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The Influence of Environmental Health Indices on Antibody Responses Against *Streptococcus agalactiae* in Bottlenose Dolphins, *Tursiops truncatus*

Kursten A. Anderson
Clemson University, kuaanderson4@gmail.com

THE INFLUENCE OF ENVIRONMENTAL HEALTH INDICES ON ANTIBODY RESPONSES AGAINST *Streptococcus agalactiae* IN BOTTLENOSE DOLPHINS, *Tursiops truncatus*

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Kursten Alyse Anderson
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Accepted by:
Dr. Charles D. Rice, Committee Chair
Dr. Peter van den Hurk
Dr. Thomas R. Rainwater
Dr. Thomas E. Schwedler

Abstract

The common bottlenose dolphin, *Tursiops truncatus*, is one of the most recognized marine mammal species. These mammals now serve as a sentinel species to evaluate and monitor ecosystem health in critical coastal regions. For example, the Atlantic bottlenose dolphin health and environmental risk assessment project (HERA) has studied these animals over the past 20 years from the Charleston harbor in South Carolina (CH) and the Indian River Lagoon (IRL) in Florida, USA, as part of an overall approach to understanding links between Oceans and Human Health (OHH). Environmental pressures, assessed as a combination of pollution exposure, habitat quality, and exposure to infectious diseases, are now considered the primary driver for the health of dolphins. Specifically, emerging infectious diseases and zoonotic pathogens have become a serious and complex threat to humans, animals, and environmental health. *Streptococcus agalactiae* is an emerging environmental disease that can also affect immunocompromised humans. One means to assessing exposure to such diseases is by examining seroconversion or seropositivity. Serum antibody levels have seldom been used with environmental species because of the general lack of immunological reagents and the intense labor associated with these methods. In this study, monoclonal antibodies (mAb) were generated against bottlenose dolphin IgG, IgM in bull shark (*Carcharhinus leucas*) and spotted eagle ray (*Aetobatus narinari*), and an on-hand mAb against loggerhead sea turtle (*Caretta caretta*) IgY was used. Serum or plasma samples were collected from animals inhabiting the same location. These reagents were used to calculate relative antibody titers against *S. agalactiae* at a dilution of 1:200 and compare

these data to antibody activities calculated over a range of serum dilutions. This part of the study demonstrates that antibody activity data are more sensitive than simple relative titers when determining antibody responses to *S. agalactiae* in all four species and across the immunoglobulin spectrum (IgM, IgY, and IgG) of marine aquatic vertebrates. The generated monoclonal antibody against bottlenose dolphin IgG was used to compare relative titers to antibody activities in bottlenose dolphin serum samples collected over the past 20 years of the HERA project to compare Charleston Harbor animals to Indian River Lagoon animals. Responses in animals from the GA Aquarium and the managed marine mammal program (US Navy) were also examined. There were few differences between the groups in terms of relative antibody titers against *S. agalactiae*, but calculated antibody activity data demonstrate differences. Previously published studies from our lab demonstrate differences in antibody responses against several other highly pathogenic bacteria when comparing the dolphin groups over the years. Compared to those data, it appears that bottlenose dolphins are not currently burdened by *S. agalactiae*, and the managed US Navy and GA Aquarium populations are very healthy.

Dedication

To my amazing partner, family, and friends who have

been with me on this journey. Thank you

for your endless love and support.

Acknowledgments

I am extremely grateful to my advisor Dr. Charles Rice for being such an amazing mentor throughout my journey at Clemson. I am truly grateful for Dr. Rice's support throughout many of the crazy, emotional, and hectic moments during my time at Clemson. Thank you, Dr. Rice, for your encouragement and mentorship to help mold me into the scientist I am today. I have learned so much from being a part of your lab, and for that, I am truly grateful. I would also like to extend my gratitude and appreciation to my committee members. Thank you for your flexibility when it came to my project, your knowledge and expertise, and for serving on my committee.

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Chapter One

Literature Review

1.1 *Tursiops truncatus background*

The common bottlenose dolphin (*Tursiops truncatus*), a member of the Order *Cetacea,* is one of the best-known and most recognized marine mammal species (Vollmer and Rosel, 2013). In the United States, they are not endangered or threatened but protected under the Marine Mammal Protection Act (National Marine Fisheries Service, 2021b). Two distinct ecotypes have been identified; an offshore, deep, cold-water ecotype and a coastal, warm, shallow ecotype (Mead and Potter, 1995). The bottlenose dolphin is widely distributed and can be found in temperate to tropical regions. They can be found on the coasts of oceanic islands and all the major continents (Reynolds, 2000).

Bottlenose dolphin sexual maturity varies from population-to-population with previous studies estimating that males mature around 7 to 17 years of age and females mature between 5 to 14 years of age. Females typically have a 12-month gestation period followed by an 18-month lactation period for captive dolphins. Dolphin calves usually will stay with their mothers for about 3 to 6 years. Their diet varies by habitat (Gannon and Waples, 2004) and feeds on prey such as crustaceans, squid, and fish (National Marine Fisheries Service, 2021a). They are of economic importance for many coastal communities providing millions of dollars each year from the whale-watching industry (Parsons et al., 2015). Also, they are a significant contributor to the employment and income in coastal towns (Parsons et al., 2003).

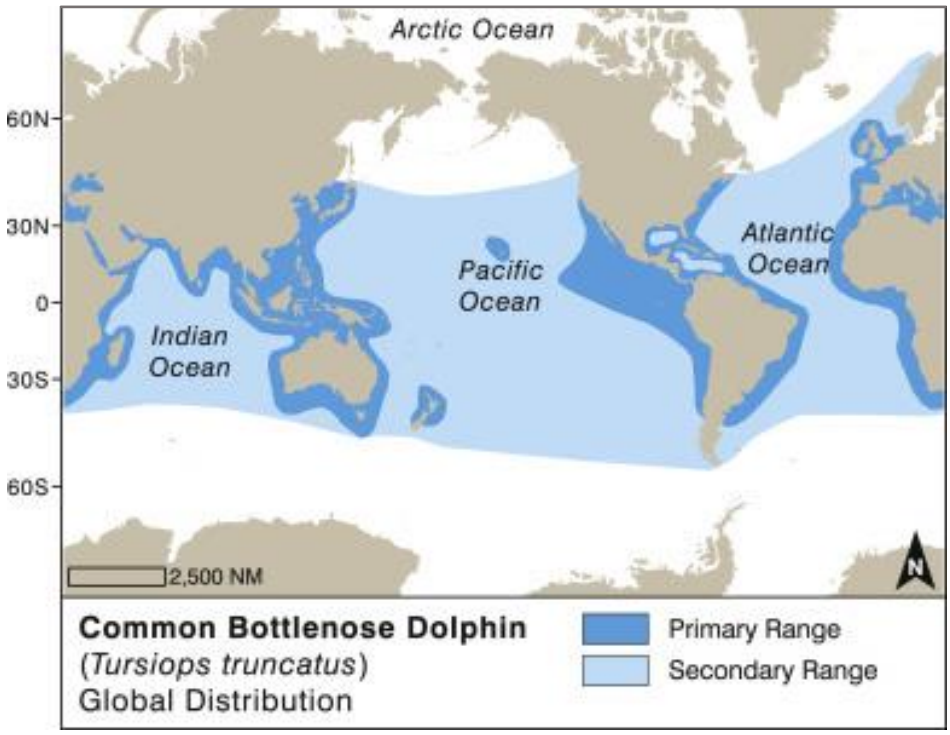

Figure 1.1. The geographical range of the common bottlenose dolphin (*Tursiops truncatus)* (International Whaling Commission, 2021).

1.2 Bottlenose Dolphin Threats

Bottlenose dolphins encounter many anthropogenic threats, pollutants and pathogens, underwater noise, and collisions with shipping vessels and boats (Fair and Becker, 2000). Pathogens are ever-present in the marine environment, and there are many different emerging viral, bacterial, fungal, and protozoal diseases that these organisms face every day.

Harmful algal blooms (HABs) have been associated with mass mortality events of dolphins, in addition to many other marine mammals (Flewelling et al., 2005; Geraci et al., 1989; Jessup et al., 2007). HABs produce harmful neurotoxins and can produce a wide range of biotoxins, including saxitoxins, brevetoxins, domoic acid, and okadaic acid. These toxins cause paralytic, neurotoxic, amnesic, and diarrhetic shellfish poisoning in humans, respectively (Landsberg, 2002). These HABs continue to be problematic worldwide and pose a significant threat to human and ecosystem health (Geraci et al., 1989)

Anthropogenic toxicants have been demonstrated to be an unrelenting issue for the marine environment. Bottlenose dolphins are exposed to many different persistent inorganic and organo-halogen toxicants that can bioaccumulate in the marine environment. The ability of these toxicants to bioaccumulate results in high tissue concentrations in bottlenose dolphins, amongst many other top marine predators (Bossart, 2011). Bottlenose dolphins have large fat stores, which allows for lipophilic toxicants to accumulate (Fair et al., 2010) and later become mobilized during periods of lactation, starvation, and fasting (Tanabe, 2002). High levels of contaminants have been

documented in marine mammals that are from coastal regions with dense human populations and increased amounts of industrial and agricultural activities (Aguilar et al., 2002; Fair et al., 2010; Houde et al., 2005). Some of the toxicants that have been documented in bottlenose dolphins include polychlorinated biphenyls, which are used in a variety of industrial and commercial purposes (Fair et al., 2010; Houde et al., 2006), perfluorinated chemicals used in water, strain, and grease repellants (Fair et al., 2010), and polybrominated diphenyl ethers commonly used in flame retardant (Fair et al., 2010; Fair et al., 2007). Persistent organohalogen toxicants have been linked to immunosuppression (Mori et al., 2008), increased susceptibility to infectious diseases (Ross, 2002), neoplasia (Newman and Smith, 2006), endocrine disruption (Porte et al., 2006), and reproductive impairment (Addison, 2011).

Brucellosis is a zoonotic disease caused by *Brucella ceti* bacteria that has caused issues for marine mammal species. Brucellosis infections have a high prevalence and are globally widespread amongst marine mammal species, as made evident by serological evidence. Marine mammal strains of brucellosis are also capable of infecting humans and livestock. Bottlenose dolphins have been noted to have *Brucella* placentitis and abortions due to infection (Miller et al., 1999).

Lobomycosis (lacaziosis) is a chronic granulomatous disease of the skin and subcutaneous tissues that occurs only been documented in dolphins and humans (Rodríguez-Toro, 1993). This disease is caused by a yeast-like fungus (*Lacazia loboi*) and has an expansive presence, with cases being reported in dolphins in the Surinam River estuary and off the coasts of North Carolina, Florida, Texas (Cowan, 1993),

southern Brazil (Simõse-Lopes et al., 1993), and between the Spain and French coasts (Symmers, 1983). In bottlenose dolphins, aggregations of white, firm raised nodules were found on the edges of pectoral and dorsal fins, fluke and caudal peduncle, and the head. Dolphins with lobomycosis have demonstrated severe diminishing of their adaptive immune response (Reif et al., 2009).

Over the past couple of decades, the cetacean morbillivirus has emerged and greatly affected the population and health of many cetacean species on a global scale (Van Bressem et al., 2014). This virus is highly contagious and lymphotropic (Shimizu et al., 2013) and has caused several mortality events in coastal *T. truncatus* populations from the Gulf of Mexico to the Atlantic coast of the United States since 1982 (Krafft et al., 1995; Taubenberger et al., 1996). Transmission of the morbillivirus is thought to occur after the inhalation of airborne virus shed by cetacean individuals, which is probable for cetaceans due to their sociable behavior and high density (Raga et al., 2008; Van Bressem et al., 1999). The pathogenesis of morbillivirus infections often leads to immunosuppression, leading to secondary viral and bacterial infections (Appel et al., 1982).

The marine environment is abundant in a wide variety of pathogens, and as previously mentioned, immunosuppression from other infections can lead to secondary bacterial infections. Previous research has found that bottlenose dolphin individuals have been exposed and have titers against many different common marine bacteria (Beck and Rice, 2003; Bossart et al., 2008; Bossart et al., 2019; Fair et al., 2017). These bacteria included *Erysipelothrix rhusiopathiae, Escherichia coli, Mycobacterium marinum, Vibrio*

carchariae, Vibrio cholerae, Vibrio parahemolyticus, and *Vibrio vulnificus*. Previous studies have reported immune responses in relative titers at a 1:200 dilution (Bossart et al., 2019) and antibody activity (units/µL) (Beck and Rice, 2003). Research on marine pathogens has yet to include the quantification of immune responses to *Streptococcus agalactiae* in bottlenose dolphins. Due to its global presence (Anshuman et al., 2018; Numberger et al., 2021), it could be a concern for bottlenose dolphins and other marine organisms in the future.

1.3 Bottlenose Dolphins and *Streptococcus agalactiae*

Streptococcus agalactiae (group B streptococcus) is a Gram-positive pathogen and has been of concern for terrestrial animals and farmed fishes. In humans and cows, it is known to cause neonatal meningitis and mastitis, respectively (Amborski et al., 1983; Baker, 1997). *S. agalactiae* has been observed to cause septicemia, necrotizing fasciitis, and large mortality events in a variety of aquatic organisms. This bacterium has been known to affect crocodiles, frogs, and an array of fish species (Amborski et al., 1983; Bishop et al., 2007). Both wild and farmed fishes have been affected by *Streptococcus,* some of which include menhaden (*Brevo ortiapatronus*), giant Queensland Grouper (*Epinephelus lanceolatus*), tilapia *(Oreochromis niloticus),* and mullet *(Liza klunzingeri)* (Bowater et al., 2012; Evans et al., 2002; Plumb et al., 1974).

Due to the ability to infect both aquatic species and terrestrial mammals, it was thought that this bacterium could also infect marine mammals (Evans et al., 2006). This was confirmed with the first known characterizations of *S. agalactiae* in marine mammal

species in the early 2000s when it was isolated from both captive and wild bottlenose dolphins (Evans et al., 2006; Zappulli et al., 2005). The captive bottlenose dolphin was diagnosed with necrotizing fasciitis, cellulitis, and myositis due to *S. agalactiae* (Zappulli et al., 2005). In the case of the wild dolphin, *S. agalactiae* was isolated from the muscle. However, no definitive link was made due to legal and ethical issues involved in marine mammal research (Evans et al., 2006). Due to its ability to adapt and survive in a wide variety of environments, more events of *Streptococcus agalactiae* are likely to occur in the aquatic environment as the main route of exposure in streptococci is from the ingestion of streptococcal contaminated materials (Bonar and Wagner, 2003; Bromage and Owens, 2002). In addition, future outbreaks in fishes could increase the transmission potential between species living in the same environment (Bromage and Owens, 2002). These outbreaks could have implications for humans and terrestrial and aquatic species as some studies have shown the disease associated with this pathogen may be caused by the same strains of *S. agalactiae* (Evans et al., 2008; Evans et al., 2009).

1.4 Bottlenose Dolphin Immune System

The immune system is a vital component of any organism allowing for protection from pathogens. Responses to those pathogens are simple physiological reactions present in all living organisms (Buchmann, 2014). The vertebrate immune system is comprised of innate and adaptive immune systems. The innate immune system responds quickly, whereas the adaptive immune system may take days or weeks to become completely activated. The adaptive immune system requires previous exposure to the antigen to have

a full immunological response in which cell-mediated and humoral immune responses are utilized (Coico and Sunshine, 2015)

Dolphin primary and secondary lymphoid organs demonstrate similarities to those of other terrestrial mammals (Beineke et al., 2006). Lymphoid tissues in bottlenose dolphins include the thymus, spleen, mucosal-associated lymphoid tissue (MALT), bone marrow, and lymph nodes (Cowan and Smith, 1999). Some lymphoid structures are unique in cetaceans, such as lymphoepithelial aggregations in the larynx and anal canal (Beineke et al., 2010). Primary lymphoid tissues consist of the bone marrow and the thymus. The bone marrow serves as the location of hematopoiesis. B cell maturation occurs in the fetal liver and switches to the bone marrow upon adulthood. T cells develop initially in the bone marrow and migrate to the thymus to fully mature (Boehm et al., 2012).

Secondary lymphoid tissues include the spleen, lymph node, and mucosal-associated tissues (Stebegg et al., 2018). These tissues are purposefully located to trap foreign antigens that enter the bloodstream, peripheral tissues, and mucosal sites (Randall and Mebius, 2014). The spleen contains both red and white pulp regions with a marginal zone to facilitate the movement of cells (Cowan and Smith, 1999). The spleen plays a role in the immune response to bloodborne pathogens (Haley, 2017). The lymph nodes are responsible for pathogens that enter through the skin barrier or other internal surfaces (gut wall, lung, tonsils). In cetaceans, the lymph nodes typically lack germinal centers (Romano et al., 1993). MALT can catch and concentrate antigens which then interact with the lymphocytes (Coico, 2015). They are responsible for pathogens that enter

through mucosal surfaces (respiratory, gastrointestinal, and urogenital tracts). Secondary lymphoid tissues facilitate the interactions between dendritic cells that have met their antigen, B cells, and T cells to initiate the adaptive immune response (Goodnow, 1997).

1.5 Bottlenose Dolphin Innate Immunity

Innate immunity provides a nonspecific response to pathogens and is present in almost all animals to survive in their environment. The innate immune system provides the first line of defense against pathogens and consists of physical barriers, lysozymes, antimicrobial peptides (AMPs), the complement system, and nonspecific leukocytes (Riera Romo et al., 2016; Zimmerman et al., 2010). Physical barriers of the innate immune system include the skin and gut mucosa, which prevent pathogen entry into the organism (Riera Romo et al., 2016). Lysozymes are antimicrobial enzymes that hydrolyze bacterial cell walls of both gram-negative and gram-positive bacteria resulting in lysis of bacteria (Callewaert and Michiels, 2010). Lysozymes have been detected in the tears, nasal mucus, saliva, blood plasm, and other tissues and secretions in mammals (Salton, 1957). Studies in marine mammals have used lysozyme activity as a marker of proinflammatory responses (Fair et al., 2017; Reif et al., 2009). AMPs are highly conserved in vertebrates and have antifungal, antiviral, and antibacterial activity through disruption and destabilizing of microbial membranes, pore formation, inhibition of intracellular targets, interference in the transcription of DNA, and inhibition of protein synthesis and folding (Riera Romo et al., 2016; Zasloff, 2002). Furthermore, AMPs can link the innate and adaptive immune response through the recruitment or activation of

immune cells: dendritic cells, T cells, and monocytes (Yang et al., 2000). These peptides can be secreted in areas that are prone to microbial infections, such as oral mucosa, gastrointestinal tract, lungs, eyes, skin, and the reproductive tract (Bals, 2000).

Other key players in innate immunity are pattern recognition receptors (PRRs) and Pathogen-Associated Molecular Patterns (PAMPs). The activation of innate defense mechanisms is reliant on PAMPs present on invading microbes. PAMPs are also vital to microbes for their survival and pathogenicity (Kumar et al., 2011). These structures are expressed and conserved in many pathogens, including bacteria and fungi (Biegańska, 2014). To identify PAMPs, PRRs are used on cells such as macrophages, dendritic cells, monocytes, and endothelial cells (Blanco and Garcia, 2008). Once the PAMPs are recognized by the PRR, the innate immune cell will become activated to destroy and clear the pathogen. Clearance can be accomplished by phagocytosis to destroy the pathogen, production of various cytokines, and activation of the adaptive immune response through antigen presentation accompanied by cytokine stimulation (Smith et al., 2019).

The complement system has been identified as the first supportive line of defense against microbial invaders and consists of serum proteins that react against pathogens through a molecular cascade. This system has three pathways that can be activated: the classical, alternative, and lectin pathways. This cascade results in opsonization, enhanced inflammatory responses, and the formation of the membrane attack complex, ultimately resulting in microbial lysis (Abbas et al., 2014; Dunkelberger and Song, 2010; Zimmerman et al., 2010). In mammals, the complement system comprises approximately

30 proteins that make up the three pathways (Smith et al., 2019). The classical pathway is activated by antibody-antigen complexes and serves as a bridge between the innate and adaptive immune systems. The alternative pathway is directly activated by pathogens and independent of antibodies. Lastly, the lectin pathway is activated by mannose-binding lectin residues on the pathogen surface (Coico, 2015; Smith et al., 2019). C3 convertase activation is induced in all three pathways, which cleaves inactive C3 into C3a and C3b, forming the membrane attack complex (Dunkelberger and Song, 2010).

Acute-phase proteins (APP) are involved in the acute-phase response (APR), which is induced by infection, trauma, or inflammation to the host (Johnson, 1997). This response is induced by proteins called cytokines which act as messengers between the local injury site and the hepatocytes that make APPs. Studies in bottlenose dolphins have utilized APPs as indicators of baseline health and to further understand immune responses in these organisms (Goldstein et al., 2006). In mammals, the main APPs are C-reactive protein (CRP), serum amyloid A and P, a2-macroglobulin, fibrinogen, and transferrin, amongst a few others (Cray et al., 2009). The majority of research has been conducted on CRP. CRP is a highly conserved serum protein (Sproston and Ashworth, 2018). During injury, infection, and inflammation, CRP levels increase dramatically in response to the damage or pathogen entry (Sproston and Ashworth, 2018). CRP is important for protection and defense from bacteria and aids in the induction of apoptosis (Sproston and Ashworth, 2018).

Major cell types that play a role in innate immunity are phagocytes (monocytes, macrophages, neutrophils, and dendritic cells), natural killer cells, epithelial cells, and

endothelial cells (Chaplin, 2010). Neutrophils, monocytes, and macrophages phagocytose pathogens and particles that have been tagged to be cleared by binding Ig or complement. These phagocytic cells release cytokines and serve as antigen-presenting cells (APCs), which present antigens to T cells connecting the innate and adaptive immune responses (Coico and Sunshine, 2015). Neutrophils are considered the main effector cell of the innate immune response and are the first cell type to respond to the site of infection (Kaiser, 2010). Natural killer cells (NK cells) assist in killing virally infected cells and cause the infected cell to undergo apoptosis (Vivier et al., 2011). Epithelial and endothelial cells express PRRs on their cell surface to recognize PAMPs from pathogens to secrete proinflammatory cytokines and release antimicrobial peptides (Nakanaga et al., 2007)

1.6 Bottlenose Dolphin Adaptive Immunity

Depending on the route of immune response induction, adaptive immunity can be further divided into cell-mediated or humoral immunity. The adaptive immune response has great specificity for target antigens (Yuan et al., 2014). These responses are based on the antigen-specific receptors on the surfaces of B- and T-lymphocytes (Chaplin, 2010). The cell-mediated immune system is centered on restricted antigen presentation to the T cell receptor (TCR) on T cells via the major histocompatibility complex (MHC), which assists the T cell in differentiating the self-component and microbial structures (Chaplin, 2010; Yuan et al., 2014). T cells play a role in antibody regulation but do not produce antibodies (Zimmerman et al., 2010). T cells are important in the identification and

destruction of infected host cells (Chaplin, 2010). The humoral immune system is mediated by the secretion of immunoglobulins (Igs) by B cells. B cells can recognize pathogens and can secrete highly specific antibodies to that pathogen when bound to an antigen.

Mammals produce five different classes of Igs: IgM, IgD, IgA, IgG, and IgE. Igs are formed by the combination of two identical heavy chains and two identical light chains held together by disulfide bonds. The heavy and light chains contain a constant region and variable region. The variable region of the heavy and light chains forms the antigen binding site (Nicholson, 2016; Zimmerman et al., 2010). The Ig heavy chain and T cell repertoire are generated by variable, diversity, and joining (VDJ) recombination events (Yuan et al., 2014). The light chain is formed through V and D gene rearrangement. This process provides a great deal of diversity amongst receptors and Igs (Collins and Watson, 2018). IgM is found in all jawed vertebrates, produced in response to gram-negative bacteria, and is very effective at activating complement (Coico, 2015). Before a B cell meets an antigen, IgD is co-expressed on the cell surface with IgM and serves as a transmembrane antigen receptor (Chen and Cerutti, 2011).

IgA in mammals is found in saliva, mucus, and gastric fluid secretions, which help protect against respiratory and gastrointestinal infections. IgG is the major antibody that aids in protectiion and immunity against bacterial or viral infections from a secondary immune response (Zimmerman et al., 2010). Lastly, IgE serves as the first line of defense against parasites and mediates allergic reactions that include systemic and localized anaphylaxis (Burton and Oettgen, 2011; Wu and Zarrin, 2014).

1.7 Role as Sentinel Species

Marine sentinel species provide a way to evaluate the aquatic ecosystem's health. Utilizing marine organisms provides early warnings about current or potential negative impacts on animal health on the individual and population level. These warnings allow for the characterization and management of potential oceanic impacts that would affect human and organismal health (Bossart, 2011). Bottlenose dolphins have inshore (coastal and estuarine) and offshore populations allowing the data collected to compare these geographical locations. Inshore populations are often near and around areas with human activities and anthropogenic pollutants (Reif et al., 2015; Wells et al., 2004). Bottlenose dolphins can serve as great sentinel species due to their long life spans, long-term coastal residencies, occupation of a high trophic level, and large blubber stores that can serve as sinks for anthropogenic toxicants (Bossart, 2011; Moore, 2008; Wells et al., 2004). This can result in biomagnification, and often toxicants are found in high concentrations in these organisms. In addition, marine mammals are sociable, charismatic megafauna that stimulate a positive response in humans making humans more likely to be attentive to ocean health matters (Bossart, 2011). Lastly, these organisms have been subject to health assessments over the past decades that included behavioral, physiological, immunological, and biochemical analyses that have provided a great deal of information about their current health and past health (Adams et al., 2014; Beck and Rice, 2003; Fair et al., 2010; Fair et al., 2017; Piwetz, 2019). With this information, scientists can gain an understanding of environmental and bottlenose dolphin population health.

1.8 Specific Aims

To date, two *Streptococcus agalactiae* events have been identified in bottlenose dolphins; one in captivity (Zappulli et al., 2005) and one free-ranging (Evans et al., 2006). This bacteria's ability to survive and adapt in a wide range of environments and hosts could pose a significant issue for bottlenose dolphins, the environment, and human health (Anshuman et al., 2018; Evans et al., 2008). Much progress in the understanding of *S. agalactiae* has been made in humans. However, there is still much to learn about the immune response in bottlenose dolphins and the epidemiology of this bacterium. To further understand *S. agalactiae* and its impact on bottlenose dolphin populations, this study plans to investigate managed-care dolphins and wild common bottlenose dolphin populations along the east and southeast coast of the United States. Bottlenose dolphin serum samples will be used to quantify serum titers and antibody activities against *S. agalactiae*. If a main protein(s) that these dolphins are recognizing can be identified, proteomics can be carried out to provide further insight into the dominant proteins involved in the immune response to this pathogen. A dominant protein could serve useful in generating a vaccine against *S. agalactiae.*

Aim 1: Investigate differences between relative titers and antibody activity utilizing bottlenose dolphin (*Tursiops truncatus*), Bull Shark (*Carcharhinus leucas*), Spotted Eagle Rays (*Aetobatus narinari*), and Loggerhead Sea Turtles (*Caretta caretta)* antibody responses to *Streptococcus agalactiae* through the generation of monoclonal antibodies against primary immunoglobulins.

Aim1a: Bottlenose dolphin serum samples have been provided by NOAA, the Georgia Aquarium, and United States Navy from dolphins along the east and southeastern seaboard of the United States and dolphins in managed human care. Bull Sharks and Spotted Eagle Rays plasma samples were collected from organisms as part of a fishery-independent survey in the Indian River Lagoon. Serum and plasma samples from these marine vertebrates will be used along with a protein A/G column to purify IgG or IgM. The purity of the Ig sample will be verified via SDS-PAGE.

Aim1b: Monoclonal antibodies against the heavy chain domain of IgG or IgM will be generated using pure Ig, balb/c mice, and previously established immunization methods from the Rice lab. Single-cell suspensions from the spleens of sacrificed mice will be fused with myeloma cells resulting in hybridomas that will be cultured, screened, and cloned. Supernatants from the hybridomas will then be used as the source of monoclonal antibodies.

Aim1c: The monoclonal antibodies will be validated using Western blot analysis. Serum samples will be diluted and subjected to SDS-PAGE. The gel will then be transferred, blocked, and probed with the generated monoclonal antibody and anti-mouse IgG - Alkaline phosphatase buffer. The blot will be developed to determine the validity of the monoclonal antibody.

Aim 1d: Develop an ELISA to quantify titers and antibody activities against *S. agalactiae* using developed monoclonal antibodies.

Aim1e: Compare relative titers and antibody activities in bottlenose dolphins, spotted eagle rays, bull sharks, and loggerhead sea turtles.

Aim 2: Determine the dominant proteins involved in the immune response against *S. agalactiae* in bottlenose dolphins.

Aim2a: Create an *S. agalactiae* lysate sample using freeze-thaw methods or sonication that will be used in the immunoprecipitation (IP) assay.

Aim2b: Immunoprecipitation assay will be performed with pooled bottlenose dolphin serum to generate a pure IgG sample bound to the agarose beads followed by the sample of bacteria lysate. Samples of the lysate before IP, the washes, after IP, and elutions will be collected.

Aim2c: Visualize and identify dominant proteins involved in the immune response utilizing SDS-page with Coomassie blue staining and Western blot analysis.

Aim 3: Determine bottlenose dolphin serum Ab titers against *Streptococcus agalactiae* utilizing enzyme-linked immunosorbent assays (ELISAs) and compare these activities to antibody titers in four different populations; Charleston Harbor,

Indian River Lagoon, Georgia Aquarium, and the United States Navy Marine Mammal Program.

Aim3a: By developing an ELISA to quantify titers using a generated monoclonal antibody against bottlenose dolphin IgG, activities of antibodies can be compared to titers.

Aim3b: ELISA data will be used to compare relative titers to antibody activities. Comparisons will be made for years and locations.

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Chapter Two

Monoclonal antibodies against bottlenose dolphin, *Tursiops truncatus*, IgG, bull shark, *Carcharhinus leucas*, and spotted eagle ray, *Aetobatus narinari*, IgM, and loggerhead turtle, *Caretta caretta*, IgY, reveal differences between relative antibody titers and antibody activities against the environmental pathogen, *Streptococcus agalactiae*

Kursten A. Anderson[1], Adam M. Schaefer[2], and Charles D. Rice[1]*

[1]Department of Biological Sciences, Graduate Program in Environmental Toxicology, Clemson University, Clemson SC USA 29634
[2]Harbor Branch Oceanographic Institute at Florida Atlantic University, Ft. Pierce, FL

2.0 Abstract

Mouse monoclonal antibodies (mAbs) were generated against bottlenose dolphin, *Tursiops truncatus*, IgG, bull shark, *Carcharhinus leucas* and spotted eagle ray, *Aetobatus narinari*, IgM. Along with a previously generated mAb against loggerhead, *Caretta caretta*, IgY, these biological reagents were used to compare the sensitivity of relative antibody titers at sample dilutions of 1:200 in buffer to calculated antibody activities across multiple serial dilutions against *Streptococcus agalactiae*. Global *Streptococcus agalactiae* and related pathogen infections are increasing and place both humans and environmental species at risk. Notably, *Streptococcus agalactiae* is now considered to be an emerging and zoonotic pathogen. Robust antibody responses were observed in each species, though some individuals differed significantly depending on whether viewed as relative antibody titers or antibody activities. The data from this study indicate that calculated antibody activities are more sensitive than relative antibody titers in determining antibody responses despite being much more labor-intensive, expensive, and time consuming. This study provides several novel antibody reagents to the greater animal health community, with a particular application to monitoring sentinel species for exposure to new emerging and zoonotic pathogens within the context of Oceans and Human Heath efforts.

2.1 Introduction

The human population living along coastal habitats has increased greatly over the last several decades. Along with this increase, so too has the impact of humans on delicate marine and estuarine habitats. Reciprocally the human population is impacted as well. This reality has led to the formation of a global community of scientists and decision-makers to engage the Oceans and Human Health (OHH) program (Bowen et al., 2006; Fleming et al., 2006; Fleming et al., 2014; Laws et al., 2008; McGowan et al., 2016). The collective OHH community links the effects of increased human fecal waste discharge, increasing eutrophication due to nutrient run-off, zones of low oxygen, noise pollution, plastic contamination, over-harvesting, rising global temperatures, and others (Bowen and Depledge, 2006; Kemp et al., 2005; Knap et al., 2002; Koelmans et al., 2014). Collectively, these factors impart significant stress to estuaries and nearby coastal ecosystems. Moreover, these environmental stressors also include the likelihood of humans coming into contact with aquatic pathogens associated with known and emerging environmental pathogens (Shuval, 2003). For example, several human zoonotic pathogens, such as *Vibrio cholera*, *Vibrio vuln*ificus, and *Vibrio parahaemolyticus,* are part of the indigenous microbial community. However, in immunocompromised humans, these bacteria and other potential pathogens can cause significant disease. An overview of the interactions between humans and aquatic pathogens from an OHH perspective has been extensively reviewed (Bienfang et al., 2011; Dyble et al., 2008).

One of the key factors in understanding and possibly managing the pathogenic component of OHH is monitoring the presence of various pathogens (Stewart et al.,

2008). Monitoring the prevalence and extent of these outbreaks is easily done by PCR using water, soil, or tissue samples (Ahmed et al., 2008; Li et al., 2020; Loge et al., 2002). However, this information may not reveal the potential for infectivity, transmission, or immunological response (Stewart et al., 2008). Another key component of monitoring for environmental pathogens is identifying a sentinel species, and to this end, bivalves have routinely served this purpose because of sediment exposures and high rate of water column filtration (Kueh and Chan, 1985). A major drawback of using sedentary sentinels to monitor for environmental pathogen prevalence is that some zoonotic or emerging pathogens could be associated with large or highly mobile animals such as bony fish, elasmobranchs, sea turtles, and marine mammals. Pathogens associated with these aquatic vertebrates may be missed in routine monitoring using bivalves or rapid PCR approaches.

Determining seropositivity and quantifying antibody responses to various pathogens of interest in humans is routine but is an under-appreciated and under-utilized tool in environmental monitoring programs. This approach is limited to only a few organisms due to a lack of species-specific antibody reagents. Also, the type of antibody responses in lower versus higher vertebrates is different in kinetics and antibody (immunoglobulin; Ig) class (Smith et al., 2019; Sun et al., 2020). Bony fish and elasmobranchs secrete IgM primarily in response to an immunogen, though other Ig classes are generated as well. One of the most essential functions of IgM is to activate complement. Serum IgM also contains natural antibodies against various carbohydrates, glycosylated proteins, and pathogen-associated molecular patterns, leading to a high

degree of cross-reactivity. In addition to IgM, reptiles such as sea turtles also secrete IgY as their mature antibody response to environmental pathogens (Rodgers and Rice, 2018; Rodgers et al., 2018). Marine mammals, like humans, generate and secrete IgG as the mature Ig response to pathogens (Beck and Rice, 2003; Nollens et al., 2007; Nollens et al., 2008). Once species-specific antibodies are available, one can develop ELISA assays to monitor antibody responses in a variety of aquatic vertebrates.

The most common method for quantifying antibody responses to pathogens in aquatic animals of interest is by determining titers, often expressed as the reciprocal of the lowest sample dilution yielding the highest optical density. Our lab has typically used 1:200 dilutions in buffer, and the associated optical density is multiplied by 200 to yield a relative titer (Beck and Rice, 2003; Fair et al., 2017; Karsten and Rice, 2006; Rodgers et al., 2018). Though seldom employed because of the intense labor and supply exhaustion, one can also quantify the activities of antibodies generated in response to pathogens (Arkoosh and Kaatari, 1990). While antibody titers reveal the relative amount of specific antibodies in a sample, this information alone does not indicate the affinity or quality of the antibody. Furthermore, circulating antibody titers will diminish over time once the specific pathogen is cleared from the animal, but the affinity of the antibody in circulation may be higher over time. By comparing antibody titers to antibody activities in a biological sample, one may inquire more information than simple seropositivity for exposure to environmental pathogens. In this study, we developed monoclonal antibodies (mAbs) against bottlenose dolphin (*Tursiops truncatus)* IgG, bull shark (*Carcharhinus leucas)* and spotted eagle ray (*Aetobatus narinari)* IgM, and used a

previously described mAb against loggerhead sea turtles (*Caretta caretta*) IgY (Rodgers and Rice, 2018) to compare titers and activities against an aquatic strain of *Streptococcus agalactiae*, an emerging environmental pathogen (Evans et al., 2008; Evans et al., 2009; Numberger et al., 2021). We also discuss the features and benefits of each approach. This study also provides additional mAbs to the scientific community for future work in these iconic and gregarious marine vertebrates.

2.2 Materials and Methods

2.2.1 Animals

Bull Sharks and Spotted Eagle Rays were captured as part of a fishery-independent survey in the Indian River Lagoon (IRL) between Brevard and Martin Counties of Florida between 2017 and 2019 (Roskar et al., 2020). Sampling was conducted each season (winter = January–March; spring = April–June; summer = July–September; fall = October–December) using a bottom longline and a monofilament gillnet. Captured elasmobranchs were identified to species level, and a blood sample (<1% of body weight) was collected from each individual via a caudal vein or wing vein (rays) puncture with an 18 or 20 gauge 1-1.5-inch needle connected to a 10 ml syringe and immediately transferred to one heparinized sodium tube and one serum separator tube. Animals were released back into the lagoon and were observed free-swimming post-release when possible. Blood samples were centrifuged for 10 minutes at 4500 rpm. Separated serum and plasma were then transferred to cryovials and stored at -80°C before shipping. Handling and sampling were conducted under protocols approved by the

Florida Atlantic University Institutional Animal Use and Care Committee (FAU AUP#16-16, 19-27). Bottlenose dolphin serum samples were randomly chosen from those stored and used in previous studies involving IRL dolphins and sent to Clemson University for previous immunological assays (Beck and Rice, 2003; Bossart et al., 2008; Bossart et al., 2011; Bossart et al., 2019; Fair et al., 2013; Fair et al., 2017). An in-hand monoclonal antibody (mAb LH-9) against loggerhead sea turtle IgY has been previously described with technical applications (Rodgers and Rice, 2018). Serum samples from loggerhead turtles were collected in the summer of 2019 during routine monitoring efforts by the South Carolina Department of Natural Resources and the Southern Atlantic Fish Management Council and shipped overnight to Clemson University under NOAA Section 10 (A) (1) (a) permits 15566 and 19621.

2.2.2. Immunoglobulins M and G purifications and monoclonal antibody production

An on-hand monoclonal antibody (mAb LH-9) against loggerhead sea turtle IgY has been previously described with technical applications (Rodgers and Rice, 2018). Immunoglobulin M (IgM) was purified from bull shark and spotted eagle ray serum samples using Protein-A columns as previously described for the Atlantic sharpnose shark, *Rhizoprionodon terraenovae,* (Karsten and Rice, 2006). This approach was previously shown to be efficient for isolation of IgM from several fish species, including elasmobranchs (Bromage et al., 2004; Karsten and Rice, 2006; Shin et al., 2006). In brief, plasma samples from several bull sharks and spotted eagle rays were pooled by species and centrifuged for 10 min at 500 × *g* to separate precipitated proteins. The

overlying serum was diluted 1:1 with 10 mM Tris–HCl, pH 8, and applied to a column of immobilized Protein-A (Pierce, Rockford IL USA) previously equilibrated with the same buffer. Purified IgM was eluted in 1 ml fractions from the column with 0.1 M glycine buffer, pH 2, and subsequently adjusted to a pH of 7.6 with 100 µL 1 M Tris–HCl, pH 8. Protein-A-purified IgM from each species was subjected to 4-20 % SDS-PAGE and subsequent Coomassie-blue staining to check for relative purity of the product.

Due to the loss of previously generated hybridomas secreting mAbs against bottlenose dolphin IgG (Beck and Rice, 2003), it was necessary to generate another source. Immunoglobulin G (IgG) was purified from bottlenose dolphin serum samples using Protein-G columns as previously described (Beck and Rice, 2003). In brief, serum samples were pooled and centrifuged for 10 min at 500 ×*g* to separate precipitated proteins. The overlying serum was diluted 1:1 with 10 mM Tris–HCl, pH 8, and applied to a column of immobilized Protein-G column previously equilibrated with the same buffer. Purified IgG was eluted from the column in 1 ml fractions with 0.1 M glycine buffer, pH 2, and subsequently adjusted to a pH of 7.6 with 100 µL 1 M Tris–HCl, pH 8. Protein-G-purified IgG was subjected to 4-20% SDS-PAGE and subsequent Coomassie-blue staining to check for relative purity of the product.

Six-week-old female Balb/c mice were housed at the Godley-Snell Animal Facility at Clemson University under IACUC approved animal use protocols. Mice were given a sub-cutaneous (s.c.) injection of 50 µg purified bull shark IgM, spotted eagle ray IgM, or bottlenose dolphin IgG in 0.9% saline containing TiterMax Gold adjuvant on day 1. Two weeks later, mice received a second s.c. immunization using Freund's incomplete

adjuvant. Subsequent boosters at 21-day intervals were given in saline via s.c. immunizations, and the final booster was given by intraperitoneal injection. Five days after the last booster immunization, mice were sacrificed using slow lethal CO_2 hypoxia, and their spleens removed using aseptic methods. Procedures for splenic plasma cell fusion with Sp02-14 myelomas and for screening, cloning, purification, and isotyping of the resulting monoclonal antibodies (mAb) have been described elsewhere (Margiotta et al., 2017; Rice et al., 1998). Subsequent testing revealed that mAbs 4F8, SER12-9, and KA-6 were most suitable for bull shark IgM, spotted eagle ray IgM, and bottlenose dolphin IgG, respectively. Each of these three mAbs were found to be an IgG_1 κ isotype.

Whole sera from each species were then diluted and subjected to SDS-PAGE and immunoblotting to verify specificity against targeted proteins of interest. Whole serum samples and purified Igs were diluted and loaded into a 4-20% MiniPROTEAN® TGX Stain-FreeTM Protein Gel (BioRad) and subjected to SDS-PAGE as previously described above. A BLUelf Prestained Protein molecular weight marker (FroggaBio) was used to serve as a reference. The proteins on the gel were then transferred to an Immun-Blot® PVDF membrane at 100V for one hour at 4°C. After the transfer, blots were blocked for 16 hours with 10% fetal calf serum in 0.01M PBS at 4°C. The blots were then probed with their respective mAbs for 1.5 hours: KA-6 against bottlenose dolphin IgG, 4F8 against bull shark IgM, or SER12-9 against spotted eagle ray IgM. The incubation was followed by three 5-minute washes with 0.05% PBST. Lastly, blots were incubated in anti-mouse IgG-Alkaline phosphatase buffer (AP) (1:1,000 in PBS; Thermo-Fisher), for one hour and following the same wash procedure as before. The blot was developed with

5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) and nitro-blue tetrazolium chloride (NBT) in AP buffer, allowing the AP activity to be visualized and recorded.

An in-hand monoclonal antibody (mAb LH-9) against loggerhead sea turtle IgY has been previously described with technical applications (Rodgers and Rice, 2018). Serum samples from loggerhead turtles were collected in the summer of 2019 during routine monitoring efforts by the South Carolina Department of Natural Resources and the Southern Atlantic Fish Management Council and shipped overnight to Clemson University under NOAA Section 10 (A) (1) (a) permits 15566 and 19621.

2.2.3 Development of enzyme-linked immunosorbent assays (ELISAs)

Streptococcus agalactiae cultures from an aquatic strain were a generous gift from Dr. John Hawke, Louisiana State University, and grown as directed (Soto et al., 2015). High bonding 96 well plates (Corning, #9018) were coated for 16 h at 4° C with 75 µL of a 0.1 mg/mL solution of poly-D-lysine in distilled water, followed by wash steps with 0.05% PBS Tween 20 (PBST). To perform the ELISAs, *S. agalactiae* was again grown at approximately 33ºC for 24 hours in tryptic soy broth media. The bacteria suspension was then spun down at 2500 rpm for 15 minutes. The supernatant was removed, and the pellet was resuspended in 20 mL of 0.01M phosphate-buffered saline (PBS). This procedure was repeated two more times, but the pellet was resuspended in 10 mL of PBS following the last centrifugation. To determine the optical density, 100 µL of the *S. agalactiae* was placed in duplicate in the wells of a 96 well plate and recorded at 600 nm. The bacterial suspension was then diluted to the established optical density of

0.200 for *S. agalactiae.* Optimized suspensions of live bacterium were then prepared, and 75 µL of the bacteria were added to wells of the 96 well plates using our lab's modifications (Beck and Rice, 2003; Karsten and Rice, 2006; Rodgers et al., 2018) of the original developers of the assay (Waterstrat et al., 1991). The plates were centrifuged at 2500 rpm for 5 minutes and then incubated at room temperature for 15 minutes. Following the 15-minute incubation, the plates were washed twice in PBS, and 100 µL of glycine-BSA (100 mM glycine with 1% BSA) was added to the plates to then incubate at room temperature for 30 minutes. The plates were washed in PBS two times and let dry at room temperature.

The dried plates each received 100 µL of 5% BSA blocking buffer, which incubated for one hour at room temperature. Serum suspensions were made by adding 10 µL of plasma from each individual animal into 990 µL of PBS in 1.5 mL snap cap tubes to yield a 1:100 dilution. As a reference sample for calculating relative titers, a composite sample for each species comprised of serum or plasma from randomly selected individuals in PBS was made at a 1:100 dilution. To each plate, 150 µL of each serum suspension and pooled standard sample were added in duplicate so that each plate could have five samples and a standard curve. Samples were serially diluted with 75 µL of PBS to yield 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200 dilutions and incubated at 4º C overnight (**Figure 2.1**). Each plate contained two wells of the composite serum sample to serve as a reference for any possible plate-to-plate variation, and two wells per plate received 75 µL PBS as an assay control. The next day, plates were washed three times with 0.05% PBST, and then 75 µL of diluted mAb were added to respective wells. Plates

were washed three times with 0.05% PBST after 2 hours of room temperature incubation. Goat anti-mouse IgG AP (1:1500) was added in 75 µL volumes to each well and incubated at room temperature for one and half hours before being washed five times with PBST. Finally, 75 µL of 1 mg/mL *p*-nitrophenol phosphate (ThermoFisher) in AP buffer were added to each well. The plates were incubated for 30 min at room temperature enzymatic reaction was stopped with 100 µL 2M NaOH. Optical densities for all plates were read at 405 nm and the data recorded.

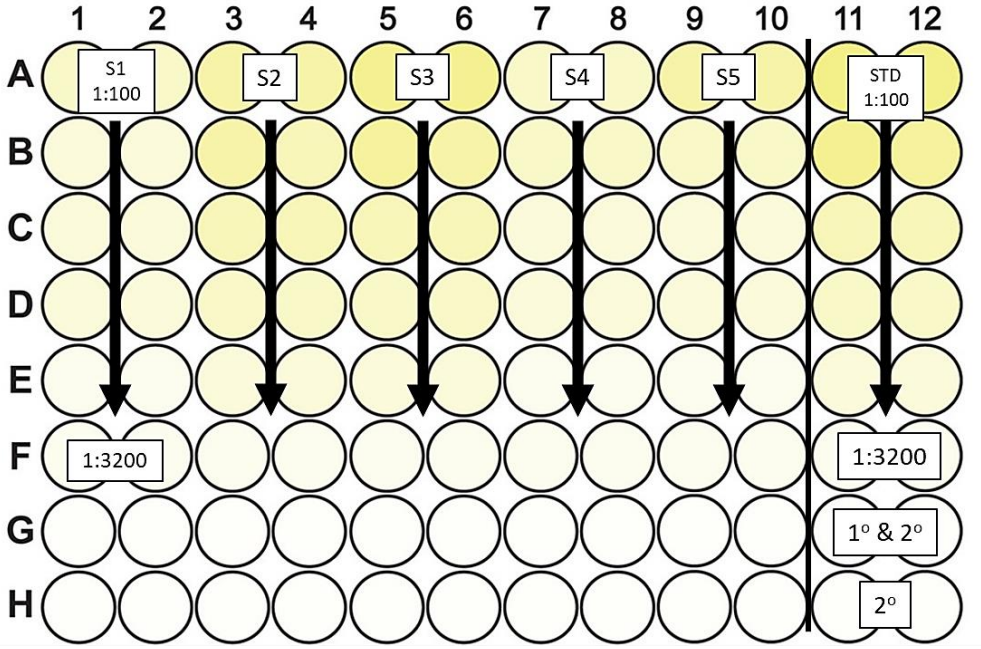

Figure 2.1. Experimental setup for ELISA. The experimental setup shown allowed for five serum samples (S) starting at a dilution of 1:100, a standard (STD) curve created from pooled serum, and four wells without serum for controls; primary antibody (1^o) and secondary antibody (2^o).

2.2.4. Determining relative titers vs. antibody activity levels

Relative antibody titers were calculated as the optical density (OD) multiplied by the serum dilution factor of 1:200 (Bossart et al., 2008; Fair et al., 2017; Karsten and Rice, 2006; Rodgers et al., 2018). Antibody activity for each sample was determined using previously published methods (Arkoosh and Kaatari, 1990) and expressed as units of activity per µL serum. In brief, dilutions were expressed as the equivalent volume of the undiluted serum determined by taking the product of the volume of dilution used (75 µL) and each of the dilution factors. The standard curve was then used to determine the volume of serum at the 50% point of the maximum O.D. obtained for the standard pooled serum, and this volume was then assumed to be the equivalent of one unit of antibody activity $\frac{1\,unit}{volume\ of\ serum}$) . Each sample was plotted against the 50% point of the maximum O.D. from the standard curve to determine the volume of test sample at that 50% point of the standard. To determine the activity for each of the test samples, the following equation was used:

$$Activity\ of\ Test\ Sample = (Activity\ of\ Standard) \times (\frac{50\%\ Volume\ of\ Standard}{50\%\ Volume\ of\ Test\ Sample})$$

2.2.5. Statistical analysis

Antibody titers from each species at 1:200 dilutions were compared to their calculated activities using a Mann-Whitney U test. Correlations between individual relative titers at 1:200 dilutions and the same individual's antibody activity were

determined by Pearson's correlation coefficient using GraphPad Prism9's statistical software. The α-value was set at 0.05 prior to the study.

2.3 Results and Discussion

In this study, we first purified bottlenose dolphin IgG using previously described techniques involving protein G columns (Beck and Rice, 2003), and purified IgM from bull shark and spotted eagle rays using purified using protein A columns, also as demonstrated in previous work (Karsten and Rice, 2006). Other methods to purify IgM were attempted as well, including a Pierce IgM purification kit (product # 44897) that capitalizes upon the ability of IgM to bind mannose-binding lectin immobilized on 4% beaded agarose, and by a stepwise dextran sulfate/$CaCl_2$ and NH_4SO_4 precipitation approaches to produce a crude IgM batch (dos Santos et al., 1997). Neither of these methods produced purified IgM to the level of the protein A method. Under reducing conditions, purified bottlenose IgG consists of the expected ≈ 55 kDa heavy chains and ≈ 25 kDa light chains (**Figure 2.2A**). Under similar conditions, purified spotted eagle ray and bull shark IgM consists of the expected ≈ 78 kDa heavy chains and ≈ 27 kDa light chains (**Figure 2.2B** and **Figure 2.2C**). Attempts to generated mAb against these immunoglobulins were successful in that mAb KA-6 is specific for the heavy chain of bottle nose dolphin IgG, and mAb SER12-9 and mAb 4F8 are specific to the light chains of spotted eagle ray and bull shark IgM, respectively. There were no attempts to determine cross-reactivity between the two mAbs against elasmobranch light chains, but based on previous studies with loggerhead sea turtles we show that mAb LH-9, which is

specific for the light chains of IgY, does react with all sea turtles tested (Rodgers and Rice, 2018). It is possible that our mAbs may recognize IgM in other elasmobranch species.

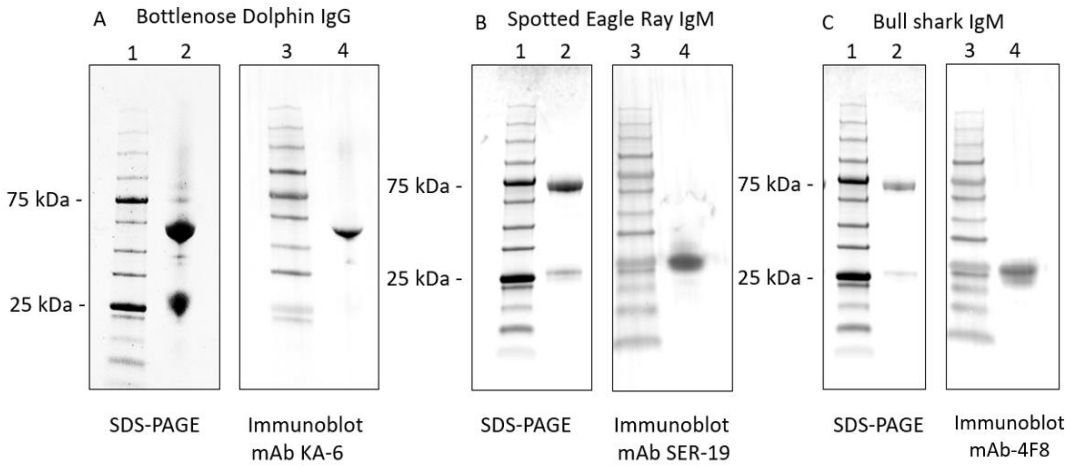

Figure 2.2. SDS-PAGE with Coomassie blue staining and Western blot analysis of purified immunoglobulins. (A) SDS-PAGE and Coomassie blue staining of purified bottlenose dolphin IgG products, followed by Western blotting with mAb KA-6. Lane 1: molecular weight marker; Lane 2: purified IgG; lane 3; molecular weight standard; lane 4 immunoblot results showing recognition of 55 kDa heavy chain. (B) SDS-PAGE and Coomassie blue staining of purified IgM products, followed by Western blotting with mAb SER 12-9. Lane 1: molecular weight marker; Lane 2: purified IgG; lane 3; molecular weight standard; lane 4 immunoblot results showing recognition of 27 kDa light chain. (C) SDS-PAGE and Coomassie blue staining of purified bull shark IgM products, followed by Western blotting with mAb 4F8. Lane 1: molecular weight marker; Lane 2: purified IgG; lane 3; molecular weight standard; lane 4 immunoblot results showing recognition of 27 kDa light chain.

Streptococcus agalactiae (group B streptococcus) is a Gram-positive bacterial pathogen known to cause septicemia, necrotizing fasciitis, and large mortality events in a variety of aquatic organisms. For example, this bacterium is known to affect crocodiles, frogs, and an array of bony fish species (Amborski et al., 1983; Bishop et al., 2007; Bowater et al., 2012; Evans et al., 2002; Plumb et al., 1974; Soto et al., 2015) and elasmobranchs (Bowater et al., 2018; Morick et al., 2020). In humans and bovines, it is known to cause neonatal meningitis and mastitis, respectively (Amborski et al., 1983; Baker, 1997).

Streptococcus agalactiae has also been isolated from both captive and wild bottlenose dolphins (Evans et al., 2006; Zappulli et al., 2005). For further information on *Streptococcal* sp infections in marine mammals, a thorough overview is available in a recent review (Numberger et al., 2021). Due to its ability to adapt and survive in a wide variety of environments, more outbreaks of *Streptococcus agalactiae* are likely to occur in the aquatic environment as the main route of exposure is from the ingestion of streptococcal contaminated materials (Bonar and Wagner, 2003; Bromage and Owens, 2002; Numberger et al., 2021). In addition, future outbreaks could have implications for humans and aquatic species as some studies suggest that disease associated with this pathogen may be caused by the same or similar strains of *S. agalactiae* (Evans et al., 2008; Evans et al., 2009; Numberger et al., 2021; Soto et al., 2015). To our knowledge, there are no previously published studies describing antibody responses against *S. agalactiae* in bottlenose dolphins, spotted eagle rays, and bull sharks collected from wild

and free-ranging animals. Our lab has examined antibody responses quantified as relative titers against *S. agalactiae* in loggerhead and Kemp's ridley turtles using general mouse polyclonal anti-sera against IgY (Rodgers et al., 2018). However, we have not examined antibody responses using specific monoclonal antibodies against sea turtle IgY.

Whole *S. agalactiae* lysates under reducing conditions were probed with plasma or serum from each species of study herein and were shown to have several proteins of varying sizes recognized by pooled serum samples after probing again with species-specific mAbs (**Figure 2.3**). Loggerhead sea turtles also recognize several proteins in similar lysates as determined previously using mouse polyclonal anti-sera against IgY (Rodgers et al., 2018). Immuno-reactivity was specific to the mAbs because no bacterial proteins were identified in the PBS control probed with secondary antibody only.

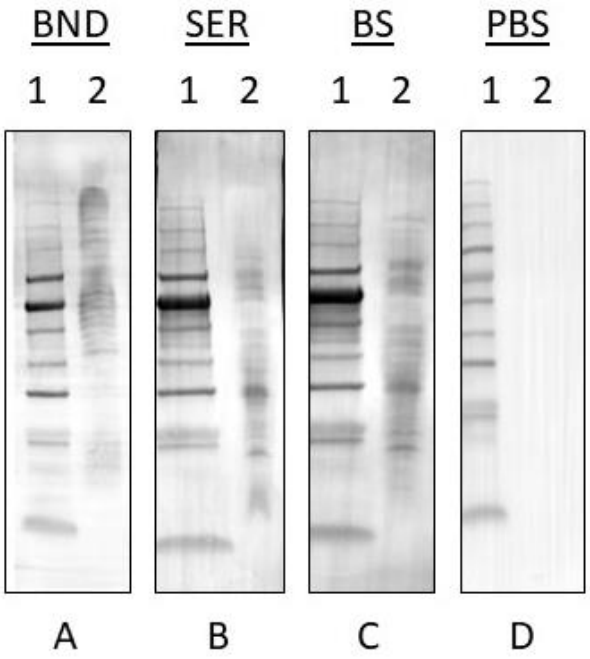

Figure 2.3. Western blot analysis demonstrating bottlenose dolphin, spotted eagle ray, and bull shark whole serum activity against *S. agalactiae* lysate using (A) mAb KA-6, (B) mAb SER-19, and (C) mAb 4F8, and PBS (D). Lane one for each Western blot contains the protein molecular weight marker; Lane 2 of each blot contains whole *S. agalactiae* lysate probed with whole serum diluted 1:100 for each organism followed by the appropriate mAbs and polyclonal secondary antibody.

When comparing how relative antibody titers and antibody activities are calculated, it is generally expected that titers would be statistically higher than activities, and this was the case except for spotted eagle ray samples (**Figure 2.4**). In general, relative titers are tightly clustered in bottlenose dolphin, loggerhead, and bull shark samples. In contrast, in spotted eagle ray samples, half had very low titers while the other half were roughly 10 times higher. Interpreting the significance of this is difficult because the sample size is very small, which is due to the sparse distribution of these animals during field work. These data may suggest that some animals have not been exposed to *S. agalactiae* or that circulating spotted eagle ray IgM is not as cross-reactive with similar antigens. As noted, antibody titers in bull sharks are tightly clustered even though the sample size is similar to what was available for spotted eagle rays. In this case, it is probable that all bull sharks sampled were previously exposed to *S. agalactiae* or that their IgM is cross-reactive with similar antigens.

Antibody activity data were less clustered than relative titers. This variability is related to varying optical density values between samples as they are serially diluted, and thus some and some samples had higher signal levels at higher dilutions. In this view, it appears that antibody activity data is more sensitive than relative titers in determining differences between samples. The highest available sample size (n=35) was for bottlenose dolphins, and thus a large distribution of activities, ranging from 1.7 to 36 Units per µl sample (**Figure 2.4A**). Activities in loggerhead samples ranged between 1.8 and 11.36 (**Figure 2.4B**), between 5.7 and 57 for spotted eagle ray samples (**Figure 2.4C**), and between 2 and 12.5 for bull shark samples (**Figure 2.4D**). Of particular note,

half of the relative titer data for spotted eagle ray samples were high and the other half low. All but one sample had activities that were very high compared to the other species examined.

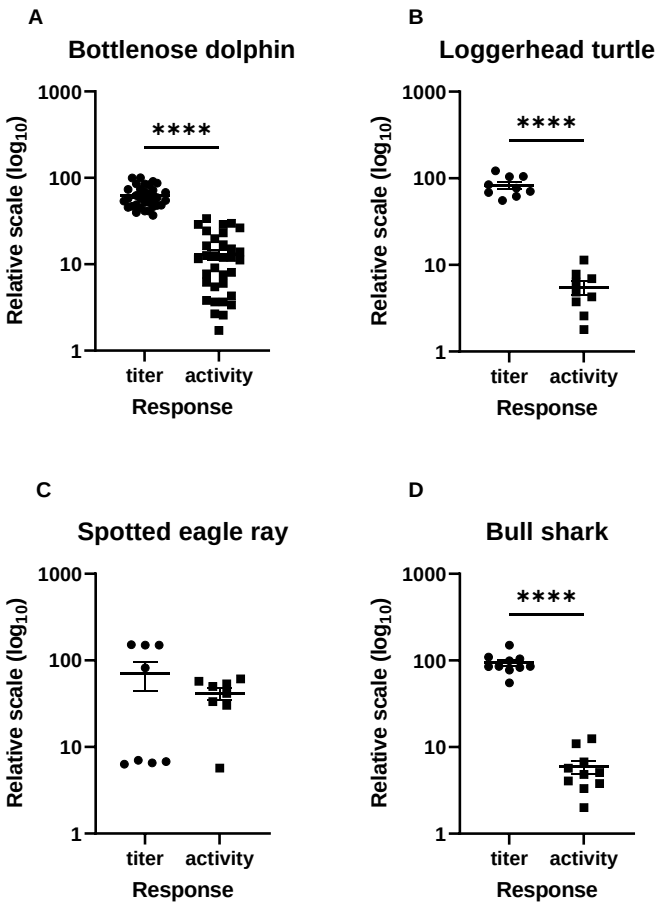

Figure 2.4. Comparison of relative antibody titers of serum or plasma dilutions at 1:200 and antibody activities (U/µl) for (A) bottlenose dolphins, (B) loggerhead sea turtles, (C) spotted eagle rays, (D) and bull sharks. Significant differences between the mean relative titer and mean activities were found for bottlenose dolphins (A), loggerhead turtle (B), and bull shark (D). Spotted eagle relative titers and antibody activities did not differ (C). *** denotes $p \leq 0.001$; **** denotes $p < 0.0001$.

One of the key questions from this study is whether antibody titers predict antibody activity in environmental species. This is important because calculating antibody activities is much more labor-intensive, requires significantly more research consumables, and is far more time-consuming. Of the four species examined in this study, only bull shark antibody activities correlated with relative titers (**Figure 2.5**). If the sample size were larger for spotted eagle rays and loggerhead turtles, there might be a better correlation. At this point, the discrepancy between relative titers and activities in spotted eagle ray samples is unclear. However, as mentioned previously, it may be associated with cross-reactivity of serum samples with similar antigens on other marine bacteria, including other *Streptococcal* sp.

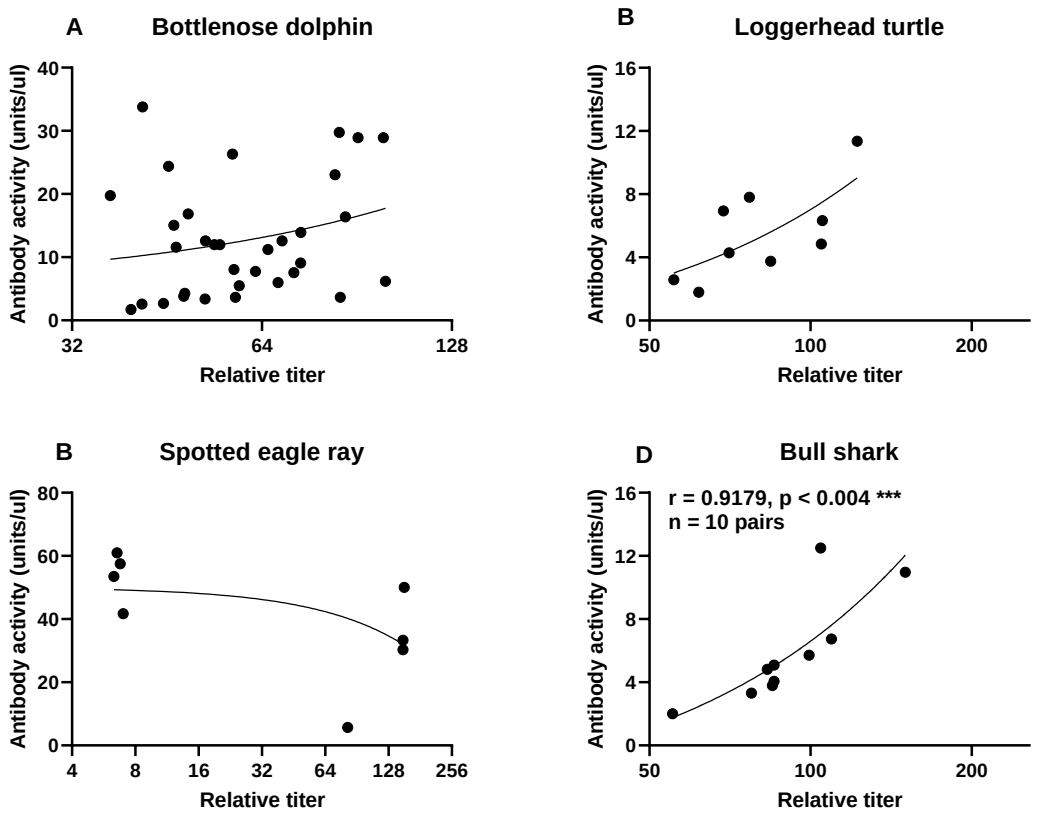

Figure 2.5. Spearman correlation analysis between relative titers of serum or plasma dilutions at 1:200 to antibody activities (U/µl) in (A) bottlenose dolphins, (B) loggerhead sea turtle, (C) spotted eagle rays, (D) and bull sharks. No significant correlations were found for relative antibody titers and antibody activities in bottlenose dolphins, loggerhead sea turtles, and spotted eagle rays. There was a positive correlation between relative antibody titers and antibody activities in bull sharks (*** denotes $p \leq$ 0.004).

In summary, we have generated novel monoclonal antibodies that are now available to the larger scientific community interested in the immunology of bottlenose dolphins, loggerhead sea turtles, spotted eagle rays, and bull sharks. These reagents will be especially useful for those interested in using these animals as sentinel species within the larger context of Oceans and Human Health. Secondary to developing these reagents, we demonstrate evidence that calculating antibody activities is a more sensitive and possibly more informative approach than calculating simple relative titers for quantifying antibody responses to pathogens of interest.

Acknowledgments

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Chapter Three

Antibody Responses against *Streptococcus agalactiae* in bottlenose dolphins, *Tursiops truncatus*

Kursten A. Anderson and Charles D. Rice

3.0 Abstract

The common bottlenose dolphin (*Tursiops truncatus)* immune response, health, and the exploration of exposure to different pathogens have been conducted as part of the Atlantic bottlenose dolphin Health and Environmental Risk Assessment (HERA) project. However, no study to date has quantified serum titers against *Streptococcus agalactiae* and compared serum titers and antibody activities. Serum from bottlenose dolphins has been collected over the past two decades from wild and managed-care populations. These samples include managed-care dolphins managed by the Georgia Aquarium (n = 34) and the United States Navy Marine Mammal Program (n=63) and wild dolphins from the Charleston Harbor in South Carolina (n =80) and Indian River Lagoon in Florida (n=163). Serum immunoglobulin G (IgG) recognition of *Streptococcus agalactiae* bacterial proteins were demonstrated through immunoprecipitation techniques. Bottlenose dolphin purified IgG recognized proteins from *S. agalactiae.* Whole bacterium-specific IgG titers and antibody activities were quantified using enzyme-linked immunosorbent assays, and significant differences were observed between bottlenose dolphin serum titers and antibody activities. Both serum titers and antibody activities demonstrated a strong correlation. Relative titers were comparable across all years of serum collection compared to significant differences present between the wild Indian River Lagoon population during sampling years. Managed-care dolphins had lower serum titers and antibody activities compared to the wild dolphins.

3.1 Introduction

Infectious diseases emerging over the decades have become a serious and complex threat for humans, animals, and environmental health (Bossart, 2011). Disease emergence has become a driver for environmental change due to its ability to cause the extinction of endangered species, alter habitats currently facing pressure from climate change and habitat fragmentation, and change ratios of predators, prey, and decomposers needed to maintain a healthy, productive ecosystem (Epstein et al., 2003). Oceanic health has been linked to human health worldwide, and the need to understand the connections between the health of humans, animals, and the environments they inhabit is critical (Bossart, 2011). Scientists have been utilizing marine sentinel species to provide a way to evaluate the aquatic ecosystem's health (Boersma, 2008; Bossart, 2011; Moore, 2008). Using this strategy, early indications about current or possible negative effects on animal health can allow for the characterization and management of impacts that could affect human and animal health (Bossart, 2011; Hazen et al., 2019).

The common bottlenose dolphin (*Tursiops truncatus*), a member of *Cetacea*, is one of the best-known and most recognized marine mammal species (Vollmer and Rosel, 2013). It has been noted that due to their life-history traits and global distribution, that they could serve as a sentinel species (Wells et al., 2004). These organisms carry out their lives immersed in water that brings with it an entirely different environment and exposure to a plethora of microbes and pathogens (Bossart et al., 2011). Previous studies have noted exposures to and diseases from viruses, fungi, and bacteria. These include the cetacean morbillivirus (Krafft et al., 1995; Taubenberger et al., 1996), the *Lacazia loboi*

fungus (Cowan, 1993), *Erysipelothrix rhusiopathiae, Escherichia coli, Mycobacterium marinum, Vibrio carchariae, Vibrio cholerae, Vibrio parahemolyticus,* and *Vibrio vulnificus* (Beck and Rice, 2003; Bossart et al., 2019). A pathogen that has not yet been studied extensively in bottlenose dolphins is *Streptococcus agalactiae.*

In the early 2000s, the first two characterizations of *S. agalactiae* in marine mammals were isolated from managed-care and wild bottlenose dolphins (Evans et al., 2006; Zappulli et al., 2005). *S. agalactiae* is a group B streptococcus strain that is a significant pathogen and has been of concern for terrestrial animals and farmed fishes. It is known to be the cause of neonatal meningitis and mastitis in humans and cows, respectively (Amborski et al., 1983; Baker, 1997). *S. agalactiae* has been observed to cause septicemia, necrotizing fasciitis, and large mortality events in a variety of aquatic organisms. This bacterium has also been documented to have harmful effects in crocodiles, frogs, and an array of fish species (Amborski et al., 1983; Bishop et al., 2007).

The immune system plays a crucial role in the pathogenesis and fate of infectious diseases in their hosts (Bossart et al., 2019). As previously noted in the literature, measuring antibody responses to pathogens is a vital tool to understand bottlenose dolphin health. Immunoglobulin M (IgM), IgG, and IgA have been confirmed to be critical constituents of the marine mammal immune system to identify and aid in the destruction of invading pathogens (Gelain and Bonsembiante, 2019). Bottlenose dolphins produce IgG, which serves as the major antibody that aids in the protection and immunity against bacterial or viral infections (Zimmerman et al., 2010). Previous studies have utilized monoclonal antibodies against bottlenose dolphin IgG to identify and gain

an understanding of significant exposure events to different marine bacteria in different bottlenose populations (Beck and Rice, 2003; Fair et al., 2017)

Bottlenose dolphin serum samples have been routinely collected from wild and managed-care dolphin facilities as a continuing effort of the Atlantic bottlenose dolphin Health and Environmental Risk Assessment (HERA) project. Wild dolphins have been sampled from two estuarine areas: the Charleston Harbor in South Carolina and the Indian River Lagoon in Florida. The Charleston Harbor site (CH) is an estuarine ecosystem that includes Ashley, Wando, and Cooper Rivers. There is an effective tidal exchange, but the estuary is surrounded by seaports, growing urban development, and population, and contains a US Naval base (Reif et al., 2008). There are two Superfund sites on the CH estuary that have been documented to be contaminated with polycyclic aromatic hydrocarbons, lead, copper arsenic, and dioxin (United States Environmental Protection Agency, 2021). The Indian River Lagoon (IRL) site is a shallow-water ecosystem that comprises 40% of the Florida east coast. The Indian River, Banana River, and Mosquito Lagoon all collectively form the IRL. The tidal exchange rates are low between the lagoon and the Atlantic Ocean, which creates a longer residence time of pollutants in the lagoon (Barile, 2018). Residential development and intense agricultural activity have resulted in increased freshwater inputs and water quality changes (Scott et al., 2002; Sigua et al., 2000). Managed-care bottlenose dolphin samples have been collected from the Georgia Aquarium facilities and the United States Navy Marine Mammal Program (MMP) pod in San Diego, California. The Georgia Aquarium dolphins in Atlanta, Georgia, are kept in a controlled artificial sea water environment.

The Georgia dolphin environment at Marineland, Florida, is outdoors with maintained natural water temperatures. Both facilities use protein skimming, filtration, and water treatment (Fair et al., 2017).

Previous work utilizing pathogen-specific responses in bottlenose dolphins has used serum antibody activities (Beck and Rice, 2003) and serum antibody titers (Bossart et al., 2019; Fair et al., 2017) to measure these responses to bacteria, fungi, and viruses. In this study herein, we aim to identify *S. agalactiae* proteins that play a role in the immune response in bottlenose dolphins through immunoprecipitation techniques, investigate differences in serum antibody titers and antibody activities to *S. agalactiae* in four different bottlenose dolphin populations, and add to the repertoire of data that has been reported for these bottlenose dolphin samples. Immunoprecipitation (IP) is a method to identify molecules that interact with specific proteins that utilize a target protein-specific antibody along with protein A/G affinity beads (Kim et al., 2013). To do this, a *S. agalactiae* culture will be grown and lysed. Immunoprecipitation will be carried out with serum from bottlenose dolphins, followed by *S. agalactiae* lysate. To observe any key immunogenic proteins, SDS-PAGE and Western blot analysis will be carried out. If there is a key immunogenic protein present that the bottlenose dolphin serum recognizes, the protein can be extracted and sequenced. Once sequenced, the protein of interest can be identified and aid in the formation of a potential vaccine against *S. agalactiae*.

3.2 Materials and Methods

3.2.1 Bottlenose dolphin samples

Bottlenose dolphin (*Tursiops truncatus)* serum samples were collected from 340 individuals starting in July of 2003 to July of 2019. These samples were collected from wild dolphins in the Indian River Lagoon in Florida (n=163) and the Charleston Harbor in South Carolina (n=80). Samples were also collected from managed-care dolphins from the Georgia aquarium (n=34) and the Marine Mammal Program pod in San Diego, California (n=63). These collections are part of an ongoing research effort for the Atlantic bottlenose dolphin Health and Environmental Risk Assessment (HERA) project.

3.2.2 Purification of bottlenose dolphin Immunoglobulin G (IgG)

Random bottlenose dolphin serum samples were used to create a pooled serum sample to be purified for IgG. The pooled serum sample was made using 500 µL of bottlenose dolphin serum was taken from 20 different bottlenose dolphin serum samples. The pooled serum was centrifuged at 2,000 rpm to rid of any cell debris in the serum samples. The supernatant was then used and filter sterilized using a 0.45 µm filter followed by a 0.2 µm filter.

To purify IgG from bottlenose dolphin serum samples, a protein G column (ThermoFisher) were used. The column was equilibrated by running 0.10 M Tris pH 8.0 through the column, then 0.01 M Trish pH 8.0, followed by the pooled serum sample. The serum was passed through the column three times. IgG samples were eluted by

running 0.05 M glycine pH 2.5 in 1 mL fractions through the column and collected. Each of these 1 mL fractions has 1.0 M Tris pH 8.0 added to the aliquot.

After collection, a standard BCA protein determination was performed for each aliquot to elucidate which fractions had IgG and the quantity present. The Pierce® BCA Protein Assay reagents (Thermo Scientific) were used to determine the amount of protein on a 96 well plate according to the manufacturer's protocol. A standard curve was generated from the known concentrations of the BCA standard wells and their optical density readings. A line of best fit was generated and employed to determine the exact amount of protein that corresponds with each optical density reading. The duplicates of the protein concentrations were averaged and considered the final protein concentration estimates for IgG.

The purity of the bottlenose dolphin IgG sample was verified via SDS-PAGE. Samples from aliquots that showed positive protein amounts were combined with 5x Laemmli sample buffer with 2-Mercaptoethanol (2-ME). These samples were boiled in water at 100^{o}C for six minutes. The samples were allowed to cool, and then the products were subjected to SDS-PAGE using 4-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (BioRad). For the gel, lane 1 contained the protein molecular weight marker (FroggaBio), followed by the pure IgG aliquot in lane 3. The gel was run at 200V for approximately 40 minutes or until the dye front reached the bottom of the gel. Once complete, the gel was stained with Coomassie blue for 16 hours and then detained using a 30% methanol/10% acetic acid solution. Once the gel was destained, the gel was imaged

using a ChemiDoc (Bio-Rad) imaging system and analyzed for purity using the molecular weight marker to reference size.

3.2.3 *Generation of a monoclonal antibody against the heavy chain domain of bottlenose dolphin IgG*

Procedures for immunizations, blood collection and spleen removal were carried out at the Godley Snell Animal facility under an approved animal use protocol. Purified IgG from bottlenose dolphins was used to immunize 6-week old female Balb/c mice using previously described methods (Rice et al., 1998). On day one, mice were administered a subcutaneous (s.c.) injection of 100 µg purified bottlenose dolphin IgG in 0.9% saline containing TiterMax Gold adjuvant. On day 14, the mice received a second s.c. injection of 50 µg of purified IgG with Freud's incomplete adjuvant (FIA). Boosters of 50 µg of purified IgG were given at 21-day intervals. On day 35, the booster was given in saline via s.c. immunization and on day 56 intraperitoneally. Five days after the last immunization, slow lethal CO_2 asphyxiation and bilateral pneumothorax were used to sacrifice the mice. The spleens were removed and dissociated into single cells by gently holding the pulp with tweezers and using a scalpel blade. Cells were washed twice via centrifugation and then fused with Sp02-Ag14 myeloma cells (ATCC) in the presence of 50% polyethylene glycol 4000 (PEG 4000) (Fisher Scientific) using previously published techniques. The 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).

3.2.4 Validation monoclonal antibody utilizing Western blot analysis

Bottlenose dolphin whole serum samples and purified IgG were diluted and loaded into a 4-20% MiniPROTEAN® TGX Stain-FreeTM Protein Gel (BioRad) and subjected to SDS-PAGE as previously described above. A BLUelf Prestained Protein molecular weight marker (FroggaBio) was used to serve as a reference. The proteins on the gel were then be transferred to an Immun-Blot® PVDF membrane at 100V for one hour at 4°C. After the transfer, blots were blocked for 16 hours with 10% fetal calf serum in 0.01M PBS at 4°C. The blots were then probed with the mAbs against bottlenose dolphin IgG (mAb KA-6), followed by three 5-minute washes with 0.05% PBS Tween 20 (PBST). Lastly, blots were incubated with goat anti-mouse IgG-Alkaline phosphatase buffer (AP) (1:1,000 in PBS; Thermo-Fisher), following the same incubation and wash procedure as the previous two steps. The blot was developed with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) and nitro-blue tetrazolium chloride (NBT) in AP buffer, allowing the AP activity to be visualized and recorded.

3.2.4 Preparation of serum prior to immunoprecipitation steps.

To purify IgG from bottlenose dolphin serum, 500 µL of bottlenose dolphin serum was taken from 20 different bottlenose dolphin serum samples. The pooled serum was centrifuged at 2,000 rpm to rid of any cell debris in the serum samples. The supernatant was then used, and filter sterilized using a 0.45 µm filter followed by a 0.2 µm filter. The filtered serum was then combined with one milliliter of 1 M Tris pH 8.

3.2.5 Streptococcus agalactiae lysate for immunoprecipitation

To create a sample of *Streptococcus agalactiae* lysate, *S. agalactiae* was grown at approximately 33ºC for 24 hours in tryptic soy broth media. The bacteria suspension was then centrifuged at 2500 rpm for 15 minutes. The supernatant was removed, and the pellet was resuspended in 20 mL of 0.01M phosphate-buffered saline (PBS). This procedure was repeated two more times. The pellet was resuspended in denaturing buffer provided in the QIAexpress Ni-NTA Fast Start kit (Qiagen). The sample was vortexed and subjected to three freeze-thaw cycles. Sonication was also used to further assist in the breaking of the bacterial walls and DNA of *S. agalactiae*.

An *S. agalactiae* sample was combined with 5x Laemmli sample buffer with 2-Mercaptoethanol (2-ME) and boiled in water at 100ºC for six minutes. The sample was allowed to cool, and then the product was subjected to SDS-PAGE using 4-20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (BioRad). For the gel, lane 1 contained the BLUelf Prestained Protein molecular weight marker (FroggaBio), followed by different amounts of the *S. agalactiae* sample across the gel; 2 µL,4 µL,8 µL,16 µL, and 30 µL. The gel was run at 200V for approximately 40 minutes or until the dye front reached the bottom of the gel. Once complete, the gel was stained with Coomassie blue for 16 hours and then detained using a 30% methanol/10% acetic acid solution. Once the gel was destained, the gel was imaged using a ChemiDoc (Bio-Rad) imaging system and analyzed for the presence of different *S. agalactiae* proteins using the molecular weight marker to reference size.

3.2.6 Immunoprecipitation

To immune-precipitate *Streptococcus agalactiae* lysate proteins, a protein G column (Thermo-Fisher) was used to capture bottlenose dolphin IgG. To equilibrate the column, three column washes of 0.1 M Tris pH 8.0 were applied to the column, followed by three column washes of 0.01 M Tris pH 8.0. Then, 10 mL of pooled bottlenose dolphin serum combined with 10 mL of 1 M Tris pH 8.0 was run through the column five times. The column was washed five times with 0.1 M Tris pH 8.0, and the *S. agalactiae* lysate solution was added to the column. The bacterial lysate solution was run through the column twice, followed by three washes with 1 ml 0.1 M Tris pH 8.0. Each wash of the sample was collected in a 15 mL centrifuge tube. Finally, IgG bound to *S. agalactiae* was eluted using six milliliters of 0.05 M glycine pH 2.5 in 500 µL fractions. To each of these 500 µL fractions, 50 µl of 1.0 M Tris pH 8.0. In addition to the 12 elution samples, 200 µL was taken from the *S. agalactiae* lysate sample before immunoprecipitation and after the immunoprecipitation.

A standard BCA protein determination was performed for each of the 12 fractions of purified IgG bound to *S. agalactiae*, the pre-and post-immunoprecipitation samples, and the three washes after the immunoprecipitation that were collected. Protein determination was performed using Pierce® BCA Protein Assay reagents (Thermo Scientific). Each sample was measured in duplicate, and the average was taken and considered the final protein concentration estimate for each of the samples.

3.2.7 Validation of Immunoprecipitation utilizing SDS-PAGE and Western blot analysis

To validate the success of the immunoprecipitation assay, the 12 fractions of *S. agalactiae* lysates bound to dolphin IgG and the pre-and post-immunoprecipitation samples were diluted and loaded into two 4-20% MiniPROTEAN® TGX Stain-FreeTM Protein Gel (BioRad) and subjected to SDS-PAGE as previously described above. A protein molecular weight marker was used to serve as a reference. One of the gels was stained according to the previously mentioned Coomassie blue staining procedure. The proteins on the second gel were transferred to an Immun-Blot® PVDF membrane at 100V for one hour at 4ºC. After the transfer, the blot was blocked for 16 hours with 10% fetal calf serum in 0.01M PBS at 4°C. The blot was then probed with the mAb against bottlenose dolphin IgG (mAb KA-6), followed by three 5-minute washes with PBST. Lastly, the blot was incubated in anti-mouse IgG-alkaline phosphatase buffer (AP) (1:1,000 in PBS; Thermo-Fisher), following the same incubation and wash procedure as the previous two steps. The blot was developed with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) and nitro-blue tetrazolium chloride (NBT) in AP buffer, allowing the AP activity to be visualized and recorded.

3.2.8 Development of enzyme-linked immunosorbent assays (ELISAs) for quantifying Streptococcus agalactiae specific IgG

To perform the enzyme-linked immunosorbent assays (ELISAs), *S. agalactiae* was again grown at approximately 33ºC for 24 hours in tryptic soy broth media. The bacteria suspension was then spun down at 2500 rpm for 15 minutes. The supernatant

was removed, and the pellet was resuspended in 20 mL of 0.01M phosphate-buffered saline (PBS). This procedure was repeated two more times, but the pellet was resuspended in 10 mL of PBS following the last centrifugation. To determine the optical density, 100 µL of the *S. agalactiae* was placed in duplicate in the wells of a 96 well plate and recorded at 600 nm. The bacterial suspension was then diluted to the established optical density of 0.200 for *S. agalactiae.*

High binding 96 well plates (Medi-sorb, ThermoFisher) were coated for 16 hours at 4° C with 50 µL of 0.1 mg/mL poly-D-lysine in distilled water in each well. The plates were then washed in 0.05% PBST two times. The optimized bacterial suspension was taken, and 75 µL of the bacteria was added to all the wells in the 96 well plates. The plates were centrifuged at 2500 rpm for 5 minutes and then incubated at room temperature for 15 minutes. Following the 15-minute incubation, the plates were washed twice in PBS, and 100 µL of glycine-BSA (100 mM glycine with 1% BSA) was added to the plates to then incubate at room temperature for 30 minutes. The plates were washed in PBS two times and let dry at room temperature.

The dried plates each received 100 µL of 5% BSA blocking buffer, which incubated for one hour at room temperature. Bottlenose dolphin serum dilutions were made by adding five µL of serum from each individual dolphin into 990 µL PBS in 1.5 mL snap cap tubes to result in a final concentration of 1:200. In addition to the individual samples, a pooled serum sample was made by combining 300 µL of the bottlenose dolphin pooled serum resource and added it into 29.7 mL of PBS, resulting in a 1:100 final concentration. To each plate, 150 µL of each serum suspension and pooled standard

sample were added in duplicate so that each plate could have eight samples and a standard curve. The remaining wells on each plate had 75 µL of PBS added to them. The samples were then serially diluted down the plate, with the last dilution being 1:3200. Four wells on each plate contained 75 µL of PBS to be used for controls (**Figure 3.1**).

The plates were then incubated with the dilutions of bottlenose dolphin serum overnight at 4°C. The following day, the plates were washed three times in 0.05% PBST via submersion, and 75 µL of monoclonal bottlenose dolphin anti- IgG (mAb KA-6) diluted 1:3 in media was added to the plates. After two hours of incubation at room temperature, plates were washed three times in 0.05% PBST. Goat anti-mouse IgG AP (1:1000) was added to the plates in 75 µL quantities and incubated at room temperature for one hour. The plates were washed five times in 0.05% PBST, and 75 µL of 1 mg/mL of p-nitrophenol in AP buffer was added to each well. After an incubation period of 16 minutes, 75 µL of 1 M sodium hydroxide solution was added to the plates to stop the enzymatic reaction. The plates were then read at 405 nm, and the data were recorded.

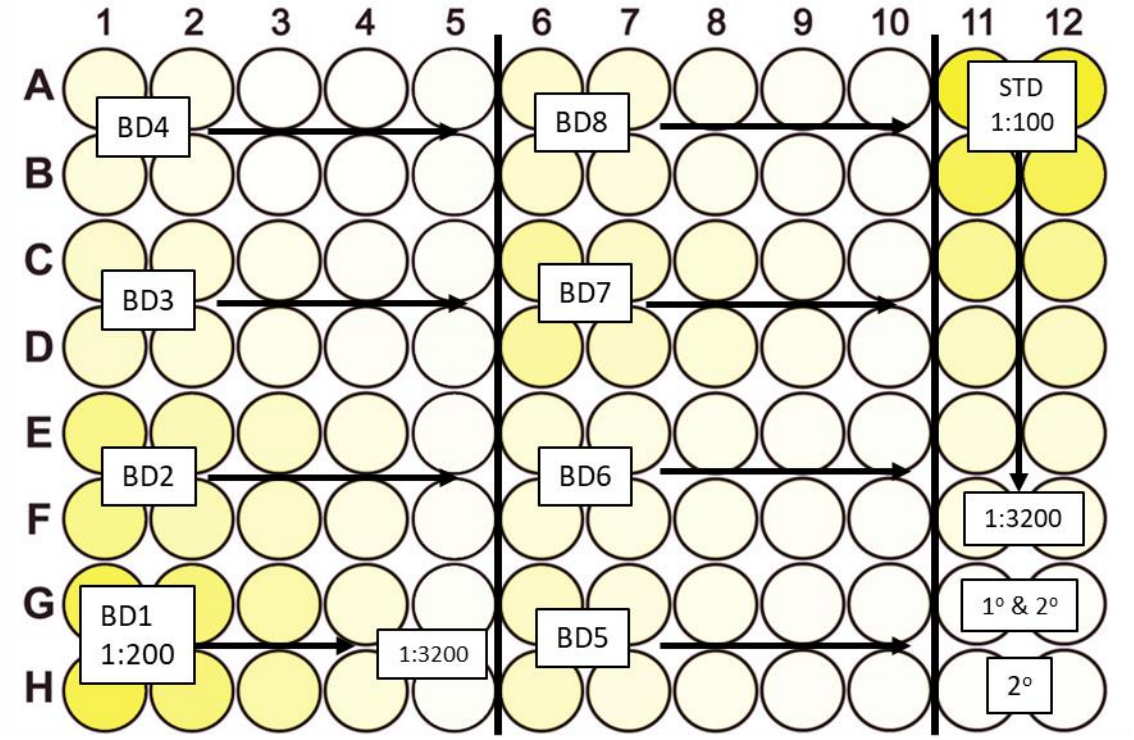

Figure 3.1. Experimental setup for ELISA. The experimental setup shown allowed for eight bottlenose dolphin (BD) samples starting at a dilution of 1:200, a standard (STD) curve created from pooled bottlenose dolphin serum, and four wells for controls; primary antibody (1^o) and secondary antibody (2^o).

Antibody activity for each sample was determined using previously published methods (Arkoosh and Kaattari, 1991). In brief, dilutions were expressed as the equivalent volume of the undiluted serum. This was calculated by taking the product of the dilution volume (75 µl) and each dilution. The standard curve generated with pooled serum was used to determine the volume of serum at the 50% point of the maximum O.D. obtained for the standard pooled serum. This volume was then assumed to be the equivalent of one unit of antibody activity ($\frac{1\ unit}{volume\ of\ serum}$). Each sample was graphed against the 50% point of the maximum O.D. from the standard curve to determine the volume of the test sample at that 50% point of the standard. Antibody activity for each of the test samples was calculated using the following equation:

$$Activity\ of\ Test\ Sample\ =\ (Activity\ of\ Standard)\ \times (\frac{50\%\ Volume\ of\ Standard}{50\%\ Volume\ of\ Test\ Sample})$$

The values for serum titers against the bacteria were calculated as relative optical density (O.D) multiplied by the serum dilution factor of 1:200 for each individual (Karsten and Rice, 2006; Rodgers et al., 2018).

GraphPad Prism's statistical software was used to compare averages using a previously set α-value of 0.05 and analysis of variance (ANOVA) with Bonferroni's multiple contrast post-hoc tests whenever differences were noted by the ANOVA. In addition, serum titer levels were compared against logarithmically transformed activities using regression analysis and Pearson's correlation to examine if bottlenose dolphin serum titers and activity were correlated.

3.3 Results

3.3.1 Analysis of Immunoprecipitation

Analysis of the SDS-PAGE of different loading volumes of *S. agalactiae* whole lysate under reducing conditions demonstrates that four to eight microliters of lysate are optimal to observe clean banding patterns (**Figure 3.2**).

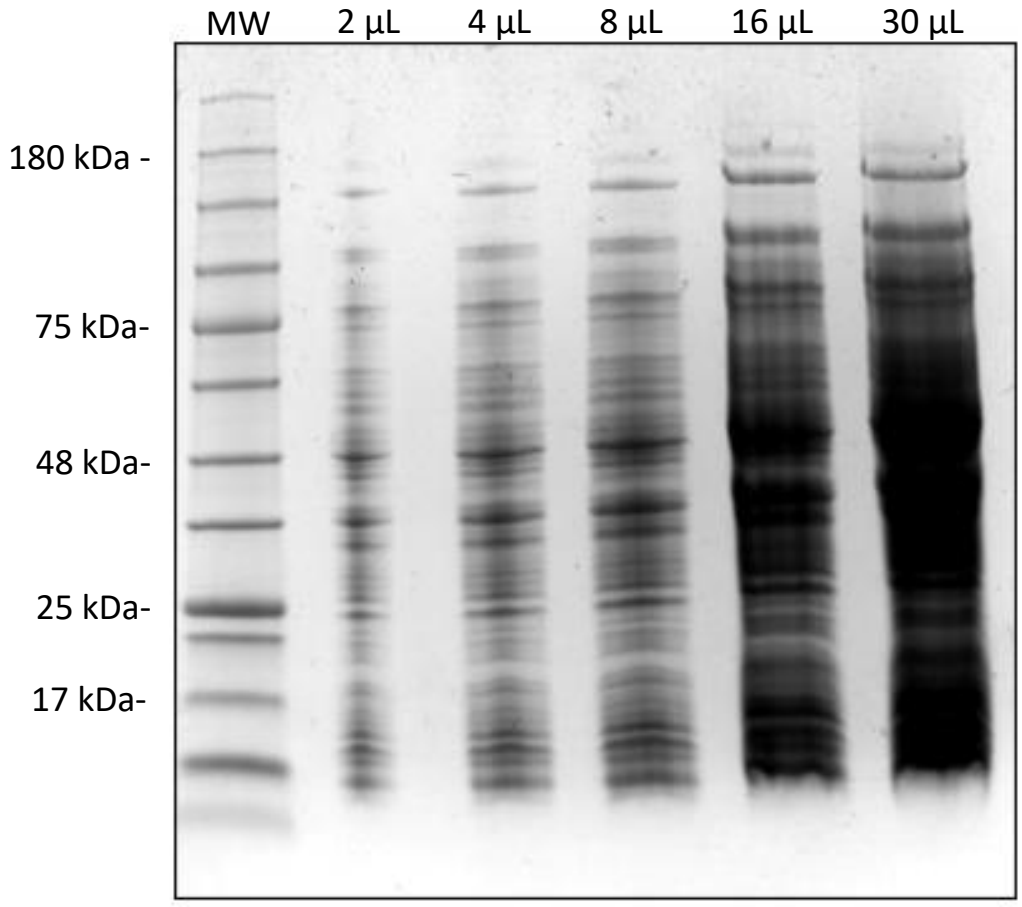

Figure 3.2. SDS-PAGE of *Streptococcus agalactiae* lysate. Lane 1 contains the protein molecular weight marker. Lane 2 contains 2 µL of lysate, lane 3 contains 4 µL of lysate, lane 4 contains 8 µL of lysate, lane 5 contains 16 µL of lysate, and lane 6 contains 30 µL of lysate.

The immunoprecipitation technique successfully immunoprecipitated *S. agalactiae* proteins interacting with bottlenose dolphin IgG (**Figure 3.3, Figure 3.4**). Lanes four through six demonstrate successful washing of the column before the elution aliquots present in lanes seven through 12. Four different dominant bands can be observed in lanes 10 and 11, which occur at approximately 75 kDa, 55 kDa, 25 kDa, and 13 kDa. The bands at 55 kDa and 25 kDa correspond to the heavy and light chain of bottlenose dolphin IgG, respectively. There also appears to be an IgG degradation product that appears just under the heavy chain of IgG. The other two bands that appear at 76 kDa and 13 kDa are likely proteins from *S. agalactiae*.

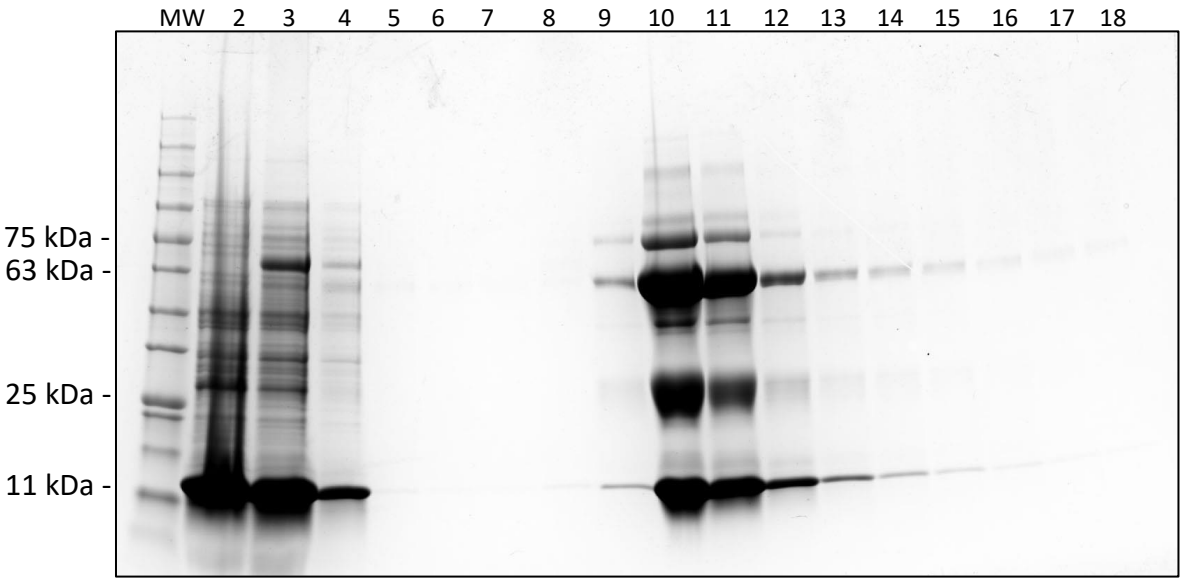

Figure 3.3. SDS-PAGE of samples from immunoprecipitation of *S. agalactiae* lysates. Lane 1 contains the protein molecular weight marker. Lane 2 contains the *S. agalactiae* lysate sample prior to immunoprecipitation. Lane 3 contains the *S. agalactiae* sample after immunoprecipitation. Lane 4 through 6 contain washes one through three. Lane 7 through lane 18 contain the 12 elutions from the immunoprecipitation assay. Four different dominant bands can be seen in lanes 10 and 11, occurring at approximately 76 kDa, 55 kDa, 25 kDa, and 13 kDa.

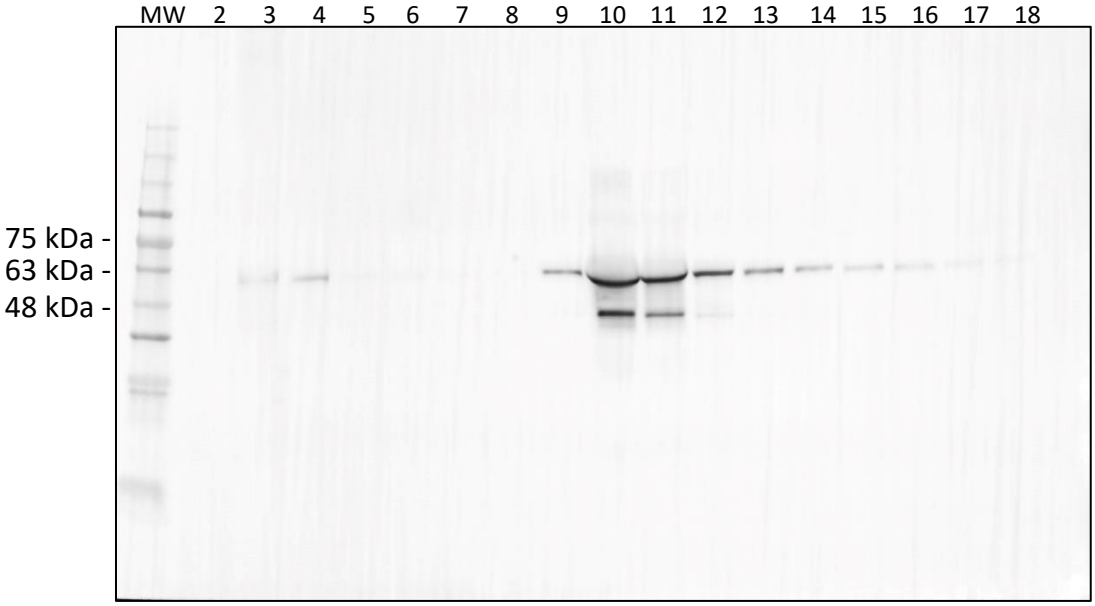

Figure 3.4. Western blot analysis of samples from immunoprecipitation of *S. agalactiae* lysates with mAb KA-6 identifies bottlenose dolphin IgG heavy chains. Lane 1 contains the protein molecular weight marker. Lane 2 contains the *S. agalactiae* lysate sample prior to immunoprecipitation. Lane 3 is the *S. agalactiae* sample after immunoprecipitation. Lane 4 through 6 contain washes one through three. Lane 7 through lane 18 contain the 12 elutions from the immunoprecipitation assay. Dominant bands can be seen in lanes 10 and 11, occurring at approximately 55 kDa and 45 kDa, bottlenose dolphin IgG heavy and degradation product, respectively.

3.3.2 ELISA results for all dolphins sampled

Statistical analysis of the serum titer levels against *S. agalactiae* demonstrates no significant differences between titer levels in sera collected during different years for all four different dolphin populations (**Figure 3.5A**). There were statistically significant differences ($p < 0.05$, $p < 0.001$, $p < 0.0001$) of serum antibody activity against *S. agalactiae* from sera collected in 2003, 2004, 2010, 2011, 2012, 2013, and 2015 (**Figure 3.5B**). Antibody activity notably increased in 2015 compared to all other years.

The relationship between sera relative titer levels and antibody activity was investigated and demonstrated a relationship between these two indices (**Figure 3.6**). A positive correlation ($r = 0.6909$) was observed, and this relationship was significant for all dolphins sampled for this study ($p < 0.0001$, $n = 340$). This data indicates that titers would indicate antibody activity, and antibody activity would indicate titers against *S. agalactiae* in bottlenose dolphin populations.

After analyzing bottlenose dolphin data as a whole, bottlenose dolphin populations were split based on their different sampling locations. Relative titers and antibody activities were analyzed for IRL (**Figure 3.7A**), CH (**Figure 3.7B**), Aq. (**Figure 3.7C**), and MMP (**Figure 3.7D**) populations. All four populations had statistically significant differences between serum relative titers and antibody activity ($p < 0.0001$).

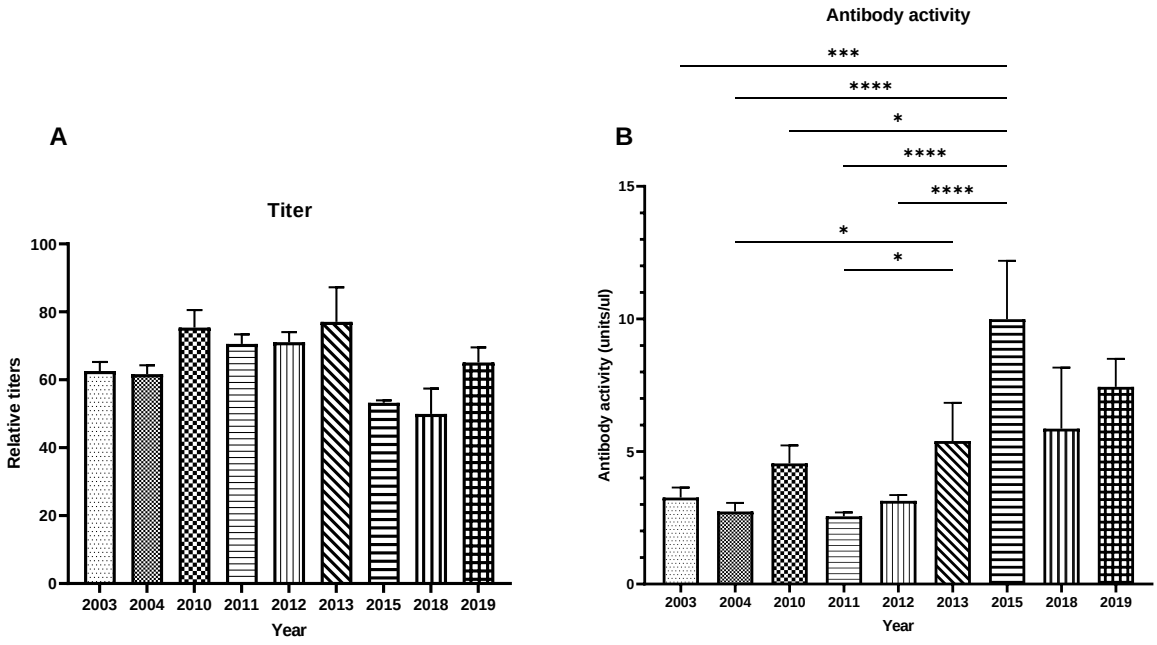

Figure 3.5. Enzyme-linked immunosorbent assay (ELISA) for antibody titers and antibody activities against *S. agalactiae* from all bottlenose dolphin samples. (A) relative antibody titers at a dilution of 1:200 and (B) antibody activities. No significant differences were seen between years of collection for relative titers. Statistical analysis indicated significant differences between antibody activities against *S. agalactiae* from sera collected in 2003, 2004, 2010, 2011, 2012, 2013, and 2015. *Indicates statistical significance of <0.05; *** indicates statistical significance of <0.001; **** indicates statistical significance of <0.0001.

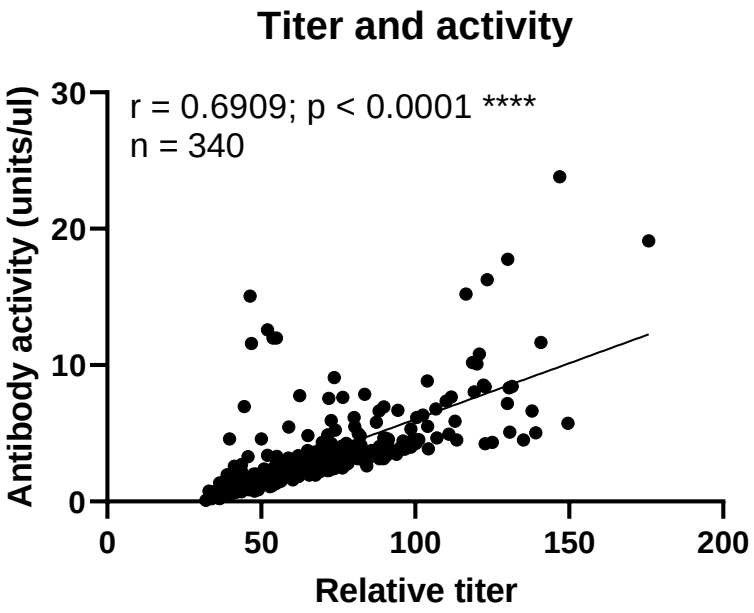

Figure 3.6. Simple correlation analysis of relative titers to antibody activities for all bottlenose dolphin populations. There was a significant correlation between antibody activity and relative titers for all dolphins sampled **(**Charleston Harbor, Indian River Lagoon, Georgia Aquarium, and Marine Mammal Program).

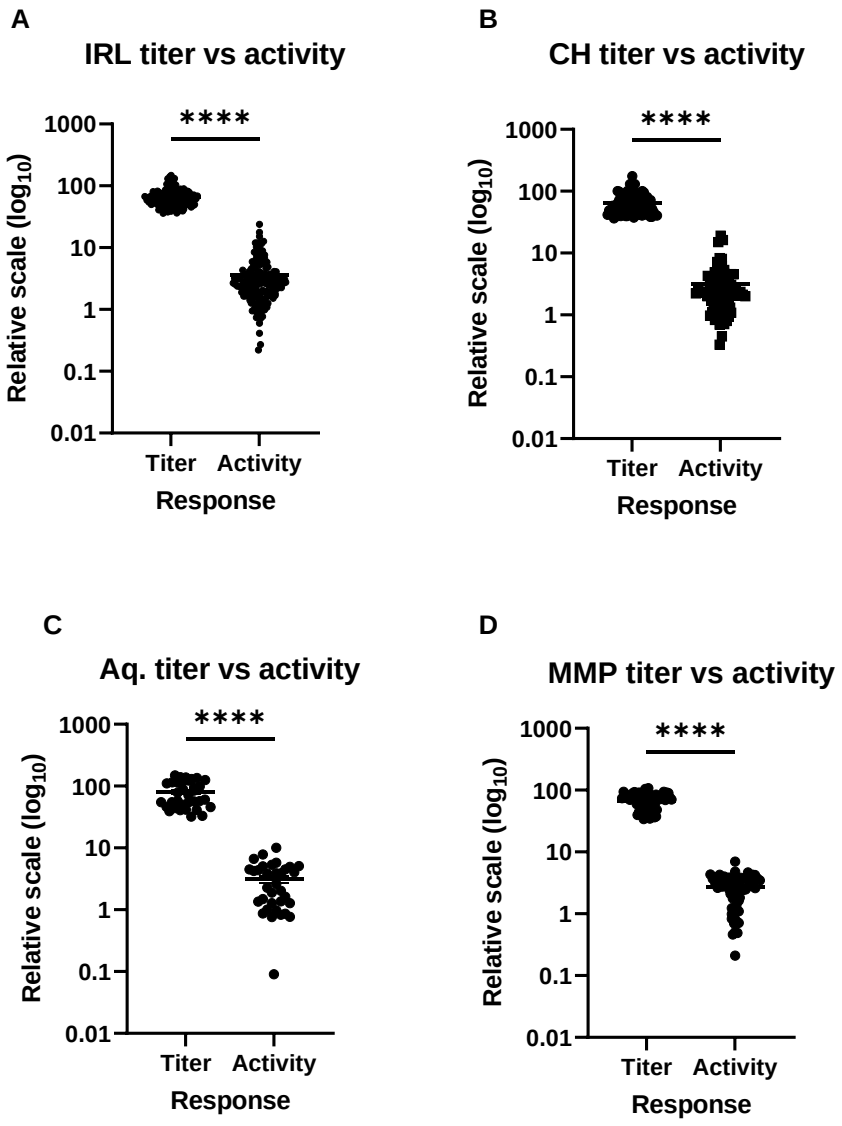

Figure 3.7. Relative titer and antibody activities in bottlenose dolphins in wild populations (Charleston Harbor (CH), Indian River Lagoon (IRL)) and managed-care (Georgia Aquarium (Aq.) and Marine Mammal Program (MMP). Significant differences between the mean relative titer and mean activities were found for the IRL (A), CH (B), Aq. (C) and MMP (D). **** indicates statistical significance of <0.0001.

Correlation analyses were also completed to determine if the same observed trends for all dolphins held true in these different populations. Dolphins from the Indian River Lagoon have a strong correlation ($r = 0.7239$) with a significant relationship between relative titer and antibody activity ($p < 0.0001$, $n = 162$; **Figure 3.8A**). Charleston Harbor dolphins have a strong correlation ($r = 0.7503$) with a significant relationship between relative titer and antibody activity ($p < 0.0001$, $n = 80$; **Figure 3.8B**). Dolphins that are kept in managed-care at the Georgia Aquarium have the strongest correlation ($r = 0.8624$) with a significant relationship between relative titer and antibody activity ($p < 0.0001$, $n = 34$; **Figure 3.8C**). Lastly, the other population of managed-care dolphins, MMP, also demonstrated a strong correlation ($r = 0.7087$) with a significant relationship between relative titer and antibody activity ($p < 0.0001$, $n = 63$; **Figure 3.8D**). These data indicate that on the population level, titers are indicative of antibody activities and vice versa.

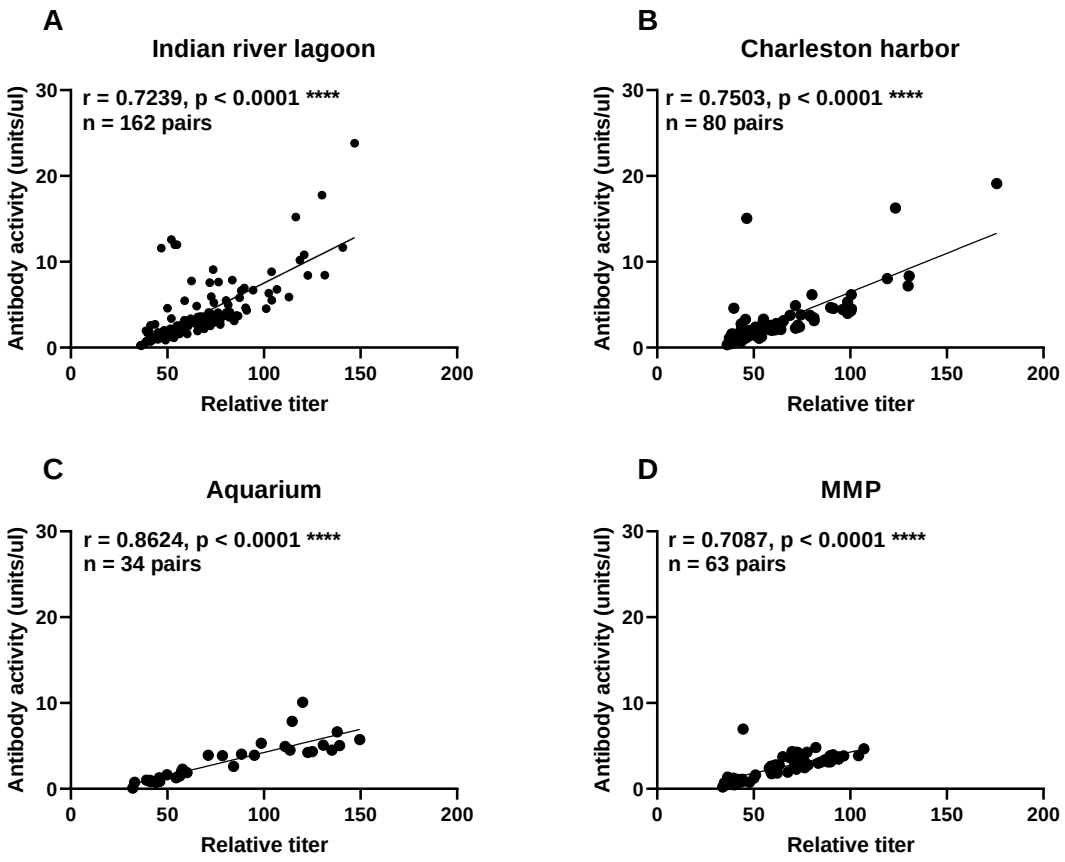

Figure 3.8. Simple correlation analysis of relative titers to antibody activities for bottlenose dolphins in wild populations (Charleston Harbor (CH), Indian River Lagoon (IRL), and managed-care (Georgia Aquarium (Aquarium) and Marine Mammal Program (MMP). Significant correlations were found between relative titers and antibody activities in bottlenose dolphins from the IRL (A), CH (B), Aq. (C) and MMP (D). The relationship between relative titers and activities was significant in all four populations ($p < 0.0001$).

3.3.3 ELISA results for the different populations across sampling dates

Statistical analysis of serum titer levels against *S. agalactiae* indicated that there are no significant differences between titer levels collected at different time points for dolphins in the wild (IRL (**Figure 3.9A**) and CH (**Figure 3.9B**) and managed-care dolphins (Georgia Aquarium (**Figure 3.9C**) and MMP (**Figure 3.9D**). Observable differences were seen in titer levels for dolphins in managed care. Dolphins from the Georgia Aquarium appear to have variable titers, whereas the MMP dolphins are more clustered together.

There were significant differences ($p < 0.05$, $p < 0.01$, $p < 0.001$) for serum antibody activity against *S. agalactiae* from sera collected in July of 2015 compared to July 2003, June 2004, June 2010, June 2011, and June 2012 for dolphins in the IRL (**Figure 3.10A**). There were no statistically significant differences for CH dolphins (**Figure 3.10B**) and managed-care dolphins (Georgia Aquarium (**Figure 3.10C**) and MMP (**Figure 3.10D**). Similar to serum titer levels, observable differences were seen in antibody activities for dolphins in managed care. Dolphins from the Georgia Aquarium appear to have variable antibody activities, whereas the MMP dolphins are clustered together.

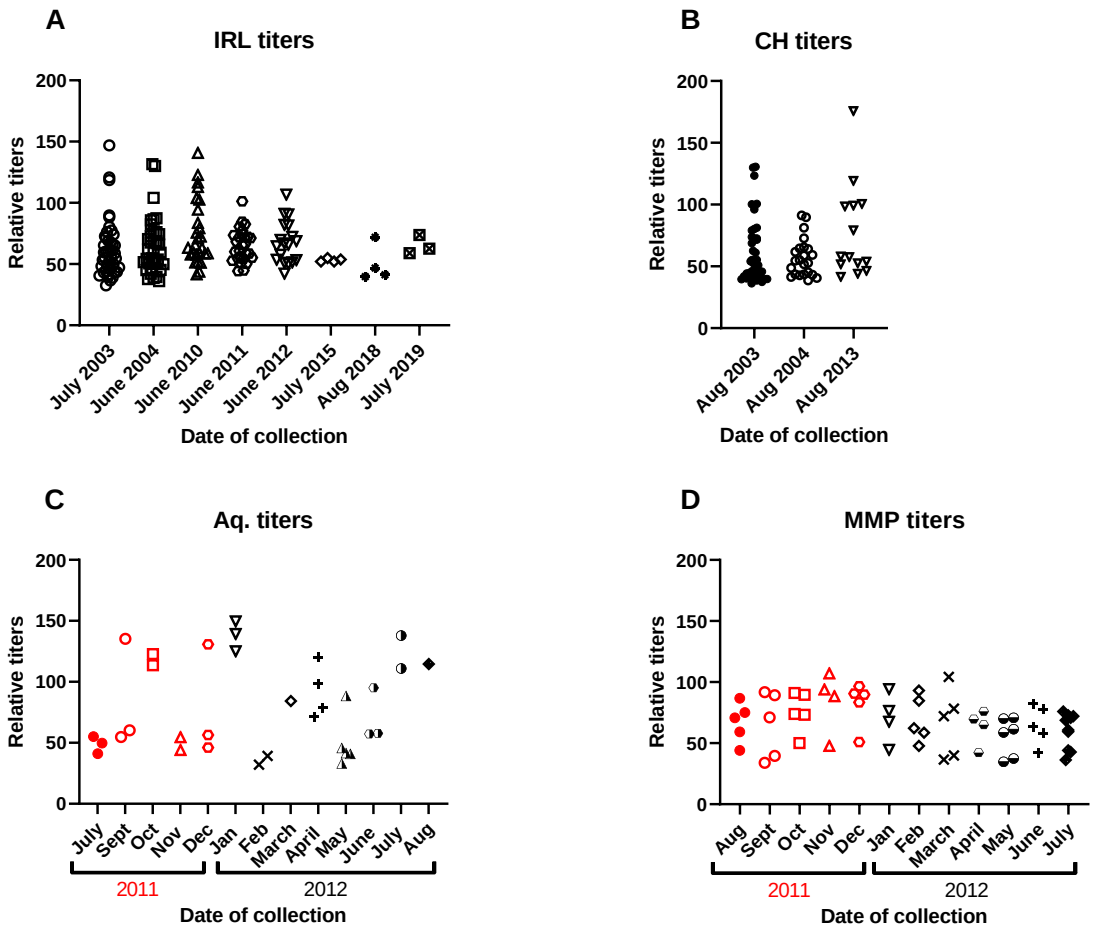

Figure 3.9. Enzyme-linked immunosorbent assay (ELISA) of relative titers against *S. agalactiae* in bottlenose dolphins from wild populations (Charleston Harbor (CH), Indian River Lagoon (IRL)) and managed-care (Georgia Aquarium (Aquarium) and Marine Mammal Program (MMP). There were no significant differences observed between sampling dates for dolphins from the IRL (A), CH (B), Aq. (C), and MMP (D).

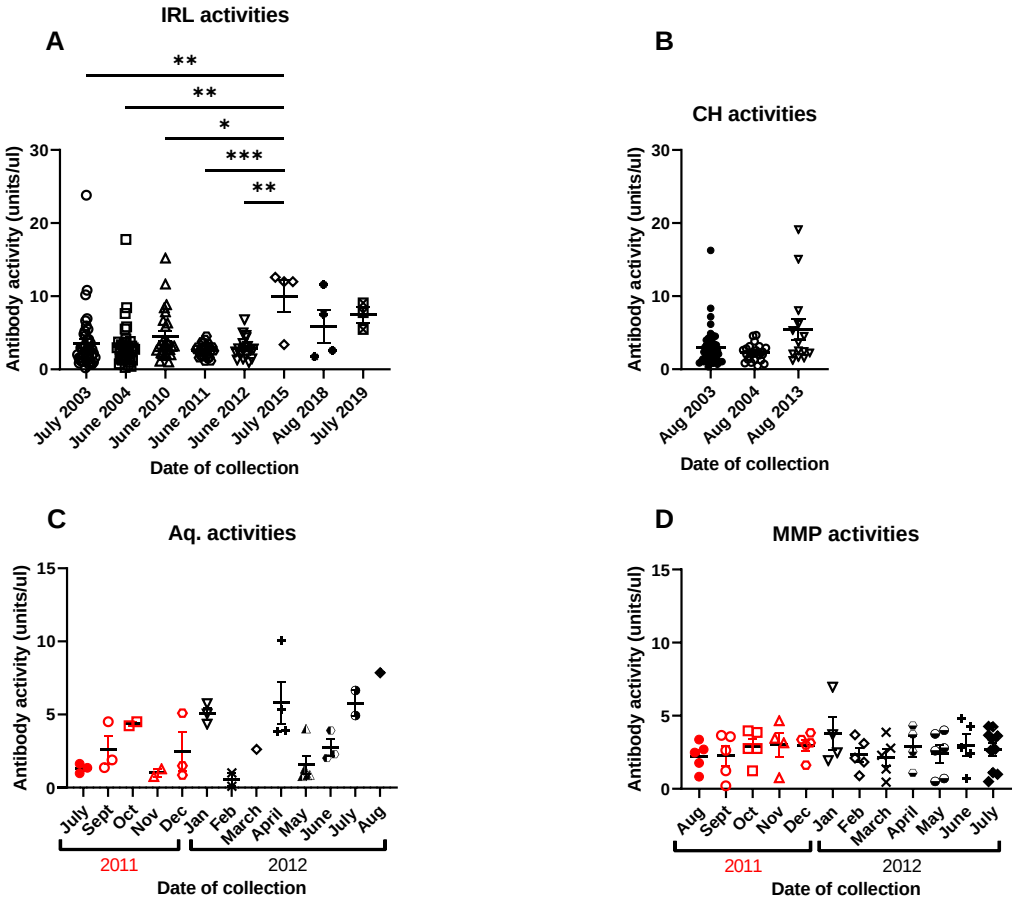

Figure 3.10. Enzyme-linked immunosorbent assay (ELISA) antibody activities against *S. agalactiae* in bottlenose dolphins from wild populations (Charleston Harbor (CH), Indian River Lagoon (IRL)) and managed-care (Georgia Aquarium (Aquarium) and Marine Mammal Program (MMP). There were significant differences observed between 2003, 2004, 2010, 2011, 2012, and 2015. There were no significant differences observed between sampling dates for dolphins from the CH (B), Aq. (C), and MMP (D). *Indicates statistical significance of <0.05; ** indicates statistical significance of <0.01; *** indicates statistical significance of <0.001.

3.3.4 ELISA results for Charleston Harbor and Indian River Lagoon in 2003 and 2004

As previously mentioned, prior work with these serum samples specifically investigated immune responses to other common marine bacteria and compared CH and IRL dolphins during 2003 and 2004. These years and locations were also investigated for this study. Statistical analyses of serum titer and antibody activity results against *S. agalactiae* in the CH and IRL did not indicate significant differences between sampling locations and years for titers (**Figure 3.11A**) and antibody activities (**Figure 3.11B**).

Differences in relative titers and antibody activities between IRL, Georgia Aquarium, and MMP dolphins for 2011 and 2012 were also investigated. Statistical analyses demonstrated significant differences for managed-care dolphins in 2012 ($p < 0.05$; **Figure 3.12A**). No significant differences were observed for serum antibody activity against *S. agalactiae* from sera collected in IRL and managed-care dolphins (**Figure 3.12B**).

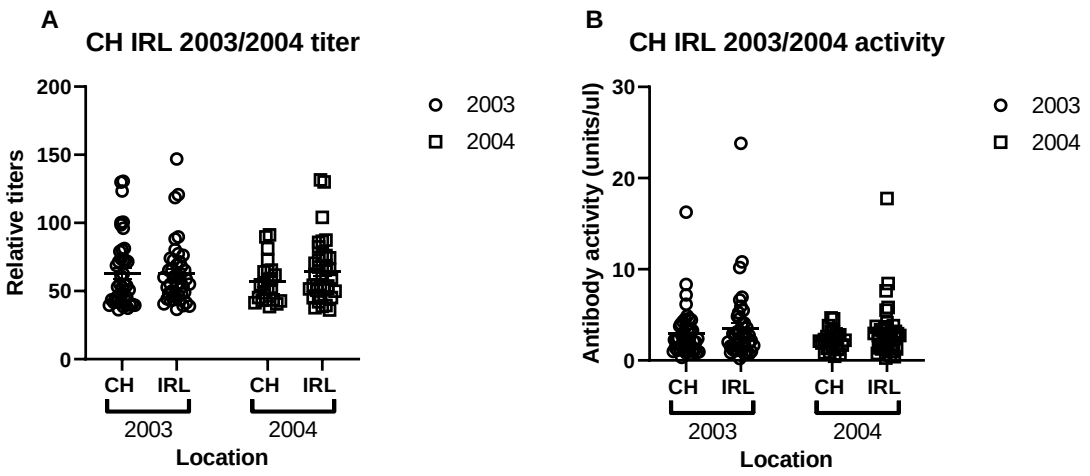

Figure 3.11. Enzyme-linked immunosorbent assay (ELISA) for relative antibody titers and antibody activities against *S. agalactiae* in the Charleston Harbor and Indian River Lagoon in 2003 and 2004 results No significant differences were observed between sampling locations and years for titers (A) and antibody activities (B).

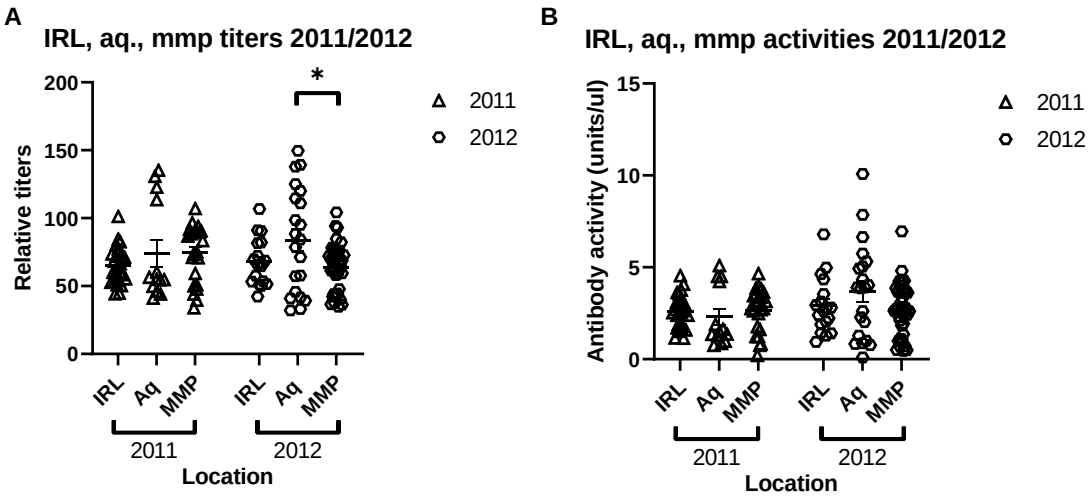

Figure 3.12. Enzyme-linked immunosorbent assay (ELISA) of relative antibody titers and antibody activities against *S. agalactiae* in the Indian River Lagoon (IRL), Georgia Aquarium (Aq), and Marine Mammal Program (MMP) in 2011 and 2012. Significant differences were observed between aquarium and MMP serum titers (A) in 2012. No other significant differences were observed between sampling locations and years for titers and antibody activities (B). * indicates statistical significance of <0.05.

3.3.5 ELISA results for wild and managed care populations across sampling dates

Differences between sampling dates for each population were investigated. Charleston harbor dolphins had no statistically significant differences between serum titer levels and serum antibody activity for different sampling years (**Figure 3.13**). Dolphins sampled from the IRL had no statistically significant differences observed for serum titer levels across sampling years (**Figure 314A**). However, there were statistically significant differences ($p < 0.05$, $p < 0.01$, $p < 0.001$) for serum antibody activity for 2015 compared to 2003, 2004, 2010, 2011, and 2012 in dolphins from the IRL. This trend was also observed for the serum antibody activities of all dolphins sampled (**Figure3.15B**). Managed-care dolphin from the Georgia aquarium serum titer level trends were observed. Both serum titer level (**Figure 3.15A**) and serum antibody activity (**Figure 3.15C**) demonstrate two different groups. Roxy, Pebbles, Sunny, Alvin, and Phebe have higher serum titer levels and antibody activities than the other individuals at the aquarium. MMP dolphins have more tightly clustered serum titer levels (**Figure 3.15B**) and antibody activities (**Figure 3.15D**) across sampling dates.

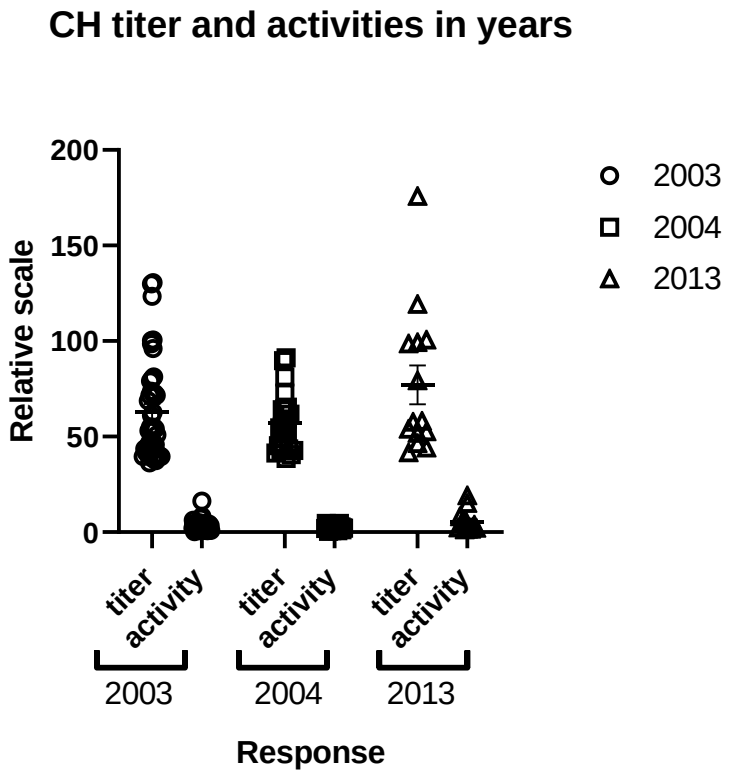

Figure 3.13. Enzyme-linked immunosorbent assay (ELISA) for relative titers and antibody activities against *S. agalactiae* in dolphins from the Charleston Harbor in 2003, 2004, and 2013. No significant differences were observed between titer-titer or activity-activity comparisons for all three years.

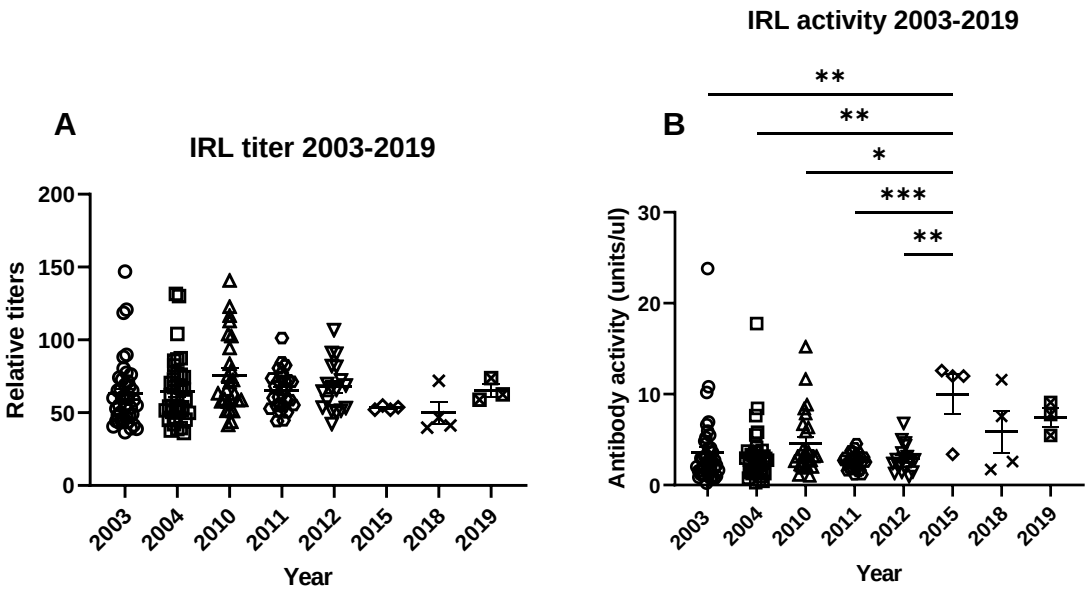

Figure 3.14. Enzyme-linked immunosorbent assay (ELISA) for antibody titers and antibody activities against *S. agalactiae* from the Indian River Lagoon by year. No significant differences were observed for relative titers(A) between years. Significant differences were observed between Indian River Lagoon (IRL) bottlenose dolphin activities (B) from the years 2003, 2004, 2010, 2011, 2012, and 2015. *Indicates statistical significance of <0.05; ** indicates statistical significance of <0.01; *** indicates statistical significance of <0.001.

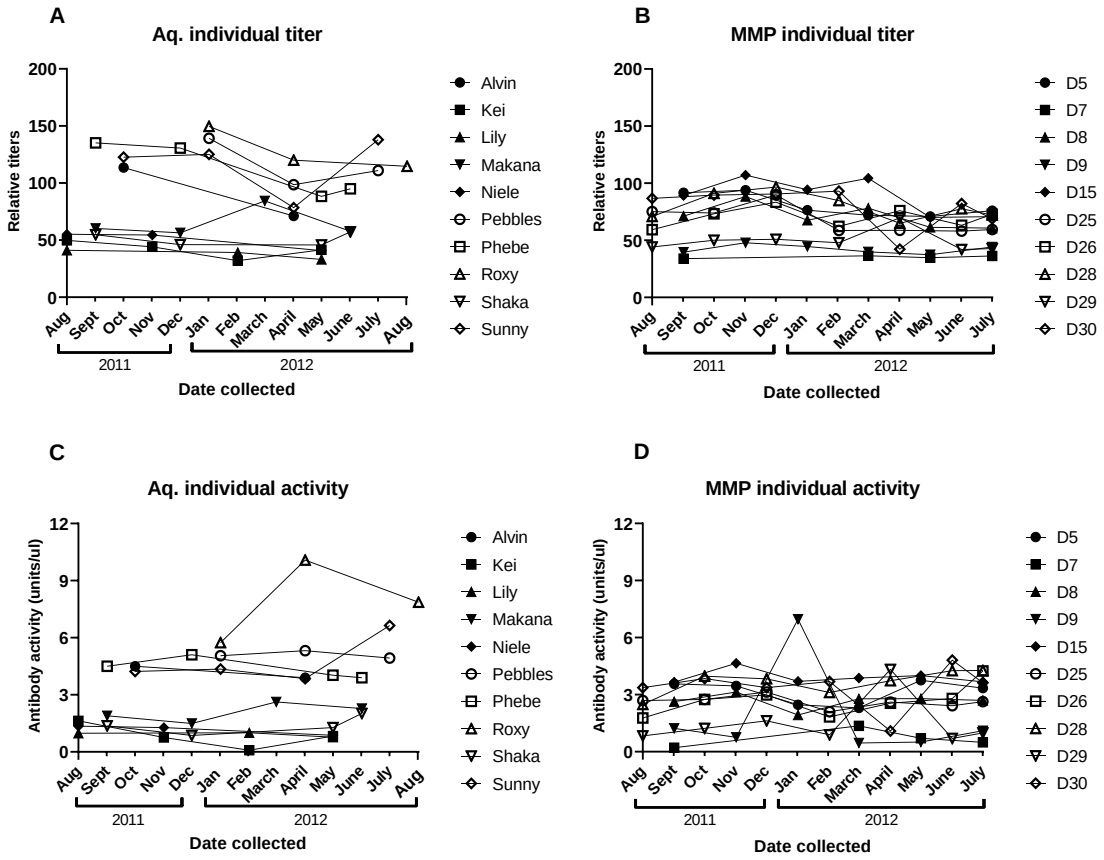

Figure 3.15. Enzyme-linked immunosorbent assay (ELISA) for antibody titers and antibody activities against *S. agalactiae* in managed-care dolphins by month/year results. The Georgia Aquarium (Aq.) dolphins were observed to have two distinct groups for both relative titers (A) and antibody activity (B). Likewise, the Marine Mammal Protection (MMP) dolphins also demonstrate two different groups for relative titers (B) and antibody activities (D).

3.3.6 ELISA results in CH (2011), IRL (2013), and GA Aquarium and MMP (2011-2012)

Lastly, the serum titer and antibody activity sampling data results against *S. agalactiae* were compared for bottlenose dolphins in wild populations in 2011 and 2013 and managed-care populations in 2011 and 2012. There were no statistically significant differences in serum titer levels between populations (**Figure 3.16A**). There were statistically significant differences ($p < 0.05$, $p < 0.01$, $p < 0.001$) in serum antibody activity between CH dolphins and the IRL, Georgia aquarium, ad MMP dolphins (**Figure 3.16B**).

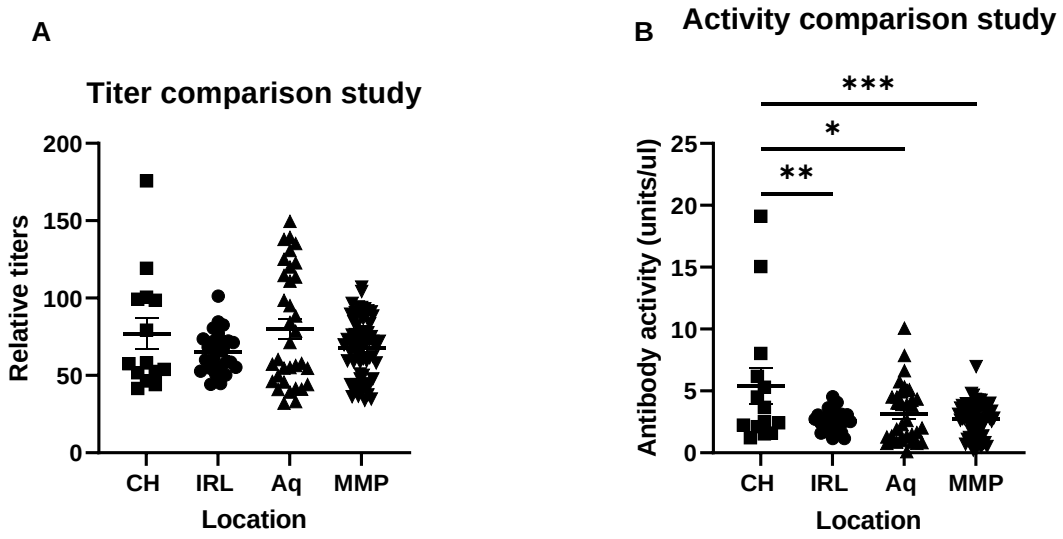

Figure 3.16. Enzyme-linked immunosorbent assay (ELISA) for antibody titers and antibody activities against *S. agalactiae* in bottlenose dolphins from wild populations (Charleston Harbor (CH), Indian River Lagoon (IRL)) and managed-care in 2011 and 2012 (Georgia Aquarium (Aquarium) and Marine Mammal Program (MMP) in 2011 and 2013. No significant differences were observed between sampling data and populations for relative titers (A). Significant differences were observed for antibody activities (B) between MMP, Aq., IRL, and CH populations. *Indicates statistical significance of <0.05; ** indicates statistical significance of <0.01; *** indicates statistical significance of <0.001.

3.4 Discussion

Upon examination of the SDS-PAGE and Western blot from the immunoprecipitation assay, it is clear that there are proteins from *S. agalactiae* that interact with bottlenose dolphin IgG. This suggests that purified IgG from bottlenose dolphin serum is binding to proteins from *S. agalactiae* lysate. The Western blot was probed with anti-bottlenose dolphin IgG (KA-6), which is specific for the heavy chain of bottlenose dolphin IgG. The banding patterns present on the blot are consistent with the expected molecular weights of the heavy chain of IgG. However, it is important to note that a degradation product appears just under the band of IgG. The degradation product has been consistent across all screening blots throughout this study and others (Nollens et al., 2007). Two other dominant proteins were also observed and had molecular weights around 76 kDa and 13 kDa. Based on their molecular weights, we can confirm that they are not bottlenose dolphin IgG or IgM proteins. It is likely that these proteins are from *S. agalactiae* and are proteins that are involved in the immune response to this pathogen in dolphins. Proteomics work should be conducted to identify these unknown and potentially key proteins further. Future work should be focused on carrying out proteomics on these proteins. If key immunogenic proteins are identified, they can serve to generate a vaccine against *S. agalactiae.*

There were no significant differences seen between years of sampling for all bottlenose dolphin samples. However, there were statistically significant differences for serum antibody activity against *S. agalactiae* between sera collected in 2003, 2004, 2010, 2011, 2012, 2013, and 2015 for all dolphins sampled. Significant differences were

observed in serum titers and antibody activity for all bottlenose dolphins and bottlenose dolphin samples divided into sampling populations. There was also a strong correlation and significant relationship between serum titers and antibody activity for all dolphin samples and bottlenose dolphins divided into their sampling populations. This demonstrates that serum titers and antibody activities may be indicative of one another. This could be useful in future experiments as antibody activity is a laborious index to determine, whereas serum titers are more feasible and quicker. However, antibody activity could provide more information when serum titers do not differ significantly.

Serum titers against *S. agalactiae* did not differ statistically for wild bottlenose dolphin populations (IRL and CH) and managed-care dolphins (Aquarium and MMP). There were significant differences for serum antibody activity against *S. agalactiae* from sera collected in July of 2015 compared to July 2003, June 2004, June 2010, June 2011, and June 2012 for dolphins in the IRL. This increase could be attributed to the poor water quality that is known for this area. The Indian River Lagoon had reports of an algal bloom during the middle of 2015, which affected many portions of the lagoon. These algal blooms are due to sewage and anthropogenic inputs. However, the sample size is small (n =4). There were no statistically significant differences between CH dolphins and managed-care dolphins (Georgia Aquarium and MMP). The dolphins from the Georgia Aquarium were more variable for both serum titers and antibody activity over the different collection time points in contrast to the MMP dolphins, which were more consistent. These differences observed between managed-care dolphin populations are likely due to differences in habitat. Georgia aquarium dolphins are kept in different

interconnected pools. The MMP dolphins are kept in a netted open water enclosure in the San Diego Bay are exposed to local conditions of the bay (Fair et al., 2017). Also, these dolphins were sampled at different time points throughout the year rather than being sampled once like the CH and IRL populations.

The wild bottlenose dolphin populations in the Charleston Harbor, SC, and Indian River Lagoon, FL, have been of focus to researchers for many years in part of a collaborative effort to assess the overall health of these two regions (Fair and Bossart, 2005). Many studies have been conducted investigating the immune responses to pathogens and the overall health of these organisms that were sampled in 2003 and 2004 (Fair et al., 2007; Goldstein et al., 2006; Reif et al., 2009). To date, no studies have been conducted investigating the immune responses in the serum samples to *S. agalactiae.* Statistical analyses did not demonstrate any significant differences in 2003 and 2004 between bottlenose dolphin serum titers or antibody activity from individuals from the CH or IRL. However, dolphins were seropositive against *S. agalactiae,* indicating exposures high enough to induce an immune response to this pathogen.

Comparisons between IRL and managed-care bottlenose dolphins for the years 2011 and 2012 were also made. In this case, the managed-care dolphins were to serve as a control. Statistical analyses demonstrated significant differences between the two managed care populations in 2012. These differences could be due to the difference in water present in their managed habitats. In addition, there are multiple samples taken from the same dolphins in the managed-care populations throughout the year. In contrast, wild dolphin samples were collected once from each organism during the

sampling period. There were no statistical differences observed between populations and years for bottlenose dolphin serum antibody activity.

Due to the extensive work that has been completed on these bottlenose dolphin samples over the dates of collection at CH and IRL, we wanted to look at all serum samples at these locations. Serum titer and antibody activity increased from 2004 to 2013, and there were no statistical differences between titer-titer and activity-activity relationships between 2003, 2004, 2013. The Charleston Harbor has been increasing in shipping activity and expanding ports. In 2010, plans for new cruise terminals were announced and finalized. In 2011, the Charleston Harbor Deepening Project began, and the port has seen increases in activity each coming year (South Carolina Ports, 2021). With increases in shipping activity, the harbor is likely to have an influx of ballast water from shipping vessels which has been documented to carry pathogens, including pathogenic Streptococcus species (Altug et al., 2012). The Indian River Lagoon bottlenose dolphin serum titers did not have statistically significant differences. However, serum antibody activity demonstrated statistically significant differences between 2015 and 2003,2004, 2010, 2011, and 2012. The antibody activity in 2015 was higher than in previous years. The sampling of the IRL bottlenose dolphin population was completed in July of 2015. In May of 2015, Hurricane Ana formed off the east coast of Florida bringing with it increased levels of rainfall. During periods of increased rainfall in which the sewer collection system could be exceeded, combined sewer overflow can result. It is possible that the combination of these two events introduced an increased amount of *S. agalactiae* and other bacteria into the marine environment.

The managed-care populations are kept in two different types of habitats while in captivity. As previously mentioned, the Georgia Aquarium dolphins are kept in three interconnected pools, and the MMP dolphins are kept in a netted area inside the San Diego Bay in California. The Georgia Aquarium dolphins are kept in consistent water parameters throughout the year, while the MMP dolphins are exposed to ambient water and air temperatures throughout the year. The serum titer levels against *S. agalactiae* in the aquarium dolphins appear to split the population into two separate groups. This is also consistent when observing serum antibody activity in aquarium individuals. Five individuals appear to have higher serum titer levels and antibody activities compared to the other five. These differences could be attributed to the life history of these dolphins, sex, age, and time spent at the aquarium. Three of the 10 Georgia Aquarium dolphins sampled came from the wild, and the remainder were born under human care (Fair et al., 2017). The MMP dolphins are more tightly clustered for both serum titer and antibody activity. Two dolphins in the MMP population were wild-caught while the others were all born in the MMP (Fair et al., 2017). This could be the reasoning for the tighter clustering and consistency of titer and antibody activity in the MMP population.

Previous research was conducted on bottlenose dolphin serum samples collected from the IRL population in July of 2011, CH population in August of 2013, the Georgia Aquarium, and MMP populations in 2011 through 2012(Fair et al., 2017). This study investigated immune and endocrine responses in these wild and managed care populations. Their findings suggested that the immune systems of wild dolphins were more upregulated than dolphins in managed care. However, their study did not include

immune responses to *S. agalactiae* (Fair et al., 2017). Using samples from those sampling years and populations to complement their previous work, no significant differences between serum titer levels for wild and managed populations were observed in this study. There were significant differences between serum antibody activity for the different bottlenose dolphin populations. The wild CH bottlenose dolphin serum antibody activity differed significantly and was greater when compared to the IRL, Georgia Aquarium, and MMP populations. These differences could be accredited to the amount of shipping and human traffic present in the Charleston Harbor, SC area. Pathogenic streptococci can be introduced to the marine environment by shipping traffic and ballast water (Altug et al., 2012). Also, animals such as seabirds (Nelson et al., 2008) and wastewater introduced through rivers and stormwater (Fries et al., 2008) have been suggested to be sources of streptococcus and other bacteria in the marine environment. The CH estuary is a very active seaport, surrounded by human development, and has three different rivers that flow in this estuary (Reif et al., 2008), which could all contribute to an increase *S. agalactiae* pathogen load.

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Chapter Four

Conclusion and Future Directions

The purpose of this research was to develop a monoclonal antibody against bottlenose dolphin (*Tursiops truncatus)* immunoglobulin G (IgG) to aid in the quantification of serum titers and antibody activities against *Streptococcus agalactiae.* The development of the monoclonal antibody against bottlenose dolphin IgG progresses the understanding of the secondary immune response in these organisms. In addition, the monoclonal antibody was employed in immunoprecipitation to determine specific protein-protein interactions between bottlenose dolphin IgG and *S. agalacatiae.* Lastly, the monoclonal antibody generated allowed for quantifying serum titers and antibody activities through the development of enzyme-linked immunosorbent assays.

In this study, three different monoclonal antibodies were successfully generated. The generated monoclonal antibody against bottlenose dolphin IgG (KA-6) recognizes the heavy chain of bottlenose dolphin IgG. The monoclonal antibodies created against bull shark IgM or spotted eagle ray IgM recognize the light chains of IgM in these species. These three antibodies could serve to be useful in furthering research to answer questions about marine organism health.

This study provides the first comparison of serum titers and antibody activity utilizing different marine vertebrates. The development of the different marine vertebrate monoclonal antibodies served useful in comparing serum antibody titers and antibody

activities in these organisms. Although the values for serum titers and antibody activity differ in terms of the range of numbers, not all titer and antibody activities differed significantly. A strong correlation was observed between bull shark serum titers and antibody activities. Due to this strong relationship, serum titers could give potential estimates for antibody activities due to the laborious process involved. Although the other three species investigated did not demonstrate strong relationships, it may be due to the low sample size during data collection. Furthermore, it appears that antibody activities are more sensitive than that of serum titers at 1:200 dilutions.

Immunoprecipitation utilizing a protein G column, bottlenose dolphin purified IgG, and *S. agalactiae* lysate demonstrated interactions between antibody and antigen proteins. The SDS-PAGE demonstrated four main proteins. Two of the protein bands observed were identified as the heavy (~55kDa) and light (~ 25 kDa) chains of bottlenose dolphin IgG. However, two bands of interest could not be identified by their molecular weights. To remedy this, these proteins should be sent off for sequencing to characterize them. Once identified and determined to be key immunogenic proteins against *S. agalactiae*, future efforts could be spent on developing a vaccine. It has been noted that many streptococci are opportunistic pathogens (Blyth et al., 2013; Díaz-Delgado et al., 2017; Louw and Tikly, 2007). As the marine environment becomes more contaminated with pollutants and pathogens, this could lead to weakened and immunocompromised bottlenose dolphins, increasing their susceptibility to infectious disease (Numberger et al., 2021). A vaccine could be useful to provide protection from future infections of *S. agalactiae* in bottlenose dolphin populations.

This study also provides baseline data for serum titer and antibody activity in the bottlenose dolphin against *S. agalactiae.* Previous research has found that bottlenose dolphin individuals have had large enough exposures to result in titers against many different common marine bacteria (Beck and Rice, 2003; Bossart et al., 2008; Bossart et al., 2019; Fair et al., 2017). These bacteria include *Erysipelothrix rhusiopathiae, Escherichia coli, Mycobacterium marinum, Vibrio carchariae, Vibrio cholerae, Vibrio parahemolyticus,* and *Vibrio vulnificus*. Previous studies have reported immune responses in relative titers at a 1:200 dilution (Bossart et al., 2019) and antibody activity (units/µL) (Beck and Rice, 2003). These are common bacteria, however, as human activities increase, more opportunistic bacteria such as *S. agalactiae* could begin to emerge and become more prevalent in the marine environment. Prior studies have not investigated immune responses to *S. agalactiae*. We measured serum antibody titers and antibody activities against *S. agalactiae* and found that dolphin samples were seropositive, demonstrating that these organisms have been exposed to quantities large enough to elicit an immune response. The relationship between serum antibody titers and antibody activities was also investigated, and we found there to be a strong correlation for all samples as well as each population. This allows for the conclusion that titers could indicate an approximate antibody activity and vice versa in bottlenose dolphins utilizing KA-6 and *S. agalactiae.* Future research should investigate other data trends with sex, age, and toxicant burden or exposure.

Wild dolphin populations are typically only sampled once. Any sample analysis of that serum sample only provides data for that specific point in time. In the case of

quantifying serum titers and antibody activity, it limits the researcher to that specific time point at serum collection. It would be worthwhile to sample the same wild dolphins over a period of time to further understand the immune response over time. With sampling conducted in that manner, we could observe to see if circulating antibodies or antibody activities increase or decrease for individuals. We could then compare that data to water quality data to determine if the toxicant or pathogen load of that water has increased or decreased. If the pathogen or toxicant load has increased in the marine environment, this could lead to dolphins and their immune systems being over inundated and possibly lead to immunosuppression. This could increase the chances for opportunistic infections of *S. agalactiae*. This could provide more insight into exposures to *S. agalactiae* and the likelihood of opportunistic exposures from *S. agalactiae*. Furthermore, although it would be laborious, it would be interesting to investigate serum titers and antibody activities to previously studied pathogens to determine if similar patterns and relationships are present.

Lastly, due to the abundance of bottlenose dolphin samples with a wide sampling date range and the generation of a monoclonal antibody against bottlenose dolphin IgG, it would be worthwhile to investigate serum antibody titers and antibody activities against the cetacean morbillivirus (CeMV). The CeMV is highly contagious, lymphotropic, and initially replicates in lymphoid tissue before infecting epithelial cells (Shimizu et al., 2013). In addition, there have been several mortality events in coastal *T. truncatus* populations from the Gulf of Mexico to the Atlantic coast of the United States since 1982 (Krafft et al., 1995; Taubenberger et al., 1996). Transmission of the morbillivirus is

thought to occur after the inhalation of airborne virus shed by cetacean individuals and is probable for cetaceans due to their sociable behavior and high density (Raga et al., 2008; Van Bressem et al., 1999). Immunoglobulin M (IgM), immunoglobulin G (IgG), and immunoglobulin A (IgA) have been confirmed to be critical constituents of the marine mammal immune system to identify and aid in the destruction of invading pathogens such as the CeMV (Gelain and Bonsembiante, 2019). From this study, we have successfully created a monoclonal antibody against bottlenose dolphin IgG, which is the major body of the secondary immune response which aids in the protection and immunity against future similar pathogens (Zimmerman et al., 2010). In the future, we could quantify serum antibodies against the CeMV to gain more information about bottlenose dolphin memory immune responses and potential exposure events and associated locations to this harmful pathogen.

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