INFLUENCE OF UV LIGHT ON VITAMIN D AND IMMUNE FUNCTION OF GREEN (*CHELONIA MYDAS*) SEA TURTLES WITH FIBROPAPILLOMATOSIS

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Sarah L. Milton, Department of Biological Science, and has been approved by all members of the 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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ABSTRACT

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Green sea turtles (*Chelonia mydas*) are an endangered species prone to a debilitating disease called fibropapillomatosis (FP). The aim of this study was to determine the influence of UV light on vitamin D levels and immune function in juvenile green sea turtles with FP. Phagocytosis, plasma vitamin D levels and viral load of ChHV5 were measured for FP- and FP+ turtles kept at the Gumbo Limbo Nature Center (GLNC) and for turtles caught at the St. Lucie power plant. Turtles kept at GLNC were housed in tanks exposed to varying amounts of UV light. Turtles brought into GLNC had lower phagocytosis compared to turtles at the St. Lucie power plant. Individuals exposed to greater UV light had higher plasma vitamin D levels and a more successful recovery. The results of this project will provide rehabilitation facilities with a mechanism to improve the recovery of animals with this disease.

INFLUENCE OF UV LIGHT ON VITAMIN D AND IMMUNE FUNCTION IN GREEN (*CHELONIA MYDAS*) SEA TURTLES WITH FIBROPAPILLOMATOSIS

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INTRODUCTION

Marine turtle fibropapillomatosis (FP) is a severe disease impacting sea turtles around the world. FP causes the growth of tumors and is a major threat to green sea turtles as in some subpopulations it affects over 60% of turtles (Herbst, 1994; Hirama and Ehrhart, 2007). Juvenile green sea turtles are particularly prone to the disease, and it appears that there is a link between the disease and their residence in nearshore areas, which present many anthropogenic threats, including temperature extremes, eutrophication, and pollution (Aguirre and Lutz, 2004). Although external tumors can be removed in a rehabilitation facility, regrowth often occurs. An enhanced treatment regime for juveniles with this disease is needed to improve their outcome and to help the population as a whole.

Anthropogenic Factors and Disease

Green sea turtle populations are found in temperate to tropical waters around the world and migrate between feeding grounds and distinct nesting beaches as adults (Spotila, 2004). Throughout their life they utilize a variety of habitats that may subject them to environmental stressors. Hatchlings from the Florida population emerge from nests on the shore and swim directly out to the Sargasso Sea (Carr, 1987). Once they have grown to about 30-40cm carapace length, juveniles migrate back to nearshore sea grass

beds, where they remain until they reach maturity (Musick and Limpus, 1997). These near shore locations were once ideal for the healthy maturation of juvenile sea turtles; however, in recent decades, they have been degraded by a variety of anthropogenic factors.

Anthropogenic factors, such as climate change, eutrophication, pollution, and diseases are impacting marine species all over the world. Climate change has led to rising temperatures and increases in storms (Pike and Stiner, 2007), and warmer sea temperatures in turn encourage algal blooms and eutrophication as well as increased zones of hypoxia (Chislock et el., 2013). Pollution and eutrophication from nutrient input have impacted marine species in multiple ways. Pollution effects can include plastic ingestion, nutrient runoff, and habitat changes. Plastic ingestion is frequently seen in marine animals as the plastics look very similar to their normal food sources. In a group of 40 loggerhead post-hatchlings that died after stranding in South Africa, 60% were killed by ingested plastics that were unable to pass through their digestive tract (Ryan et al. 2016). Excessive nutrient runoff can also affect nearshore organisms as it has increased harmful algal blooms (Phlips et al., 2003; Gobler and Sunda, 2012). In 2011, a super bloom of brown algae in the Indian River Lagoon, FL resulted in up to an 87% reduction of seagrass coverage (Indian River Lagoon 2011 Consortium, 2015). Loss of these seagrass beds, a primary food source for many organisms, will result in food web changes. In Hawaii, a nonnative macroalgae has become the main food source for green sea turtles in nearshore areas after dominating native seagrass beds (Van Houtan et al., 2010). The increase of pollution in nearshore areas has also led to the emergence of diseases. Loggerhead sea turtles (*Caretta caretta*) are often affected by “Debilitated

Loggerhead Syndrome," which results in anemic and/or moribund individuals (Wyneken et al., 2006). Sea turtles also become infected with bacterial or viral infections.

Florida's Indian River Lagoon (IRL) is a polluted nearshore area that has experienced outbreaks of various diseases. Lobomycosis is a fungal infection in bottlenose dolphins (*Tursiops truncatus*) that causes dermal lesions to form on large portions of the body (Murdoch et al., 2008). It is one of the more prevalent lesions that can form and is thought to be endemic to the IRL (Reif et al., 2006; Reif et al., 2013; Murdoch et al., 2008; Bossart et al., 2015). Bacterial diseases have also been shown to be a major threat. Studies have found a variety of bacterial strains in *T. truncatus* and have shown that the majority of dolphins that have died from infectious disease, died from a bacterial infection (Morris et al., 2011; Bossart et al., 2003; McFee and Lipscomb, 2009). One disease that is significantly impacting green sea turtles in the IRL is marine turtle fibropapillomatosis (FP).

FP is a debilitating disease that mainly affects juvenile green sea turtles. FP was first described in 1938 in an adult green turtle captured from the Florida Keys (Lucke, 1938; Smith and Coates, 1938). Since then, FP has spread worldwide and affects over 60% of green sea turtles in some subpopulations (Herbst, 1994; Hirama and Ehrhart, 2007). Although FP primarily affects green sea turtles, it has been confirmed in all species of hard shelled marine turtles (Smith and Coates, 1938; Harshbarger, 1991; Barragan and Sarti, 1994; D'Amato and Moraes-Neto, 2000; Aguirre et al., 1999; Limpus et al., 1993). FP is characterized by benign fibroepithelial tumors that can be present on the eyes, skin, and mouth cavity as well as internally (Jones et al., 2016). These tumors vary in size ranging from 0.1 to 30 cm and can differ in morphology from smooth-

surfaced to cauliflower-shaped (Jones et al., 2016). The main virus associated with this disease is a herpes virus known as Chelonian herpesvirus 5 (ChHV5), previously Chelonid fibropapilloma-associated herpesvirus (Adams and Carstens, 2010). FP has also been associated with papillomavirus and retrovirus (Aguirre and Lutz, 2004); however, ChHV-5 appears to be the virus presenting in the majority of cases. It is thought that ChHV5 co-evolved with marine turtles and has been around for millions of years (Herbst et al., 2004). Since the virus has been around a long time and the tumors only started becoming more prominent about 80 years ago, environments affected by anthropogenic factors are likely contributing to the increase in FP (Jones et al., 2016). Along with an increase of FP, the combination of ChHV5 and environmental conditions may also impact a sea turtle's immune system.

Immunosuppression

Vertebrate immune systems are comprised of both the adaptive and innate immune response. The innate immune system is more ancient and widespread in the animal kingdom and generates the initial response against an invading pathogen involving phagocytotic cells, blood proteins, and physical and chemical barriers (Owen et al., 2013). Adaptive immunity, which evolved more recently, is the delayed response that relies on immunologic memory as it recognizes specific antigens (Owen et al., 2013). Each form of immunity has different types of leukocytes that are activated in response to a pathogen. Leukocytes of the innate immune system include monocytes and the granulocytes: heterophils (equivalent to the mammalian neutrophil), eosinophils and basophils (Campbell, 2006). Monocytes and heterophils are the main phagocytotic cells

that respond to inflammation and infection (Owen et al., 2013), with heterophils making up about 55% of the blood population in sea turtles (Lutz et al., 2001). Eosinophils are known to target larger parasitic organisms through phagocytosis (Tizard, 2009). Basophils are typically found in low numbers and respond to allergies by producing histamine (Owen et al., 2013). The primary leukocytes of the adaptive immune system are lymphocytes, which come in two major types: B and T lymphocytes (Mansour et al., 1980; Campbell, 2006). The numbers and ratios of these various leukocytes can be used to determine the status of the immune system.

Turtles that develop FP often have a compromised immune system. Studies have examined the immune system of green sea turtles with FP found along the coast of Florida by looking at their leukocyte counts (Lutz et al., 2001; Cray et al., 2001). Lutz et al. (2001) determined that immune function was much lower in turtles from highly polluted areas and in turtles with FP. In both studies, FP turtles had higher heterophil counts and lower lymphocyte counts than non-papilloma turtles (Lutz et al., 2001; Cray et al., 2001). Similar findings have been seen in Hawaii as well (Aguirre et al., 1995; Work et al., 2001). Both studies found higher heterophil counts and lower lymphocyte counts in turtles with FP compared to non-FP turtles (Aguirre et al., 1995; Work et al., 2001). More recent work has shown that turtles from more polluted environments also had reduced phagocytic capacity (Fig. 1 and 2, Sposato, 2014 MS Thesis). Together, these data suggest that the immune systems of FP turtles are being affected by or are impacting the course of the disease in some way. Determining a way to aid immune system function may lead to better survival of juveniles with this disease.

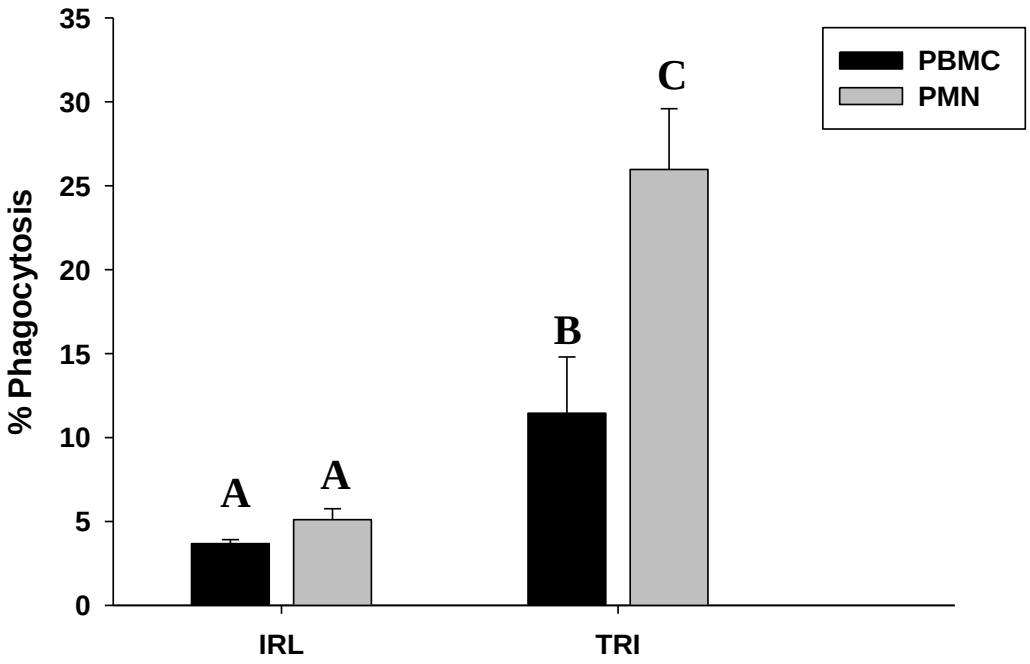

Figure 1: Rates of phagocytosis from turtles captured in the Indian River Lagoon (n=107) compared to turtles captured from the Trident Basin, Cape Canaveral (n=27). Rates were lower in IRL compared to TRI (Sposato, 2014 MS Thesis).

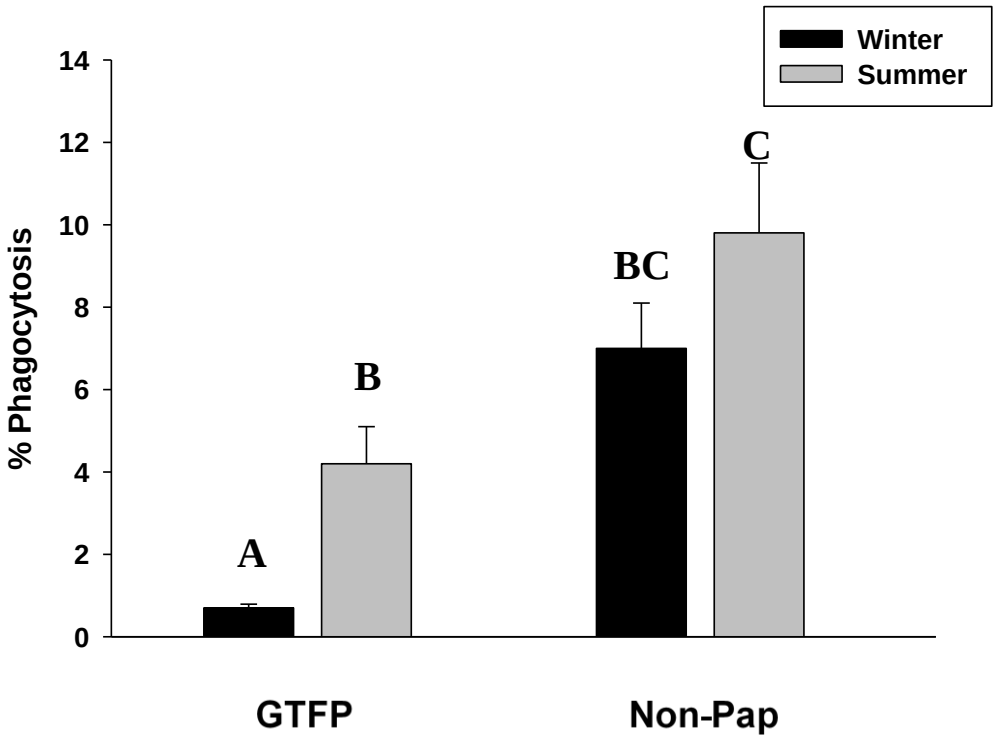

Figure 2: Seasonal variations in phagocytosis within the Indian River Lagoon are lower in turtles with FP compared to turtles without tumors (Sposato, 2014 MS Thesis).

Importance of Vitamin D

One potential way to increase immune competence is by increasing Vitamin D (vit D) levels as it has recently been linked to immune system function. Vit D is an essential nutrient for all vertebrates that can be obtained by consuming foods containing vit D or by synthesis in skin tissues after exposure to ultraviolet (UV) irradiation (Chao et al., 2015). When obtained through UV irradiation, vitamin D_3 is produced by the conversion of 7-dehydrocholesterol to cholecalciferol (Beard et al., 2010). Once obtained, the body converts vit D to various forms including 25-hydroxyvitamin D (25-OH-D) and

1,25-dihydroxyvitamin D (1,25-(OH)2-D). The liver generates 25-OH-D from the reaction of 25-hydroxylases with vit D (Prietl et al., 2013), and it is the inactive form that is used to test for vit D deficiency (Chao et al., 2015). From there, 25-OH-D is further converted to the active form 1,25-(OH)2-D by 1-α-hydroxylase (CYP27B1) (Prietl et al., 2013). The production of the active form allows vit D to fulfill its many functions. Vit D traditionally plays an essential role in bone mineralization and calcium homeostasis by increasing the transport and absorption of calcium by the intestine (Barrett et al., 2010). Vit D deficiency in humans and reptiles, as well as other vertebrates, can lead to metabolic bone disease (Christodoulou et al., 2013; George, 1997; Hoby et al., 2010). More recently, vit D has been found to play a major role in both the adaptive and innate immune systems.

Effects of vit D on immune system function have been seen in multiple species. In humans, 1,25-(OH)2-D can potentially increase cathelicidin release, which has been shown to inhibit vaccinia virus, herpes simplex virus type 1, and retrovirus replication (Chao et al., 2015). Cathelicidin is an antimicrobial peptide that is a natural defense in most living organisms (De Smet and Contreras, 2005). It has also been shown that production of cathelicidin from vit D could influence a response against intracellular *M. tuberculosis* (Liu et al., 2007). In mice with mammary cancer, vit D has been shown to inhibit tumor growth (Valrance et al., 2007). Links between vit D and immune function have also been observed in European sea bass. Sea bass administered high levels of vit D had greater immune function and phagocytotic capabilities than the non-supplemented group (Dioguardi et al., 2017). Increasing vit D levels could lead to better immune function and potentially could affect sea turtles with FP in a similar way.

As with most vertebrates, reptiles obtain vit D through the food they consume and through UV irradiation. Many species rely on UV irradiation for vit D as not all diets are rich in vit D. Studies have shown that supplemental UV irradiation can help boost vit D levels in reptiles (Acierno et al., 2008; Acierno et al., 2007, Oonincx et al., 2010). Corn snakes exposed to UVB and UVA radiation had higher vit D levels than control animals (Acierno et al., 2008). A study in red-eared sliders exposed to UVB radiation also had higher vit D levels compared to the control (Acierno et al., 2007). In a study on juvenile bearded dragons, UVB supplementation resulted in vitamin D levels 5-18 times higher than in vitamin D dietary supplemented groups (Oonincx et al., 2010). Of all the species of marine turtle, green sea turtles are one of the only species known to come ashore to bask in the sun without nesting (Spotila, 2004). This basking behavior may help with regulating temperature, avoiding mates, and escaping predators (Spotila, 2004). It is possible that this behavior could also increase vitamin D levels. Purgley et al. (2009) monitored the vitamin D levels of captive green sea turtles that were kept in outdoor and indoor facilities. The turtles that were kept inside without any exposure to ultraviolet irradiation had lower vitamin D levels than those kept outside (Purgley et al., 2009). A study looking at the basking behavior of green sea turtles with FP found that turtles with FP basked more frequently than turtles without FP, suggesting that this behavior could be used to enhance their immune response (Swimmer, 2006). It is possible that sea turtles obtain vitamin D from basking in the sun and that light availability could influence their vitamin D levels. Vitamin D supplementation could potentially be used with current treatments to better the outcome of juveniles with FP.

The current treatment for FP is tumor removal surgery. Although this method is effective, regrowth of the tumors often occurs. The objective of this study was to determine if increased levels of UV light increase vit D levels in sea turtles in a rehabilitation center for FP treatment, and if this in turn will boost immune function in these animals. Phagocytotic capability was determined as one measure of immune system status, in FP individuals exposed to light treatment and those not exposed, as well as in individuals without FP. Many rehabilitation facilities have enclosures that are exposed to limited natural UV light. The results of this project may provide rehabilitation facilities with a mechanism to improve outcome in animals with fibropapillomatosis, through vit D and UV light supplementation, and will benefit the survival and conservation of the species.

Specific Aims

1) **To determine the variations in health parameters in FP- versus FP+ turtles**

   Hypothesis 1.1: Hematology parameters will vary between FP- and FP+ turtles upon intake.

   HO1.1: Hematology parameters will not vary between FP- and FP+ turtles upon intake.

   Hypothesis 1.2: Blood chemistry parameters will vary between FP- and FP+ turtles upon intake.

   HO1.2: Blood chemistry parameters will not vary between FP- and FP+ turtles upon intake.

   Hypothesis 1.3: Rates of phagocytosis will be lower in FP+ turtles upon intake.

HO1.3: Rates of phagocytosis will be higher in FP+ turtles upon intake.

HO1.3a: Rates of phagocytosis will be the same in FP+ and FP- turtles upon intake.

2) **To examine the impacts of UV light therapy on vitamin D levels.**

   Hypothesis 2.1: Vitamin D levels will be higher in turtles exposed to greater levels of UV light.

   HO2.1: Vitamin D levels will be lower in turtles exposed to greater levels of UV light.

   HO2.1a: Vitamin D levels will be the same in turtles exposed to greater levels of UV light.

3) **To determine the effects of UV light therapy and vitamin D on immune function.**

   Hypothesis 3.1: Rates of phagocytosis will be higher in turtles exposed to greater levels of UV light.

   HO3.1: Rate of phagocytosis will be lower in turtles exposed to greater levels of UV light.

   HO3.1a: Rates of phagocytosis will be the same in turtles exposed to greater levels of UV light.

   Hypothesis 3.2: Rates of phagocytosis will be positively correlated with vitamin D.

   HO3.2: Rates of phagocytosis will be negatively correlated with vitamin D.

   HO3.2a: Rates of phagocytosis will not be correlated with vitamin D.

4) **To determine if UV light therapy reduces regrowth of fibropapillomatosis in sea turtles.**

Hypothesis 4.1: Tumor regrowth will be less in turtles exposed to greater levels of UV light.

HO4.1: Tumor regrowth will be greater in turtles exposed to greater levels of UV light.

HO4.1a: Tumor regrowth will be the same in turtles exposed to greater levels of UV light.

5) **To determine if UV light exposure influences viral load of ChHV5**

Hypothesis 5.1: Viral load amount will vary depending on tumor location.

HO5.1: Viral load amount will not vary depending on tumor location.

Hypothesis 5.2: Viral load amount will increase with greater exposure to UV light.

HO5.2: Viral load amount will decrease with greater exposure to UV light.

HO5.2a: Viral load amount will not change with greater exposure to UV light.

MATERIALS AND METHODS

To test these hypotheses, I compared vitamin D levels, immune function, viral load, and blood chemistry in turtles without evident FP tumors (FP-) vs turtles with evident tumors (FP+) kept in varying UV light conditions at the rehabilitation facility at the Gumbo Limbo Nature Center (GLNC). I also compared the samples collected from turtles in rehabilitation to presumed healthy turtles caught at the St. Lucie Power Plant by the Inwater Research Group (IRG). This study was conducted in collaboration with rehabilitation staff at GLNC (Marine Turtle Permit #084), with the researchers at Inwater Research Group (Marine Turtle Permit #125) and under Marine Turtle Permit #053.

GLNC Candidate Selection and Care

Juvenile green turtles (*C. mydas*) appropriate for the study (see below) that were FP- and FP+ were randomly selected upon admittance to GLNC into one of four groups (FP- control, FP- treatment, FP+ control, or FP+ treatment). All turtles brought to GLNC were assessed by the attending veterinarian and GLNC staff to determine inclusion in the study. To minimize variability due to secondary anomalies, turtles with severe secondary injuries (i.e. invasive boat strike, deep lacerations from constricting entanglement, etc.) were excluded. Turtles with minor secondary injuries (healed boat strike, superficial entanglement, etc.) were considered for inclusion. Turtles included in this study were juveniles ranging from >25cm to <60cm straight carapace length.

All turtles were cared for by GLNC rehabilitation staff and received routine veterinary care. Animals are offered a consistent diet of up to 5% BW daily of the same variety of silversides, squid, shrimp, caplin, and green vegetation as directed by the attending veterinarian. Every attempt to eliminate variation due to diet was made by providing the same food for all individuals. Standard medical interventions were made as needed; these included fluids, antibiotics, and vitamins. Routine care also includes administration of a Vitamin D gel capsule treatment (5000 ICU), which likely altered vitamin D levels. The dose was standardized and used as a baseline for all turtles in the study, with UVB treatment potentially increasing vitamin D levels further.

IRG Candidate Selection and Sample Collection

Turtles captured at the St. Lucie Power Plant by IRG are individuals that were entrained into the outflow area. The turtles were collected by IRG and sampled for various projects. Body measurements were taken, blood was drawn (~5ml), and the turtle was tagged and then released. All individuals included from that source were healthy FP-juveniles ranging from >25cm to <60cm straight carapace length.

UV Light Treatment

UV light treatment was only given to turtles kept at GLNC. Turtles were exposed to varying amounts of UVB depending on the location of the tank (Fig. 3). Turtles from the treatment groups were kept in tanks that were exposed to full sunlight and turtles in the control groups were housed in tanks that are covered, and therefore receive limited amounts of light. The amount of light the tanks received daily depended on the sun

exposure each day and varied with cloud cover and time of year. The UVA and UVB radiation were measured with a HOBO radiometer-photometer at 30-minute intervals at the water's surface in all tanks to compare maximum potential UVB exposure. Although the maximum amount of UVB exposure was measured, the amount of UVB radiation each turtle received varied depending on behavior, as they would often spend time submerged in the water during the day. All turtles were monitored by rehabilitation staff for adverse reactions to the treatment; however, no such reactions occurred.

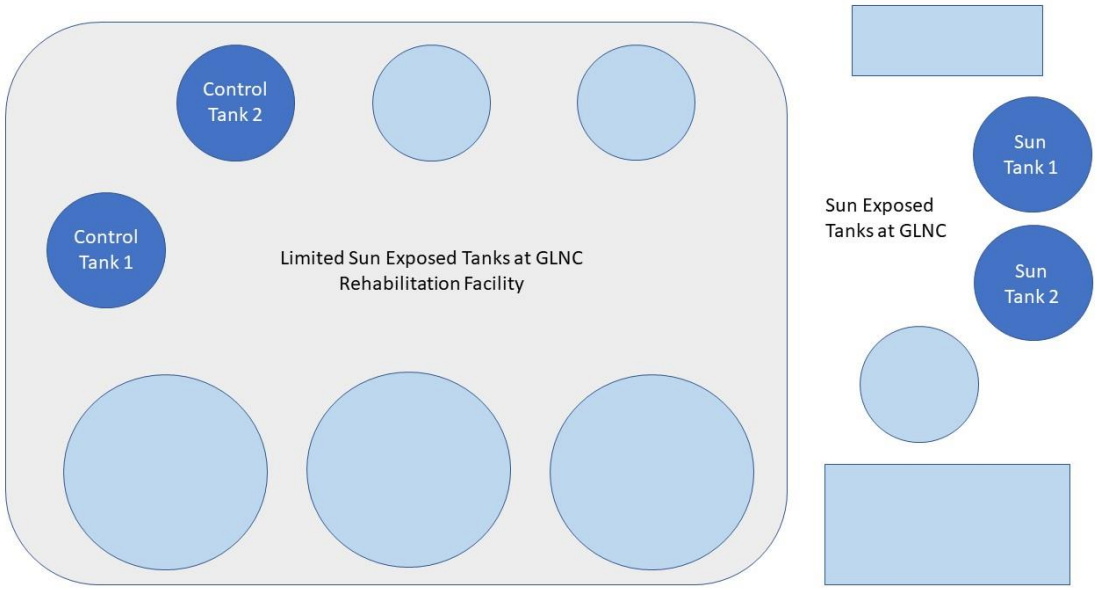

Figure 3: Diagram of the rehabilitation facility at GLNC. Brown gray square represents the portion of the rehab facility located underneath an overhang. Dark blue tanks represent tanks selected in the study. Light blue tanks are other tanks located in the rehab facility.

Blood Sample Collection

All samples from rehabilitation turtles were collected by GLNC staff under MTP #084. Blood samples were taken upon intake, prior to release and at 2-month intervals if the animal remained longer than 8-12 weeks (Fig. 4). These samples were taken using a vacutainer and appropriate size needle (21 or 22 gauge). Prior to blood sampling, turtles were restrained, and skin was disinfected 3 times with alternating applications of betadine and isopropyl alcohol. ~5 mL of blood was drawn from the dorsal cervical sinus and transferred to various containers (size-dependent - samples drawn were significantly less than the safe maximal amount of blood that can be drawn from a healthy reptile, determined to be 4% of body weight converted to volume, or 3 mLs/kilo, (Lillywhite and Smits, 1984; Smits and Kozubowski, 1985)). Blood was drawn regularly during the rehabilitation process for in house blood chemistry analysis and for cell blood count (CBC) and differentials, which were determined by the University of Miami Health Systems Comparative Pathology Laboratory.

~200 µL of blood was placed in 2 mL cryovial and flash frozen for viral load analysis. These samples were stored at -80°C until transferred to Florida Atlantic University Harbor Branch Campus for analysis. ~ 2.5 mL of blood was placed in a lithium-heparin tube was spun down to separate the plasma. ~1.5 mL of plasma was collected and stored at -80°C until shipped to Animal Health Diagnostic Laboratory at Michigan State University to measure 25-hydroxyvitamin D_3 concentrations. The remaining blood sample was placed in a sodium-heparin vacutainer. These samples were placed in a test tube rack to prevent contact with ice packs after collection and transported to the lab at Florida Atlantic University to determine immune responsiveness.

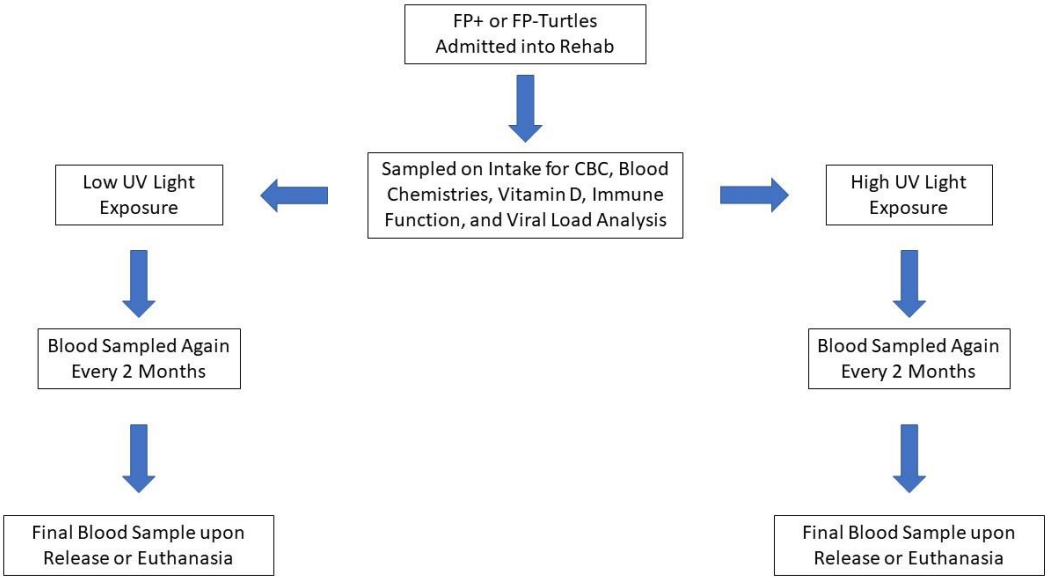

Figure 4: Flow chart of experimental design and sample collection for rehab turtles brought into GLNC.

Tumor collection and Regrowth Monitoring

Tumor samples from FP+ turtles were collected post-surgery and post-euthanasia for viral load analysis. These samples were flash frozen and stored at -80°C until transferred to Florida Atlantic University Harbor Branch Campus for analysis. Turtles were monitored post-surgery for any potential regrowth. The individuals were checked every two weeks and the number of tumors that regrew were recorded.

Immune Function Analysis

Separation of Whole Blood

Whole blood was layered using 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. After layering, the sample was centrifuged at 400 relative centrifugal force (RCF) for 5 minutes, followed by 800 RCF for 15 minutes. Each layer was then transferred to 1.75mL microcentrifuge tube and washed three times with 1x phosphate buffered saline (PBS) with 5% heat inactivated fetal bovine serum (HI FBS) at 4°C. Cell viability was determined by staining 10µL of cell solution with 10µL of Trypan Blue using standard hemocytometer methodology. Each sample was then be diluted with RPMI with 5% HI FBS to bring the concentration to $1x10^6$ cells/mL.

Flow Cytometry Phagocytosis Assay

After separating the blood, phagocytosis was measured using flow cytometry. Each sample was incubated for one hour at room temperature with a suspension of fluorescent latex beads labeled with fluorescein isothiocyanate (FITC). In brief, 0.75mL of FITC particles (diluted to 40uL/mL in RPMI) were added to 1.5mL of cells (cell concentration of $1x10^6$ cells/mL) in 50mL conical tubes. After the incubation period, phagocytosis was stopped by placing samples on ice. Leukocytes were then washed three times with 1x PBS plus 5% HI FBS to wash off any non-internalized and nonspecifically bound beads. The samples were then fixed using a Becton, Dickinson and Company (BD) Cytofix fixative and stored in FACS Buffer (1x PBS plus 3% HI FBS and Na Azide) for up to one week before analysis. A BD FACSCalibur high-speed digital bench-top cell sorter was used to evaluate fluorescence, and phagocytosis was measured at 480 nm to

detect the leukocytes which had engulfed the fluorescein marked beads. Forward scatter (FS) and side scatter (SS) was used to identify the different cell types according to their patterns of size and granularity. Samples were gated to include all leukocytes and left out any debris.

Viral Load Analysis

DNA Extraction

Blood and tumor tissue samples were used for viral load analysis. A Qiagen DNeasy Blood and Tissue Kit was used to extract DNA from the samples. For blood samples, 10µL of blood was mixed with 20µL of proteinase K and 190µL of PBS in a 1.5mL microcentrifuge tube. These samples were then vortexed and incubated at 56°C for 10 minutes to lyse the blood cells. For tumor samples, the tissue was cut into small pieces (<25mg) and mixed with 200µL of tissue lysis buffer containing proteinase K in a 1.5mL microcentrifuge tube. The samples were then vortexed and incubated at 56°C until completely lysed. After these samples were lysed, they were washed 2 times with washing buffers. An elution buffer was used to collect the DNA into solution. The DNA concentration was then measured using absorbance spectrophotometry (Nanodrop), and the absorption ratios at 260nm versus 280nm were evaluated to ensure DNA purity.

qPCR Reaction

Quantitative Polymerase Chain Reaction (qPCR) was run on all extracted DNA to determine the viral load content of ChHV5. qPCR reaction solutions were created by mixing 10µL of SensiFAST^{TM} Probe Lo-ROX, 0.8 µL (0.32 µM) each of forward and reverse ChHV5 UL30 primers, 0.2 µL (0.32 µM) of fluorescent probe, and 8.2 µL of

extracted DNA. Primers used in this study were originally develop by Page-Karjian et al. (2015). All qPCR reactions were carried out using a MX3000 qPCR instrument and the following reaction conditions: 10 min at 95°C (polymerase activation step), and 40 cycles each of 30 s at 95°C (denaturation step), 1 min at 55°C (primer annealing step), and 1 min at 72°C (elongation step). All samples were done in triplicate. Positive and negative controls were also run in triplicate. Samples were considered positive if average ChHV5 copy number/mg DNA was equal to or higher than the assay's analytical limit of detection.

Statistical Analysis

Comparisons were made between control and treatment animals, and between FP- and FP+ groups. Data were tested for normality utilizing Shapiro-Wilks test, and One-way Anovas and t-tests were used to detect differences between groups. Data that were not normally distributed were analyzed by non-parametric tests: Kruskall-Wallace with Dunn's test and Wilcoxon Rank Sum Test respectively. Correlations were also run to compare rates of phagocytosis with vitamin D levels and to compare these values to blood chemistry parameters and body condition index. Pearson correlations were run when data were normally distributed and Spearman Rank correlations were run when data were not normally distributed. Fisher's exact test were used to compare survival regrowth occurrence.

RESULTS

Hematology

Pack cell volume (PCV), white blood cell count (WBC), and leukocyte differentials were measured for all samples collected from turtles in rehabilitation. Only PCV was measured for non-rehab (FP-) turtles from the St. Lucie power plant. PCV values upon intake ranged from 20-39.5% in FP- rehab turtles, 5-27% in FP+ rehab turtles and 24.5-42.5% in non-rehab FP- turtles (Fig. 5). PCV values were highest in non-rehab FP- turtles and lowest in FP+ rehab turtles. Since FP- rehab turtles are the intermediate value, there was no significant difference between rehab FP- turtles and non-rehab FP- or rehab FP+ ($p > 0.05$); however, there was a significant difference between FP+ rehab turtles and non-rehab FP- turtles ($p < 0.001$). WBC count ranged from 4,400-19,800 cells in FP- rehab turtles and 7,300-49,000 cells in FP+ rehab turtles (Fig. 6). Median WBC count was higher in FP+ rehab turtles compared to FP- rehab turtles; however, there was no significant difference between the two ($p > 0.05$). Leukocyte populations were consistent between FP- rehab turtles and FP+ rehab turtles (Fig. 7). The most frequent cells seen in both turtle groups were heterophils, followed by lymphocytes, monocytes, eosinophils and basophils. Although there was a difference in the percentage of cells seen in each group of turtles, there was no significant difference in leukocyte populations between FP- rehab turtles and FP+ rehab turtles ($p > 0.05$).

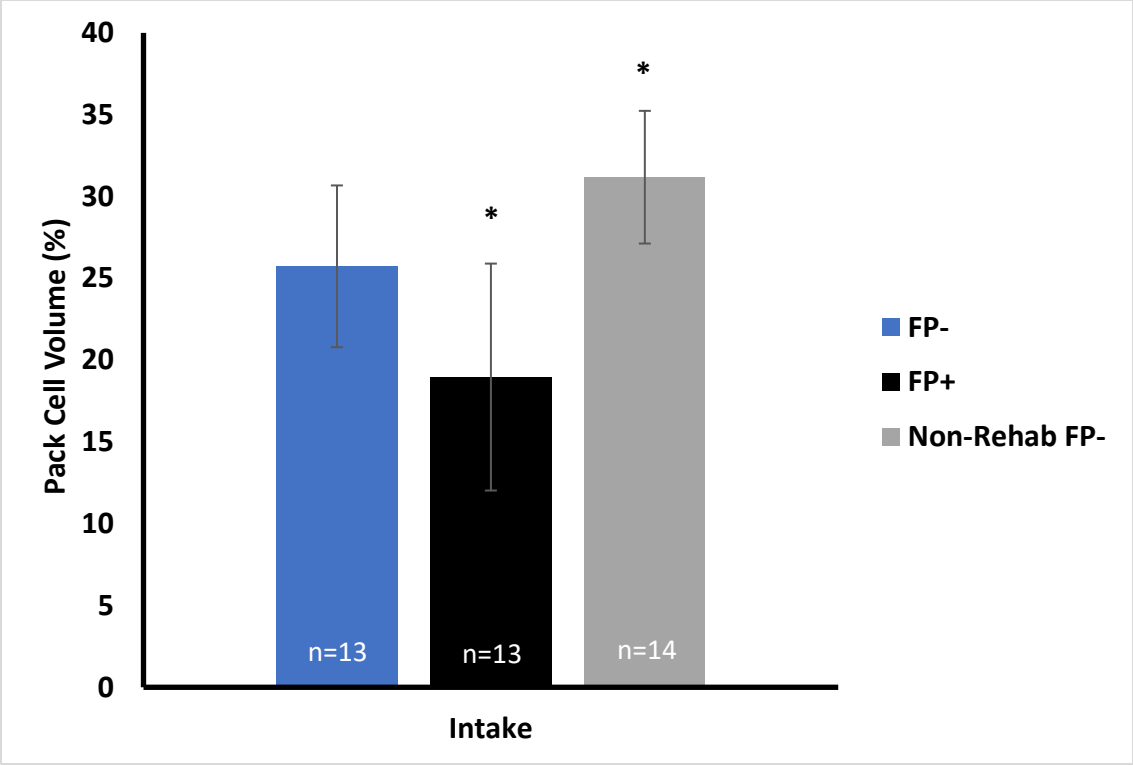

Figure 5: Intake pack cell volume (PCV) for rehab and non-rehab turtles. * Rehab FP+ turtles PCV values were significantly different from non-rehab FP- turtles ($p < 0.001$). Error bars are one standard deviation from the mean.

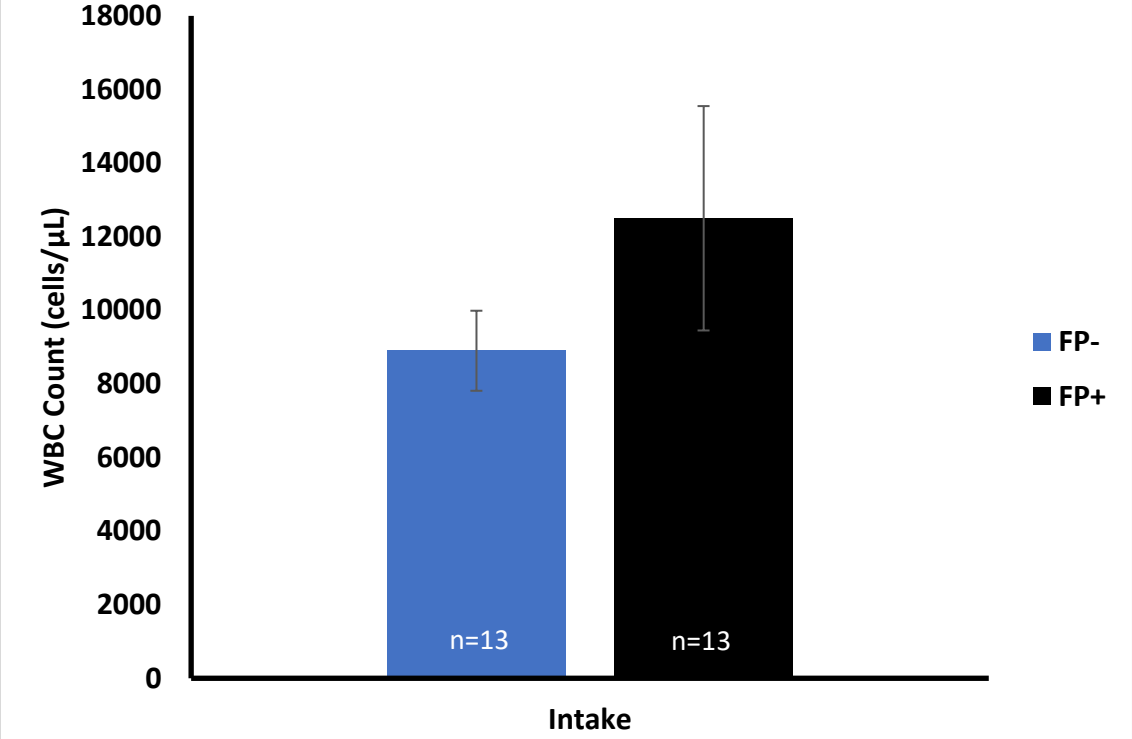

Figure 6: Intake white blood cell count for rehab turtles. There was no significant difference for WBC counts between FP+ and FP- turtles ($p > 0.05$). Error bars are one standard error from the median.

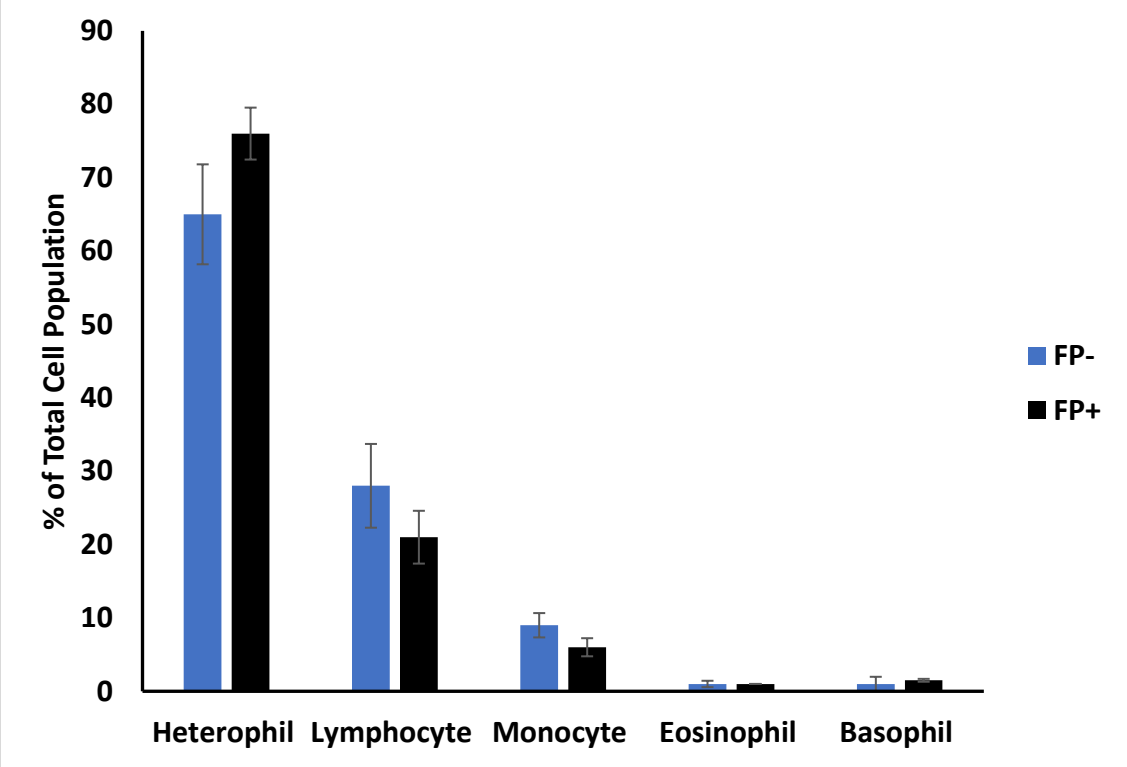

Figure 7: Intake cell differentials for rehab turtles. There was no significant difference between FP- (n=13) and FP+ (n=13) turtles for all cell types ($p > 0.05$). Error bars are one standard error from the median.

Blood Chemistry

Blood chemistry values were measured upon arrival for sea turtles brought into rehab at GLNC as part of standard intake procedures. Chemistry parameters, except calcium and cholesterol, were not significantly different between FP- and FP+ turtles (Table 1). Calcium values were significantly higher in FP- rehab turtles compared to FP+ rehab turtles ($p < 0.001$). Cholesterol values were also significantly higher in FP- rehab turtles compared to FP+ rehab turtles ($p < 0.05$).

Table 1: Intake blood chemistry values for rehab turtles. Calcium and cholesterol values in FP- rehab turtles were significantly different than FP+ values ($p < 0.05$). All other blood chemistry values were not significantly different ($p > 0.05$). * Medians were used for these chemistries due to non-normalized data.

Chemistry Parameter	FP- Mean/ Median (n=13)	FP- Range	FP+ Mean/ Median (n=14)	FP+ Range	Significance?
Glucose	122.5	75-183	91.1	16-208	n.s.
BUN	42.5	2-105	59.1	13-130	n.s.
Uric Acid*	0.9	0.5-4.5	1.45	0.4-3.3	n.s.
Phosphorus	7.0	4.7-11.8	7.63	2.7-10.5	n.s.
Calcium*	7.15	5.4-11.1	5.2	4.1-6.3	$p < 0.001$
Sodium	154.5	146-161	153.4	149-159	n.s.
Potassium	4.2	2.9-4.7	4.3	3.4-5.2	n.s.
Na:K Ratio*	36	33-53	36	30-46	n.s.
Chloride	129.9	122-141	125.3	116-135	n.s.
Total Protein	3.8	1.9-5	3.6	2.3-4.7	n.s.
Albumin	1.3	0.8-2	1.1	0.5-1.7	n.s.
Globulin	2.4	1.1-3.6	2.4	1.5-3.3	n.s.
Albumin:Globulin Ratio	0.55	0.4-0.7	0.53	0.3-0.8	n.s.
ALT*	14	10-102	13	2-343	n.s.
ALP*	20	10-52	18.5	10-93	n.s.
GGT*	0	0-3	0	0-1	n.s.
Bilirubin*	0.1	01-0.4	0.1	0.1-0.5	n.s.
Cholesterol*	84.5	32-152	21	6-246	$p < 0.05$
Amylase	421	178-795	394	77-903	n.s.
Lipase*	16	10-33	21	10-88	n.s.
Osmolality	317.3	288-339	312.9	300-328	n.s.

Flow Cytometry Phagocytosis Assays

Phagocytosis was measured using a flow cytometry assay, which measures the number of cells that engulfed at least one fluorescent bead (FITC). In Figure 8, flow cytometry analyses of leukocyte phagocytosis are shown. Control samples that did not include FITC beads are represented by histograms and scatterplots (Fig. 8a). When

incubated with FITC beads, a subset of the gated leukocytes, which is either below the bar in the histogram or within the gate of the scatter plot, shows a relative fluorescence for the engulfed beads (Fig. 8b). The rate of phagocytosis was quantified based upon 10,000 events or individual cells detected by the flow cytometer.

Phagocytosis upon intake varied among FP+ rehab turtles, FP- rehab turtles, and in wild caught FP- non-rehab turtles (Fig. 19). Phagocytosis ranged from 1.08-7.97% in FP- rehab turtles, from 1.18-14.00% in FP+ rehab turtles, and from 4.08-13.90% in FP- non-rehab turtles. Median immune function for FP- rehab turtles was significantly less than FP+ rehab turtles and FP- non-rehab turtles ($p < 0.01$). Immune function for FP+ rehab turtles was also significantly less than FP- non-rehab turtles ($p < 0.03$). Seasonal variations for phagocytosis upon intake were present (Fig. 10). Phagocytosis was greater in winter than summer for FP+ rehab turtles, but the opposite was seen in FP- rehab turtles, where phagocytosis in winter was lower than in summer. Non-rehab FP- turtles had high rates of phagocytosis in winter similar to the FP+ rehab turtles, but no samples were taken in the summer to compare to. There was no significant difference in phagocytosis in summer versus winter for FP- rehab turtles ($p > 0.05$); however, there was a significant difference in phagocytosis in summer versus winter months for FP+ rehab turtles ($p < 0.01$).

Samples taken immediately prior to release for phagocytosis were only collected for rehab turtles. FP- rehab turtles experienced an increase in phagocytosis between intake and release, where the mean phagocytosis increased from 3.33% to 5.06%. FP+ rehab turtles experienced a slight decrease in phagocytosis, where the mean phagocytosis decreased from 6.21% to 5.79% (Fig. 11). Although these changes exist, the data were

highly variable and thus there was no significant difference between intake and release for FP- or FP+ rehab turtles ($p > 0.05$).

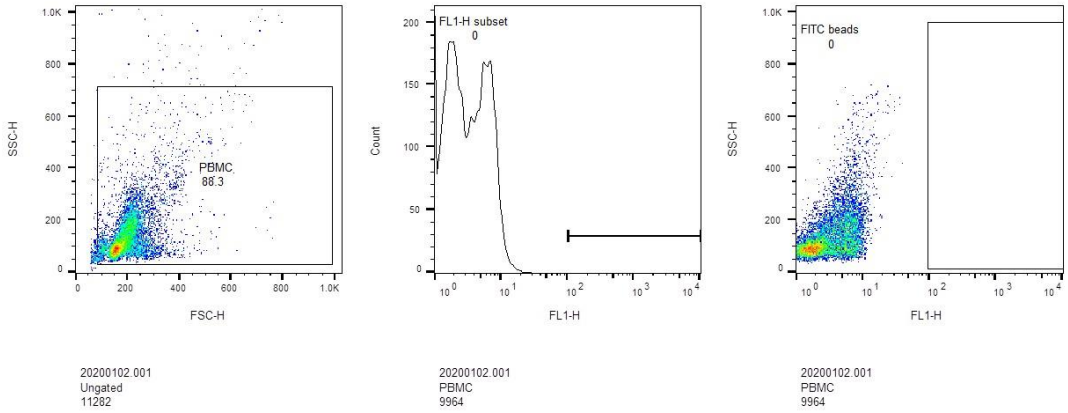

Figure 8a: Scatterplot and histogram (center) representations for all cell populations without FITC beads present.

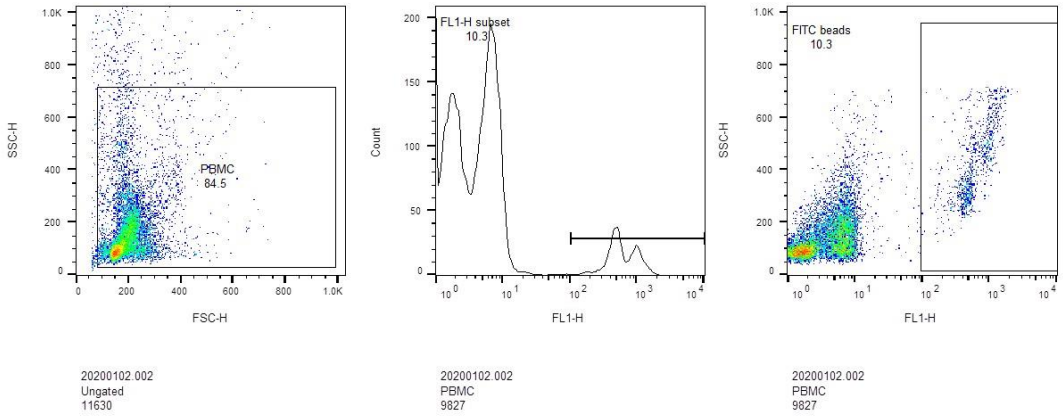

Figure 8b: Scatterplot and histogram (center) representations for same cell population (14a above) with FITC beads present. 10.3% of cells engulfed the FITC beads.

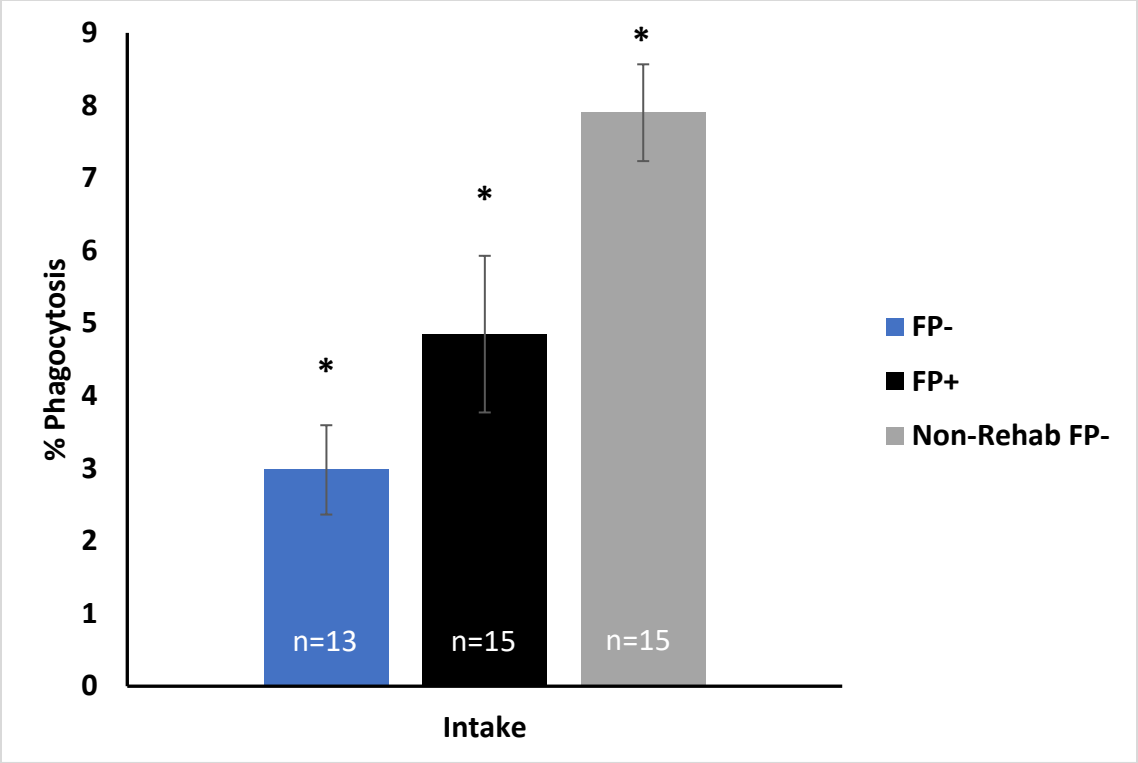

Figure 9: Intake values of phagocytosis. * Immune function for FP- rehab turtles was significantly less than FP+ rehab turtles and FP- non-rehab turtles ($P < 0.01$), and immune function for FP+ rehab turtles was also significantly less than FP- non-rehab turtles ($P < 0.03$). Error bars are one standard error from the median.

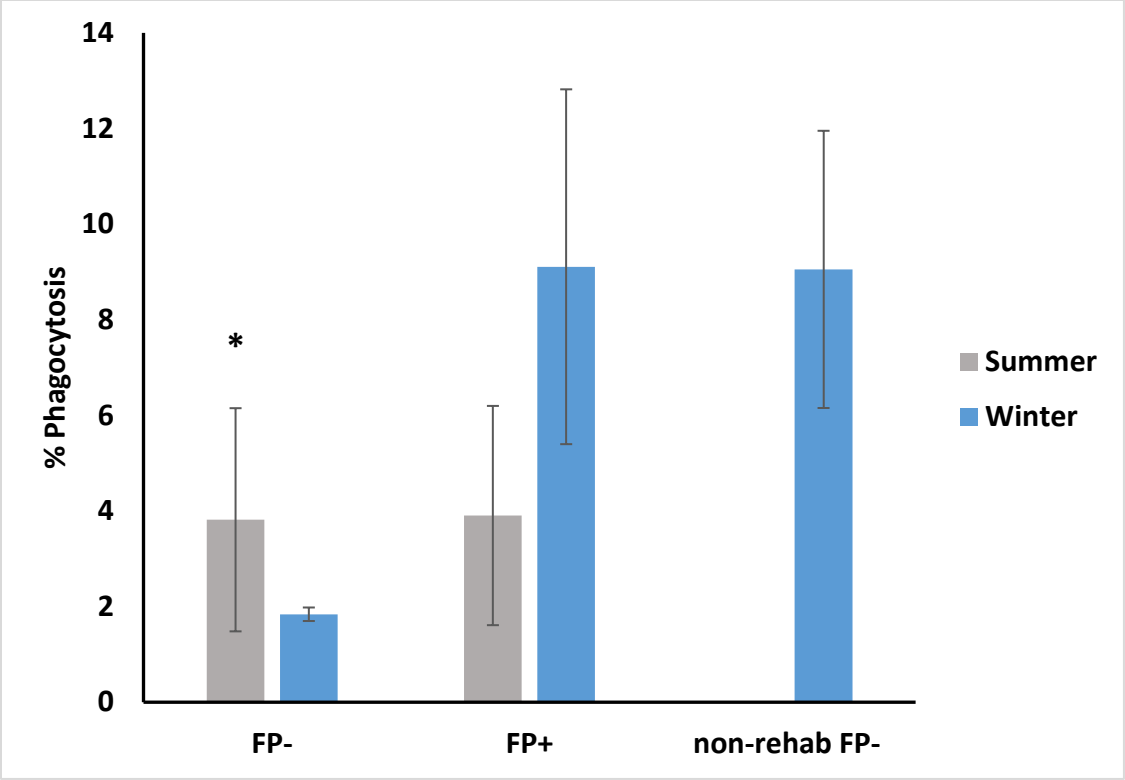

Figure 10: Seasonal variations in phagocytosis upon intake for FP- and FP+ rehab turtles and FP- non-rehab turtles. * Phagocytosis during the winter was significantly higher than during the summer for FP+ rehab turtles ($p < 0.01$). There was no significant difference between winter and summer phagocytosis for FP- rehab turtles ($p > 0.05$). Error bars are one standard deviation from the mean.

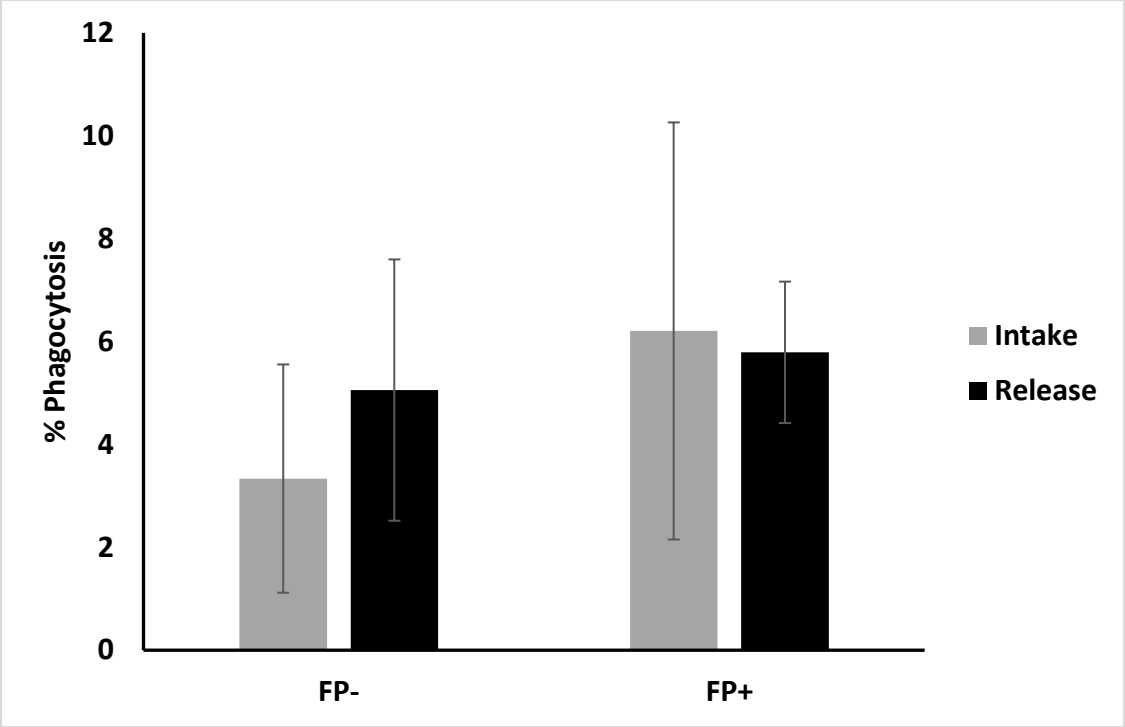

Figure 11: Change in phagocytosis upon release for FP- and FP+ rehab turtles. There was no significant change in phagocytosis from intake to release for either group ($p > 0.05$). Error bars are one standard deviation from the mean.

UV Light Exposure Conditions

UV light exposure varies throughout the rehabilitation facility at GLNC. UV intensity varies diurnally as well as throughout the year (Fig. 12). The daily median for tanks included in this study ranged from 54,649 Lux to 184,720 Lux in low UV light tanks and from 356,438 Lux to 429,084 Lux in high UV light tanks (Fig. 13). There was a significant difference in the daily median UV intensity among all 4 selected tanks ($p < 0.001$). Seasonal variations are present in each tank (Fig. 14). In both sun tanks, UV intensity was significantly greater in summer months compared to winter months ($p < 0.01$); however, UV intensity was significantly greater in winter months compared to

summer months in control tank 2 ($p < 0.01$). It is possible that control tank 2 had greater sun exposure in the winter due to its location at the facility and the angle of the sun at that time of year. There was no significant difference between winter and summer months for control tank 1 ($p > 0.05$).

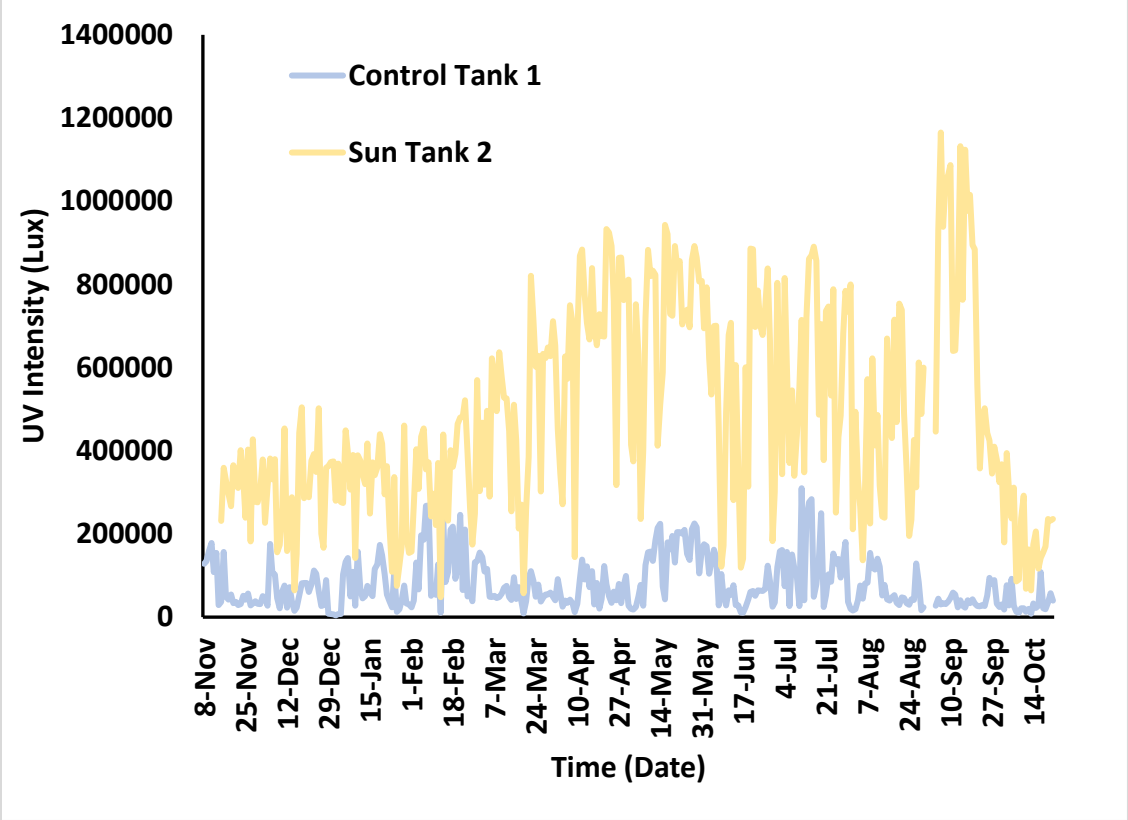

Figure 12: Daily fluctuations for control tank 1 and sun tank 2 over a year time span.

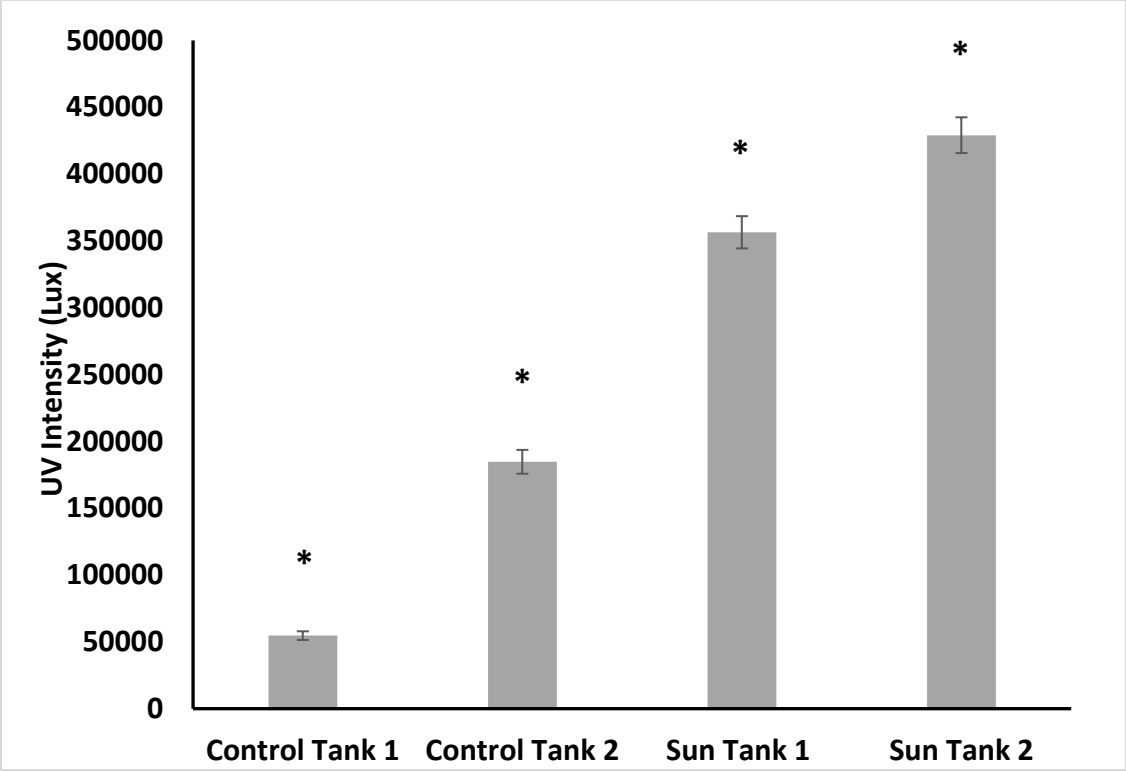

Figure 13: Daily median UV intensity for all treatment tanks. * All tanks are significantly different from one another ($p < 0.001$). Error bars are one standard error from the median.

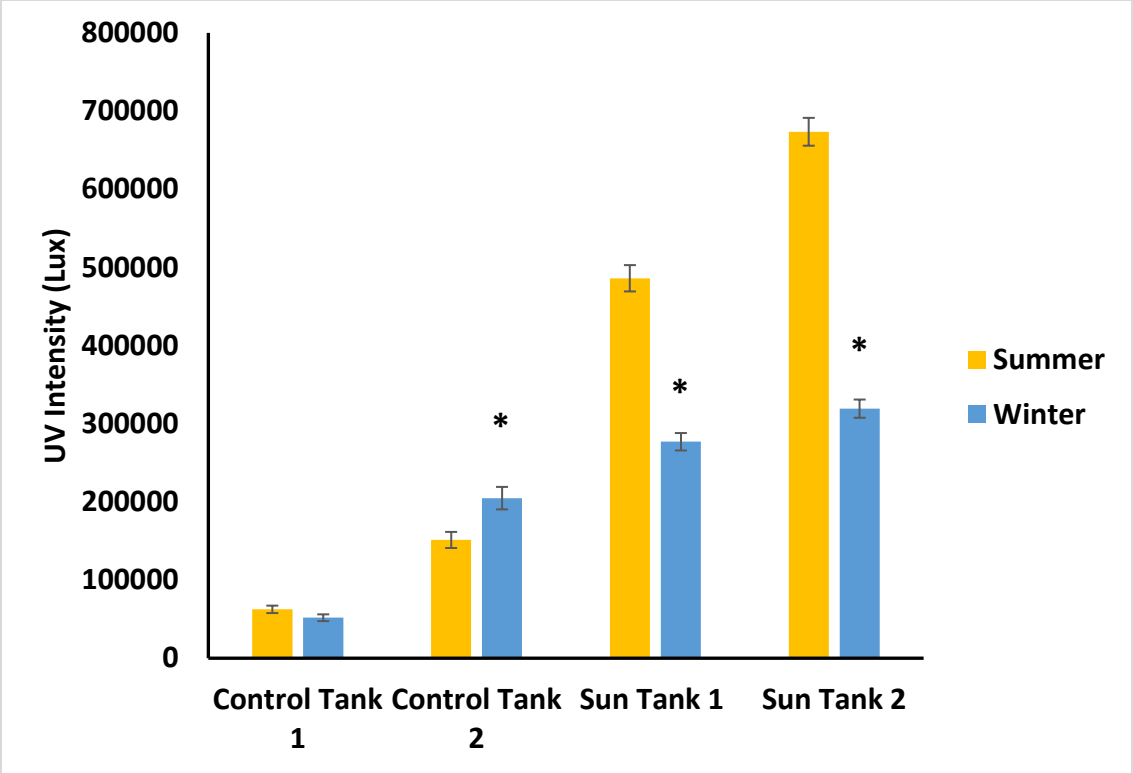

Figure 14: Seasonal variations in daily median UV intensity for all treatment tanks. * Significant seasonal variations were seen in control tank 2, sun tank 1, and sun tank 2 ($p < 0.01$). Error bars are one standard error from the median.

Vitamin D, Parathyroid Hormone, and Ionized Calcium

Parathyroid hormone, ionized calcium, and plasma 25-hydroxyvitamin D levels were determined for all samples taken. Parathyroid hormone levels ranged from 0-1 pmol/L in FP- rehab turtles, 0.3-1.9 pmol/L in FP+ rehab turtles, and 0.3-2.8 pmol/L in non-rehab FP- turtles (Fig. 15). Median parathyroid hormone levels were highest in FP+ rehab turtles and lowest in both rehab and non-rehab FP- turtles. There was no significant difference between non-rehab FP- and rehab FP+ turtles or between non-rehab FP- and FP- rehab turtles ($p > 0.05$); however, there was a significant difference between FP+

rehab turtles and FP- rehab turtles ($p < 0.02$). Conversely, Vitamin D levels were significantly lower in FP+ turtles compared to both groups of FP- turtles (Fig. 16, $p < 0.001$). FP- rehab turtles had the highest vit D levels on intake and FP+ rehab turtles had the lowest vit D levels. Similar to Vit D, ionized calcium levels were significantly lower in FP+ rehab turtles upon intake compared to both groups of FP- turtles (Fig. 17, $p < 0.01$). Ionized calcium levels were slightly higher in FP- rehab turtles compared to non-rehab FP- turtles, but there was no significant difference ($p > 0.05$).

As the objective of this study was to determine if UV light influenced vitamin D levels and light levels varied significantly depending on which tanks the turtles were kept in, we examined the change in vitamin D over time. FP+ turtles admitted in GLNC for rehab had low vitamin D levels as mentioned above and often stayed longer in rehab compared to FP- turtles, as their injuries/disease required longer intensive care. FP+ turtles kept in high UV light conditions had a greater increase in vitamin D levels over time compared to the FP+ turtles kept in the low UV light conditions (Fig. 18a). There was a significant increase in vit D for FP+ turtles exposed to high UV light when their recovery lasted longer than 60 days ($p < 0.05$). FP+ turtles kept exposed to low UV light conditions experienced a slight increase in vitamin D, but it was not significant ($p > 0.05$). A similar trend was seen in FP- rehab turtles (Fig. 18b). FP- turtles kept in high UV light conditions had greater vitamin D levels over time compared to those kept in low UV light conditions; however, the change over time was not significant ($p > 0.05$).

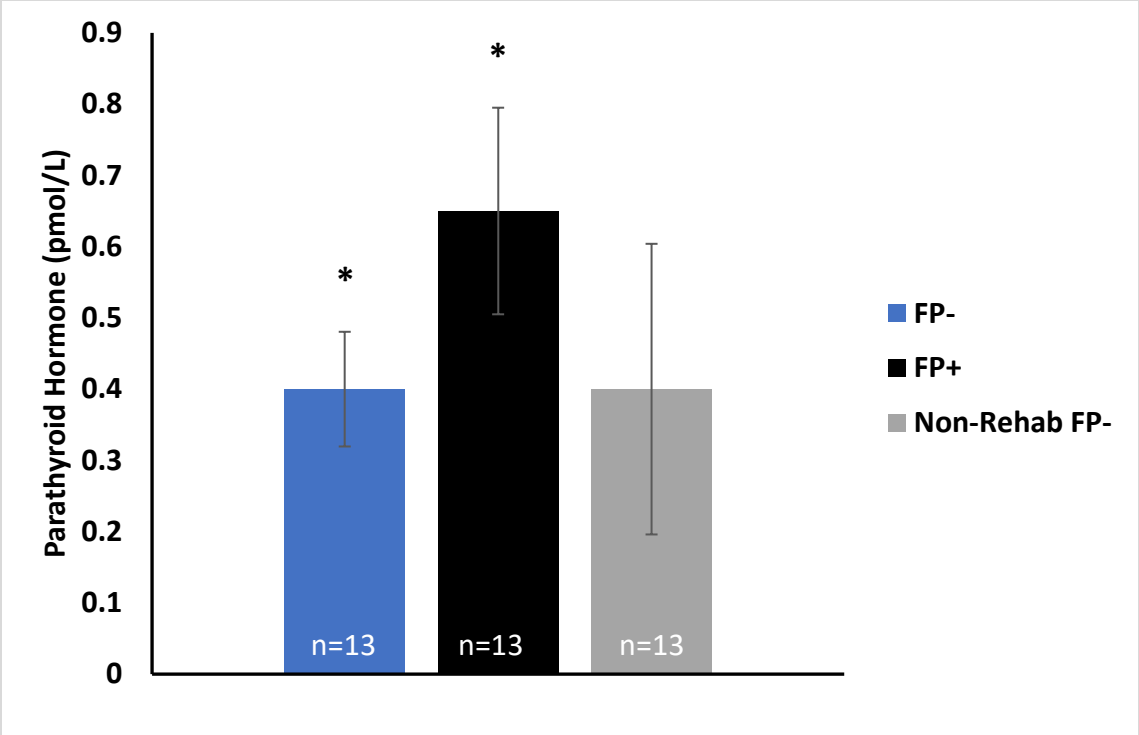

Figure 15: Parathyroid hormone levels on intake for rehab and non-rehab individuals. * There was a significant difference between FP- rehab turtles and FP+ rehab turtles ($p < 0.02$). There was no significant difference between non-rehab FP- turtles and either FP- rehab turtles or FP+ rehab turtles ($p > 0.05$). Error bars are one standard error from the median.

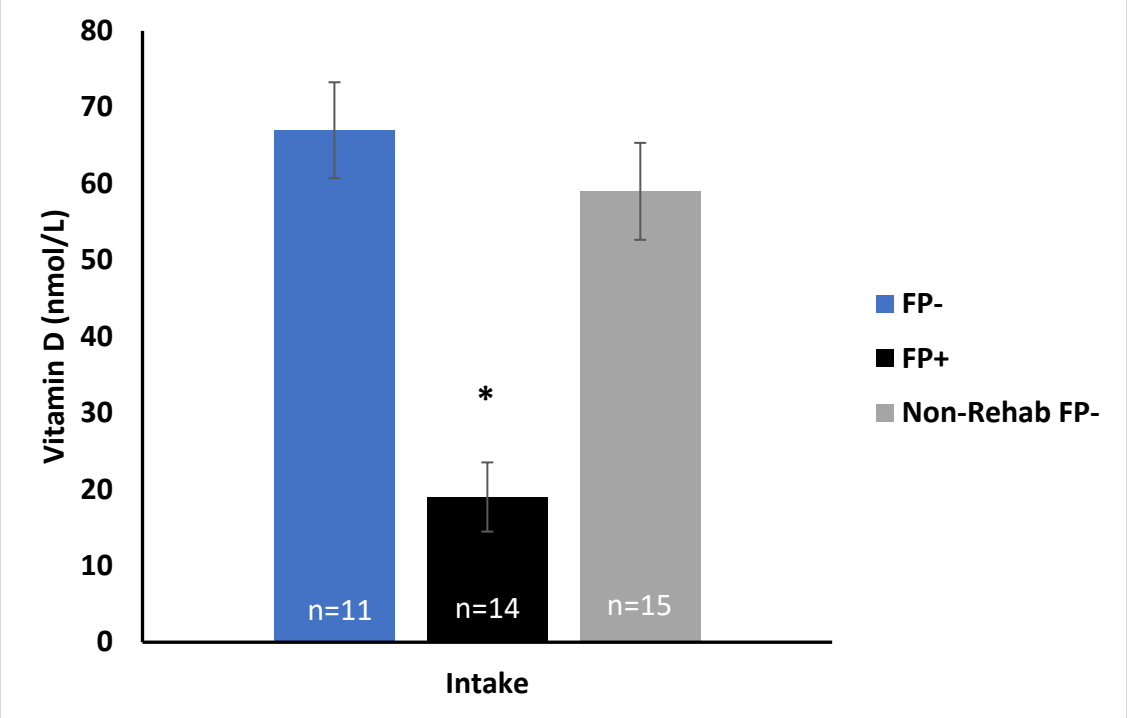

Figure 16: Intake values of plasma vitamin D. * Vitamin D levels for FP+ rehab turtles was significantly less than FP- rehab turtles and FP- non-rehab turtles ($p < 0.001$). Error bars are one standard error from the median

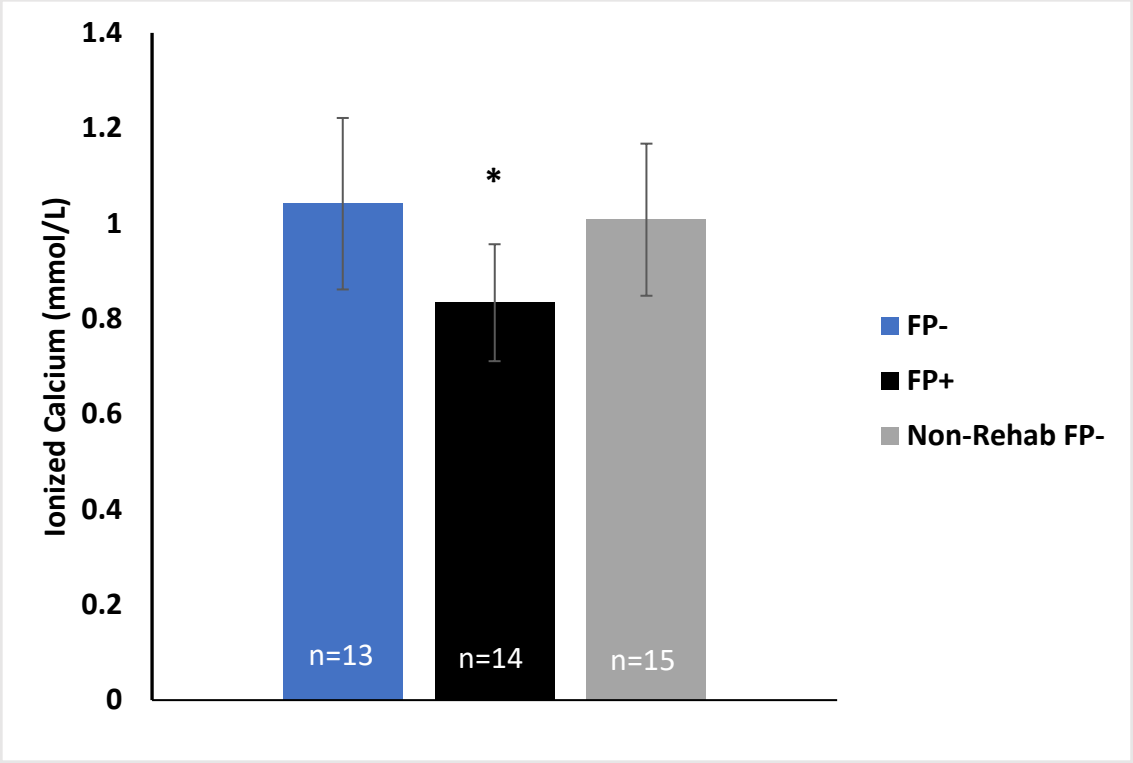

Figure 17: Intake ionized calcium values for all individuals. * Ionized calcium values for FP+ rehab turtles were significantly lower than both FP- rehab turtles and non-rehab FP- turtles ($p < 0.01$). There was no significant difference between FP- rehab turtles and non-rehab FP- turtles ($p > 0.05$). Error bars are one standard deviation from the mean.

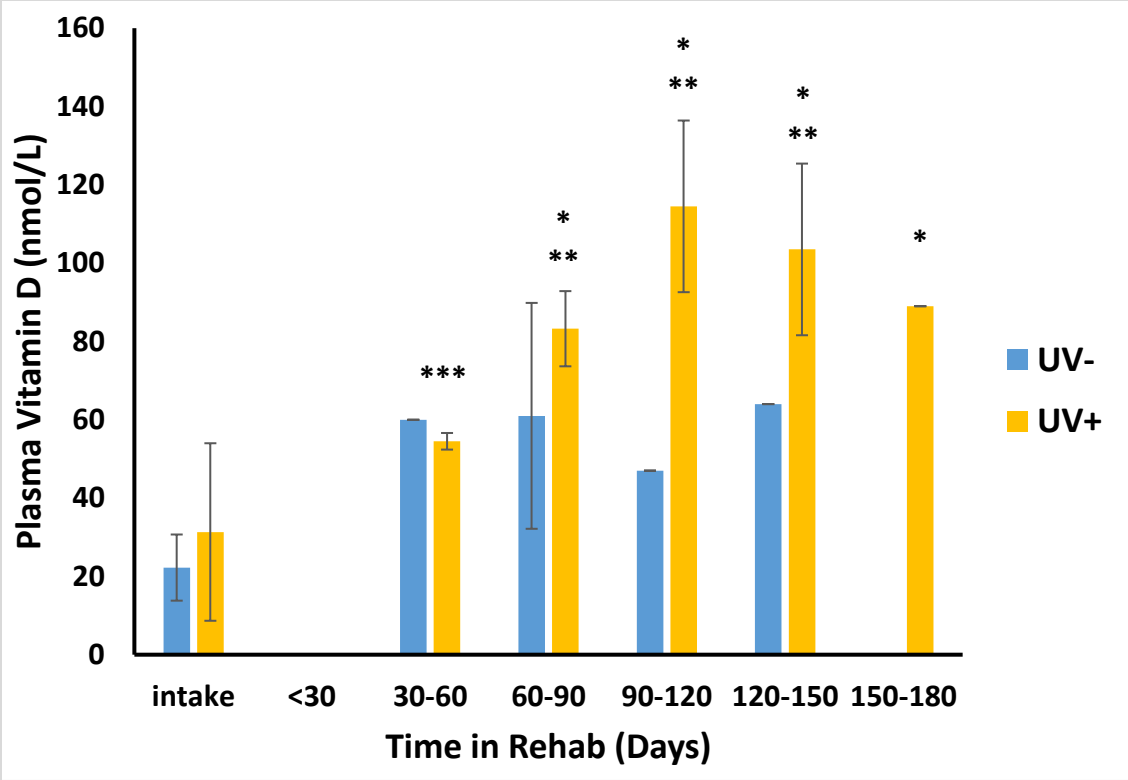

Figure 18a: Change in plasma vitamin D levels over time in FP+ rehab turtles. * are significantly different from UV- intake values. ** are significantly different from UV+ intake values. *** are significantly different from the group of turtles kept for 90-120 days in UV+ conditions.

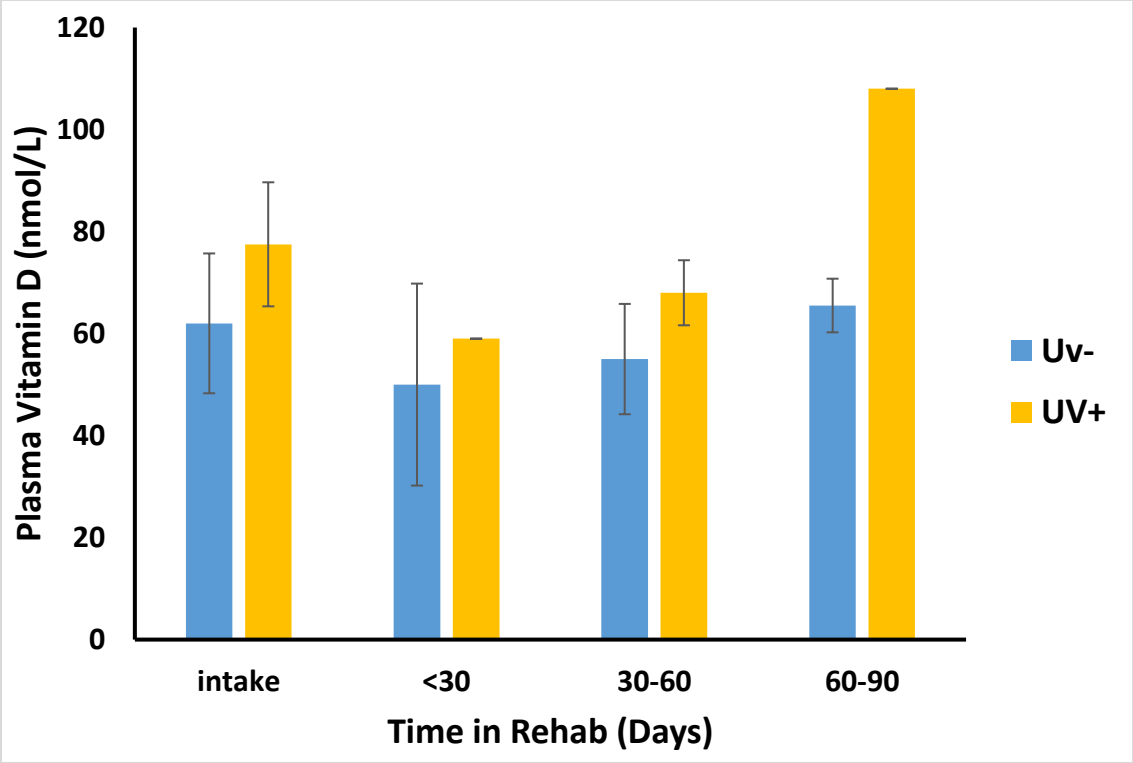

Figure 18b: Change in plasma vitamin D levels over time in FP- rehab turtles. There were no significantly different changes in vitamin D levels over time.

Body Condition Index

Body condition index (BCI) was measured for all individuals, as individuals' conditions varied greatly upon intake. To see if these variations potentially affected immune function or vitamin D levels, I ran correlations (Fig. 19 and Fig. 20). There was no significant correlation between BCI and immune function ($p > 0.05$) or between BCI and vitamin D levels ($p > 0.05$).

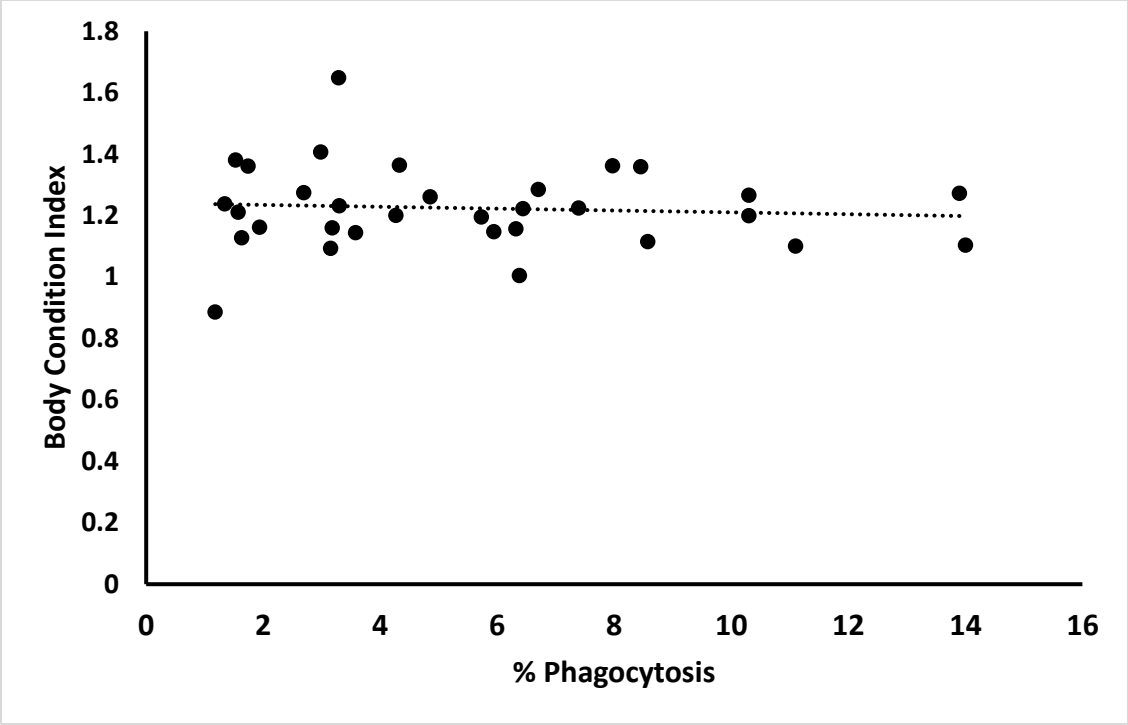

Figure 19: Correlation between body condition index and immune function. There is no significant correlation between BCI and % phagocytosis ($p > 0.05$).

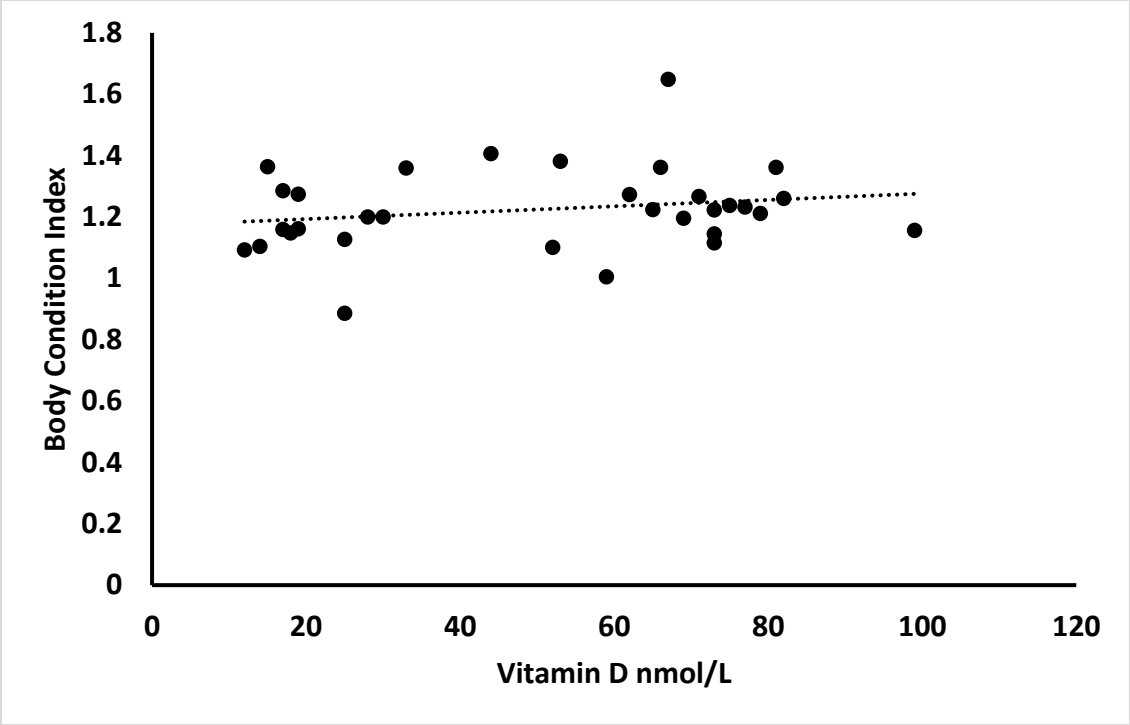

Figure 20: Correlation between body condition index and plasma vitamin D levels. There is no significant correlation between BCI and vit D ($p > 0.05$).

Immune Function, Vitamin D, and Blood Chemistry Parameters

I ran correlations between blood chemistry parameters and both vitamin D and immune function (Table 2). Glucose, calcium, sodium, total protein, albumin, globulin, ALT, ALP, cholesterol, amylase, and osmolality were all significantly correlated with vitamin D ($p < 0.05$). Only one parameter, phosphorus, was significantly correlated with immune function ($p < 0.05$). There was also a slight positive correlation between phagocytosis and vitamin D levels, but it was not significant (Fig. 21, $p > 0.05$).

Table 2: Blood chemistry parameters correlated with plasma vitamin D levels and phagocytosis. Glucose, calcium, sodium, total protein, albumin, globulin, ALT, ALP, cholesterol, amylase, and osmolality were all significantly correlated with vitamin D ($p < 0.05$). The other parameters were not significantly correlated ($p > 0.05$). Immune function was only correlated with phosphorus ($p < 0.05$). All other parameters were not correlated with phagocytosis ($p > 0.05$). ** indicates statistical significance.

Chemistry Parameter	Significance with Vitamin D (P-Value, Cor. Coeff)	Significance with Immune Function (P-Value, Cor. Coeff)
Glucose	2.184e-07, 0.6108 **	0.6859, -0.0533
BUN	0.3953, 0.1117	0.0927, 0.2190
Uric Acid	0.2786, -0.1421	0.8509, 0.0248
Phosphorus	0.4305, 0.1152	0.0236, 0.3229 **
Calcium	5.101e-05, 0.5452 **	0.6669, -0.063
Sodium	0.0191, 0.3337 **	0.9596, 0.0074
Potassium	0.6619, 0.0641	0.1486, 0.2095
Na:K Ratio	0.9217, -0.0144	0.1231, -0.2233
Chloride	0.2127, 0.1812	0.7359, -0.0494
Total Protein	3.146e-05, 0.5139 **	0.5113, 0.0872
Albumin	1.403e-06, 0.5769 **	0.2638, 0.1466
Globulin	0.0007, 0.4309 **	0.5098, 0.0875
Albumin:Globulin Ratio	0.2659, 0.1472	0.7241, 0.0469
ALT	0.0001, 0.4721 **	0.4509, 0.0992
ALP	0.0026, 0.3816 **	0.2857, 0.1401
GGT	0.1647, 0.2017	0.4589, 0.1083
Bilirubin	0.0641, 0.2666	0.8874, -0.0208
Cholesterol	4.959e-07, 0.6528 **	0.2895, 0.156
Amylase	0.0262, 0.3175 **	0.149, 0.2092
Lipase	0.0733, -0.2581	0.9451, -0.0101
Osmolality	0.001, 0.4688 **	0.186, 0.1985

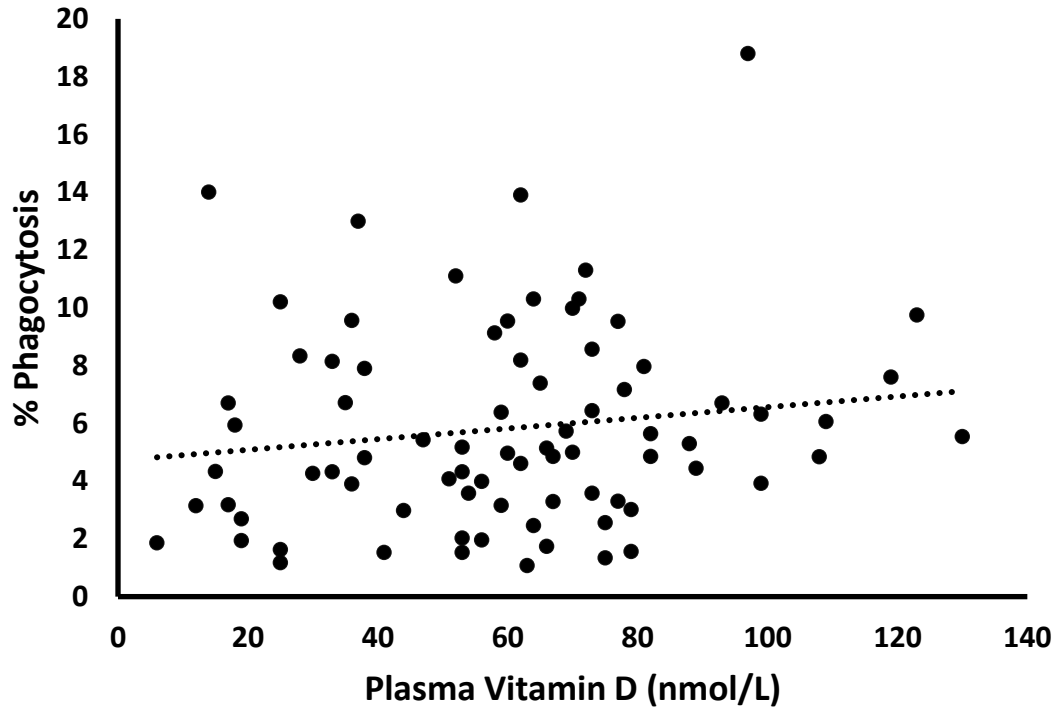

Figure 21: Correlation between immune function and plasma vitamin D level. There is no significant correlation between the two (Cor. Coeff. = 0.164, p = 0.1487).

Regrowth

Since one of the objectives of this study was to determine the influence of UV light and vitamin D on tumor regrowth, I monitored the turtles who had tumor removal surgery for any possible regrowth. Of the 7 turtles that underwent surgery, 4 turtles experienced regrowth (Table 3 and 4). All of the turtles that experienced regrowth had a Balazs tumor score of 3 on intake. The 3 turtles that did not experience regrowth had tumor scores of 1, 2, and 3. The number of regrowth areas ranged from 5 to 13. Turtles kept in high UV light conditions experienced less regrowth than those kept in low UV light conditions; however, the sample size is too small to determine any statistical significance.

Table 3: Regrowth occurrence for FP+ turtles kept in low UV light conditions.

Turtle	Tumor score	Regrowth?	# of regrowth areas
Padfoot	3	Yes	5
Thor	3	Yes	13

Table 4: Regrowth occurrence for FP+ turtles kept in high UV light conditions.

Turtle	Tumor score	Regrowth?	# of regrowth areas
Wisteria	3	Yes	unknown
Rowling	3	Yes	9
Yucca	2	No	n/a
Black Panther	1	No	n/a
Gamora	3	No	n/a

Viral load

Viral load was measured for blood samples from all turtles and for tumor samples from turtles that had tumor removal surgery or were euthanized. All blood samples were negative for ChHV5. Tumor samples that were positive for ChHV5 were split into four tumor groups: soft tissue tumors, hard tissue tumors, eye tumors, and regrowth tumors. Eye tumors had the greatest viral load of ChHV5 and regrowth tumors had the lowest viral load (Table 5). Viral load of regrowth tumors was significantly lower than viral load of soft tissue, hard tissue and eye tumors ($p < 0.01$). There was no significant difference among soft tissue tumors, hard tissue tumors, and eye tumors ($p > 0.05$).

Since one of the objectives of this study was to determine the effects of UV light exposure on ChHV5 viral load, we examined the viral load in each tumor type based on UV light exposure. Viral load was higher in turtles exposed to low UV light conditions for soft tissue tumors and eye tumors but was lower for hard tissue tumors and regrowth tumors (Table 6). There was no significant difference in viral load between high UV light conditions and low UV light conditions for hard tissue tumors, eye tumors, or regrowth tumors ($p > 0.05$); however, there was a significant difference in viral load between high UV light conditions and low UV light conditions for soft tissue tumors ($p < 0.05$).

Table 5: Median viral load for 4 tumor types: soft tissue, hard tissue, eye, and regrowth. Regrowth tumor viral load was significantly less than soft tissue, hard tissue, and eye tumors ($p < 0.01$). There was no significant difference among soft tissue tumors, hard tissue tumors, and eye tumors ($p > 0.05$).

	Soft Tissue	**Hard Tissue**	**Eye**	**Regrowth**
Median copy number per µg DNA (range)	1.04×10^8 (2.79×10^3 – 6.01×10^8)	1.08×10^8 (3.67×10^7 – 1.41×10^9)	1.33×10^8 (3.92×10^7 – 2.85×10^9)	2.61×10^7 (2.59×10^2 – 8.47×10^7)

Table 6: Median viral load for 4 tumor types (soft tissue, hard tissue, eye, and regrowth) under high UV light and low UV light conditions. There was a significant difference in viral load between high UV light and low UV light conditions for soft tissue tumors ($p < 0.05$). There was no significant difference in viral load between high UV light conditions and low UV light conditions for hard tissue tumors, eye tumors, or regrowth tumors ($p > 0.05$).

	Soft Tissue	**Hard Tissue**	**Eye**	**Regrowth**
High UV Light Median copy number per µg DNA (range)	$7.65x10^7$ ($2.79x10^3$ – $2.98x10^8$)	$2.13x10^8$ ($8.97x10^7$ – $3.35x10^8$)	$7.85x10^7$ ($3.92x10^7$ – $2.85x10^9$)	$2.98x10^7$ ($2.59x10^2$ – $5.49x10^7$)
Low UV Light Median copy number per µg DNA (range)	$1.79x10^8$ ($4.34x10^7$ – $6.01x10^8$)	$1.08x10^8$ ($3.67x10^7$ – $1.41x10^9$)	$2.30x10^8$ ($1.53x10^8$ – $2.74x10^8$)	$1.46x10^7$ ($1.33x10^4$ – $8.47x10^7$)

Survival statistics

The endpoint of the individuals brought into rehab varied. FP- individuals were all released (12/12), compared to FP+ individuals, where a majority were euthanized or died and only a few were released (5/14). When looking at the effect on UV exposure on FP+ survival, the survival of FP+ turtles appears to increase with the amount of UV light (Fig. 22); however, the samples size is too small to determine any statistical significance.

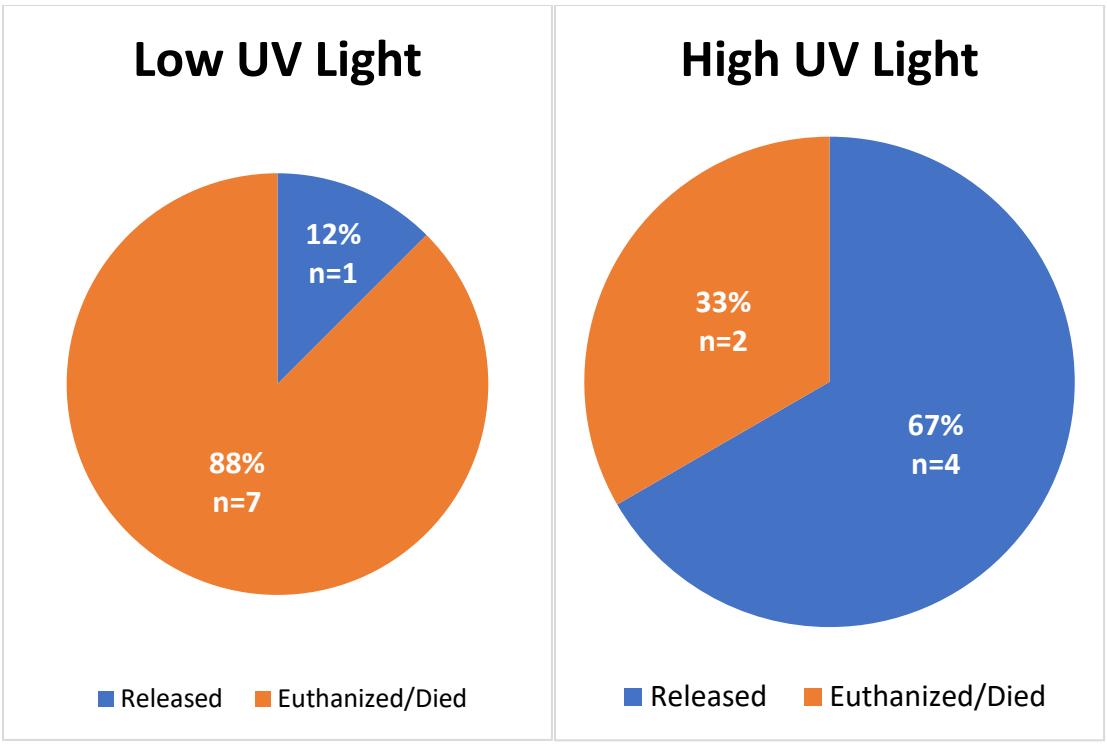

Figure 22: Survival rate in high versus low UV light tanks. Survival varied with UV light conditions.

DISCUSSION

This study focused on the effect of UV light on vitamin D levels and immune function in green sea turtles with FP. FP is a disease that affects up to 60% of individuals in some sub-populations (Herbst, 1994; Hirama and Ehrhart, 2007). When brought into rehabilitation facilities, the disease is treated with tumor removal surgery. Although the tumors can be removed, the outlook for turtles with this disease is poor. A new treatment to help better the outcome of FP+ turtles is needed. The aims of this study were to (1) determine the variations in health parameters in FP- versus FP+ turtles, (2) to examine the effects of UV light on vitamin D levels, (3) to determine the effect of UV light and vitamin D on immune function, (4) to determine if UV light therapy influences regrowth of fibropapilloma tumors in sea turtles and (5) to examine the effects of UV light on viral load of ChHV5.

Hematology and Blood Chemistry Parameters

It was important to determine if there were any variations in health parameters for the turtles in this study, because turtles brought into GLNC come from all over the east coast of Florida. They are exposed to different environmental conditions and may be suffering from a variety of health issues. The turtles that come into the St. Lucie power plant also likely experience differences in their environment. Pack cell volume (PCV or hematocrit) varied between rehabilitated turtles and non-rehab FP- turtles (Fig. 5). All individuals admitted into rehab were dealing with some type of disease or injury, which

likely is the cause of the reduced PCV (Bloodgood et al., 2019). Bloodgood et al. (2019) found that turtles brought into rehab had significantly lower PCV levels compared to wild individuals. In this study, the only significant difference was between FP+ rehab turtles and FP- non-rehab turtles, where FP+ rehab turtles had the lowest PCV value and non-rehab FP- turtles had the greatest. These trends in FP+ turtles are similar to other studies (Zwarg et al., 2014; Aguirre et al., 1995; Work and Balazs, 1999; Hirama et al., 2014). FP+ turtles from Brazil had lower PCV values compared to healthy individuals (Zwarg et al., 2014). FP+ turtles in Hawaii also had significantly lower PCV values compared to FP- turtles (Aguirre et al., 1995). There was also a trend with tumor score in Hawaiian turtles, where turtles with a Balazs tumor of score of 3, which is the most severe tumor score, had the lowest PCV values especially in comparison to FP- individuals (Work and Balazs, 1999). Free-living turtles from the Indian River Lagoon have also been shown to have an inverse relationship between PCV values and tumor score, with the lowest PCV values seen in turtles with a tumor score of 3 (Hirama et al., 2014). These individuals with FP, including the ones in this study, are in poor condition and often anemic, which results in lower PCV values (Aguirre et al., 1995; Zwarg et al., 2014). Other hematological parameters are also influenced by this disease.

White blood cell count varies between FP- and FP+ individuals. Although there was no significant difference between FP- and FP+ WBC count values, FP+ turtles had a higher WBC count (Fig. 6), which is primarily due to the greater number of heterophils seen in the total cell population (Fig. 7). Other studies have seen varying results when comparing WBC counts in FP+ and FP- turtles (Cray et al., 2001; Aguirre et al., 1995). Similar to this study, Cray et al. found that turtles with FP had higher WBC counts

compared to turtles without FP (2001). Aguirre et al., on the other hand, saw higher WBC counts in FP- turtles compared to FP+ turtles. Higher WBC counts typically suggest the body is fighting off some type of infection (Owen et al., 2013). Differences seen in these studies could potentially be due to the varying environmental conditions the animals are exposed to.

The data on WBC differentials showed that FP+ rehab turtles have a higher portion of heterophils compared to FP- rehab turtles (Fig. 7). Although the difference between FP+ and FP- turtles was not significant, it follows the same trends as previous studies. Cray et al. (2001) found that captive FP- turtles leukocyte populations were made up of 55.4% heterophils and diseased FP+ turtle leukocyte populations were made up of 66.7% heterophils. In this study, the means values were slightly higher than the study by Cray et al., but the trend was the same. Page-Kajian et al. (2014) found that in FP+ turtles the percentage of heterophils was still greater than that of lymphocytes (48.43% and 40.86%, respectively), but the difference between the two were not as great compared to this study. The heterophil percentage in that study was also much lower than the FP+ rehab turtles from this study. Data from wild caught, presumably healthy individuals from throughout the United States, showed proportion of heterophils ranging from 10.5-74% and the proportions of lymphocytes ranges from 26-69% (Cray et al., 2001; Work and Balazs, 1999; Anderson et al., 2011; Bloodgood et al., 2019; Aguirre et al., 1995). Work and Balazs (1999) and Aguirre et al. (1995) even showed greater proportions of lymphocytes compared to heterophils in healthy individuals. Heterophils are one of the main phagocytotic cells that responds to inflammation and infection (Owen et al., 2013). The lower heterophil counts in these studies suggest these individuals are healthy as

increased heterophil counts indicates that the individuals could be having an inflammatory response or, in the case of FP+ turtles, be responding to tumors (Varela, 1997). Outside of differentials, some blood chemistry parameters varied between FP+ and FP- rehab turtles.

A majority of the blood chemistry parameters showed no variation between FP- and FP+ rehab turtles, but calcium and cholesterol were significantly lower in FP+ turtles compared to FP- turtles (Table 1). Previous studies showed similar results, where FP+ turtles had significantly lower calcium and cholesterol (Aguirre et al., 1995; Aguirre and Balazs, 2000). Reduction in these chemistry parameters is typically associated with severe debilitation, which is common for turtles with FP; in this study, both calcium and cholesterol were significantly correlated with body condition index. Although hematology and blood chemistry parameters are important for determining the heath of an individual, it is also important to determine how the immune system is being affected in turtles with FP.

Immune Function

Immune function is another health parameter that varies between FP+ and FP- turtles. Previous studies showed reduced immune function in FP+ turtles compared to FP- turtles (Cray et al., 2001; Sposato, 2014 MS Thesis). Lymphocyte proliferation, which is part of the adaptive immune system, is significantly reduced in FP+ turtles compared to FP- turtles (Cray et al., 2001). The innate immune system has also been shown to be affected. Both FP+ and FP- turtles living in polluted environments experience reduced phagocytosis compared to those living in more pristine conditions (Sposato, 2014 MS

Thesis). Rates of phagocytosis in FP+ turtles were shown to be significantly lower than FP- turtles (Sposato, 2014 MS Thesis). In this study, FP- rehab turtles had the lowest rate of phagocytosis, FP+ rehab turtles had the second highest rate of phagocytosis, and FP- non-rehab turtles had the highest rate of phagocytosis (Fig. 9). Although these results are different than the studies previously mentioned, many variables come into play to cause this variation. All of the turtles brought in GLNC are sick or injured in some way. It is possible that the immune systems of the FP- turtles are compromised by these injuries. The turtles are also from various locations and have different health/body conditions upon intake. Although there is an unexpected difference between the two groups of turtles in rehab, these turtles still show reduced immune function compared to the non-rehab FP- turtles.

There were also seasonal variations in immune function upon intake. Phagocytosis was lower in the summer for FP+ turtles, but lower in the winter for FP- turtles (Fig. 10). There is a large amount of variation in the data for FP+ individuals, which could help explain the differences from the FP- individuals. Since these animals are from different locations in Florida, it is likely that the environmental conditions are also influencing these variations seen, as sea turtles, like other reptiles, are poikilotherms meaning their internal body temperature is regulated by their external environment (Braun-McNeill et al., 2008). A previous study in green sea turtles with and without FP showed that turtles typically have higher phagocytosis in summer months compared to winter months (Sposato, 2014 MS Thesis). There have also been seasonal differences in immune function in other reptiles and poikilothermic vertebrates (Muñoz et al., 2000; Collazos et al., 1995). In the turtle *M. capsica,* lymphocyte proliferation was increased in

winter and spring months compared to summer and autumn months (Muñoz et al., 2000). Tench fish also experience seasonal variation in immune function, where phagocytosis is highest in winter and spring compared to summer and autumn (Collazos et al., 1995). Since these animals rely on the environment for thermoregulation, it is possible that environmental conditions in the summer months cause warmer temperatures that contribute to reduced immune function in these animals.

When looking at phagocytosis upon release, we see an increase in immune function in FP- rehab turtles and a slight decrease in immune function for FP+ turtles (Fig. 11). The increase seen in the FP- turtles suggests an improvement in their immune system, which is likely due to the animals being healed. Although the FP+ turtles see a slight reduction in immune function, the difference is not significant and likely contributes to the individual being healthier than it was upon intake.

Baseline Vitamin D Levels

It was also important in this study to determine the effect of UV light exposure on plasma vitamin D levels in green sea turtles. Vitamin D can be obtained through the skin when it is exposed to UV light or through the consumption of foods that contain vitamin D_2 or D_3 (Lockau and Atkinson, 2018). It is used for a number of functions in the body, including bone mineralization and calcium uptake. Mammals and other reptiles show greater vitamin D levels when exposed to sunlight. Dairy cows exposed to natural sunlight or artificial UVB light for 73 days had increased plasma vitamin D levels (Jakobsen et al., 2015). Red eared sliders provided UV supplementation had significantly higher plasma vitamin D levels after 30 days compared to individuals only kept in

ambient light (Acierno et al., 2007). Corn snakes also show higher plasma vitamin D levels when exposed to supplemental UV light (Acierno et al., 2008). Since green sea turtles are reptiles, vitamin D is a necessary nutrient (Donoghue, 2006); however, few studies have looked at baseline levels in this species or the effects of UV exposure on vitamin D.

The baseline values for vitamin D, as well as ionized calcium and ionized calcium levels, have only been determined in green sea turtles in a few studies. Purgley et al. (2009) found that adult captive green turtles exposed to sunlight had vitamin D levels ranging from 61-69 nmol/L, while captive adults kept indoors had levels ranging from 5-53 nmol/L. Purgley et al. (2009) also showed that the longer the animals were kept indoors without exposure to UV the lower the plasma vitamin D levels. Two other studies compared vitamin D levels, parathyroid hormone, and ionized calcium levels in rehabilitated versus wild caught juvenile green sea turtles (Stringer et al., 2010; Bloodgood et al., 2019). Stringer et al. (2010) had turtles that were rehabilitated for anywhere from 75- 329 days depending on the individual and were exposed to limited UVB light. The only vitamin D supplementation these turtles received was an oral vitamin D_3 supplement given 3 days per week (Stringer et al., 2010). Rehabilitated turtles had lower median vitamin D and ionized calcium values compared to wild caught individuals (Stringer et al., 2010). Parathyroid hormone levels were greater in rehabilitated turtles, compared to wild caught individuals (Stringer et al., 2010). Although there was no significant difference in vitamin D levels between rehabilitated turtles and wild caught turtles (Stringer et al, 2010). the same trend is seen where less exposure to natural UV light resulted in lower vitamin D levels. Bloodgood et al. (2019) found that

upon admittance rehabilitated turtles had lower vitamin D, parathyroid hormone, and ionized calcium levels compared to free-ranging individuals.

Vitamin D, parathyroid hormone, and ionized calcium levels in this study are similar to those mentioned above. When comparing FP- rehab turtles to FP- non-rehab turtles, there was no significant difference in vitamin D levels, parathyroid hormone, and ionized calcium levels (Fig. 11, 12, and 13). Ionized calcium levels were comparable to the baseline levels determined by Stringer et al. (2010) and Bloodgood et al. (2019); however, vitamin D levels were much higher and parathyroid hormone levels were much lower than both studies. The variation seen, especially in the FP- rehab turtles could be due to the fact that most of the FP- rehab turtles were turtles hooked by fisherman on the pier and were relatively healthy. Environmental conditions could have also caused variation from the other studies. The turtles used by Stringer et al. (2010) were all from North Carolina, where UV intensity varies from that of Southern Florida. It was shown in other vertebrates that latitude has an effect on plasma vitamin D levels, where higher latitudes tend to produce lower plasma vitamin D levels (Leary et al., 2017; Southworth et al., 2013). To our knowledge, the rehabilitated turtles included in the previous studies were not FP+ turtles, so determining baseline values in those turtles is needed.

In this study, we found lower vitamin D and ionized calcium levels in FP+ turtles brought into rehab at GLNC compared to the FP- individuals (Fig. 11 and 12). We also found that parathyroid hormone levels were higher in FP+ compared to FP- individuals (Fig. 13). Parathyroid hormone helps to regulate the amount of calcium in the blood, so when levels are high ionized calcium levels tend to be low, which is seen here (Nussey and Whitehead, 2001). Vitamin D, as mentioned previously, helps with calcium uptake in

the body, especially when it comes to bone mineralization (Nussey and Whitehead, 2001). Since vitamin D and ionized calcium levels are low and parathyroid hormone levels are high in FP+ turtles, it is likely that these individuals are not able to obtain enough vitamin D or calcium, which could potentially be contributing to the poor health of individuals with FP. Adding UV light supplementation to the rehabilitation process of FP+ and FP- turtles could positively influence vitamin D levels.

Vitamin D levels increased over time in both FP+ and FP- turtles and when the turtles were exposed to higher levels of UV light (Fig. 14a and 14b). The turtles exposed to lower UV light had an increase in plasma vitamin D levels, which is likely due to the oral vitamin D supplementation that these turtles were given. The turtles exposed to higher UV light had a much greater increase in plasma vitamin D levels than just the oral supplement alone, as the individuals are obtaining vitamin D from two sources instead of just one. A study in bearded dragons also found that individuals given an oral supplementation and were exposed to high UV light had much higher plasma vitamin D levels than the oral supplement alone (Oonincx et al, 2010). The changes in vitamin D were only significant in the FP+ turtles; however, the FP- turtles experienced the same trend. The smaller change in the FP- turtles is likely due to their short stay at GLNC. These individuals also started with higher vitamin D levels upon intake compared to the FP+ turtles. Knowing that vitamin D in green sea turtles does increase with exposure to greater UV light, understanding the role it plays in their immune system is key.

Vitamin D and Health Parameters

Vitamin D plays an important role in the body and the immune system. Studies have shown that vitamin D can help benefit both innate and adaptive immunity. Vitamin D helps innate immune cells, such as monocytes, respond to infection; for instance, it can reduce viral load of herpes zoster virus in humans (Prietl et al., 2013; Chao et al., 2015). Vitamin D supplementation has also been shown to boost the antimicrobial response of monocytes and macrophages against *M. tuberculosis* (Liu et al., 2007). The adaptive immune system is also influenced as increased vitamin D often helps to reduce inflammation and benefit individuals with autoimmune diseases such as multiple sclerosis (Prietl et al., 2013; Cantorna et al., 2012). Vitamin D helps to activate lymphocytes, such as regulatory T-cells, which can suppress proinflammatory responses that can prevent over activity of other immune cells (Prietl et al., 2013). Other vertebrates besides humans have seen benefits of vitamin D to their immune system. European sea bass given oral vitamin D supplements have increases in rates of phagocytosis when given larger amounts (Dioguardi et al., 2017). This study focused on the innate immune system and used phagocytosis as a measurement of immune function. There was a slight positive trend between phagocytosis and vitamin D (Fig. 21); however, it was not significant. It is possible that vitamin D could have an effect on other parts of the immune system in green sea turtles, as phagocytosis is only one measurement of immune function. For the innate immune system, monocytes seem to be the main cell that vitamin D enhances (Prietl et al., 2013; Borges et al., 2011). Since monocytes only make up a small portion of immune cells in sea turtles, monoclonal antibodies could be used to select for these specific cells in-vitro and test the effect of vitamin D on their

function. In the adaptive immune system, vitamin D is shown to increase lymphocyte proliferation and differentiation (Prietl et al., 2013; Borges et al., 2011). Future studies could look at this in sea turtles as another form of immune function to test. Even though immune function in this study was not affected, other health parameters were influenced by vitamin D.

Various blood chemistry parameters were significantly correlated with vitamin D levels (Table 2). Cholesterol and glucose had the strongest positive correlation with vitamin D. Cholesterol plays many roles in the body including hormone production, cell membrane structure development, and vitamin D synthesis (Bloch, 1991; Yeagle, 1991; Prietl et al., 2013). Vitamin D has been shown to reduce LDL-cholesterol and increase total cholesterol by increasing HDL-cholesterol in humans, which helps reduce risk of heart disease (Faridi et al., 2017; Mosca et al., 2019). In this study, cholesterol levels of the rehabilitated turtles on intake were lower than those of other studies (Bloodgood et al., 2019; Swimmer, 2000).The positive correlation seen in this study between vitamin D and cholesterol is like due to the fact that there needs to be sufficient cholesterol in the skin to synthesized vitamin D (Prietl et al., 2013). Glucose is also important in the body as it is necessary for proper cell function by providing energy through the production of ATP (Olson and Pessin, 1996). Vitamin D has been shown to play a role in controlling glucose homeostasis and reducing glucose levels in diabetic patients (Yousefi Rad et al., 2014; Foroughi et al., 2016). It is likely that vitamin is helping regulate the amount the glucose in the blood of these turtles. Glucose levels of these rehabilitated turtles upon intake were comparable to levels found in wild-caught Hawaiian turtles by Swimmer

(2000) but were higher than those determined for turtles brought into a rehabilitation facility by Bloodgood et al. (2019).

Albumin levels and ALT were also positively correlated with vitamin D. Albumin functions to transport molecules that are not water soluble, to regulate osmotic pressure, and to provide antioxidant capabilities (Vincent, 2009). Previous studies found a significant positive correlation between albumin and vitamin D and linked deficiencies in both to reduced muscle strength and balance (Yonemura et al., 2000; Kwon et al., 2007). The albumin levels in this study were higher than those determined for both rehabilitated and free-ranging turtles by Bloodgood et al. (2019) but were comparable to albumin levels analyzed for FP- and FP+ turtles in Hawaii by Aguirre and Balazs (2000). Alanine aminotransferase (ALT) is a catalyst for the production of oxaloacetate and plays a role in proper liver function (Kim et al., 2008; Price and Alberti, 1979). Typically, high levels of ALT are associated with liver disease or other diseases in the body (Kim et al., 2008). In patients with liver disease, vitamin D levels tend to be low, which puts ALT levels at risk for being elevated (Skaaby et al., 2014); however, in healthy humans ALT levels are positively correlated with vitamin D levels (Shehata and Qayyum, 2016). ALT levels in this study were comparable to values determined for FP- and FP+ turtles in Hawaii by Swimmer (2000) but were higher than those determined by Aguirre and Balazs (2000). Although these correlations do not signify a causational relationship, vitamin D and these parameters likely improve the health of these animals in rehab.

Regrowth, Viral Load and Survival

Regrowth of FP tumors is a common occurrence for turtles that have tumor removal surgery. One study showed that 50% of individuals that underwent tumor removal surgery experienced regrowth (Page-Karjian et al., 2019). Of the turtles that underwent surgery in this study, 57% of individuals experienced regrowth. All of the turtles that experienced regrowth had a Balazs tumor score of 3 upon intake. It is possible that tumor score could affect regrowth; however, the sample size is small, and a previous study found no significant relationship between tumor score and regrowth occurrence (Page-Karjian et al., 2019). There was a difference in regrowth depending on the exposure to UV light the turtles received (Table 3 and 4). Those exposed to high UV light conditions experienced less regrowth compared to those exposed to low UV light conditions. A previous study in mice showed reduced tumor growth when exposed to UV light (Valrance et al., 2007). It is possible that a similar effect is being shown in the rehab turtles at GLNC; however, the samples size is too small to determine any significant relationship. One thing to keep in mind is that every veterinarian uses different techniques to remove tumors, so there could be some variability in comparison to other studies. The most common form of tumor removal surgery is by CO_2 laser; however, some individuals use a cauterizer or scalpel blade to remove the tumors. The amount of tissue that is removed around the tumor also depends on the veterinarian, which can influence the amount of regrowth as well. In this study, only one veterinarian, Dr. Maria Chadam, conducted all of the tumor removal surgeries. Dr. Chadam used a combination of scalpel blades, cauterizer, and CO_2 laser to remove the tumors and chose which method to use based on the location and vascularization of the tumor, as well as the

tolerance of the animal while under anesthesia. Besides regrowth, viral load also appears to be influenced by UV light.

Viral load was measured for blood and tumor samples. All blood samples were negative for ChHV5 and the qPCR resulted in copy numbers that were not calculable. These results are similar to a study conducted by Page-Karjian et al. (2015), where blood samples had low viral DNA compared to other tissue types, such as tumor and skin tissue. To our knowledge few studies have compared viral load amount of various tumor types from green sea turtles with FP. Viral load of regrowth tumors was significantly less than soft tissue, hard tissue and eye tumors (Table 5). K. Yetsko (personal communication, December 7, 2019) also found lower viral load amounts in regrowth tumors compared to external tumors. It is possible that the lower ChHV5 amount seen in regrowth tumors is because the tumors are freshly grown, as K. Yetsko showed established tumors have greater viral load compared to newer tumors (personal communication, December 7, 2019) We also compared viral load in these tumor types based on UV exposure (Table 6). Regrowth and hard tissue tumors had higher viral load in turtles exposed to high UV light conditions, compared to soft tissue and eye tumors, which had lower viral load in turtles exposed to high UV light conditions. The only significant difference was seen in soft tumor tissues. Vitamin D supplementation has been shown to reduce viral entry of human immunodeficiency virus in humans (Jiménez-Sousa et al., 2018). Vitamin D has also been shown to contribute to reduced viral load of herpes zoster and herpes simplex virus type 1 in humans by increasing the release of cathelicidin (Chao et al., 2015). It is possible that this is what is occurring in the soft tissue and eye tumors of these turtles. Since the tumors collected were from various

individuals, it is also possible that each turtle is responding differently to the UV light treatment. Some of the turtles might be seeing a benefit of UV light on the viral load of their tumors, while others that are severely immunocompromised might not see as great of an effect. The sample size for the hard tissue tumors and regrowth were much lower, so the true relationship in these tumor types would be seen with a larger set of samples.

The survival of green sea turtles with FP is significantly reduced compared to FP- turtles brought into rehabilitation facilities. One previous study showed that 75% of turtles admitted into rehabilitation facilities for FP either died or were euthanized (Page-Karjian et al., 2019). Another study found that 77.3% of turtles treated for FP died or were euthanized (Stacey et al., 2017). In this study, 64% of FP+ turtles died or were euthanized. UV light appears to have an effect on the survivorship of turtles with FP (Fig. 22). 88% of FP+ turtles kept in low UV light conditions died or were euthanized, where only 33% of FP+ turtles kept in high UV light conditions died or were euthanized. It is likely not UV light alone that is contributing to this increase in survival. Although the turtles were randomly assigned to tanks, it is possible that there were some trends that could have led to variation in survival. Three of the FP+ turtles placed in the low UV light tanks were determined to have internal tumors a few weeks after admittance, which likely increased the number of euthanized individuals in this group. Some of the turtles also came in with minor secondary conditions that could have contributed to their survival. It is also possible that the sun tanks exposed to greater UV light provided warmer water temperatures, which could have permitted increased basking behavior. Veterinarians at each rehabilitation facility also use their own judgement when it comes to euthanasia, and their criteria could differ. It is also possible that other health

parameters and the standard treatment of antibiotics and vitamins could have contributed to the survival of these individuals.

Green sea turtles are an endangered species and prone to a number of factors that are detrimental to their survival. Fibropapillomatosis is one disease that affects these turtles worldwide. Although the disease can be treated with tumor removal surgery, the outlook of turtles with this disease is poor and often results in euthanasia. When turtles are stable enough to undergo surgery, they usually experience regrowth of the tumors. Individuals with FP demonstrate reduced immune function and variations in blood chemistry parameters. One potential method to better the outcome of turtles with this disease is increased exposure to UV light. Although a cure for this disease is yet to be discovered, increased plasma vitamin D levels from exposure to high UV light has the potential to boost immune function and contribute to improved recovery.

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