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EVALUATION OF IMMUNE FUNCTION IN TWO POPULATIONS OF GREEN SEA TURTLES (*CHELONIA MYDAS*) IN A DEGRADED VERSUS A NONDEGRADED HABITAT

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ABSTRACT: There is a strong correlation between degraded marine habitats and the prevalence of diseases such as green turtle fibropapillomatosis (GTFP) in coastal populations. In GTFP, small to large tumors grow on the turtle's soft tissues and shell, while internal nodules may also occur. The disease primarily affects juvenile green sea turtles (*Chelonia mydas*) that reside in nearshore waters. As a link has been shown between environmental pollution and immune suppression in a variety of animals, the objective of our research was to compare innate and adaptive immune responsiveness in green sea turtles from a severely degraded and a more pristine habitat, which differ greatly in rates of GTFP. We quantified phagocytosis by flow cytometry and performed in vitro stimulation analysis to measure activity of both the innate and adaptive immune systems in wild-caught Florida green turtles. Sea turtles from the degraded environment, both with and without visible cutaneous tumors, exhibited significantly reduced phagocytosis and stimulation indices than did those from the less polluted environment. Our results suggest that environmental factors may contribute to the development of GTFP and thus can impact the health of sea turtle populations.

Key words: Adaptive immune function, flow cytometry, green turtle fibropapillomatosis (GTFP), innate immune function, lymphocyte proliferation, phagocytosis.

INTRODUCTION

Sea turtles are bound to coastal habitats during several developmental stages, thus the integrity of the nearshore ecosystem may play a critical role in their welfare. Pollutants in nearshore habitats, including carcinogens and heavy metals (Perrault et al. 2011; Sinaei and Bolouki 2017; Speer et al. 2018), polychlorinated biphenyls (Camacho et al. 2013), harmful algal blooms (Perrault et al. 2014, 2017a), and agricultural runoff (Van Houtan et al. 2010; Bossart 2011) may threaten sea turtle health. These anthropogenic factors are linked to infectious diseases in coastal wildlife (Schaefer et al. 2011; Vilela et al. 2016; Bossart et al. 2019). In marine mammals, these include tumors and disease in beluga whales (*Delphinapterus leucas*; Lair et al. 2016; Iqbal et al. 2018) and deaths among California sea lions (*Zalophus californianus*) from toxic algal blooms (Lefebvre et al. 2016, 2018) and infectious disease (Seguel et al. 2019). In sea turtles, a high incidence of green turtle fibropapillomatosis (GTFP) is associated with degraded habitats (Herbst and Klein 1995; dos Santos et al. 2010). Investigators have therefore examined the associations between GTFP and various environmental factors including the prevalence of tumor-promoting toxins (Landsberg et al. 1999; Arthur et al. 2008), persistent organic pollutants (Keller et al. 2014; Sanchez-Sarmiento et al. 2017), dietary changes (Van Houtan et al. 2014), and ultraviolet radiation (Duffy et al. 2018), in addition to the presence of herpesvirus (Lackovich et al. 1999; Rodenbusch et al. 2014).

While considered the most likely etiologic agent for GTFP, chelonid alphaherpesvirus 5 has coexisted with sea turtle populations for 300 million yr, with no evidence for a recent increase in virulence correlating to the increased disease prevalence over the past century (Herbst et al. 2004; Lawrance et al. 2018). The virus is present worldwide (Greenblatt et al. 2005) in all hard-shelled sea turtle species (Quackenbush et al. 1998; Alfaro-

Núñez et al. 2014; Chaves et al. 2017) and in clinically healthy turtles (Page-Karjian et al. 2012; Alfaro-Núñez and Gilbert 2014). This suggests that the virus is widespread, but can be latent or subclinical (Alfaro-Núñez et al. 2016), and that expression of the disease is multi-factorial, involving interactions between the virus, host, and environment (Jones et al. 2016; Duffy and Martindale 2019). One factor that may affect host-virus interactions is a decrease in immune competence linked to pollutants, as has been shown in many animals including shellfish (Jiang et al. 2017), fish (Martyniuk et al. 2016; Chen et al. 2019), freshwater turtles (Ming-ch'eng Adams et al. 2016), and marine mammals (reviewed in Desforges et al. 2016). Cray et al. (2001) found reduced immune responsiveness in captive green sea turtles (*Chelonia mydas*) exhibiting GTFP compared to animals without evident tumors, though some have suggested that immune suppression is a result of, rather than a contributing agent to, the disease (Work et al. 2001).

We examined immune function in two populations of noncaptive turtles, comparing resident turtles from an area of poor water quality with those in a more pristine environment. Florida's Indian River Lagoon (IRL) is a heavily polluted estuary with high levels of heavy metals and persistent organic pollutants (Wang et al. 1992; Durden et al. 2007; Fair et al. 2010) and eutrophication (Lapointe et al. 2015; Barile 2018). As with other animals in the IRL, resident juvenile green turtles exhibit high rates of disease, with approximately 50% of green turtles showing tumors (Hirama and Ehrhart 2007; Lawrance et al. 2018). However, animals from the more pristine Trident Basin (TRI), located near Cape Canaveral, Florida, have essentially no GTFP (Hirama and Ehrhart 2007). While most previous papers on immune function in sea turtles examined adaptive immunity (Cray et al. 2001; Work et al. 2001; Keller et al. 2014; Rousselet et al. 2017), innate immune function is also likely to be important (Rousselet et al. 2013); thus, we examined aspects of both innate and adaptive function in the two populations. Adaptive immunity involves the specific recognition of antigens and the development of memory cells and, in turtles, is most often measured by lymphocyte proliferation (Rousselet et al. 2013), while innate immunity acts as an initial defense mechanism against pathogenic agents and involves natural killer cells and phagocytic cells. We hypothesized that cells from both the innate and adaptive immune system would show reduced function in animals from the more polluted habitat.

MATERIALS AND METHODS

Collections sites

The IRL is a 250-km long shallow estuary on the east-central coast of Florida, with minimal water exchange with the Atlantic Ocean. The TRI is a man-made embayment located near the mouth of an inlet with a strong tidal flux, is located on government property, and experiences little pollution. The TRI is considered a representative location for a pristine, unimpacted population and is free of GTFP (Hirama and Ehrhart 2007). Because turtles captured in the IRL may or may not have internal nodules, and thus gross observation cannot determine if they are GTFP positive, for the purposes of this study we categorized animals captured in the IRL by the presence or absence of visible tumors (VT+ and VT–, respectively) without presumption of viral infection.

Animal capture and handling

Blood samples were obtained from 87 green turtles captured at TRI (n=27) and in the IRL (n=60) as part of ongoing tag-recapture studies at those locations (Florida Fish and Wildlife Conservation Commission permit no. FWC MTP186 and National Marine Fisheries Service permit no. NMFS 14506). Animals were sampled in 1999 and 2001 (n=52) and from 2011 to 2013 (n=35) across all seasons. Animals were caught in a 152-m tangle net, dip netted, and removed to a boat for data collection. Animals were released following collection of biologic and morphometric data, blood collection, and tagging. All immunologic analyses were performed under Florida Fish and Wildlife Conservation Commission permit no. FWC MTP053 and approved by the Florida Atlantic University Institutional Animal Care and Use Committee. For each study, 2–5 mL of blood were drawn from the dorsal cervical sinus and transferred to a sodium-heparin Vacutainer (BD Biosciences, Franklin Lakes, New Jersey, USA). Blood samples were chilled above ice (approximately 4 C) for transportation to Florida Atlantic University for analysis.

Hematology

Heparinized whole blood smears were stained using Diff Quick differential stain (Sigma-Aldrich, St. Louis, Missouri, USA) or a 1:20 diluted Giemsa stain (Sigma-Aldrich). Leukocytes were counted under 40× magnification and recorded as a percentage of the total leukocyte population. Additionally, an aliquot of 10 μL of whole blood was centrifuged at 27,950 × G for 5 min to determine packed cell volume (PCV).

Separation of whole blood

Whole blood was layered on a discontinuous Percoll gradient (GE, Pittsburgh, Pennsylvania, USA): a 60% layer to restrict monocytes and lymphocytes (peripheral blood mononuclear cells; PBMCs) and a 75% layer to separate granulocytes (polymorphonuclear cells; PMNs). Each layer was washed twice in 1× phosphate-buffered saline. The viable cell yield was determined by standard trypan blue hemocytometry. Each sample was then diluted with phosphate-buffered saline to bring the concentration to 1×10^6 cells/mL.

Phagocytosis assay

Samples were incubated with a suspension of 1.0 μm fluorescein isothiocyanate (FITC)-labeled latex beads (Spherotech, Lake Forest, Illinois, USA). Then 50-μL of FITC-labelled beads were added to cells (100 μL PBMCs and PMNs) in Hank's balanced salt solution (Fisher Scientific, Waltham, Massachussetts, USA) in 96-well plates. After 1 h, phagocytosis was interrupted by placing samples on ice. Leukocytes were then gently layered on cold 10% bovine serum albumin (VWR, Radnor, Pennsylvania, USA) and centrifuged at 800 × G at 4 C for 8 min to elute noninternalized and nonspecifically bound beads. The percent of cells containing phagocytosed beads was evaluated using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (BD Biosciences). Fluorescence was measured at 480 nm. Cells alone, without fluorescent beads, were used as a negative control. Forward scatter vs. side scatter plots were used to identify PMN, PBMC, and red blood cell populations by size and granularity (Rousselet et al. 2013). For each sample, 20,000 events were collected.

In vitro lymphocyte proliferation

Lymphocytes were isolated from whole blood with Histopaque-1077 (Sigma-Aldrich) and washed three times. The viable cell yield was determined and each sample diluted with complete media to 1×10^6/mL. Lymphocytes were incubated at 37 C and 5% CO_2 with phytohemagglutinin or concanavalin A—considered to be T-cell mitogens; lipopolysaccharide (LPS; a B-cell mitogen); phorbol 12-myristate 13-acetate (PMA) + ionomycin; or pokeweed (PWM) mitogen (both a T- and B-cell mitogen), with complete media as a negative control (Cray et al. 2001). All stimulating agents were from Sigma-Aldrich. After 48 h, cells were pulsed with 20 μL of Promega CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin). Optical densities were then taken for each plate utilizing the Wallac 1250 Betaplate liquid scintillation counter (Perkin Elmer, Waltham, Massachusetts, USA) every hour for 3 h. Stimulation indices were calculated using counts per minute (cpm) as [mean mitogen cpm]/[mean medium only cpm].

Statistical analysis

Statistical comparisons were made both between sites, time of year (season), and VT+ vs. VT− status. Data were tested for normality and equal variance of residuals, with appropriate analyses for parametric (Shapiro-Wilkes) or nonparametric (Kruskal-Wallis and Dunn's) data to detect differences between groups. Statistics were run on Sigmaplot software version 12.1 (IBM SPSS Statistics for Windows, Armonk, New York, USA).

RESULTS

Hematology

Leukocyte differential and PCV were determined for each sample. In both IRL and TRI groups, heterophils comprised the largest fraction of the leukocyte population; lymphocytes made up the next largest fraction followed by eosinophils. Monocytes made up the smallest fraction (Fig. 1). Interestingly, the percentage of circulating monocytes was significantly higher in VT+ (IRL) turtles (P=0.002) than TRI turtles. There were no other significant differences in the relative proportions of circulating leukocytes among the populations (P=0.119), nor was mean PCV significantly different between TRI and IRL turtles either with or without tumors.

Phagocytosis assays

Flow cytometry revealed that both the PBMC and PMN fractions of sea turtle white blood cells are capable of phagocytosis (Fig.

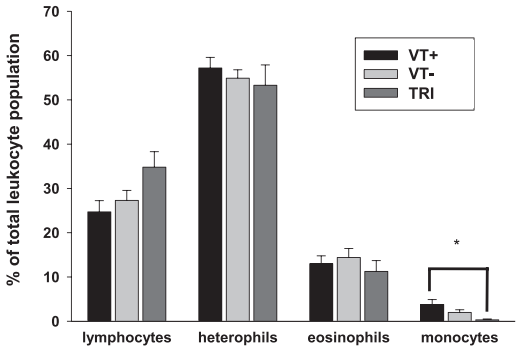

FIGURE 1. Predominant leukocyte populations for green (*Chelonia mydas*) turtles from two locations in Florida, USA: the Indian River Lagoon (high prevalence of green turtle fibropapillomatosis [GTFP]; with [VT+] and without [VT–] visible tumors) and the Trident Basin (TRI) where GTFP is rare. Animals were sampled in 1999 and 2001 and from 2011 to 2013 across all seasons. There were no differences in the percentage composition of the leukocytes between turtles from the two locations (P=0.866), except in the monocyte population. *Monocyte levels are significantly higher in TRI turtles than in VT+ (P=0.002). There is no statistical difference (P=0.119) between VT+ turtles and turtles without tumors (VT– and TRI).

2). The mean percentage of phagocytosis for PBMCs and PMNs across all groups was 4% and 8.2%, respectively. Overall, immune function as determined by phagocytosis was significantly related to geographic location, tumor status, and season.

Phagocytosis was significantly higher for both PBMC and PMN populations from TRI turtles than for leukocytes from VT+ or VT– animals captured in the IRL (P<0.05; Fig. 3). Regarding tumor status, the PBMCs in turtles with GTFP (consisting of animals from both the TRI population and IRL VT–) exhibited a higher percentage of phagocytosis than did those animals with visible tumors (P< 0.001). Within the IRL population, PBMC activity was significantly higher in VT– animals than in VT+ turtles (P<0.05); PMN activity in the IRL population also appeared to be higher in VT– compared to VT+ animals, but the difference was not statistically significant (Fig. 3).

Additionally, there was a seasonal difference in phagocytic capacity: for leukocyte populations in IRL turtles (both VT+ and VT–), there was a higher fraction of phagocytically active cells in the summer samples (June–August) than in winter (January–March; Fig. 4). In winter, samples from TRI turtles had significantly higher percentages of phagocytosis (mean 10.5%) compared to both VT– (3.5%, P<0.05) and VT+ (0.7%, P<0.001) turtles from the IRL. The TRI

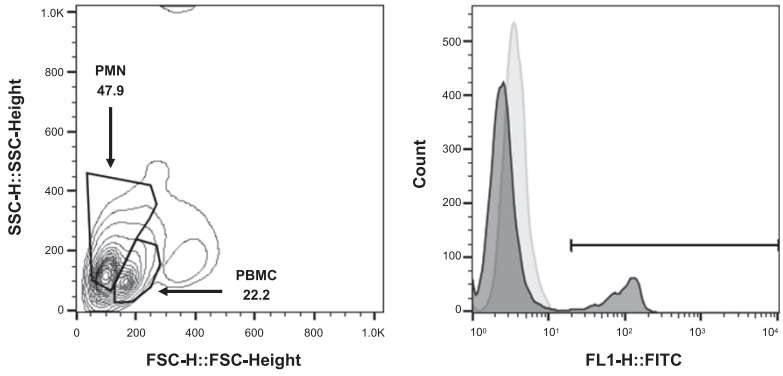

FIGURE 2. Representative analysis of flow cytometry results for phagocytes of green turtles (*Chelonia mydas*) sampled in the Indian River Lagoon, Florida, USA, where there is a high prevalence of green turtle fibropapillomatosis. Animals were sampled from 2011 to 2013 across all seasons. Contour plot (left panel) shows forward scatter (FSC) vs. side scatter (SSC), with areas containing peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) outlined. Histogram (right panel) shows each cell population peak and representative gate for cells that have phagocytosed fluorescein isothiocyanate-labelled (FITC+) beads. The darker gray represents the PMNs and the lighter gray represents the PBMCs. Each sample represents 20,000 events.

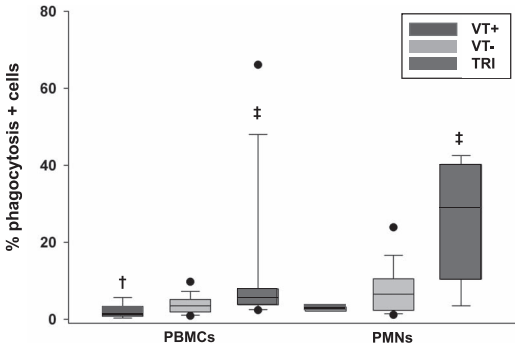

FIGURE 3. Box and whisker plots showing percentage of phagocytic-positive cells in the peripheral blood mononuclear cell (PBMC) and polymorphonuclear cell (PMN) layers from all green turtles (*Chelonia mydas*) captured in the Indian River Lagoon (IRL; n=60) and Trident Basin (TRI; n=27), Florida, USA. Animals were sampled from 2011 to 2013 across all seasons. Leukocytes of TRI turtles had significantly greater phagocytosis by both the PBMC and PMN subpopulations than either IRL group ($P<0.05$). Within the IRL, the PBMCs had greater phagocytosis in turtles without visible tumors (VT–) than did those animals with visible tumors (VT+; $P<0.001$). †=Within same white blood cell (WBC) population, significantly different from both VT+ and TRI turtles. ‡=Within same WBC population, significantly different from both VT+ and VT– turtles.

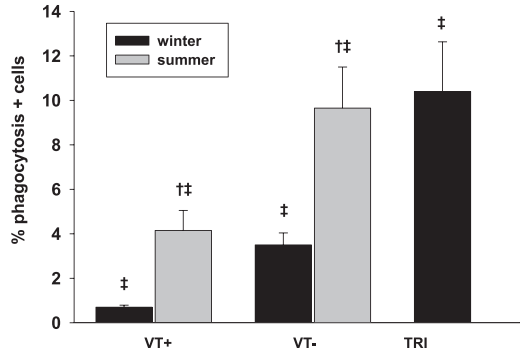

FIGURE 4. Mean percentages of phagocytosis in leukocytes collected in winter vs. summer in green (*Chelonia mydas*) turtles of the Indian River Lagoon (IRL, Florida). Animals were sampled from 2011 to 2013. There were no summer data collected from the Trident Basin (TRI, Florida), but winter rates of phagocytosis are significantly higher ($P<0.001$) than in animals from the IRL. Additionally, IRL turtles without visible tumors (VT–) exhibited higher rates of phagocytosis than did those with visible tumors (VT+) during summer months (P=0.023). ‡=Significantly different between groups in same season. †=Significantly different between seasons for same group.

population was not sampled in the summer months. Additionally, VT– turtles in the IRL exhibited significantly higher percentages of phagocytic cells than did VT+ turtles during both the summer and winter months (P=0.023); VT+ turtles exhibited the lowest percentages of phagocytosis regardless of season ($P<0.001$).

In vitro lymphocyte proliferation

Sea turtle leukocytes responded most strongly to PMA + ionomycin, with a lesser response to either LPS or PWM (Fig. 5). The only mitogen resulting in a statistically significant difference between TRI turtles and both VT– and VT+ turtles in the IRL was PMA + ionomycin (P=0.045). Additionally, VT+ turtles had stimulation indices that were significantly lower than those of nonpapilloma turtles from either the IRL or TRI when pulsed with LPS or PWM ($P<0.05$). The median stimulation index in response to phytohemagglutinin for leukocytes from VT+ turtles was lower than in TRI animals, but the difference was not significant. Overall responsiveness was low in response to concanavalin A, and there was no difference between turtle

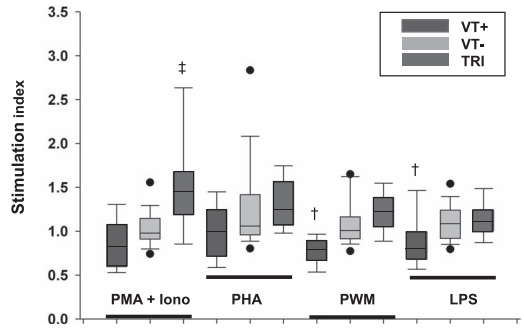

FIGURE 5. Box and whisker plots showing stimulation indices for green sea turtle (*Chelonia mydas*) lymphocytes exposed to respective mitogens. ‡=Significantly different from Indian River Lagoon (IRL, Florida) turtles both with (VT+) and without visible tumors (VT–). †=Significantly different from both Trident Basin (TRI, Florida) and IRL VT–. Stimulating agents included phytohemagglutinin (PHA), concanavalin A, lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) + ionomycin, or pokeweed mitogen (PWM), with complete media as a negative control.

populations (data not shown). When IRL VT+ and VT– turtles were grouped to focus on location rather than tumor status, IRL animals on the whole did not respond as vigorously to either PMA + ionomycin or PWM as did those animals from TRI ($P<0.045$).

DISCUSSION

Our results indicate that both adaptive and innate immune function are compromised in green turtles captured in the highly polluted IRL, where historical rates of GTFP in juvenile green turtles are ≥50% (Hirama and Ehrhart 2007; Lawrance et al. 2018). Within the IRL group, immune function was generally lower in VT+ turtles than in VT– turtles. By comparison, turtles from the TRI basin are free of GTFP, and both the innate and adaptive branches of immune function exhibit greater immune competence. This correlation suggests that location or habitat quality may contribute to immune competence in green turtles; thus, disease prevalence may reflect ecosystem health. While Work et al. (2001) suggested that immune suppression is a result rather than a contributing agent to the disease, our data suggest that habitat quality, disease state, and immune function are intertwined, forming a positive feedback loop wherein polluted environments impact the immune system and make animals more prone to the expression of GTFP, which in turn further compromises the immune system. Links between pollution and disease have been well described in mammalian studies (Scholin et al. 2000; Lefebvre et al. 2016, 2018; Lair et al. 2016; Iqbal et al. 2018; Seguel et al. 2019). In Florida, for example, an overabundance of nitrogen in the IRL encourages the proliferation of the fungus *Paracoccidioides brasiliensis* (Durden et al. 2009) and increasing numbers of Atlantic bottlenose dolphins (*Tursiops truncates*) with chronic mycotic dermal infection (Vilela et al. 2016; Bossart et al. 2019), while pollutants in Charleston Harbor, South Carolina, have been linked to the spread of infectious disease in their resident dolphin population (Bossart et al. 2017; Reif et al. 2017).

Similar to resident dolphin populations, juvenile green turtles are intrinsically bound to coastal habitats and subject to a variety of ecologic stressors that influence the health of both the ecosystem and its inhabitants. This is especially true in the IRL, an estuary experiencing declining health due to multiple ecologic stressors. The IRL experiences very little tidal mixing with the Atlantic Ocean; freshwater inputs and nutrient pollution have exacerbated harmful algal blooms (HABs; Gobler and Sunda 2012; O'Neil et al. 2012), including a brown algae bloom in 2011 that resulted in the loss of approximately 60% of total seagrass cover (St. Johns River Water Management District 2012) and a blue-green algal bloom in 2019 that increased toxic microcystin levels. Such HAB events are associated with declining health in resident *Tursiops* populations (Twiner et al. 2011). Other organisms and estuaries are also affected: decreased salinity in the IRL has also resulted in, for example, an overabundance of a toxic fungus, *Aphanomyces invadens*, which creates lesions in the skin and muscle tissue of fish (Vandersea et al. 2006; Sosa et al. 2007), while novel neoplasias have recently been detected in fish in other Florida estuaries (Kiryu et al. 2018). Prevalence of GTFP is generally associated with degraded habitats and nearshore environments worldwide (Milton and Lutz 2003; dos Santos et al. 2010), and juvenile green turtles in the IRL have shown an increase in GTFP prevalence during long-term monitoring (Aguirre and Lutz 2004). An examination of stress responses at the molecular level also suggests that green turtles in the IRL are physiologically stressed. Whether they have visible tumors or not, levels of cellular stress markers are higher in these animals than in TRI turtles (Deming 2008).

Hematology

Studies in other aquatic organisms have shown impacts by pollutants such as HABs and heavy metals on immune function, including in manatees (*Trichechus manatus*; Walsh et al. 2015), freshwater turtles (Walsh

et al. 2019), and sea turtles (Walsh et al. 2010; Perrault et al. 2014, 2017a, 2017b), while differences in circulating leukocytes in response to pollution have been demonstrated in fish (Marchand et al. 2020). One aspect of immune competence that might reflect ecosystem health is a difference in circulating leukocyte populations, and previous studies have shown variable heterophil to lymphocyte ratios based on sampling site (Aguirre et al. 1994; Cray et al. 2001; Lutz et al. 2001). We found that heterophils comprised the majority of white blood cells, as reported in other sea turtle studies (Rousselet et al. 2013; Muñoz et al. 2014; Rossi et al. 2016); interestingly, we observed an increase in the mean heterophil population from 34% (in 1999) to 53% (in 2014) in the TRI turtles, possibly indicating some other physiologic stress. In reptiles, heterophils and monocytes are thought to be the first line of immune defense (Rousselet et al. 2013), although a subset of B-cells that normally produce antibodies has also been shown to have phagocytic capabilities in freshwater turtles (Zimmerman et al. 2009). Monocytes are also an important component of the innate immune response and comprise up to 5% of the leukocyte population in IRL turtles with visible tumors, 2% in IRL turtles without apparent tumors, and 0.8% in turtles from the TRI, though in one study of captive loggerheads, monocyte levels were greater than eosinophil numbers (Rousselet et al. 2013).

While the small fraction of monocytes in the leukocyte population compared to the heterophil population would suggest that monocytes are not the primary phagocytic cell in juvenile green sea turtles, Rousselet et al. (2013) reported that monocytes had the highest phagocytic activity in captive juvenile loggerhead turtles, while Rossi et al. (2016) found that in green turtles with GTFP, the lymphocyte and monocyte populations had significantly more phagocytic activity than did the heterophils. Differences between studies are likely to result from a variety of factors including use of wild caught vs. captive turtles, the length of residence in rehabilitation facilities, the selection of phagocytic target and opsonization, and whether other stimulants were used (e.g., Zymosan A from yeast [Rossi et al. 2016]). Although overall monocyte numbers were low in this study, there was a significant difference between IRL animals with tumors and the TRI turtles, suggesting that this white blood cell subpopulation is upregulated in cases of active GTFP. Increased monocyte numbers in reptiles are thought to indicate chronic conditions such as bacterial or parasitic infections, inflammation, and neoplastic diseases (Stacy et al. 2011).

Phagocytosis

Flow cytometry revealed that both monocytes and heterophils were capable of ingesting FITC beads. Phagocytosis by different fractions of the white blood cell populations, including heterophils, monocytes, and eosinophils, has been reported previously (Rossi et al. 2009, 2016; Rousselet et al. 2013; Muñoz et al. 2014). Rousselet et al. (2013) reported similar levels of phagocytosis for both monocytes and heterophils in juvenile loggerhead turtles. Though Rossi et al. (2016) reported in one study that lymphocytes and monocytes had greater phagocytic activity than did granulocytes in green turtles, method differences may have resulted in different ratios of leukocytes: the heterophil percentage in the Rossi et al. (2016) study (10%) was much lower than the lymphocyte population (88.3%), suggesting that few heterophils were retrieved. In our study, phagocytic activity varied dependent on where the animals were captured, tumor status, and time of year.

While there was no significant difference in the circulating heterophil percentages between IRL and TRI turtles, heterophils (the main component of the PMN layer in our study) from TRI exhibited the highest percentages of phagocytosis, indicating that immune cells in those turtles from a more pristine environment perform better than those from a degraded habitat. Even within the IRL population, VT– turtles showed better immune competence than did those with visible tumors, supporting the hypothesis

that diseased states may hamper immune function, as suggested by Work et al. (2001).

Within the IRL, tumor-bearing animals are more prevalent in the summer months (Hirama and Ehrhart 2007) despite the fact that, as shown in this study, phagocytosis is significantly higher in summer than winter, suggesting better immune function in the warmer months. Many factors could result in higher apparent rates of GTFP in the summer IRL population, from a different group of resident turtles to a higher growth rate of some unknown disease vector such as leeches (Herbst and Klein 1995). Among green turtles from the IRL, VT– animals did exhibit significantly higher percentages of phagocytosis in both seasons than did VT+ turtles (Fig. 4).

In vitro lymphocyte proliferation

The lymphocyte proliferation assays also showed a significant difference in adaptive immune function between the IRL and TRI turtles. Cells from TRI turtles exhibited the highest stimulation indices with PMA + ionomycin and PWM, both of which are reflective of a response from both B- and T-cells, though because we did not separately test the B- and T-cells in this study, there was no way to differentiate their activity. Lymphocytes from IRL turtles exhibited distinctly reduced lymphocyte proliferation in response to mitogen stimulation compared to TRI turtles. For the majority of mitogens, the only significant difference was between VT+ turtles in the IRL and TRI animals; the IRL VT– animals consistently showed lower responses than TRI animals but were higher than VT+ turtles, suggesting that both T and B lymphocytes from the IRL turtles with visible tumors were relatively unresponsive. The lack of significant differences may be due in part to the overall low responsiveness of turtle leukocytes to the mitogens, as was seen in earlier studies (Lutz 2001; Cray et al. 2001). The reagent that generated the strongest response in all populations (PMA + ionomycin) was also the one that showed a significant difference not only between TRI and VT+ animals, but also between TRI and VT–, and between VT+ and VT– turtles in the IRL. Previous studies of adaptive immune responses in sea turtles have shown correlations to suppressed lymphocyte proliferation in turtles exposed to organochlorides, disease (GTFP), and degraded environments (Cray et al. 2001; Lutz et al. 2001; Keller et al. 2006). Negative effects on immune function by various contaminants in sea turtles have been demonstrated specifically for mercury (Day et al. 2007), other heavy metals (Camacho et al. 2013), and polychlorinated biphenyls (Rousselet et al. 2017), while both papilloma disease and herpes viral infections are associated with reduced immune competence in animals (Nicholls and Stanley 2000), from oysters (de Lorgeril et al. 2018) to dogs (Sundberg et al. 1994) and cows (Jones 2019).

Together, the results of the phagocytosis and lymphocyte proliferation assays substantiate the hypothesis that turtles from more pristine habitats are better able to mount a robust defense against pathogens, while those from degraded habitats exhibit reduced immune function that may make them more prone to disease. Defects in innate immune function are likely to increase susceptibility to pathogenic infection, as host leukocytes would be unable to clear microbial cells and dead or damaged host cells. Additionally, stimulation of the adaptive immune system by the innate response may be reduced (Abbas et al. 2012). This study supports the earlier findings of Cray et al. (2001) that an altered adaptive immune function is associated with tumor development and, in fact, strongly suggests that immune responsiveness may be linked to environmental health, not just tumor status. We also found that VT– turtles from either the TRI or IRL site showed better immune competence than those with visible FP tumors, even within the highly polluted IRL, agreeing with Work et al. (2001) that reduced immune function follows disease. Our results, however, also indicate that reduced immune competence may initially permit disease, and disease status in turn may then further hinder immunocompetence. Such a vicious cycle could explain why certain locations have such

a high incidence of disease, while other areas have clinically healthy turtles that test positive for chelonid alphaherpesvirus 5 (Page-Karjian et al. 2012; Alfaro-Núñez and Gilbert 2014). These results are not surprising because immunosuppression is known to stimulate further tumor growth and increase infection risk (Schreiber et al. 2011). Our study indicates that where increased incidence of disease exists, gross observation alone does not necessarily indicate that wildlife populations are healthy, which in turn may reflect overall ecosystem health.

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