Humoral Immune Responses in Loggerhead (Caretta caretta) and Kemp's Ridley (Lepidochelys kempii) Sea Turtles

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HUMORAL IMMUNE RESPONSES IN LOGGERHEAD (CARETTA CARETTA) AND KEMP’S RIDLEY (LEPIDOCHELYS KEMPII) SEA TURTLES

A Dissertation
Presented to
the Graduate School
of Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biological Sciences

by
Maria L. Rodgers
May 2016

Accepted by:
Dr. Charles D. Rice, Committee Chair
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Abstract

Sea turtles are a unique group of marine organisms that live in tropical and temperate waters across the globe. They are one of just a few groups of marine reptiles that currently inhabit oceanic environments, and therefore fill an important and distinct role in marine ecosystems. Though much research has been conducted on sea turtle nesting and ecology, comparatively little research has been done on sea turtle immunology, especially in turtles other than green sea turtles (*Chelonia mydas*). Using a simple technique, immunoglobulin Y (IgY), which is found in reptiles, birds, and amphibians, was purified. Using standard techniques, polyclonal antisera and monoclonal antibodies were made against two forms of IgY (known as IgY and IgY(ΔFc)) for both loggerhead (*Caretta caretta*) and Kemp’s ridley (*Lepidochelys kempii*) sea turtles. Following antibody production, serum samples from 60 loggerhead and 30 Kemp’s ridley individual turtles were examined to look for antibodies specific to 9 marine bacterial species (*Escherichia coli*, *Vibrio cholera*, *V. parahaemolyticus*, *V. anguillarum*, *V. vulnificus*, *Mycobacterium marinum*, *Erysipelothrix rhusiopathiae*, *Streptococcus agalactiae*, and *Brevundimonas vesicularis*). It was found that serum titer levels against these bacteria can vary significantly in regards to factors such as gender and year of serum collection. In addition, some correlations were found between total serum protein levels and serum titers against bacteria, and between straight carapace length and serum titers against bacteria. Measurements taken from these individual turtles (packed cell volume, glucose, and total protein) are similar to measurements seen in other wild, healthy turtles of the same species. This indicates that the bacterial data is
not reflective of special or unusual circumstances (such as disease or injury), but is what can be expected from healthy, wild turtles from these populations. This is the first study of its kind to examine serum titers against marine bacteria, and therefore offers clues to the species of bacteria that sea turtles may be exposed to in their natural environments. Moreover, this study lends insight into the nature and function of the truncated form of IgY, known as IgY(ΔFc).
Dedication

To all of the dreamers, misfits, and so-called “crazy ones”: may you never give up on your dreams or yourself.
Acknowledgements

First and foremost, I must thank my parents, Bill and Dorothy, for their unconditional love and support, and for encouraging me to always pursue my dreams and passions, no matter what. You never gave up on that wide-eyed six year old who insisted that fish have ears, and that has truly been everything. To my sister, Christine, who has been a constant source of friendship throughout my life: thank you. You are the wisest person that I know, and you have deeply touched my heart and soul, and showed me the path when I have been uncertain. All of my grandparents played an essential role in my learning and development, and I am so grateful to them. To my college friends, especially Kelley Winship: you have filled me with laughter and fun, and made me realize that home is people, not a place. To my graduate school friends, especially Namrata Sengupta, Sarah Au, Ramiya Kumar, Ray Liu, and Vipul Pai Raikar: you have led me on numerous adventures in every sense of the word, thank you!

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Chapter 1: Literature Review

Species Overview

Loggerhead turtles are a sea turtle species with a wide habitat range that encompasses more than 50 countries worldwide, as seen in Figure 1 (National Marine Fisheries Service, 2009). They can grow to lengths of more than 1 meter and typically weigh around 110 kilograms (National Oceanic and Atmospheric Administration, 2015a). Their diet consists of gastropods, sponges, cephalopods, fish, and a variety of other prey (Ernst and Lovich, 2009). As it true with all sea turtles, loggerheads lay their eggs in nests on beaches. It is estimated that in the United States, there are 68,000-90,000 loggerhead nests laid per year (National Oceanic and Atmospheric Administration, 2015a). Current data suggests that loggerhead turtles reach sexual maturity between 10 and 39 years of age (Casale and Tucker, 2015). This species of sea turtle is currently labeled as “vulnerable” from the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species (Casale and Tucker, 2015). This means that, in the near future, this species faces a high risk of becoming extinct in the wild (IUCN Red List, 2001). In the United States of America, all populations of loggerhead sea turtles are currently listed as either “endangered” or “threatened” (U.S. Fish & Wildlife Service, 2015).
Figure 1. Loggerhead sea turtle habitat range (National Marine Fisheries Service, 2009).
In contrast, Kemp’s ridley turtles are a species mainly the waters off of two countries: the United States and Mexico, as seen in Figure 2 (Marine Turtle Specialist Group, 1996). They are smaller than loggerheads with a typical adult size of 60-70 centimeters and weighing less than 50 kilograms (National Oceanic and Atmospheric Administration, 2015b). They share a similar diet to loggerheads, which consists of crabs, mollusks, jellyfish, and fish (National Oceanic and Atmospheric Administration, 2015b). Kemp’s ridley turtles mostly nest in Mexico, but there have been some small nests sited in Texas, Florida, and North and South Carolina (National Oceanic and Atmospheric Administration, 2015b). Kemp’s ridley turtles are defined as critically endangered via The IUCN Red List of Threatened Species, meaning that, in the immediate future, these turtles are at an extremely high risk of extinction in the wild (Marine Turtle Specialist Group, 1996).
Figure 2. Kemp’s ridley turtle range (National Marine Fisheries Service, 2007).
Evolutionary Relationships of Sea Turtles

Throughout the years, there have been questions and debates as to the evolutionary relationships of the current seven living sea turtle species to one another. A 2008 study supported the idea that flatback sea turtles (*Natator depressus*) and green sea turtles (*Chelonia mydas*) have a sister-taxon relationship (Naro-Maciel et al., 2008). In addition, this study also supported the previous notion of a sister-taxon relationship between Kemp’s ridley (*Lepidochelys kempii*) and olive ridley (*Lepidochelys olivacea*) sea turtles, with a close relationship between these two species and loggerhead sea turtles (*Caretta caretta*) (Bowen et al., 1993; Dutton et al., 1996; Naro-Maciel et al., 2008).

Sea Turtle Threats

Though both turtle species are thought to be experiencing slow population recovery, concerns exist over threats due to entanglement in fishing gear and diseases and strandings. Due to their life history (late maturing, and very few young succeeding into adulthood), sea turtles are especially at risk of becoming extinct when populations have diminished. One difficulty with assessing the state of sea turtle populations is that it is very difficult to obtain data on hatchling survivorship, therefore, population fluxes (positive or negative) are often not realized for years, or even decades (Gardner and Eva, 2005). Sea turtle health has been a hot topic in recent years with potentially deadly diseases such as fibropapillomatosis receiving attention from the media and the public.
In the past few years, sea turtles have come into the news again as nearly 1200 turtles stranded in Cape Cod and the surrounding areas in the late months of 2014—most of them Kemp’s ridley (Gorman, 2014). Although some strandings occur every winter from dropping sea water temperatures, the sheer number of turtle strandings this past year was staggering, especially considering that there were just over 400 turtles stranded in the year 2012 (Gorman, 2014). Thus far, no answers have been found as to what causes some turtles “cold strand”, or why the number of strandings sharply increased in 2014.

Due to the fact that sea turtles are ectotherms, extreme temperatures can cause physiological stress responses to occur. When exposed to low temperatures, it has been shown that immunoglobulin production in reptiles decreases (Zapata et al., 1992). In some turtles, primary humoral immune responses during the summer months are sub-optimal, despite having a robust secondary response during this period (Leceta and Zapata, 1986). However, it should be noted that further studies need to examine the impacts of seasonal variation on the immune system of different types of reptiles, because some species may experience different effects than other species. In addition, there is evidence to suggest that mating periods can also impact immune responses in turtles (Leceta et al., 1989; Leceta and Zapata, 1985).

Fibropapillomatosis is a disease of sea turtles that is characterized by raised masses (tumors) on the turtle’s skin (usually soft skin), where each mass can be 0.1 cm to well over 30 cm in size (Herbst, 1994). These masses can become so large that they inhibit the turtle from foraging and swimming, which can ultimately lead to death (Herbst, 1994). Interestingly, some turtles have tumors that shrink over time, while others have
tumors that continue to grow in size, but there is not yet an explanation for why some turtles recover and others do not (Herbst, 1994). This disease tends to impact certain groups (particularly juvenile turtles) more than others, but it is still unclear why this is the case (Herbst, 1994). Strong evidence suggests that the cause of this disease in sea turtles is a herpesvirus, but there is no conclusive evidence as to the exact etiology of this disease (Herbst, 1994). It is possible that a genetic predisposition for the disease is present in some turtles; however, current research has not delved into this hypothesis (Herbst, 1994). There may be an immune dysfunction present in turtles that contract and succumb to fibropapillomatosis, but further studies need to be conducted to solidify this hypothesis (Herbst, 1994).

Sea Turtle Immunology

The immune system is a necessary component for organisms to fight infections and invasions from bacteria, viruses, fungi, and parasites. Reptiles have innate, cell-mediated, and humoral immune functions (Gardner and Eva, 2005). The reptilian immune system has some of the same elements as the mammalian immune system, but also offers its own unique features. As in mammals, reptiles have bone marrow (for hematopoiesis and B-cell maturation), a thymus (for T-cell maturation), as well as a spleen (for removal of old red blood cells and synthesis of antibodies), and gut-associated lymphoid aggregates (GALT) (which helps concentrate antigens and expose them to lymphocytes) (Keller et al., 2005).
Innate immunity does not recognize nor respond specifically to pathogens and does not generate immunological memory, but it does act as a primary response unit against foreign invasion in the body (Gardner and Eva, 2005). An important component of the reptilian innate immune system are lysozymes, which are enzymes that lyse bacteria by hydrolyzing their cell wall, and have varying degrees of success depending upon their host and which bacteria they come into contact with (Araki et al., 1998; Gayen et al., 1977; Ingram and Molyneux, 1983; Thammasirirak et al., 2006). Another innate immune response element, β-defensin-like protein, has been found in several reptiles, including the loggerhead turtle, but little is understood about its exact functionality (Zimmerman et al., 2010b).

In addition to other innate immunity components, it has been confirmed that reptiles have a complement system that is comprised of at least two pathways: classical and alternative (Sunyer and Lambris, 1998). The complement system is made up of proteins which opsonize or directly lyse bacteria in the plasma (Zimmerman et al., 2010b). When opsonized, the bacteria are coated with proteins which allow macrophages to recognize them as foreign invaders and then phagocytose them (Zimmerman et al., 2010b).

Furthermore, reptiles have various leukocytes which aid their innate immune responses. Among these are: monocytes, macrophages, heterophils, basophils, and eosinophils, each with varying functionalities (Zimmerman et al., 2010b). Both monocytes and macrophages serve similar functions: they process and present antigens as well as release cytokines (Coico et al., 2003). Heterophils have similar function in reptiles as neutrophils have in mammals: they aid in suppression of microbial invasions.
and have a hand in inflammatory responses (Zimmerman et al., 2010b). Basophils have immunoglobulins on their surfaces which are specific to certain antigens; these cells release histamine once they are triggered by the antigen that they have the receptors for (Zimmerman et al., 2010b). Despite knowing that eosinophils are present in reptiles, their function remains unknown (Zimmerman et al., 2010b).

During inflammatory responses, reptiles undergo different steps depending on whether the pathogen impacts the system extracellularly or intracellularly (Zimmerman et al., 2010b). If the immune system encounters an extracellular pathogen, heterophilic granulomas are formed for heterophils to accumulate and then these cells are degranulated and undergo necrosis which causes macrophages to respond to the area (Montali, 1988). If the immune system encounters an intracellular pathogen, the response is similar except that a histiocytic granuloma will be formed to attract and accumulate macrophages, and the central macrophages will undergo necrosis (Montali, 1988). Cytokines and chemokines are molecules which direct inflammation, but little is still known about these molecules in reptiles (Zimmerman et al., 2010b). In mammals, an important group of chemokines is interleukins (ILs), which act to guide the migration of lymphocytes and phagocytic cells (Coico et al., 2003). In terms of cytokines, an important class in mammals is interferons (IFNs), which are able to interfere with, and inhibit, replication of viruses (Coico et al., 2003).

For interleukins, it has been shown that there exists an IL-8 homolog in some reptiles (such as the Chinese soft-shelled turtle), an IL-1-like molecule in a species of wall lizard, an IL-2-like molecule in diadem snakes, and an interferon (IFN) in a tortoise and turtle.
species (El Ridi et al., 1986; Galabov and Velichkova, 1975; Mathews and Vorndam, 1982; Mondal and Rai, 2001; Zhou et al., 2009). The exact function of IL-8 still remains a mystery, but it has been shown that mRNA production of this chemokine is upregulated after an episode of bacterial infection in the Chinese soft-shelled turtle. IL-1 is thought to be connected with regulation of behavior after pathogenic infections in western fence lizards, and it is hypothesized that the production of this chemokine is related to temperature and reproductive phases of reptiles (Mondal and Rai, 2001, 2002). It is suggested that IL-2 is necessary for some cell proliferation in reptiles, as it has been seen that an IL-2-like molecule enhances mitogenesis of thymocytes in diadem snake (El Ridi et al., 1986). Finally, IFN has been seen in certain cells (kidney and peritoneal) and tissues (heart) after infection with a virus, but nothing further is known (Galabov and Velichkova, 1975; Mathews and Vorndam, 1982).

**Cell-Mediated Immunity**

It has been previously established that reptiles have functional T cells in the forms of cytotoxic-like T cells and regulatory T helper cells, each with unique functions (Burnham et al., 2005; Pitchappan and Muthukkaruppan, 1977). Cytotoxic-like T cells kill cells that have become infected by either a virus or bacteria and also attack abnormal cells, such as those associated with cancer (Coico et al., 2003). The role of T helper cells in reptiles is unclear, as reptiles do not have germinal centers or lymph nodes as in mammals (Zimmerman et al., 2010b). For these cell-mediated immunity features, it has been seen
that sex differences occur (typically females have stronger cell-mediated immune responses) and that contaminants in the environment also alter proliferation of cells, which varies in accordance with the contaminant (Ansar Ahmed et al., 1985). For example, loggerhead sea turtle lymphocyte proliferation is positively correlated with blood concentrations of polychlorinated biphenyls (PCBs), but negatively correlated with mercury levels (Day et al., 2007; Keller et al., 2006).

**Humoral Immunity**

An integral part of adaptive immunity is the ability to produce antibodies via B cell stimulation from a specific antigen (Coico et al., 2003). The basic structure of antibodies is well preserved in jawed vertebrates: two identical heavy chains, two identical light chains, and disulfide bonds joining the two (Zimmerman et al., 2010b). However, there is recent evidence to suggest that sea turtles may have some asymmetrical immunoglobulins (Work et al., 2015). It is important to note that contained on each heavy and light chain, there is one constant region and one variable region (Zimmerman et al., 2010b). The variable regions are essential to the ability of organisms to respond specifically to pathogens because the antigen binding site is formed by the variable region of each chain (Zimmerman et al., 2010b). The way that so much diversity is able to be obtained in the variable region of each chain is via rearrangement of specific gene segments: variable (V), diversity (D), and joining (J) gene segments are rearranged to make variable regions in heavy chains, and V and D gene segments are rearranged to
make variable regions in light chains; this provides for an incredible amount of genetic diversity (Turchin and Hsu, 1996).

Turtles and other reptiles (as well as birds) produce at least two immunoglobulins: immunoglobulin M (IgM) which occurs in all jawed vertebrates, and immunoglobulin Y (IgY) which is believed to be what mammalian immunoglobulin G (IgG) and immunoglobulin E (IgE) are derived from (Brown, 2002; Natarajan and Muthukkaruppan, 1985). IgM is the first Ig to respond when an infection arises: it is specifically produced when Gram-negative bacteria infiltrate the body and is excellent at activating complement (Coico et al., 2003). On the other hand, IgY responds after IgM but is produced in larger quantities and acts as the main defense mechanism toward infection (Warr et al., 1995).

One isoform of IgY in reptiles has two heavy and two light chains with a total molecular weight of approximately 180 kDa (this form is known simply as IgY) (Chartrand et al., 1971; Warr et al., 1995). It is, however, important to note that another isoform of IgY exists which is a truncated form of approximately 120 kDa (known as IgY(ΔFc)) (Figure 3) (Leslie and Clem, 1972). Reptiles and birds may just have the 180 kDa IgY isoform, the 120 kDa IgY(ΔFc) isoform, or a coexistence of both isoforms (Leslie and Clem, 1969). The heavier isoform of IgY consists of two heavy chains (known as 7S IgY, each has a molecular weight of approximately 63 kDa) which have one variable and four constant regions (Munoz et al., 2013). The IgY(ΔFc) isoform consists of two heavy chains (known as 5.7S IgY, each has a molecular weight of approximately 33.8 kDa) which have one variable and two constant regions (Munoz et
al., 2013; Warr et al., 1995). The light chain for both IgY and IgY(ΔFc) has a molecular weight of approximately 23.1 kDa (Munoz et al., 2013).

Due to its presence in diverse groups of species, IgY is difficult to generalize about because in many groups, little is still known about the function of this molecule (Warr et al., 1995). In addition, the two isoforms of IgY do not always possess the same functional abilities. For example, in animals that possess both IgY and IgY(ΔFc), or solely IgY, IgY is transferred to the embryo thorough the yolk, but in animals solely producing IgY(ΔFc), it is transmitted to the embryo via the yolk-sac (Warr et al., 1995). In ducks, the approximate ratio of IgY to IgY(ΔFc) in serum is 3:5, but this ratio may vary when examined in other species (Lundqvist et al., 2006). In addition, it is unclear of the exact role that IgY(ΔFc) plays in the immune function of animals which possess it, especially since it is not present in all animals which contain IgY. Investigations concerned with finding whether or not specific species contain IgY(ΔFc) could potential lead to a better understanding of evolutionary relationships in birds, reptiles, and amphibians.
Figure 3. Structure of the two isoforms of IgY. Adapted from: Warr et al., 1995
Chapter 2: Purification of Immunoglobulin Y (IgY) and anti-IgY Antibody

Production

Abstract

In order to elucidate the exact role that IgY and IgY(ΔFc) play in bacterial infections, first IgY needed to be purified using readily available serum samples. Though some methods to purify IgY exist, these methods are often expensive, can require egg yolk, and can require large equipment such as high speed centrifuges. The technique that was developed ran sea turtle serum through protein A/G columns that were previously equilibrated with 0.10 M Tris (pH 8.0) followed by 0.01 M Tris (pH 8.0). Columns were then eluted with 0.05 M glycine at pH 3.0, and eluted in 1 mL fractions with 100 µL of 1.0 M Tris pH 8.0 added to each fraction. Through experimentation, it was found that the majority of IgY was collected in the first and second fractions, and little-to-no IgY was collected in subsequent fractions. SDS-PAGE gels were able to confirm that IgY was purified, as bands were visualized at 68 kDa (IgY heavy chain), 37 kDa (IgY(ΔFc) heavy chain), and 22 kDa (IgY light chain), which aligns with values of IgY in sea turtles from the literature.

Introduction

Though sea turtle immunology has been fairly well studied in certain sea turtle species (such as the green sea turtle), other species (such as Kemp’s ridley sea turtles) have suffered from a lack of research. Green sea turtles have been disproportionately
studied because they have the highest prevalence of the disease marine turtle fibropapillomatosis (Aguierre and Lutz, 2004). However, it is important to understand immunology for all sea turtles species. This can allow for a better understanding of healthy baseline values for various immunological measurements and biomarkers, which can lead to better diagnoses and care strategies. For turtles that arrive at turtle hospitals when they are experiencing illness and/or injury, this will be especially important. This purified a sea turtle immunoglobulin (immunoglobulin Y) and produced antibodies against this immunoglobulin to give researchers and veterinarians the ability to expand upon the number and type of immunological assays which can be performed to measure sea turtle health.

An integral part of adaptive immunity is the ability to produce antibodies via B cell stimulation from a specific antigen (Coico et al., 2003). The basic structure of antibodies is well preserved in jawed vertebrates: two identical heavy chains, two identical light chains, and disulfide bonds joining the two (Zimmerman et al., 2010b). It is important to note that contained on each heavy and light chain, there is one constant region and one variable region (Zimmerman et al., 2010b). The variable regions are essential to the ability of organisms to respond specifically to pathogens because the antigen binding site is formed by the variable region of each chain (Zimmerman et al., 2010b).

Turtles and other reptiles (as well as birds and amphibians) produce at least two immunoglobulins: immunoglobulin M (IgM) which occurs in all jawed vertebrates, and immunoglobulin Y (IgY) which is believed to be what mammalian immunoglobulin G (IgG) and immunoglobulin E (IgE) are derived (Benedict and Pollard, 1972; Brown,
IgM is the first immunoglobulin to respond when an infection arises: it is specifically produced when Gram-negative bacteria infiltrate the body and is excellent at activating the complement system (Coico et al., 2003). On the other hand, IgY responds to infection after IgM, but is produced in larger quantities and acts as the main defense mechanism toward infection (Warr et al., 1995).

One isoform of IgY in reptiles has two heavy and two light chains with a total molecular weight of approximately 180 kDa (this form is known simply as IgY) (Warr et al., 1995). It is, however, important to note that another isoform of IgY exists which is a truncated form of approximately 120 kDa (known as IgY(ΔFc)) (Figure 3) (Leslie and Clem, 1972). Reptiles and birds may just have the 180 kDa IgY isoform, the 120 kDa IgY(ΔFc) isoform, or a coexistence of both isoforms (Leslie and Clem, 1969). The heavier isoform of IgY consists of two heavy chains (known as 7S IgY, each has a molecular weight of approximately 63 kDa) which have one variable and four constant regions (Munoz et al., 2013). The IgY(ΔFc) isoform consists of two heavy chains (known as 5.7S IgY, each has a molecular weight of approximately 33.8 kDa) which have one variable and two constant regions (Munoz et al., 2013; Warr et al., 1995). The light chain for both IgY and IgY(ΔFc) has a molecular weight of approximately 23.1 kDa (Munoz et al., 2013).

Due to its presence in diverse groups of species, IgY is difficult to generalize about because in many groups, little is still known about the function of this molecule (Warr et al., 1995). In addition, the two isoforms of IgY do not always possess the same
functional abilities. For example, in animals that possess both IgY and IgY(ΔFc), or solely IgY, IgY is transferred to the embryo thorough the yolk, but in animals solely producing IgY(ΔFc), it is transmitted to the embryo via the yolk-sac (Warr et al., 1995). Therefore, it is important to research IgY further in hopes of understanding its functional abilities. The objectives for this study were to: 1. Purify IgY from sea turtle serum, 2. Generate polyclonal antisera against IgY, and 3. Generate monoclonal antibodies against IgY.

Materials and Methods

Purification of IgY from Sea Turtle Serum

In order to purify IgY from sea turtle serum, a methodology was developed to isolate immunoglobulin Y (IgY) from sea turtle serum using 1 mL protein A/G columns (ThermoFisher). First, the columns were equilibrated by running 10 volumes 0.10 M Tris pH 8.0 through the column, followed by 10 volumes of 0.01 M Tris pH 8.0. Then, 3 mL of pooled serum samples from each sea turtle species along with 300 µL of 1.0 M Tris at pH 8.0 were added to their respective columns and allowed to run through three times each. Finally, IgY was eluted using 10 mL of 0.05 M glycine at a pH of 3.0 in 1 ml fractions. To each of these 1 mL fractions, 100 µL of 1.0 M Tris pH 8.0 added.

Upon collection of the purified IgY product, a standard BSA protein determination was performed for each aliquot to elucidate which fractions held IgY, and
how much was present. Protein determination was performed in a 96 well plate by using Pierce® BCA Protein Assay reagents (Thermo Scientific). Two wells of each of the following concentrations of bovine serum albumin standard (Thermo Scientific) were created by using a serial dilution methodology with phosphate buffered saline (PBS): 2 mg/mL, 1 mg/mL, 0.50 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.0625 mg/mL. 10 µL of the purified IgY product was then added to remaining wells (each aliquot in duplicate). Immediately after the addition of the product to the wells, a 50:1 ratio of Pierce® BCA Protein Assay Reagent A: Pierce® BCA Protein Assay Reagent B was added to all wells, such that each well received 200 µL. The 96 well plate was then incubated at 30ºC for 30 minutes, and the optical density at 562 nm was recorded for each well. A standard curve was generated from the known concentrations of the BSA standard wells and their respective optical density readings. Upon creation of the standard curve, a line of best fit was added, and the equation of this line was used to determine the exact amount of protein that corresponded with each optical density reading. Since each sample was measured in duplicate, the average of these two wells were taken, and that amount was considered the final protein concentration estimate for each specific individual sample.

From aliquots that showed positive protein amounts, 16 µL of sample was taken and combined with 4 µL of 5x Laemmli sample buffer with 2-Mercaptoethanol (2-ME) and boiled in 100º C water for 8 minutes. Once cooled, 20 µL of the product were subjected to SDS-PAGE using 4-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (BioRad). For each gel, lane 1 contained 12 µL of Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards molecular weight marker (BioRad),
followed by 20 µL of IgY from aliquot 1 (corresponding to the first elution) in lane 2, and 20 µL of IgY from aliquot 2 (corresponding to the second elution) in lane 3. Two gels were run: one for loggerhead samples, and the other for Kemp’s ridley samples, at 180 V for approximately 40 minutes. Upon completion, the gels were stained with Coomassie blue for 16 hours and then destained using a 30% methanol/10% acetic acid solution for three hours. Gels with obvious bands were then dried and imaged, using the molecular weight marker as a size reference.

In addition, one SDS-PAGE 4-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gel (BioRad) was run. For this gel, lane 1 contained 12 µL of Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards molecular weight marker (BioRad), followed by 20 µL of IgY purified from loggerhead sea turtles in lane 2, and 20 µL of IgY purified from Kemp’s ridley sea turtles in lane 4. This gel was run at 180V for approximately 40 minutes, and then stained with Coomassie blue for 16 hours and destained with a 30% methanol/10% acetic acid solution for three hours. This gel was then dried and imaged, using the molecular weight marker as a size reference.

**Generation of polyclonal antisera against IgY**

Purified IgY (from the method above) from both sea turtle species were used to immunize 6-week old female Balb/c mice using previously described general methods (Rice et al., 1998). Mice were immunized subcutaneously (SC) with 100 µg of purified IgY in a volume of 100 ul PBS mixed 1:1 with 100 ul TiterMax® Gold adjuvant on day
1. On day 14, mice were immunized again using Freud’s incomplete adjuvant, and these mice received booster immunizations on days 35 and 56 via intraperitoneal injections. Five days later, mice were sacrificed by slow lethal CO$_2$ asphyxiation, followed by bilateral pneumothorax according to approved IACUC AUP, and were then bled to collect blood. Blood was allowed to clot at room temperature for 2 hours, then centrifuged for 20 minutes at 10,000 g to collect overlying serum as the source of polyclonal serum.

Serum samples were tested for specificity against purified IgY samples by western blotting. IgY was subjected to SDS-PAGE as described above, then transferred onto two Immun-Blot® PVDF (polyvinylidene fluoride) membranes (BioRad) at 100 V for 60 minutes on ice. Lane 1 of these blots contained the molecular weight marker (Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards molecular weight marker (BioRad)), followed by loggerhead IgY in lane 2, and Kemp’s ridley IgY in lane 4. Upon transfer, these blots were blocked at 4°C for 16 hours with 10% fetal calf serum in PBS. Next, the blots were subjected to whole polyclonal anti-sera (pAb) diluted at a ratio of 1:500 in PBS to probe for IgY: this anti-sera was incubated with the blots for one hour at room temperature. Blots were then washed three times each, for five minutes each time with PBS tween20 solution. Next, the blots were probed with goat anti-mouse IgG conjugated with alkaline phosphatase (ThermoFisher, 1:1000) for one hour at room temperature before the washing procedure was again repeated. Finally, alkaline phosphatase activity was visualized with Nitro Blue Tetrazolium (Nitro BT) and 5-bromo-4-chloro-3'-indolyphosphate (BCIP) in AP buffer.
Generation of monoclonal antibodies against IgY

Mice were immunized with purified IgY using the same conditions and schedules as described above. Spleens were removed from the mice and the tissue was dissociated into single cells by gently grasping the pulp with tweezers and using a scalpel blade. Cells were washed twice via centrifugation, then fused with Sp02-Ag14 myeloma cells (ATCC) in the presence of 50% polyethylene glycol 4000 (PEG 4000) (Fisher Scientific) using previously published techniques (Rice et al., 1998). Resulting hybridomas were cultured, screened, and cloned as previously described (Rice et al., 1998). Supernatants from cloned hybridomas were used as the source of monoclonal antibodies (mAb). Resulting mAbs were then tested against purified IgY samples by both ELISA and western immunoblotting as described in the literature.

Results

Using the protein A/G approach, IgY from both species of sea turtles was eluted to a high degree of purity (Figure 4). For both loggerhead and Kemp’s ridley turtles, obvious banding was present for both aliquots at approximately 63 kDa (corresponding to the heavy chain of IgY, known as 7S IgY), 33.8 kDa (corresponding to the heavy chain of IgY(ΔFc), known as 5.7S IgY or the truncated form of IgY), and at 23.1 kDa (corresponding to the light chains of both isoforms of IgY). No other strong banding
patterns were present, indicating that IgY was the primary product purified using this novel approach.

Mouse anti-sera against purified IgY from both species recognized proteins in whole turtle serum samples from both loggerhead and Kemp’s ridley turtles, with some additional bands being recognized as well (Figures 5 and 6). Of particular note, both the heavy chain and truncated form of IgY were recognized, but only traces of the light chain could be detected. Higher molecular weight proteins of approximately 150 kDa and 72 kDa were also recognized by the pAb anti-IgY sera, but these proteins were not obvious in purified IgY (Figure 4). It is clear that anti-IgY made against loggerhead IgY recognizes the IgY components in Kemp’s ridley samples, and vice versa, meaning that polyclonal anti-sera shows cross-reactivity in this particular case.

Attempts to make monoclonal antibodies against purified IgY resulted in mAb named LH 2-6 which recognizes the light chains of loggerhead IgY, but also recognizes the same protein in Kemp’s ridley IgY. In addition, this study produced the first known mAb (mAb KR-11) against the heavy chain of the truncated form of IgY in Kemp’s ridley turtles, and this antibody also recognizes the same product in loggerhead sea turtles, implying some level of cross-reactivity (Figures 7 and 8).
Figure 4. This SDS-PAGE gel shows a molecular weight marker in the first lane, purified loggerhead IgY in the second lane, and purified Kemp’s ridley IgY in the fourth lane. Obvious bands appear at approximately 63 kDa (corresponding to the heavy chain of IgY, known as 7S IgY), 33.8 kDa (corresponding to the heavy chain of IgY(ΔFc), known as 5.7S IgY), and 23.1 kDa (corresponding to the light chains of both forms of IgY).
Figure 5. Western blot analysis of the binding of loggerhead polyclonal antisera to purified sea turtle IgY. Lane one is molecular weight marker, lane two is purified loggerhead IgY, and lane four is purified Kemp’s ridley IgY. The blot was probed with loggerhead polyclonal antisera from mice, and then with goat anti-mouse IgG AP NaN₃. Obvious bands appear at 150 kDa, approximately 77 kDa and 63 kDa, and around 23 kDa.
Figure 6. Western blot analysis of the binding of Kemp’s ridley polyclonal antisera to purified sea turtle IgY. Lane one is molecular weight marker, lane two is purified loggerhead IgY, and lane four is purified Kemp’s ridley IgY. The blot was probed with Kemp’s ridley polyclonal antisera from mice, and then with goat anti-mouse IgG AP NaN₃. Obvious bands appear at 150 kDa, approximately 77 kDa and 63 kDa, and around 37 kDa.
Figure 7. Western blot analysis of the binding of monoclonal antibody LH 2-6 to purified IgY from sea turtles. Lane one is molecular weight marker, lane two is purified loggerhead IgY, and lane four is purified Kemp’s ridley IgY. The blot was probed with the monoclonal antibody LH 2-6 made in mice, and then was probed with goat anti-mouse IgG AP NaN₃. Obvious bands appear at approximately 23 kDa for both sea turtles species, which is consistent with the weight of the light chain of IgY.
Figure 8. Western blot analysis of the binding of monoclonal antibody KR-11 to purified IgY from sea turtles. Lane one is molecular weight marker, lane two is purified loggerhead IgY, and lane four is purified Kemp’s ridley IgY. The blot was probed with the monoclonal antibody KR-11 made in mice, and then was probed with goat anti-mouse IgG AP NaN3. Obvious bands appear at approximately 37 kDa for both sea turtles species, which is consistent with the weight of the Fab’ fragment (the heavy chain of IgY(ΔFc)).
Discussion

In this study, it is shown that IgY can be purified from sea turtle serum using standard protein A/G columns. More traditional methods of IgY purification include extraction from eggs, and even molecular weight size exclusion assays. The protein A/G method is beneficial because protein A/G columns are a fraction of the cost of IgY columns designed for purifying IgY from eggs. Moreover, protein A/G columns require serum, rather than egg yolks, meaning that whole eggs can be spared for IgY purification in sea turtles, which is especially relevant as many sea turtle species are considered to be endangered. In addition, this methodology can be readily applied to purify IgY from freshly collected serum in the field, and the aliquots can be frozen for further analysis later.

Generation of polyclonal antisera and monoclonal antibodies in sea turtles is important because it allows for immunological techniques and assays (such as Western blotting and ELISA) to be performed. Monoclonal antibodies, though time-consuming to initially make, can provide, essentially, a limitless supply of antibodies once they are created, and cells producing these monoclonal antibodies can be easily shipped to other laboratories and veterinary clinics. The availability of readily accessible antibodies for sea turtles will greatly increase the number of immunological assays that can be performed on sea turtles, both in field settings and in laboratory and clinical scenarios. With this, more robust data sets on baseline measurements (such as quantifications of titers in sea turtle serum against bacteria) can be achieved, which will lead to a better understanding of the parameters that separate healthy sea turtles from sick and/or injured
sea turtles, allowing rehabilitation centers improved estimates of when their sea turtles can safely be released back into the wild.

One reason that the monoclonal antibody against loggerhead light chain IgY (LH 2-6) also recognized Kemp’s ridley light chain IgY could be because these species are relatively closely related. A 2008 study that examined evolutionary relationships in marine turtles agreed with previous work by finding that there is a close affiliation between the *Lepidochelys* genus (which includes Kemp’s ridley and olive ridley turtles) and loggerhead sea turtles (Bowen et al., 1993; Dutton et al., 1996; Naro-Maciel et al., 2008). This may be good news for laboratories and clinics, because this proposes that monoclonal antibodies may not need to be produced for every single sea turtle species, just for several species that cross-react.

These results suggest that loggerhead and Kemp’s ridley turtles are closely related, and one set of monoclonal antibodies may be sufficient to perform immunological assays in these two species, and very likely in olive ridley turtles as well, due to the fact that they share the same genus with Kemp’s ridley sea turtles. However, further research is needed to understand whether or not monoclonal antibodies produced against loggerhead sea turtles, for example, would cross react for other sea turtle species that were less related to them (such as hawkbill, green, and leatherback turtles).
Chapter 3: Antibody Responses to Marine Bacterial Pathogens

Abstract

Though several studies have explored the presence of bacteria in sea turtles, no study to date has used previously produced antibodies in serum to determine prior exposure of sea turtles to bacteria found in their environments. Nine marine bacteria (Escherichia coli, Vibrio cholera, V. parahaemolyticus, V. anguillarum, V. vulnificus, Mycobacterium marinum, Erysipelothrix rhusiopathiae, Streptococcus agalactiae, and Brevundimonas vesicularis) were grown and lysed, and that lysate was run out on 4-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (BioRad). The gels were transferred onto an Immun-Blot® PVDF membrane (BioRad), and one set of blots was probed with whole serum from each species, and the other set was probed with purified IgY. Polyclonal IgY antisera was used to detect the presence of bacterial components. Strong banding patterns were found against E. coli, V. parahaemolyticus, M. marinum, and E. rhusiopathiae in both species. However, when mAb KR-11 (anti-truncated IgY) or mAb LH 2-6 (anti-IgY light chain) were used instead of anti-serum, few bacterial specific components could be detected, except for a prominent M. marinum and B. vesicularis protein.
Introduction

An important component to understanding overall sea turtle health is the ability to recognize potential previous infections that sea turtles have had which may have compromised their immune systems, even if only for a short time period. Several studies have been carried out in our lab to examine antibody responses in both fish and bottlenose dolphins against 9 ubiquitous marine bacteria (Beck and Rice, 2003), and include *Escherichia coli*, *Vibrio cholera*, *V. parahaemolyticus*, *V. anguillarum*, *V. vulnificus*, *Mycobacterium marinum*, *Erysipelothrix rhusiopathiae*, *Streptococcus agalactiae*, and *Brevundimonas vesicularis*. Conditions for growing these bacteria, adhering bacterial to ELISA plates, and conditions for the ELISAs are routine, and provide the opportunity to examine antibody responses to these bacteria in sea turtles.

*E. coli* represent a diverse group of bacteria that are commonly found in the marine environment. This bacterium is so common, that both freshwater and marine sources are frequently tested to quantify the concentrations in the water (US Environmental Protection Agency, 2016). Many strains of *E. coli* are harmless, though some strains are pathogenic and can cause illness or even death in humans (Centers for Disease Control and Prevention, 2015). Previously, this species has been isolated from nasal and cloacal samples of nesting green sea turtles from Tortuguero National Park in Costa Rica and loggerhead sea turtles in the central Mediterranean Sea, in liver and lung samples from a juvenile green sea turtle, and in cloacal and upper respiratory tract swabs from leatherback turtles (*Dermochelys coriacea*) (Centers for Disease Control and Prevention, 2015; Foti et al., 2009; Raidal et al., 1998; Santoro et al., 2006, 2008). A study from
2009 found that *E. coli* was present in green sea turtle (*Chelonia mydas*) eggs, and accounted for nearly 15% of total antibiotic resistant isolates found in the eggs—the only species that contributed higher percentages were *Pseudomonas aeruginosa* and *Salmonella arizonae* (Al-Bahry et al., 2009). Overall, *E. coli* is one of the most commonly cultured bacteria from diseased sea turtles (Lutz and Musick, 1997).

*Vibrio cholerae* is another bacterium commonly found in aquatic environments which has strains that can lead to the deadly condition of cholera. This bacterium is prominent in marine and brackish environments, and is prone to attaching to copepods and other chitinous organisms (Colwell et al., 1981; Huq et al., 1983). *Vibrio parahaemolyticus* is also able to be found in marine and brackish water environments (Colwell et al., 1977). This *Vibrio* species can cause illness and has been implicated as the cause of gastroenteritis from improperly prepared seafood (Sizemore et al., 1975). Furthermore, previous studies have found the presence of *V. parahaemolyticus* in the oral cavity and impacted intestine of juvenile sea turtles, as well as from nasal samples (Chuen-Im et al., 2010; Santoro et al., 2006). *Vibrio anguillarum* is a pathogenic bacterium that infects marine fish to cause the disease vibriosis (Milton et al., 1995). *V. vulnificus* is a disease-causing pathogen contracted by ingestion of contaminated seafood or exposure to infected seawater with an open wound and represents a serious human health concern (Centers for Disease Control and Prevention, 2013).

The bacterium *Mycobacterium marinum* infects humans opportunistically via lesions (Bhaty et al., 2000). This species has been previously isolated from reptiles and has been implicated as a zoonotic agent which can cause cutaneous nodular disease in
humans (Aguirre et al., 2006). It has previously been cultured from a head-started Kemp’s ridley sea turtle with a lung infection (Lutz and Musick, 1997).

*Erysipelothrix rhusiopathiae* is a bacterial species which has been seen in both commercially important animals, such as turkeys and chickens as well as wildlife, such as fish, mollusks, and crustaceans (Klauder, 1926; Murase et al., 1959; Wood, 1975).

*Streptococcus agalactiae* is able to cause septicemia in fish, rays, saltwater crocodiles, and dolphins (Amborski et al., 1983; Bishop et al., 2007; Evans et al., 2006; Plumb et al., 1974; Zappulli et al., 2005). Finally, *Brevundimonas vesicularis* is a marine bacteria that has rarely infected immunocompromised humans (Shang et al., 2011).

It is essential to study reptiles and their immune system because they are unique in the fact that they are ectotherms, and therefore may respond very differently to environmental stressors such as bacteria, pollutants, and abiotic threats than mammals or other types of animals (Zimmerman et al., 2010b). In addition, studying these animals can give scientists a better idea of what to expect when environmental disasters (such as oil spills) occur: their immune systems vary responses depending upon season (and with temperature), so seasonality and yearly weather patterns may be an important factor in determining illness and mortality after such events.

The impacts of seasonality on immune system parameters in sea turtles is not an area that has generated much research, though some studies have delved into the seasonality question in other turtle species. A 2010 study on red-eared sliders (*Trachemys scripta*) found that immunoglobulin levels steadily increased from the beginning of the sampling
period in late April through August, with a modest decline in September (Zimmerman et al., 2010a). This same study also found that plasma bacterial killing capacity was highest in late May and early June, and reached a minimum in early August (Zimmerman et al., 2010a).

More research is needed to determine whether or not these same types of patterns would be seen in sea turtles throughout the summer months, and even throughout all four seasons. With the looming threat of climate change, it is important to understand how sea turtles may respond to pathogens that will be found in their environments under higher temperature conditions than are currently present. The objectives for this study were to: 1. Determine if whole serum and purified IgY from both sea turtle species co-recognize dominant bacterial antigens, and 2. Develop enzyme-linked immunosorbent assays (ELISAs) for quantifying bacteria-specific IgY in circulation.

**Materials and Methods**

*Determination: do whole serum and purified IgY co-recognize dominant bacterial antigens?*

To determine previous exposure of individual sea turtles from both loggerhead and Kemp’s ridley turtle species, 20 µL of each of the aforementioned nine ubiquitous marine bacteria were inoculated into 25 mL of their specified growth medium: *E. coli* was inoculated into Luria broth, *V. cholera* and *V. parahaemolyticus* into nutrient broth with
3% NaCl, *V. anguillarum*, *B. vesicularis*, and *V. vulnificus* into marine broth, *M. marinum* into Middlebrook broth, *E. rhusiopathiae* into tryptic soy broth with 5% defibrinated sheep red blood cells, and *S. agalactiae* into tryptic soy broth.

These bacteria were grown at approximately 37ºC for 24-48 hours in 50 mL tubes, then each tube was spun down at 3000 rpm for 15 minutes, and the supernatant was poured off and frozen at -20º C. 20 mL of 0.01 M PBS was added to all of the bacterial pellets, and these were re-suspended via vortexing and again spun down at 3000 rpm for 15 minutes. This procedure was repeated twice more, with 4 mL of PBS being used the last time instead of 20 mL. Then, 120 µL of bacterial suspension was added to 30 µL of 5x Laemmli sample buffer with 2-ME and boiled for 8 minutes in order to generate bacterial lysates. On a 4-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gel (BioRad), 12 µL of Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards molecular weight marker (BioRad) was added to the first well in each of two separate 4-20% gels, and then the lysate samples (25 µL per well) were added in the following order: *E. coli*, *V. cholerae*, *V. parahaemolyticus*, *V. anguillarum*, *V. vulnificus*, *M. marinum*, *E. rhusiopathiae*, *S. agalactiae*, and *B. vesicularis*. Both gels were run at 180 V for approximately 40 minutes. Proteins in the two gels were then transferred onto an Immun-Blot® PVDF membrane at 35 V for 16 hours at 4º C, then blocked for 1 hour with blocking buffer at room temperature.

100 µL of serum from each sea turtle species was then added into separate 50 mL tubes and brought up to 20 mL with PBS to form a pooled sample. The blots were incubated at room temperature for 2.5 hours with this serum/PBS mixture, and then were
washed three times with PBS tween20 for five minutes each time. 20 mL of each turtle’s polyclonal anti-IgY was made by adding 100 µL of anti-IgY to 20 mL of PBS and the blots were incubated in these solutions for one hour at room temperature and then washed three more times with PBS tween20 for five minutes each time. Goat anti-mouse IgG (ThermoFisher, 1:000 in PBS) was then added to the blots and they were again incubated at room temperature for one hour and then washed three times, for five minutes each, with PBS tween20. Next, the blots were developed with NBT and BCIP in AP buffer and alkaline phosphatase activity visualized and recorded.

A similar process was undertaken to ensure that bands which were visualized were a result of IgY binding. To perform this, the above procedure was carried out, but using purified IgY instead of whole serum. 400 µL of purified IgY from each sea turtle species was diluted in 25 mL of PBS (to serve as the primary antibody) and incubated with the respective blot for two hours. Following incubation, blots were washed three times in PBS tween20 and mouse anti-turtle IgY sera diluted in PBS to a 1:500 was then applied to the respective blots for one hour. After washing three more times with PBS tween20, mouse anti-mouse IgG AP was applied for one hour and then the blots were washed again three times in PBS tween20. Finally, each blot was developed with Nitro Blue Tetrazolium (Nitro BT) and 5-bromo-4-chloro-3’-indolyphosphate (BCIP) (at a 2:1 ratio) in AP buffer and bands were then visualized.

To examine whether or not the monoclonal antibodies LH 2-6 (a monoclonal antibody against 7S IgY heavy chain) and KR-11 (a monoclonal antibody against 5.7S IgY heavy chain) would express differential binding patterns to the marine bacteria,
Western blot analysis was again performed as described above. Bacterial lysates were subjected to SDS-PAGE and immunoblotting, and probed with species-specific purified IgY for one hour at room temperature (two blots for each species) and then washed three times with PBS tween20 for five minutes each time. Next, either mAb KR-11 or mAb LH 2-6 was added to the blots for one hour at room temperature (one blot from each species was probed with KR-11, and one from each species was probed with LH 2-6). After that time, each blot was washed three times with PBS tween20 for five minutes each, and then goat-anti-mouse IgG AP was added for one hour at room temperature. Blots were washed again three times in PBS tween20 for five minutes each time, and then all four blots were developed with Nitro BT and BCIP (at a 2:1 ratio) in AP buffer, and bands were visualized.

*Development of enzyme-linked immunosorbent assays (ELISAs) for quantifying bacteria-specific IgY in circulation.*

To perform enzyme-linked immunosorbent assays (ELISAs), the previously mentioned nine marine bacteria were again grown at approximately 37°C for 24-48 hours in their specific medias and then spun down at 3000 rpm for 15 minutes. The supernatant was then removed and the pellet was resuspended in 20 mL of 0.10 M phosphate buffered saline (PBS), and this procedure was repeated once more. After the second resuspension, the bacteria were once again spun down at 3000 rpm, but when the supernatant was removed this time, they were then re-suspended in 10 mL PBS. 100 µL of each bacteria
was placed into three separate wells of a 96 well plate and the optical densities for each were recorded at 600 nm. Bacterial suspensions were diluted to optimal optical densities (which were previously established for all nine of these marine bacteria in our laboratory) in 15 mL of total suspension, using PBS. The established optical densities were as follows: 0.200 for *E. coli*, *V. cholerae*, *V. anguillarum*, *V. vulnificus*, *E. rhusiopathiae*, *S. agalactiae*, 0.100 for *V. parahaemolyticus*, and *B. vesicularis*, and 0.050 for *M. marinum*.

High bonding 96 well plates (Medi-sorb, ThermoFisher) were coated for 16 hours at 4° C with 50 µL of poly-D-lysine in distilled water in each well. The optimized suspensions were then taken and 50 µL of each bacteria were added to wells such that three 96 well plates were coated for each bacterial species. The plates were centrifuged at 3000 rpm for 5 minutes and then 50 µL of 0.5% glutaraldehyde was added to each well. The plates were then spun down again for 5 minutes at 3000 rpm. All plates were incubated at room temperature for 15 minutes and then washed twice with PBS. After washing, to each well, 100 µL of glycine-BSA (100mM glycine with 1% BSA) was added and the plates were then incubated for 30 minutes at room temperature. For each plate, two more washes with PBS were performed and then two washes with distilled water were performed. The plates were kept at room temperature for two hours to dry thoroughly, and then stored in plastic wrap for 36 hours at room temperature. After storage, every well of each plate received 100 µL of 5% BSA blocking buffer, which remained in the wells for two hours at room temperature, and then each plate was washed twice with PBS tween20.
Loggerhead and Kemp’s ridley serum suspensions were made by adding 10 µL of serum from each individual turtle into 990 µL of PBS in 1.5 mL snap cap tubes. In addition, one tube of combined loggerhead serum was made by combining loggerhead serum from 10 randomly selected loggerhead individuals and adding 990 µL of PBS, and one tube of combined Kemp’s ridley serum was made using the same procedure. 75 µL of each serum suspension was added, in duplicate, to the respective wells and incubated at 4º C overnight. The next day, plates were washed two times with PBS, then 75 µL of mouse polyclonal anti-loggerhead or anti-Kemp’s ridley IgY (1:500 dilution) were added to respective wells. Loggerhead anti-IgY was made by adding 200 µL of anti-IgY in 100 mL of PBS with 0.50 grams of sodium azide; Kemp’s ridley anti-IgY was made by adding 100 µL of anti-IgY in 50 mL of PBS with 0.25 grams of sodium azide. Plates were washed two times with PBS after two hours of room temperature incubation. Goat anti-mouse IgG AP (1:1000) was then added in 75 µL quantities to each well and incubated at room temperature for 1.5 hours before being washed four times with PBS. Finally, 100 µL of 1mg/mL of p-nitrophenol (Fisher) in AP buffer was added to each well, and then the plates were all read after 30 minutes at 405 nm and data were recorded.

The values for serum titers against bacteria, calculated as relative optical density (O.D.) at a 1:200 dilution, from individual turtles were then organized into categories based on gender and year of serum collection, and the titers levels were averaged. These averages were then compared to one another by analysis of variance (ANOVA) with Bonferroni's multiple contrast post-hoc tests. GraphPad Prism’s statistical software was used for this analysis, and the α-value was set at 0.05 prior to the experiment (Fisher,
In addition, serum titer levels were compared against straight carapace length using regression analysis to examine if the size of a turtle correlated with its amount of serum titers for each bacterial species.

Results

Circulating antibodies against the nine examined bacterial lysates in loggerhead turtles indicate that some proteins are more immunogenic (seen with more distinct banding patterns) than other proteins (Figure 10). For example, there was one dominant protein revealed in *E. coli*, one very high molecular weight protein from *V. cholerae*, two very dominant bands in *V. parahaemolyticus*, no dominant bands in *V. anguillarum*, one band in *V. vulnificus*, a very distinct band from *M. marinum*, one dominant lower molecular weight protein in *E. rhusiopathiae*, and one faint band from *B. vesicularis*. Likewise, circulating antibodies against these nine bacterial lysates in Kemp’s ridley sea turtles recognized similar protein bands, with similar intensity (Figures 11). Similar results are seen when these same bacterial proteins were probed with purified IgY in both species (Figures 12 and 13), but some of the bands were more intensely stained, indicating the possibility that using purified IgY improves the signal by removing interfering proteins from whole serum that could lead to spurious cross-reactivity.

Having mAbs specific to IgY light chains and the truncated form of IgY allows us to determine the possible role, if any, of these fragments in recognizing bacterial proteins, and thereby having a role in immunity. When purified loggerhead IgY was used as the probe for bacterial proteins, followed by detection using anti-light chain mAb LH 2-6,
recognition was reduced to a dominant band in *M. marinum* lysates and *B. vesicularis* lysates (Figure 14). For the most part, the same binding patterns are observed when using Kemp’s ridley IgY as the probe and LH 2-6 for detection, except that an additional high molecular weight protein in *E. coli* can been seen (Figure 16). Using purified IgY from loggerhead turtles as the probe, and mAb KR-11 for detection, distinct bands for *M. marinum* and *B. vesicularis* were observed (Figure 15), but more bands were recognized when Kemp’s ridley IgY was used as the probe followed by detection with mAb KR-11 (Figure 17).

Overall, there were no significant differences in relative antibody titers against the nine bacteria found between either of the species for any of the sampling years included in this study (2011, 2012, 2013) (Figure 18). However, as shown in Figure 19, in 2013 loggerhead males and females showed significantly higher titer levels versus in 2011 and 2012 males and females for *V. vulnificus*. In addition, 2013 loggerhead males and females showed significantly higher titer levels for *V. parahaemolyticus* when compared to females sampled in 2011. Finally, 2013 loggerhead females showed significantly higher titer levels for *E. coli* when compared to their 2012 loggerhead female counterparts. Conversely, the data from Kemp’s ridley turtles shows no significant differences between genders or years for any of the serum titers against bacteria. However, it should be noted that for Kemp’s ridley samples, there were no males represented in 2011, and only one represented in 2012. In 2011, there were three turtles from unidentifiable genders, and there were two of these turtles in 2012. Therefore, 2013
is the only one of the three years that has titer data for Kemp’s ridley turtles that includes both male and female individuals.

When regression analyses were performed to examine the linear relationship between an individual sea turtle’s straight carapace length (SCL) and level of titers against particular bacteria, there were some correlations. For Kemp’s ridley sea turtles, as seen in Figure 21, there is a strong positive correlation between straight carapace length of females sampled in 2011 and their serum titer levels against *E. coli* ($R^2 = 0.5106$). Conversely, Figure 22 shows a strong negative correlation between straight carapace length of Kemp’s ridley females sampled in 2013 and their titer levels against *V. anguillarum* ($R^2 = 0.8654$). However, the small sample size from these data should be noted, and results should be interpreted with caution.

There were also some correlations found between straight carapace length and serum titers against bacteria in loggerhead sea turtles. Male loggerhead turtles sampled in 2011 showed a strong negative correlation between straight carapace length and *V. anguillarum* titers, as seen in Figure 23 ($R^2 = 0.5004$). There were several strong correlations between the straight carapace length for male loggerhead sea turtles sampled in 2012 and several levels of serum titers against bacteria. Figure 24 shows a strong positive correlation between these males and titers against *E. coli* ($R^2 = 0.6343$). Strong negative correlations were found between straight carapace length of these loggerhead males sampled in 2012 and titers against *E. rhusiopathiae* ($R^2 = 0.5877$), *V. parahaemolyticus* ($R^2 = 0.6906$), and *V. vulnificus* ($R^2 = 0.7896$) (Figures 25, 26, and 27,
respectively). One important note is that the sample size for 2012 males was only four individuals, so results should be interpreted with caution.
Figure 9. Western blot analysis of nine marine bacterial lysates probed with loggerhead whole serum diluted 1:200. Lane one is the molecular weight marker (Kaleidoscope, BioRad), lane two is *E. coli* lysate, lane three is *V. cholerae* lysate, lane four is *V. parahaemolyticus* lysate, lane five is *V. anguillarum* lysate, lane six is *V. vulnificus* lysate, lane seven is *M. marinum* lysate, lane eight is *E. rhusiopathiae* lysate, lane nine is *S. agalactiae* lysate, and lane ten is *B. vesicularis* lysate.
Figure 10. Western blot analysis of nine marine bacterial lysates probed with Kemp’s ridley whole serum. Lane one is the molecular weight marker (Kaleidoscope, BioRad), lane two is *E. coli* lysate, lane three is *V. cholerae* lysate, lane four is *V. parahaemolyticus* lysate, lane five is *V. anguillarum* lysate, lane six is *V. vulnificus* lysate, lane seven is *M. marinum* lysate, lane eight is *E. rhusiopathiae* lysate, lane nine is *S. agalactiae* lysate, and lane ten is *B. vesicularis* lysate.
Figure 11. Western blot analysis of nine marine bacterial lysates probed with purified loggerhead IgY. Lane one is the molecular weight marker (Kaleidoscope, BioRad), lane two is *E. coli* lysate, lane three is *V. cholerae* lysate, lane four is *V. parahaemolyticus* lysate, lane five is *V. anguillarum* lysate, lane six is *V. vulnificus* lysate, lane seven is *M. marinum* lysate, lane eight is *E. rhusiopathiae* lysate, lane nine is *S. agalactiae* lysate, and lane ten is *B. vesicularis* lysate.
Figure 12. Western blot analysis of nine marine bacterial lysates probed with purified Kemp’s ridley IgY. Lane one is the molecular weight marker (Kaleidoscope, BioRad), lane two is *E. coli* lysate, lane three is *V. cholerae* lysate, lane four is *V. parahaemolyticus* lysate, lane five is *V. anguillarum* lysate, lane six is *V. vulnificus* lysate, lane seven is *M. marinum* lysate, lane eight is *E. rhusiopathiae* lysate, lane nine is *S. agalactiae* lysate, and lane ten is *B. vesicularis* lysate.
Figure 13. Western blot analysis of nine marine bacterial lysates probed with purified loggerhead IgY followed by LH 2-6 monoclonal antibody. Lane one is the molecular weight marker (Kaleidoscope, BioRad), lane two is *E. coli* lysate, lane three is *V. cholerae* lysate, lane four is *V. parahaemolyticus* lysate, lane five is *V. anguillarum* lysate, lane six is *V. vulnificus* lysate, lane seven is *M. marinum* lysate, lane eight is *E. rhusiopathiae* lysate, lane nine is *S. agalactiae* lysate, and lane ten is *B. vesicularis* lysate.
Figure 14. Western blot analysis of nine marine bacterial lysates probed with purified loggerhead IgY followed by KR-11 monoclonal antibody. Lane one is the molecular weight marker (Kaleidoscope, BioRad), lane two is *E. coli* lysate, lane three is *V. cholerae* lysate, lane four is *V. parahaemolyticus* lysate, lane five is *V. anguillarum* lysate, lane six is *V. vulnificus* lysate, lane seven is *M. marinum* lysate, lane eight is *E. rhusiopathiae* lysate, lane nine is *S. agalactiae* lysate, and lane ten is *B. vesicularis* lysate.
Figure 15. Western blot analysis of nine marine bacterial lysates probed with purified Kemp’s ridley IgY followed by LH 2-6 monoclonal antibody. Lane one is the molecular weight marker (Kaleidoscope, BioRad), lane two is *E. coli* lysate, lane three is *V. cholerae* lysate, lane four is *V. parahaemolyticus* lysate, lane five is *V. anguillarum* lysate, lane six is *V. vulnificus* lysate, lane seven is *M. marinum* lysate, lane eight is *E. rhusiopathiae* lysate, lane nine is *S. agalactiae* lysate, and lane ten is *B. vesicularis* lysate.
Figure 16. Western blot analysis of nine marine bacterial lysates probed with purified Kemp’s ridley IgY followed by KR-11 monoclonal antibody. Lane one is the molecular weight marker (Kaleidoscope, BioRad), lane two is *E. coli* lysate, lane three is *V. cholerae* lysate, lane four is *V. parahaemolyticus* lysate, lane five is *V. anguillarum* lysate, lane six is *V. vulnificus* lysate, lane seven is *M. marinum* lysate, lane eight is *E. rhusiopathiae* lysate, lane nine is *S. agalactiae* lysate, and lane ten is *B. vesicularis* lysate.
Figure 17. Overall enzyme-linked immunosorbent assay (ELISA) serum titer results for both Loggerhead and Kemp’s ridley sea turtles for the nine studied marine bacteria. Upon statistical analysis of the results, no significant differences were found for any species in any year.
Figure 18. Loggerhead sea turtle-specific ELISA results against the nine marine bacteria, broken down into categories of gender and year. Significant differences were seen for *V. vulnificus*, *V. parahaemolyticus*, and *E. coli*. * represents significance of p<0.05; ** represents significance of p<0.01.
Figure 19. Kemp’s ridley sea turtle-specific ELISA results against the nine marine bacteria, broken down into gender and year. No significant differences were seen between genders or year of collection for any of the bacterial species that were examined in this study.
Figure 20. The relationship between straight carapace length and serum titer levels against *E. coli* from Kemp’s ridley female turtles in 2011.

\[ y = 0.0439x + 1.6609 \]

\[ R^2 = 0.5106 \]
Figure 21. The relationship between straight carapace length and serum titer levels against *V. anguillarum* from Kemp’s ridley female turtles in 2013.

\[ y = -0.0394x + 4.074 \]

\[ R^2 = 0.8654 \]
Figure 22. The relationship between straight carapace length and serum titer levels against *V. anguillarum* from loggerhead male turtles in 2011.
Figure 23. The relationship between straight carapace length and serum titer levels against *E. coli* from loggerhead male turtles in 2012.

$y = 0.023x + 0.9489$

$R^2 = 0.6343$
Figure 24. The relationship between straight carapace length and serum titer levels against *E. rhusiopathiae* from loggerhead male turtles in 2012.
Figure 25. The relationship between straight carapace length and serum titer levels against \textit{V. parahaemolyticus} from loggerhead male turtles in 2012.

\[ y = -0.0525x + 5.5545 \]

\[ R^2 = 0.6906 \]
Figure 26. The relationship between straight carapace length and serum titer levels against *V. vulnificus* from loggerhead male turtles in 2012.

\[ y = -0.0486x + 5.7122 \]

\[ R^2 = 0.7896 \]
Discussion

Upon examination of western blots with bacterial lysates that were probed with whole serum from either turtle species versus purified IgY from either turtle species, there were no striking differences in banding patterns. This suggests that IgY in the serum that is binding to the lysates, and the other sea turtle immunoglobulin (IgM), is probably not binding as much. This finding is useful because it means that in clinical or laboratory settings, veterinarians and/or researchers can examine prior bacterial exposure in sea turtles without necessarily needing purified IgY to probe the Western blots. Using serum for this purpose would be more cost efficient and could potentially lead to receiving results faster (due to not having to go through the process of purifying IgY). In addition, leftover serum from sea turtles that has been properly stored can be tested with relative ease, and this data can be added to existing data from that individual.

The blots that were probed with either LH 2-6 or KR-11 showed overall weaker binding patterns than the blots probed with polyclonal antisera from either of the sea turtle species. However, there was one notable exception: the band seen at approximately 50 kDa in *B. vesicularis*. Very little is still known about the exact functionality and purpose of IgY(ΔFc), but results from these enzyme-linked immunosorbent assay suggest that perhaps this truncated form of IgY does not play a large role in the immune response in sea turtles toward bacterial infections. However, there is still much to be learned about this unique immunoglobulin, and further studies are needed in order to elucidate its exact function and purpose in the immune system of sea turtles. Additional research into
IgY(ΔFc) can possibly serve to help examine evolutionary relationships between species, as not all animals with IgY also have this truncated form.

An interesting finding for both the LH 2-6 and KR11 monoclonal antibodies, is that they showed the same binding patterns between the two species, confirming the results from previous experiments from our laboratory that these antibodies cross react between these two sea turtle species. Again, this is likely due to the fact that loggerhead turtles are closely related to the ridley turtles (both Kemp’s and olive) (Brown, 2002; Dutton et al., 1996; Naro-Maciel et al., 2008). However, a previous study that produced monoclonal antibodies against these immunoglobulins in green sea turtles found that the monoclonal antibody produced against 5.7S was species specific (no cross reactivity), but that the light chain monoclonal antibody that was produced cross reacted with loggerhead, hawksbill, Kemp’s ridley, and olive ridley turtles, but did not cross react with leatherback sea turtles (Herbst and Klein, 1995).

It is possible that light chain-specific antibodies are not useful because the light chain is often concealed near the contact point between the IgY molecule and pathogen (its epitope), therefore, either a polyclonal antibody against whole IgY, or a monoclonal antibody against the heavy chain is preferred since this portion of the IgY molecule is more freely available for detection. Whether the truncated form of IgY is functional in some capacity for certain pathogens, and thereby selected for in evolution, remains unclear. Further studies into this truncated form in species that contain it are necessary to help answer these basic, fundamental questions.
Despite the fact that there was no significant difference in quantity of serum titers against bacteria when comparing sea turtle species and year of serum collection, some significant differences were seen in loggerhead sea turtle serum titers when examining year of serum collection and gender. A statistically significant increase (p<0.01) of serum titers against *V. vulnificus* was found in the serum of loggerhead males and females collected in 2013 in comparison with 2011 and 2012 serum. In addition, there was a statistically significant increase (p<0.05) of serum titers against *V. parahaemolyticus* when examining serum from male and female loggerhead turtles in 2013 versus their 2011 female counterparts. Finally, a statistically significant increase (p<0.05) was observed in serum titers for *E. coli* in loggerhead female serum collected in 2013 versus loggerhead female serum collected in 2012.

Originally, it was thought that these results could possibly be attributed to 2013 having a higher global sea surface temperature in the months of June, July, and August versus those months in 2012 and 2011 (National Oceanic and Atmospheric Administration, 2016). However, none of these same patterns were observed when examining the Kemp’s ridley turtle data, indicating that rising sea surfaces temperatures are likely not the sole cause of the increase in titers for several bacterial species in 2013.

We hypothesized that other factors, such as life stage, could possibly be a driving factor behind the observed differences in serum titer levels. Therefore, we explored straight carapace length (SCL) in individual turtles against their titers for all bacteria species. Straight carapace length is an important factor to explore because there is evidence to suggest that certain diseases of sea turtles, such as fibropapillomatosis,
impacts turtles in certain size ranges more than others (Herbst, 1994). Though some
correlations between straight carapace length and levels of serum titers were seen, these
results should be interpreted with caution due to small sample sizes. However, it is
interesting to note that some correlations between size and serum titer levels were
positive, while others showed a negative relationship. Again, this may be a result of
differential habitat use at various life stages in sea turtles.
Chapter 4: Determination of blood and serum health parameters

Abstract:

In order to determine whether or not the previous serum titer results from both turtle species may correspond to healthy, wild turtle populations, packed cell volume, glucose levels, and total protein amounts were determined for each individual turtle. This data was compared to previously existing data on these parameters in both healthy, wild loggerhead and healthy, wild Kemp’s ridley turtles, and no difference was found. This implies that the turtles from our samples are indeed representative of healthy, wild populations of turtles. Furthermore, lysozyme activity was measured for each individual turtle, but no significant differences were found in terms of gender or sampling year for loggerhead sea turtles, though some significance was seen for Kemp’s ridley sea turtles. Finally, parameters such as total protein, lysozyme activity, and straight carapace length were examined to determine any relationships with serum titers, and some correlations were seen for total protein and serum titer levels.

Introduction:

Lysozymes are enzymes that lyse bacteria by hydrolyzing their cell wall, and have varying degrees of success depending upon their host and which bacteria they come into contact with (Araki et al., 1998; Gayen et al., 1977; Ingram and Molyneux, 1983; Thammasirirak et al., 2006). Lysozyme activity has been found to be significantly and
negatively correlated with both concentrations of 4,4’-dichlorodiphenyl dichloroethylene and the sum of chlordanes in whole blood in loggerhead sea turtles (Keller et al., 2006). However, lysozyme activity has been significantly and positively correlated with concentrations of mercury (Hg) in the blood of loggerhead turtles in samples from 2001, but not samples from 2003 (Day et al., 2007). In addition, lysozyme activity has been significantly and positively correlated with loggerhead sea turtles that were rescued upon showing symptoms of brevetoxicosis versus healthy loggerhead turtles in captivity (Walsh et al., 2010).

It is important to determine the blood and serum chemistry values for packed cell volume (PCV), glucose, and total protein, because these results can assist in indicating the health of wild sea turtle populations. In addition, these type of baseline values are useful when comparing the health of captive turtles with wild turtles in order to determine readiness for release of turtles who have been under veterinary care for illness or injury. The objectives for this study were to 1. Determine: serum lysozyme activity for each individual sea turtle, and to compare glucose levels, hematocrit/packed cell volume (PCV), and total serum protein with levels from healthy, wild sea turtles.
Materials and Methods:

*Determinations of serum lysozyme activity, and comparison of glucose levels, hematocrit/packed cell volume (PCV), and total serum protein with previously found values for healthy, wild loggerhead and Kemp’s ridley sea turtles.*

To quantify lysozyme activity, 1 mg/mL of hen egg white lysozyme was prepared in 0.1 M pH 5.9 phosphate buffer in accordance with the methods of Walsh, Leggett, Carter, & Colle, 2010. *Micrococcus lysodeikticus* solutions were made just before use with 50 mg of lyophilized cells in 100 mL of the pH 5.9 0.1 M phosphate buffer. A standard curve was generated by serially diluting the hen egg white lysozyme to achieve concentrations from 0-40 µg/mL and then adding 25 µL of the dilution per well in triplicate in a 96 well plate. For each plasma sample (30 from Kemp’s ridley and 60 from loggerhead), 25 µL of plasma was added in quadruplicate to a 96 well plate, and then 175 µL of *M. lysodeikticus* was added to three of the plasma wells and all of the standard curve wells. Absorbance was measured at 450 nm immediately and then again after a 5 minute room temperature incubation. The absorbance at 5 minutes was subtracted from the original absorbance to quantify change in absorbance. In addition, the absorbance from the sample well without *M. lysodeikticus* was subtracted from the average absorbance of the other three wells, and then this value was converted into hen egg white lysozyme concentration via linear regression using the generated standard curve. Averages were taken for each category (which was composed of species, gender and year
of serum collection, for example: loggerhead females from 2011). These averages were then compared to one another by analysis of variance (ANOVA) with Bonferroni’s multiple contrast post-hoc tests. GraphPad Prism’s statistical software was used for this analysis, and the α value was set at 0.05 prior to the experiment (Fisher, 1925).

Glucose levels were determined for wild-caught turtles from the South Carolina Department of Natural Resources by using a handheld glucose meter. These levels were then analyzed to determine whether they vary significantly with total carapace length (turtle size) and if there is a correlation between bacterial levels from ELISAs and glucose levels. In addition, hematocrit/packed cell volume (PCV) and total serum protein were analyzed against bacterial levels and total carapace length. Straight carapace length was also measured for each individual turtle by the South Carolina Department of Natural Resources by using a pair of calipers. Packed cell volume (PCV) was also determined by the South Carolina Department of Natural Resources by using hematocrit tubes and a centrifuge. Finally, total protein was determined by the South Carolina Department of Natural Resources by using a refractometer.

**Results:**

There were no statistically significant differences observed in loggerhead sea turtle serum lysozyme activity when comparing year of sample collection and turtle gender. However, in Kemp’s ridley sea turtles, there was a significant difference in
serum lysozyme activity between females collected in 2012, and those collected in 2013, but no other significance was observed for the Kemp’s ridley sea turtle data.

Strong correlations ($R^2=0.6693$) were seen between total protein levels and titer levels against *E. coli* in serum collected from all Kemp’s ridley male turtles (including 2011, 2012, and 2013). In addition, a strong correlation ($R^2=0.6242$) was found between total protein levels and titers against *M. marinum* in serum collected from all Kemp’s ridley turtles sampled from 2011-2013.

Due to the fact that other blood chemistry levels (such as glucose, packed cell volume, and total protein) from both loggerhead and Kemp’s ridley sea turtles used in this study are comparable to those observed in other studies (*Table 1*) the lysozyme results and correlations between total protein levels and levels of some serum titers are likely representative of healthy, wild sea turtles from these populations.
Loggerhead Lysozyme Activity

Figure 27. Loggerhead sea turtle average lysozyme activity comparison between genders and year of serum collection. No statistically significant differences were detected.
Figure 28. Kemp’s ridley sea turtle average lysozyme activity comparison between genders and year of serum collection. Statistical significant at the p<0.05 level was detected between 2012 Kemp’s ridley female turtles and 2013 Kemp’s ridley female turtles.
Table 1. Comparison of Blood Chemistry Values from Loggerhead and Kemp’s Ridley Sea Turtles (Chesapeake Bay data from (George, 1997); New York Bight data from (Carminati et al., 1994)).

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Sample size</th>
<th>Packed cell volume (%)</th>
<th>Glucose (mg/dl)</th>
<th>Total protein (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chesapeake Bay</td>
<td>50</td>
<td>29</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Southeastern USA</td>
<td>60</td>
<td>33.5</td>
<td>115</td>
<td>3.98</td>
</tr>
<tr>
<td></td>
<td>New York Bight</td>
<td>60</td>
<td>31.1</td>
<td>115</td>
<td>0.992</td>
</tr>
<tr>
<td></td>
<td>Southeastern USA</td>
<td>30</td>
<td>32.4</td>
<td>153</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Table 1. Comparison of Blood Chemistry Values from Loggerhead and Kemp’s Ridley Sea Turtles (Chesapeake Bay data from (George, 1997); New York Bight data from (Carminati et al., 1994)).
Figure 29. The relationship between total protein and serum titer levels against *E. coli* from Kemp’s ridley male turtles from 2011, 2012, and 2013.

\[ y = 2.2307x - 5.5258 \]

\[ R^2 = 0.6693 \]
Figure 30. The relationship between total protein and serum titer levels against *M. marinum* from Kemp’s ridley male turtles from 2011, 2012, and 2013.

\[ y = 2.7475x - 6.195 \]
\[ R^2 = 0.6242 \]
Discussion

As seen in Table 1, the means and standard deviations for packed cell volume, glucose, and total protein, were similar to values for these parameters that have previously been shown for both species. The external data were taken in healthy, wild, juvenile loggerhead and Kemp’s ridley sea turtles, which reflects the subjects of this study. This means that all results from this study (including serum titer results, lysozyme activity measurements, etc.) are likely reflective of what is to be expected in populations of healthy, wild loggerhead and Kemp’s ridley turtles taken in and around these sampling sites during the sampling years of 2011, 2012, and 2013.

There were no significant differences between years or genders in terms of lysozyme activity for loggerhead sea turtles (Figure 27). Significant increased or decreases in lysozyme activity in groups of sea turtles could be associated with increased or decreased ability of those turtles to lyse bacteria, because lysozymes are a set of enzymes that are able to lyse and destroy bacteria by hydrolyzing their cell wall.

However, as seen in Figure 28, there was a statistically significant difference between Kemp’s ridley females from 2012 and Kemp’s ridley females from 2013. Although the titer levels from the nine bacteria that we examined were not statistically significant between these two groups, it is possible that 2013 Kemp’s ridley females were exposed to other bacterial species in larger quantities than their 2012 female counterparts.

There were some positive correlations between total protein levels and serum titers for Kemp’s ridley males sampled from all available years (the sample size for all males constituted seven individuals). As seen in Figures 29 and 30, positive correlations
were found when examining the relationship between total protein levels and serum titer levels against *E. coli* (R²=0.6693), as well as the relationship between total protein levels and serum titer levels against *M. marinum* (R²=0.6242).

These results are of interest because the only strong relationships that were seen between total protein levels and bacterial titers were in male Kemp’s ridley sea turtles. In order to further investigate this phenomenon, a larger number of samples encompassing a broader range of collection years need to be assessed to determine if this trend is unique to male Kemp’s ridley sea turtles with serum collections taken in 2011, 2012, and 2013, or if this is an overall trend relating to Kemp’s ridley sea turtles.
Chapter 5: Conclusions and Future Directions

Conclusions

Our studies show that Protein A/G columns are an effective way to purify IgY from serum. This method for purification results in a product containing the heavy chain of IgY around 68 kDa, the Fab’ fragment from IgY(ΔFc) around 37 kDa, and a light chain, which is likely from both IgY and IgY(ΔFc), at around 22 kDa. Therefore, the products from this purification method are able to be used to produce polyclonal antisera and monoclonal antibodies against any of these IgY portions.

The monoclonal antibody made against the loggerhead sea turtle light chain of IgY and/or IgY(ΔFc), LH 2-6, recognizes the light chain fragment in both loggerhead and Kemp’s ridley turtles. In addition, the monoclonal antibody produced against the Kemp’s ridley Fab’ fragment from IgY(ΔFc) recognizes this fragment in both Kemp’s ridley and loggerhead turtles. This is likely a result of the relatively close evolutionary relationship between these two turtle species that has been previously found in the literature (Naro-Maciel et al., 2008).

Western blot analysis revealed that there are certain bacteria which both species of sea turtle have produced antibodies against that are still present in the serum, such as: E. coli, V. parahaemolyticus, M. marinum, and S. agalactiae. However, loggerhead sea turtles showed strong binding against V. vulnificus, while Kemp’s ridley turtles did not share this same binding pattern, suggesting differential exposure of these two sea turtles to this particular bacteria species. The commonalities in the binding between the two
species could be due to their overlap in habitat range, while the differences could be due to the variable usage of this habitat between the species.

When Western blot analyses were performed on the bacterial lysates and the blots were probed with the KR-11 antibody, very weak binding patterns were present. This could indicate that the Fab’ portion plays little-to-no role in bacterial infection responses within the immune system, but more studies are needed to verify this idea.

In addition, some significant differences were seen in loggerhead sea turtles regarding serum titers when accounting for gender and year of serum collection. Differences between genders could be due to female turtles coming into contact with bacteria in the sand while they are nesting on the beaches, while differences between years of serum collections could be due to variable sea surface temperatures, and other biotic and abiotic factors. Habitat usage in sea turtles varies depending on the species and the life stage, therefore, this likely plays a large role in what bacteria sea turtles are being exposed to in the wild.

Our investigation into how size impacts serum titers against bacteria showed some interesting results: there were some strong correlations between turtles in various categories (when accounting for factors such as gender, size, and year of sampling) and titers levels against certain bacteria. However, these correlations varied: some were strongly positive (such as between straight carapace length and serum titer level against E. coli in Kemp’s ridley female turtles in 2011), while others were strongly negative (between straight carapace length and serum titer levels against V. anguillarum in
loggerhead male turtles in 2011). In addition to size, some titer level variation for some groups of sea turtles was able to be explained with total protein levels.

Future Directions

In order to compare results of monoclonal antibody production with that of a previous study, monoclonal antibody specificity should be tested to see if the monoclonal antibodies cross react with other sea turtles in the family Cheloniidae, and if they cross-react with leatherback sea turtles (Dermochelys coriacea) from the family Dermochelyidae (Herbst and Klein, 1995). The Herbst and Klein study produced monoclonal antibodies in green sea turtles for light chain IgY, heavy chain IgY, and the Fab’ portion (which is the heavy chain of IgY(ΔFc). They found that their monoclonal antibody against 5.7S IgY (the Fab’ fragment) did not cross-react with any other sea turtle species that they tested, but they did not test cross-reactivity against flatback turtles, which is relatively closely related to the green sea turtle (Herbst and Klein, 1995, Naro-Maciel et al., 2008). The green sea turtle monoclonal that they produced against 7S IgY (the heavy chain of the full form of IgY) cross-reacted with all other turtle species tested (Herbst and Klein, 1995). Finally, their light chain monoclonal antibody cross reacted with all sea turtle species from the family Cheloniidae (Herbst and Klein, 1995).

Our results may vary from the Herbst and Klein study because loggerhead and Kemp’s ridley sea turtles are closely related, which allows for a higher chance of cross-reactivity for monoclonal antibodies between the two species. It is possible that the
Herbst and Klein study did not find cross-reactivity for their 7S IgY antibody because they did not examine whether this antibody cross reacted with the close relative of the green sea turtle, the flatback sea turtle (Naro-Maciel et al., 2008).

Using the IgY purification technique that our work has established, leatherback sea turtle serum should be tested to see if it also contains both isoforms of IgY: as of this writing, this work has not yet been undertaken. From an evolutionary standpoint, this research is worthwhile, because it may help to further elucidate the evolutionary relationships between sea turtle species and other reptile species.

In order to verify the trends found between standard carapace length and serum titers, and standard carapace length and total protein, more samples are needed. Though the results received from this experiment provided for interesting discussion, in order to truly understand if a variable such as standard carapace length can have an impact on levels of serum titers or total protein in serum, more individual turtles (in particular, male turtles) would need to be analyzed in order to provide a more robust analysis of these potential relationships.

Researching historically lesser-studied sea turtle species (such as the ridley turtles, flatback turtles, and leatherback turtles) can shed light onto some important questions, such as: why are some turtle species prone to contracting certain diseases or prone to stranding, while others are not? Exploring these areas of research and continuing to ask and answer questions about sea turtle immune health is a vital step in protecting these important species from extinction.
Further research is also needed to determine the exact function of IgY(ΔFc). Our results suggest that the Fab’ portion of this IgY isoform does not play a strong role in helping the immune system of sea turtles fight off bacterial infections. It is possible that this particular immunoglobulin is better apt to assist the immune system with viral infections, but additional studies are crucial to determining whether or not this is the reality, and if IgY(ΔFc) behaves the same in all species where it is present.

Comparing serum parameters and serum titer quantities from healthy wild turtles, healthy captive turtles, and sick or stranded turtles would serve as an interesting indication of immune status for sea turtles. Results from this type of work could be used as an additional parameter to help determine if previously sick or stranded sea turtles are ready to leave rehabilitation facilities to be released back into the wild. In addition, this type of research would shed light onto what bacteria sea turtles commonly encounter in their environment, and how this changes with factors such as sea turtle age, nesting location (for female turtles), and how this changes during natural and unnatural events, such as El Niño years, oil spills, and future climate change scenarios.

Due to the poikilothermic nature of turtles, they have variable immune responses depending on seasonal temperature variations within their habitats (Leceta and Zapata, 1986; Zapata and Torroba, 1992; Zimmerman et al., 2010a). In future experiments, it will be important to determine the exact impacts that seasonal variations have on sea turtles specifically, because this can help researchers better understand the susceptibility of sea turtles to infections. Furthermore, this type of research can lend better understanding to how normal baseline immunological parameters for sea turtles will vary in accordance
with seasonal variations, and can also help to begin to unravel the potential impacts that climate change will have on the immune responses of sea turtles.
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