ECOSYSTEM HEALTH AND ENVIRONMENTAL INFLUENCES ON INNATE IMMUNE FUNCTION IN THE LOGGERHEAD (*CARETTA CARETTA*) AND GREEN (*CHELONIA MYDAS*) SEA TURTLE

by

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A Thesis Submitted to the Faculty of

The Charles E. Schmidt College of Science

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Florida Atlantic University

Boca Raton, Florida

December 2014

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Sarah L. Milton, Department of Biological Sciences, and has been approved by the members of her supervisory committee. It was submitted to the faculty of The Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ACKNOWLEDGEMENTS

I wish to thank the many contributors that made this research possible: Dr. Lew Ehrhart and Dr. Kate Mansfield of the University of Central Florida and their indefatigable crew for supplying most of the samples used in this study; to Mike Bressette and the Inwater Research Group for samples from the St. Lucie Power Plant and to Dr. Kirt Rusenko and the veterinary staff at Gumbo Limbo for retrieving blood "on the fly."

My sincere gratitude to Greg Bossart who's "tank side manner" inspired my research path which led me to the Physiology lab of Peter Lutz and Sarah Milton, and later to Patricia Keating and James Hartmann - their contributions throughout this endeavor have been innumerable.

Lastly, I would like to extend heartfelt gratitude to my family for all of their patience and support through my latest metamorphosis and to Rene Varela, my friend and partner in sea turtle immunosuppression, a big long distance hug. And to Olena, for patiently teaching me Sigma Plot and the intriguing conversations about life, the universe, and everything!

ABSTRACT

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Title: Ecosystem Health and Environmental Influences in Innate Immune Function In The Loggerhead (*Caretta Caretta*) and Green (*Chelonia Mydas*) Sea Turtle

Thesis Advisor: Dr. Sarah L. Milton

Degree: Master of Science

Year: 2014

Loggerhead (*Caretta caretta*) and green (*Chelonia mydas*) turtles recruit to nearshore environments as juveniles. These often degraded habitats are associated with emerging diseases such as green turtle fibropapillomatosis (GTFP), however there are few studies on immune function in sea turtles. The objective of this research was to quantify phagocytosis of the innate immune system by flow cytometry and compare levels between animals from a degraded habitat (the Indian River Lagoon, FL) to a more pristine environment (the Trident Basin, Port Canaveral, FL), and across a range of temperatures. While *in vitro* temperatures did not alter rates of phagocytosis, it was higher in samples obtained in the summer than winter. Rates of phagocytosis in sea turtles with GTFP and from degraded environments with increased prevalence of GTFP were low compared to animals from the more pristine environment, suggesting that the environment can alter innate immunological function and thus contribute to the development of disease.

DEDICATION

This manuscript is dedicated to Peter L. Lutz, who even now compels me to question these remarkable marine reptiles. He was a Scotsman, a physiology teacher, and a mentor who enjoyed gamboling. You and your sense of humour are missed.

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INTRODUCTION

Sea turtles are remarkable, long- lived, air- breathing reptiles that have occupied the marine realm for over 100 million years. Sea turtles migrate thousands of miles to inhabit different environments during their life histories. Every species of sea turtle commences its life history on a natal beach, after a 60 – 80 day incubation period (depending upon egg chamber temperatures). Hatchlings then go into a "frenzy" period whereby the yolk supplies the necessary energy and nutrients to propel them through a barrage of predators to a developmental habitat in the Sargasso sea; there they will traverse the oceanic gyres for an unknown period of time called the "lost years" (Carr, 1952). Immature turtles then return to coastal shallow waters where they forage in relatively safe habitats such as lagoons and estuaries. Two species of sea turtles are known to migrate to Florida's coastal waters in order to feed as juveniles averaging 45cm in length: the green turtle (*Chelonia mydas*) and the loggerhead (*Caretta caretta*). The juveniles then leave the developmental habitat moving to other (pelagic) habitats before maturation (Ehrhart, 1983).

Sea turtles reach maturity at approximately 20 -35 years of age depending on the species and occupy specific ecological niches depending on their diet. Adults return to natal beaches to breed off shore and the life history is repeated.

As sea turtles are intrinsically bound to coastal habitats during several stages of their life histories, ecosystem health may play an integral physiological role in their survival. While Florida's climate is ideal for sea turtles, it also attracts millions of

endotherms, namely, humans. Thus, terrestrial and marine ecosystems have been impacted by anthropogenic factors such as habitat destruction and fragmentation, urban development, and inundation by pollutants and contaminants. Nearshore habitats pose numerous anthropogenic threats to sea turtles such as agricultural run-off, carcinogenic pollutants, polychlorinated biphenyls (PCBs), harmful algal blooms and accumulation of inorganic compounds such as nitrogen and phosphorus (Bossart, 2011; Durden et al., 2009). Each of these ecological stressors influences species interactions and ecosystem health.

Of increasing concern is how anthropogenic impacts affect the emergence of infectious disease within Florida's coastal wildlife populations. The long term consequences of agricultural run-off, industrial waste and increasing human populations along our coasts are likely contributing factors for emerging diseases for those species that recruit to or reside in near shore habitats (Bossart, 2011). In Florida's Indian River Lagoon (IRL), examples include Atlantic bottlenose dolphins (*Tursiops truncates*) that have been diagnosed with Lobomycosis (*Lacazia loboi*), a fungal disease characterized by multifocal white nodules while fish species appear to have lesions with destruction of flesh. These lesions are associated with a toxic fungus, *Aphanomyces invadens*. The favorable environmental conditions necessary for these fungal agents to proliferate include decreased salinity in the case of *A. invadens* and an overabundance of nitrogen in the case of Lobomycosis (Durden et al., 2009). In fact, disease states in turtles have prompted some researchers to consider sea turtles as biological indicators of the overall health of the environment as they exhibit tremendous site fidelity throughout their life histories (Aguirre and Lutz, 2004).

As in the IRL, many of these emerging diseases are associated with degraded habitats; abiotic components within the marine ecosystem may facilitate novel disease epidemics and compromise immune function in marine organisms (Mydlarz et al., 2006). These factors create a challenge in determining etiological agents of disease as the driving environmental stressor(s) may act synergistically to compromise immune function in the host. For example, increased nitrogen levels have recently been linked to green turtle fibropapillomatosis (GTFP) in Hawaii (Van Houtan et al., 2010). Excessive nitrogen loads can alter plant composition of an ecosystem, generally displacing native species and allowing recruitment of non-native and/or invasive species. Van Houtan et al. (2010) found that nonnative macroalgae have become so dominant in the nearshore habitats of Hawaii, that it has become the predominant diet of the green turtle (*Chelonia mydas*) (Van Houtan et al., 2010). Additionally, plants in high nutrient areas store excessive nitrogen in the form of arginine, an amino acid essential for viral growth and replication (Van Houtan et al., 2010). Blood biochemistry values were also investigated from Hawaiian juvenile green turtles: two healthy wild populations compared to turtles afflicted with GTFP (Aguirre and Balazs, 2000). Turtles with moderate to severe tumor loads exhibited increased levels of blood urea nitrogen (BUN) compared to turtles with minimal tumor loads. Therefore, abiotic factors, such as nitrogen are significantly correlated to land use as an environmental stressor and disease expression in green turtles (Aguirre and Balazs, 2000; Van Houtan et al., 2010). Juvenile green sea turtles often forage in degraded estuarine systems, and may lack the physiological competence to combat pathogenic burdens. The environment could thus play a significant role in the immunological function (or lack thereof) within the population (Aguirre and Lutz, 2004).

Pollutants such as organochlorides (OC) and PCBs may also make sea turtles more vulnerable to disease by altering immune responsiveness. Keller et al. (2006) postulated that organochlorides may modulate immune function in loggerhead (*Caretta caretta*) turtles, as they found that OC exposed peripheral blood leukocytes showed an enhanced immune response to mitogenic stimulation. They suggested that this increased immune effect was related to a hypersensitivity to the contaminant (Keller et al., 2006). As this result was unexpected, the authors conclude that any type of disruption to the immune response, either compromised or enhanced, may have negative effects. Exposure to pesticides such as OCs and PCBs have been found to depress immune function in sea turtles (Keller et al., 2004) and other aquatic vertebrates such as the Brown bullhead (*Ameiurus nebulosis*) (Iwanowicz et al., 2009). Brown bullhead fish exposed to Aroclor 1248 (A1248), a contaminant found in their natural habitat, exhibited significantly decreased respiratory oxidative burst activity. After 3 weeks of exposure to the contaminant, leukocytes were extracted from the kidneys and assessed for their ability to destroy a known salmoniid pathogen, *Yersnia ruckeri*. Results indicated that functional immune responses in contaminated bullhead were reduced after A1248 exposure as bactericidal activity decreased by 10 – 15% when compared to those fish that served as the controls (Iwanowicz et al., 2009).

And as green and loggerhead turtles spend their early years in bays and lagoons (Ehrhart, 1983), these animals have been shown to be exposed to higher nutrient pollutant burdens than those living in areas of less anthropogenic impact. Evidence has been found linking near shore foraging habitat of Hawaiian green turtles and the occurrence of toxic dinoflagellates, particularly from the genus *Prorocentrum*. Members of this group are

known to produce okadaic acid, which is a tumor-promoting toxin that has been found in tissue samples from turtles afflicted with GTFP (Landsberg et al., 1999). Additionally, there are known correlations between near-shore residency and increase in incidence of disease in marine mammals such as the West Indian manatee (*Trichechus manatus*) and the Atlantic bottle-nose dolphin (*Tursiops truncates*) that inhabit degraded ecosystems. In an investigation involving 13 stranded dolphins from the IRL, pathological results indicated the dolphins died from infectious bacterial diseases including pneumonia and meningitis (Bossart et al., 2003).

Specific Diseases

Sea turtles worldwide are faced with numerous stressors, including predation, parasites, habitat degradation and loss, and debilitating diseases. Two diseases in particular affect sea turtle populations: loggerhead populations have been plagued by "Lethargic Loggerhead Syndrome" (LLS) where stranded turtles are found listless, anemic and/or moribund, while juvenile green turtles increasingly suffer from fibropapilloma disease (GTFP) characterized by multiple debilitating though benign tumors. As these tumors reach up to 30cm in diameter, they increase the difficulty of foraging for food, avoiding predators, and increase the probability of entanglement and drowning in nets (Milton and Lutz, 2003; Witherington and Ehrhart, 1989). The juvenile turtles that are found washed up onshore are often emaciated and anemic. A concurrent investigation conducted in Hawaii and the IRL (1998-2000) revealed that hematocrit values were lower in green turtles with GTFP and their lymphocytes exhibited an inability to proliferate *in vitro* when stimulated with various mitogens (phytohemagglutin (PHA), Concanavalin A (ConA) known T-lymphocyte mitogens and lipopolysaccharide

(LPS) a known B-lymphocyte mitogen (Lutz et al., 2001; Work et al., 2000). Results indicate that green turtles with GTFP are immunocompromised from these habitats.

While GTFP appears to affect juvenile greens more than any other age class, and LLS appears to target juvenile loggerheads, neither the causes of these diseases nor their cures are known. The most likely etiological agent for GTFP is a novel, cheloniid herpes virus, fibropapilloma-associated turtle herpesvirus (FPTHV). Viral particles from a herpes virus were found in more than 95% of the DNA from tumor tissues in a study of Florida green turtles (Herbst, 1994) Several investigators have also found viral particles which were purified from fibropapillomas in order to analyze and map the genome of another novel virus, sea turtle tornovirus 1 (STTV1), a single stranded DNA virus that the authors suggest may have coinfection capabilities along with FPTHV (Ng et al., 2009). Of the 27 turtles with FP in that study, only 2 were STTV1 positive (Ng et al., 2009).

Viruses are the most numerous biological agents in the ocean and it was proposed that the virus associated with FP has mutated from a previously innocuous virus (Furhman, 1999; Herbst et al., 2004). However, recent sequencing of the chelonid fibropapilloma-assoiciated herpes virus genome has shown that it is most closely related to a non-oncogenic herpes virus responsible for lung-eye-throat disease also found in sea turtles (Herbst et al., 2004). This result lends to the hypothesis that the increase of fibropapillomatosis is due to an environmental factor(s) that may induce increased susceptibility to the disease (Herbst et al., 2004; Jones, 2004).

As there are a multitude of biotic and abiotic factors that are associated with GTFP, it is clear that our understanding of disease in sea turtles is limited by our lack of basic knowledge of sea turtle physiology and immune function.

Along with GTFP and LLS, sea turtles are subjected to numerous other bacterial and viral diseases (George, 1997). However, our ability to treat disease is hampered by the fact that scientists and veterinarians know little about the immune function of sea turtles and the response to environmental stressors such as temperature, inadequate food supply and anthropogenic burdens such as agricultural run-off (Lutz et al., 2001). It is widely known that immune function varies with age, sex and nutritional status of the animal (Borysenko and Lewis, 1979; Casal et al., 2009; De Guise et al., 1995; Duguy, 1970; Fournier et al., 2000; Keller et al., 2005; Pienaar, 1962). Some diseases, including GTFP and LLS, appear to affect juveniles more than any other age class, though this variable may be due to habitat preference.

Juveniles or those in poor body condition (malnourished or heavy parasite loads) also may be less responsive to immune challenge; the immune response of 2-year-old soft shelled turtles against bacterial challenge increased with the increasing size, for example, but was depressed under starvation conditions (Yang et al., 1999). Environmental insults may also compromise the sea turtle immune system thus opening the animal to infectious agents, reducing in turn animal viability and fitness.

The immune system is comprised of two major components: innate immunity (or natural/native immunity) and adaptive immunity. The innate immune response is the first line of defense a host has against invading pathogens and is comprised of physical and chemical barriers, phagocytic cells and blood proteins that make up the complement system. The innate immune response exhibits the following characteristics: 1) it is non-specific, 2) exposure to foreign bodies leads to immediate maximal response, 3) there is no immunologic memory and 4) it is found in nearly all life forms. The most notable

feature of the innate immune response is that it is poised to respond immediately upon insult or injury to the host (Abbas et al., 2012). The leukocytes of the innate immune response include monocytes and granulocytes: heterophils (the turtle equivalent to the mammalian neutrophil), eosinophils and basophils. Monocytes and heterophils are primarily phagocytic white blood cells that participate in inflammatory responses. Heterophils are associated with parasitic disease and microbial infection and represent up to 55% of the circulating peripheral blood populations in sea turtles (Lutz et al., 2001). Eosinophils are also recruited to sites of inflammation and are primarily responsible for the destruction of large parasites by phagocytosis (such as leeches found on sea turtles) and allergic reactions (Tizard, 1996). Basophils are by far the least numerous of all the leukocytes in the blood; they too accompany inflammatory events especially during allergy season as their granules contain histamine.

The adaptive immune response is characterized by the following: 1) exhibits a specific response against foreign bodies as it recognizes specific antigens, 2) there is a lag time between exposure and the immune response, 3) exposure leads to immunologic memory and 4) it is only found in jawed vertebrates. The primary leukocytes involved in the adaptive response are lymphocytes and there are many types: B and T lymphocytes and natural killer cells (although this subset is also considered part of the innate immune repertoire). Previous studies of adaptive immune responses in sea turtles have shown correlations to suppressed lymphocyte numbers in sea turtles exposed to organochlorides, disease (GTFP) and degraded environments (Cray et al., 2002; Keller et al., 2000; Lutz et al., 2001). *In vitro* lymphocyte response to mitogens revealed that juvenile green turtles sampled from three different habitats exhibited striking differences. Lutz et al. (2001)

found that lymphocytes from the IRL exhibited a pronounced ten-fold decrease in lymphocyte proliferation in response to stimulation as compared to green turtles from the Trident Basin a habitat where there is zero disease prevalence (Lutz et al., 2001). And as sea turtles are long lived, highly migratory animals that face numerous threats in degraded habitats, elucidating their immune function becomes of considerable importance if we are to ensure their survival.

Immune Response/Physiology and Temperature

Each of the seven extant species of sea turtles has unique characteristics that allow them to inhabit a specific ecological niche and tolerate environmental factors that influence their physiologic states (Aguirre and Lutz, 2004; Hendrickson, 1980). The most influential abiotic factor in the pathogenesis of disease and the physiologic state of most ectothermic marine organisms is that of temperature. Ocean temperatures dictate a species range (as in the case of coral reefs and fishes), and can affect the incidence or prevalence of disease (Barber, 2004; Bruno et al., 2007). Thus as ectotherms, sea turtles are also heavily influenced by the environment in which they live: temperature regulates their metabolism and plays a physiologically significant role, from how long they can withstand anoxia, to how behaviorally they may induce a fever in response to pathogens (Hutchison and Maness, 1979; Nilsson and Lutz, 2004).

Sea turtle immune function at the cellular level needs to be better elucidated as their physiology differs from birds and mammals, in that sea turtles are ectotherms and therefore rely on environmental factors to regulate their metabolism. Seasonal fluctuations in photoperiod and water temperatures may thus adversely affect their immune function (Southwood et al., 2003). Optimal temperatures for sea turtles lie

between 15°C and 30°C (Southwood et al., 2003). Below 15°C, sea turtles decrease foraging efforts, and reduce swimming activity (Moon et al., 1997). If temperatures drop below 10°C too quickly, turtles become buoyant and extremely listless. These cold stun events affect the smaller juvenile size class more rapidly than larger turtles and there may be a species difference as well. Witherington and Ehrhart observed that green turtles are more susceptible to cold stun events than loggerhead turtles, possibly due foraging range limits (Witherington and Ehrhart, 1989).

Immune responses in reptiles in general are known to be suppressed during colder winter months (Zapata et al., 1992); lymphocyte numbers in circulating blood populations are highest during the spring and summer and lowest during the winter (Kanakambika and Muthukkaruppan, 1972). And in softshell turtles, Interleukin – 2 (IL-2) activities of splenocytes increase with temperature from 15–25 °C (Guo, 2001). However, while the immunological performance of reptiles increases during the warmer summer months (Duguy, 1970), seasonal responses in sea turtle immune function have not been intensely investigated.

With the growing concern with climate change, increasing temperatures are likely to contribute to an alteration in immune function as well. One unfortunate by product of increasing global temperatures is an increase of disease due to human impacts that have altered the marine environment in favor of pathogens and created shifts to naïve hosts (i.e. Morbillivirus from dogs to pinnipeds and ceteaceans) (Kim et al., 2005). Boyd and Burnett (1999) reported that increased seasonal temperatures were accompanied by increased carbon dioxide levels and low pH levels in an estuarine habitat, creating hypoxic conditions which rendered benthic sessile invertebrates such as the oyster,

Crassostrea virginica unable to mount an immune response against pathogens. With global temperatures increasing, the combined impacts of hypoxia and pH will ultimately affect all marine organisms' ability to defend themselves against pathogens as the ocean becomes more acidic (Mydlarz et al., 2006). Presently, it is unknown how sea turtles will cope with hyperthermic stress in the aquatic realm. But ultimately, increasing temperatures may have a synergistic effect by contributing to the status of the environment and the health of its inhabitants (Knowlton and Jackson, 2008).

To date, several investigators have looked at adaptive immune response in the context of environmental stressors such as organochloride exposure (Keller et al., 2000, 2004, 2006) and fibropapillomatosis (Cray et al., 2002; Lutz et al., 2001; Work et al., 2000). However, there have been few investigations (Rossi et al., 2009) that define the innate (nonspecific) immune system of sea turtles other than identification of white blood cell populations (Casal et al., 2009; Casal and Orós, 2007). Innate immunity utilizes complement proteins and leukocytes that respond immediately and non-specifically to viral and bacterial pathogens. The primary defense mechanisms of the innate immune response include phagocytosis of pathogens and the respiratory burst, primarily by macrophages or neutrophils (heterophils in reptiles and avian literature). The ingested pathogen particles are destroyed by enzymes of the phagolysosome (Kuby, 2000), and with the highly toxic reactive oxygen intermediates of the respiratory burst (Janeway et al., 2001; Kuby, 2000). It is highly probable that sea turtle leukocytes will function in much the same manner as their avian and mammalian counterparts: leukocytes will be recruited to sites of injury or infection, and leukocytes such as monocytes and heterophils will phagocytose foreign matter. As nonspecific defenses are evolutionarily ancient, it is

likely that the sea turtle as a "living fossil" relies heavily on these responses, but they have not been investigated.

Any defects in innate immune function therefore are likely to increase susceptibility to pathogenic infection as host leukocytes would be unable to clear microbial cells and damaged or dead host cells. Additionally, tissue repair would not ensue nor would the innate response stimulate the adaptive immune response if necessary (Abbas et al., 2012). An example of an inherited phagocytic disease found in mammals such as the Persian cat (*Felis domesticus*) and the killer whale (*Orcinus orca*) is Chediak-Higashi Syndrome (CHS). In this disease, leukocytes have impaired mobility as well as morphological defects in either the cytoplasmic granules of neutrophils and eosinophils and may cause death through acute hemorrhage due to a deficiency in platelets (Tizard, 1996).

Since sea turtles encounter several different habitats that are known to alter physiological competence, it becomes crucial to understand their basal immune responses in light of anthropogenic burdens and global climate change.

As six of the seven sea turtle species are in the family Cheloniidae, immune function studies are likely to benefit all sea turtle species. Immunology is an exponentially emerging science in mammalian physiology; therefore, it is highly likely that we will be able to apply emerging techniques to further elucidate the reptilian immune system. By interpreting how aspects of these habitats influence the immune function of sea turtles, we will be better equipped to perhaps prevent disease in the wild and manage their diseases in captivity with the hope of releasing them back into the wild after rehabilitation.

Hypotheses

The objective of this research, then, is to quantify leukocyte function in the innate immune response in two species of sea turtle: the loggerhead (*Caretta caretta*) and the green turtle (*Chelonia mydas*). Innate immune response will be determined by separating and identifying sea turtle leukocytes in a flow cytometer while measuring their capacity for phagocytosis. Flow cytometry has been utilized to characterize cell populations, phagocytosis, and respiratory burst in numerous other organisms, including clams (Donaghy et al., 2009; Lambert et al., 2003), oysters (Goedken and De Guise, 2004), trout (Shelley et al., 2009), birds (Tell et al., 1997), and beluga whales and dolphins (De Guise et al., 1995). Sea turtles are known to possess leukocytes similar to avian and mammalian species (Casal and Orós, 2007; Cray et al., 2002; Lutz et al., 2001) and while little data exists for reptiles and birds in comparison to mammals, much as been inferred from mammalian studies (Harmon, 1998). Unfortunately, the mechanisms behind the sea turtles' innate (non-specific) immune response are largely unknown. As non-specific defenses evolved relatively early, it is likely that the sea turtle as a "living fossil" relies heavily on this modality in addition to mounting an adaptive response. I will examine immune function in three groups of turtles at different seasons and temperatures, and compare function in turtles from areas where GTFP is prevalent versus more pristine environments with a low prevalence of GTFP. By elucidating the basics of innate immune function in sea turtles, I will gain a better understanding of their physiology in degraded near shore habitats as well as predict responses to ever increasing global temperatures. To acquire a basic understanding of the innate immune system in sea turtles, I examined three specific hypotheses:

1. Leukocytes of the innate immune system in sea turtles, including the heterophil (the equivalent of the mammalian neutrophil), and monocyte/macrophage, are capable of phagocytosis. I investigated the rates of phagocytosis of both the heterophil and the monocyte to create baseline data for functional analysis of the innate immune system. This was the first investigation utilizing flow cytometry for the study of sea turtle heterophils.
2. The functional capacity of the innate immune system in sea turtles varies with temperature. My study will examined the effects of seasonal variations on immune function both by collecting data across the seasons, and through direct manipulations to determine the effects of temperature alone on innate immune function. I incubated sea turtle leukocytes at different temperatures to determine their phagocytic abilities over a range of physiologically relevant temperatures, including 4, 10, 22, 30 and 37°C. This was to determine if there is an optimal range for sea turtle immune response when faced with pathogens over the temperature range turtles are known to encounter.
3. Sea turtles with GTFP, or from areas where GTFP is prevalent, have compromised innate immune responses compared to animals from less polluted environments.

This study compared blood samples from turtles with and without fibropapillomas for phagocytic capabilities at temperatures that approximate those the turtle may encounter in the wild. Sea turtles with evident tumors from the Indian River Lagoon (IRL) have been shown to have greater physiological stress than those from less polluted areas. Deming (2008) biopsied tumor tissue and healthy tissue from IRL turtles and found

increased expression of two heat shock proteins (or stress response proteins), HSP72 and GRP96, which are up-regulated under many stressor conditions (thermal, oxidative, pathogenic infection and OCs) (Deming, 2008), thus suggesting that IRL turtles are under physiological stress compared to animals from a more pristine environment. As animals from the IRL have been shown to have decreased lymphocyte responses to mitogenic stimulation regardless of tumor load (Lutz et al., 2001; Sposato et al., 2002) I expected to find that innate responses in animals from the IRL are immunocompromised regardless of tumor status, suggesting an increased susceptibility to GTFP or other diseases.

MATERIALS AND METHODS

To test these hypotheses, I examined immune function in three groups of turtles over the course of 18 months, examined cellular responses at different temperatures, and compared immune function in turtles from areas where GTFP is prevalent versus more pristine environments with a low prevalence of GTFP. Seventy-eight sea turtles (*Chelonia mydas* and *Caretta caretta*) were sampled (dependent on seasonal variability and catch rates) from those captured at the Florida Power and Light plant (SLPP) in Martin County, Florida, the Port Canaveral Trident Basin (TRI), and the Indian River Lagoon (IRL), Brevard County, Florida as part of ongoing tag/recapture studies at those locations. Turtles were sampled seasonally over 18 months (2013-2014) to examine seasonal fluctuations as an environmental variable.

Collections Sites

The Indian River Lagoon (IRL) is a degraded 250 kilometer long estuary on the east coast of Florida with minimal tidal flux or water exchange with the Atlantic Ocean. The run-off from citrus groves, cattle ranches, and storm sewers result in high levels of contaminants. Many species in this system are experiencing emerging health problems, including a number of fish species and the resident bottlenose dolphin population (Bossart, 2011). Turtles in the IRL suffer from high rates of GTFP, with up to 70% of the population exhibiting external tumors (Ehrhart, 1991; Hirama and Ehrhart, 2000). The Trident Basin (TRI) is a man-made embayment located near the mouth of an inlet with a strong tidal flux. TRI is located on government property and experiences little

anthropogenic pollution. This is thus considered a representative location for a pristine, unimpacted population. At the St. Lucie nuclear power plant (SLPP), turtles are captured in standing nets as part of their sea turtle protection plan, to prevent animals from being swept into the power plant intakes. Disease prevalence is not generally associated with this location and turtle origins are largely unknown. Animals were released following collection of biological data (parasite loads and FP incidence), sampling (morphometrics and blood collection), and tagging (flipper tags and electronic PIT).

Blood Collection

For this study, 2-4mL of blood was drawn from the dorsal cervical sinus and transferred to a Sodium-heparin vacutainer. This is significantly less than the safe maximal amount of blood that can be drawn from a healthy reptile, determined to be 5-8% of body weight converted to volume (Lillywhite and Smits, 1984; Smits and Kozubowski, 1985). Prior to blood sampling, turtles were restrained, and skin disinfected with an application of isopropyl alcohol. Blood samples were placed in a test tube rack to prevent contact with ice packs after collection and then transported to Florida Atlantic University.

Hematology

Heparinized whole blood mounts were made at the time of bleeding and later stained utilizing Diff Quick differential stain or a 1:20 diluted Geimsa stain. Leukocytes were then counted under 40X magnification and recorded as a percentage of the total leukocyte population. Additionally, an aliquot of 10uL of heparinized whole blood was used to determine packed cell volumes.

Separation of Whole Blood

Whole blood was layered on a discontinuous Percoll gradient: a 60% density gradient to restrict monocytes and lymphocytes and a 75% density gradient to prevent the granulocytes from forming a buffy coat. Each layer was then transferred to a 15mL Falcon tube and washed two times in 1X phosphate buffered saline (PBS). The viable cell yield was then determined by staining 10µL of cell solution with 90µL of Trypan Blue using standard hemocytometer methodology. Each sample was then diluted with PBS to bring the concentration to 1×10^6 cells/mL.

Flow Cytometry Phagocytosis Assay

Phagocytosis was measured by flow cytometry for each following one hour incubation with a suspension of fluorescent latex beads labeled with fluorescein isothiocyanate (FITC). In brief, 50µL of FITC particles were added to cells (100µL agranulocytes and granulocytes in Hank's Balanced Salt Solution [HBSS]) in 96 well plates. Varying temperatures from 4°C to 37°C were utilized to incubate the cells to determine an optimal range for phagocytosis in the sea turtle. After the incubation period, phagocytosis was interrupted by placing samples on ice. Leukocytes were then gently layered on cold bovine serum albumen (BSA), and centrifuged at 800g for 8 minutes at 4°C to wash off any non-internalized and nonspecifically bound beads. Fluorescence was evaluated with a BD FACSCalibur high-speed digital bench-top cell sorter. Phagocytosis was measured at 480 nm as the fluorescein marked beads are engulfed by various leukocytes. Forward scatter (FS) and side scatter (SS) were used to identify the different cell types according to their patterns of size and granularity. Gates were set around each of the populations: heterophils, lymphocytes and red blood cells.

Statistical Analysis

Statistical comparisons were made both between sites, time of year (seasonal temperatures) and papilloma vs. non-papilloma groups. Data were tested for normality utilizing One-way Anovas and Paired t-tests to detect differences between groups. Data that did not meet these conditions were analyzed by non-parametric tests: Kruskall-Wallace and Dunn's test respectively.

RESULTS

Hematology

Leukocyte differentials and packed cell volumes (PCV) were determined for each sea turtle sampled (n = 78) between January 2013 and March 2014. Figure 1 depicts the distribution of the predominant white blood cell types found in loggerheads (*Caretta caretta*) and green (*Chelonia mydas*) turtles. In both species, heterophils comprise the largest fraction (58% in *C. caretta* and 50% in *C. mydas*) of the leukocyte population (Fig. 1). Lymphocytes made up the next largest fraction, (22% in *C. caretta* and 33% in *C. mydas*) followed by eosinophils (15% in *C. caretta* and 9% in *C. mydas*) while monocytes made up the smallest fraction (0.1% in *C. caretta*; data not shown and 0.8 to 2.3% in *C. mydas* depending on health status in *C. mydas*; Fig. 2). Between species, loggerheads (*C. caretta*) exhibit lower percentages of circulating lymphocytes and eosinophils, but higher percentages of heterophils than green turtles (*C. mydas*) (Fig. 1). Among the green turtle populations from the Indian River Lagoon and the Trident Basin, there was no difference in the predominant circulating leukocyte values; however, a significant difference exists in circulating monocyte populations (Fig. 2). Additionally, there was no difference in packed cell percentages between IRL turtles with visible tumors (Pap) and those animals without visible tumors (Non-pap) (Fig. 3).

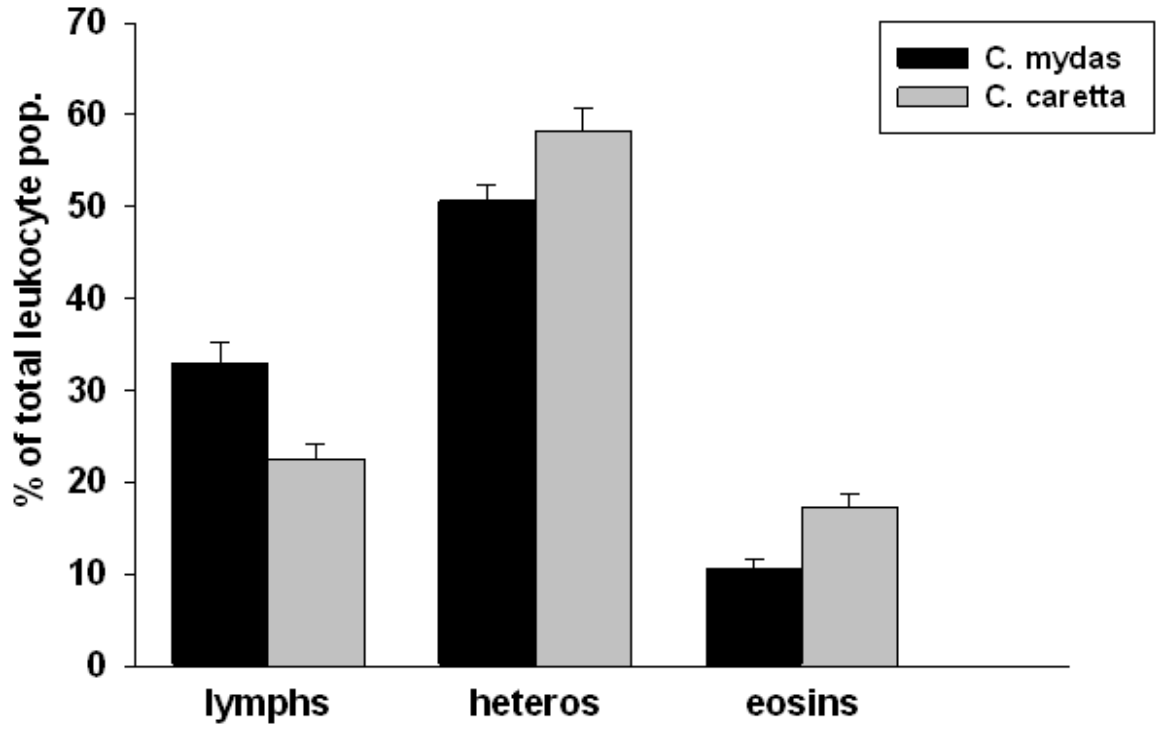

Figure 1. Distribution of the predominant (greater than 5%) leukocytes in loggerhead (*Caretta caretta*) (n = 16) and green (*Chelonia mydas*) (n = 62) sea turtles. There is no difference between the two species with respect to individual circulating white blood cell subpopulations.

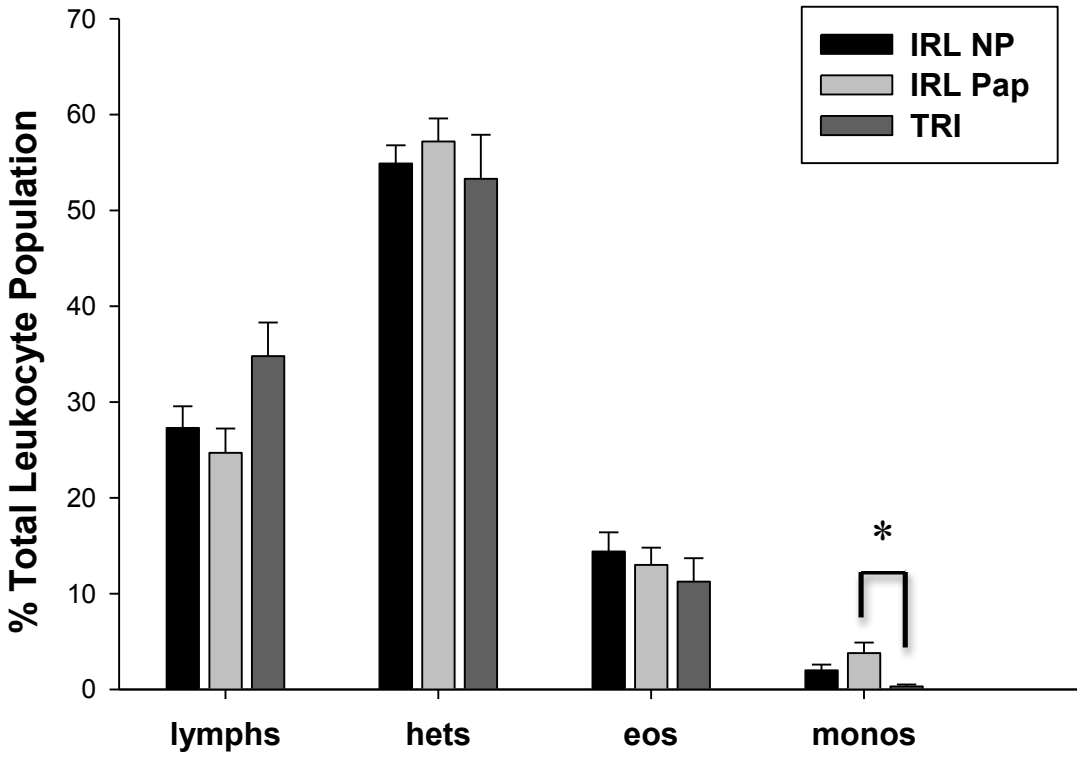

Figure 2. Predominant leukocytes percentages for green (*Chelonia mydas*) turtles from the Indian River Lagoon (with and without visible tumors) and the Trident Basin where GTFP is rare. There were no differences between the two locations (P = 0.866), except in the monocyte population. * Monocyte levels are significantly higher in TRI turtles than in IRL turtles exhibiting papilloma tumors (P = 0.002).

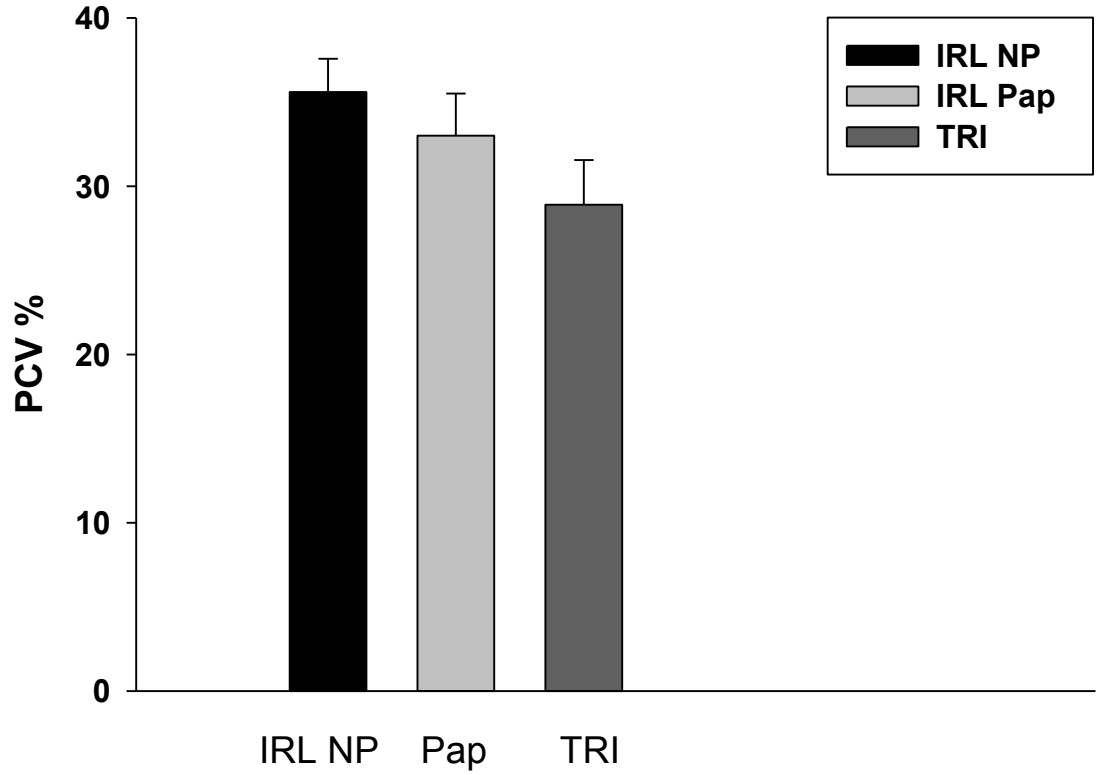

Figure 3. Packed cell volumes of green (*Chelonia mydas*) turtles from the Indian River Lagoon and the Trident Basin. There is no statistical difference (P = 0.119) between those turtles with visible tumors (Pap) compared to turtles without tumors (NP and TRI).

Flow Cytometry Phagocytosis Assays

Flow cytometry revealed that as proposed in Hypothesis I, sea turtle white blood cells are indeed capable of phagocytosis; this was true of both the monocytes/lymphocytes and the heterophils, and the ingestion of FITC labeled beads by the cells could be detected as an increase in fluorescence. Representative flow cytometry analyses of leukocyte phagocytosis are shown in Figure 4. The leukocytes were gated based on unstimulated control samples as represented by scatterplot and histograms (Fig. 4A). Leukocytes that ingested FITC labeled beads are shown as a subset with relative fluorescence with both a histogram (as shown below the bar) and a scatterplot (as shown

within the gate or box) (Fig. 4B). The rate of phagocytosis was quantified based upon 20,000 events or individual cells detected by the flow cytometer.

To determine if leukocytes of the innate immune system are affected by various abiotic and biotic factors, rates of phagocytosis were quantified and compared based on the following parameters: leukocyte subpopulations, *in vitro* temperatures, seasonal sampling, species differences, health status (tumored vs. no visible tumors), and location.

Rates of phagocytosis for each white blood cell population (PBMCs and PMNs) from all turtles sampled in both winter and summer, averaged 4% and 8.2% respectively (Fig. 5). There was no statistical difference in rates of phagocytosis between the combined PBMC and the PMN subpopulations (Fig. 5), suggesting that the PMN cells play as important a role in sea turtle immune defense as the lymphocytes of the adaptive immune response.

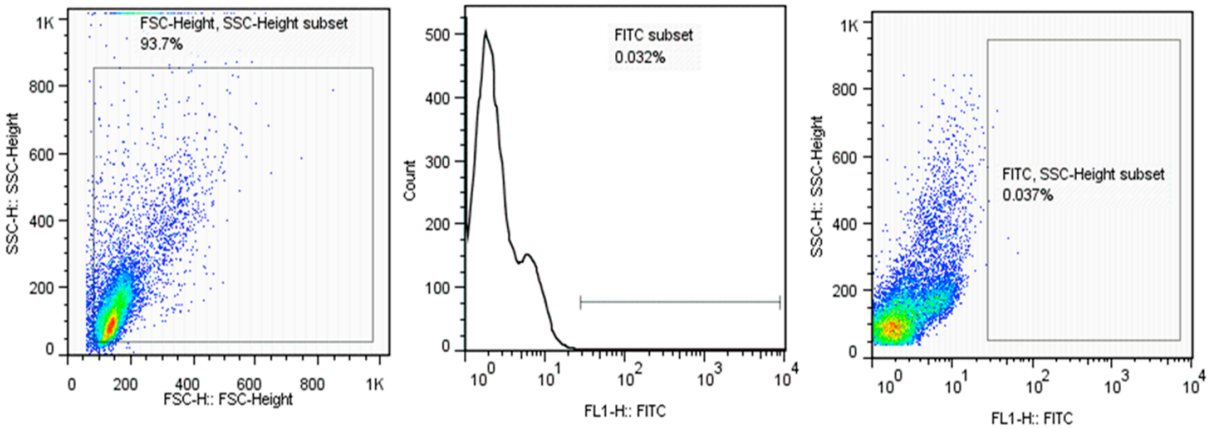

Figure 4A. Scatterplot and histogram (center) representations of the PMN subpopulation without fluorescent beads. (Negative Controls were performed on all samples to ensure cell viability.)

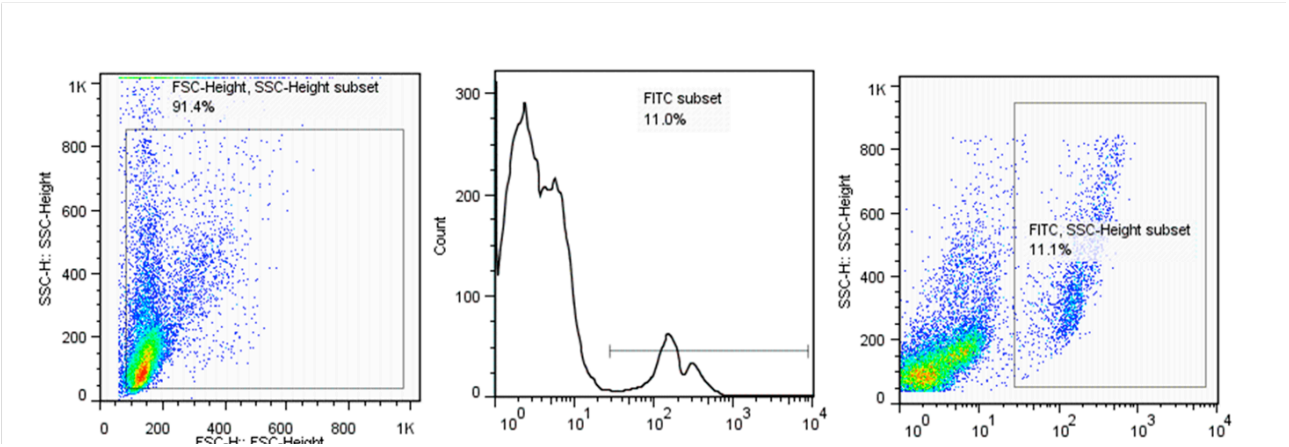

Figure 4B. Scatterplot and histogram (center) representations of the same PMN subpopulation above (4A) with fluorescent beads detected and labeled as FITC subset (11% of heterophils consumed fluorescent beads). The histogram subset (center) is indicated under the bar and the scatterplot subset is indicated within the box (right).

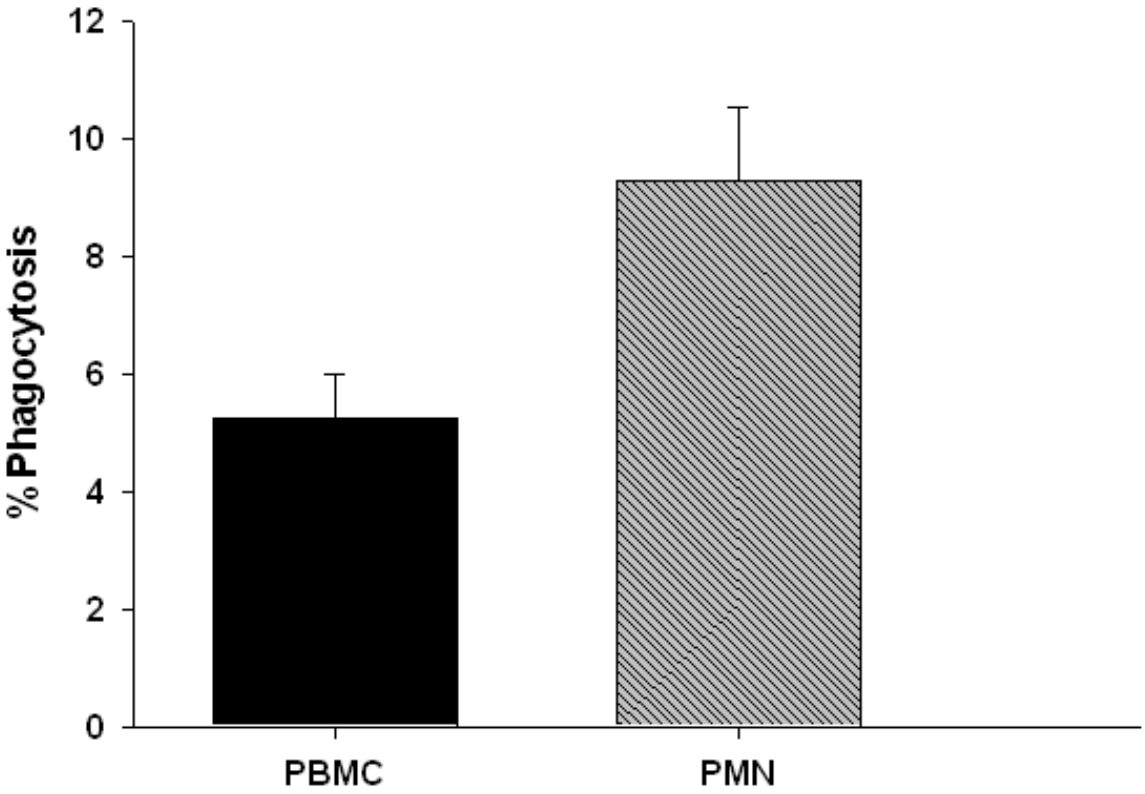

Figure 5. Rates of phagocytosis of the monocyte and lymphocyte (PBMC) layer (n = 40) and heterophils (PMN) (n = 30) of both species combined. There were no significant differences between PBMC and PMN subpopulations (P= 0.866).

Species

Overall, rates of phagocytosis were low in the sea turtles sampled. Less than three percent of loggerhead PBMCs engulfed FITC beads while green turtle PBMCs exhibited rates of phagocytosis averaging 4.9% of cells (Fig. 6). Loggerhead PMNs averaged 1.8% phagocytosis while on average 12.8% green turtle PMNs engulfed FITC beads (Fig. 6). There was no statistical difference in rates of phagocytosis between the loggerhead PBMC and PMN subpopulations or between either subpopulation of loggerhead cells and the green PBMC subpopulations. However, green turtle PMNs showed significantly greater rates of phagocytosis than loggerhead PMNs ($P < 0.005$). and green turtle PBMCs

(Fig. 6) further supporting the idea that heterophils are an important component of sea turtle immune function.

Green turtles leukocyte populations also showed a seasonal difference: greens sampled in the summer exhibited higher rates of phagocytosis than loggerheads in the summer or winter months (Fig. 7). While rates of phagocytosis in the winter only averaged 3% in the loggerheads and greens, in summer rates of phagocytosis in the green turtles increased to 9% (Fig. 7), thus rates of phagocytosis were also significantly different between summer and winter greens.

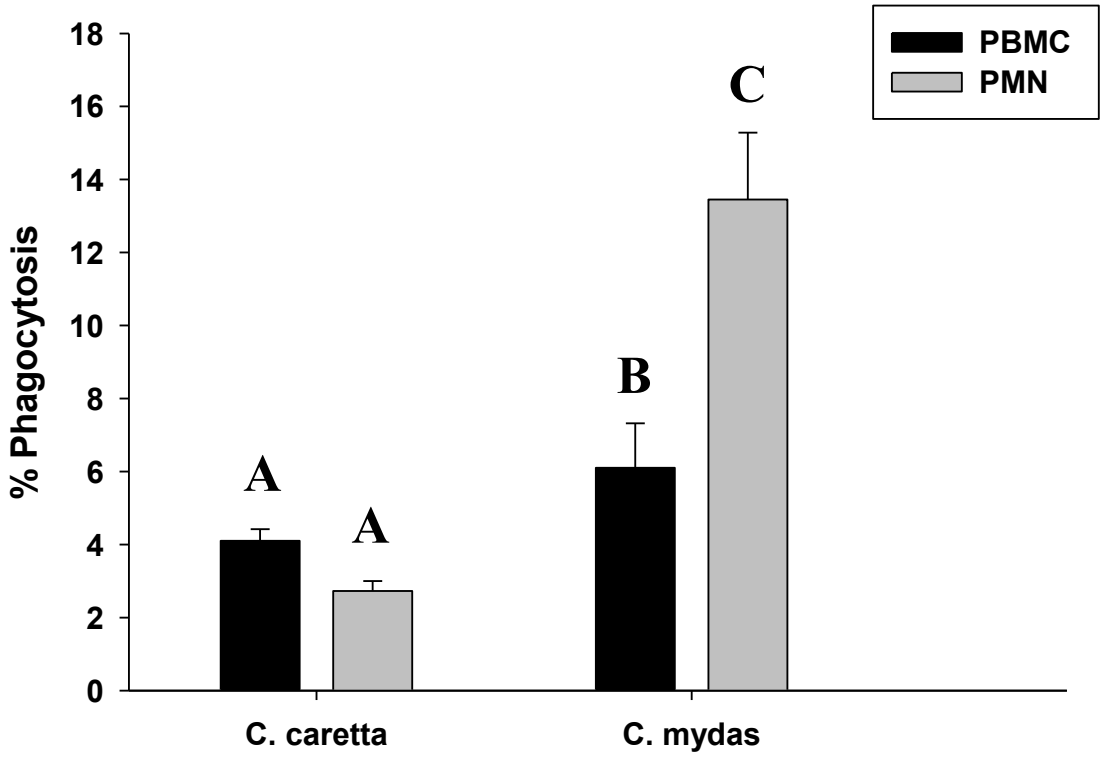

Figure 6. Rates of phagocytosis between loggerhead (*C. caretta*) and green (*C. mydas*) turtles and their respective leukocyte subpopulations. Monocytes and lymphocytes are denoted as PBMC (loggerhead n = 53 and green n = 80) whereas the heterophils are denoted as PMN (loggerhead n = 35 and green n = 55). ***** Green turtle PMN subpopulations had significantly higher rates of phagocytosis than loggerheads ($P < 0.001$).

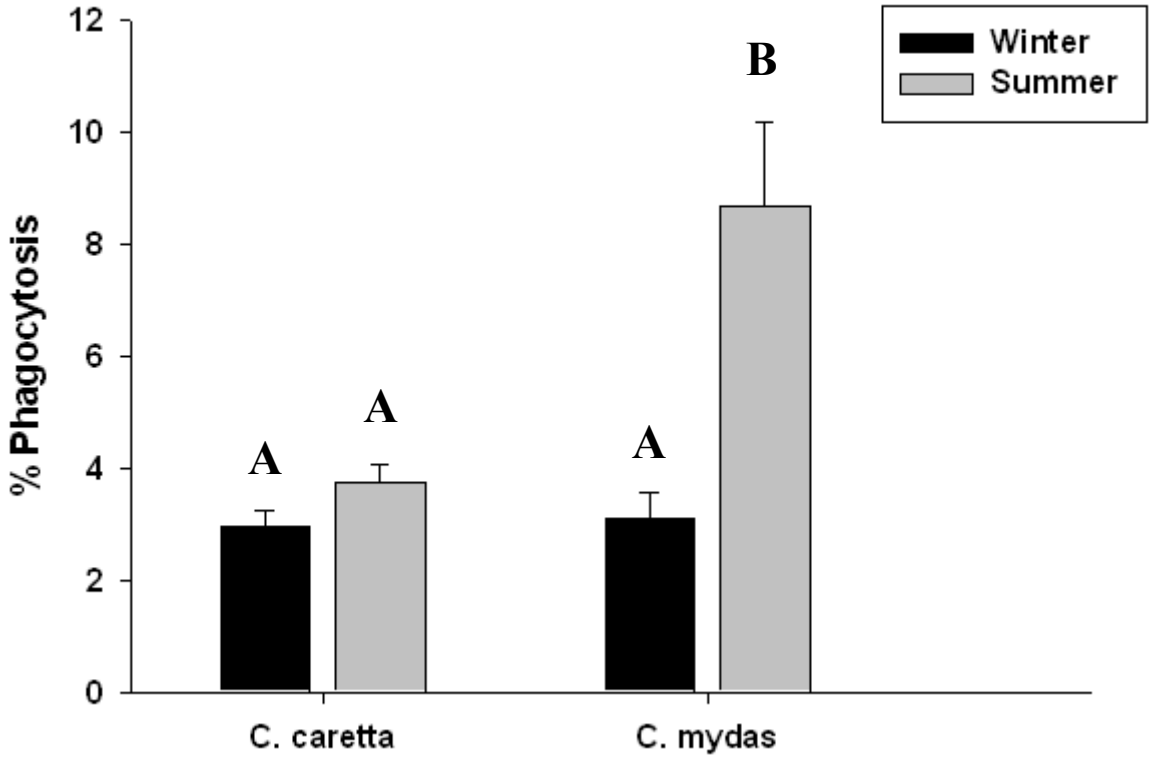

Figure 7. Seasonal rates of phagocytosis in loggerhead (*C. caretta*) (Winter n = 52 and Summer n = 44) and green (*C. mydas*) turtles (Winter n = 48 and Summer n = 21) sampled in the winter and summer. Different letters indicate significant difference in rates of phagocytosis in the green turtle between where A represents the winter and B represents summer months ($P < 0.001$) as well as between green and loggerhead turtles during the summer, C ($P<0.05$).

Temperature and Season

Because sea turtles are ectotherms and the incidence of disease varies seasonally in reptiles, I hypothesized that rates of phagocytosis would vary with temperature. Surprisingly, however, there were no statistical differences between PBMC and PMN subpopulations incubated over a range of *in vitro* temperatures (Fig. 8). Cells incubated for one hour at temperatures ranging from 4°C to 37°C exhibited similar rates of phagocytosis ranging from 2.7% to 4.5% for the PBMC layer and 1.9% to 6.7% for the

PMN layer (Fig. 8). Rates of phagocytosis were significantly different between species, however, with green turtle leukocytes exhibiting higher rates (6% on average) than loggerheads (3% on average) over the range of *in vitro* temperatures (Fig. 9) but this is most likely a function of either species and/or seasonal differences rather than temperature differences.

Over the *in vitro* temperature range investigated, non-papilloma green turtles also exhibited higher rates of phagocytosis ranging from 4.3% to 19.3% compared to those animals with GTFP with 1.6% of cells exhibiting phagocytosis on average ($P = 0.002$) (Fig. 10). Across the range of temperatures, there was no statistical difference between TRI green turtles and IRL non-papilloma turtles; however, there was a difference between non-papilloma turtles from both locations when compared to those green turtles with visible tumors (Fig. 11).

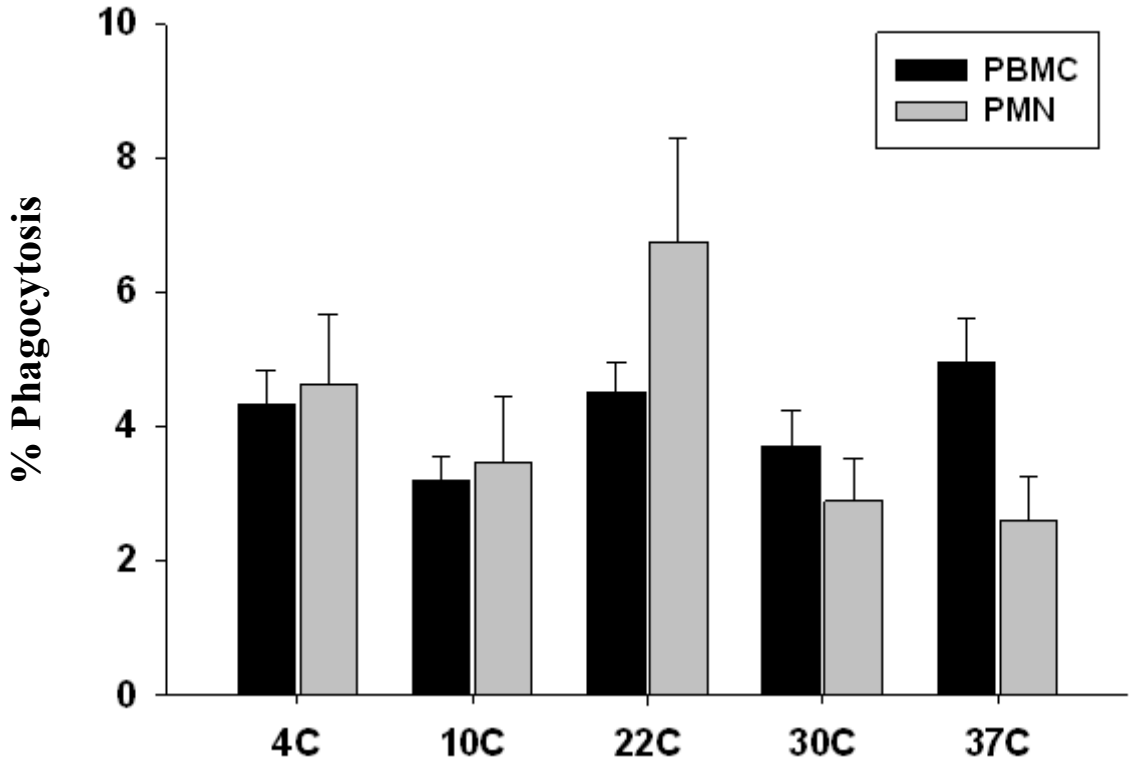

Figure 8. Rates of phagocytosis of monocyte and lymphocyte subpopulation (PBMC) and the heterophil subpopulation (PMN)) in turtle leukocytes incubated *in vitro* over a range of biologically relevant temperatures (n = 21 at 4ºC, n = 41 at 22ºC and n = 13 at 37ºC). There is no statistical difference in the subpopulations with respect to *in vitro* temperatures (P=0.907).

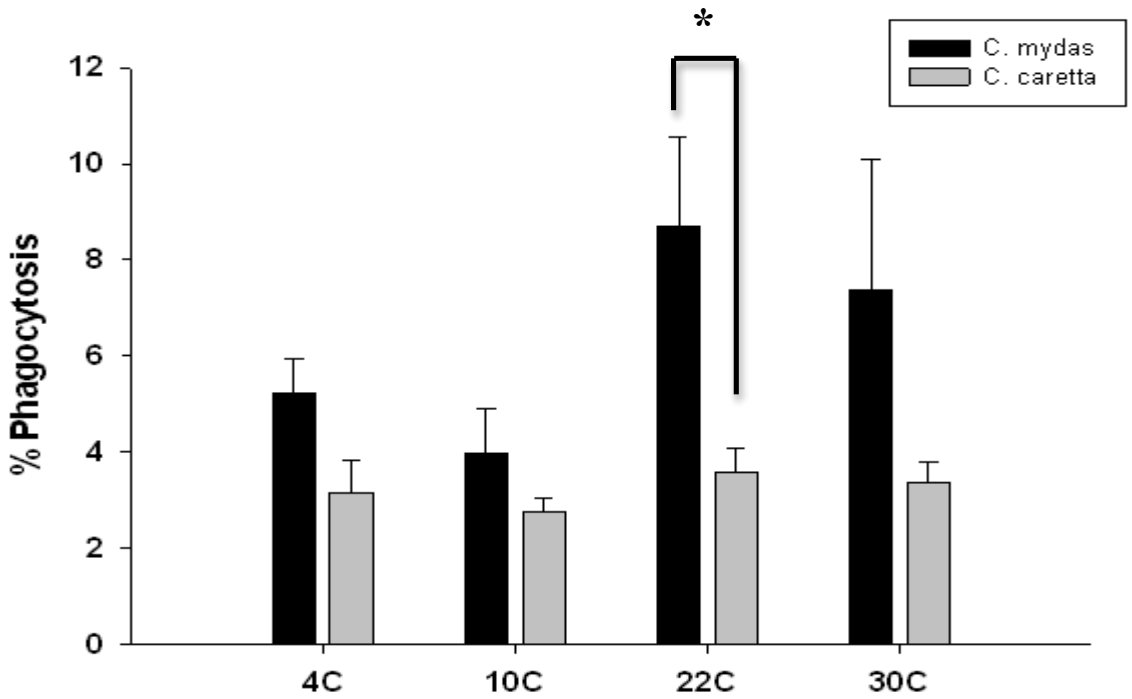

Figure 9. Rates of phagocytosis for loggerhead (*C. Caretta*) (n = 24 at 22ºC) and green (*C. mydas*) turtles (n = 45 at 22ºC) showed no difference across most biologically relevant temperatures. * Green turtles perform significantly better than loggerheads at 22ºC (P = 0.045); there were no differences between species at other temperatures.

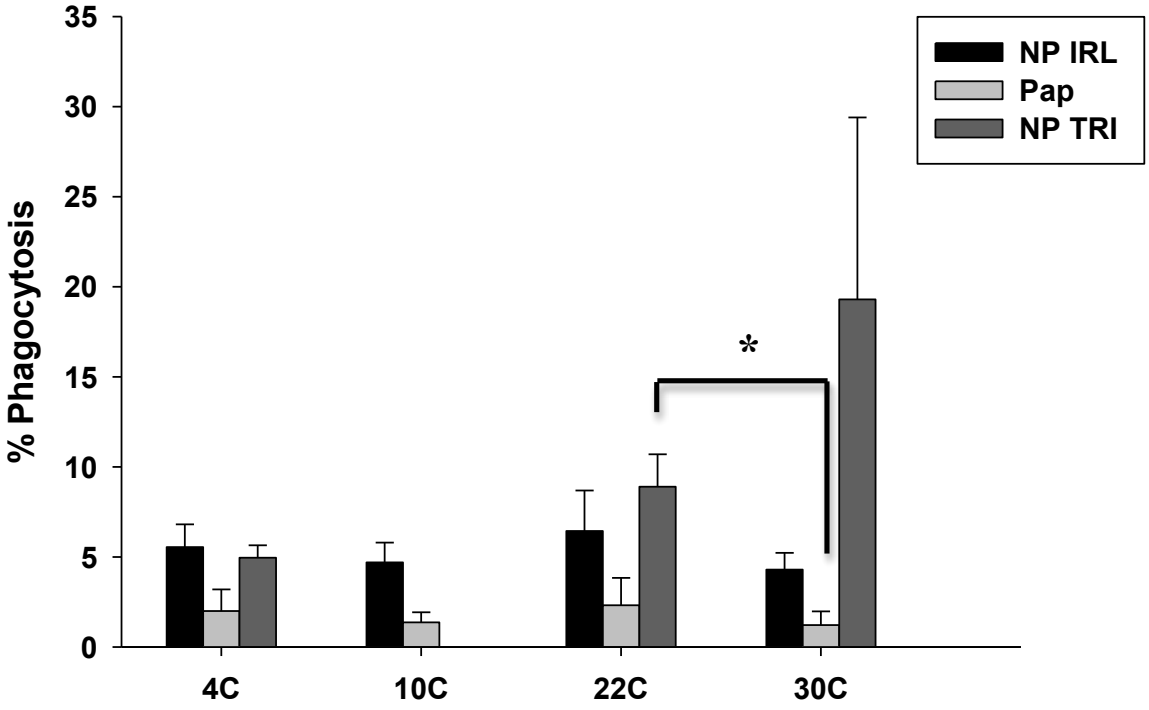

Figure 10. Rates of phagocytosis of green (*C. mydas*) turtles within two distinct habitats. Turtles from the Indian River Lagoon (IRL) are further subdivided by health status (NP n = 40 and Pap n = 4); no turtles from the Trident Basin (TRI) (n = 45) exhibit tumors. TRI turtles show the highest rates of phagocytosis compared to either group from within the IRL, but only at 22ºC (*) is there a significant difference (P=0.002) over 30ºC Pap turtles.

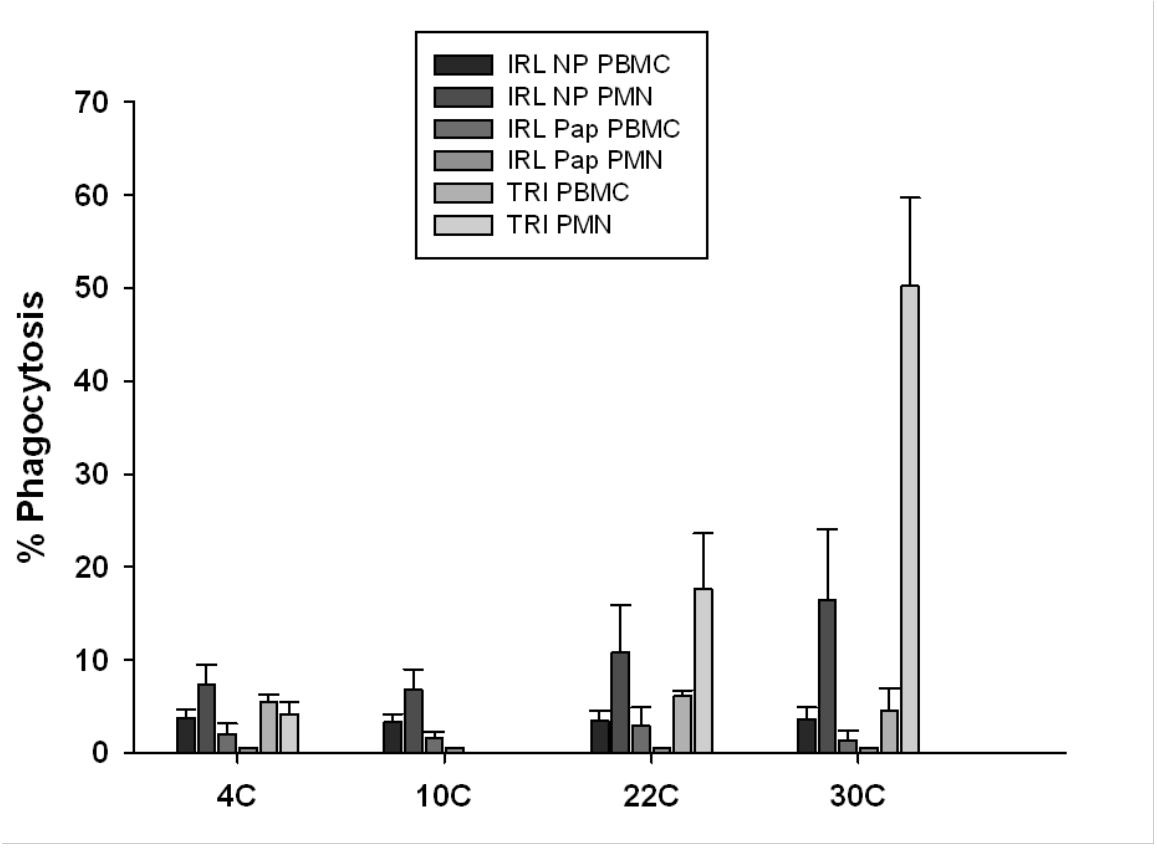

Figure 11. Rates of phagocytosis of green turtle white blood cells from two environments with and without tumors. There is no difference between *in vitro* temperatures and PBMC sub-populations ($P = 0.26$) or the PMN subpopulation ($P = 0.175$).

There is a slight seasonal difference with regard to *in vitro* temperatures at 4°C, 10°C, and 37°C with rates of phagocytosis ranging from 2.7% to 7% in the winter and 4% to 7% in the summer (Fig. 12), but no difference at 22°C and 30°C. Surprisingly, the only significance lies at the lower end of the temperature range ($P < 0.001$ at 4ºC).

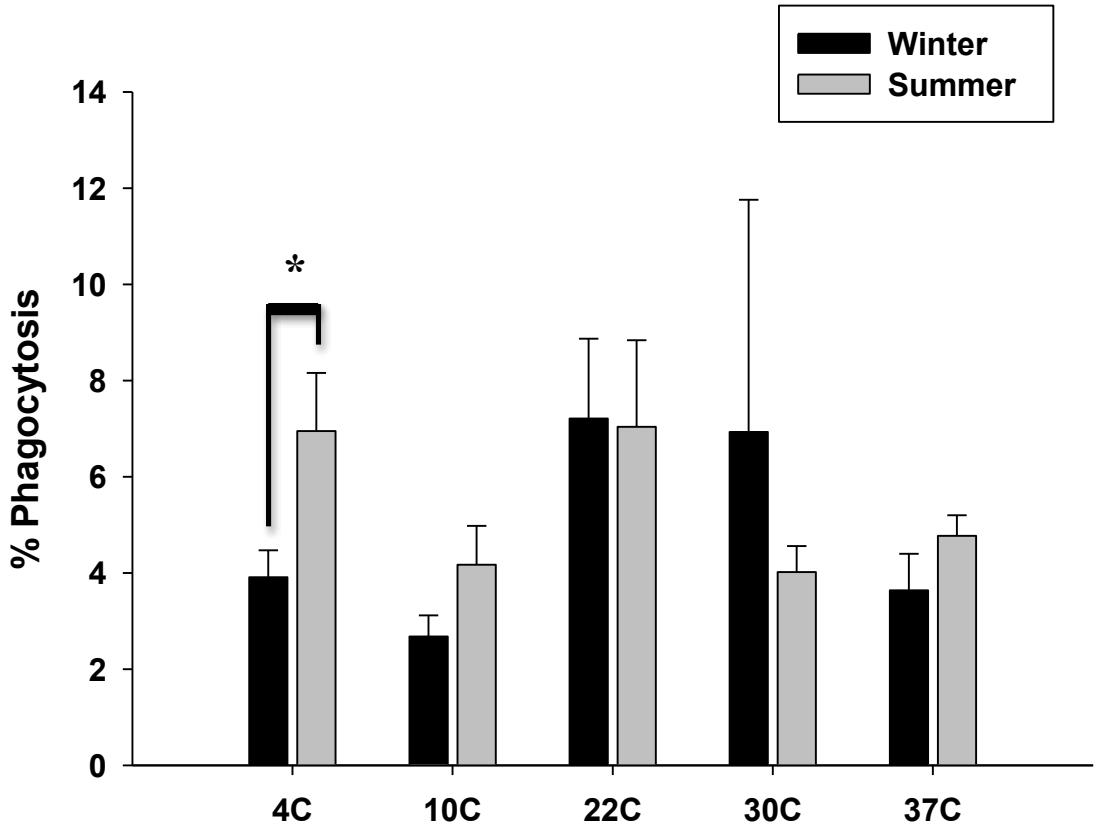

Figure 12. Rates of phagocytosis between both species (n = 70) sampled in winter and summer. There is no seasonal difference at the upper temperatures however there is a difference at the lower temperatures (*P < 0.001).

Rates of phagocytosis by turtle PBMCs are comparable in both seasons averaging 5%; however, green turtle PMNs sampled during the summer exhibited higher rates of phagocytosis than those sampled during the winter (Fig. 13).

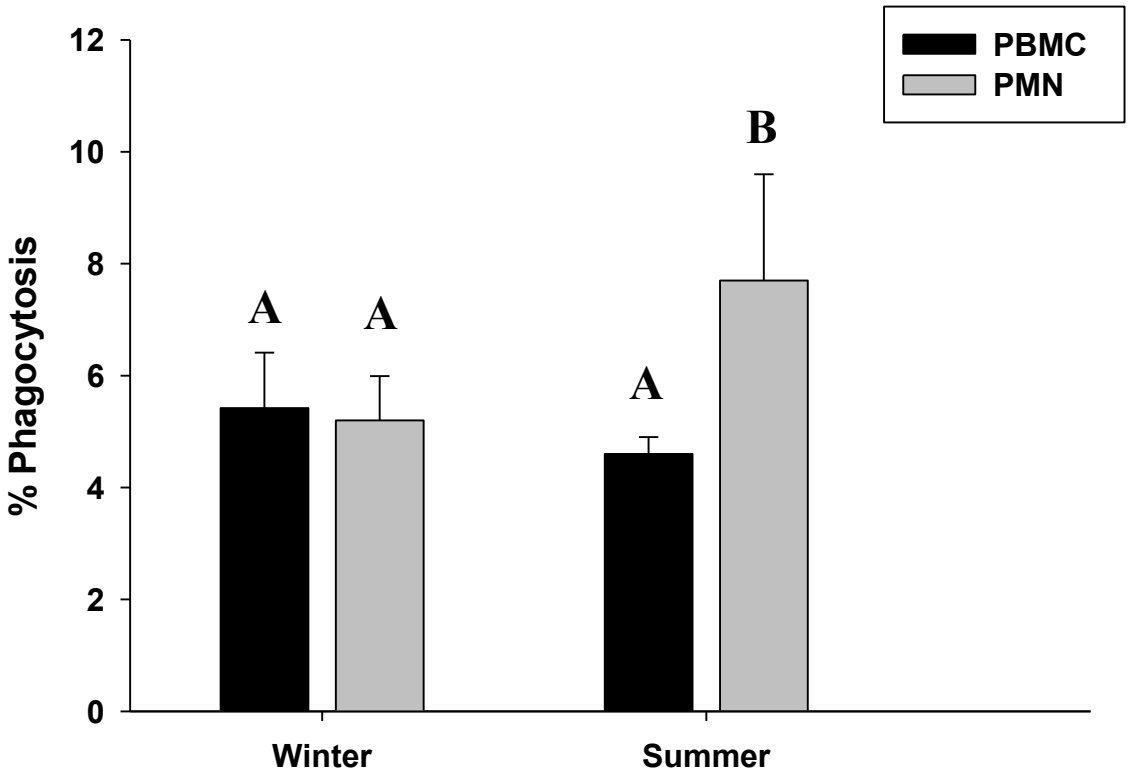

Figure 13. Seasonal rates of phagocytosis of the separate leukocyte subpopulations from both species combined. There is no difference between the PBMC and PMNs in the winter months; however, the summer PMN subpopulations are significantly higher than all other groups (P = 0.037). Different letters are significantly different from each other.

There also exists a seasonal difference pertaining to location with regards to rates of phagocytosis, with those turtles from the TRI displaying higher winter rates of 10.5%, compared to both non-papilloma (3.5%) and papilloma (0.7%) turtles from the IRL (Fig. 14). Leukocytes obtained during the winter from TRI turtles showed increased rates of phagocytosis over both non-papilloma ($P < 0.05$) and papilloma (GTFP) turtles ($P < 0.001$) from the IRL; additionally, non-papilloma turtles from the IRL exhibited higher rates of phagocytosis over papilloma turtles sampled from the same location during the summer months ($P = 0.023$) (Fig.14). Due to the significant differences within the

degraded IRL, we tested for seasonal differences with regard to health status. Results indicate that those turtles with visible tumors (GTFP) exhibit lower rates of phagocytosis during both the winter and summer months ($P < 0.001$) (Fig. 15).

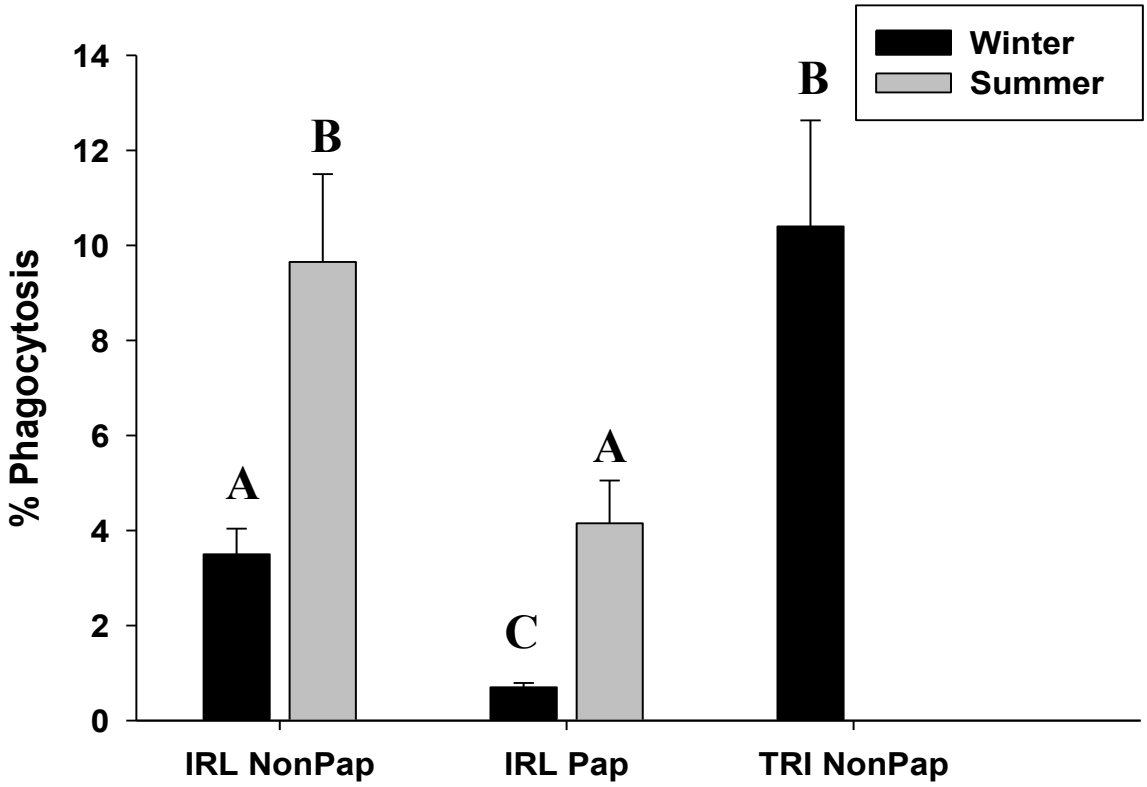

Figure 14. Seasonal rates of phagocytosis from green (*C. mydas*) turtles of the IRL (n = 41 and n = 10 for winter; Non-pap and Pap turtles; n = 16 and n = 4 for summer Non-Pap and Pap turtles respectively), and TRI (n = 45). Different letters indicate significant differences. There was no summer data from the TRI, but winter rates of phagocytosis are significantly higher ($P < 0.001$) than those animals from the IRL. Additionally, IRL Non-pap turtles exhibit higher rates of phagocytosis than Pap turtles during summer months ($P = 0.023$).

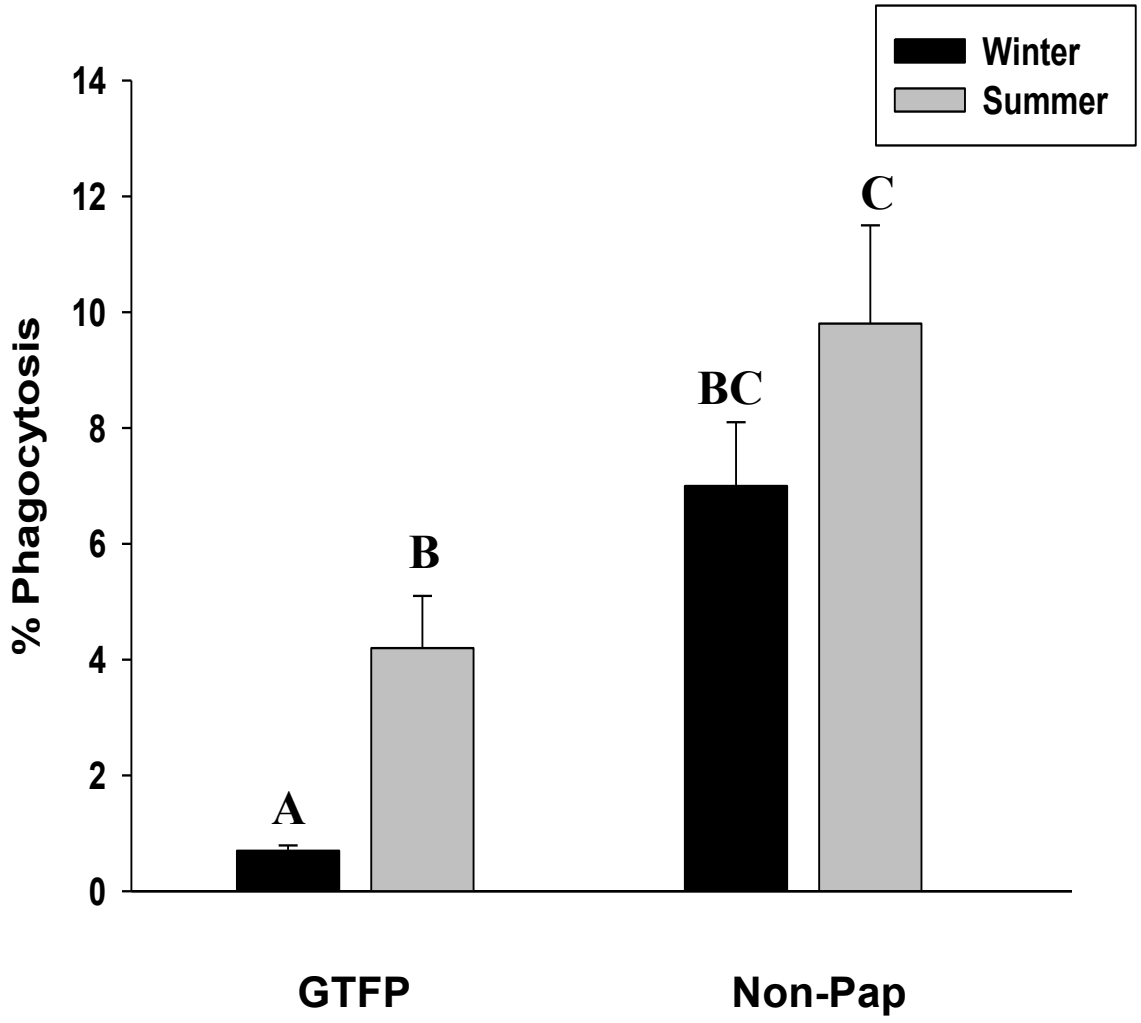

Figure 15. Seasonal rates of phagocytosis within the IRL between green (*C. mydas*) turtles with visible tumors (GTFP) and those without (Non-tumored) ($P < 0.001$). Different letters represent significantly different rates of phagocytosis.

Health Status and Location

As hypothesized, location played a significant role in immune function. Rates of phagocytosis by both the PBMC and PMN subpopulations of TRI turtles were statistically higher than those animals from the IRL, while within the TRI animals, the PMN layer had greater rates of phagocytosis than the PBMCs (Fig. 16).

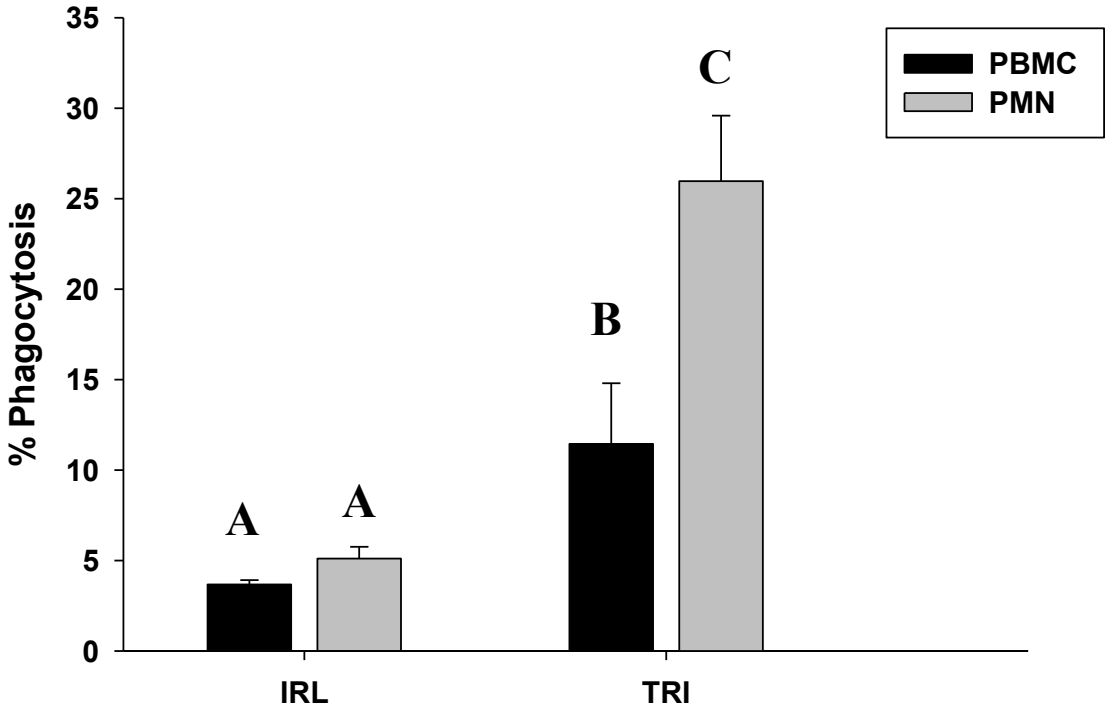

Figure 16. PBMC layer vs. PMN layer rates of phagocytosis from all turtles captured in the IRL (n = 107) and TRI (n = 27). Different letters are significantly different from each other. The PBMC subpopulation has lower rates of phagocytosis between the two locations ($P < 0.05$) than the PMN subpopulation ($P < 0.001$).

Turtles from the IRL exhibit comparable rates of phagocytosis no matter the subpopulation, averaging 4.9%.

The impact of living in the degraded IRL on the health of the animals is further evidenced when comparing turtles from the IRL with and without visible tumors to turtles from the TRI: green turtles with visible tumors have much lower rates of phagocytosis, averaging 2.7% compared to the TRI where there is no prevalence of

disease; in the Trident Basin, rate of phagocytosis averaged 17.2% between the two leukocyte subpopulations (Fig. 17).

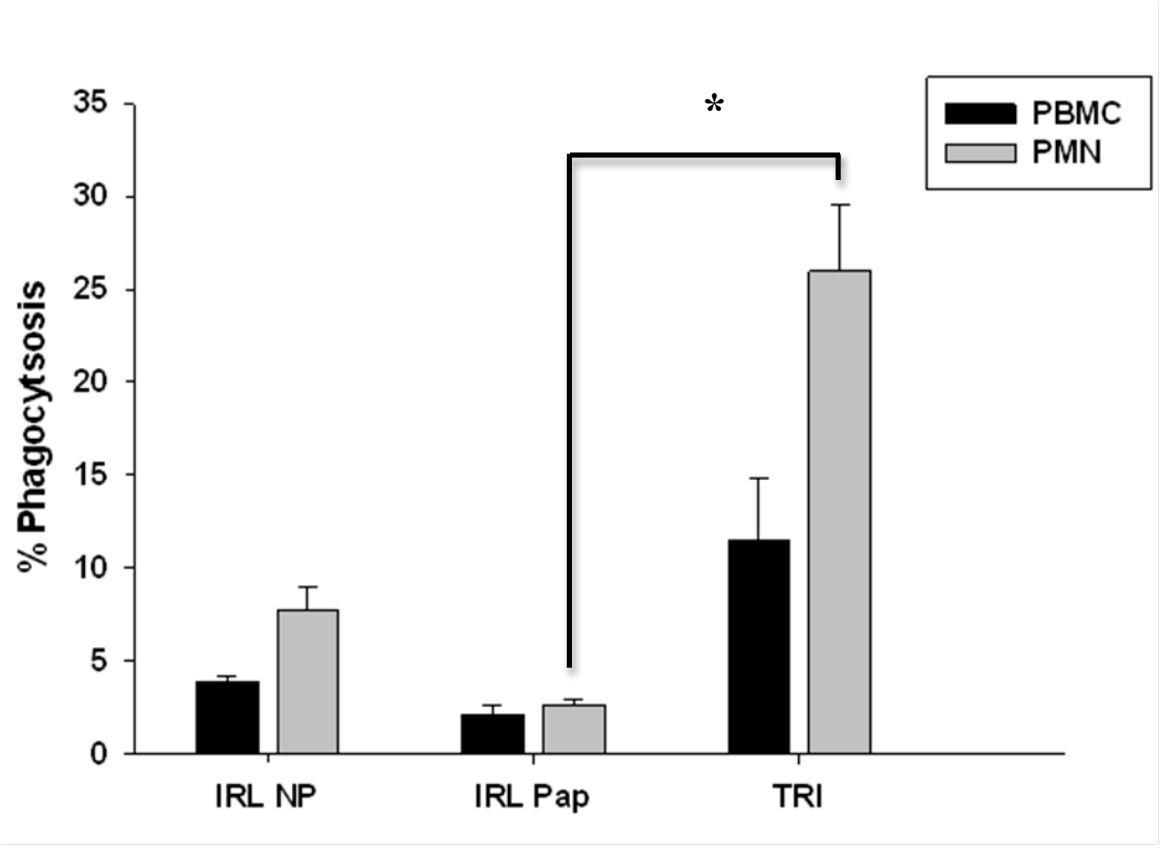

Figure 17. Rates of phagocytosis of each leukocyte subpopulation obtained from green (*C. mydas*) turtles from the IRL and TRI. There is no significance between the PBMC subpopulation (n = 13) and the PMN layer (n = 3) in turtles with visible tumors; however, there is a difference between TRI PMNs and IRL Pap PMNs (* $P < 0.001$).

DISCUSSION

This investigation focused on the innate immune response of wild sea turtle populations through quantification of phagocytosis utilizing flow cytometry. I hypothesized that sea turtles as "living fossils" would rely heavily on the innate immune system due to their ectothermic physiology and the fact that non-specific responses are evolutionarily ancient, but their capability to respond to pathogens would still be similar to birds and mammals. Previous studies have shown that sea turtle adaptive immune responses are comparable to birds and mammals with respect to *in vitro* blastogenic assays (Cray et al., 2002; De Guise et al., 1996; Zhu et al., 1999). I also hypothesized that animals from the Indian River Lagoon, as an environmentally degraded habitat, and/or animals that were diseased, would show reduced innate immune responsiveness; previous results have indicated that the adaptive immune response in sea turtles is compromised in animals from near shore declining ecosystems and also that the adaptive immune response is suppressed in those that exhibited disease states such as GTFP (Lutz et al., 2001; Varela, 1997). While the *in vitro* blastogenic assay is an effective tool to determine whether lymphocytes are capable of mounting an immune response, it does not elucidate the function of heterophils which comprise the majority of circulating leukocytes in adult loggerhead and juvenile green sea turtles (Bradley et al., 1998; Lutz et al., 2001).

For the purposes of this study, rates of phagocytosis were analyzed based upon the species of the turtle (loggerhead or green), the location where the turtle was captured (Indian River Lagoon, IRL and the Trident Basin, TRI), the season that sampling

occurred (winter or summer), health status (green turtles only; those with evident GTFP vs. turtles without), the leukocyte sub-population (monocytes/PBMC layer or heterophils/PMN layer), and a range of *in vitro* temperature (4°C to 37°C).

Hematology

First, it was important to determine if there were any significant differences in the leukocyte populations in animals from the different locations, or in diseased vs non-papilloma turtles. Leukocyte differentials were performed to quantify the percentages of targeted circulating white blood cells from each species. These white blood cells were then retrieved from the discontinuous Percoll gradient. Heterophils, lymphocytes, eosinophils, and monocyts were counted as the total circulating leukocyte populations of loggerheads and green turtles from the Indian River Lagoon (IRL) and the Trident Basin (TRI) (greens only). Heterophils are known phagocytic granulocytes and represent up to 50% of the leukocyte differential in chelonids (Stacy et al., 2011); increased heterophil counts in turtles may also be indicative of nonspecific inflammatory responses, stress, and tumor loads (Varela, 1997).

Aguirre et al. (1995) found that juvenile green turtles from Hawaii without tumors had initial heterophil counts of 16% of the total circulating white blood cell population at the time of bleeding while juvenile greens with GTFP had heterophil counts of 30% (Aguirre et al., 1995). A previous study in Florida found that heterophil percentages averaged 55% in green turtles from declining habitats such as the IRL, while the TRI green turtle heterophils made up 33% of the circulating white blood cell population (Lutz et al., 2001). This lends support to the hypothesis that these differences in circulating leukocyte populations are attributable to animals in poor health, perhaps due to degraded

environmental conditions. In this study, heterophils comprise the majority of white blood cells averaging 55% of the leukocyte population in loggerheads and 50% in green turtles with no differences between the two species for any of the white blood cell types. Thus my results are similar to the IRL animals examined in previous studies (Cray et al., 2002; Lutz et al., 2001), though in this study the heterophil population was also high in green turtles from the Trident Basin despite that 15 years ago in this wild population heterophils only made up 34% of the white blood cells (Lutz et al., 2001), which suggests these animals too, may be under physiological stress. These differences may also be a result of the particular age class sampled, species differences, or the near shore environment where juvenile and sub adult turtles are found. Depending on the age class, loggerheads have been shown to have markedly different leukocyte populations. For example, Bradley found that heterophils make up approximately 32% of the circulating leukocytes in juvenile loggerheads while Casal et al. found that the adults have twice as many heterophils (Bradley et al., 1998; Casal et al., 2009).

In addition to determining the phagocytic capabilities of heterophils, I wanted to quantify monocyte function. These white blood cells generally comprise 0 – 10% of the circulating leukocyte population (Stacy et al., 2011) in reptiles and a study on Brazilian turtles has recently suggested that they are the primary phagocytic cell in that population (Rossi et al., 2009). However, due to the nature of the reagents used to retrieve the leukocytes for flow analysis in that study, the heterophil subpopulation was not investigated. Another aspect to consider is that not only are monocytes retrieved from utilizing standard reagents such as Histopaque 1077 as in the Rossi et al. (2009) study or a 60% Percoll gradient as used in this protocol, but lymphocytes are also found in this

same “buffy coat.” Recently, a subset of B cells that normally produce antibodies has been identified in teleost fishes and amphibians and turtles; these phagocytic B-1 cells were thought to have evolved from a phagocytic precursor (Zimmerman et al., 2009). In that study, isolated leukocytes were incubated with fluorescent beads and analyzed with flow cytometry. Results indicated that freshwater turtles (*Trachemys scripta*) do possess this same subset of B cells that exhibit phagocytic capabilities (Zimmerman et al., 2009).

It may be that sea turtles also possess B-1 cells and if this is the case, the rates of phagocytosis that were observed in the PBMC layer of this study most likely included both B-1 cells and monocytes as lymphocytes comprise 30% of circulating white blood cells.

Monocytes comprise up 5% of the leukocyte population in IRL turtles with visible tumors, 2% in IRL turtles without tumors, and 0.8% in turtles from the TRI, in line with previous studies; the small fraction of monocytes in the leukocyte population compared to the monocytes would suggest that monocytes are not the primary phagocytic cell in juvenile sea turtles. Though numbers of monocytes are low, there was a significant difference between IRL animals with tumors and the TRI turtles, suggesting that this white blood cell subpopulation is upregulated when GTFP is present. Increased monocyte percentages in reptiles could indicate chronic infections such as bacterial or parasitic, inflammation, and neoplastic disease (Stacy et al., 2011).

Since heterophils make up such a large percentage of the sea turtle leukocyte population, it seems likely that they play a key role in phagocytosis. While there have been previous studies utilizing flow cytometry to examine monocyte function and cell morphology, this is the first investigation into the function of the sea turtle heterophil.

While there were no differences in the circulating heterophil percentages between the IRL and the TRI turtles, heterophils from the TRI exhibited higher rates of phagocytosis (see below) indicating that those turtles from a more pristine environment perform better than those from the degraded IRL.

Additionally, green turtles without visible tumors function better than those turtles with GTFP, signifying that diseased states do hamper immune function; since there were no large differences in leukocyte subpopulations, differences in rates of phagocytosis are likely to result from actual differences in phagocytic capacity rather than population differences.

Phagocytic Capacity

Species

Flow cytometry revealed sea turtle white blood cells are indeed capable of phagocytosis; this was true of both the monocytes/lymphocytes and the heterophils, as shown by the ingestion of FITC labeled beads by the cells. This is the first study to show that sea turtle heterophils are capable of phagocytosis. Rates of phagocytosis in the loggerhead PBMC and PMN layers (Fig. 6) are comparable at between 2.5 and 3.8% but while rates decreased slightly in the winter (Fig. 7) the difference was not significant. Rates of phagocytosis for green turtles in the summer were significantly higher than either the greens in winter or the loggerheads in summer. This was not surprising since this seasonal difference of suppressed immune responses during winter months has been reported in other reptiles (Zapata et al., 1992). The lower rates of phagocytosis observed in summer loggerhead turtles may be due in part to age and class sizes of the turtles captured as well as where they were captured.

Temperature and Season

Sea turtles are long-lived ectotherms that encounter a wide range of environmental temperatures. There are seven extant species of sea turtles and each inhabits a specific ecological niche (Hendrickson, 1980); these niches allow sea turtles to tolerate environmental factors that not only influence their physiology (Aguirre and Lutz, 2004) but possibly, their immune function as well.

While many factors of reptilian physiology are strongly affected by temperature, it is unclear how temperature influences sea turtle immunity. Recent studies in ecological immunity emphasize the importance of the innate immune system (phagocytosis, inflammation, NK cells) in vertebrates, as opposed to the adaptive immune response (the antibody response) (Matson 2006; Zimmerman et al., 2010). In ectothermic vertebrates especially, innate immunity may be more important due to their slow metabolic rates and relatively slowly acquired immune response which is the premise of this research.

The most influential abiotic factor in the pathogenesis of disease and the physiologic state of most ectothermic marine organisms is that of temperature, which affects multiple cellular and physiological processes. Over the course of a year, sea turtles can be exposed to extremes of temperature from the cold northern ocean to tropical seas. Depending on the species, sea turtles exhibit different physiological "strategies" in order to tolerate extreme temperatures. The effect of reduced temperatures was examined in captive juvenile Kemp's ridleys (*Lepidochelys kempi*) and green (*Chelonia mydas*) turtles. Cold temperatures reduce activity levels; below 15°C, both species decreased foraging efforts, but the green turtles appeared to "tolerate" temperatures between 15ºC and 11ºC with some movement whereas the Kemp's ridleys

remained motionless at the bottom of their tanks (Moon et al., 1997). The experimental design included housing these animals at temperatures as low as 5ºC, but one of the ridleys died at 8.7ºC (the average water temperature of a "cold stun" event). Interestingly, wild populations of Kemp's ridleys exhibit seasonal off shore movement leaving shallow coastal habitats during the winter (Musick and Limpus, 1996) when temperatures reach lower critical limits to avoid rapid decreases in water temperature whereas juvenile green turtles recruit to shallow near shore habitats such as the Indian River Lagoon where water temperatures generally remain within 18ºC to 25º (Ehrhart, 1983; Mendonca, 1983).

In another study, it was shown that sea turtles rely on environmental factors such as temperature and photoperiod to regulate their metabolism. Results indicated that optimal water temperatures for metabolic rate and cardiac output in sea turtles lies between 15°C and 30°C (Southwood et al., 2003). Higher temperatures may induce other stressors, such as reduced dive time due to increases in oxygen consumption, or increased production of reactive oxygen species during breathold dives.

Additionally, increases in temperature are correlated to disease states in benthic invertebrate species such as the oyster, *Crassostrea virginica* found in estuarine habitats. Results indicated that increased water temperatures contributed to altered immune function where the oyster was unable to mount an immune response against pathogens (Boyd and Burnett, 1999). Therefore, it is likely that seasonal fluctuations and extremes in water temperatures also affect sea turtle immune function.

To determine if temperature directly influenced phagocytic capacity of sea turtle leukocytes, I incubated heterophils and monocytes with fluorescent latex beads at biologically relevant temperatures ranging from 4 - 37°C for one hour. It was expected

that since metabolism in heterotherms varies with temperature, rates of phagocytosis would increase at warmer temperatures, thus explaining in part why immune responsiveness is generally reduced in the winter in a variety of reptiles (Duguy, 1970; Guo, 2001; Zapata et al., 1992). Surprisingly, there were no *in vitro* temperature differences in rates of phagocytosis for either species of sea turtle. Any significant differences in temperature are most likely attributable to either the location where the turtles were sampled or the health status of the individuals (Fig. 10).

There were no *in vitro* temperature differences pertaining to the individual subpopulations except at 4ºC (Fig. 12). This implies that if temperature does not have an acute effect on cell physiology, the seasonal effects may be a long term acclimation related to temperature, photoperiod, or other factors (Southwood et al., 2003). There was evidence of seasonal differences: heterophils exhibit increased rates of phagocytosis during the summer (Fig. 13) An additional factor is that there were no extreme temperature events during this study that may have had adverse physiological repercussions such as cold stunning which may have yielded different results.

And of course, the cells were incubated with the beads at each temperature for only one hour. This may not have been sufficient time to alter the molecular and cellular components of the immune response from their baseline acquired at either the turtle's environmental temperature or from maintaining the cells in cool conditions for transport following collection (from 4 to 7 hours prior to experimental incubations). However, molecular pathways studied in freshwater turtles respond within1 hr to such stressors as anoxia (Kesarju et al., 2009), with strong temperature differences impacting such

responses (Stecyk et al., 2012) so reptiles are certainly able to alter cellular metabolism quickly.

Health Status and Location

Sea turtles are intrinsically bound to coastal habitats that pose numerous anthropogenic threats to their survival. Ecological stressors, such as habitat destruction and fragmentation, and inundation by pollutants and contaminants may influence the health of an ecosystem, which will in turn influence the health of the inhabitants.

This is especially true in the Indian River Lagoon, an estuary experiencing declining health due to an accumulation of these ecological stressors. Located along the eastern coast of Florida, it spans 250 km of central Florida and has very little tidal mixing with the Atlantic Ocean. Freshwater inputs and nutrient pollution have exacerbated harmful algal blooms (HABs) (Gobler and Sunda, 2012; Phlips et al., 2004). A brown algae bloom in 2011 resulted in a loss of 19,000 hectares of seagrass, or approximately 60% of total seagrass cover in the IRL (St. Johns Water Management District [SJRWMD], 2013). In addition, these HAB events have been associated with health declines in resident Atlantic bottle-nose dolphin (*Tursiops truncates*) populations (Fire et al., 2007, 2011). Dolphins are also presenting with Lacaziosis in the southern IRL where decreased salinity allows for opportunistic fungal infections (Durden et al., 2009).

The IRL's oceanic shores attract a high influx of nesting adult sea turtles from March through October, while juveniles recruit to nearshore habitats to forage in the winter (Aguirre and Lutz, 2004). Juvenile green turtles also experience the effects of ecological stressors as long term monitoring of these animals have documented an increase in neoplastic tumors and the prevalence of Green Turtle Fibropapillomatosis

(GTFP). Greater than 70% of the IRL's green turtle population has GTFP (Aguirre and Lutz, 2004; Hirama and Ehrhart, 2000). An examination of stress responses at the molecular level also suggests that green turtles in the IRL are physiologically stressed, since levels of such stress markers as the heat shock proteins are higher in these animals than in turtle captured in the Trident Basin, whether they had visible tumors or not (Deming, 2008).

Within the IRL, GTFP is more prevalent in those green sea turtles captured during summer months (L. M. Ehrhart, personal communication, August 20, 2008), despite the fact that, as shown in this study, rates of phagocytosis are significantly higher in the summer than winter. Among green turtles from the IRL, those without visible tumors exhibit significantly higher rates of phagocytosis in both seasons than turtles with GTFP (Fig. 14). Additionally, TRI turtles sampled in the winter exhibit the highest rates of phagocytosis (Fig. 14). This substantiates the hypothesis that turtles from more pristine habitats where disease is rare are more able to mount a robust defense against pathogens, while those from degraded habitats are physiologically stressed and immunosuppressed.

The data show that sea turtles that inhabit degraded environments exhibit reduced innate immune function, which may make them more prone to disease. This is apparent *in vitro* whether the turtles exhibit tumors or not. Similar results have been found with the adaptive immune response (Cray et al., 2002) where lymphocytes from green turtles with GTFP of the IRL did not respond as vigorously to mitogenic stimulation (Lutz et al., 2001).

Utilizing flow cytometry to quantify innate immune function has proved a valuable tool in quantifying phagocytosis in loggerhead and green turtles. And although

temperature did not yield the expected results, a temperature difference might have been observed if a cold-snap and subsequent cold stun event provided longer term acclimation to lower temperatures and thus allowed any slower-developing responses to unfold. Photoperiod could not be manipulated in wild populations, but seasonal differences were observed in green turtle heterophil function. Health status and degraded habitats clearly impact immune function in these animals as rates of phagocytosis are significantly higher in turtles from the TRI compared to the IRL. This is especially evident for the granulocyte layer, likely because heterophils make up ~50% of the WBC population whereas monocytes make up only 1-4%. While this study highlights the importance of the innate immune system as a key defense mechanism in juvenile sea turtles, it is clear that further research is needed to further elucidate sea turtle immune function.

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