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STUDIES OF THE ASSOCIATION BETWEEN IMMUNOSUPPRESSION AND FIBROPAPILLOMATOSIS WITHIN THREE HABITATS OF *CHELONIA MYDAS*

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ABSTRACT

Green turtle fibropapillomatosis (GTFP) is a severely debilitating disease which continues to threaten sea turtles worldwide. While GTFP has been investigated intensively, little has been ascertained as to its cause. In this investigation we examined the association between immunosuppression and fibropapillomatosis occurring on the east coast of Florida in three separate habitats: the Trident Basin at Port Canaveral Naval Base, outside of Titusville; the central part of the Indian River Lagoon; and a reef adjacent to the lagoon site south of the Sebastian Inlet. In vitro immunoassays, leukocyte cell differentials, clinical chemistries, hematology and serum protein electrophoresis from healthy and fibropapilloma turtles were utilized to determine health status of wild green sea turtles. The results of this investigation will provide greater understanding of health assessment of wild sea turtles and how environmental factors may affect the status of their health in relation to fibropapillomatosis.

The immune response of sea turtles is poorly understood much less how it responds to environmental stress. We performed in vitro lymphocyte proliferation assays, serum protein electrophoresis, clinical chemistries, packed cell volumes, and leukocyte differentials on blood taken from healthy sea turtles to provide a profile of their immune system. The results will provide baseline data with which to compare that of sea turtles with fibropapillomas. Additionally, we performed in vitro lymphocyte proliferation assays during the winter to determine if suboptimal temperatures directly suppress the proliferation of lymphocytes of green sea turtles. These results will provide insights into the response of the immune system of sea turtles to environmental stress.

In vitro lymphocyte stimulation indices indicated that green sea turtles from the Indian River Lagoon exhibited the lowest indices of the three groups. Sea turtles from the Trident Basin exhibited the highest stimulation indices while Sebastian Inlet Reef turtles had slightly lower stimulation indices. Papilloma turtles exhibited significantly lower levels of glucose, calcium, ALKP, AST (SGOT), and LDH. There were no significant differences between non-papilloma and papilloma turtles with respect to PCV, phosphorus, uric acid, and GGT levels.

The results from this investigation indicate that immunological differences attributable to green turtle fibropapilloma disease exist in wild populations. Whether immunosuppression is a contributing factor of GTFP or is instead an effect of the disease is an extremely important etiological question that must be addressed if we are to ensure the viability of *Chelonia mydas*.

Key words: Fibropapilloma, immunosuppression, mitogen, clinical chemistry

INTRODUCTION

Green turtle fibropapillomatosis (GTFP) is a severely debilitating disease characterized by epizootic tumors of the skin, flippers, periocular tissues, carapace and plastron as well as internal nodules which interfere with organ or systemic function (Campbell, 1996). The tumors, which vary in size and morphology, can decrease fitness by limiting the turtle's capacity to move and find food and may ultimately be fatal. GTFP has reached epidemic proportions worldwide and has affected all of the Cheloniids (Aguirre, et al., 1999). While GTFP has been investigated intensively, little has been ascertained as to its cause. Possible causal agents of this disease include parasites and herpesvirus (Quackenbush et al., 1999; Herbst, 1998). Pollution, phycotoxins, water temperatures, and genetic factors have also been suggested as variables of etiology (Landsberg, et al., 1999; Balazs and Pooley, 1991). While anthropogenic events may be implicated in the initiation and/or the expression of the disease, it is necessary to determine where in the cascade of events the etiological agent lies. In the context of understanding the etiology of this disease it is important to determine the role of the sea turtle's immune system in the expression of GTFP. Previous studies conclusively indicate that immunosuppression is strongly correlated with fibropapillomatosis in captive green sea turtles but this remains to be confirmed in wild populations (Varela, 1997). The purpose of this investigation was to determine if in fact this correlation exists in the wild. Our objective was to look at wild fibropapilloma infected green sea turtles and non-papilloma turtles to look at immunosuppression and blood chemistries. Preliminary results indicate that individuals from wild populations of *Chelonia mydas* respond to varying concentrations of mitogens in the same manner as captive green turtles. That is, lymphocytes from healthy sea turtles proliferate to a greater degree than those of GTFP turtles. Our understanding of green sea turtle immune function is essential if we are to ensure their viability as a species.

OBJECTIVES

The objective of this study was to describe the immune function of wild green sea turtles, to determine their immunocompetence when threatened with antigenic burden, and to describe their physiological status as shown by blood chemistry analysis.

MATERIALS AND METHODS

Eighty-three juvenile green turtles (*Chelonia mydas*) from three separate populations were sampled between November and April. The populations included: The Port Canaveral Trident Basin where there is zero prevalence of fibropapillomatosis ($n = 19$), a group from south

of the Sebastian River Inlet where there is a 5% prevalence of GTFP ($n = 38$), and another group from the Indian River Lagoon in Brevard County, Florida where ~67% of all the turtles evidence external fibropapillomas ($n = 26$) (Fig. 1). With the assistance of Dr. Lew Ehrhart (University of Central Florida) *Chelonia mydas* were captured in a 500-ft net and brought on board a 17-ft Boston Whaler for morphological measurements and blood samples. Individuals were released immediately following their work-up. For this study, 2-5 ml of blood was drawn from the dorsal cervical sinus of each turtle and transferred to a sodium heparin 3 cc vacutainer. In vitro lymphocyte proliferation, leukocyte differentials, serum protein gel electrophoresis, clinical chemistries, and packed cell volumes were performed at the Department of Pathology, University of Miami, School of Medicine for all turtles taken at each site. These diagnostic tests will help describe health status of sea turtle populations in areas of anthropogenic burden.

The Trident Basin turtles were utilized as health standard controls with which to compare the Indian River Lagoon and Sebastian Inlet Reef turtles, as they have no known history of fibropapilloma disease.

In Vitro Lymphocyte Proliferation

Lymphocytes were isolated from whole blood by using Histopaque-1077 and washed three times. The viable cell yield was then determined by staining 10 ul of cell solution with 200 ul of Trypan Blue using standard hemocytometer methodology. This yielded an exact count in millions of cells. Each sample was then diluted with complete media to bring the concentration of lymphocytes to 1X106/ml. The lymphocytes were then stimulated with varying concentrations of Phytohemagglutinin, Pokeweed mitogen, PMA, Ionomycin, Concanavalin A and lipopolysaccharide, and complete media as a triplicate control. Lymphocyte concentrations of 100 ul were plated on 96-well flat bottom culture plates with 100 ul of each mitogen and incubated at 37°C. After 48 hours cells were pulsed with 20 ul of Promega CellTiter 96®AQueous One Solution Cell Proliferation Assay. Optical densities were taken for each plate utilizing the Wallac 1205 Betaplate liquid scintillation counter every hour for 3 hours. Stimulation indices of lymphocyte proliferation were then determined after averaging mitogen triplicates. Statistical comparisons were made both between papilloma and non-papilloma groups and between sites.

Leukocyte Differentials

Heparinized whole blood mounts were made at the time of bleeding and later stained utilizing Diff Quick differential stain. Leukocytes were then counted under 100X magnification and recorded as a percentage of the total leukocyte population.

Serum Protein Electrophoresis

Serum protein values were obtained by the Beckman Paragon Electrophoresis System including the SPE II protein electrophoresis kit and dryer. Each gel was evaluated by densitometer yielding values for total protein: albumin %, alpha-1 globulin %, alpha-2 globulin %, beta-globulin %, gamma-globulin %, and albumin/globulin ratios.

Clinical Chemistry

Clinical chemistries were performed as a UM Advanced Avian Panel on the Kodak Ektachem System with total protein assessed by refractometer. Glucose (Glu), calcium (Ca), phosphorus (P), uric acid (Uric), alkaline phosphatase (ALKP), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK) and gamma-glutamyltransferase (GGT) were assessed for each sea turtle. Packed cell volumes (PCV) were obtained by centrifuging blood in microcapillary tubes and read as a percentage of the total blood volume.

RESULTS

In Vitro Lymphocyte Proliferation

Turtles from the Indian River Lagoon with cutaneous papillomas ($n = 26$) had stimulation indices that were significantly lower than non-papilloma turtles from the Trident Basin ($n = 19$) and Sebastian Inlet Reef ($n = 38$) indicating that their immune function is severely challenged. Reef turtles responded to stimulation of mitogens slightly higher than the Lagoon turtles but not as well as those turtles from the Trident Basin. This trend was observed with the following mitogens: ConA 5 ug/mL ($P = 0.006$) (Fig. 2), LPS 100 ug/mL, LPS 50 ug/mL $P = 0.002$) (Fig. 3), PHA 10 ug/mL (Fig. 4), PWM 10 ug/mL, PWM 5 ug/mL ($P = 0.006$) (Fig. 5), and the lymphocyte polyclonal stimulator combination of PMA 25 nM/Ionomycin 0.3 nM ($P = 0.036$) (Fig. 6).

White Blood Cell Differentials

Papilloma turtles show statistically significant higher heterophil counts and lower lymphocyte counts than the non-papilloma and control groups ($P = <0.001$). Fifty-six per cent of the leukocytes identified are heterophils (on average) for the papilloma group while non-papilloma turtle heterophil percentages averaged 35% (Fig. 7). Papilloma turtles also exhibited lower lymphocyte percentages (32%) with the non-papilloma turtles of the Trident Basin having the highest lymphocyte percentages (55%). Lymphocyte and heterophil ratios are maintained when eosinophils increase. Normally these leukocytes account for 1-2% of the total circulating population but increase in response to parasitic burden.

Serum Protein Electrophoresis

Serum protein electrophoresis was performed on papilloma and non-papilloma turtles from the three locations. Albumin and gamma globulin are the most interrelated serum proteins; the other serum proteins account for very little of the total protein. Trident Basin turtles exhibited the highest levels of albumin, and the lowest levels of gamma globulin for the three groups (P = 0.002). Papilloma turtles expressed lower gamma globulin percentages and higher albumin percentages than non-papilloma turtles (Fig. 8).

Clinical Chemistry

We found that non-papilloma turtles have statistically significant higher glucose levels than papilloma turtles (P = 0.001). Differences in glucose levels between the three locations are also statistically significant (P = 0.028) (Fig. 9). Both papilloma and non-papilloma turtles exhibited normal values for calcium; however, there is a statistically significant difference between location for calcium levels (P = <0.001). Phosphorus levels were not statistically different between papilloma and non-papilloma turtles ranging from 6.5 mg/dL to 7.2 mg/dL; however, there is a statistically significant difference between the three locations (P = <0.001) (Fig. 10). It is unlikely that this is of biological significance since all values were within normal ranges. Uric acid levels were not statistically different between papilloma and non-papilloma turtles. Papilloma turtles had statistically significant lower levels of ALKP (P = 0.001) (Fig. 11), AST (P = 0.005) (Fig. 12), and LDH (P = 0.006) (Fig. 13) compared to non-papilloma turtles which may be biologically significant (P = 0.001). There is no significant differences in CK between papilloma and non-papilloma turtles (P = 0.154). Papilloma turtles exhibit slightly lower levels of GGT; however, there were no statistical differences. There were no significant differences in PCV between non-papilloma and papilloma turtles. Papilloma turtles exhibit the widest range of packed cell volume from 14% to 40% (Fig. 14).

DISCUSSION

This investigation focused on determining whether an association exists between immunosuppression and fibropapilloma disease in wild populations of green sea turtles. The results of this study indicate a strong correlation between immunosuppression and fibropapillomatosis in wild populations of *Chelonia mydas*. In vitro lymphocyte proliferation assays indicated a significant difference of immune function between the three locations. Sea turtles from the Trident Basin at Port Canaveral exhibited the highest stimulation indices of all three sites while turtles from the Indian River Lagoon exhibited the lowest stimulation indices. Lymphocytes from healthy sea turtles proliferate to a greater degree than those of GTFP turtles, a pattern similar to that found in captive green turtles (Varela, 1997). Hence, wild sea turtles with GTFP may be immunosuppressed. This is an important finding since many of the papilloma associated pathologies could be secondary to a compromised immune system (Duncan, 1975;

Sundberg et al., 1994). This study supports the earlier findings of Cray et al. (1999) that an altered immune function is associated with tumor development. The results are not surprising since immunosuppression is known to increase the risk of infection and stimulate further growth of tumors (Kuby, 2000). Results from immunological data indicate that where increased incidence of disease exists, superficial observation alone does not necessarily indicate that non-papilloma turtles are healthy.

To further support the in vitro lymphocyte proliferation assays, white blood cell differentials were performed to substantiate the number of lymphocytes retrieved during the Ficoll-hypaque technique. Heterophils, lymphocytes, and eosinophils were counted as the total circulating leukocyte populations of papilloma and non-papilloma turtles. Heterophils are primarily phagocytic granulocytes which participate in inflammatory responses associated with parasitic diseases and microbial infection. Heterophils can represent up to 40% of the leukocyte differential (Campbell, 1996). Papilloma turtles show statistically significant higher heterophil counts and lower lymphocyte counts than the non-papilloma group. Fifty-six per cent of the leukocytes identified were heterophils (on average) for the papilloma group while non-papilloma turtle heterophil percentages averaged 45%. Papilloma turtles also exhibited lower mean lymphocyte percentages (25%) while non-papilloma turtles of the Trident Basin have the highest lymphocyte percentages (55%). Lymphocyte and heterophil ratios are maintained in papilloma and non-papilloma turtles when eosinophil levels increase. Normally these leukocytes account for 1-2% of the total circulating population but increase in response to parasitic burden. While Trident Basin turtles exhibited normal eosinophillic values, approximately 20% of the turtles from the Indian River Lagoon and Sebastian Inlet Reef locations expressed significantly higher eosinophil levels ranging from 10% to 32% of the total circulating leukocyte population. Therefore, significant differences exist in leukocyte composition between papilloma and non-papilloma turtles.

Serum protein electrophoresis is used as a nonspecific diagnostic tool to determine immune function. Serum proteins may change drastically in cases of disease. Albumin constitutes 35-50% of the total serum protein in animals: increases in albumin levels may represent dehydration in the animal while decreases in albumin may be due to malnutrition, liver disease, or gastroenteropathies (Kaneko, 1980). Gamma globulin represents the immunoglobulins of the animal, but these are not separated out utilizing SPE II methodology. Decreases in gamma globulin levels may represent agammaglobulinemia while increases may represent chronic disease, liver disease, or myeloma (Kaneko, 1980). Turtles from the lagoon and reef exhibited higher levels of gamma globulin and lower levels of albumin, thus indicating possible liver disease, myeloma, or malnutrition. Trident Basin turtles exhibit normal levels of albumin at 34% and lower levels of gamma globulin.

Clinical chemistry values have not been previously described comparing papilloma and non-papilloma sea turtles. Clinical chemistries are utilized to assess the overall health or physiological state of sea turtles (George, 1997). Our results indicate significant differences in clinical chemistry values that indicate diseased animals were physiologically challenged. Non-papilloma turtles have statistically higher glucose levels than papilloma turtles. While

glucose levels were significantly different between the three locations and by status, this does not necessarily indicate a biological difference, as glucose levels can vary widely under normal conditions. Calcium levels of non-papilloma turtles averaged 2.3 mmol/L while papilloma turtles averaged 1.8 mmol/L indicating no statistical significance between groups; however, there is a statistically significant difference between the three locations. There were also no significant differences in phosphorus or uric acid levels between papilloma and non-papilloma turtles. We also tested for standard liver enzymes used in veterinary medicine (Kramer, 1980). These isoenzymes are normally paired to determine health standards for each individual. For instance, if ALKP levels increase in juveniles while GGT levels remain normal, then the animal is considered stressed. If ALKP and GGT decrease simultaneously, on the other hand, there may be liver dysfunction. GGT is an enzyme used to measure obstructive processes in hepatobiliary tissues (Cornelius, 1980). ALKP and GGT levels were significantly lower in papilloma turtles; however, GGT levels were not statistically significantly lower. GGT levels of papilloma turtles averaged less than 5 U/L; reef turtles averaged 6.2 U/L while Trident Basin turtles averaged 8.1 U/L. Papilloma turtle GGT levels could not be statistically determined because of detectable levels of GGT in the serum. This may be of biological significance due to the presence of visceral nodules found in papilloma turtles' livers. AST in conjunction with CK are useful as an index of hepatic or muscular cell damage (Kramer, 1980). LDH is utilized as heart and muscle isoenzymes in veterinary diagnostics but is not used as a diagnostic tool for reptiles (Doug Mader, personal communication). And while hematocrit values may be utilized for general health assessment (nutritional state), they are clearly not useful indicators for papilloma disease. Normal hematocrit range for green turtles is between 26% and 32% (Wells and Baldwin, 1994).

Immune function may also vary with age, sex, or nutritional state of the animal (Pienaar, 1962; Duguy, 1970; Borysenko and Lewis, 1979). GTFP appears to affect juveniles more than any other age class. This variable may be due to the habitats they prefer. Green turtles spend their early postpelagic years in bays and lagoons and move to other habitats before maturing (Ehrhart, 1983). The consequences of choosing nearshore and shallow lagoons are many. Areas of eutrophication due to agricultural run-off may be rich in phycotoxins such as Pfeisteria, a known tumor-producing epiphyte of the turtle grass (Thallassia) on which green turtles feed (Landsberg et al., 1999). Juveniles may not possess normal innate immunity to combat these ingested toxins which would afflict the liver. These toxins would then accumulate, resulting in visceral tumors, later to be manifested as cutaneous tumors.

Viruses and parasites also affect the variability of lymphocyte numbers in the circulating blood population (Jacobson et al., 1981). In this scenario opportunistic agents can cause disease in immunosuppressed individuals (Kuby, 2000). Herpesviruses, for example, characteristically colonize tumors and tissues of debilitated and perhaps immune impaired animals. (Lackovich, 1999). And some viruses are capable of eluding immune response altogether by changing their antigens or causing generalized immunosuppression by directly infecting lymphocytes (Kuby, 2000). Whether immunosuppression is a contributing cause of fibropapillomatosis or is instead an effect of the disease is an extremely important etiological question that must be addressed.

This study was performed primarily during the colder winter months when immune function in reptiles in general is known to be suppressed (Zapata, 1992). For example, lymphocyte numbers in circulating blood populations are highest during the spring and summer and lowest during the winter (Kanakambika, 1972). Seasonal fluctuations may also play a role in sea turtle lymphocyte proliferation as in other reptiles. Further work, therefore, is necessary to determine if the immune function of papilloma turtles is also relatively nonfunctional during warmer summer months. However, while the immunological performance of sea turtles should increase during the summer months (Duguy, 1970), tumor growth is also reportedly more prolific (Herbst, 1994). Under these circumstances, immune-suppressed papilloma turtles could be at a severe disadvantage in respect to both GTFP and other etiological agents. It is important therefore, from an animal health point of view, to determine what role temperature plays in the immune function of sea turtles. We need to know, for example, the relationship between GTFP, immune response, and temperature as an environmental stressor. Our ongoing study will gather such information from wild populations to determine the effects of seasonal variations of immune status relative to temperature as an environmental stressor affecting immunosuppression.

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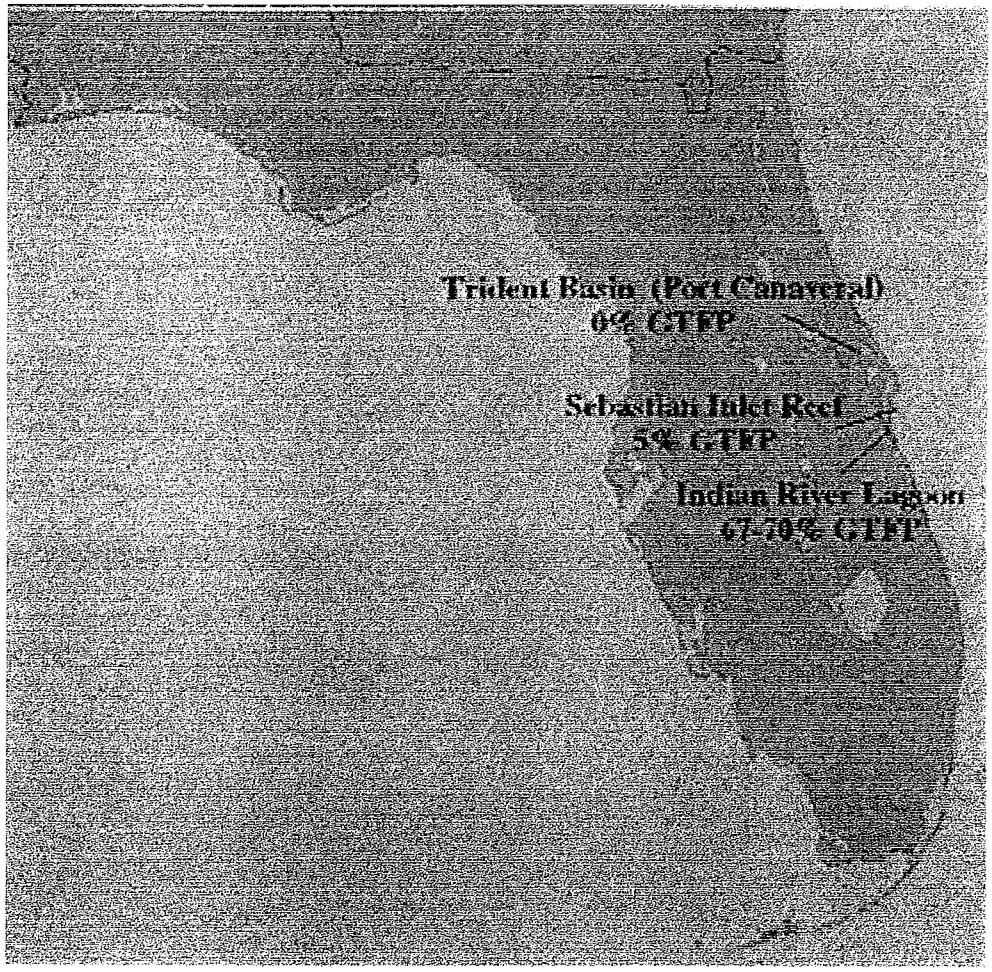

Figure 1. Prevalence of fibropapillomatosis at three Florida sites.

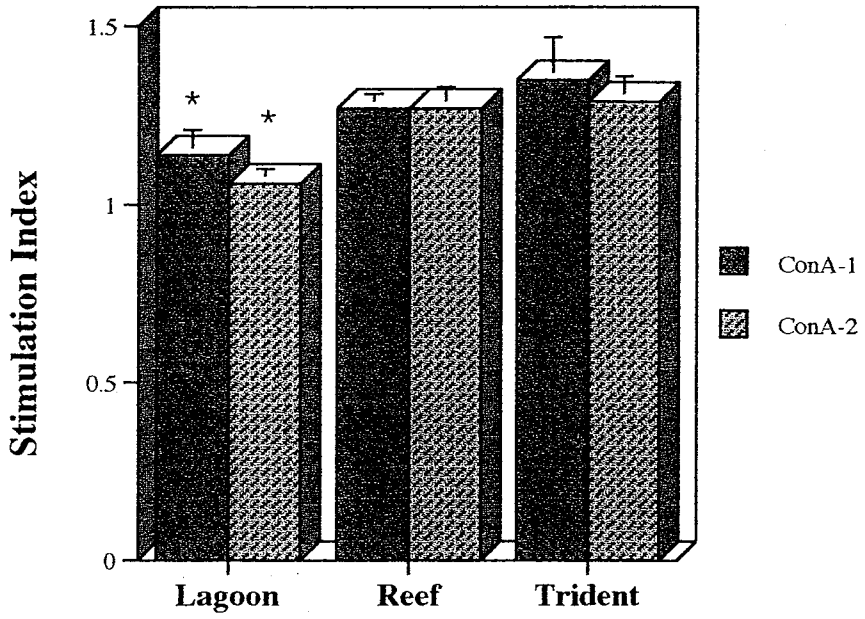

Figure 2. Stimulation indices of Concanavalin A-1 (10uM/ml) and ConA-2 (5uM/ml). Lagoon turtles did not respond as well to ConA-2 compared to turtles of the reef and Trident Basin (P=0.006). * indicates statistical difference from reef and Trident groups.

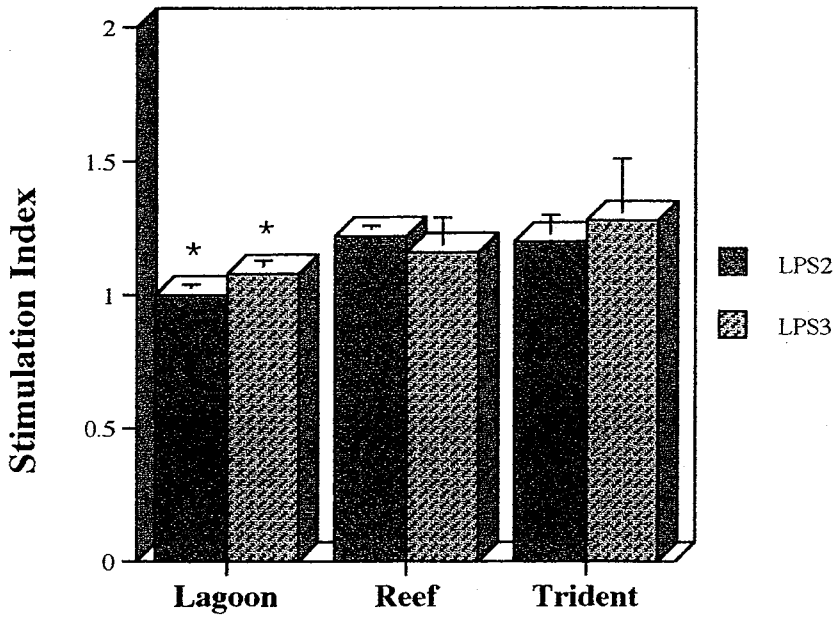

Figure 3. Lagoon turtles did not respond as well as the reef and Trident Basin turtles to LPS. * indicates statistical significance (P=0.002) vs. reef and Trident Basin turtles.

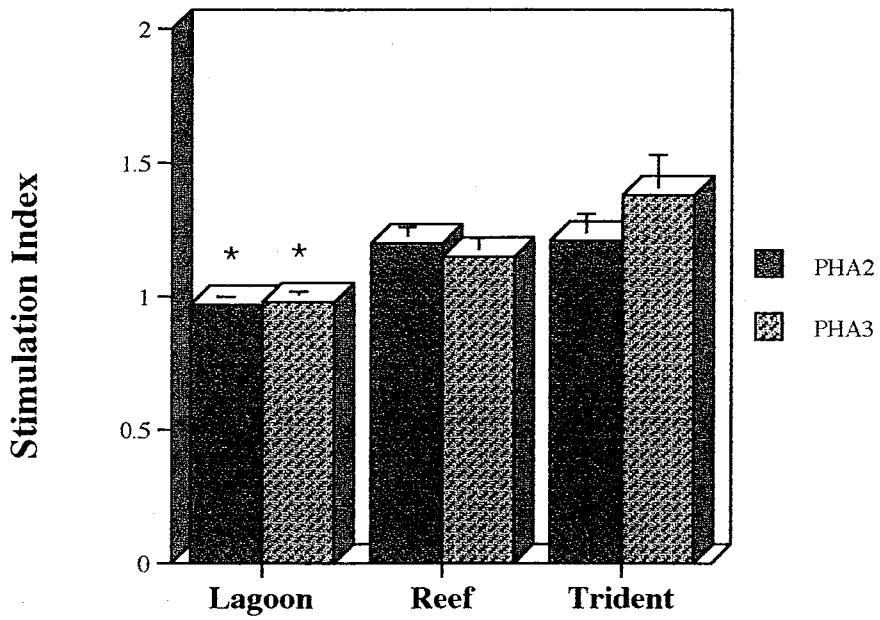

Figure 4. Lymphocytes from Lagoon turtles did not show as strong of a response to PHA as turtles from the reef or Trident Basin. * indicates statistical significance P=<0.001.

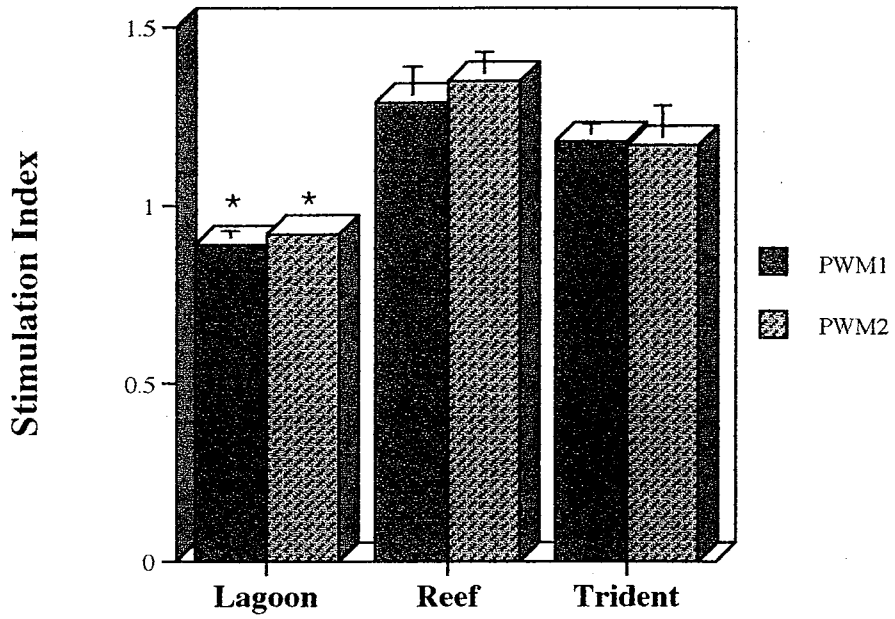

Figure 5. Lymphocytes from Lagoon turtles did not respond to PWM as vigorously as turtles from the reef or Trident Basin. * indicates a statistical difference (P=0.006) vs. reef and Trident Basin turtles.

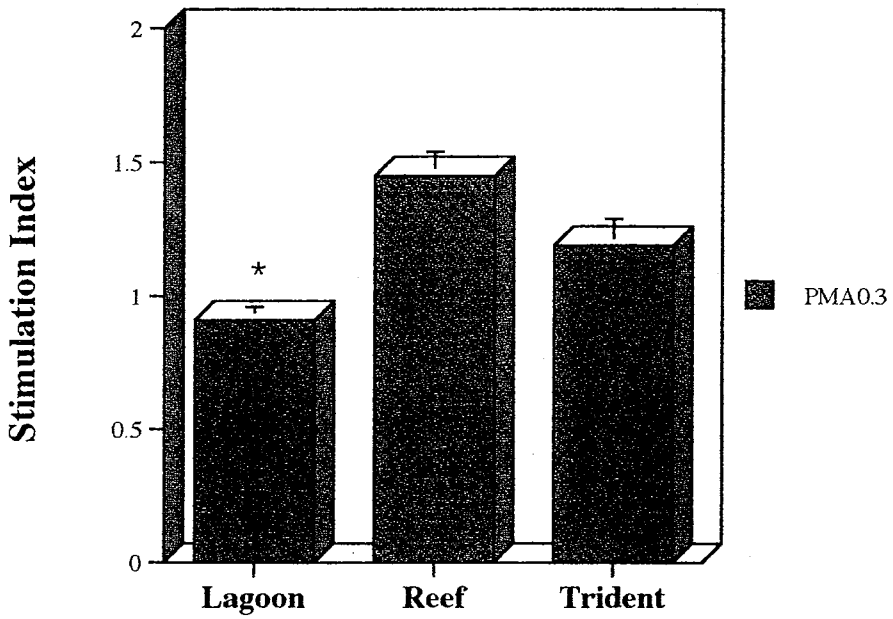

Figure 6. Stimulation indices of the lymphocyte polyclonal stimulator Ionomycin 0.3uM + PMA. Lagoon turtles did not respond as vigorously to PMA as the reef and Trident Basin turtles did. * indicates statistical significant difference (P=0.036) vs. Trident Basin turtles and (P=0.002) vs. reef turtles.

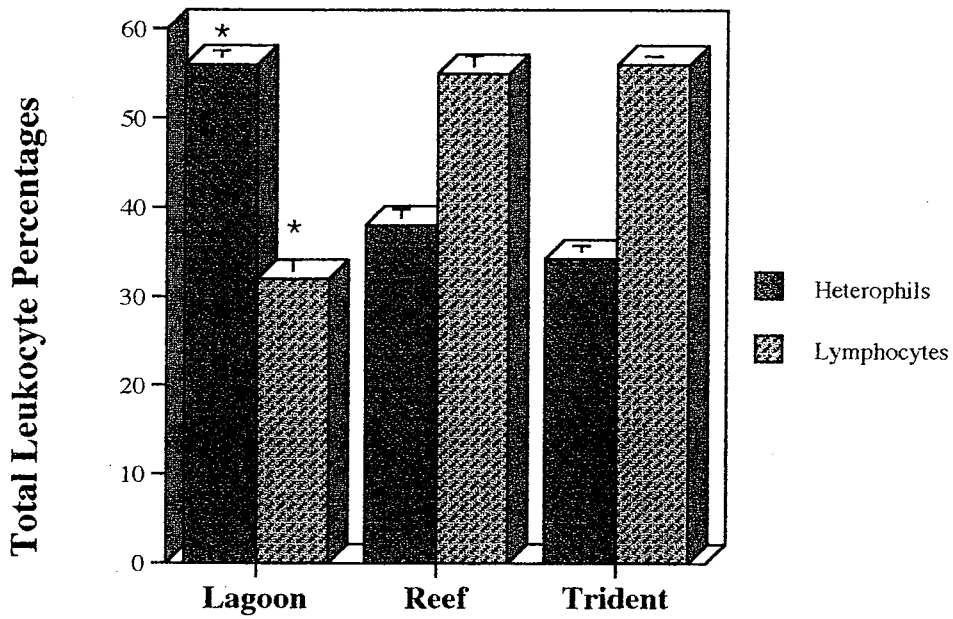

Figure 7. Heterophil and lymphocyte percentages from the three locations. Lagoon turtles show statistically higher heterophil counts and lower lymphocyte counts than reef and Trident Basin turtles. * indicates statistically significant difference (P=<0.001).

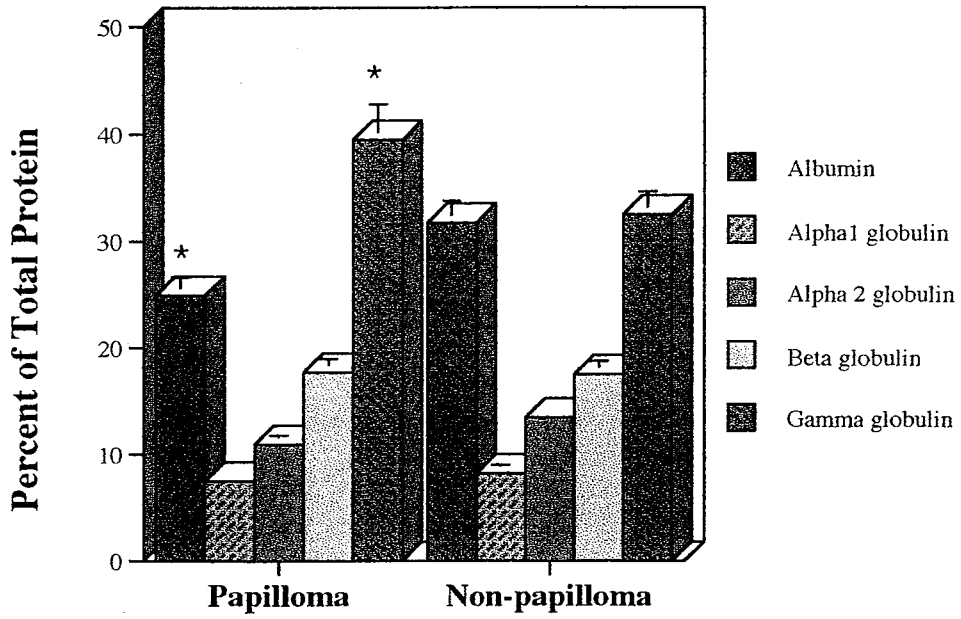

Figure 8. Serum protein levels for papilloma and non-papilloma sea turtles. Papilloma turtles exhibited lower gamma globulin levels and higher albumin percentages than non-papilloma turtles. * indicates statistical significance P=0.002.

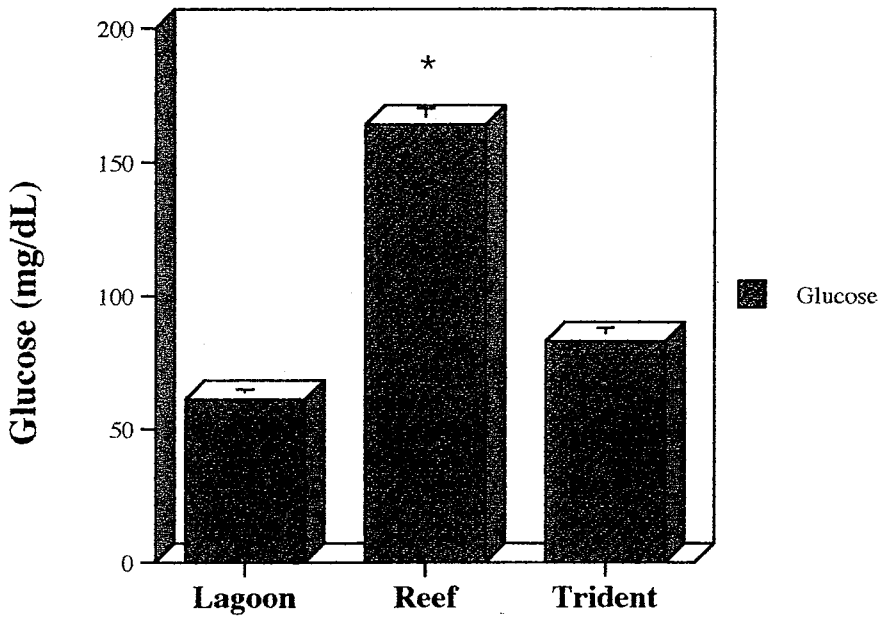

Figure 9. Reef turtles exhibited the highest glucose levels of the three groups. The biological significance of this is yet to be determined. * indicates statistical difference (P=0.001).

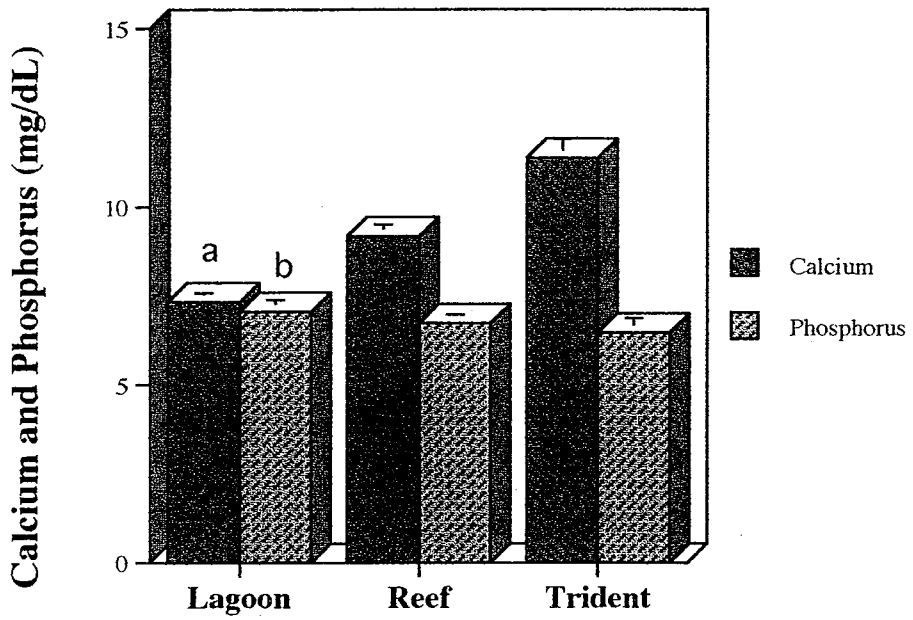

Figure 10. Lagoon turtles exhibited the lowest levels of calcium of the three groups. While phosphorus levels were all within normal range, there was statistical significance between the three locations. a represents statistical significance of calcium (P=<0.001) and b represents statistical significance of phosphorus (P=<0.001).

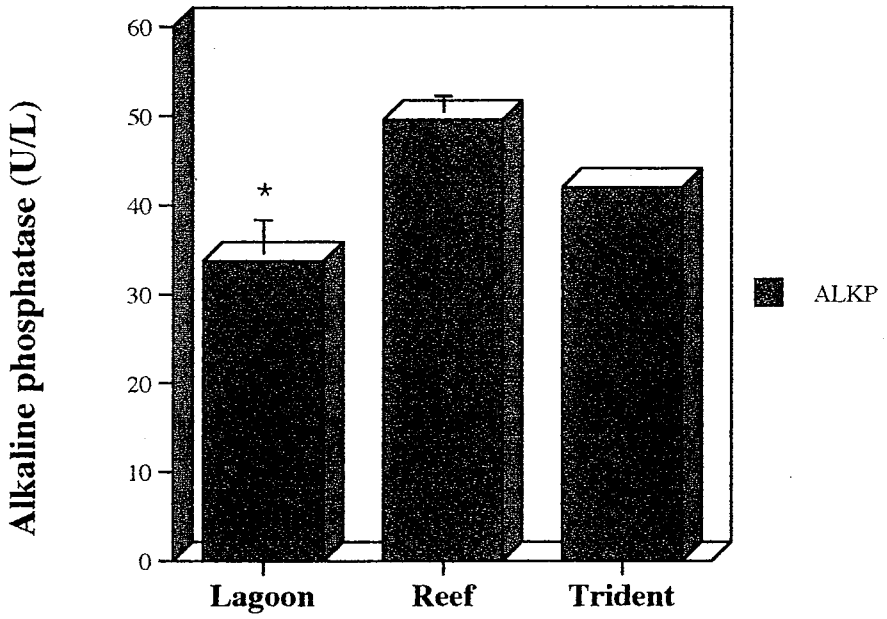

Figure 11. Lagoon turtles exhibit statistically significantly lower levels of ALKP. * indicates statistical difference (P=0.001).

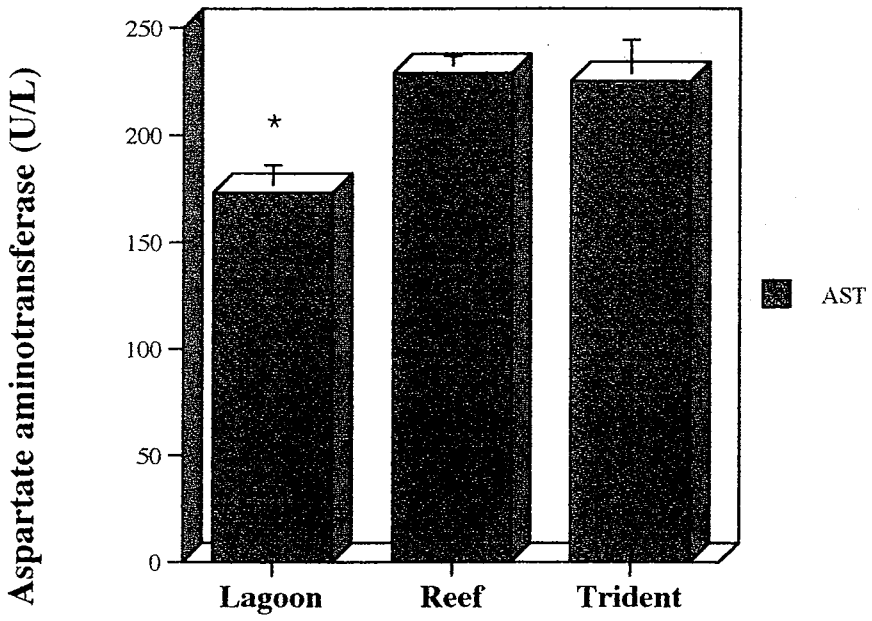

Figure 12. Lagoon turtles exhibited the lowest AST levels of the three groups. * indicates statistical significance at P=0.005.

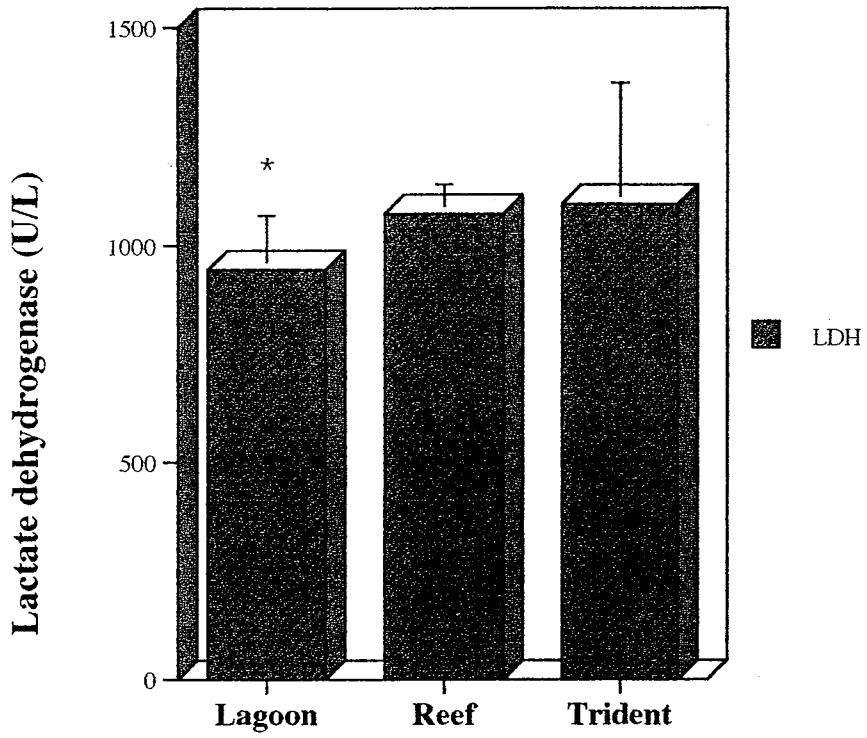

Figure 13. Lagoon turtles exhibited statistically significantly lower levels of LDH compared to reef and Trident Basin turtles. * = statistically significant difference (P=0.006).

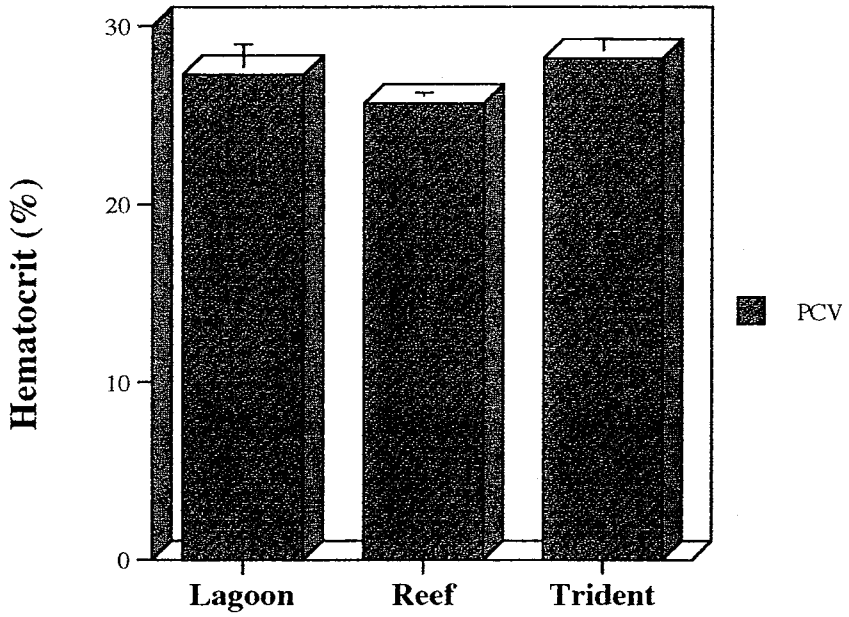

Figure 14. Packed cell volumes of the three locations. There were no significant differences between the three sites.