyst.com

Home
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Accessed August 17, 2015

>Acknowledgements

The Downtown Aquarium in Denver was instrumental in this project. A huge thank you to all of the curators (Ken Yates, Tom Fenske, Rob Brynda, Meiling Roitz and Jessica Miller). There were also several individuals that contributed to this project. It is with their help that I was able to perform physical exams and necropsies; have access to equipment and interpret ultrasound imaging; collect blood samples, interpret counts and morphology; and learn the process of preparing and interpreting tissue samples for histology. We hope the information provided here helps other facilities maintain and care for their stingrays.

Physical Exams and Necropsy
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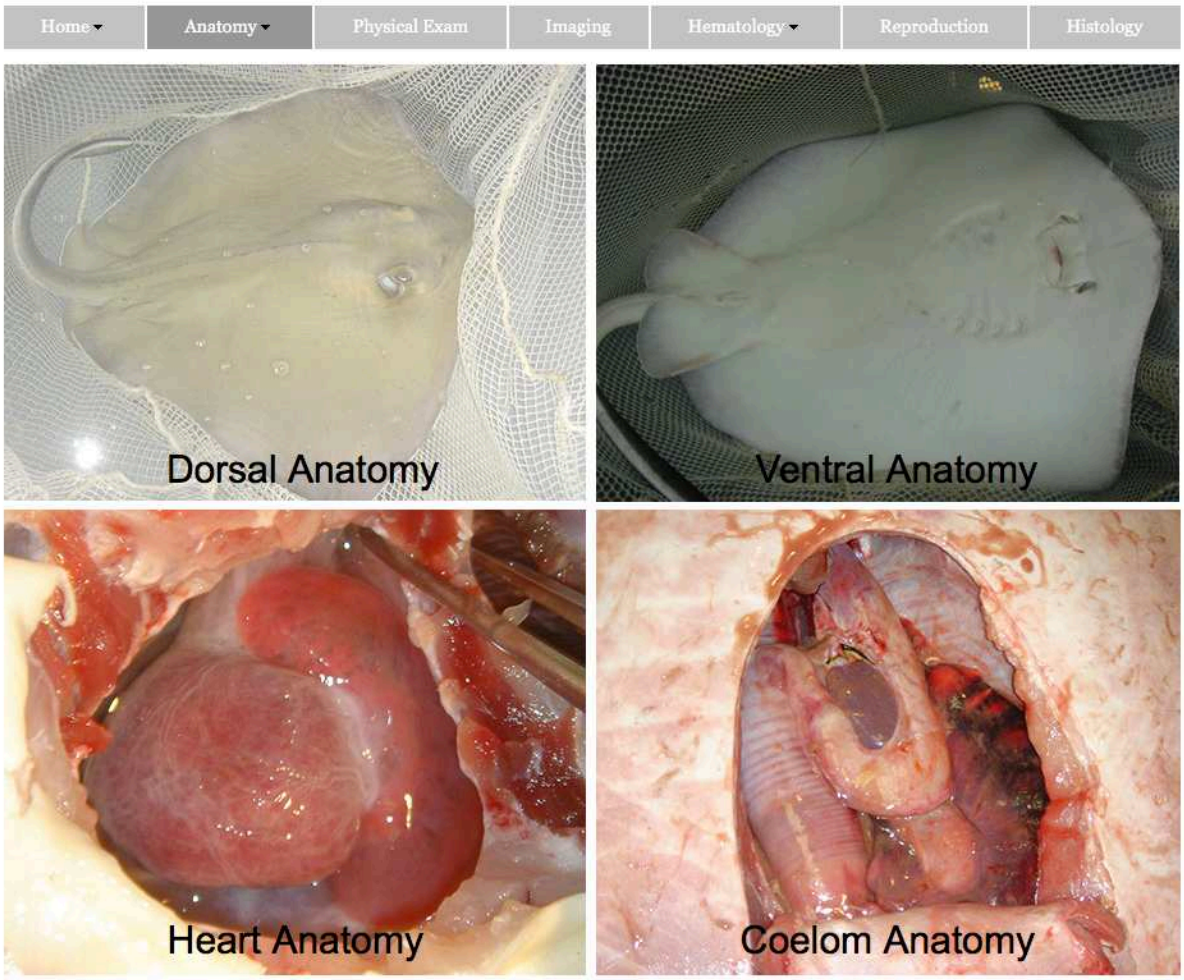
Karen A. Fox, DVM, PhD, Diplomate ACVP
Karamjeet Pandher, DVM, PhD, Diplomate ACVP

Anatomy – Example page



Elasmobranch Necropsy Procedure

Physical Exam – Example page

Home | Anatomy | Physical Exam | Imaging | Hematology | Reproduction | Histology

Physical Examination

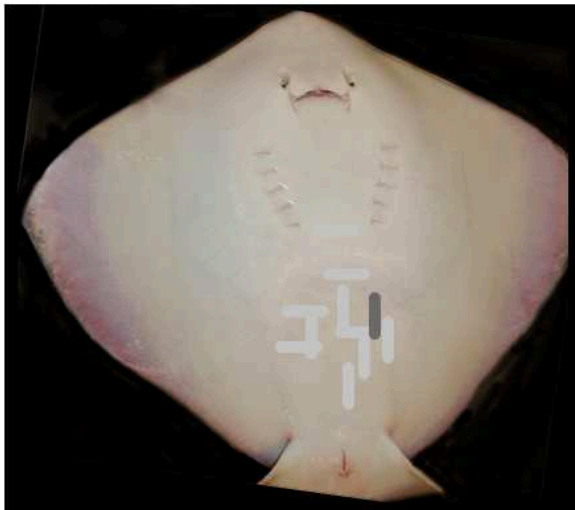
- Capture
- PIT Tag Reader
- Implanting PIT Tag
- Tonic Immobilization
- Measuring Wingspan
- Measuring Small Liver
- Acknowledgments

With the stingray either in dorsal (shown here) or ventral recumbency the wingspan may be measured using a tape measure. The tape measure should be held close to the body while holding the ends of the tape at each wing tip.

Imaging – Example page

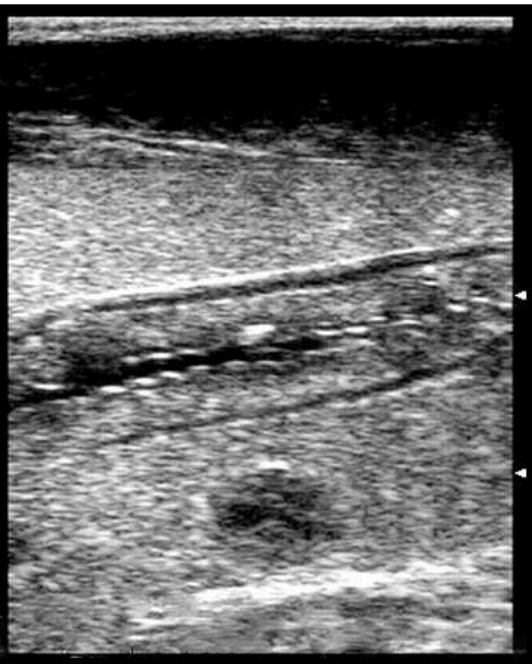
Home | Anatomy | Physical Exam | Imaging | Hematology | Reproduction | Histology

Ultrasonographic Imaging



The image above represents a stingray in dorsal recumbency with various locations marked for ultrasound imaging. Click on the linear transducer location to display a corresponding image to the right. Hovering over major organs will highlight their margins for identification.

A special thank you to Ray Parham and the Colorado State University radiology department for use of the ultrasound and imaging storage equipment; as well as to Tawni Silver for her expertise on equipment use and interpretation.



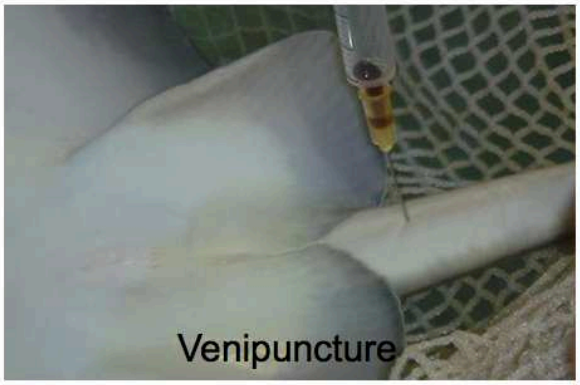
This is an image with the transducer in a sagittal orientation to the left of midline at midcoelom. The liver, stomach, and epigonal organ are seen. The long axis of the stomach is located just left of midline. The epigonal organ is closely associated with the ovary in this species and when imaged may be seen completely surrounding follicles.

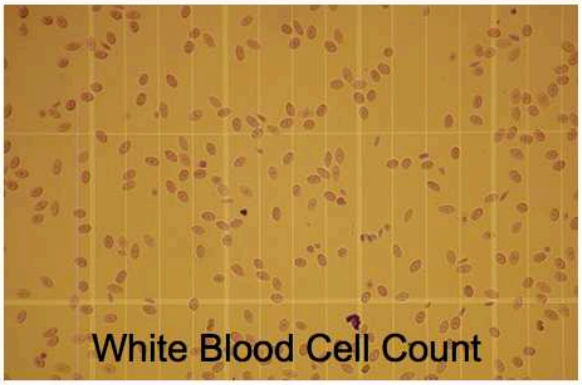
When hovering over the image the liver highlights blue, the stomach highlights yellow, and the epigonal organ highlights green.

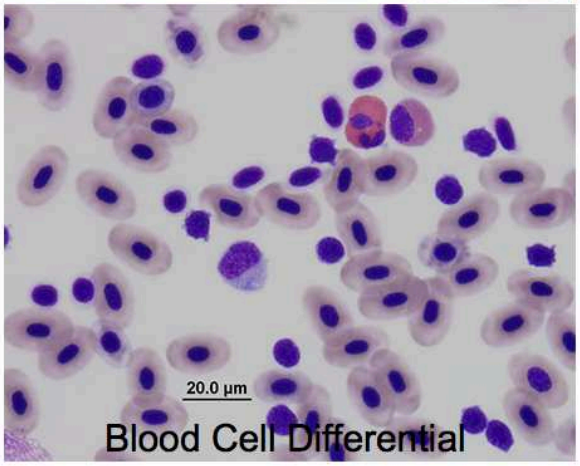
Hematology – Example page

Home | Anatomy | Physical Exam | Imaging | Hematology | Reproduction | Histology

Hematology





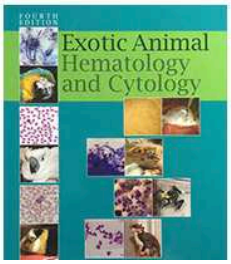


Hematology Reference Values

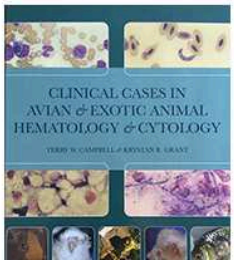
	Reference[a]
WBC x10³/ul	13.6 (3.8-27.9)
G_1 (Heterophils) x10³/ul	4.8 (1.0-8.9)
G_2 (Neutrophils) x10³/ul	0.3 (0-0.9)
G_3 (Eosinophils) x10³/ul	0.75 (0.1-3.1)
Basophils x10³/ul	0.1 (0-0.5)
Lymphocytes x10³/ul	8.25 (1.1-30.1)
Monocytes x10³/ul	0.2 (0-1.0)
Plasma protein g/dl	6.1 (5.3-8.6)
PCV %	26 (21-36)
Thrombocytes	

Reference Values

Exotic Animal Hematology and Cytology, 4th Edition by *Terry W. Campbell*



Clinical Cases in Avian & Exotic Animal Hematology & Cytology by *Terry W. Campbell* and *Krystan R. Grant*



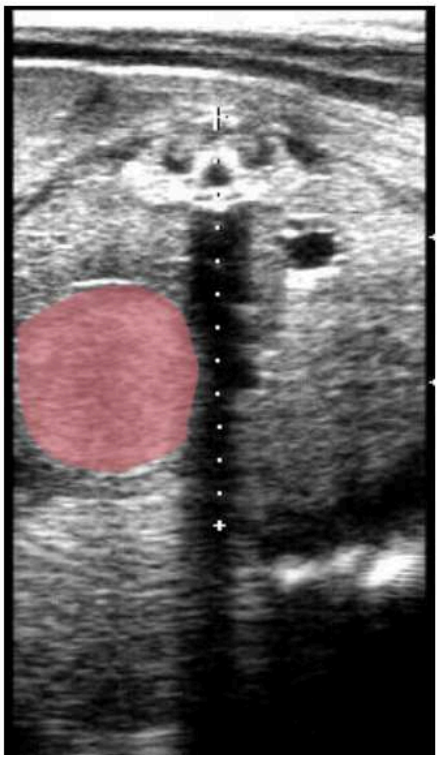
Reproduction – Example page

Home | Anatomy | Physical Exam | Imaging | Hematology | Reproduction | Histology

Reproductive Stages

- Trophonemata
- Early gestation - Egg Capsule
- Egg capsule and trophonemata
- Egg Yolk Sac - Trophonemata
- Cranial aspect of Fetus
- Caudal aspect of Fetus
- Post-gestation
- Cystic ovaries

All images were captured using a commmercial ultrasound unit (Aloka SSD-900v) and 7.5 MHz linear array transducer. The overall gain, time gain compensation, and depth settings were adjusted in order to maximize image resolution and organ visualization. Hovering over the image will reveal colored overlays to help with identification of certain organs



This image is obtained with the transducer in a transverse position relative to the fetus on the left side of the pregnant stingray.

This image shows the caudal aspect of the fetus (coelomic cavity of the fetus). Within the uterine wall (blue overlay) the fetus occupies the majority of the space. The large and small + symbols along with the dotted line denote the overall thickness of the fetus (3.92 cm). The cartilage forming the spine at the dorsal aspect of the fetus produces an acoustic shadow (yellow overlay). The gall bladder (green overlay) and spiral intestine (pink overlay) are also distinctly seen here.

This particular stingray pupped one month after this exam was conducted.

Histology – Example page

Home | Anatomy | Physical Exam | Imaging | Hematology | Reproduction | Histology

Histology

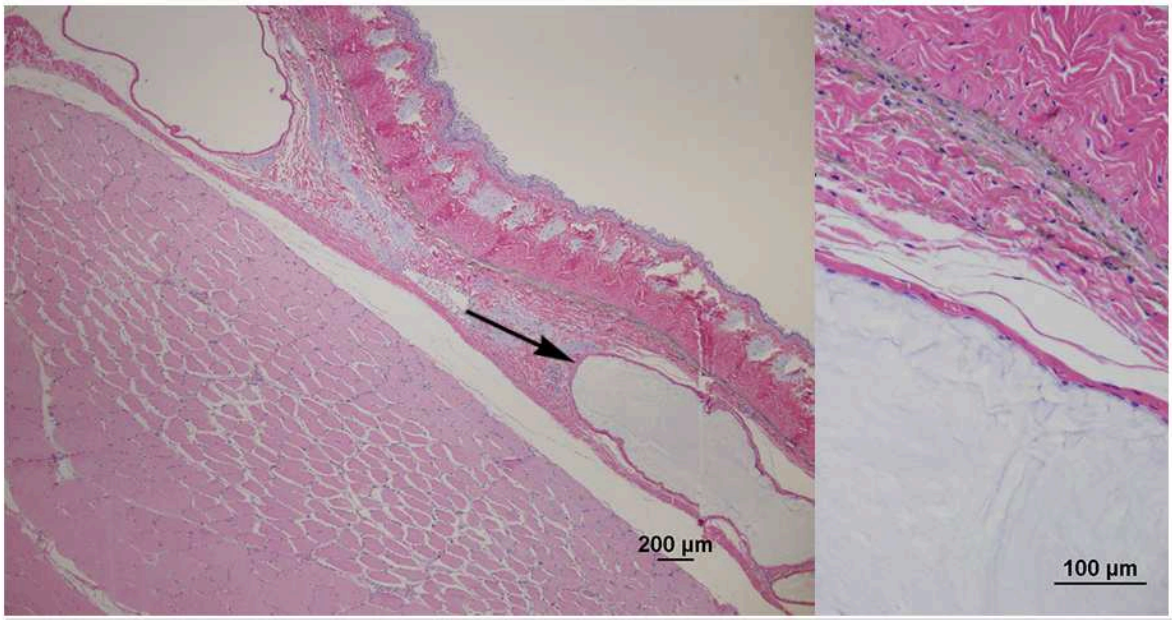
Krystan R. Grant, DVM © 2015

Eye

Skin

Ampullae of Lorenzini

The ampullae of Lorenzini are specialized electrosensory receptors located in the skin on the head and wings. Their use includes detecting changes in electric fields as a result of nearby prey and for navigation and orientation (Bleckmann and Hofmann, 1999). The images below show cross sections of the ampullary canals in the skin (arrow). The ampullary canal walls and inside consist of squamous epithelium (resistance of 6 million ohms/cm) and mucopolysaccharides (20-25 ohms/cm, similar to seawater), respectively (Bleckmann and Hofmann, 1999).



Muscle

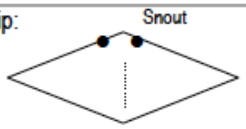
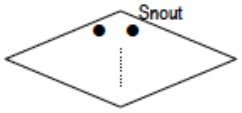
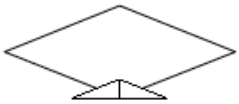
Brain - Cerebrum

Brain - Inferior Colliculus

Brain - Optic nerve

Heart

APPENDIX 5: Physical Examination Form

Identification	PIT Tag #:	Location of chip: (Dorsal) 	
Blood Collection (Tail vein)	☐ Blood smear ☐ Blood smear ☐ Green top tube ☐ Green top tube	Notes:	
Physical Exam			
Location	☐ Touch pool	☐ Quarantine #	☐ Other: Tank #
Species	☐ Southern	☐ Cownose	☐ Freshwater
Color			
Sex	☐ Male	☐ Female	
Size	Wingspan:	Snout to vent:	
BCS (out of 5) Vitals	(Flesh slope from the spine) 1 2 3 4 5	HR:	RR: WT:
Integument	 		
Measurements (cm)	Pectoral to pelvic girdle:	Liver (Distance to cd cart):	Liver: Coelom (%):
Ultrasound Exam			
Liver	☐ Sagittal: mid, LT, RT (gb) ☐ Transverse: mid, LT, RT (gb) ☐ Spleen comparison		
Stomach	☐ Sagittal ☐ Transverse		
Spiral colon	☐ Sagittal ☐ Transverse		
Pancreas	☐ Sagittal ☐ Transverse		
Epigonal organ	☐ Sagittal ○ Left ○ Follicles ○ Measure follicles		
Uterus	☐ Sagittal ☐ Measure uterus ☐ Trephonemata?		
Free Fluid	☐ No ☐ Yes ☐ Coelom tap		
Comments			

APPENDIX 6: Elasmobranch Necropsy Procedure

Elasmobranch Necropsy — Krystan R. Grant, DVM

Data collection and external examination

Gather supplies:

1
- Gloves
- Ruler or tape measure
- Scissors
- Forceps
- Bone cutter
- Scalpel blades
- 10% buffered formalin
- Microscopes slides
- Sterile syringe
- Culturette

Data collection:

2
- Record history - presumptive cause of death, location found, health status, exhibit/quarantine
- PIT tag number?
- Weight and length (sharks measure snout to caudal notch; rays measure discwidth)
- Male or female?

Note: The necropsy should be completed as soon as possible after death. If a necropsy cannot be performed do NOT freeze the specimen. Keep in a cooler or on ice.

External examination:

3
- Integument (**SKIN** including fins and tail) - note any lesions (type and location), unique markings.
- Examine **EYES** (3a) - note clarity (clear, cloudy), color, or possible trauma
- Examine **MOUTH** (3b/c) - note any discoloration or blockage (it may be difficult to pry open)
- Examine **GILLS** - note color (pink, red, blanched) and presence or absence of blood
- Examine **CLOACA** for any parasites or discharge - if so then collect a sample for cytology

Incision

The approach to the internal organs is a ventral midline incision in small sharks. The approach in larger sharks is the same with the addition of two perpendicular incisions at both ends, making an "I" incision, and retracting or removing the flaps. The approach in stingrays is to cut along the pectoral and pelvic cartilaginous margins and make a circular window.

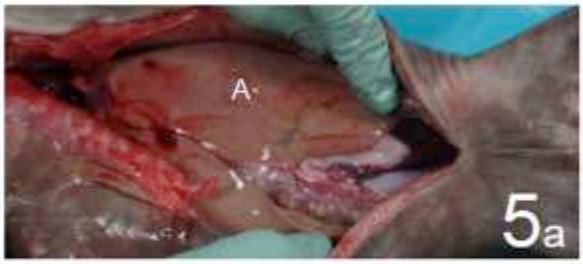
4 Shark skin is made up of tiny denticles, making it very difficult to cut through. To begin the incision, pinch the skin with forceps (or forefinger and thumb, but be careful not to cut yourself) and make a stab incision with a scalpel or sharp knife (4a). Continue the incision cranially and caudally with either the scalpel, knife or scissors (4b). The normal liver in elasmobrachs is ventral and large so be careful not to cut the liver while making the initial incicsion (4c). The incision should span the entire length of the shark from mouth to cloaca (4d). Do not touch the internal cavity. Cultures and fluid should be collected before touching any internal orgrans. The pectoral (4e) and pelvic (4f) cartilaginous girdles may need to be cut to access organs cranial and caudal to them, respectively.

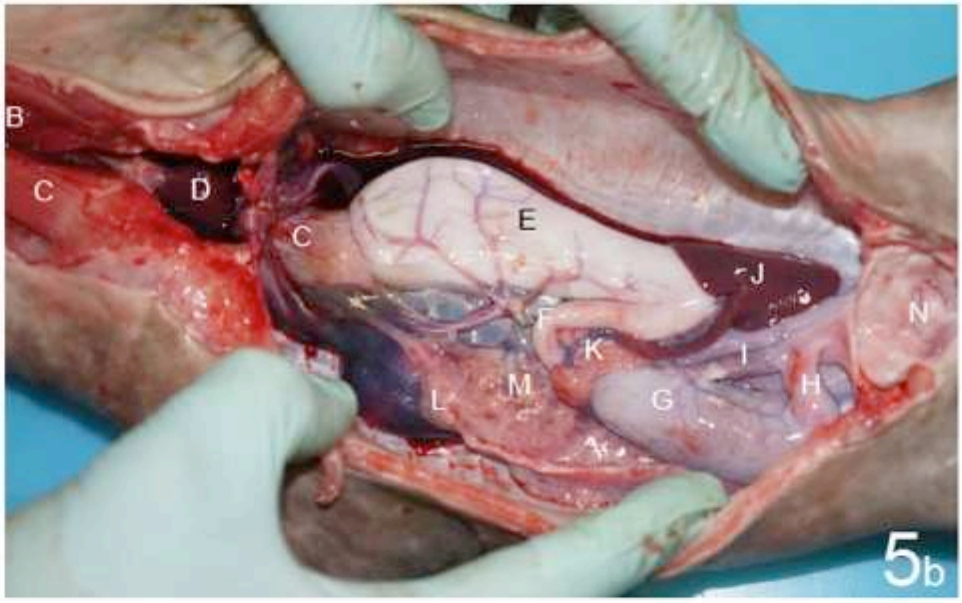
Anatomy

5 As mentioned, the liver in elasmobranchs is typically located along the ventral coelom and is quite large. Many times it will be the only organ seen upon initial view of the opened coelomic cavity (5a). Once the liver is removed (5c), most of the other internal orgrans can be seen (5b). The liver is the organ used in elasmobranchs for storing fat therefore its gross appearance should be large and tan-colored (5d).

Key for Images 5a/5b:

A. Liver
B. Thyroid
C. Esophagus
D. Heart
E. Stomach
F. Proximal intestine
G Spiral intestine
H. Rectocolon
I. Rectal gland
J. Spleen
K. Pancreas
L. Epigonal organ
M. Ovary
N. Cloaca
Not pictured: Reproductive tract, kidney, brain



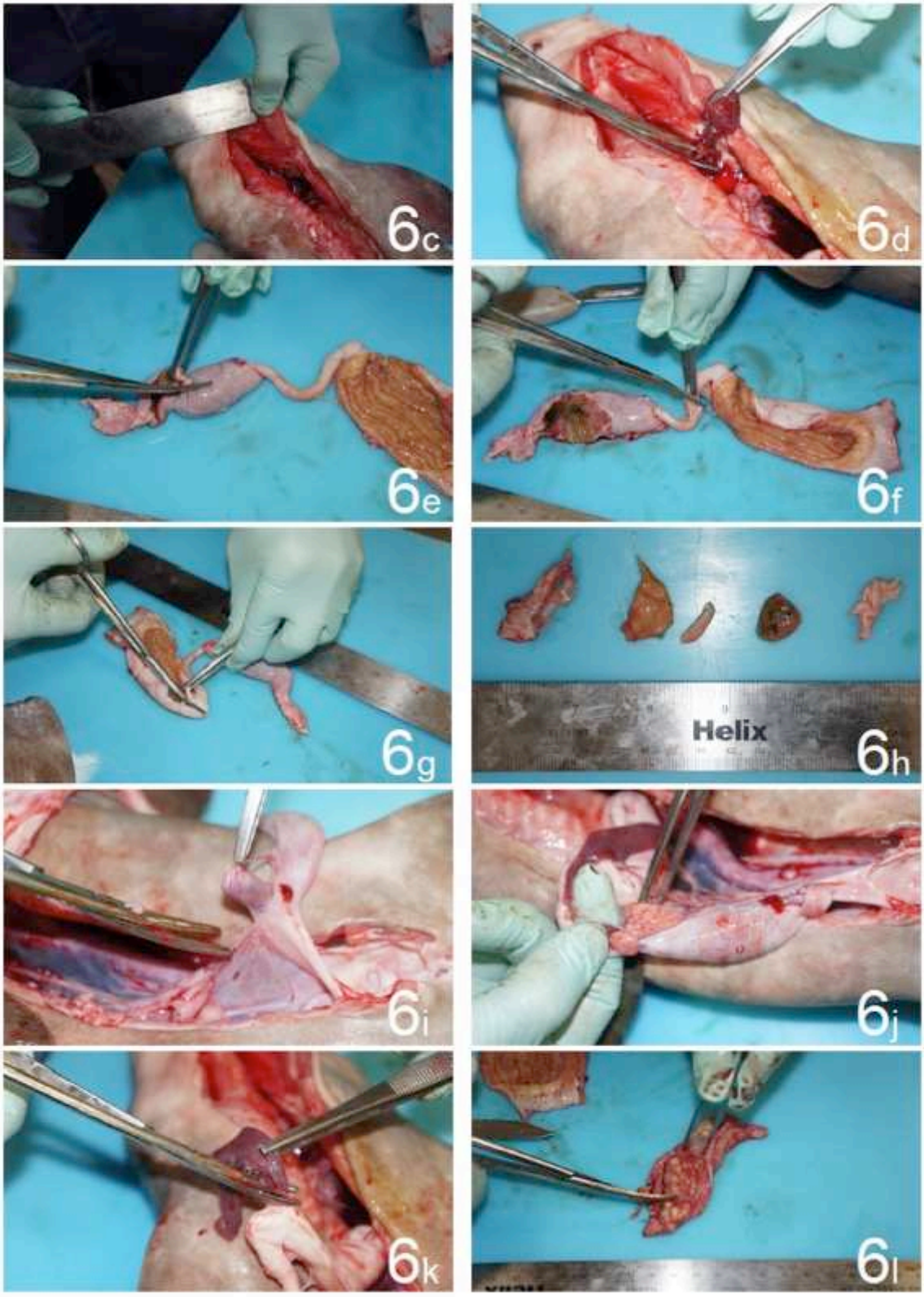


Sample Collection

6 Each organ should be properly identified and assessed for any gross pathology or abnormalities (masses, discoloration, size discrepencies, trauma, parasites, odor, obstructions, torsions, or other lesions). If potential disease is present consider taking impressions (on a slide away from formalin) and culture samples. Samples for histopathology should be no larger than **1 cm by 1 cm** and placed in 10% buffered formalin (6a and 6b). In some cases, the entire organ may be placed in formalin. Multiple samples may be placed in the same formalin container. If an organ appears abnormal, take a sample of the abnormal section as well as a sample from the apparent normal section. Organs for sample collection should include:

- **Liver** - large organ seen first when opening the coelom. It should be tan or beige and have sharp edges (6a).
- **Gall bladder** - attached to the liver, green and filled with bile.
- **Thyroid** - small, flattened gland caudal to the mouth (6c).
- **Heart** - just cranial to pectoral cartilaginous girdle (6d).
- **Esophagus** (and leydig organ) - extended from the distal oral cavity to the stomach. The leydig organ is usually located along the outer surface at the distal end (not in all species).
- **Stomach** - open stomach and inspect contents (6e).
- **Proximal spiral intestine** - connects stomach and body of spiral intestine (6f).
- **Spiral intestine** - open spiral intestine and inspect contents (6g).
- **Distal spiral intestine** (rectocolon)- connects body of spiral intestine to cloaca
- Gastrointestinal samples for histopathology (pictured left to right in 6h): esophagus, stomach, proximal spiral intestine, spiral intestine, distal spiral intestine (rectocolon).
- **Rectal gland** - small gland found near outer surface of distal spiral intestine (6i)
- **Pancreas** - small, tan organ located on the outer surface of the proximal spiral intestine (6j).
- **Spleen** - reddish organ located near pancreas (6k).
- **Epigonal organ** - varies in size (dynamic organ) and may be closely associated with the gonad, may be reddish or tan and bilateral (6l).
- **Gonad** (ovary or testis) - may be uni- or bilateral depending on species and may be in close proximity to epigonal orgran
- **Reproductive tract** (oviduct, oviducal gland, uterus) - be sure to check for egg development and fetuses. This organ may also be uni- or bilateral depending on species (6m).
- **Kidney** - retroperitoneal, bilateral organ located near spinal column (6n).
- **Eye** - carefully remove one eye and place in formalin (6o).
- **Gill** - red organ found inside gill slits (6p).
- **Skin** (and ampuli of Lorenzini) - collect any portion, ampuli of Lorenzini is a special sensory organ on the ventral surface.
- **Muscle** - collect any 1 cm by 1 cm portion and place in formalin.
- **Brain** - shave skin and cartilage to access the brain, should be light yellow to white. Carefully remove and place entire brain in formalin if the animal is small as in this example. Larger brains may need to be sectioned (6q-6t).

Sample Collection (continued)



Sample Collection (continued)