Serum Protein Analysis as an Immune Assessment Tool in South Florida Nurse Sharks, Ginglymostoma cirratum

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SERUM PROTEIN ANALYSIS AS AN IMMUNE ASSESSMENT TOOL IN SOUTH FLORIDA NURSE SHARKS, GINGLYMOSTOMA CIRRATUM

By

Leila Atallah Benson

A THESIS

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SERUM PROTEIN ANALYSIS AS AN IMMUNE ASSESSMENT TOOL IN SOUTH FLORIDA NURSE SHARKS, *GINGLYMOSTOMA CIRRATUM*

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Assessment of shark health can provide valuable insight into overall population health. In this study, immunological assessment tools are reviewed for their use with wild shark populations. Nurse sharks, *Ginglymostoma cirratum*, were non-lethally blood sampled from wild individuals in south Florida. Reference ranges are given for the first time for serum protein electrophoresis, which included five fractions that correspond closely with other elasmobranch species. Differences between serum and plasma may be negligible for protein electrophoresis, and therefore, plasma may be preferred in field settings. Reference ranges for other biometric and blood parameters were also established. The granulocyte to lymphocyte ratio for nurse sharks was found to be $1.17 \pm 0.18$. Protein isolation and identification techniques were used to determine if c-reactive protein (CRP) or related proteins might be used as immunological biomarkers. Protein isolation yielded two putative serum protein fractions of interest, a complement component C3 protein and a pentraxin domain-containing molecule. Future studies may allow development of antibody assays to biomarkers such as C3 and CRP to effectively measure these proteins in shark blood samples from wild individuals. Use of these biomarkers may be examined in
combination with other immune health parameters to assess the health of wild shark populations and relate these to ecological conditions in the environments they inhabit.
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Introduction

Immunological health can provide substantial information about wild populations and their environments (Pedersen & Babayan 2011). In order to measure health parameters in wild sharks, effective methods must be established and baseline data must be collected for each species (Arnold 2005). Once reference ranges are established, concentrations of certain health biomarkers can be used as proxies for shark health.

The current study had three specific aims: 1) provide insight into possible immunological assessment tools for use with wild shark populations, 2) provide reference ranges for nurse sharks, *Ginglymostoma cirratum*, found in south Florida as an initial species of interest, and 3) isolate and characterize candidate biomarkers such as c-reactive protein in nurse sharks as a first step in developing health biomarker assays that could potentially be used routinely in the future to assess shark health in the wild from blood samples.

The process of assessing health can be particularly difficult in wild animal populations. In captive situations, animals can be sampled multiple times and changes in condition can be monitored, providing information as to what caused the abnormality. In most wild populations, one encounter is the only guarantee you have with any sampled animal, and therefore, assays must be able to provide information with only a small snapshot of data. There are multiple immune assessment tools used throughout human and mammalian medicine that can be drawn upon to develop ways to clinically evaluate wild shark populations (Van Beaumont 1972, Keller et al. 2000, Tothova et al. 2016). To address the study objective (1), the first part of this thesis reviews common immunological tools found
throughout the literature and is aimed at establishing what is feasible for use with wild sharks.

One immune assessment tool accessible with wild sharks is serum protein analysis (Haman et al. 2012, Cray et al. 2015). Many proteins found in serum samples are already used in mammalian medicine as biomarkers for health. Accordingly, to address study objective (2), the second part of this thesis was aimed at generating reference ranges for five different fractions of serum protein, based on mammalian serum protein electrophoresis fractions (Cray et al. 2015). This is the first study to provide protein electrophoresis reference ranges for the nurse shark. Peripheral blood smears were also used to provide differential leukocyte counts for 19 individuals, and these reference ranges are also given, along with three fitness measurements, precaudal length, fork length, and total length, for all 50 sharks, four fitness measurements, lateral span, frontal span, proximal span, and caudal keel, for 41 sharks, and three other physiological parameters, glucose, hematocrit, and lactate, for 34, 32, and 10 sharks respectively. Together these reference ranges provide a clearer picture of what average nurse sharks look like in south Florida.

Finally, to address study objective (3), the final part of this thesis attempts to identify candidate biomarkers found among serum proteins that might be suitable for clinical use. One such candidate commonly used in human clinical medicine, c-reactive protein (CRP), was targeted in this study in the nurse shark. C-reactive protein is an acute phase protein, which is used as a biomarker for mammalian inflammatory diseases. After isolation and characterization, this protein could potentially be used as a biomarker for shark health.
Chapter 1: Immune assessment tools in the conservation of wild sharks

Background

Sharks are dependent on their immune systems to protect them against pathogens and disease (Janeway & Medzhitov 2002). In wild populations, measuring components of immunological activity can provide a way to assess the health of sharks and relate those assessments to population dynamics (Keller et al. 2000). Measures of immunocompetence in individual sharks may reveal trade-offs that exist within populations responding to various immune stressors, such as an increased pathogen or parasite load, habitat degradation, or other environmental disturbances (Luster et al. 1992). An initial step in determining the immunocompetence of wild sharks is establishing baseline measurements within and across species. Abnormal results can then be used as biomarkers of poor health status in shark populations.

Immunological studies in sharks have been conducted using model organisms in the laboratory, such as the small-spotted catshark, Scyliorhinus canicula, and the nurse shark, Ginglymostoma cirratum (Morrow et al. 1982, Roux et al. 1988, Li et al. 2015). These animals have been used primarily as models for studying the evolution of the human immune system, given that elasmobranchs are among the earliest groups of vertebrates to exhibit a fully functional innate and adaptive system comparable to that found in mammals (Rauta 2012). Both innate and adaptive components of the shark immune system have now been described in detail and many of the pathways for immune reactivity are well known (Lopez et al. 1974, Diaz et al. 1999, Fujita 2002, Flajnik & Du Pasquier 2004, Dooley & Flajnik 2005). However, much of this information has been derived from only small or
juvenile stages, of a few species of shark, used as models because of the ease with which they are kept in captivity and/or handled in laboratory settings. Very little is known about other shark species in terms of their immune system and immune reactivity. There is sparse information available about the susceptibility of wild sharks to disease following injuries associated with being caught as by-catch, or associated with other environmental stresses and/or behavioral patterns. It is not known whether the immunological reactivity seen in model organisms is uniform across different species not yet examined. Evidence suggests that there is a high degree of variation in the immune systems of different species which might be explained by the length of evolutionary time between divergences among various shark lineages (Marchalonis et al. 1998, Watts et al. 2001).

One of the challenges for studying the immune system of migratory sharks is the ability to collect tissue samples and conduct field immune assessments. Current monitoring programs, that include tagging studies, present an opportunity to investigate the immune status of wild sharks and gain further understanding of their immune system. Immune status might then be used to evaluate the well-being of wild sharks under various stresses, in diverse habitats, and at different levels of maturity. However, before such studies can really take shape and provide critical information for the conservation of each species, baseline data for various immunological parameters must be established (Arnold 2005). Once baseline information is established, a methodology can be developed to apply these immune assessments to current monitoring protocols. This would allow investigators to assess the health and immune status of wild sharks and relate their immune condition to their age, maturity, habitat use, migration pattern, behavior, reproductive activity, and anthropogenic stressors. Here, current methods used in the measurement of health
parameters in sharks are briefly reviewed. The purpose of this review is to highlight the role of shark immune response in health, explore the development of immune assessment tools in shark studies, and discuss the application of these tools in shark conservation.

**Current Health Assessment**

The health of wild sharks has only recently been measured, while studies conducted in captive animals have been published over the last few decades as case studies and as part of care manuals (Fange et al. 1981, Crow et al. 1991, Smith et al. 2004, Walsh & Luer 2004, Bodine et al. 2004, Marancik 2012). Captive studies are not necessarily representative of conditions sharks face in the wild, but the methods used in their health assessment may be a useful guide as the transition is made to conducting assessments from samples collected in the field. In captive populations, baseline data can be obtained by repetitive sampling of the same individuals over time, typically with a small number of animals. In the wild, researchers are often guaranteed only one interaction with the individual, only receiving a snapshot of that individual, and an even smaller snapshot of the population. With species in which sampling of large numbers of the population is difficult, this can produce a serious problem with establishing baseline values.

Immunological functions may differ between populations of the same species depending on environment and prey, creating generalization across populations difficult (Le Moullac & Haffner 2000, Cheng & Chen 2000, Kau et al. 2011). This may also be an issue when trying to compare captive baselines with wild baselines. Therefore, to create accurate baseline levels and evaluate potential differences between and within populations, as many individuals within a population and as many populations as possible should be
sampled. There are studies that have attempted to assess effects of fishing and/or handling on stress physiology, but measures of physiological stress have been reviewed elsewhere and are beyond the scope of this review (Wells et al. 1986, Hoffmayer & Parsons 2001, Gallagher et al. 2014). Similarly, there have been investigations measuring the nutritional and/or metabolic status of sharks in the wild, using various measurements and relating these variables to ecological conditions such as foraging behavior and habitat use (Bushnell et al. 1989, Lowe 2001, Dowd et al. 2006). Other studies have focused on measures of body condition using biometric measurements and hematological parameters that can be measured in both captive and wild populations using non-invasive techniques (Arnold 2005, Irschick & Hammerschlag 2015, Fu et al. 2016). Interest in these health assessment tools has grown, with more sharks being kept in captivity in various aquaria settings, and with an increased interest in monitoring and conserving wild populations. In general, the number of papers related to health assessment in sharks has significantly increased over the last 20 years (Figure 1.1).

**Figure 1.1:** Literature published on shark health parameters. Number of research papers published between 1996 and 2016 on shark health parameters found using several databases including PubMed, Google Scholar, and Web of Science. A total of 65 papers were found and were inclusive of those related to stress physiology and studies done in captive populations.
Measures of Body Condition

The development of a reliable measure of body condition for a species is critical for predicting how a population is likely to respond to either natural or anthropogenic changes in their immediate environment (Irschick & Hammerschlag 2014). In earlier studies, the livers of sharks were used to measure body condition following capture, often as part of fisheries management actions. The weight of the liver can yield clues, a heavier liver may indicate the animal has higher energy reserves since more fatty acids are stored, therefore suggesting it is a relatively healthy animal. However, it depends on the overall weight of the individual, since that can skew results of shark liver weight as a measure for body condition (Kohler et al. 1996). The overall size of shark liver is a very accurate metric of health, but challenges remain in estimating liver size in live sharks (Irschick & Hammerschlag 2014). Measuring body condition in live sharks has been addressed in several ways, although relatively few studies have addressed this directly.

A traditional approach to body condition is to consider the condition factor as mass per unit length. It is assumed that this measure is related to evolutionary fitness as increases in body condition should be correlated with greater energy reserves, which should allow for increased survivability and reproductive capacity (Dibattista et al. 2007). Often, measurements of mass are not available in field studies and in those studying large sharks; the condition factor is taken instead as the relationship between body girth and total body length. Overall body condition has also been addressed by relating condition indices to energy stores. Specifically, the relationship between triglycerides and the metric of overall body condition has been evaluated (Gallagher et al. 2014). In this study, twenty-eight tiger sharks, *Galeocerdo cuvier*, were captured, measured, and blood samples were taken and
used to measure triglyceride values. Results showed a significant positive relationship between condition and triglyceride values, suggesting that future health assessment research can use this method, especially in large sharks, when using other techniques may not be possible (Gallagher et al. 2014).

One of the challenges associated with the measurement of girth is that it is extremely difficult to measure the total circumference of a captured shark. In large predatory sharks, a simple metric has been developed to quantify body condition based on four measurements of dorsal side only body girth measurements relative to body length, and is called the scan condition analysis, or SCA (Irschick & Hammerschlag 2014). SCA may be used as a universal method for all sharks, as well as marine mammals. Measurements of different body dimensions used to calculate an index of condition include pre-caudal length, lateral span, frontal span, proximal span, and caudal keel circumference, considering the tapered shape of sharks (Irschick & Hammerschlag 2014). In all cases, accurate measures of body condition in the health assessment of captured wild sharks should include biometric data as well as hematological data that may include fatty acid analysis, triglyceride levels, ketone bodies, and other measures of nutritional and/or metabolic status.

**Critical Blood Analytes**

Blood chemistry reference values have only been determined for a relatively small number of species and those values are often based on small samples sizes (Haman et al. 2012). Thus far, there is evidence that suggests that baseline hematologic and biochemistry values may be significantly different between species, and those differences may be due, at least in part, to environmental and/or ecological conditions within different habitats. A recent study of wild caught sharks compared health indices of three species: thirty Atlantic
sharpnose, *Rhizoprionodon terraenovae*, thirty-one bonnetheads, *Sphyra tiburo*, from the western Atlantic, and thirty spiny dogfish, *Squalus acanthias*, from the eastern Pacific (Haman et al. 2012). All three species have minimally overlapping habitats with different foraging ecologies and two populations inhabit coastal and estuarine waters, but under very different conditions. Significant differences in basic hematologic and biochemistry values were found, with interspecies differences observed in all plasma chemistry except a few plasma proteins and all plasma trace nutrients were different amongst species except for iron (Haman et al. 2012). However, some plasma chemistry variables were not analyzed because they were outside the detectable range (Haman et al. 2012). The study highlights the need to standardize methodology for blood chemistry analyses so that differences between species and among populations can be explored further. Very little work has been published relating changes in blood chemistry values to disease susceptibility in any elasmobranch species, so further work in this area is also required (Hattingh et al. 1974, Skomal & Mandelman 2012). Health assessments of wild sharks are currently hindered by the lack of clinical pathology reference values.

An important step in establishing useful blood chemistry reference values is to standardize collection methods (Harms et al. 2002). Blood gas, pH, and lactate values are useful in assessing the health of fish immediately after collection. Portable clinical analyzers (PCAs) may present a unique opportunity to measure these parameters in the field more effectively, as they are portable and can obtain boat-side results in minutes (Gallagher et al. 2010). PCAs have been shown to reliably assess blood gases and acid base profiles of teleosts, but equivalent data is not yet available across elasmobranch species, although PCA use has been validated for relative accuracy in select blood gas properties...
for two species of ectothermic shark. PCAs, designed for use in mammals, process samples at 37°C, so it is necessary to obtain species-specific equations to convert blood values obtained at 25°C, or other possible temperatures. Gallagher et al. 2010, used PCAs with sandbar sharks and dogfish, and found they were able to convert collected measurements using these species-specific equations, but that these equations would have to be developed depending on the species and temperature. By comparing data obtained from this method with previously obtained measures, it was shown that hematologic parameters can be converted, with confidence, using the equations provided by Mandelman and Skomal (2009). However, the application of this technique for other elasmobranch species should be used with caution, since values seem to be species-specific and temperature dependent (Gallagher et al. 2010). PCAs have also been used for blacktip, bull, great hammerhead, lemon, and tiger sharks. However, validity of PCAs in these species was not reached due to species-specific equations not being obtained (Gallagher et al. 2014).

It is important to measure these parameters in a wider range of species to validate methods; many studies have been done in captive populations. Additionally, it would be beneficial to validate measures at different stages in life history, and for populations in diverse habitats. Some studies have used the PCA technique to investigate critical analytes in specimens categorized by their habitat use as either pelagic, benthic, or intermediate (Atkins et al. 2010, Kinney et al. 2011, Naples et al. 2012, Stoot et al. 2014). Species in these environments may differ in their tolerance to oxygen level conditions and other factors that may be reflected in their blood chemistry (Naples et al. 2012).
**Shark Immune Responses**

Sharks have a robust set of immune responses that are similar to that observed in higher vertebrates, such as mammals, and that provide sharks substantial protection from disease (Raschi & Tabit 1992, Alexander & Ingram 1992, Mann et al. 2014, Andre et al. 2015). A shark’s first line of defense is its tough skin, covered in dermal denticles, which presents a significant physical barrier to infection (Raschi & Tabit 1992). These placoid scales have specialized, micropatterned arrangements that decrease the incidence of bacterial invasion and have been used as a template in engineering antibacterial surfaces (Mann et al. 2014). Shark skin also contains a robust layer of mucus, consisting of lysins, agglutinins, and antimicrobial factors (Alexander & Ingram 1992). Second lines of defense include innate immune recognition molecules. Soluble forms of immune recognition molecules include lectins and other molecules that bind antigen. Cell bound forms of innate immune recognition include toll-like receptors (TLRs) and other recognition receptors on the surface of leukocytes (Andre et al. 2015, Butcher 1991). Leucine rich repeats on TLRs bind to specific bacterial ligands and include a suite of innate responses, such as the complement cascade (Takeda et al. 2003). Complement cascades release bioactive peptides and clear infections through membrane attack complexes that lyse cell membranes (Müller-Ederhard 1988). Immune recognition in leukocytes also leads to phagocytosis and downstream immunological responses.

**Innate Immune Responses**

Cellular signaling induces recruitment of cells and inflammation at sites of infection (Hanada & Yoshimura 2002). Chemokines are released and help recruit neutrophils, monocytes, and other immune responsive cells responsible for cell-mediated responses,
including phagocytosis. Responding cells express receptors that recognize microbial ligands and are induced to produce additional signaling molecules like cytokines. Cytokines are cell to cell messenger molecules that induce further innate responses, such as the complement cascade and the production of acute phase proteins. Acute phase proteins (APPs) are released and targeted to areas of inflammation where they aid in acute responses (Marchand et al. 2005, Zlotink & Yoshie 2000, Black 2002). Measuring specific APP concentrations at a given time can be used as biomarkers of inflammation and overall health (Eckersall & Bell 2010).

**Adaptive Immune Responses**

Innate immune cells can further drive immune responses by presenting antigenic material to cells of the adaptive immune system to induce the production of antibody and T cell responses. Sharks possess adaptive immunity and produce antibody molecules that exhibit exquisite specificity to infectious agents (Carroll 2004, Vesely et al. 2011). V, D, and J germ-line encoded segments rearrange, utilizing RAG1 and RAG2 genes, within their clusters to create specific antibodies (Schulter & Marchalonis 2003, Rumfelt et al. 2000, Dooley & Flanjnik 2006). Shark immunoglobulins produced in response to infection as well as natural antibody can make up a significant portion of plasma protein. Measurements of plasma proteins may include innate immune proteins like complement, inflammatory mediators, like acute phase proteins, as well as, immunoglobulin and total protein.

**Shark Immune Assessment**

Sharks possess all of the major features of innate and adaptive immune function seen in humans, and have been used as model organisms since they are the most evolutionarily distant taxonomic group to share the same molecular mechanisms underlying these
features, with their last shared ancestor existing 450 million years ago (Rauta 2012). In fact, many of the genes and proteins of the shark immune system have high rates of similarity and identity to those found in mammals (Saavedra et al. 1989, Greenberg et al. 1993, Diaz et al. 1999). Given these similarities, tools used to assess human immune function may be used to inform projects aiming to measure and monitor the immunological health of sharks (Keller et al. 2000). Using clinical assessment methods from human populations may also be beneficial, in that they are usually minimally invasive and non-destructive, which may be particularly important in threatened shark populations.

**Clinical Hematology**

Clinical hematological assays are used to assess the condition of the body. They are among the initial measures used to assess physiological status in an organism. These measures include hematocrit, erythrocyte sedimentation rate, and measures of hemoglobin. Hematocrit is used to measure the amount of blood cells relative to plasma, the protein fraction of the blood. This information provides insight as to how healthy an individual is, by indicating how successfully they can produce red blood cells (Van Beaumont 1972). Erythrocyte sedimentation rate (ESR) measures how quickly red blood cells settle within an hour, which nonspecifically measures infection and inflammation (Plebanini & Piva 2002). Hemoglobin is the protein in blood that carries oxygen. A hemoglobin test measures how much of this protein is present in whole blood (Van Kampen & Zijilstra 1966). A lack of hemoglobin may point to decreased health or a medical condition, such as anemia. These tests are all part of a normal human blood panel. Examining all three tests together can provide an idea of the overall clinical health of an individual. In combination with other hematological and immunological parameters, they may provide a non-invasive way to
evaluate an organism clinically using only a small sample of blood, ideal for field collection.

**Peripheral Blood Smears**

Differential leukocyte counts are performed on peripheral blood smears for the enumeration of peripheral blood leukocyte types, or white blood cells. The differential blood count is used routinely as a clinical tool in both human and animal medicine (Centers for Disease Control Laboratory Manual 2013-2014). Following infection and inflammation leukocyte levels are often increased, and differential counts can be used to assess the infection and/or inflammation status of an animal. Shark blood cells were characterized in 1983 by Hyder et al. in nurse sharks. Shark blood possesses nucleated red blood cells, as well as leukocyte cells not present in human blood. Thrombocytes are spindle shaped leukocytes that assist in clotting (Hyder et al. 1983). Multiple other kinds of leukocytes are found in shark blood, including granulocytes, such as neutrophils, coarse eosinophilic granulocytes and fine eosinophilic granulocytes, lymphocytes, and monocyte-macrophages, several of which are phagocytic (Hyder et al. 1983, Smith et al. 2014).

The site of hematopoiesis in sharks are the leydig and epigonal organs, unlike mammals whose take place in bone marrow, which sharks lack (Oguri 1983, Fange & Mattisson 1981, Dean & Summers 2006). The Leydig organ contains a reticulum with leukocytes that are less mature than leukocytes found in the blood (Mattisson & Fange 1982). However, immature leukocytes are found circulating throughout the blood, like blast-like cells and mitotic cells (Hyder et al. 1983). This can make blood counts more difficult than in their mammalian counterparts, because it is difficult to define which leukocytes are which in
their immature state. Therefore, standardizing the methods used in identifying shark leukocytes in each species is required.

Peripheral blood slides prepared in the field can be taken back to the laboratory and viewed under light microscopy to establish reference intervals for sharks. These average reference values can then be compared to abnormal blood cell ratios to examine differences in the individual sampled. Arnold 2005 created a standardized technique for complete blood counts in elasmobranchs. This standardization was done with five captive sandbar sharks, *Carcharhinus plumbeus*. Like most other tests covered in this review, baseline data for different populations, as well as a species, must be obtained. Once baselines are obtained, observations such as leukocytopenia, a decrease in white blood cells, or off-balance granulocyte to lymphocyte ratios can detect health problems among individuals in specific populations (Reichardt et al. 1999, Yamanaka et al. 2007). Specific populations may be effected by their habitat location and use, such as sharks living near coastal cities, experiencing anthropogenic effects.

**Leukocyte Functional Assays**

Functional assays for immune reactivity are traditionally used to measure immunocompetence in both medical and veterinary settings. When available, isolated and/or cultured cells derived from an animal are used as targets in measuring functional responses such as phagocytic activity, proliferative or chemotactic responses to various antigens, and changes in gene expression and subsequent release of cytokines/chemokines in response to stimulus. However, these studies require fresh cells to be harvested, isolated, and maintained in a laboratory setting for several hours, if not days. Field sampling presents several limitations to conducting such studies. Most immunoassays performed as part of
clinical assessments require access to individuals for repeated measurements, or the isolation of cells and/or tissues that cannot be obtained from studies where wild animals are typically only encountered once and minimally invasive techniques are used (Keller et al. 2000). The lack of repeatability available with wild sharks makes a lot of assays difficult to perform in the field.

First, separating target cells such as peripheral blood leukocytes from whole blood while aboard a vessel is problematic and difficult to do. Second, shark blood contains a high level of urea as part of its osmoregulatory mechanism (Goldstein et al. 1968, Epstein et al. 1983). The high urea content influences the structure and functional capability of shark immune proteins (Forster & Goldstein 1976, Rahman et al. 2015). It also presents a challenge when conducting studies with shark cells, since normal activity can only be observed under ideal urea concentrations. Given the nature of field conditions, it is difficult to keep samples in a state conducive to isolation and maintenance in the laboratory and it is also necessary to develop reagents specific to shark osmolality. This is important to consider when trying to perform any functional cellular assay, such as proliferation, chemotaxis, or phagocytosis (Lopez et al. 1997, Saliki & Lehenbauer 2001, Toetsch et al. 2009, Bossart et al. 2011). Other functional assays can include measures of antibody responses using the whole animal where an antigen is introduced and then later, the antibody response is measured by measuring the antibody titer in the blood of the animal (Voller et al. 1978). This would require at least two encounters with the animal of interest, which is not possible under normal field sampling conditions. Recapture rates are typically too low and the timing between encounters entirely unpredictable, when they do occur.
**Protein Electrophoresis**

Serum protein electrophoresis measures the amount of proteins present in a serum sample. Serum is obtained from whole blood after the blood clots and the clotting protein fibrinogen and other clotting proteins are removed. Cells are also removed via centrifugation, leaving behind the liquid serum. Plasma is collected via the same process, but without allowing time for clotting to occur, keeping clotting proteins as part of the plasma sample. Both plasma and serum can be applied to protein electrophoresis techniques to observe and measure the major protein components present. These can include many protein components including albumin, fibrinogen, and immunoglobulin (Longsworth et al. 1939). Clinical reference values for the levels of protein fractions of interest and the ratios between various proteins are established in both medical and veterinary settings for species of interest. Abnormal protein ratios can point to disease and are used clinically as a biomarker of overall health (Tothova et al. 2016). In wild animals, plasma is usually obtained from blood collected because it is easier to process using centrifugation than serum, which requires time for blood clotting. The presence of clotting proteins in plasma may skew the way in which proteins are fractionated in electrophoresis, so it becomes necessary to establish whether these differences are negligible for each species studied (Tothova et al. 2016).

The use of protein electrophoresis in clinical and veterinary medicine is routinely carried out as a diagnostic tool. In captive animals, it is often a component of routine health assessments. For animals that are kept in captivity, some data is available on reference values, but they are still lacking for many species of shark due to issues of sample size and standardization of methods. For many species, the number of samples is still too low to
establish normal baseline measures and the interpretation of the results are difficult. In the last few decades, efforts have also been made using electrophoresis in wild animal populations as a measure of health status since it only requires a small volume of blood to perform these tests (Cray & Tatum 1998, Haman et al. 2012).

Studies in avian medicine have demonstrated the utility of protein electrophoresis to indicate disease states in bird populations as well as link physiological well-being to environmental variables such as habitat degradation, urban development, and other anthropogenic stressors (Cray & Tatum 1998, Tatum et al. 2000, Naylor et al. 2017). The success of such analyses is dependent on obtaining sufficient sample sizes in each population to establish clinical norms. In the ocean, these techniques have been applied to wild pinniped and cetacean populations that are protected and, therefore, can only be sampled intermittently and with non-invasive techniques (Zenteno-Savin et al. 1997, Brock et al. 2013, Paez-Rosas et al. 2016). Results indicate that abnormalities in protein electrophoresis profiles can provide clues to these animals’ hydration status, nutritional status, infection state, and/or susceptibility to disease (Gray et al. 2005, Marquez et al. 2007, Bossart et al. 2011).

Protein electrophoresis has been used to clinically assess elasmobranch samples using fractionation based on conventional mammalian standards. In studies by Krol et al. 2014, Cray et al. 2015, and Hyatt et al. 2016, electrophoresis profiles were determined using serum or plasma collected, and applied to beta gels, which were then scanned using software to identify protein fractions. Five fractions were established using conventional mammalian placements, but referred to as numbered fractions since electrophoresis in sharks may not be comparable to mammals: albumin (1), alpha 1 (2), alpha 2 (3), beta- (4),
and gamma-gobulins (5). In all three studies, albumin levels were negligible and beta-fractions were the most elevated, a consistent result across elasmobranch protein electrophoresis (Krol et al. 2014, Cray et al. 2015, Hyatt et al. 2016). Krol et al. 2014 found no significant difference between protein electrophoresis of captive whitespotted bamboo sharks, *Chiloscyllium plagiosum*, with physical abrasions and those who appeared healthy. However, they did find that females had higher beta-fractions and total protein than males (Krol et al. 2014). Cray et al. 2015 found in a captive cownose ray, *Rhinoptera bonasus*, population, abnormal individuals showed significant differences in medians of total protein, albumin, and alpha 2-fractions compared to normal individuals. They also found that leeched individuals showed significant differences in medians of albumin and alpha 2-fractions (Cray et al. 2015). Similar to Cray et al.’s results, Hyatt et al. 2016 found that in captive bonnethead sharks, *S. tiburo*, abnormal individuals showed decreased fractions of alpha 1 and alpha 2, and increased gamma-fractions to healthy sharks (Krol et al. 2014, Cray et al. 2015, Hyatt et al. 2016). This evidence shows that anomalies among immunoglobulin fractions and total protein may indicate infections, or stressors that may cause increased susceptibility to disease, such as malnourishment.

**Protein Immunoassays**

Immonoassays used routinely in laboratory investigations include the use of antibody based methods. Immunoflorescence, which involves attaching florescent labels to antibodies, helps identify infectious disease and auto-immune disorders (Nairn 1976). RIAs, or radioimmunoassays, use isotopes attached to antigens, for sensitive steroid assays (Sonksen 1974, Cameron et al. 1975). However, both these assays have limitations,
including ease, time, and cost (Voller et al. 1978). ELISAs, or enzyme linked immunosorbent assays, use enzymes linked to antigens or antibodies so that the complexes may show both immunological and enzymatic activity. Degradation of the enzyme provides an amplification factor that can detect enzymes accurately and sensitively (Voller et al. 1978).

Most available commercial assays are based on mammalian systems, often using human plasma-based standards. It is necessary to validate these assays for use on non-mammalian species, so that the results obtained are accurate and reliable. Not only may validity fail, but there may be some cross-reactivity that does not follow true predictions, failing to provide accurate measurements of immunological components (Ravanat et al. 1995). Some validation has been done in the measurement of stress metabolites in shark studies. Mills et al. 2010 successfully validated enzyme immunoassay kits to measure plasma 11-ketotestosterone in wild populations of blacktip reef sharks, *Carcharhinus melanopterus*. The kits used rabbit and mouse components, and reactivity with shark plasma was not guaranteed. Mills et al. could show validation through dose-response curves parallel with the kit standards, high accuracy obtained from spike recovery determination, and high precisions from intra-and inter-assay variation studies. Future examination of 11-ketotestosterone between individuals and populations can provide information on reproduction levels (Mills et al. 2010). Mills et al. was also able to validate plasma cortisol using an EIA kit in clownfishes, *Amphiprion percula*, and anemonefishes, *Amphiprion chrysopterus*. Corticosterone is created as a response to stress, and has trade-offs with immune functions, providing a better understanding of overall fitness of an organism (Grossman 1985, Foldstad & Karter 1992). Unfortunately, validity of the cortisol
EIA kit for *C. melanopterus* was not reached in this study, but may be able to tell us important information if validation is reached in the future.

**Antibody Titers**

In many species, clinical tests can be performed to determine the antibody titer present in the animal in response to various antigens of interest. This can be done to show the capacity of an animal to respond to an inoculating agent by producing a robust adaptive response in the form of antibodies present in serum (Dooley & Flajnik 2005). Unfortunately, these kinds of antibody titer tests are dependent on the ability to prime the animal with the antigen of interest and then measure the antibody response to that antigen some time later. In studies of wild sharks, where individual animals are seen only once in the field, such tests are not applicable. Alternatively, the natural serum IgM level of sharks to known antigens of interest can be tested in serum using various protein techniques such as ELISA and western blotting. However, although this may yield information about exposure of sharks to specific antigens, it may not provide a clear picture of their immunocompetence (Marancik et al. 2012).

**Acute Phase Proteins**

Acute phase proteins (APPs) are a set of proteins that are commonly measured as part of clinical assessments in human health. These small proteins are produced in the liver and fluctuate in expression depending on infection and injury (Samols et al. 2002). There are both positive and negative APPs. Positive APPs increase with inflammation and destroy the invaders responsible, while negative APPs decrease concentrations during inflammation (Gruys et al. 2005). This decrease in APPs can be used as a marker of inflammation (Eckersall & Bell 2010). There are many APPs used clinically, such as
haptoglobin, serum amyloid A, ferritin, and fibrinogen, because APPs increase or decrease quickly when immunity is compromised.

C-reactive protein (CRP) is a positive APP that is a routinely measured parameter in human blood panels (Robey et al. 1983). This protein has been characterized in both the dusky smooth-hound dogfish, *Mustelus canis*, and the Atlantic sharpnose shark, *R. terraenovae*, through chromatography in 1983 and 2004 respectively (Robey et al. 1983, Karsten & Rice 2004). In both cases, like the EIA with *C. melanopterus*, collected serum samples were pooled by population and not examined on the individual level. Karsten & Rice compared three separate populations, concluding that the two suburban populations of *R. terranova* had less CRP present than the urban population. Additionally, this study showed that the urban population was significantly different across the solstices, with higher CRP expressed in the summer. It would provide further insight if individuals were measured and compared within the populations, instead of pooling samples. This study highlights the importance of baseline data, and how it alters between populations. “Healthy” levels of a given population, may not be a standard for other populations within the species. Further discrepancies can be seen in captive versus wild populations.

In whitespotted bamboo sharks, *C. plagiosum*, serum was harvested from captive individuals and CRP, Serum Amyloid A (SAA), and Haptoglobin (HP), were quantitated using the Randox Daytona analyzer and canine APPs (Krol. et al. 2014). In captive bonnethead sharks, *S. tiburo*, CRP and HP were quantitated using the Randox Daytona analyzer and canine APPs (Hyatt et al. 2016). Using this analyzer, individual shark APP measurements were compared. In *C. plagiosum*, three sharks that had noticeable abrasions did not have significantly increased CRP expression compared to sharks without abrasions.
Females were found to have higher total protein, but all sharks portrayed relatively similar, “healthy,” measurements (Krol et al. 2014). This was not the case for bonnethead sharks. In *S. tiburo*, individuals with a known infectious disease showed significantly increased CRP and HP, validating APPs as biomarkers for infection in this species (Hyatt et al. 2016). This method of analysis is like that discussed earlier with ELISAs, the comparison of shark to mammalian components must be regarded cautiously. Mammalian components may react unexpectedly with non-mammal organisms and results may not be an expected, true reaction.

**Other Potential Biomarkers**

Complement is a specialized defense system important to immune function (Nonaka & Sylvia 2000). It consists of serine proteases that act as a part of an enzymatic cascade to release bioactive peptides and lead to the clearance of infectious agents (Müller-Eberhard 1988). Shark complement is comparable to that observed in mammals, all three complement pathways, classical, alternative, and lectin, are functional (Jensen et al. 1981, Shin et al. 2007, Endo et al. 1998). Each of these pathways contain several proteins that can potentially be used as biomarkers. For example, C1q is a complement component that binds with CRP, so examining fluctuating levels of the protein can give insight into an individual’s health. Similarly, the central protein in complement, C3, is produced *de novo* in the liver under inflammatory conditions (Alper et al. 1969). Measures of C3 might also be used to evaluate the health status of wild sharks (Flierl et al. 2011, Hamid et al. 2012). C3 and other shark complement proteins have already been isolated and characterized in the literature (Smith 1998, Graham et al. 2009). This may make them very good targets for
use as biomarkers, as antibodies can be raised against species-specific complement proteins to be used to quantify them in shark samples.

Recently, another potential biomarker for inflammation, Hemopexin (Hx) was isolated from blood plasma in nurse sharks, *G. cirratum*, and characterized (Dooley et al. 2010). Hemopexin (Hx) is a plasma protein produced in the liver as an APP and binds heme which provides the body with protection against oxidative damage. The expression of Hx in sharks was found to be induced by inflammation, and serum protein levels were increased at elevated environmental temperatures, as well as, during infection (Dooley et al. 2010). A functional analysis showed that shark Hx binds heme like mammals, but was found at unusually high levels. Furthermore, Dooley et al. 2010 observed several unknown proteins that also had very high levels in blood plasma. Further studies on measuring plasma levels of these proteins might also be useful in the study of health in wild sharks. To validate these immunoassays, baseline data will be required for each species of interest. Species differ in what is average, so studies being performed on disparate species may not tell us much about another species, given the evolutionary divergence present among shark lineages.

**Immune Assessment Tools in Shark Conservation**

Wild sharks rely on their immune systems to keep them healthy throughout their range of habitats, in which they experience many environmental stressors, including parasites, pathogens, temperature variability, and anthropogenic factors. Pollutants and/or other toxicological stressors may also be present in the environments that sharks inhabit and be influencing the level of stress on their immune capacity (Lohman & Belkin 2014).
Immunocompromised individuals might then be more susceptible to disease, or may experience immune dysregulation, where a heightened state of inflammation is maintained to the detriment of host tissues (Tomanek 2011). Chronic inflammation can lead to physiological problems, especially in long-lived organisms like sharks (Rich & Romero 2005). Furthermore, additional energy invested in heightened inflammation or immune defense may mean that less energy is available to other systems in the body and/or the dietary needs of the animal may change (Martin 2009).

In other groups of vertebrates, it is well established that trade-offs can exist between immune defense, foraging activity, and reproductive success (Norris & Evans 2000). Populations that are experiencing malnourishment or other stressors related to poor habitat may have to invest additional energy in foraging for food at the expense of immune defense, potentially making them more susceptible to disease (Martin 2009). Immune activity, primarily driven by protein, can be energetically costly (Lochmiller & Deerenberg 2000, Martin et al. 2008). There may be sublethal impacts in populations experiencing these kinds of ecological trade-offs that are too subtle to be observed using current monitoring techniques.

Identifying which immunological assessment tools are optimal for examining wild populations may help study at-risk populations and aid in shark conservation. Reliably measuring immune parameters, across populations, may provide a way to assess whether the population is experiencing any tradeoffs linked to their habitat use. Baseline data for individuals in these populations will allow investigators to identify anomalies. These abnormalities will hopefully be something which can be studied further and addressed, giving valuable insight to keeping wild shark populations healthy. Utilizing these tools in
shark conservation programs may help us determine what habitats are beneficial for sharks, and what areas might need more protection from anthropogenic stressors.
Chapter 2: Serum protein analysis of south Florida nurse sharks, *Ginglymostoma cirratum*

**Background**

Proteins found in peripheral blood carry out many critical biological functions for vertebrates. The proteins present and their relative concentrations can be used as proxies for health parameters in clinical settings (Jain et al. 2011). One effective way to characterize the proteins present in a blood sample is through protein electrophoresis (Gammopathies 2005). In veterinary medicine, protein electrophoresis is used clinically to assess health in mammals and avian species (Cray & Tatum 1998, Ecerksall 2008). Due to previous studies on elasmobranch protein electrophoresis, we know that elasmobranchs tend to have five fractions of blood proteins (Hamman et al. 2012, Cray et al. 2015, Hyatt et al. 2016). These fractions are based on mammalian reference values. Accepted elasmobranch reference values have yet to be developed. Reference ranges for specific species are important to understand what protein parameters and/or electrophoretic patterns correspond to average versus abnormal individuals (Haman et al. 2012). The only way to establish reference ranges is to collect blood from many individuals of a given species. In captive settings, the advantage is the relative ease with which multiple samples can be obtained from the same set of individuals over time. This is especially beneficial because repeated measurements can reveal changes in health status or corresponding variation in blood parameters as they relate to other health patterns observed. However, because in most cases, captive settings do not match the natural setting closely, the data obtained is often not applicable to animals in the wild. Collection of reference ranges using wild populations is more difficult, but not impossible. With wild populations, there is most
likely only one opportunity to work with and collect from an individual animal. The data collected from wild populations, however, is much more likely to reflect baseline health patterns for a given species in its natural habitat.

Nurse sharks, *Ginglymostoma cirratum*, are a hearty species of shark common to Florida and the Caribbean, and have a wide distribution across the Atlantic (Castro 2000). Nurse sharks are found in tropical to subtropical waters, and feed benthically on fish and crustaceans (Compagno 2001). The species global extinction risk level is listed as data deficient on the International Union of the Conservation of Nature Red List, due to a lack of knowledge about their migratory behaviors and gene flow between different populations (IUCN 2006).

The overall aim of this study is to provide average protein electrophoresis and peripheral blood cell reference ranges for the nurse shark in south Florida. In order to achieve this, nurse shark serum protein electrophoresis profiles were examined and peripheral blood smears were counted. Furthermore, this study determined if any other parameters were correlated with the protein profiles, and may be used as a proxy for protein electrophoresis. Physical measurements were examined to see if body span was related to protein, as physically fitness may relate to immunological health. Body condition may be compromised if increased immunological functions are occurring, because it is biologically costly to have increased immune response. Glucose, lactate, and hematocrit, physiological parameters often used in stress studies, were also investigated to see if they can be used as a proxy for protein electrophoresis. However, it was unexpected that these homeostatic parameters would affect underlying immunological status. Granulocyte to lymphocyte ratios, which look at the proportion of some immunological cells, were also compared for
use as a proxy to protein electrophoresis. Lastly, this study evaluates the differences between serum and plasma protein electrophoresis, to see if they are similar enough to use interchangeably when looking at shark proteins. Serum collection requires additional processing time, therefore plasma may be more efficient to use if difference in protein between the two is negligible.

**Methods**

**Field Collection**

All nurse sharks were caught off the coast of Miami, Florida, USA, at sites known to have shark presence from past success fishing in the area (Figure 2.1). Sampling occurred from 2014 to 2017, across both wet and dry seasons. Sharks were targeted using a circle-hook drumline system, as described by Gallagher et al. 2014 (Figure 2.2). Although other species were caught, only nurse sharks were considered in this study. Sharks were brought onto a slightly submerged platform and restrained until all data collection took place. In order to see if body condition correlated to protein electrophoresis, seven physical measurements were taken using a transect tape: precuadal length (PCL), fork length (FL), total length (TL), lateral span, frontal span, proximal span, and caudal keel. Precuadal length (PCL), lateral span, frontal span, proximal span, and caudal keel were used in the scan condition analysis equation as described by Irschick & Hammershlag 2015. A total of 50 sharks were used in this study. After blood and other scientific measurements were collected, the sharks were released in good condition.
Figure 2.1. Sampling sites of nurse sharks in southeast Florida. Stars represent generalized sampling locations, not individual shark capture locations, there may be multiple sharks from one starred sampling site.

Figure 2.2. Drumline system used to capture sharks as described in Gallagher et al. 2014: (a) bullet floats; (b) large floating poly-ball; (c) rope attaching float to submerged weight; (d) ~18 kg cement weight; (e) hook timer; (f) main line of ~410 kg test monofilament; (g) 4 m double-stranded leader of ~410 kg test monofilament; (h) 16/0 5°-offset circle hook.
**Blood Collection**

In order to collect blood to perform the protein electrophoresis and measure physiological parameters, blood was collected using 18-gauge x 3” needles and 10 ml syringes. 10 ml of whole blood was taken via venipuncture of the caudal vein. In order to examine the differences between plasma and serum, four ml of the whole blood was centrifuged immediately (1,500 rpms for two minutes) and plasma was collected. The plasma sample was then placed on ice until being stored at -20° C. The rest of the whole blood was placed on ice and allowed to clot undisturbed. Within 24 hours, the samples were centrifuged (1,500 rpm for 10 minutes) and the pellet discarded. The resulting supernatant was then aspirated and placed in a new tube, undergoing centrifugation again (1,500 rpm for 10 minutes). The resulting serum was collected and stored at -20° C until further analysis.

In order to see if physiological parameters, usually related to capture stress, could be use as proxies for protein electrophoresis, whole blood was used for clinical glucose and lactate meter readings, and hematocrit was collected in the field. In order to collect hematocrit, a drop of blood was placed in a capillary tube and spun at 1,500 rpm for two minutes. The resulting hematocrit tube was read using a hematocrit graph.

Nurse sharks, when captured via the aforementioned system, tend to rest on the bottom and not fight. Given this capture tactic, these sharks tend to experience less capture stress. It was therefore expected that the parameters collected in this study were close to an average, unstressed baseline.
Peripheral Blood Smears

In order to provide reference ranges for peripheral blood smears, blood smears were performed for 19 sharks. These were prepared in the field, in triplicate, for each shark. A drop of blood was placed on a slide, allowed to dry, and was then stained using Ricca Chemical Giesma Stain (Catalog number 3250-4). Blood counts were done in the lab on smears by the same individual (Matt Bernanke) based on a 100-differential white blood cell count, using light microscopy under oil emersion, 1,000x magnification. Leukocytes were identified and enumerated (Haines & Arnold 2014).

Five different cell types were counted in the blood smears: neutrophils, heterophils (fine eosinophil granulocytes, FEG), eosinophils (course eosinophil granulocytes, CEG), monocytes, and lymphocytes. Granulocytes were also calculated from the addition of the neutrophils, heterophils, and eosinophils. In order to see if the granulocyte to lymphocyte ratio could be used as a proxy for protein electrophoresis, the granulocyte to lymphocyte ratio was calculated. Thrombocytes were also enumerated (#cells per 100 leukocytes).

Total Solids

In order to cut protein fractions from total protein, total solids were determined using a non-temperature compensated refractometer, Schuco, Japan (Cray et al. 2015).

Protein Electrophoresis

To determine the fractions of protein, samples were analyzed using an electrophoresis analysis system following the manufacturer’s instructions, using gels that accompany the system (SPIFE 3000 systems, Split Beta gels; Helena Laboratories Inc., Beaumont, TX). This system was previously used with avian samples (Cray et al. 2011). The gels were scanned and analyzed by manufacturer software. Using mammalian fraction delimits,
albumin, alpha 1, alpha 2, beta-, and gamma-globulins were identified, but referred to as fractions 1-5, respectively (Cray et al. 2015). Percentages were determined using the same software, and absolute values (g/dL) were obtained by multiplying the calculated percentages with the total solids concentration (Cray et al. 2015).

**Statistical Analysis**

Data was analyzed for normality using the Shapiro-Wilk test. As the majority of the parameters were not normally distributed, nonparametric tests were used throughout the analysis. The Spearman test was used to analyze correlations between fraction 4, the major fraction, and total protein and the following parameters: body condition, glucose, hematocrit, lactate, and the granulocyte to lymphocyte ratio. The Spearman test was also used to correlate lengths and spans. The Wilcoxon signed rank test was used to analyze plasma versus serum protein fractions. Significance was set at $P < 0.05$. These analyses were conducted using R (3.4.1) and MATLAB R2017a.

**Results**

**Morphology and Length**

Pre-caudal length, fork length, and total length were measured for all 50 nurse sharks. The mean total length of sampled nurse sharks was 230.45 cm (range: 154-289 cm). Lateral span, frontal span, proximal span, and caudal keel were measured for 41 of the nurse sharks sampled. The mean lateral span was 56.87 cm (range: 33-81 cm); mean frontal span was 55.48 cm (range: 36-76 cm); mean proximal span was 39.32 cm (range: 20-64); and mean caudal span was 26.15 cm (range: 10-39 cm) (Table 2.1). Total length was positively correlated to both pre-caudal length and fork length, but showed no significant correlation
to lateral span. Lateral span was positively correlated to frontal span, proximal span, and caudal keel. All individuals used for reference intervals looked physically fit upon sampling, meaning that they had no noted physical ailments, no discoloration, were not lean, and had no obvious injury.

Table 2.1. Reference intervals for the morphology and length of nurse sharks, *Ginglymostoma cirratum*.

<table>
<thead>
<tr>
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<th>n</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>LRL</th>
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</thead>
<tbody>
<tr>
<td>Lateral Span</td>
<td>41</td>
<td>56.87 ± 12.28</td>
<td>59</td>
<td>33</td>
<td>81</td>
<td>53.64 ± (43.24-64.04)</td>
<td>60.09 ± (45.01-75.18)</td>
</tr>
<tr>
<td>Frontal Span</td>
<td>52</td>
<td>55.48 ± 10.37</td>
<td>58</td>
<td>36</td>
<td>76</td>
<td>52.75 ± (43.97-61.53)</td>
<td>58.20 ± (45.47-70.93)</td>
</tr>
<tr>
<td>Proximal Span</td>
<td></td>
<td>39.32 ± 9.48</td>
<td>40</td>
<td>20</td>
<td>64</td>
<td>36.82 ± (28.80-44.85)</td>
<td>41.81 ± (30.17-53.45)</td>
</tr>
<tr>
<td>Caudal Keel</td>
<td></td>
<td>26.15 ± 5.19</td>
<td>28</td>
<td>10</td>
<td>39</td>
<td>24.78 ± (20.39-29.18)</td>
<td>27.51 ± (21.14-33.89)</td>
</tr>
<tr>
<td>Pre-Caudal Length</td>
<td>50</td>
<td>161.28 ± 20.10</td>
<td>164.5</td>
<td>110</td>
<td>188</td>
<td>156.51 ± (139.24-173.79)</td>
<td>166.05 ± (141.89-190.20)</td>
</tr>
<tr>
<td>Fork Length</td>
<td></td>
<td>183.00 ± 21.47</td>
<td>188</td>
<td>131</td>
<td>220</td>
<td>177.91 ± (159.46-196.36)</td>
<td>188.09 ± (162.29-213.89)</td>
</tr>
<tr>
<td>Total Length</td>
<td></td>
<td>230.45 ± 28.79</td>
<td>236</td>
<td>154</td>
<td>289</td>
<td>223.62 ± (198.88-248.37)</td>
<td>237.28 ± (202.68-271.87)</td>
</tr>
</tbody>
</table>

*All measurements are in centimeters. Caudal Keel, PCL, FL, and TL were not normally distributed. No outliers were removed. Values in parenthesis are 90% confidence intervals. SD = standard deviation; LRL = lower reference limit; URL = Upper reference limit.

**Biometric Parameters**

Glucose was taken for 34, hematocrit was taken for 32, and lactate was taken for 10 of the nurse sharks sampled. The mean glucose level was 37 mg/dL (range: 22-56 mg/dL); mean hematocrit was 20.06 % (range: 13.5-28.5 %); mean lactate was 2.84 mmol/L (range: 0.9-12 mmol/L) (Table 2.2).
Table 2.2. Reference intervals for blood parameters of nurse sharks, *Ginglymostoma cirratum*.*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>LRL</th>
<th>URL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>34</td>
<td>37.00 ± 7.84</td>
<td>37</td>
<td>22</td>
<td>56</td>
<td>34.72 ± (28.18-41.27)</td>
<td>39.28 ± (29.41-49.14)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>32</td>
<td>20.06 ± 3.52</td>
<td>19.5</td>
<td>13.5</td>
<td>28.5</td>
<td>19.01 ± (16.08-21.93)</td>
<td>21.12 ± (16.65-25.59)</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>10</td>
<td>2.84 ± 3.38</td>
<td>1.4</td>
<td>0.9</td>
<td>12</td>
<td>0.88 ± (-1.58-3.35)</td>
<td>4.80 ± (-0.76-4.04)</td>
</tr>
</tbody>
</table>

*Lactate was not normally distributed. No outliers were removed. Values in parenthesis are 90% confidence intervals. SD = standard deviation; LRL = lower reference limit; URL = Upper reference limit.

**Peripheral Blood Smears**

Differential leukocyte counts were conducted for 19 nurse sharks on duplicate blood smears. Neutrophils had a mean of 11.21 neutrophils (range: 8-15); eosinophils had a mean of 26.42 (range: 18-31); monocytes had a mean of 0.63 (range: 0-1); heterophils had a mean of 15.53 (range: 7-29); and lymphocytes had a mean of 46.05 (range:38-52) (Table 2.3). The number of granulocytes was calculated by adding neutrophils, eosinophils, and heterophils together. The mean number of granulocytes was 53.16 (range: 48-62). The mean granulocyte to lymphocyte ratio was calculated to be 1.17 (range: 0.92-1.63). Lymphocytes and Granulocytes were negatively correlated (Spearman, r = -0.986, p = 0.000) (Figure 2.3). The mean number of thrombocytes per 100 leukocytes was found to be 156.05.
Table 2.3. Reference intervals for differential leukocyte counts of nurse sharks, *Ginglymostoma cirratum*.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>LRL</th>
<th>URL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>11.21 ± 2.07</td>
<td>11</td>
<td>8</td>
<td>15</td>
<td>10.39 ± (8.75-12.02)</td>
<td>12.03 ± (9.17-14.90)</td>
</tr>
<tr>
<td>CEG</td>
<td>26.42 ± 2.87</td>
<td>27</td>
<td>18</td>
<td>31</td>
<td>25.28 ± (23.01-27.55)</td>
<td>27.56 ± (23.59-31.54)</td>
</tr>
<tr>
<td>Monocyte</td>
<td>0.63 ± 0.60</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0.39 ± (-0.08-0.87)</td>
<td>0.87 ± (0.04-1.70)</td>
</tr>
<tr>
<td>FEG</td>
<td>15.53 ± 4.98</td>
<td>15</td>
<td>7</td>
<td>29</td>
<td>13.54 ± (9.61-17.48)</td>
<td>17.51 ± (10.61-24.41)</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>46.05 ± 3.69</td>
<td>46</td>
<td>38</td>
<td>52</td>
<td>44.59 ± (41.67-47.50)</td>
<td>47.52 ± (42.41-52.63)</td>
</tr>
<tr>
<td>Granulocyte</td>
<td>53.16 ± 3.72</td>
<td>52</td>
<td>48</td>
<td>62</td>
<td>51.68 ± (48.75-54.61)</td>
<td>54.64 ± (49.49-59.78)</td>
</tr>
<tr>
<td>Granulocyte: Lymphocyte</td>
<td>1.17 ± 0.18</td>
<td>1.13</td>
<td>0.92</td>
<td>1.63</td>
<td>1.10 ± (0.95-1.24)</td>
<td>1.24 ± (0.99-1.49)</td>
</tr>
</tbody>
</table>

*Analyses conducted on 19 samples. Granulocytes include neutrophils, CEG, and FEG. CEG and monocytes were not normally distributed. No outliers were removed. Values in parenthesis are 90% confidence intervals. SD = standard deviation; LRL = lower reference limit; URL = Upper reference limit.

Figure 2.3. Granulocytes to Lymphocytes, Spearman, r = -0.986, p = 0.000

Serum Protein Electrophoresis

Using protein delimits defined in Cray et al. 2015, 5 fractions were defined (Figure 2.4). Serum Fraction 1 had a mean of 0.10 g/dL (range 0.01-0.28 g/dL); fraction 2 had a...
mean of 0.08 g/dL (range 0.03-0.2 g/dL); fraction 3 had a mean of 0.78 g/dL (range 0.25-1.28 g/dL); fraction 4 had a mean of 3.46 g/dL (range 0.76-5.28 g/dL); fraction 5 had a mean of 0.28 g/dL (range 0.05-1.07); and total solids had a mean of 4.68 g/dL (range 1.10-6.80) (Table 2.4). In the majority of sharks, fraction 4 was presented as a high, broad peak. This peak made up a mean of 73% of the total solids in the serum. Fraction 4 and total solids were positively correlated (Spearman, r = 0.981, p = 0.000) (figure 2.5). Glucose was not correlated to fraction 4 (Spearman, r = 0.119, p = 0.503). Glucose was not correlated to total solids (Spearman, r = 0.1046, p = 0.556). Hematocrit was not correlated to fraction 4 (Spearman, r = -0.264, p = 0.1447). Hematocrit was not correlated to total solids (Spearman, r = -0.303, p = 0.0914). Lactate was not correlated to fraction 4 (Spearman, r = 0.006, p = 0.987). Lactate was not correlated to total solids (Spearman, r = -0.134, p = 0.712). The granulocyte to lymphocyte ratio also had no correlation to fraction 4 (Spearman, r = 0.166, p = 0.497). The granulocyte to lymphocyte ratio also had no correlation to total solids (Spearman, r = 0.096, p = 0.696). Body condition was not significantly correlated to fraction 4 (Spearman, r = -0.247, p = 0.119). Body condition was also not significantly correlated to total solids (Spearman, r = -0.258, p = 0.103).
Figure 2.4. Electrophoretograms of 12 of the nurse sharks sampled, representative of the 50 sharks sampled. The y-axis is the concentration of protein. The protein started from the left side of the x-axis and moved to the right, when an electrical current was applied.
Table 2.4. Reference intervals for serum protein electrophoresis values of nurse sharks, *Ginglymostoma cirratum*.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>LRL</th>
<th>URL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Fraction 1 (g/dL)</td>
<td>0.10 ± 0.07</td>
<td>0.09</td>
<td>0.01</td>
<td>0.28</td>
<td>0.08 ± 0.14</td>
<td>0.12 ± (0.04-0.20)</td>
</tr>
<tr>
<td>(%)</td>
<td>2.22 ± 1.47</td>
<td>2.00</td>
<td>0.14</td>
<td>5.77</td>
<td>1.87 ± (0.60-3.13)</td>
<td>2.57 ± (0.80-4.34)</td>
</tr>
<tr>
<td>Serum Fraction 2 (g/dL)</td>
<td>0.08 ± 0.03</td>
<td>0.07</td>
<td>0.03</td>
<td>0.2</td>
<td>0.07 ± (0.05-0.10)</td>
<td>0.09 ± (0.05-0.13)</td>
</tr>
<tr>
<td>(%)</td>
<td>1.77 ± 0.57</td>
<td>1.73</td>
<td>0.74</td>
<td>3.42</td>
<td>1.64 ± (1.15-2.13)</td>
<td>1.91 ± (1.22-2.59)</td>
</tr>
<tr>
<td>Serum Fraction 3 (g/dL)</td>
<td>0.78 ± 0.27</td>
<td>0.81</td>
<td>0.25</td>
<td>1.28</td>
<td>0.72 ± (0.49-0.95)</td>
<td>0.85 ± (0.52-1.17)</td>
</tr>
<tr>
<td>(%)</td>
<td>16.81 ± 3.56</td>
<td>16.97</td>
<td>7.38</td>
<td>25.05</td>
<td>15.96 ± (12.90-19.03)</td>
<td>17.65 ± (13.37-21.94)</td>
</tr>
<tr>
<td>Serum Fraction 4 (g/dL)</td>
<td>3.46 ± 0.96</td>
<td>3.65</td>
<td>0.76</td>
<td>5.28</td>
<td>3.23 ± (2.40-4.05)</td>
<td>3.68 ± (2.53-4.84)</td>
</tr>
<tr>
<td>(%)</td>
<td>73.73 ± 4.12</td>
<td>73.39</td>
<td>66.36</td>
<td>83.46</td>
<td>72.75 ± (69.21-76.30)</td>
<td>74.71 ± (69.76-79.66)</td>
</tr>
<tr>
<td>Serum Fraction 5 (g/dL)</td>
<td>0.28 ± 0.16</td>
<td>0.26</td>
<td>0.05</td>
<td>1.07</td>
<td>0.24 ± (0.11-0.38)</td>
<td>0.32 ± (0.13-0.52)</td>
</tr>
<tr>
<td>(%)</td>
<td>5.44 ± 1.42</td>
<td>5.17</td>
<td>3.30</td>
<td>10.06</td>
<td>5.10 ± (3.88-6.33)</td>
<td>5.78 ± (4.07-7.49)</td>
</tr>
<tr>
<td>Serum Total Solids (g/dL)</td>
<td>4.68 ± 1.29</td>
<td>5.00</td>
<td>1.10</td>
<td>6.80</td>
<td>4.38 ± (3.27-5.48)</td>
<td>4.99 ± (3.44-6.53)</td>
</tr>
</tbody>
</table>

*Analyses conducted on 50 samples. Fractions 1, 2, 5, and Total Solids were not normally distributed. No outliers were removed. Values in parenthesis are 90% confidence intervals. SD = standard deviation; LRL = lower reference limit; URL = Upper reference limit.
Figure 2.5. Fraction 4 vs. Total Solids, Spearman, $r = 0.981$, $p = 0.000$

**Serum Versus Plasma Protein Electrophoresis**

There was no significant difference between serum and plasma fractions 1, 2, 3, 4, or total solids. There was a significant difference between serum and plasma fraction 5 ($p = 0.0365$). Figure 6 compares serum and plasma electrophoresis profiles for two sharks.
Figure 2.6. The serum and plasma electrophoretograms for 2 sharks. Serum and plasma electrophoretograms are paired for each shark. The images on the left are the serum electrophoretograms and the plasma electrophoretograms are on the right.

**Discussion**

In order to understand the health of a population, baseline values need to be collected from average individuals of the population (Arnold 2005). The collection of the parameters collected in this study, from average individuals, can be used together to create an accepted range, within which an individual from the population is considered normal. These ranges can then be used to compare to sampled individuals in the future, to observe any deviations, hopefully providing insight into that individual's health status. Collecting baseline data is difficult in wild populations due to the short amount of interaction with a specimen, and no promised second interaction.
To better understand the representation of average sampled nurse sharks in the area, length and span measurements, glucose, hematocrit, lactate, and differential blood counts are provided for a number of these sharks. These parameters, along with the protein electrophoregrams, provide ranges that average nurse sharks may fall into.

**Morphology and Length**

The total length may not predict the width of an individual, therefore span measurements should be taken. Given the correlation between frontal span, proximal span, and caudal keel, individuals become larger isometrically, like previously seen (Robinson & Motta 2012). If there is not enough time to measure all 4 spans, it may be possible to measure a few and know that the missed spans will correlate with the measured spans.

Body condition could be compromised if continuous immunological functions are expressed, because it is energetically costly to launch immune responses. However, in this study nurse shark body condition was not correlated with fraction 4 or total solids, and therefore cannot be used as a proxy for protein electrophoresis.

**Biometric Parameters**

Given that there was no significant correlation to fraction 4 or total solids, glucose, hematocrit, and lactate cannot be used as proxies for protein fractions. This was as expected, as short-lived situational stress should not affect underlying immunological status (Moberg 2000). There have been multiple studies examining these concentrations in correlation to capture stress, but not many studies establishing what baselines levels of these parameters are (Cliffman & Thurman 1984, Spargo 2001, Mandelman & Skomal 2009, Brooks et al. 2012, Jerome 2016). Haman et al. did find hematocrit and glucose reference ranges for Atlantic sharpnose sharks, *Rhizoprionodon terranovae*, bonnethead
sharks, *Sphyrna tiburo*, and spiny dogfish, *Squalas acanthias*. They found that these sharks had hematocrit medians of 22, 25, and 20, respectively (Haman et al. 2012). They also found glucose medians of 167, 175, and 42, respectively (Haman et al. 2012). As seen from these previous studies, and this study, reference ranges of these parameters are species-specific and must be determined for each species individually. These reference values may also be specific to population location, and future studies should aim to test this.

**Peripheral Blood Smears**

Granulocytes are part of innate defenses and operate when they come into contact with foreign substances (Borregaard et al. 2007). Lymphocytes contain antibodies and are active when specific antigens are present (Haines & Arnold 2014). Healthy mammals have both innate cells and lymphocytes present in the blood, on guard for invaders, but tend to have less lymphocytes due to less antigens present in the body (Evans 2008). The individuals sampled in this population show relative health, as seen by the significant negative granulocyte to lymphocyte ratio. Given the narrow range of the granulocyte to lymphocyte ratio, this range may be a reliable health indicator for nurse sharks in south Florida. In this study, the GLR was not correlated with protein levels, and may therefore not be used as a proxy for protein electrophoresis, but may be used as a health indicator independently.

**Serum Protein Electrophoresis**

Trivial levels of albumin were found in both serum and plasma electrophoresis, which is in accordance with previous reports in the literature for elasmobranchs (Ballantyne 1997, Metcalf & Gemmell 2005, Cray et al. 2015). Fraction 1 was a minor band identified near mammalian albumin, and is consistent with high-density lipoproteins (Metcalf & Gemmell 2005, Cray et al. 2015). Fractions 2 and 3 were insignificant across the sample size, and
have been seen to follow this pattern in other elasmobranch species, showing that these fractions do not follow normal mammalian fractionations and may not be very important in elasmobranch species (Cray et al. 2015). Fraction 4, which is similar to mammalian Beta fractions, showed the most amount of protein. In past studies on elasmobranchs this fraction has been defined with two peaks associated with very low-density lipoproteins and low-density lipoproteins (Metcalf & Gemmell 2005). However, in the nurse shark there was only one broad peak. Fraction 5 was seen in the mammalian gamma range, this may be the immunoglobulin M response found in other elasmobranch species (Cray et al. 2015). A small fraction 5 is most likely normal for average sharks. Sharks whose immune systems are actively fighting may show increased fraction 5, due to the increase of immunoglobulins present to fight infection (Cray 2015). This would probably lead to a shift in other fraction concentrations, to compensate for more immunoglobulin (Cray et al. 2015). Generally, nurse shark electrophoretograms corresponded with the values reported in Atlantic sharpnose, bonnethead, spiny dogfish sharks, and cownose rays (Hamman et al. 2012, Cray et al. 2015).

Fraction 4 (Beta) and total solids were positively correlated, this is because the majority of protein was seen in fraction 4. This is seen consistently throughout elasmobranch populations, such as in the cownose ray (Hamman et. al. 2012, Cray et al. 2015) (Figure 2.7). In mammals, the majority of beta fraction is composed of transferrin and beta-lipoproteins, but can also hold immunoglobulins and complement proteins, such as C-reactive protein (Gammopathies 2005). This fraction may be beneficial to analyze further in future studies for biomarkers of wild shark health.
Serum Versus Plasma Protein Electrophoresis

In mammals, fraction 5, which was the only fraction to have a significant difference between serum and plasma, tends to hold the majority of immunoglobulins (Gammopathies 2005). If immunoglobulins are the focus when performing protein electrophoresis, care should be taken to decide if plasma or serum will be used. If immunoglobulin is not the primary focus, given that there were negligible differences between 4 of the 5 fractions, serum and plasma should give similar results. This can assist in field situations where collecting plasma is more accessible, without sacrificing results. Plasma and serum are used relatively interchangeably in assays in mammals and may be acceptable to be used with relative interchangeability in sharks (Sullivan et al. 2005, Dawson et al. 2011, Oddoze et al. 2012, Nissum & Foucher 2014). In this study, paired blood samples for the same individuals were used to determine any differences in plasma and serum electrophoresis. This is the first time this comparison has been made in a wild animal population.
Summary

This study presents important baseline data that can assist in monitoring the health of nurse shark populations in south Florida. There is still much to be done, as different species may possess different baseline levels and reference ranges, and therefore collection must be done species by species. In south Florida, average nurse sharks have a mean granulocyte to lymphocyte ratio of 1.17, minimal protein concentration in electrophoresis fractions 1, 2 and 5, a mean protein concentration of 0.78 g/dL for fraction 3, and the majority of protein comprised in fraction 4, with a mean protein concentration of 3.14 g/dL. Future studies should look for deviations from these baselines to examine sick or abnormal individuals. It may also be beneficial to examine nurse shark populations elsewhere and see if these ranges match. In this study, it has been shown that body condition and other biometric parameters are not indicative of protein concentrations, therefore as many variables should be measured as possible to continue to develop a thorough picture of shark health. There were also negligible differences between serum and plasma electrophoresis, and, in the field, plasma may be sufficient.

Through continued observation of shark baselines, immune biomarkers may be established, providing the ability to assess immune health across shark populations. These biomarkers may become a proxy for overall environmental stability in the area. With increased sampling and collaboration, it is possible to better understand what a healthy shark population looks like and establish standardized ways to monitor them using various clinical assessments.
Chapter 3: Serum proteins as health biomarkers in nurse sharks, *Ginglymostoma cirratum*: Investigating c-reactive protein as a candidate molecule

**Background**

Proteins found in animal serum are often utilized as biomarkers of disease and inflammation. Acute phase proteins (APPs) are serum proteins that significantly increase or decrease with inflammation (Gruys et al. 2005). Many APPs are measured clinically in human medicine (Ye et al. 2003, Beard et al. 2006, Bozinovski et al. 2008, Duvoix et al. 2013). C-reactive protein (CRP) is an APP in the pentraxin family. It is a calcium dependent molecule with a pentameric structure, consisting of five, non-covalently associated protomers (Black et al. 2004). In humans, its gene is known to be located on the first chromosome (1q21-q23), have 224 amino acids, and possess a molecular mass of 25,106 Da (NCBI GenBank). Each protomer contains a phosphocholine recognition site made up of calcium ions and a hydrophobic pocket (Thompson et al. 1999). Glu-18 is found opposite the pocket, and recognizes charged choline nitrogen (Black et al. 2003). The other side of the pentamer binds to C1q and can kick off complement (Agrawal et al. 2001). CRP is produced by the liver in response to inflammatory cytokines secreted from immune cells (Pepys & Hirschfield 2003).

The phosphocholine binding sites recognize and bind phosphocholine residues on various bacterial species (Hack et al. 1997). When a cell wall is damaged, through deterioration or apoptosis, phosphocoline inside the cell becomes exposed (Hack et al. 1997). In healthy host cells, phosphocholine is not accessible for binding, therefore, binding only occurs between CRP and non-healthy cells or invaders (Hack et al. 1997). When the phosphocoline binding site is occupied, C1q recognizes CRP and they bind,
initiating the classical complement pathway (Black et al. 2004). The classical pathway starts an enzymatic cascade, which ultimately leads to opsonization of abnormal or invasive cells, through the formation of C3 convertase (Mold et al. 1999). CRP can also interact with other ligands, resulting in the clearing of apoptotic cells with phosphocholine exposed, binding of nuclear antigens, increased phagocytosis, the release of cytokines, and the blockage of activating signals by the crosslinking of FcγRIIb (Marnell et al. 2005).

William Tillet and Thomas Francis discovered CRP in 1930, by finding an isolate from human patients with pneumococcus infections (Tillet & Francis 1930). Seventeen years later, Maclyn McCarty described it as increasing in patients during inflammatory states (McCarty 1947). Values of CRP reflect ongoing inflammation and tissue damage (Pepys & Hirschfield 2003). It is usually unaffected by most food and drug consumption, and is therefore used in human blood panels as a non-specific biochemical inflammatory biomarker (Pepys & Hirschfield 2003). CRP is used in clinical medicine as a sensitive detector of sepsis and is used to monitor post-operative patients (Castelli et al. 2004, Ozhato et al. 1992, Owen et al. 2007). Chronic inflammation is often the underlying pathology in many disease states (Khansari et al. 2009). Stress can also sometimes induce chronic inflammation (Cohan et al. 2012). CRP is up-regulated during inflammation, and is used successfully in human medicine as a biomarker to help monitor these chronic states and inflammatory diseases, including cardiovascular disease, metabolic syndrome, colon cancer, and atherosclerosis (Black et al. 2004).

In order to monitor wild shark populations, it would be beneficial to successfully measure an established biomarker for inflammation as a proxy for physiological well-being. As CRP is a well-established clinical biomarker in human health, it is possible to
utilize the understanding of this protein to assess wild animal populations. In 1983, Robey et al. isolated CRP using affinity chromatography from dusky smooth-hound dogfish, *Mustelus canis*, serum and evaluated the concentrations of protein. They found that the concentrations were higher than the concentrations normally found in humans, reaching levels only present in patients with acute inflammation (Robey et al. 1983). Given the drastic concentration difference present in shark serum, they speculated that CRP may play an important role in shark immunity. Shark CRP has now been isolated in the dusky smooth-hound dogfish and the Atlantic sharpnose shark, *Rhizoprionodon terraenovae*, and has been detected in the white-spotted bamboo shark, *Chiloscyllium plagiosum*, using mammalian antibodies (Robey et al. 1983, Karsten & Rice 2004, Krol et al. 2014). Isolation and characterization of this protein is an important initial step in the ability to measure this biomarker in wild shark populations.

In 2004, Karsten and Rice took serum samples of three different populations, Brunswick, GA, Charleston, SC, and Beaufort, SC, of the Atlantic sharpnose shark. Following the methods set forth by Robey et al. 1983; they pooled shark samples from each population to isolate the CRP protein. They developed a quantitative capture ELISA using polyclonal anti-sera against the putative CRP protein fraction and used it to measure the CRP concentration in serum samples. They then compared the concentrations across populations. They found that sharks from the Charleston, SC population had significantly higher levels of CRP than the other two populations. It is speculated that this population was exposed to a more industrial harbor, and therefore exposed to more contaminants than the other populations (Karsten & Rice 2004).
Seasonal differences were observed when Karsten & Rice (2004) recollected shark serum from the Charleston population in the fall. They found that summer CRP levels were significantly higher than the fall. Given the spatial and temporal differences, these findings suggest that CRP concentrations could potentially be used to track habitat pollution conditions of sharks. For example, summer conditions typically have higher pollution concentrations, leading to harmful algal blooms and other environmental conditions, and may cause an increase in CRP (Wells et al. 2015).

However, in both the Robey et al. 1983 and Karsten and Rice 2004, study CRP was isolated but never molecularly confirmed. Without molecular confirmation, it is still unclear if shark CRP was actually measured in either study.

In field settings, there is often only one guaranteed chance to sample any individual from a population. Therefore, creating a quick, effective way of measuring CRP would be ideal. The development of an antibody for the detection of this protein could provide an assay that could be performed easily and measure shark CRP concentration levels across different populations and, perhaps, across species depending on the level of cross-reactivity observed. Once baseline data has been collected, CRP levels may be used as a biomarker of wild shark population immunological health.

The use of biomarkers, such as CRP, to successfully assess and monitor shark health could prove to be a vital step in comprehending shark stressors, such as human activities. Anthropogenic stressors, such as overfishing or increased pollutants, may be placing sharks in states of heightened stress, and possibly causing chronic inflammation (Harvell et al. 1999, Burek et al. 2008). These effects can potentially be linked to dietary and migratory changes in sharks. With more understanding of shark’s immunological responses to these
factors, connections may be made to their physiological and ecological responses, leading to a more informed management plan for reducing these effects.

Since isolating CRP in the dusky smooth-hound dogfish and Atlantic sharpnose shark, CRP has been measured in the whitespotted bamboo shark, via a canine-CRP reaction on a clinical analyzer (Krol et al. 2014). This method of detection, however, must be approached with caution, as using mammalian CRP standards for shark samples may result in low levels of sensitivity and/or false cross-reactivity. For the majority of shark species, CRP protein has not been isolated and in those studies mentioned above where CRP has been used, characterization of the protein has never been done, and very little baseline data is available. The goal of this study was to determine whether c-reactive protein could be effectively isolated and measured in the serum of wild nurse sharks, *Ginglymostoma cirratum*, found in south Florida. Further study can then allow investigators to develop a reliable way to measure CRP and evaluate its candidacy as an immunological biomarker for inflammation. This may then be used to assess wild shark populations and their habitats.

The overall goal of this study was to measure CRP in wild shark species. One attempt to achieve this goal was through the measuring of multiple species using the canine-CRP reaction on a clinical analyzer, as used by Krol et al. 2014. Another attempt to achieve this goal was to isolate CRP using a new chromatographic separation media for CRP isolation, *p*-Aminophenyl Phosphoryl Choline Gel, and confirm the functionally significant structural features of the protein by determining the amino acid sequence of CRP for three shark species using protein identification techniques. This will allow future studies to focus on the development and standardization of shark-specific antibody based assays that can be used to measure CRP levels in wild sharks as part of routine health assessments.
Utilizing this protein concentration measurement method, investigators should be able to measure and determine baseline serum CRP levels within south Florida shark populations. These baseline levels can then be used to identify abnormal individuals and populations, and hopefully determine whether certain concentrations of CRP correlate with other physiological and ecological conditions sharks may be exposed to.

**Methods**

*Field Collection*

All sharks were caught off the coast of Miami, Florida, USA, at sites known to have shark presence from past success fishing in the area. Sampling occurred from 2014 to 2017, across both wet and dry seasons. Sharks were targeted using a circle-hook drumline system (Gallagher et al. 2014). Sampled individuals were brought onto a slightly submerged platform and restrained until all data collection took place.

Blood was collected using 18-gauge x 3” needles and 10 mL syringes. 10 mL of whole blood was taken via venipuncture of the caudal vein. Whole blood was placed on ice and allowed to clot undisturbed. Within 24 hours, the samples were centrifuged (1,500 rpm for 10 minutes) and the pellet discarded. The resulting supernatant was then aspirated and placed in a new tube, undergoing centrifugation again (1,500 rpm for 10 minutes). The resulting serum was collected and stored at -20° C until further analysis.

*Canine-CRP Analysis*

In seeking to establish whether mammalian assays could be used to detect and measure CRP across species, a canine specific assay was used. Shark serum samples were run on the Randox Daytona analyzer (Kearneysville, West Virginia, 25430, USA), using a
purified canine CRP reagent (Life Diagnostics, West Chester, Pennsylvania, 19380, USA),
and Randox high-linearity CRP reagents, as previously described, were used to quantitate
CRP (Kjelgaard-Hansen et al. 2003, Klenner et al. 2010, Krol et al. 2014). If CRP
concentrations were detected, serial dilutions were performed to see if the concentrations
were consistent.

A total of 110 sharks were run from 10 different species: nurse, *Ginglymostoma cirratum* (n=50), blacktip, *Carcharhinus limbatus* (n=19), sandbar, *Carcharhinus plumbeus* (n=17), lemon, *Negaprion brevirostris* (n=8), bull, *Carcharhinus leucas* (n=8),
tiger, *Galeocerdo cuvier* (n=3), great hammerhead, *Sphyrna mokarran* (n=2), scalloped
hammerhead, *Sphyrna lewini* (n=1), Atlantic sharpnose, *Rhizoprionodon terraenovae*
(n=1), and blacknose, *Carcharhinus acronotus* (n=1) sharks.

**Chromatography**

In order to isolate shark CRP, a modified protocol was developed using Thermo
Scientific’s Immobilized *p*-Aminophenyl Phosphoryl Choline Gel (Catalog number 20307,
Volanakis et al. 1978).

A 5 mL column was packed with the *p*-Aminophenyl Phosphoryl Choline Gel, allowed
to settle, and equilibrated for 24 hours in binding buffer (0.1 M Tris, 0.1 M NaCl, 1 mM
CaCl$_2$, pH 8). A serum sample was dialyzed in binding buffer for 24 hours. Once
equilibrated, the serum sample, at room temperature, was placed directly on top of the
column bed. The column was run to allow the sample to be in direct contact with the beads.
The sample was given an hour incubation time, and was washed with binding buffer. 2 mL
fractions were collected from the column, with an average flow rate of 51 drops per minute,
and measured spectrophotometrically at 280 nm, until a reading of zero was obtained,
indicating that all the unbound protein was collected. Bound proteins on the column were then eluted with about 36 mL of elution buffer (0.1 M Tris, 0.1 NaCl, 2 mM EDTA, pH 8). 2 mL collections, with an average flow rate of 75 drops per minute, were taken from the column and measured spectrophotometrically until a reading of zero was obtained, indicating that all the bound protein had come off the column. To concentrate protein, fractions were pooled for both pre-and post-elution peaks, resulting in about 6 mL subsets. Fractions were pooled based on protein peaks for spectrophotometric readings. Pooled peaks were then frozen, lyophilized, and re-suspended in 2 mL of binding buffer. They were then stored at -20° C in 0.1 mL aliquots until they could be run on a gel.

In some cases, flow rate of the column was slowed significantly. Some serum samples were therefore run in 10-mL columns or were run as batches using a 100-mL beaker. For the batch runs, the beads were swirled with each addition of buffer and allowed to settle. The supernatant was then taken from the top, this collection was then run on the spectrophotometer. Beads were washed in-between uses with 6M Guanidine. Bead sets were used for a maximum of 10 samples before switching to a new set. Three p-Aminophenyl Phosphoryl Choline Gel sets were used over the course of this study.

**SDS-PAGE Gel Electrophoresis**

SDS-PAGE gel electrophoresis was carried out to confirm the isolation and purity of CRP. Lonza PAGEr GELS, 4-20% Tris-Glycine, Cat. No. 59511, were used. Samples were loaded into wells and allowed to run at 125 V for 2 hours. Protein samples were compared to AMRESCO Protein MW Marker, Bluestep™ Broad Range, Prestained (Code K973-0.5 mL) and BioVision, CRP, Human Recombinant from VWR (Catalog number 10007-236). The protein standard consisted of 9 bands ranging from 9.0-200.0 kDa. After the gel ran,
it was subject to staining using Laemmeli Coomassie Blue Stain covering the gel on a shaker for 1.5 hours, and then destained using Laemmlie Fix/Destain covering the gel on a shaker for 1.5 hours, discarding and replacing the destain every .5 hours (Laemmlie 1970).

**Protein Identification**

Once the CRP-like molecule was isolated by SDS-PAGE, the CRP bands containing the concentrated protein were excised and sent to the Interdisciplinary Center for Biotechnological Research (ICBR), University of Florida, and analyzed using LC-MS/MS to obtain protein sequence identification.

**Results**

**Canine-CRP Analysis**

The Randox Daytona analyzer recognized a few shark samples with slightly elevated concentrations of CRP. There was no detection of elevations above 40 mg/L for any nurse, lemon, tiger, great hammerhead, or sharpnose shark. There were detections for three sandbar sharks with CRP concentrations of 60.16 mg/L, 123.07 mg/L and over 220.00 mg/L, two blacktip sharks with CRP concentrations of 50.56 mg/L and 74.57 mg/L, one bull shark with a CRP concentration of 54.34 mg/L, one scalloped hammerhead shark with a CRP concentration of 52.49 mg/L, and one blacknose shark with a CRP concentration of 47.45 mg/L. Unfortunately, when performing serial dilutions, concentrations were not following expectations, and these results could not be confirmed.

**Isolation of C-Reactive Protein**

A total of twelve nurse sharks, one blacktip shark, and two sandbar sharks were run on Immobilized p-Aminophenyl Phosphoryl Choline Gel column chromatography. Nurse
sharks were run on the first two sets of beads, and the blacktip and sandbars were run on the third set of beads. The first and second set of beads showed variation in the amount of post elution peaks obtained and the post elution peak absorbance levels observed. The degree of variation made it difficult to isolate putative protein bands from many of the columns run in this study. The third set of beads showed no post elution peaks and no proteins were isolated from these columns.

Two runs of the 23 successfully separated a putative CRP-like protein that could be identified using SDS-PAGE and subsequent analysis. The first run, via batch, for nurse sharks yielded a significant pre-elution peak with an absorbance of 2.842, and three post-elution peaks of interest, as indicated by Figure 3.1a. The second run, via the 5 mL column, for nurse sharks yielded a significant pre-elution peak with an absorbance of 2.853, and one post elution peak of interest, as indicated by Figure 3.1b.

![Figure 3.1a and 3.1b](image)

**Figure 3.1a and 3.1b.** (1a) The first run post-elution buffer chromatogram. Run via batch. Pre-elution buffer absorbance was 2.842. The red ovals indicate the fractions which were pooled together as 1 post elution peak. (1b) The second run post-elution buffer chromatogram. Run via 5 mL column. Pre-elution buffer absorbance was 2.853.

The peaks of interest were concentrated via lyophilization and then run on SDS-PAGE gels and compared to recombinant human CRP as a standard. The first nurse shark sample, Nurse 1, showed two bands, one consistent with human recombinant CRP, around 24 kDa, and another band higher than where CRP would be expected, around 100 kDa (Figure 2).
The banding around where CRP was expected, 24 kDa, was excised and sent to ICBR for identification.

Figure 3.2. SDS-PAGE gel electrophoresis photo of Nurse 1. From left to right: molecular weight ladder, with 9 bands ranging from 9-200 kDa, the CRP human recombinant standard at 23 kDa, the post elution peak 1 and post elution peak 2 for Nurse 1, showing banding around 100 kDa.

Nurse 1 showed over 95% similarity to peptides of Keratin type II cytoskeletal 8 in Callorhinichus mili and Alpha 2-macroglobulin (Fragment), secreted IgW heavy chain, Haptoglobin, Novel antigen receptor, Complement component factor 1 and C3 complement component in Ginglymostoma cirratum. The C3 protein sequence matched the Nurse 1 sample by 100% with at least 13 peptides providing identity (Figure 3.3).
Figure 3.3. The protein sequence of C3 complement component for nurse sharks, *Ginglymostoma cirratum*, with which Nurse 1 matched 100%. Matching peptides are highlighted in the amino acid sequence.

The peak from the second run of interest, Nurse 2, was placed on SDS-PAGE gels and compared to recombinant human CRP as a standard. Nurse 2 showed one band consistent with human recombinant CRP, around 23 kDa (Figure 4). This protein fraction was more purified than Nurse 1, as very little banding was observed other than the CRP-like protein band of interest. This band was excised and sent to ICBR for characterization.
Figure 3.4. SDS-PAGE gel electrophoresis photo of Nurse 2. From left to right: molecular weight ladder, with 9 bands ranging from 9-200 kDa, diluted whole serum of Nurse 2, the pre-elution peak of Nurse 2, the post elution for Nurse 2, showing banding around 23 kDa, and CRP human recombinant standard at 23 kDa.

Nurse 2, the column run, showed over 95% similarity of two peptides with fibrinogen beta chain precursor (*Rattus norvegicus*), one peptide with Jeltraxin precursor (*Esox lucius*), and one peptide with von Willebrand factor type A, EGF, and pentraxin domain-containing protein (*Xiphophorus maculatus*). Using a Basic Local Alignment Search Tool (BLAST) the peptides of the pentraxin domain protein sequence was seen to have 100% identity probability with Southern platyfish, *Xiphophorus maculatus*, molecular weight 65 kDa, Accession XP_014325421.1 (NCBI).

**Discussion**

**Canine-CRP Analysis**

Only eight sharks, across five species showed elevated concentrations of CRP when analyzed using the canine CRP assay, out of a total of 110 sharks, across 10 species. CRP
levels in the sharks tested in this study was low, which may mean that the sharks tested were primarily healthy sharks that did not have high levels of chronic inflammation. Of course, it is unclear as of yet whether CRP spikes in response to inflammation in sharks in the same way as it does in other vertebrates. Assuming that shark CRP does behave similarly to mammalian CRP, low levels of CRP would mean that chronic inflammation is not extremely prevalent in these populations, and these sharks are relatively healthy. However, the limitation of using canine CRP means that the interaction taking place is not necessarily indicative of CRP, and could be a protein that is CRP-like enough to react with the canine CRP antibody.

A low level of sensitivity may be observed as the recognition sequence or binding site of the antibody to mammalian CRP may be different than that found in shark CRP. An antibody developed against shark CRP specifically, instead of canine, would have much higher affinity for the CRP molecule and be a much more sensitive test for the presence of CRP. It is possible that CRP levels were underestimated in this study due to lack of cross-reactivity with the mammalian antibody used in the canine assay. Valid results could be obtained with an assay developed for the exact structure of shark CRP. It is possible this assay would have to be developed for each individual species, depending on how much variation exists among CRP proteins in different shark species. Alternatively, it may be found that there is sufficient cross-reactivity among shark CRP to use a single antibody assay across different species. Without further isolation and characterization, it cannot be confirmed that the concentrations ran on the Randox Analyzer are of shark CRP. When characterization of shark CRP is done, it can then be used in similar analyzers and give valid results for sharks. Furthermore, the assays performed were not validated in terms of
repeatability because dilution runs did not correspond to expected protein levels in these serum samples. It is unclear if this is due to the lack of affinity between the antibody in the assay and the CRP ligands present, or due to low quality in the serum samples. It indicates, however, that results for CRP and other biomarkers in sharks established using mammalian assays should be viewed with caution as there may be underlying problems associated with lack of cross-reactivity and low sensitivity.

**C-reactive Protein as a Candidate Biomarker**

The Immobilized p-Aminophenyl Phosphoryl Choline Gel column chromatography utilized in this study gave highly variable results depending on the bead set used and the exact methodology utilized (i.e. whether a 5 mL column or batch run was employed). The results were also variable across different shark serum samples, even within the same species. Although chromatographic beads were carefully stripped, washed, and re-equilibrated between samples, protein residue from previous runs or some other problem in bead activity was present as chromatographic patterns were different and not predictable throughout the study. As you can see in Figure 1, the batch run (1a) showed much more variation across protein fraction absorbance values than the column run (1b).

These inconsistencies suggest that different techniques should be used in future studies to isolate CRP from shark serum. The chromatographic method used by Robey et al. could not be replicated as the beads used in that study are no longer commercially available. Several manufacturers are attempting to resolve the problems of inconsistency associated with these beads. It seems clear from this study that the CRP binding sites, presumably phosphocholine residues, are not working efficiently. The isolation in the post-elution peak of other prominent serum proteins like C3 of the complement cascade indicate that this
method is not producing reliable isolation of CRP. Although shark CRP could not be isolated and identified in this study, it may be worth refining this methodology, as protein isolation did occur and a pentraxin domain containing molecule was isolated in the Nurse 2 sample, and may be a few steps away from isolating the target protein, CRP.

Nurse 1, when placed on a gel, showed two bands (Figure 2). The lack of pure isolation may be due to the nature of a batch run, where protein may have gotten stuck on the beads during supernatant removal. This did not give as clean an isolation as hoped. The band around 24 kDa, was characterized at ICBR, and found to be C3. C3 is an important complement component that is synthesized from the presence of CRP earlier in the complement reaction chain, and alpha 2-macroglobulin is most likely the ancestral gene for C3 (Sottrup-Jensen et al. 1985). C3 is used in human medicine as a biomarker for diseases, such as, amyotrophic lateral sclerosis, Parkinson’s disease, myocardial infarction, and ALS (Goldknopf et al. 2006, Carter et al. 2009, Ganesalingam et al. 2011). C3 could potentially be used as a biomarker for nurse shark health as well. Future studies should utilize the isolated C3 and make an antibody to easily test C3 concentrations in the field. Once baseline concentrations are established, C3 may be a successful biomarker in wild sharks.

The second peak of interest, when placed on a gel, showed a single band at 23 kDa (Figure 4). Compared to the multiple peaks found in Nurse 1, the isolation of this one protein in Nurse 2, is probably due to running the sample through the chromatography column, where things are more linear, and unbound protein likely made its way out before the gel was eluted. This result shows that even given column difficulties, results will
probably be more defined using the column method, rather than with a batch run. Future studies should aim to use column chromatography, if feasible.

The Nurse 2 protein isolated showed similarity to a fibrinogen precursor, a jeltraxin precursor, and a pentraxin domain-containing protein. Fibrinogen beta chain is a protein that assists in coagulation, and fibrinogen is also commonly used as a biomarker for inflammation and disease, along with CRP (Ziakas et al. 2006, Vila et al. 2008, Windsperger & Lehner 2013). However, as column chromatography took place with serum samples, most fibrinogen beta chains should have been removed through the clotting process. This could also be used as a biomarker for wild sharks, but would require different methodology to obtain accurate concentrations. As there are functional assays that can be used to detect the level of fibrinogen present in a sample, this may make a useful biomarker if it can be studied carefully in sharks. The measurement of fibrinogen would not require the use of antibody if it can be quantified in a functional assay. Jeltraxin is a glycoprotein related to CRP and another acute phase serum protein, serum amyloid P (Peavy et al. 2003). Given its similarities to acute phase proteins of interest, it is expected that jeltraxin concentrations would be correlated with inflammation, and may therefore be used as a biomarker. However, since jeltraxin has only been found in frog egg jelly and not in the human genome, and its precursor has only been discovered in some vertebrates, much more information must be obtained on this protein before it can successfully be used as a biomarker (Tadiso et al. 2011). It does provide further evidence, though, that the protein isolated in the Nurse 2 sample was very similar in structure to a CRP molecule and may well be CRP, although it could not be identified using the methods in this study.
Unfortunately, CRP has yet to be characterized within the nurse shark. However, given the isolation of a pentraxin molecule, the Immobilized p-Aminophenyl Phosphoryl Choline Gel column chromatography may be working, but may require follow-up chromatography to obtain a purified protein fraction. It may also be beneficial to pool samples for a higher concentration of CRP for initial isolation. Given the proximity this method has gotten to the isolation of CRP, it should continue to be further investigated, however different methodology may be more effective. Moreover, the study identified other potential candidate molecules that may be studied further and used as clinical biomarkers for sharks sometime in the future.

**Future Direction**

The Immobilized p-Aminophenyl Phosphoryl Choline Gel column chromatography could be optimized, or other commercial products like this could be experimented with, in order to isolate CRP. Another option is to make a resin with phosphocholine fixed to sepharose beads. Unfortunately, this method is not easy unless it is produced by a lab that is experienced in making fixed resins. Another option is to explore other potential biomarkers such as fibrinogen or C3, which were both found in Nurse 1, or serum amyloid P, another APP similar to CRP.

Once CRP or another biomarker is isolated and characterization is complete, it is hopeful that an antibody can be produced off-site by a company specializing in antibody development. These polyclonal antibodies can then be used to measure these biomarkers in shark serum samples across various populations. Depending on the cross reactivity of the antibody, it may be possible to measure a chosen biomarker in plasma samples from sharks collected in previous years.
Summary

Having an established immune biomarker in wild shark populations can be used to both assess and monitor shark population health. Better understanding of baseline health parameters for wild sharks can help us be more attentive when measurements are significantly different from or fall outside baseline ranges. The attempted isolation and characterization of c-reactive protein was not completed within this study. C3 and a pentraxin domain-containing protein, however, were isolated and characterized. CRP remains a potential target for isolation and measurement, since there is potential that the pentraxin domain-containing protein is nurse shark CRP. Use of these biomarkers could prove to be a vital step in comprehending shark health and related stressors in these populations, hopefully leading to better management of these species.
Conclusion

The assessment of immunological health of wild sharks provides insight into overall population health and environmental stressors. This thesis reviewed immunological assessment tools and their potential to be used with wild shark populations. The study provides serum protein electrophoresis reference ranges for healthy nurse sharks in south Florida, as well as, reference ranges for other blood and biometric parameters. C-reactive protein as a potential biomarker in the nurse shark was examined by attempting to isolate the protein from serum. C3 and a pentraxin domain-containing molecule were isolated. These results suggest that other serum proteins, such as fibrinogen and C3, might make useful targets for future studies as potential clinical biomarkers. It shows that the isolation of CRP may be possible, and the potential for developing an antibody assay for the measurement of a protein biomarker should be explored further. Future work should continue to explore the development of these health assessment tools in wild sharks. Clinical measurements of immunocompetence and health in these animals can then be related to relevant ecological and environmental conditions.
Literature Cited


Appendix A – General Experimental

Chapter 2:

For Peripheral Blood Smear Staining – Ricca Chemical Giesma Stain (Catalog number 3250-4)

For Protein Electrophoresis – SPIFE 3000 systems, Helena Laboratories Inc., Beaumont, TX

For Protein Electrophoresis – SPIFE® Split Beta gels SPE (Catalog number 3422)

Chapter 3:

For Canine CRP Analysis – Randox Daytona Analyzer, Kearneysville, West Virginia, 25430, USA

For Canine CRP Analysis – Randox high-linearity CRP reagents, Kearneysville, West Virginia, 25430, USA

For Canine CRP Analysis – Purified Canine CRP reagent, Life Diagnostics, West Chester, Pennsylvania, 19380, USA

For Chromatography – Thermo Scientific’s Immobilized p-Aminophenyl Phosphoryl Choline Gel (Catalog number 20307)

For Chromatography – Binding Buffer (0.1 M Tris, 0.1 M NaCl, 1 mM CaCl₂, pH 8)

For Chromatography – Elution Buffer (0.1 M Tris, 0.1 NaCl, 2 mM EDTA, pH 8)

For SDS-PAGE Gel Electrophoresis – Lonza PAGEr GELS, 4-20% Tris-Glycine (Catalog number 59511)

SDS-PAGE Gel Electrophoresis – AMRESCO Protein MW Marker, Bluestep™ Broad Range, 9 bands ranging from 9.0-200.0 kDa, Prestained (Code K973-0.5 mL)

SDS-PAGE Gel Electrophoresis – BioVision, CRP, Human Recombinant from VWR (Catalog number 10007-236)

SDS-PAGE Gel Electrophoresis – SDS-PAGE running buffer Laemmli – 100mL PAGE 10x running buffer (30.0 g Tris, 144.0 g Glycine, into 1 L ddH₂O), 10 mL SDS solution, up to 1 L ddH₂O)
For Staining of Gel Electrophoresis – 1.875 g Coomassie Brilliant Blue (R250), 227 mL Methanol, 94 mL Glacial Acetic Acid, 179 mL ddH2O

For Destaining of Gel Electrophoresis – 100 mL Glacial Acetic Acid, 375 mL Methanol, 25 mL Glycerol, 500 mL ddH2O
Appendix B – Statistical Tests Performed in Chapter 2

Shapiro-Wilk test for normality – R (3.4.1)
Spearman test to analyze correlations – MATLAB R2017a
Wilcoxon signed rank test to analyze plasma versus serum protein fractions – MATLAB R2017a
Paired t test to analyze plasma versus serum protein fractions. – MATLAB R2017a
Significance was set at $P < 0.05$
Appendix C – Box-and-Whisker Plots of Nurse Sharks, *Ginglymostoma cirratum*, Protein Fraction and Granulocyte:Lymphocyte Reference Ranges

Serum Fractions

![Box plot for Serum Fraction 1](image1)

**Figure C.1.** The box plot for serum fraction 1. The red + represents an outlier.

![Box plot for Serum Fraction 2](image2)

**Figure C.2.** The box plot for serum fraction 2. The red + represents outliers.
Figure C.3. The box plot for serum fraction 3.

Figure C.4. The box plot for serum fraction 4. The red + represents outliers.
Figure C.5. The box plot for serum fraction 5. The red + represents outliers.

Figure C.6. The box plot for serum total solids. The red + represents an outlier.
Plasma Fractions

Figure C.7. The box plot for plasma fraction 1. The red + represents outliers.

Figure C.8. The box plot for plasma fraction 2.
Figure C.9. The box plot for plasma fraction 3. The red + represents an outlier.

Figure C.10. The box plot for plasma fraction 4.
Figure C.11. The box plot for plasma fraction 5. The red + represents outliers.

Figure C.12. The box plot for plasma total solids. The red + represents outliers.
Granulocyte:Lymphocyte

**Figure C.13.** The box plot for granulocytes to lymphocytes. The red + represents an outlier.
## Appendix D – Reference Ranges of Plasma

Table D.1. Reference intervals for plasma protein electrophoresis values of nurse sharks, *Ginglymostoma cirratum*.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>LRL</th>
<th>URL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma Fraction 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/dL)</td>
<td>0.08 ± 0.07</td>
<td>0.07</td>
<td>0.00</td>
<td>0.35</td>
<td>0.06 ± (0.00-0.12)</td>
<td>0.10 ± (0.01-0.19)</td>
</tr>
<tr>
<td>(%)</td>
<td>1.59 ± 1.46</td>
<td>1.25</td>
<td>0.02</td>
<td>6.93</td>
<td>1.17 ± (-0.05-2.38)</td>
<td>2.01 ± (0.18-3.85)</td>
</tr>
<tr>
<td><strong>Plasma Fraction 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/dL)</td>
<td>0.09 ± 0.04</td>
<td>0.08</td>
<td>0.01</td>
<td>0.18</td>
<td>0.04 ± (0.04-0.11)</td>
<td>0.10 ± (0.04-0.16)</td>
</tr>
<tr>
<td>(%)</td>
<td>1.69 ± 0.57</td>
<td>0.81</td>
<td>0.43</td>
<td>3.65</td>
<td>1.46 ± (0.78-2.13)</td>
<td>1.92 ± (0.91-2.94)</td>
</tr>
<tr>
<td><strong>Plasma Fraction 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/dL)</td>
<td>0.80 ± 0.29</td>
<td>0.83</td>
<td>0.22</td>
<td>1.43</td>
<td>0.71 ± (0.47-0.95)</td>
<td>0.88 ± (0.51-1.24)</td>
</tr>
<tr>
<td>(%)</td>
<td>14.97 ± 3.55</td>
<td>15.31</td>
<td>6.95</td>
<td>21.94</td>
<td>13.94 ± (10.98-16.90)</td>
<td>16.00 ± (11.54-20.47)</td>
</tr>
<tr>
<td><strong>Plasma Fraction 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/dL)</td>
<td>4.01 ± 1.20</td>
<td>4.26</td>
<td>1.40</td>
<td>6.96</td>
<td>3.66 ± (2.65-4.66)</td>
<td>4.36 ± (2.84-5.87)</td>
</tr>
<tr>
<td>(%)</td>
<td>74.61 ± 5.00</td>
<td>74.11</td>
<td>65.77</td>
<td>85.38</td>
<td>73.16 ± (68.99-77.33)</td>
<td>76.06 ± (69.77-82.36)</td>
</tr>
<tr>
<td><strong>Plasma Fraction 5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(g/dL)</td>
<td>0.36 ± 0.12</td>
<td>0.36</td>
<td>0.13</td>
<td>0.70</td>
<td>0.32 ± (0.22-0.42)</td>
<td>0.39 ± (0.24-0.55)</td>
</tr>
<tr>
<td>(%)</td>
<td>6.88 ± 2.15</td>
<td>6.42</td>
<td>3.62</td>
<td>13.04</td>
<td>6.26 ± (4.46-8.05)</td>
<td>7.51 ± (4.80-10.21)</td>
</tr>
<tr>
<td><strong>Plasma Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Solids (g/dL)</strong></td>
<td>5.34 ± 1.46</td>
<td>5.70</td>
<td>1.80</td>
<td>9.00</td>
<td>4.92 ± (3.70-6.12)</td>
<td>5.76 ± (3.93-7.60)</td>
</tr>
</tbody>
</table>

*Analyses conducted on 34 samples. Fractions 2 and 5 were not normally distributed. No outliers were removed. Values in parenthesis are 90% confidence intervals. SD = standard deviation; LRL = lower reference limit; URL = Upper reference limit.