PLASMA LEVELS OF VITAMINS A AND E IN MARINE TURTLES (*CHELONIA MYDAS* AND *CARETTA CARETTA*)

by

KAREN PATRICIA FRUTCHEY
B.S. University of Central Florida, 1995

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Biology
in the College of Arts and Sciences
at the University of Central Florida
Orlando, Florida

Spring Term
2004

ABSTRACT

Insight into blood values for free-ranging populations of chelonians is very incomplete. A better understanding of marine turtle ecological physiology is necessary for population health assessments and conservation management plans. Vitamins A and E are fat-soluble compounds required by vertebrates. Vitamin A is required for growth, differentiation and integrity of epithelial tissue, bone remodeling, reproduction and vision. Vitamin E is a constituent of cell membranes where it is an antioxidant and free radical scavenger. Both nutrients are required for reproduction and immune system function. I determined concentrations of vitamins A and E in plasma of blood samples from *Caretta caretta* and *Chelonia mydas* using high performance liquid chromatography (HPLC).

The first investigation focused on vitamin concentrations in nesting *Caretta caretta* and *Chelonia mydas* in the Archie Carr National Wildlife Refuge, Melbourne Beach, Florida, USA. Vitamin A concentrations were significantly higher in the herbivore, *Chelonia* than in the carnivore, *Caretta.* Plasma vitamin A concentrations decreased in *Caretta* as the nesting season progressed, while they remained constant in *Chelonia*. Plasma vitamin A concentrations only decline after liver stores of the vitamin are depleted, so it appears *Caretta* did not feed during the nesting season. Plasma vitamin concentrations in *Chelonia* remained stable during the nesting season, so it is not clear whether green turtles fed or fasted as the season progressed. Plasma vitamin E concentrations were significantly higher in *Caretta* than in *Chelonia.* This suggests that

marine turtles follow the previously established chelonian pattern of carnivores having greater circulating concentrations of vitamin E than herbivores.

The second study focused on vitamin concentrations in subadult *Caretta* and juvenile *Chelonia* from three Atlantic Coastal habitats: Indian River Lagoon (IRL), the Nearshore Reef and Trident Submarine Basin. The disease fibropapillomatosis (FP) is prevalent in turtles from the IRL and the Nearshore Reef but not Trident Submarine Basin. Vitamin E concentrations differed significantly among turtles with different degrees of FP affliction. Turtles moderately afflicted with had significantly lower plasma concentrations of vitamin E compared with turtles not afflicted with the disease and as a result, may have compromised immune status. In contrast, however, severely afflicted turtles did not have lower circulating levels of vitamin E compared with those of mildly afflicted turtles. Vitamin A varied significantly among *Chelonia* from the three different developmental habitats. Turtles from the IRL had significantly higher concentrations of circulating vitamin A than turtles from both the Reef and Trident Basin. *Chelonia* on the Reef had significantly higher circulating concentrations of vitamin E than turtles in Trident Basin. *Chelonia* in the IRL had vitamin E concentrations that did not differ significantly from either the Nearshore Reef or Trident Basin turtles. Plasma vitamin E concentrations increased with decreasing straight-line carapace length in *Chelonia*. This is one of the largest scale vitamin studies conducted on reptiles and will contribute to conservation of marine turtles by providing baseline data on vitamins A and E. This information is useful for wildlife rehabilitators and zoos and aquaria with captive marine turtles. Effects of reproduction and disease on plasma concentrations of vitamins A and E are also discussed in this thesis.

ACKNOWLEDGMENTS

I thank my committee members: Dr. L. M. Ehrhart, Dr. P. C. H. Pritchard, Dr. E. S. Dierenfeld, Dr. H. O. Whittier and Dr. W. K. Taylor. I cherish my years doing research as a member of the UCF Marine Turtle Research Group and I appreciate Dr. Ehrhart giving me the chance to do so. I also thank him for funding this vitamin research and my travel to meetings. Dr. Pritchard has been a mentor and friend for several years. I thank him and his Chelonian Research Institute for educating me in not only marine turtles but the whole chelonian group and for providing me with a research assistantship. Without the encouragement and support of Dr. Whittier and Dr. Taylor I would not have pursued my dream of continuing my education. I am extremely grateful to have Dr. Ellen Dierenfeld as a committee member. Her expert advice was absolutely essential to make sure this research was carried out as accurately and efficiently as possible. She also always was available on a daily basis to answer all the questions I had regarding this work.

Joan Deutsch spent considerable time on the lab-work for this project and for that I am extremely grateful. I also thank Jasmine Thomas for all of her assistance with the lab portion of this project. John Behler and the WCS Reptile House Crew provided housing while I was in New York. Dr. Glen Cunningham gave me space in his lab at the UCF Department of Chemistry and Dr. Thomas Selby allowed me to continue using the lab when he took it over. I am also thankful for all the assistance from the staff of the UCF Biology Department.

Many people were involved in the data collection for this project and without their effort, this project would not have been possible. I offer my sincerest gratitude to all the members of the UCF Marine Turtle Research Crew; every person that worked on the project from 2000-2002 was involved in data collection for this thesis research. In 1996, Dean Bagley, Karen Holloway-Adkins and Linh Uong taught me most of what I know now about marine turtles, their biology and how to work with them in the field. Dean Bagley and Karen Holloway-Adkins showed me how to obtain blood samples from turtles. The beach work for this study was particularly challenging and I am especially thankful to Shigetomo Hirama, Ann Marie Maharaj, Karen Holloway-Adkins, Dean Bagley and Rachel Hirschmann and the others that assisted me in the field with this study. Ann Marie Maharaj and Kelly Roberts spent many hours collecting blood from turtles and dealing with the meticulous processing procedure that was necessary for this project.

I am very thankful for all the time Mario Mota worked with me on statistical analyses. Dr. John Fauth assisted with the final statistical analysis. Stacy Kubis spent a great amount of time assisting me with PowerPoint presentations for meetings. I thank the National Marine Fisheries Service and the Florida Fish and Wildlife Conservation Commission for permitting this research.

Most of all, I thank my husband Jack Frutchey, for providing me with his love and support though this entire process. More than anyone else, he bore the brunt of the spectrum of emotions I projected as I went through this process, and he was always patient loving and caring. Thank you so much Jack, I could never have done this without you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE: VITAMINS A AND E IN NESTING MARINE TURTLES

Introduction

Insight into blood values for free-ranging populations of chelonians is very incomplete. Gaining a better understanding of marine turtle ecological physiology is necessary, considering the impact such information has on conservation management plans and population health assessments (Bolten and Bjorndal, 1992, Lutz and Dunbar-Cooper, 1987, Raphael, et al., 1994). The purpose of this study is to determine the concentrations of vitamins A and E (nutrients critical for reproduction in vertebrates) circulating in the plasma of nesting *Caretta caretta* and *Chelonia mydas*, to investigate whether these vitamins are depleted as the nesting season progresses, to see if there are differences in vitamin concentrations between the species, and to investigate if there is a relationship between plasma levels of vitamins and clutch size and hatching success.

Vitamins are diverse organic compounds that have been identified as necessary micronutrients for the health and normal growth of many animal species. During the early 1800s, William Prout, an English physician, described three principles which provide complete nutrition for animals: a saccharine principle (carbohydrates), an oily principle (fats) and an albuminous principle (proteins). Later, the discovery was made that these principles alone were not enough to provide complete nutrition; such a diet lacked other organic compounds necessary in trace amounts. One of the first of these micronutrients discovered was given the name “vitamine” by Funk in 1912 and since

then the term vitamin has been used to describe a group of unrelated organic compounds essential in the animal diet (Maynard and Loosli, 1969).

Vitamin A, or retinol ($C_{20}H_{30}O$), (Frickel, 1984) is a fat soluble growth factor found in foods and is required by vertebrates for survival. This vitamin is acquired directly from the diet or through the conversion of dietary carotenoids into active vitamin A. Only plants and bacteria have the ability to biosynthesize carotenoids (pro-vitamin A) which in turn can be enzymatically converted into retinol within the mucosa of the intestine, and in some cases the liver (Pitt, 1985). Vitamin A active compounds also include retinal and retinoic acid, but in this paper I focus on the most active chemical form, retinol. Retinol is stored in the liver, which is responsible for maintaining the vitamin at a relatively constant concentration in blood plasma. A drop in plasma retinol will only be observed if liver reserves of vitamin A are considerably depleted. Growth, differentiation and integrity of epithelial tissue, bone remodeling, vision and reproduction all rely on a supply of vitamin A (Arnhold, et al., 2000, Underwood, 1984).

Both a deficiency and an excess of retinol can be detrimental to animals. Characteristics of vitamin A deficiency in vertebrates include: lack of appetite, slowed growth, weight loss, drying and keratinization of membranes, infections, loss of sight, nephrosis, nerve degeneration, hydrocephalus, cessation of spermatogenesis, resorption of fetuses and reduced resistance to parasites. Vitamin A deficiency has been determined in both fee-ranging animals as well as through controlled laboratory studies (Holladay, et al., 2001, Underwood, 1984). An excess of vitamin A induces thinning and fracturing of bones, hair loss, hepatic dysfunction, lysis of lysosomes and erythrocytes, and death (Pitt, 1985); vitamin A toxicity is rare in free-ranging animal populations (Robbins, 1983).

Vitamin E, derived from its most biologically active form as α-tocopherol ($C_{29}H_{50}O_2$), is found naturally in grasses, algae, vegetables, fruits, seeds, nuts and oils. Three other tocopherol isomers and 4 tocotrienols also show vitamin E activity (Diplock, 1985). Animal tissues are generally poor sources of this nutrient and are influenced by the dietary intake of vitamin E by the prey species. α-Tocopherol is primarily located in intracellular membranous organelles where it functions as a free radical scavenger, protecting unsaturated fatty acids embedded in these membranes from lipid peroxidation. Animals consuming diets containing high amounts of polyunsaturated fatty acids (PUFAs) have a higher requirement of vitamin E in order to prevent chain reaction formation of free radicals as a result of PUFA/ free radical interactions (Kasparek, 1980). Vitamin E protects vitamin A from oxidative degradation (Jennings, 1970) both at the cellular and intestinal level (Underwood, 1984).

Deficiency of vitamin E may cause muscle wasting (Diplock 1985), hemolysis of erythrocytes, damage to microsomal and mitochondrial membranes, blood vessel lesions and cardiomyopathy (Nelson, 1980). α-Tocopherol is also essential for normal reproduction and deficiency may cause the death and resorption of fetuses in female rats and has been proven to result in degenerative changes in the testes, resulting in permanent sterility in male rats (Maynard and Loosli, 1969). An excess of vitamin E may cause instability of cellular and sub-cellular membranes, resulting in the leakage of lysosomal enzymes (Jennings, 1970).

Vitamin A has many crucial roles in the reproduction and development of vertebrates. Dietary retinol is required in mammals for spermatogenesis (Chytil, 1994, Galdieri, 1994, Thompson, 1969, Underwood, 1984) within the testis and for the

maturation of spermatozoa in the epididymis (Packer and Wolgemuth, 1999). Vitamin A maintains the testes in roosters, rats, guinea pigs and hamsters (Thompson, 1969). Retinol regulates Sertoli cell metabolism and affects the secretion of androgen binding protein and transferring by these cells (Galdieri, 1994). A deficiency of vitamin A in animals may result in vesicular glands being smaller than normal and may cause a reduction of androgen production in the interstitial cells of the testes (Jennings, 1970, Thompson, 1969). Rats deficient in retinol lose the germinal epithelium of the testes, prohibiting the formation of normal, mature sperm (Jennings, 1970, Thompson, 1969). Lesions of the male reproductive system as a result of vitamin A deficiency are reversible with the addition of the vitamin to the diet (Packer and Wolgemuth, 1999, Pitt, 1985, Thompson, 1969).

Most of the research regarding the vitamin A requirement for reproduction in female animals has been done on rats and farm animals. Vitamin A maintains pregnancy for a full gestation (Chytil, 1994, Underwood, 1984) in the female rat and a deficiency can result in the permanent keratinization of vaginal epithelium, reduced rate of cell division in the fetus and placenta (Maynard and Loosli, 1969, Pitt, 1985) and subsequent resorption of fetuses (Pitt, 1985, Thompson, 1969). Deficiency status in sows results in disruption of the estrus cycle and premature birth of weak or dead pigs. In deficient sheep, lambs die within the uterus or shortly after birth. Cattle fed for an extended period on dry pasture (very low carotenoid content) give birth to dead or weak calves and frequently retain the placenta resulting in an infectious abortion (Maynard and Loosli, 1969). A similar study showed that dried range feeding could cause corpora lutea not to regress and ovarian follicles to become atretic in young heifers (Jennings, 1970).

Vitamin A has been proven necessary for development of vertebrate embryos. Retinol is transferred by birds to their eggs and subsequently stimulates the development of the embryo (Thompson, 1969). Rat and quail embryos deprived of vitamin A exhibit severe abnormalities in the development of cranial neural crest cells, diaphragm, lung cardiovascular tissue, genitourinary tract and the eye (Packer and Wolgemuth, 1999). Normal organogenesis in a developing fetus is reliant on placental supply of retinol (Chytil, 1994, Underwood, 1984). In avian embryos (domestic hen and quail), vitamin A has a role in the development of the veins that connect the heart with the extraembryonal vascular system and tissue (where the blood forms) (Perrotta, et al., 2003, Thompson, 1969, Zile, 1999).

Retinol is required for hatchability of the fertile eggs of fowl (Underwood, 1984). Egg production and hatchability in bobwhite quail increased with addition of vitamin A to a diet deficient in the nutrient (Robbins, 1983). Hens deficient in retinol show a significant decrease in egg production and this is more likely to occur before hatchability is affected (Thompson, 1969). Japanese quail require vitamin A to maintain development beyond 48 hours and subsequently hatch (Pitt, 1985).

Vitamin E is also important for vertebrate reproduction and development. Male rats deficient in vitamin E suffer permanent testicular lesions (Maynard and Loosli, 1969, Scott, 1970) as a result of the degeneration of testicular tubular cells (Jennings, 1970), agglutination of mature sperm cell bodies, nuclear break-up in spermatids (and secondary spermatocytes) and the atrophy of the germinal epithelium (Pitt, 1985). Deficiency also causes sterility in male guinea pigs, hamsters, rabbits, chickens, dogs, monkeys (Mason

and Horwitt, 1972, Pitt, 1985, Scott, 1970) and pigs (Nelson, 1980); however, only after prolonged deficiency are lesions observed in mice (Mason and Horwitt, 1972, Pitt, 1985).

In female rats, vitamin E prevents death and resorption of the fetus when added to a diet deficient in the nutrient (Scott, 1970). In deficient female rats, reproduction carries on as normal through implantation (Mason and Horwitt, 1972) but the animals are unable to rear their young (Jennings, 1970). Such a progression may occur because in these animals, production of thyrotropic and lactogenic hormones ceases before gonadotropic hormone production stops. In more severe deficiency states, luteinising activity suppression results in the resorption of fetuses (Jennings, 1970). Fetal resorption also occurs in vitamin E deficient mice and guinea pigs (Pitt, 1985) and swine (Nelson, 1980). Other problems related to vitamin E deficiency in the female rat include interference with the implantation of the ovum and a uterine eclamptic syndrome which preclude rats from carrying a pregnancy to term (Mason and Horwitt, 1972). In mice, an absence of vitamin E causes changes in the ovary including the presence of less interfollicular tissue, absence of neutral fat, smaller and more numerous corpora lutea, and fewer primordial ova (Mason and Horwitt, 1972).

It has been concluded that vitamin E directly acts to maintain embryonic growth (Pitt, 1985). Vitamin E is necessary for the development of vertebrate embryos and deficiency causes degeneration of embryonic vasculature in rats, chickens, turkeys, cows and sheep (Scott, 1970). Several other developmental problems are observed in embryonic vitamin E deficient rats: retarded development of the ectodermal cavity, no formation of ectoplacental and amniotic cavities, diminished hemopoietic activity in the liver, and abnormal yolk sac and allantois formation (Mason and Horwitt, 1972, Pitt,

1985). Although there are many manifestations of vitamin E deficiency in the embryo, the one most likely to cause death is defects in fetal blood vessels (Nelson, 1980, Pitt, 1985). A deficiency of vitamin E also results in low hatchability in poultry (Maynard and Loosli, 1969).

Very few studies have investigated vitamins and their relationship to reproduction in reptiles. Two studies have previously been conducted on captive and wild alligators (*Alligator mississippiensis*) to determine plasma vitamin E concentrations and to investigate if there is a relationship between reproductive success and vitamin concentrations (Lance, et al., 1983, Lance, et al., 2001); one of these studies also investigated plasma levels of vitamin A (Lance, et al., 2001). Clearly, more information is needed on vitamins and their influence on reptile reproductive success and this study of nesting *Caretta caretta* and *Chelonia mydas* will provide important baseline information about plasma concentrations of vitamins A and E in marine turtles in a reproductive state.

Methods

Survey area and methods

This study was conducted within the boundaries of the Brevard County section of the Archie Carr National Wildlife refuge on the Atlantic coastal beach of Central Florida, USA (Figure 1). This study area is approximately 2 km south of the southern boundary of the Town of Melbourne Beach and is bounded by GPS coordinates: (80º30.34'W, 28º02.33'N) to the north and (80º27.25'W, 27º52.43'N) to the south. Night-time surveys were conducted 5 to 7 nights a week from 28 April through 3 September, 2000. An all-

terrain vehicle (ATV) outfitted with a dim, red-filtered headlight was used to traverse the 21 km study area. Turtles were sampled for this study in the order they were encountered. The turtles were allowed to oviposit before they were tagged, measured and checked for damage or signs of disease (including fibropapillomas). Measurements taken included: curved carapace length (OCL), measured nuchal notch to carapace tip, and curved carapace width (OCW), measured across the widest part of the carapace, perpendicular to the longitudinal axis. In most instances straight-line measurements were not obtained because of the difficulty of carrying a forester's tree caliper on the ATV. When these measurements were obtained, they included: straight-line carapace length (SCL), straight-line carapace width (SCW) and head width (HW). Eggs were counted as they were dropped by the turtle in order to get an accurate clutch size with minimal disturbance to the eggs. On a few occasions when an egg count was not obtained as the turtle was laying, eggs were excavated and counted within 10 hours of deposition. Nest locations were marked by first locating the clutch and then placing 2 numbered stakes in the dune (one hidden in dune vegetation and the other near the dune edge in plain view) both in line with the clutch and perpendicular to the dune line. Measurements were taken from both stakes to the exact clutch location in order to facilitate finding the nest in the future. After the hatchlings emerged (approximately 60 days post deposition), the clutch was located and the eggs were excavated. Data were recorded as to how many eggs hatched and the stage of development unhatched eggs attained.

Blood collection and processing

After oviposition, blood samples from *Caretta* and *Chelonia* were collected from the bi-laterally located dorsal cervical sinus using the method previously described by Owens and Ruiz (1980). Before blood collection was attempted on a turtle, its skin was thoroughly cleaned with an alcohol swab. Vacutainers® (7ml) containing lithium heparin and sterile 1 1/2" 20 gauge needles were utilized for blood collection.

Extreme care was taken when handling blood samples potentially containing retinoids and tocopherols. Vitamins A and E can be destroyed or isomerized by bright daylight, bright electrical lighting and excessive heat (Abe and Matsumoto, 1993, Barua and Furr, 1998). Since samples were obtained from turtles at night and minimal light from a small flashlight was used while taking each sample, excessive light and heat were not of concern. The samples were centrifuged immediately at 3000 RPM and 1100 x g using a portable centrifuge or were placed on ice and centrifuged within twenty minutes of collection time. Blood was centrifuged for ten minutes to achieve complete separation of erythrocytes from blood plasma. Hemolyzed samples were not used in this study because hemolysis of erythrocytes can cause metal ion contamination of plasma, resulting in reduction of vitamin compounds through oxidation (Barua and Furr, 1998; Abe and Matsumoto, 1993). The plasma layer was carefully removed from the top with a small, disposable, polyethylene pipette and transferred to a sterile 1.5 ml cryovial. Vial size was selected to fit the plasma sample in order to minimize the amount of air in contact with the plasma. Exposure to air or oxygen can result in oxidation of these vitamins into inactive compounds. Cryovials were then placed on ice in a cooler until transfer to a freezer kept at -10 C and stored a maximum of seven months before analysis. During the

summer nesting season of 2000, an attempt was made to get 10 samples from each species each week for 13 weeks during the oviposition period between 28 April and 28 August for *Caretta* and for 16 weeks between 6 June and 4 September for *Chelonia*. The total number of blood samples obtained was 160 for *Caretta* and 79 for *Chelonia*.

Laboratory methods

For the analysis of turtle plasma samples for retinol and α-tocopherol, I used a modification of the method used by Storer (1974). Laboratory procedures took place under diffuse yellow lighting to avoid isomerization of the vitamins by UV light. Each sample was removed from the freezer, thawed and then vortexed for 30 seconds. With a pipette, 200 µl of plasma was transferred from the cryovial into a 10 x 75 mm disposable borosilicate culture tube (referred to as test tube below). To precipitate proteins in the plasma sample, 200 µl of HPLC grade ethanol with 0.1% BHT was then added. Next, 200 µl of HPLC grade hexane with 0.025% BHT was added to the test tube. The test tube was then quickly capped with a polyethylene cap and vortexed for 30 seconds. Vortexing was followed by centrifuging the tube for 15 seconds. Next, the cap of the test tube was removed and 200 µl of the hexane-based supernatant was removed and transferred into a 0.5 ml microcentrifuge tube which was placed under a light stream of high-purity grade N_2 gas. The hexane was left to evaporate to dryness. The sample was reconstituted with 200 µl HPLC grade methanol with 0.025% BHT and the cap on the microcentrifuge tube closed tightly. The sample was then vortexed for 10 seconds, poured into a HPLC crystal, covered with tin-foil and placed in an auto sampler. A Series 400 Perkin Elmer (PE) HPLC system with a 15-cm C18 reverse phase column was

used to analyze samples. HPLC grade methanol and water (98:2 vol/vol) was used as the mobile phase with a flow rate of 2.0ml/min. α-Tocopherol was monitored using a PE LS-1 fluorescence detector (excitation wavelength 280 nm, emission >310 nm); retinol was detected at 325 nm on a Perkin-Elmer Model LC-95 UV/visible spectrophotometer. HPLC chromatograms were produced and interpreted using PE Turbochrom 4 software on an IBM compatible computer.

Statistical analysis

Statisticians debate how to report results of analyses that include samples reported as below detection limits (BDL), not-detectable (ND) or non-quantifiable (NQ). Here, I used a method (Hecht and Honikel, 1995) that suggests if ND samples are < 60% of the total sample size, using limit of detection (LOD)/2 is a reasonable prediction of their mean. Limits of detection for retinol and α-tocopherol in this study were 0.02 μg/ml; therefore, 0.01 μg/ml was used for samples reported as ND. Very few samples fell into this category (6 of 239 observations).

I estimated correlations of vitamins A and E separately for each species. I used multiple linear regression to test the null hypotheses that plasma concentrations of vitamins A and E did not vary with species, date, OCL, and species x date, date x OCL and species x OCL interactions. Vitamin E values were log-transformed ($\log_{10}(x + 1)$) to meet normality assumptions. I used the multiple linear regression to test the null hypotheses that hatching success and clutch size did not vary with species, date, OCL, plasma retinol concentration, plasma α-tocopherol concentration, or species x date and date x OCL interactions. Vitamin E values were not log-transformed for these two

hypothesis tests; I removed any effects that did not explain substantial deviance ($F \leq 1.00$) in vitamin concentrations from the models. I defined hatching success as the proportion of eggs with hatchlings that successfully extracted themselves from their egg shells. I used JMP version 5.1 (SAS Institute, Inc., 2003), with $\alpha = 0.05$ for all statistical analyses and hypothesis tests.

Results

Correlation of vitamins A and E

Plasma concentrations of vitamins A and E were positively correlated in *Caretta* ($r = 0.3635$, $n = 160$, $P = 0.0001$) and *Chelonia* ($r = 0.6381$, $n = 79$, $P = 0.0001$; Figure 2). Vitamin A (measured as µg/ml retinol) concentrations averaged (mean ± 1 SE) 0.43 ± 0.02 µg/ml and ranged between 0.01 - 1.20 µg/ml in *Caretta*, and in *Chelonia* averaged 0.52 ± 0.04 µg/ml and ranged between 0.04 - 1.74 µg/ml. Vitamin E (measured as µg/ml α-tocopherol) concentrations averaged 12.98 ± 0.65 µg/ml and ranged between 0.01 - 48.64 µg/ml in *Caretta*, and in *Chelonia* averaged 6.08 ± 0.40 µg/ml and ranged between 0.90 - 25.31µg/ml.

Vitamins A and E in nesting Chelonia *and* Caretta

Vitamin A concentrations in *Chelonia* were (LS mean ± 1 SE) 0.52 ± 0.04 µg/ml, while in *Caretta* they were 0.43 ± 0.02 µg/ml. *Chelonia*, which is herbivorous, had significantly higher circulating concentrations of vitamin A than the carnivorous *Caretta* (Table 1). Seasonal changes in retinol concentrations varied significantly between the

species (significant species x date interaction, Table 1). In *Caretta*, retinol concentrations decreased as the nesting season progressed while in *Chelonia* they remained stable (Figure 3). The regression equation for the decline of vitamin A in *Caretta* was: Plasma vitamin A (μg/ml) = 0.793 - 0.002 (Julian date).

Mean transformed ($\log_{10}$(vitamin E + 1)) concentrations of plasma vitamin E were 1.09 ± 0.03 μg/ml in *Caretta* and they were 0.75 ± 0.05 μg/ml in *Chelonia*. *Caretta* had significantly higher concentrations of vitamin E than *Chelonia* (Table 2). However, when species were analyzed separately, this relationship was significant only in *Caretta* (Figure 4).

Relationship between vitamins, clutch size and hatching success

There was a significant species by date interaction on hatching success (Table 3), which remained stable as the season progressed in *Caretta* (Hatching success = 60.59 + 0.06 (Julian date); $F_{1,121} = 0.05$, $P = 0.48$) but declined in *Chelonia* (Hatching success = 179.68 – 0.54 (Julian date); $F_{1,28} = 13.38$, $P = 0.0010$). Vitamin concentrations did not affect hatching success of either species (Table 3).

Clutch size varied significantly with both date and OCL (Table 4). Clutch size decreased as the nesting season progressed and larger turtles deposited larger clutches: There was no significant relationship between plasma vitamin concentration and clutch size in *Caretta* or *Chelonia* (Table 4).

Discussion

Correlation of vitamins A and E

The significant correlation between vitamins A and E in the plasma of nesting marine turtles observed in this study has been previously observed in another chelonian species. A positive correlation has been reported for the Aldabra Giant Tortoise (*Geochelone gigantea*) ($r = 0.72$, $P < 0.005$, $n = 12$) (Ghebremeskel, et al., 1991). Although vitamins A and E do interact *in vivo*, mentions of their correlation in plasma are infrequent in the literature. Vitamin E protects vitamin A from oxidation at intraluminal and cellular levels (Underwood, 1984). Many other studies address the relationship of the two vitamins, but these usually include pharmacological dosing of the nutrients in laboratory animals. The relationship between retinol and α-tocopherol may not be of major nutritional significance within the range of normal dietary intakes (Draper, 1980) and therefore may be of only anecdotal interest in this study of vitamins in nesting marine turtles.

Vitamin A in nesting Chelonia *and* Caretta

Plasma vitamin A concentrations were higher in *Chelonia* than in *Caretta*. This may be because of differences in dietary habits between the two species and consequently the way turtles obtain vitamin A. *Chelonia* is a herbivorous genus, feeding primarily on seagrasses throughout most of its range but consuming algae in places where seagrasses are not prevalent (Mortimer, 1995). Herbivores obtain retinol by converting dietary vitamin A pre-cursors, (such as carotenoids) in the mucosa layer of the intestine or in

some cases the liver (Pitt, 1985) to active vitamin A through enzyme activity. In humans, the enzyme which facilitates this conversion by cleaving β-carotene is carotene 15,15'-dioxygenase (Vogel, et al., 1999). The presence of this enzyme has not been confirmed in *Chelonia*, but the ability of *Chelonia* hatchlings to convert β-carotene to vitamin A has been proven (Patterson, 1974).

Caretta, primarily a carnivore, consumes a variety of marine invertebrates and occasionally some plant material (Bjorndal, 1997). Carnivores obtain preformed vitamin A from their animal-tissue diets (Pitt, 1985). Further investigation is required to gain a better understanding of vitamin A metabolism in *Caretta*.

Many species (cows, ferrets, chickens) exhibit markedly different characteristics in vitamin A pre-cursor (carotenoid) absorption and metabolism (Vogel, et al., 1999); however, few references regarding metabolism of retinol and its pre-cursors in reptiles exist. Species-specific selective absorption of carotenoids have been observed in common green iguanas (*Iguana iguana*) (Raila, et al., 2002) and are suspected in panther chameleons (*Furcifer pardalis*) (Dierenfeld, et al., 2002). Some mammalian species such as ferrets and cows have higher fasting concentrations of retinyl esters compared with other species such as humans (Vogel, et al., 1999). If indeed both *Caretta* and *Chelonia* in this study are not feeding during the nesting season, they have significantly different fasting concentrations of retinol.

Plasma concentrations of vitamin A decreased significantly as the nesting season progressed in *Caretta* while remaining stable in *Chelonia*. In *Caretta*, plasma concentrations of retinol decreased by 0.002 μg/ml each day as the nesting season progressed. A decline in plasma retinol is not observed until liver stores of the vitamin

are depleted (Underwood, 1984) and since a decline was observed, it appears that *Caretta* nesting in the Archie Carr National Wildlife Refuge may not be feeding during internesting periods and that liver stores are marginal in status. Other studies have concluded that *Caretta* does not feed during the nesting season. In a study using time temperature recorders placed in stomachs of nine loggerheads, it was determined that nesting turtles drank water after nesting but did not feed during the internesting period (Tanaka, et al., 1995). Hundreds of Australian *Caretta* gastrointestinal tracts have been observed by laparoscopy and determined to be largely empty throughout the nesting season (Limpus and Limpus, 2003); with these observations it was concluded that *Caretta* do not forage during the internesting period and live on fat reserves accumulated before migration. Seasonal decline in *Caretta* retinol concentrations is of interest because this may indeed point to turtles' need to take one or more seasons off to replenish lost stores of fat soluble nutrients (possibly including fat soluble vitamins A and E) before remigrating to nesting grounds. For *Caretta* from ACNWR, vitamin A may be a limiting nutrient for reproductive output.

A decline in plasma vitamin A was not observed in *Chelonia*; however, these nesting turtles may be experiencing retinol depletion below the threshold of detection in blood plasma. The best method of determining vitamin A status of an animal is to determine vitamin A content of the liver (Pitt, 1985). Obtaining liver biopsies on nesting *Chelonia* was deemed excessively invasive and was not done.

It has been concluded that *Chelonia* does not feed near the nesting beaches and nutrients necessary for an entire nesting season are obtained and stored while on feeding grounds (Bjorndal, 1985). This observation could be true for *Chelonia* from ACNWR

but without a significant drop in plasma vitamin A observed, it is difficult (in terms of vitamin A condition) to confirm fasting. However, this condition may indicate better overall nutritional status and other markers are needed to confirm turnover rate of this nutrient.

It may be possible that *Chelonia* feed during the internesting period in some places. Some reproductive turtles from Raine Island, Australia had small amounts of food in their digestive tracts (including some animal material). Other studies have recorded evidence of feeding in adult female *Chelonia* in the vicinity of a nesting beach; however, confirmation of reproductive status of turtles was not done as it was in the Australian study (Tucker and Read, 2001). Perhaps they will eat if food is available; often it is not.

Vitamin E in nesting Chelonia *and* Caretta

Plasma vitamin E concentrations were higher in *Caretta* than in *Chelonia* and in this manner both species follow the previously established chelonian pattern of omnivores having higher concentrations of α-tocopherol than herbivores. Herbivorous forms such as *Gopherus polyphemus*, *Malacochersus tornieri* and *Testudo graeca nikolskii* have lower plasma vitamin E concentrations (mean ± 1 SD (μg/ml)) 1.56 ± 1.04, 2.34 ± 1.17 and 4.66 ± 2.62, respectively, than reported in omnivores, such as *Sternotherus odoratus* and *Clemmys muhlenbergii*, 6.64 ± 2.10 and 17.23 ± 0.03, respectively (Dierenfeld, et al., 1999). Higher concentrations of plasma vitamin E also occur in carnivorous as compared with herbivorous birds. Eighteen species of raptors (*n*

= 247) averaged 69.14 ± 6.32 μmol/l while fifteen species of cranes fed a grain based diet (*n* = 274) averaged 15.24 ± 3.15 μmol/l (Dierenfeld, 1994).

Larger *Caretta* had higher concentrations of circulating vitamin E; this may be because nesting turtles from ACNWR with a greater carapace length lay larger clutches (Osegovic, 2001). The need for mobilization of fat soluble resources to be contributed to these larger clutches may require increased circulating lipids and α-tocopherol in larger *Caretta*.

Vitamin E concentrations did not decline in either *Caretta* or *Chelonia* as the nesting season progressed. Unlike plasma concentrations of vitamin A, circulating concentrations of vitamin E seem to be a better indicator of α-tocopherol status of the animal (Dierenfeld, et al., 1999). Although there was no change in plasma vitamin E, it is still difficult to say with confidence that nesting turtles were feeding to maintain vitamin E concentrations. Stable vitamin levels may be a result of a slower turnover of stored nutrients to yolks. In animals studied thus far, the body has a great capacity to store vitamin E. Storage is primarily in the liver but may also occur in other organs and tissues. Female rats which were born to mothers that consumed diets with a liberal supply of α-tocopherol, have enough to carry them through their first pregnancy (Maynard and Loosli, 1969). Rats raised on a diet rich in vitamin E and subsequently placed on a deficient diet may produce three to four litters before stores of the nutrient are depleted (Maynard and Loosli, 1969). With other vertebrates' proven ability to store vitamin E and a lack of a significant drop of plasma concentrations observed in turtles from this study, it is still unclear if vitamin E is a limiting nutrient for reproductive output for marine turtles nesting at ACNWR.

Relationship of vitamins with clutch size and hatching success

The observation in this study that vitamins A and E did not have an effect on clutch size in *Caretta* and *Chelonia* is similar to observations in other studies that looked at clutch size in relation to other aspects of marine turtle nutrition. Captive *Chelonia* from the Cayman Turtle Farm fed a high quality pelleted diet showed increased clutch frequencies and shorter interbreeding intervals than in wild populations but mean clutch size was not affected by the higher quality diet (Bjorndal, 1985). Bjorndal (2003) has concluded for *Caretta* that nutrients are most influential on clutch frequency and interbreeding interval, and have a smaller effect on clutch size.

Plasma concentrations of the two vitamins were not related to hatching success in nests deposited by *Caretta* and *Chelonia*. Hatchability of alligator eggs was lower in captive alligators fed fish with lower circulating concentrations of vitamin E compared with wild alligators although, low hatching success was attributed to other problems related to captivity in addition to vitamin E status (Lance, et al., 1983). Hatchability of captive Peregrine Falcon (*Falco peregrinus*) eggs increased after quail they were fed were supplemented with vitamin E (Dierenfeld, et al., 1989). It is important to note that in this study on nesting marine turtles from Archie Carr National Wildlife Refuge, all nests were left *in situ* and were exposed to a wide array of biotic and abiotic factors. Future research on vitamin concentrations and hatchability of marine turtle eggs should include samples of eggs incubated in a controlled environment.

In summary, this study established base-line values for plasma concentrations of vitamins A and E (nutrients necessary for reproduction in vertebrates) in nesting marine turtles. Based on vitamin A data presented here, it appears that *Caretta* probably fasts

during the nesting season and undergoes depletion of fat-soluble nutrients. However, vitamin E data in *Caretta* and the concentrations of both vitamins observed in *Chelonia* are inconclusive in relation to fasting status, although, related to clutch size and hatching success in other species, concentrations of vitamins A and E were not correlated with either in this study. Plasma concentrations of vitamins A and E in *Caretta* and *Chelonia* are similar to those reported for other chelonians.

CHAPTER TWO: VITAMINS A AND E IN JUVENILE MARINE TURTLES

Introduction

Insight into blood values for free-ranging populations of chelonians is very incomplete. Gaining a better understanding of marine turtle ecological physiology is necessary, considering the impact such information has on conservation management plans and population health assessments (Aguirre and Balazs, 2000, Bolten and Bjorndal, 1992, Lutz and Dunbar-Cooper, 1987, Raphael, et al., 1994). The purpose of this study is to determine the concentrations of vitamins A and E (nutrients critical for immune function in vertebrates) circulating in the plasma of juvenile *Chelonia mydas* and subadult *Caretta caretta*. Also investigated are the differences in concentrations of vitamins between species, different populations and healthy individuals and those afflicted with fibropapillomatosis (FP).

Fibropapillomatosis is a debilitating and potentially fatal non-cancerous tumor-causing disease which afflicts marine turtles from several locations worldwide including Florida, Hawaii, Indonesia, Australia, Mexico, Brazil and Costa Rica. Tumors are most frequently found on the soft skin of turtles, especially in the dorsal cervical, inguinal and axillary regions of the body (Jacobson, et al., 1989). Tumors often form on eyelids and directly on the conjunctiva, occluding the vision. Internal fibromatous tumors also occur with this disease in the kidneys and gastrointestinal tract (Cray, et al., 2001). Although no specific etiological agent has been isolated as the cause for the disease, some that are suspect include genetic predisposition, oncogenic viruses, immune suppression, chronic

stress, UV light, exposure to toxic chemicals and nutritional deficiencies (Aguirre, et al., 1995, Hirama, 2001). Many studies mention herpesviruses as the most likely etiologic agent (Herbst, et al., 1998, Lackovich, et al., 1999, Quackenbush, et al., 2001) With the etiology of FP being unknown, it is even more critical that basic knowledge be obtained about aspects of marine turtle physiological aspects such as hematological values since it is necessary to be able to distinguish between normal and handicapped or non-functional physiology (Lutz, 1998).

Vitamins are diverse organic compounds that have been identified as necessary micronutrients for the health and normal growth of many animal species. Vitamin A, or retinol ($C_{20}H_{30}O$), (Frickel, 1984) is a fat soluble compound found in foods that is required by vertebrates for growth, differentiation and integrity of epithelial tissue, bone remodeling, reproduction, vision and immune function (Underwood, 1984). Vitamin E, derived from its most biologically active form as α-tocopherol ($C_{29}H_{50}O_2$), is primarily located in intracellular membranous organelles where it functions as a free radical scavenger, protecting unsaturated fatty acids embedded in these membranes from lipid peroxidation (Kasparek 1980). Both vitamins A and E are necessary for proper immune function of many vertebrates.

Retinol is required in various metabolic pathways involved in immune function of animals. Animals and humans suffer from increased frequency and severity of parasitic, viral and bacterial infections when suffering from vitamin A deficiency (Dennert, 1984). Without a supply of vitamin A, mouse thymocytes fail to multiply and subsequently die (Ross, 2000). In rats, vitamin A is necessary for intracellular killing and phagocytic functions of neutrophils and macrophages (Perrotta, et al., 2003). Vitamin A deficient

rats have severely lowered immunoglobulin (Ig) responses to T-dependent antigens (Perrotta, et al., 2003). Deficiency also results in the keratinization of mucous membranes, resulting in an increased susceptibility to infections in rats (Pitt, 1985). Retinol may have a role in the regulation of lymphocyte turnover (Perrotta, et al., 2003).

Vitamin E is required for immune response and disease resistance in vertebrates. Most research to determine effects of vitamin E on immune response has been with animals either in a deficiency state or given pharmacological doses (vitamin levels significantly higher than in the normal diet) of the nutrient (Tengerdy, 1980). Vitamin E's main function as a membrane-protecting antioxidant is emphasized in immune function since the vitamin is found in higher concentrations in immune cells, probably because these cells are more exposed to oxidative damage (Beharka, et al., 1997). Although this vitamin has a role in both cellular and humoral immunity, it is most important for the phagocytic ability of cells during affliction with infectious disease (Tengerdy, 1989). Vitamin E increases the proliferation, chemotaxis and bactericidal abilities of polymorphonuclear leukocytes (PMN) (Tengerdy, 1989).

Animals deficient in vitamin E suffer in various ways physiologically including their immune function. Deficiency results in reduced ability of the immune system to produce an antibody response to antigens, respond to infectious organisms and to have a delayed-type hypersensitivity (DTH) reaction (Beharka, et al., 1997).

In pharmacological doses, vitamin E increases humoral antibody production (in particular IgG) against antigens in mice, rabbits, guinea pigs, turkeys and chickens (Tengerdy, 1980). A high vitamin E diet increases the cellular immune response: antibody-dependent cell-mediated cytotoxicity (ADCC) of mice (Watson and Petro,

1982). Both cell-mediated immune function and resistance to infectious diseases increased in elderly humans, farm livestock and laboratory animals given diets containing more than five times the recommended amount of vitamin E (Beharka, et al., 1997).

Methods

Study areas

Turtles used for this study were captured at three different developmental habitat locations (Figure 5). The Indian River Lagoon (IRL) study area is located in northern Indian River County, about 3 km south of Sebastian Inlet in an area referred to as South Bay (27º 25' 45" N, 80º 26' 30" W). Juvenile *Chelonia mydas* captured at this site average 41.6 cm straight-line carapace length (SCL) (Ehrhart, et al., 2001). *Caretta caretta* captured at this IRL study site average 64.8 cm SCL and are categorized as sub-adults by Ehrhart (1985). Since 1982, prevalence of fibropapillomatosis in *Chelonia* from this study area has ranged between 50-70% of annual captures (Hirama, 2001) and in *Caretta* has ranged from 0-12% of annual captures (Ehrhart, et al., 2001).

The Nearshore Reef study area is located nearshore to northern Indian River County, FL on the opposite side of the barrier island from the IRL site in the area of Ambersand Beach (27º 49' 11" N, 80º 25' 31" W). Reefs at this location consist of beach rock and colonies of dwelling tubes created by sabellariid polychete worms. This reef system is commonly covered with an algal mat. Juvenile *Chelonia mydas* captured at this study site average 42.6 cm in carapace length (Ehrhart, et al., 2001). From 1989 through

1996, no FP was recorded; however, since 1997, prevalence of the disease in *Chelonia* has ranged between 8-21% (Hirama, 2001).

Trident Basin is a man-made basin of approximately .8 square kilometers, lined with granite rock rip-rap, covered with an algal mat. This basin is situated within the Port Canaveral Shipping Channel in Brevard County, FL (28º 25'0" N, 80º 17' 30" W) and is oceanic in nature. Juvenile *Chelonia mydas* captured at this location are considerably smaller than those at the other 2 study sites, averaging 30.9 cm SCL (Ehrhart and Bagley, 2002). Different theories have been proposed as to why only smaller turtles are found in this basin: the sparse algal mat is unable to satisfy the nutritional requirements of turtles of larger size classes, the ecological geography of the species may result in smaller *Chelonia mydas* leaving the pelagic zone may recruit first to northern areas (Trident Submarine Basin) and then move south (IRL and Nearshore Reef), and that algae at this location are more readily accessible to foraging by smaller turtles since lush algal growths are found in smaller recesses in the rock rip-rap (Redfoot, 1997). No incidence of FP has been recorded in *Chelonia* from the Trident Submarine Basin (Hirama, 2001).

Turtle capture and handling

For the three in-water study areas, turtles were captured using entanglement nets and dip nets. The tangle net used in the Indian River Lagoon study site was an approximately 450 m long, multifilamentous nylon twine mesh net of 40 cm stretch from knot to knot. The mesh was suspended from a braided polyethylene top line and has a number 30 continuous lead core bottom line. Bullet-shaped floats were placed at equally spaced intervals and facilitated observation of entangled turtles by "dancing." This net

was tended continuously by pulling hand-over-hand on the top line while standing on the bow of a small boat. By tending the net continuously, turtles were removed from the net shortly after entanglement, minimizing capture stress.

In Trident Submarine Basin, two separate nets were used, together having the same length as the above mentioned net. One of the nets had smaller mesh (30.5 cm stretch knot to knot). Floats were attached and nets were tended as mentioned above. In addition, turtles at this study area were captured with long-handled dip nets.

The Nearshore Reef study site, with oceanic conditions being unpredictable in nature, required varying netting methods. Length of net deployed depended on oceanic conditions. Again, the net was continuously monitored, in this case by snorkelers, or was immediately retrieved in the case of "striking the net." More details on above mentioned netting procedures are discussed elsewhere (Ehrhart and Ogren, 1999).

After capture, turtles were either kept on the boat or brought to shore. Turtles were tagged, measured (straight-line carapace length (SCL) was obtained), weighed, lavaged, checked for damage or disease and photographed. Turtles were categorized as to FP status into the following categories: 0 (no sign of FP affliction), 1 (mildly afflicted), 2 (moderately afflicted) and 3 (severely afflicted) (Hirama, 2001). Blood samples for analysis of vitamins A and E were also acquired at this time. Turtles were returned immediately to original capture locations.

Blood collection and processing

Blood samples (2-3 ml) from *Caretta* and *Chelonia* were collected from the bi-laterally located dorsal cervical sinus using the method previously described by Owens

and Ruiz (1980). Before blood collection was attempted on a turtle, its skin was thoroughly cleaned with an alcohol swab. Vacutainers® (7ml) containing lithium heparin and sterile 1", 22 gauge needles (or 1 ½" 20 gauge for larger *Chelonia* and *Caretta*) were utilized for blood collection.

Extreme care was taken when handling blood samples potentially containing retinoids and tocopherols. Vitamins A and E can be destroyed or isomerized by bright daylight, bright electrical lighting and excessive heat (Abe and Matsumoto, 1993, Barua and Furr, 1998). Blood samples were taken in a shaded area protected from direct UV light. Samples were centrifuged immediately at 3000 RPM and 1100 x g using a portable centrifuge or were placed on ice and centrifuged within twenty minutes of collection time. Blood was centrifuged for ten minutes in order to achieve complete separation of erythrocytes from blood plasma. Hemolyzed samples were not used in this study because hemolysis of erythrocytes can cause metal ion contamination of plasma, resulting in reduction of vitamin compounds (Abe and Matsumoto, 1993, Barua and Furr, 1998). The plasma layer was carefully removed from the top with a small, disposable, polyethylene pipette and transferred to a sterile 1.5 ml cryovial. Vial size was selected to fit the plasma sample to minimize the amount of air in contact with the plasma. Exposure to air or oxygen can result in oxidation of these vitamins into inactive compounds. Cryovials were then placed on ice in a cooler until transfer to a freezer kept at -10 C and stored a maximum of 2 years before analysis.

Laboratory methods

The laboratory method used for analysis of turtle plasma samples for retinol and tocopherol was a modification of the method by Storer (1974). Laboratory procedures took place under diffuse yellow lighting to avoid isomerization of the vitamins by UV light. Each sample was removed from the freezer, thawed and then vortexed for 30 seconds. With a pipetter, 200 µl of plasma was transferred from the cryovial into a 10 x 75 mm disposable borosilicate culture tube (referred to as test tube in the following text). In order to precipitate proteins in the plasma sample, 200 µl of HPLC-grade ethanol with 0.1% BHT was then added. Next, 200 µl of HPLC-grade hexane with 0.025% BHT was added to the test tube. The test tube was then quickly capped with a polyethylene cap and vortexed for 30 seconds. Vortexing was followed by centrifuging the tube for 15 seconds. Next, the cap of the test tube was removed and 200 µl of the hexane based supernatant was removed and transferred into a microcentrifuge tube which was placed under a light stream of high-purity grade N_2 gas. The hexane based sample was left to evaporate to dryness. The sample was reconstituted with 200 µl HPLC-grade methanol with 0.025% BHT and the cap on the microcentrifuge tube closed tightly. Each microcentrifuge tube was wrapped tightly with at least 2 layers of parafilm and placed in a microcentrifuge tube cryo-box for shipping.

Samples were shipped overnight on dry-ice to the Wildlife Conservation Society's Department of Wildlife Nutrition, Bronx, NY. Samples were then vortexed for 10 seconds, poured into a HPLC crystal, covered with tin-foil and placed in an auto sampler. A Series 400 Perkin Elmer HPLC system (Perkin-Elmer, Inc., Norwalk, CT) with a 15-cm C18 reverse phase column was used to analyze samples. HPLC-grade methanol and

water (98:2 vol/vol) was used as the mobile phase with a flow rate of 2.0ml/min. α-Tocopherol was monitored using a PE LS-1 fluorescence detector (excitation wavelength 280 nm, emission >310 nm); retinol was detected at 325 nm on a Perkin-Elmer Model LC-95 UV/visible spectrophotometer. HPLC chromatograms were produced and interpreted using PE Turbochrom 4 software on an IBM compatible computer.

Statistical analysis

I used two different regression models to determine the effects of FP severity, SCL, species, and year on plasma vitamin concentrations with variables. Comparison 1 (Figure 6) only included turtles from Indian River Lagoon because it was the only study site where both *Chelonia* and *Caretta* were captured. Within this site, I tested the null hypotheses that plasma concentrations of vitamins A and E did not vary with species, FP severity, SCL and year. Comparison 2 (Figure 6) included *Chelonia* from 3 different study sites which have different levels of FP affliction. I used 2 different models within this comparison because different variables had significant effects on each vitamin. After removing variables with *F*-ratios ≤ 1.00 from the full models, I used multiple linear regression to test the null hypothesis that circulating concentrations of vitamin E did not vary with location, FP severity, turtle SCL, date and the location x SCL interaction. Similarly, I tested the null hypothesis that plasma retinol concentrations did not vary with location, FP severity and year. I log transformed ($\log_{10}(x + 1)$) vitamin E values to meet normality assumptions and used Tukey (HSD) post-hoc tests to compare any significant means.

I estimated correlations of vitamins A and E in the plasma for all marine turtles sampled, using separate correlations for each species. All statistical analyses were performed using JMP version 5.1 (SAS Institute, Inc., 2003), with $\alpha = 0.05$ for all hypothesis tests.

Results

I collected blood samples from 29 *Caretta* and 282 *Chelonia* from 24 May 2000-8 September 2002. All 29 subadult *Caretta* were from IRL and 5 (17.2%) had mild (severity category 1) FP. I sampled 143 juvenile *Chelonia* from IRL; 67 (46.9%) were not afflicted with FP, 53 (37.1%) were mildly afflicted, 19 (13.3%) were moderately afflicted (severity category 2) and 4 (2.8%) were severely afflicted (severity category 3). I obtained 72 blood samples from *Chelonia* on the Nearshore Reef; 18 (25%) were from turtles mildly afflicted with FP (severity category 1) and remaining turtles were not afflicted. I obtained 67 blood samples from Trident Basin *Chelonia*; none had FP.

Relationship between vitamins A and E in the plasma of Chelonia *and* Caretta

In *Caretta* from Indian River Lagoon, plasma concentrations of vitamins A and E were uncorrelated ($r = -0.116$, $n = 29$, $P = 0.5478$, Figure 7). Juvenile *Chelonia* from 3 different habitats showed a positive correlation between plasma concentrations of vitamins A and E ($r = 0.385$, $P = 0.0001$, Figure 7). Vitamin A (measured as µg/ml retinol) concentrations averaged 0.63 ± 0.04 µg/ml and ranged between 0.07 - 1.06 µg/ml in *Caretta* from IRL, and *Chelonia* from the 3 study sites averaged 0.61 ± 0.01 µg/ml and ranged between 0.05 - 1.31 µg/ml. Vitamin E (measured as µg/ml α-tocopherol)

concentrations averaged 4.78 ± 0.51µg/ml and ranged between 1.22 – 12.66 µg/ml in *Caretta*, and in *Chelonia* averaged 3.87 ± 0.13 µg/ml and ranged between 0.64 - 12.41µg/ml.

Vitamin A in juvenile Chelonia *and subadult* Caretta *in Indian River Lagoon*

Plasma vitamin A concentrations in *Chelonia* were (LS mean ± SE) 0.67 ± 0.04 µg/ml, and in *Caretta* were 0.56 ± 0.05 µg/ml. Retinol concentrations were significantly higher in *Chelonia* than *Caretta* (Table 5). Plasma concentrations of retinol were not significantly affected by SCL, FP status or year.

Vitamin E in juvenile Chelonia *and subadult* Caretta *from Indian River Lagoon*

Vitamin E concentrations in *Chelonia* were (LS mean ± 1 SE) 0.57 ± 0.03 µg/ml, and in *Caretta* were 0.68 ± 0.05 µg/ml. Plasma concentrations of α-tocopherol significantly differed among turtles with different levels of FP affliction (Table 6) and Tukey's HSD test showed turtles with a FP category of 2 had significantly lower circulating concentrations of vitamin E than turtles not afflicted with the disease (Figure 8). Turtles mildly afflicted (severity category 1) or severely afflicted (severity category 3) were not significantly different from each other in their plasma vitamin E content. *Caretta* had significantly higher plasma concentrations of vitamin E than *Chelonia* ($F_{1,164}$ = 6.68, P = 0.0106, Table 6). Year affected vitamin E concentrations ($F_{2,164}$ = 3.69, P = 0.0270, Table 6); but, Tukey's HSD test was unable to determine which years differed (Figure 9). Turtle SCL had no effect on vitamin E concentrations in turtles from Indian River Lagoon (Table 6).

Vitamin A in juvenile Chelonia *from three different study sites*

Retinol concentrations varied significantly among *Chelonia* from three different study sites ($F_{2,274} = 14.84$, $P < 0.0001$, Table 7). Plasma vitamin A concentrations (LS mean ±1 SE) were 0.65 ± 0.03 µg/ml in IRL, 0.50 ± 0.04 µg/ml on the Nearshore Reef, and 0.51 ± 0.04 µg/ml at Trident Basin; turtles from Indian River Lagoon had significantly higher concentrations of circulating vitamin A than turtles in both the Nearshore Reef and Trident Basin (Figure 10). Plasma concentrations of vitamin A did not vary significantly with FP status or year (Table 7).

Vitamin E in juvenile Chelonia *from three different study sites*

Plasma vitamin E concentrations (LS mean ±1 SE) were: 0.61 ± 0.03 µg/ml in IRL, 0.69 ± 0.04 µg/ml on Nearshore Reef, and 0.52 ± 0.05 µg/ml at Trident Basin. Vitamin E concentrations varied significantly between turtles from different habitats ($F_{2,272} = 7.66$, $P = 0.0006$, Table 8). Tukey's HSD test showed *Chelonia* on Nearshore Reef had significantly higher circulating concentrations of vitamin E than in Trident Basin (Figure 11). *Chelonia* in Indian River Lagoon had vitamin E concentrations that did not differ significantly from either Nearshore Reef or Trident Basin (Figure 11). Vitamin E concentrations varied significantly with turtle size (SCL, Table 8); the smallest *Chelonia* had circulating concentrations of vitamin E almost an order of magnitude higher than the largest turtles (Figure 12). Plasma concentrations of vitamin E did not vary significantly with FP severity, date and the location x SCL interaction (Table 8).

Discussion

Relationship between vitamins A and E in the plasma of Chelonia *and* Caretta

The significant correlation between vitamins A and E in the plasma of *Chelonia* observed in this study has been previously observed in another chelonian species. A positive correlation has been reported for the Aldabra Giant Tortoise (*Geochelone gigantea*) ($r = 0.72$, $P < 0.005$, $n = 12$) (Ghebremeskel, et al., 1991). *Caretta* in this study are in contrast to this observation in that the two vitamins had no significant relationship in plasma. Although vitamins A and E do interact *in vivo*, mentions of their correlation in plasma are rare in the literature. Vitamin E protects vitamin A from oxidation at intraluminal and cellular levels (Underwood, 1984). Many other studies address the relationship of the two vitamins, but these usually include pharmacological dosing of the nutrients in laboratory animals. The relationship between retinol and α-tocopherol may not be of major nutritional significance within range of normal dietary intakes (Draper, 1980) and therefore may be only of anecdotal interest in this vitamin study in juvenile and subadult marine turtles.

Vitamin A in juvenile Chelonia *and subadult* Caretta *from the Indian River Lagoon*

Plasma vitamin A concentrations were higher in *Chelonia* than in *Caretta*. This may be because of differences in dietary habits between the two species and consequently in the way turtles obtain vitamin A. *Chelonia* is a herbivorous species, feeding primarily on seagrasses throughout most of its range but consuming algae in places where seagrasses are not prevalent (Mortimer, 1995). Herbivores obtain retinol by converting

dietary vitamin A pre-cursors, (such as carotenoids) in the mucosa layer of the intestine or in some cases the liver (Pitt, 1985). In humans, the enzyme which facilitates the conversion by cleaving β-carotene is carotene 15,15'-dioxygenase (Vogel, et al., 1999). The presence of this enzyme has not been confirmed in *Chelonia*, however; the ability of *Chelonia* hatchlings to convert β-carotene to vitamin A has been proven (Patterson, 1974). *Caretta*, a carnivore, consumes a variety of marine invertebrates and occasionally some plant material (Bjorndal, 1997). Carnivores obtain preformed vitamin A from their animal tissue diets (Pitt, 1985). Further investigation is required to gain a better understanding of vitamin A metabolism in *Caretta*.

Many species (cows, ferrets, chickens) exhibit markedly different characteristics in vitamin A pre-cursor (carotenoid) absorption and metabolism (Vogel, et al., 1999); however, few references regarding metabolism of retinol and its pre-cursors in reptiles exist. Species specific selective absorption of carotenoids has been observed in the common green iguana (*Iguana iguana*) (Raila, et al., 2002) and suspected in panther chameleons (*Furcifer pardalis*) (Dierenfeld, et al., 2002). Different absorption of carotenoids and retinol may be the reason for the differences observed in plasma vitamin A concentrations between *Caretta* and *Chelonia*.

Plasma vitamin A concentrations did not vary with size (SCL) of turtles in the IRL. Although vitamin requirements have been demonstrated to change at different life stages in some animals (Jennings, 1970), this has either not occurred in these turtles or has not had a detectable effect on circulating concentrations of retinol.

There was no difference in vitamin A concentrations between turtles with different levels of FP affliction. Other studies mention that one of the underlying causes

of FP may be related to nutritional deficiencies (Cray, et al., 2001, Hirama, 2001) but in respect to vitamin A in IRL turtles, this appears not to be the case. There is an abundance of available algae for *Chelonia* in the IRL (Holloway-Adkins, 2001, Redfoot, 1997), and this seems to be reflected in the plasma levels of vitamin A in these animals. Therefore, it is unlikely that these animals are suffering from any adverse immune related functions (epithelial keratinization, decreased killing abilities of macrophages and neutrophils and decreased turnover of lymphocytes) as a result of a vitamin A deficiency. As concluded in other studies, immunosupression (here in terms of vitamin A status) is not a necessary prerequisite for FP expression (Work, et al., 2001). A study of desert tortoises (*Xerobates agassizii*) afflicted with chronic upper respiratory disease also found no difference in serum vitamin A concentrations between ill and control animals (Jacobson, et al., 1991).

Another possibility for the similarity in vitamin A concentrations in FP and control turtles in this study may be related to stress. In a study of *Chelonia* in Hawaii, it was concluded that turtles afflicted with FP were chronically stressed compared with turtles without the disease based on elevated plasma corticosterone concentrations (Aguirre, et al., 1995). *In vitro* studies show corticosterone increases rat heptatoma cell secretion of retinol binding protein (the carrier molecule for retinol in the plasma) to concentrations 2 to 3 times normal (Underwood, 1984). FP afflicted turtles in the IRL may have elevated corticosterone concentrations as observed in Hawaii turtles with the disease. If marine turtle cells have the same reaction to elevated corticosterone, this may result in higher than expected plasma concentrations of vitamin A. However, other *in vivo* animal studies showed increased stress due to physiological or environmental

factors, resulted in lower plasma concentrations of retinol even in the presence of increased glucocorticoid hormone concentrations (Underwood, 1984). Clearly, future studies on vitamins in marine turtles should include investigations of hormones which may indicate evidence of physiological stress and affect observed concentrations of vitamins.

Vitamin E in juvenile Chelonia *and subadult* Caretta *from the Indian River Lagoon*

Vitamin E concentrations did vary in juvenile turtles in relation to their FP status. Turtles with a FP severity of 2 had significantly lower plasma concentrations of vitamin E compared with turtles not afflicted with the disease and as a result, may have compromised immune status. Turtles in category 2 may have difficulty responding to disease primarily with mononuclear phagocytic cell functions (Meydani and Blumberg, 1993). In Hawaii, turtles of category 2 and 3 had significantly lower cell proliferation indices in the presence of concanavalin A (ConA) and phytohaemagglutinin (PHA) (Work, et al., 2001). Similar decreased lymphocyte proliferations were observed in captive turtles with FP compared with a disease free captive population using PHA, ConA and lipopolysaccaride (Cray, et al., 2001). Both of these previous studies conclude that the depressed immune status of these turtles may be a result of FP. In the present study, the lower circulating concentrations of vitamin E, also may be a result the disease.

In contrast, however, turtles of category 3 did not have lower circulating levels of vitamin E compared with those of turtles of category 1. Plasma concentrations of vitamin E may vary widely in some animals (Dierenfeld, 1994), so elevated concentrations of α-tocopherol in category 3 turtles may be an effect of sample size ($n = 4$). The previously

mentioned study of desert tortoises also showed no significant difference in serum vitamin E levels between tortoises afflicted with chronic respiratory disease and control animals (Jacobson, et al., 1991).

Plasma vitamin E concentrations were significantly higher in *Caretta* than in *Chelonia* and in this manner both species follow the previously established chelonian pattern of omnivores having higher concentrations of α-tocopherol than herbivores. Herbivorous species such as *Gopherus polyphemus*, *Malacochersus tornieri* and *Testudo graeca nikolskii* have lower plasma vitamin E concentrations (mean ± 1 SD (μg/ml)) 1.56 ± 1.04, 2.34 ± 1.17 and 4.66 ± 2.62, respectively, than in omnivores *Sternotherus odoratus* and *Clemmys muhlenbergii*, 6.64 ± 2.10 and 17.23 ± 0.03, respectively (Dierenfeld, et al., 1999). Higher concentrations of plasma vitamin E also occur in carnivorous as compared with herbivorous birds, eighteen species of raptors (n = 247) averaged 69.14 ± 6.32 μmol/l while fifteen species of cranes fed grain based diets (n = 274) averaged 15.24 ± 3.15 μmol/l (Dierenfeld, 1994).

Vitamin A in juvenile Chelonia *from three different developmental habitats*

Plasma vitamin A concentrations were significantly higher in *Chelonia* from IRL compared with the Nearshore Reef and Trident Basin. The drift algae species *Gracilaria* spp, *Bryothamnion seaforthii*, *Acanthophora spicifera* and *Hypnea spp* (red algae) were very abundant in the IRL (Holloway-Adkins, 2001). For Trident Basin, it has been suggested that limited biomass could be part of the reason why that study site supports *Chelonia* of smaller size classes. A greater biomass per square meter in the IRL compared with Trident Basin (Redfoot, 1997), with more food availability, may result in

higher plasma concentrations of vitamins as a result of increased dietary intake by turtles. Turtles from the Nearshore Reef also have an abundance of forage available as compared with Trident Basin (Redfoot, 1997) so it is not clear why Nearshore Reef turtles have lower circulating concentrations of vitamin A. Further research is needed on vitamin content of diet items of *Chelonia*. Although literature exists on vitamin content of marine algae, studies focus primarily on species used by humans for food (Jensen, 1969, Sánchez-Machado, et al., 2002) and not by marine turtles.

As observed in *Caretta* and *Chelonia* from the Indian River Lagoon, there was no significant difference in plasma concentrations of vitamin A in *Chelonia* from all three study areas in relation to their FP status. Possible reasons for this observation are discussed above.

Vitamin E in juvenile Chelonia *from three different developmental habitats*

Plasma concentrations of vitamin E were higher in smaller *Chelonia* (shorter SCL). Vitamin requirements have been demonstrated to change at different life stages in some animals (Jennings, 1970), and this may have an effect on plasma concentrations of vitamins. Smaller animals may have to mobilize more vitamins for growth compared with larger animals. Whole body composition of neonate bearded dragons contain more vitamin E than in older lizards (Cosgrove, et al., 2002).

Vitamin E concentrations did differ significantly in relation to location. α-Tocopherol concentrations were significantly higher in turtles from the Nearshore Reef as compared with Trident Basin. *Chelonia* feeding on the reef have access to forage of a wider species diversity as compared with turtles from Trident Basin (Holloway-Adkins,

2001). Some species present in the diet of turtles feeding on Nearshore Reef may have higher concentrations of vitamin E than those species available to Trident Basin turtles. Again, it is clear that further investigation into the vitamin content of the diet items of *Chelonia* is required. Another reason for the differences between turtles from these locations may be nutrient limitation in the Trident Basin turtles. These *Chelonia* forage on algae growing on rocks in a man-made embayment of 1 km^2 (Redfoot, 1997) while Nearshore Reef *Chelonia* forage on algae growing on a worm rock reef system which stretches many kilometers along the east coast of Florida. The algal mat on the rocks of Trident Basin is more closely cropped compared with the algae found at Nearshore Reef (Redfoot, 1997). This may indicate that Trident Basin turtles are constantly grazing over the same area because of limited available forage and as a result have lower amounts of vitamins available to them.

Unlike the observation of *Caretta* and *Chelonia* having different concentrations of vitamin E in relation to FP status, *Chelonia* from the three study areas combined showed no difference in plasma α-tocopherol concentrations in relation to disease status. In choosing to combine turtles from Trident Basin (which do not exhibit the disease), Nearshore Reef (which has few, mildly afflicted individuals) and IRL (which has more individuals afflicted and greater severity), I believe that more important issues come to light from the results of this study. Although disease status may affect vitamin E concentrations in a population, other factors such as forage availability and species composition may have a greater effect on this nutrient in *Chelonia*. Not only should diet items of *Chelonia* be investigated for vitamin content, factors affecting the species

composition and nutrient quality of diet items should be investigated such as environmental pollutants and climate effects.

In summary, this study established base-line values for plasma concentrations of vitamins A and E (nutrients necessary for proper immune system function in vertebrates) in juvenile *Chelonia* and sub-adult *Caretta*. More severe disease affliction (category 2) was associated with lower concentrations of circulating plasma vitamin E, suggesting that the disease itself may deplete this nutrient, rather than a lower overall status of vitamin E as a predisposing factor to incidence of FP. Other factors such as species, location and size (SCL), appeared to override disease in having effects on plasma concentrations of vitamins and should always be considered in future vitamin studies in marine turtles. Location effects on plasma vitamin content in turtles are especially interesting and emphasize the need for more research on vitamin content of turtle diet items and abiotic factors, such as climate and pollution that may have an effect on the nutrient content of those items.

APPENDIX
FIGURES AND TABLES

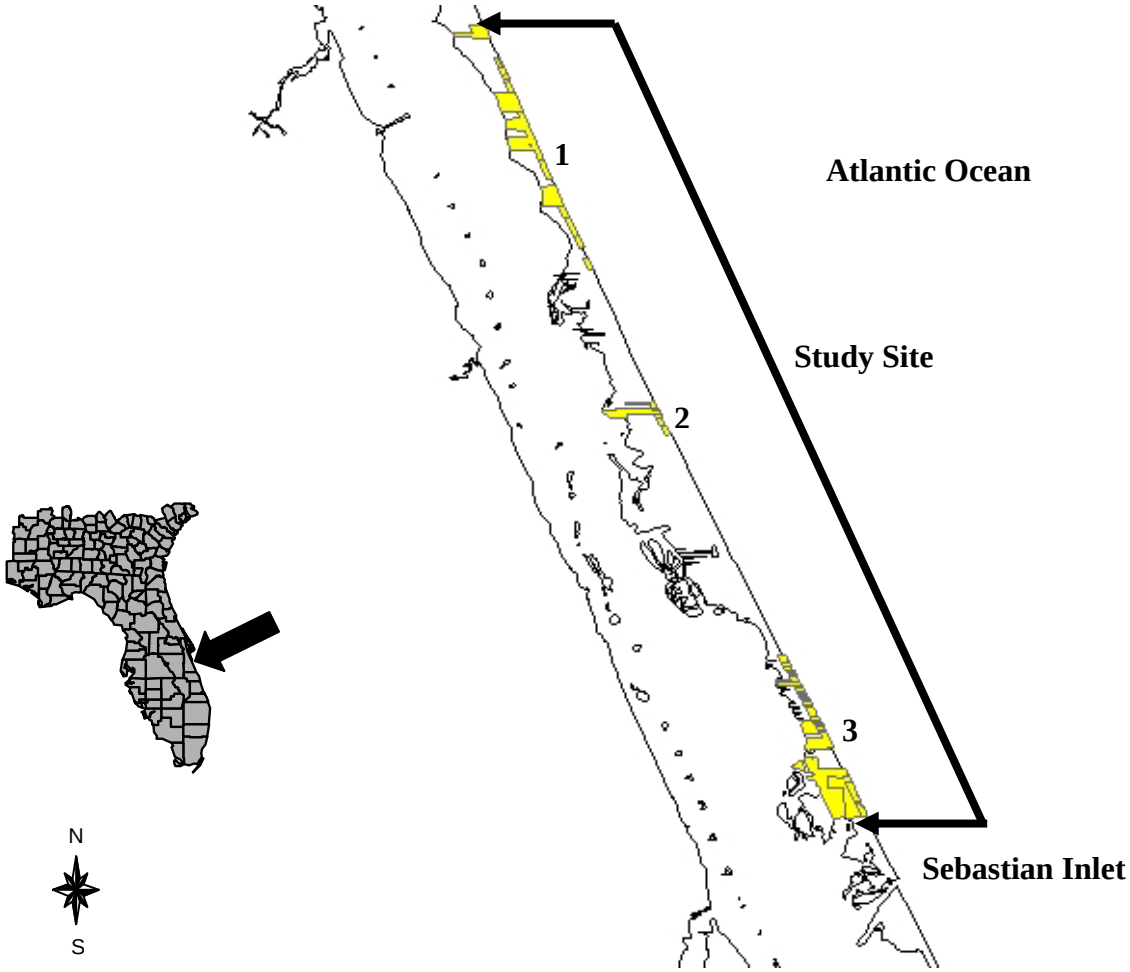

Figure 1. Location of the 21 km study site within the boundaries of the Archie Carr National Wildlife Refuge in south Brevard County, Florida. Shaded regions are three core refuge areas.

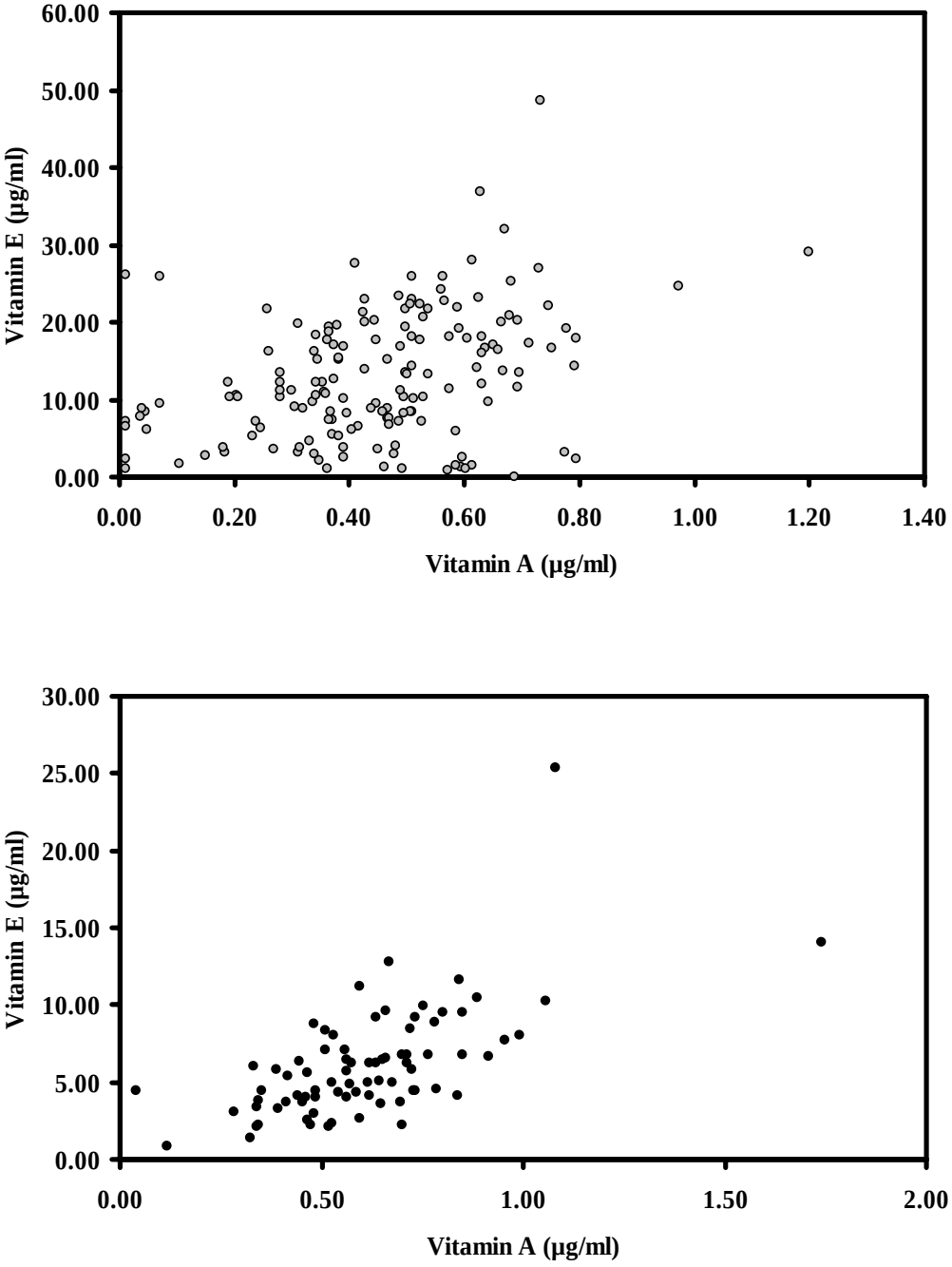

Figure 2. Positive correlations between vitamins A and E in the plasma of 160 *Caretta caretta* (top) and *Chelonia mydas* (bottom) that nested at Archie Carr National Wildlife Refuge, Brevard County, Florida.

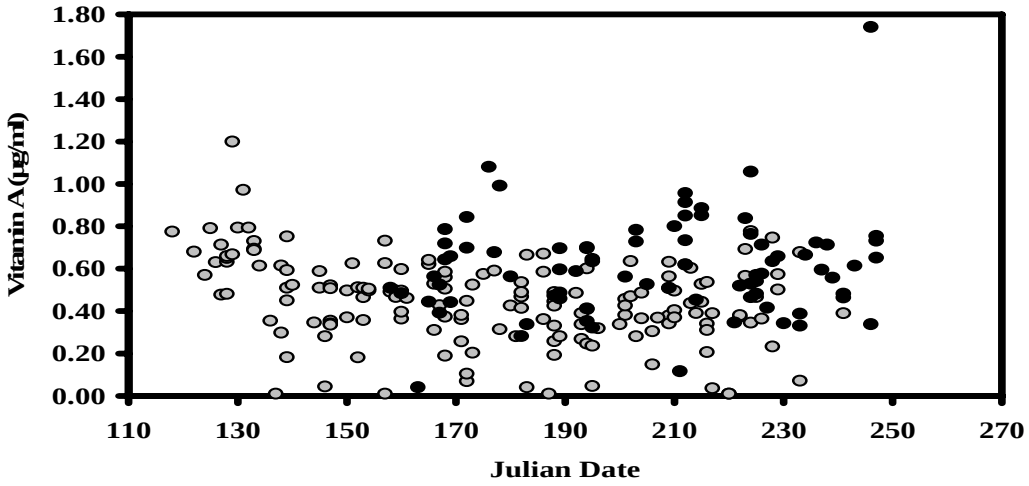

Figure 3. Plasma concentrations of vitamin A as a function of date in 160 *Caretta caretta* and 79 *Chelonia mydas* at Archie Carr National Wildlife Refuge, Brevard County, Florida. Whole model: *Caretta* (gray circles) $R^2 = 0.10$, $F_{1,158} = 17.26$, $\beta = -0.002$, $P = 0.0001$; *Chelonia* (black circles) $R^2 = 0.021$, $F_{1,77} = 1.62$, $\beta = 0.001$, $P = 0.2073$.

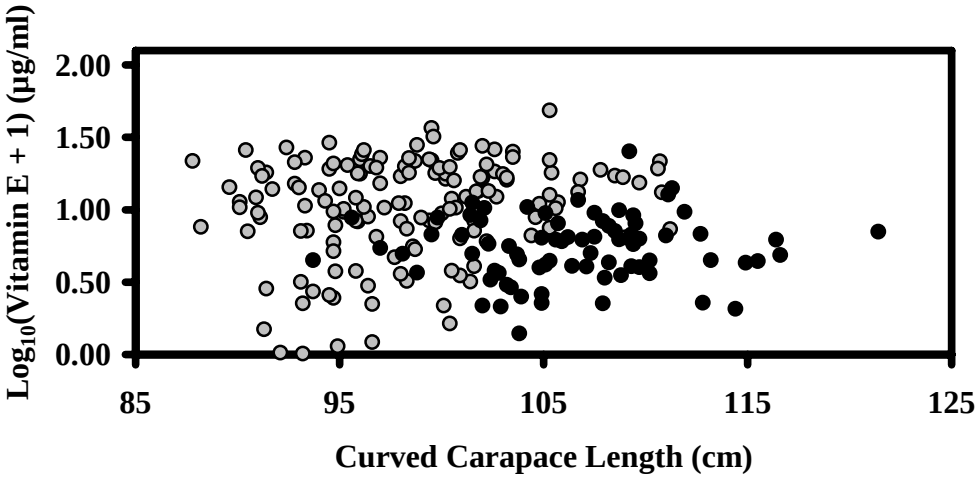

Figure 4. Plasma concentrations of vitamin E as a function of curved carapace length in 152 *Caretta caretta* and 78 *Chelonia mydas* nesting at Archie Carr National Wildlife Refuge, Brevard County, Florida. Whole model: *Caretta* (gray circles) $R^2 = 0.04$, $F_{1,150} = 5.47$, $\beta = 0.012$, $P = 0.0207$; *Chelonia* (black circles) $R^2 = 0.01$, $F_{1,76} = 0.71$, $\beta = 0.003$, $P = 0.4019$.

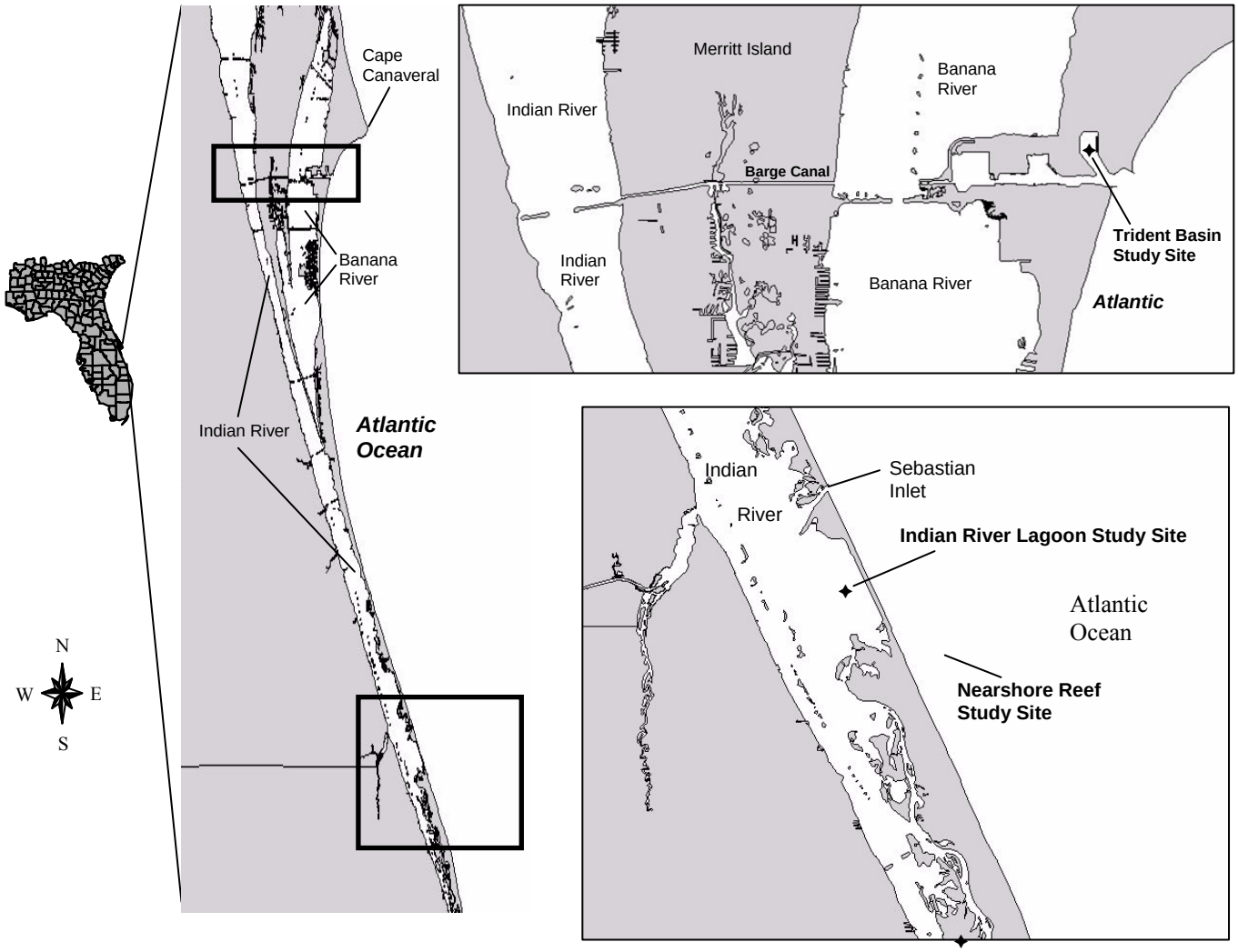

Figure 5. Locations of Indian River Lagoon, Nearshore Reef and Trident Basin study sites on Florida's Atlantic Coast.

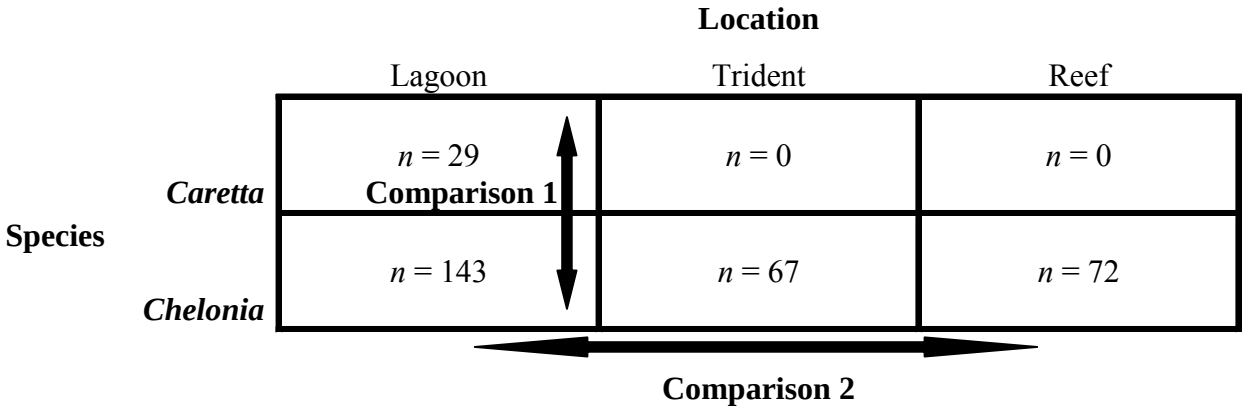

Figure 6. Comparison of plasma vitamins A and E among different groups of marine turtles. Comparison 1 included only turtles in Indian River Lagoon to address differences between species. Comparison 2 contrasts *Chelonia mydas* from three different study areas to address habitat-specific differences within a single species.

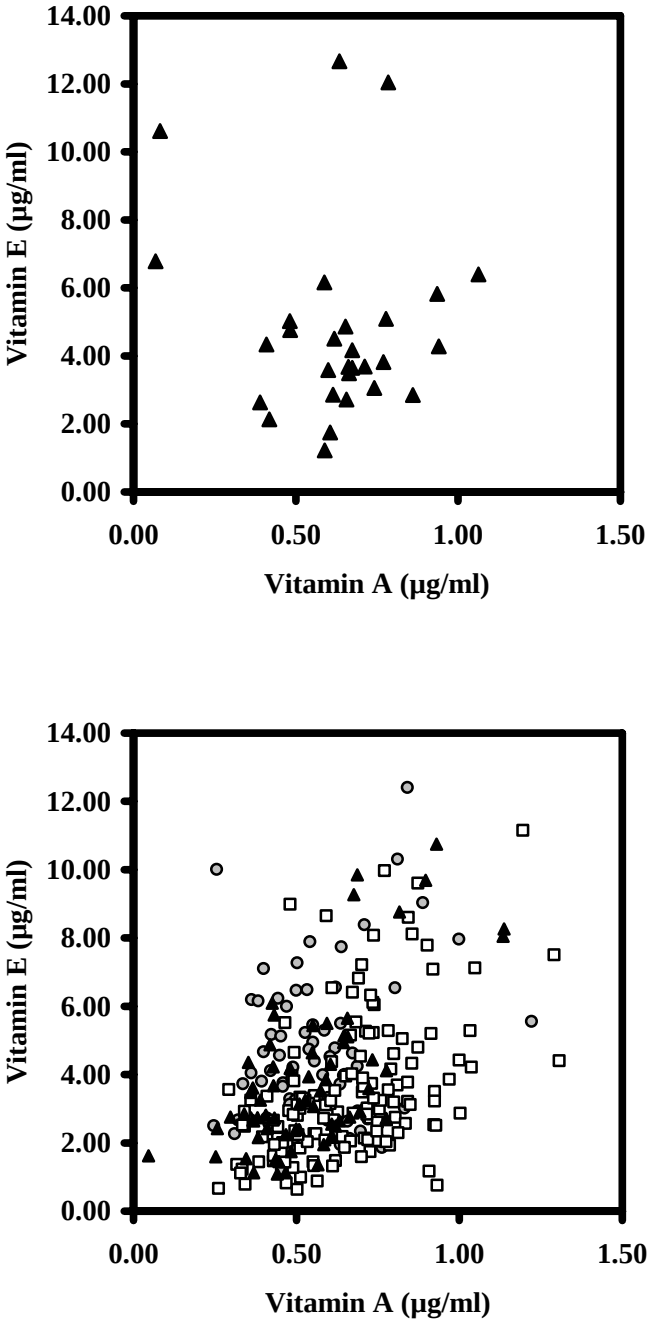

Figure 7. Relationship between vitamins A and E in the plasma of subadult *Caretta caretta* (top) and juvenile *Chelonia mydas* (bottom) in Brevard and Indian River Counties, Florida. There was no significant correlation between the two vitamins in *Caretta caretta*. There was a positive correlation between the two vitamins in *Chelonia mydas* from 3 different study sites: Nearshore Reef (gray circles), Indian River Lagoon (open squares) and Trident Submarine Basin (black triangles).

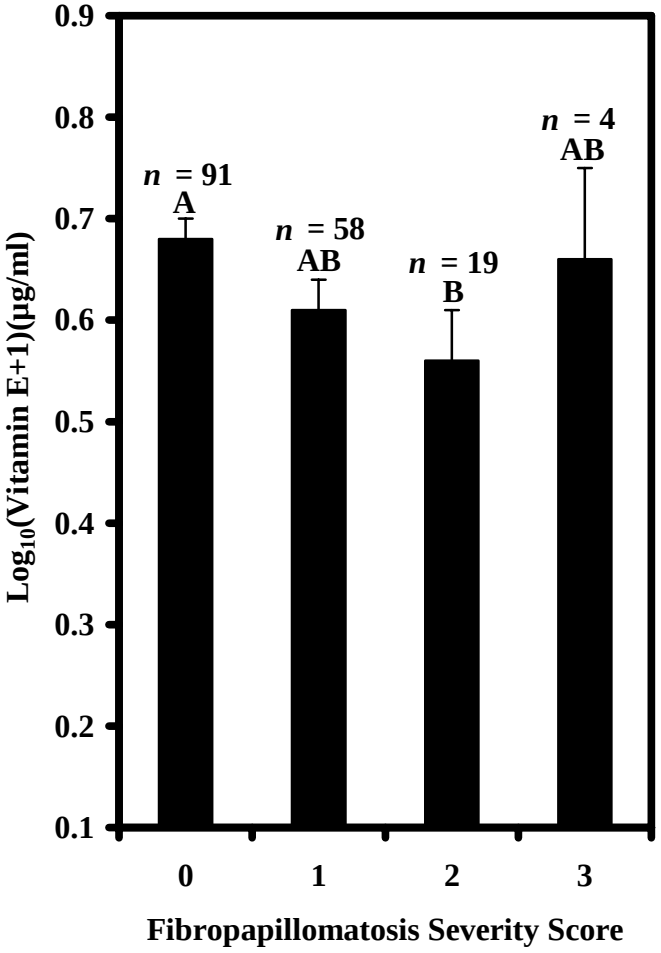

Figure 8. Mean (± 1 SE) plasma concentrations of vitamin E in *Chelonia mydas* and *Caretta caretta* with different fibropapillomatosis severity scores in Indian River Lagoon, Indian River County, Florida. Groups not connected by the same letter had significantly different means.

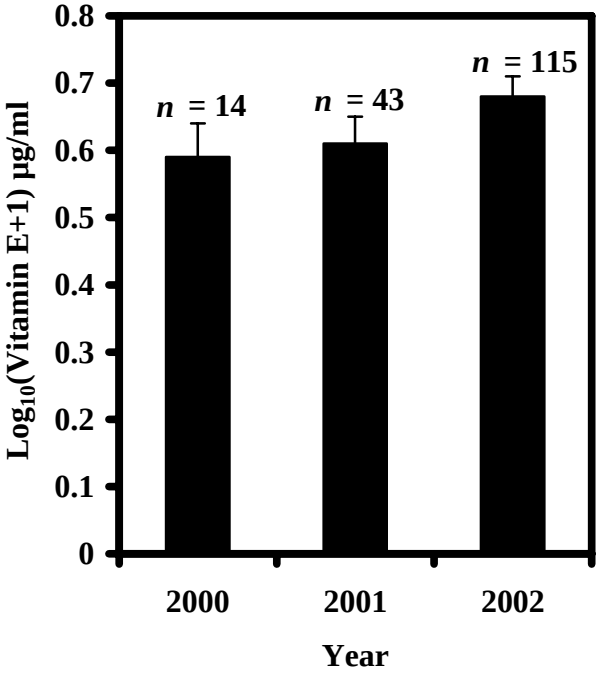

Figure 9. Mean (± 1 SE) plasma concentrations of vitamin E in *Chelonia mydas* and *Caretta caretta* sampled in different years in Indian River Lagoon, Indian River County, Florida. Differences among means were significant statistically ($P < 0.05$) but could not be separated using Tukey's HSD test.

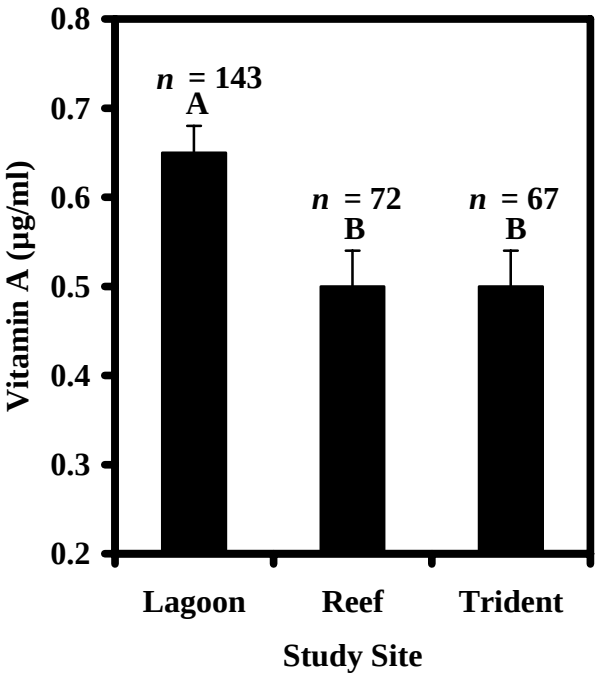

Figure 10. Mean (± 1 SE) plasma concentrations of vitamin A in *Chelonia mydas* from Indian River Lagoon, Nearshore Reef and Trident Submarine Basin. Groups not connected by the same letter had significantly different means.

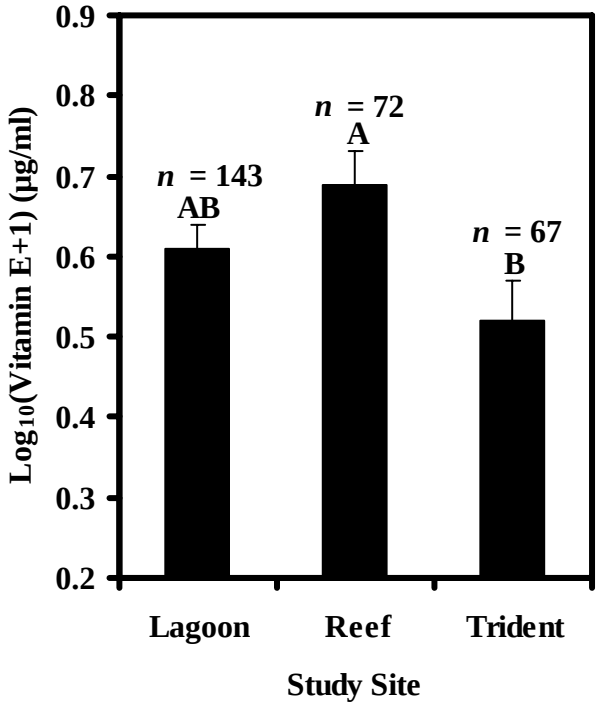

Figure 11. Mean (± 1 SE) plasma concentrations of vitamin E in *Chelonia mydas* from Indian River Lagoon, Nearshore Reef and Trident Submarine Basin. Groups not connected by the same letter had significantly different means.

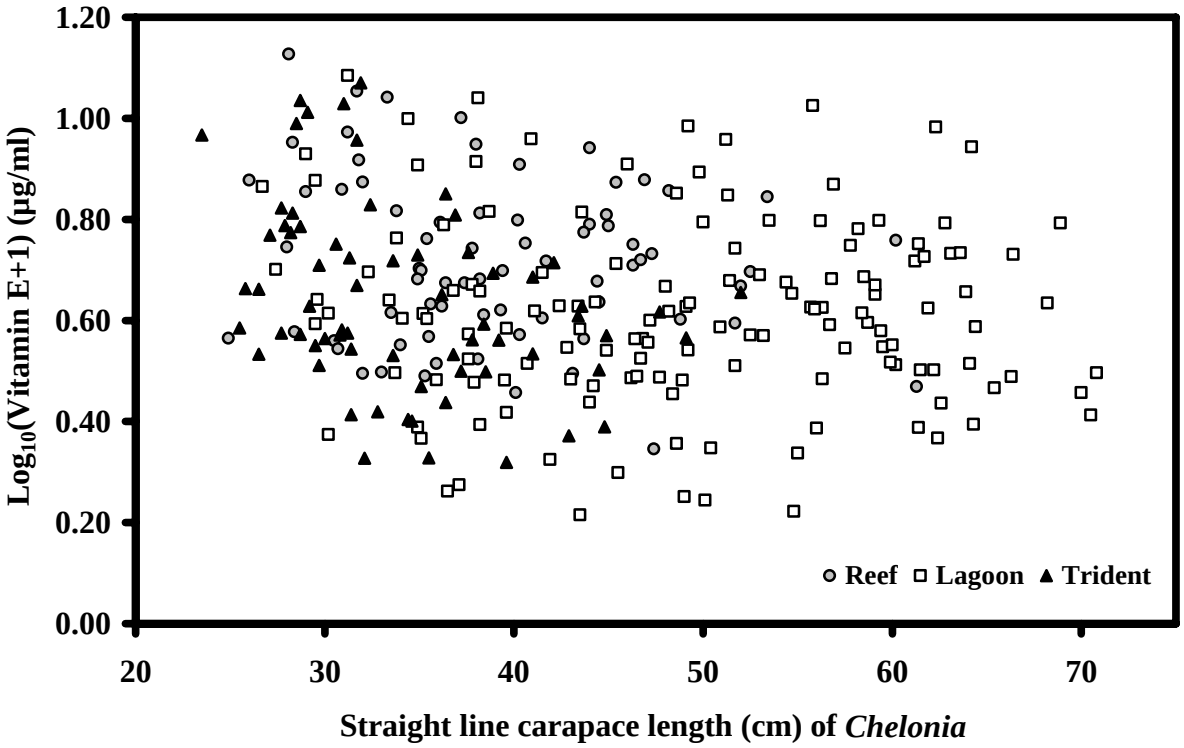

Figure 12. Relationship between plasma vitamin E and straight line carapace length in *Chelonia mydas* from the Indian River Lagoon, Nearshore Reef and Trident Submarine Basin. Circulating concentrations of vitamin E declined significantly with increasing straight line carapace length. Whole model: $R^2 = 0.11$, $F_{1,272} = 11.11$, $\beta = -0.005$, $P = 0.001$.

Table 1. Results of multiple linear regression on plasma concentrations of vitamin A (μg/ml retinol) in nesting *Caretta caretta* and *Chelonia mydas* in the Archie Carr National Wildlife Refuge, Brevard County, Florida. Table entries are sources of variation, degrees of freedom (df), sums of squares (SS), *F*-ratios (*F*), and *P*-values (*P*); OCL = Curved carapace length. Betas indicate strength and direction of each effect on plasma concentrations of vitamin A. Whole model: $F_{6,223} = 9.87$, $P = 0.0001$, $R^2 = 0.21$.

Source	df	SS	*F*	*P*	Beta
Species	**1**	**0.200**	**4.82**	**0.0291**	**-0.05**
Date	1	0.029	0.69	0.4077	-0.0004
OCL	1	0.150	3.61	0.0586	0.006
Species x date	**1**	**0.219**	**5.28**	**0.0225**	**-0.001**
Date x OCL	1	0.043	1.03	0.3111	0.0001
Species x OCL	1	0.130	3.14	0.0776	-0.005
Error	223	9.238			
Total	229	11.692			

Table 2. Results of multiple linear regression on $\log_{10}(x + 1)$ transformed plasma concentrations of vitamin E in nesting *Caretta caretta* and *Chelonia mydas* in Archie Carr National Wildlife Refuge, Brevard County, Florida. Entries are as in Table 1. Whole model: $F_{6,223} = 8.58$, $P = 0.0001$, $R^2 = 0.19$.

Source	df	SS	*F*	*P*	Beta
Species	**1**	**2.311**	**29.71**	**<0.0001**	**0.**17
Date	1	0.011	0.14	0.7111	0.0003
OCL	**1**	**0.415**	**5.34**	**0.0218**	**0.01**
Species x Date	1	0.097	1.25	0.2653	-0.001
Date x OCL	1	0.137	1.76	0.1856	-0.0001
Species x OCL	1	0.015	0.19	0.6632	0.002
Error	223	17.346			
Total	229	21.350			

Table 3. Results of multiple linear regression on hatching success of nests of *Caretta caretta* and *Chelonia mydas* in Archie Carr National Wildlife Refuge, Brevard County, Florida. Entries are as in table 1. Whole model: $F_{7,138} = 1.94$, $P = 0.0676$, $R^2 = 0.09$.

Source	df	SS	*F*	*P*	Beta
Species	1	962.341	1.39	0.2408	-5.20
Date	1	2516.964	3.62	0.0588	-0.19
OCL	1	1.895	0.00	0.9584	0.02
Vitamin A	1	37.818	0.05	0.8157	3.01
Vitamin E	1	8.836	0.01	0.9103	-0.03
Species x Date	**1**	**3643.764**	**5.25**	**0.0234**	**0.24**
Date x OCL	1	2273.215	3.28	0.0724	-0.02
Error	138	95698.980			
Total	145	105121.870			

Table 4. Results of multiple linear regression on clutch size of nests of *Caretta caretta* and *Chelonia mydas* in Archie Carr National Wildlife Refuge, Brevard County, Florida. Entries are as in Table 1. Whole model: $F_{7,140} = 5.56$, $P = 0.0001$, $R^2 = 0.22$.

Source	df	SS	*F*	*P*	Beta
Species	1	40.491	0.07	0.7856	-1.06
Date	**1**	**3448.695**	**6.33**	**0.0130**	**-0.22**
OCL	**1**	**9526.048**	**17.48**	**<0.0001**	**1.80**
Vitamin A	1	408.588	0.75	0.3880	9.86
Vitamin E	1	1258.425	2.31	0.1309	0.42
Species x Date	1	1087.606	2.00	0.1600	0.13
Date x OCL	1	397.288	0.73	0.3946	-0.01
Error	140	76291.246			
Total	147	97513.047			

Table 5. Results of multiple linear regression on plasma concentrations of vitamin A (μg/ml retinol) in subadult *Caretta caretta* and juvenile *Chelonia mydas* from Indian River Lagoon, Indian River County, Florida. Entries are as in table 1. Whole model: $F_{4,164} = 1.65$, $P = 0.0359$, $R^2 = 0.07$.

Source	df	SS	*F*	*P*
Species	**1**	**1.180**	**4.47**	**0.0359**
SCL	1	0.073	1.81	0.1804
FP severity code	3	0.161	1.34	0.2643
Year	2	0.096	1.19	0.3070
Error	164	6.597		
Total	171	7.060		

Table 6. Results of multiple linear regression on $\log_{10}(x + 1)$ transformed plasma concentrations of vitamin E in subadult *Caretta caretta* and juvenile *Chelonia mydas* in Indian River Lagoon, Indian River County, Florida. Entries are as in table 1. Whole model: $F_{4,164} = 3.68$, $P = 0.0221$, $R^2 = 0.14$. SCL = Straight-line carapace length.

Source	df	SS	*F*	*P*
FP severity code	**3**	**0.315**	**3.29**	**0.0221**
SCL	1	0.060	1.87	0.1732
Species	**1**	**0.213**	**6.68**	**0.0106**
Year	**2**	**0.236**	**3.69**	**0.0270**
Error	164	5.238		
Total	171	6.061		

Table 7. Results of multiple linear regression on plasma concentrations of vitamin A (μg/ml retinol) in juvenile *Chelonia mydas* from Indian River Lagoon, Nearshore Reef and Trident Basin study sites. Entries are as in table 1. Whole model: $F_{7,274} = 4.89$, $P = 0.0001$, $R^2 = 0.11$.

Source	df	SS	*F*	*P*
Location	**2**	**1.065**	**14.84**	**<0.0001**
FP Severity	3	0.230	2.14	0.0959
Year	2	0.141	1.96	0.1431
Error	274	9.830		
Total	281	11.057		

Table 8. Results of multiple linear regression on $\log_{10}(x + 1)$ transformed plasma concentrations of vitamin E in juvenile *Chelonia mydas* from the Indian River Lagoon, Nearshore Reef and Trident Basin study sites. Whole model: $F_{9,272}$, $P = 0.0001$, $R^2 = 0.11$. SCL = Straight-line carapace length.

Source	df	SS	*F*	*P*
Location	**2**	**0.417**	**7.66**	**0.0006**
FP severity code	3	0.157	1.71	0.1655
SCL	**1**	**0.341**	**11.11**	**0.0010**
Julian date	1	0.053	1.71	0.1915
Location x SCL	2	0.154	2.51	0.0831
Error	272	8.343		
Total	281	9.399		

LIST OF REFERENCES

Abe, K. and A. Matsumoto. 1993. Quantitative determination of tocopherols. Pages 13-19 *in* Vitamin E-Its Usefulness In Health and Curing Diseases. M. Mino, H. Nakamura, A. T. Diplock, and H. J. Kayden, eds. Karger, Tokyo.

Aguirre, A. A. and G. H. Balazs. 2000. Blood biochemistry values of green turtles, *Chelonia mydas*, with and without fibropapillomatosis. Comparative Haematology International. 10:132-137.

Aguirre, A. A., G. H. Balazs, T. R. Spraker, and T. S. Gross. 1995. Adrenal and hematological responses to stress in juvenile green turtles (*Chelonia mydas*) with and without fibropapillomas. Physiological Zoology. 68(5):831-854.

Arnhold, T., H. Nau, and R. Ruehl. 2000. Vitamin A. Pages 3-49 *in* Modern Analytical Methodologies in Fat- and Water-Soluble Vitamins. W. O. Song, G. R. Beecher, and R. R. Eitenmiller, eds. John Wiley and Sons, Inc., New York.

Barua, A. B. and H. Furr. 1998. Properties of retinoids: Structure, handling and preparation. Pages 3-28 *in* Retinoid Protocols. C. P. F. Redfern, ed. Humana Press, Totowa.

Beharka, A., S. Redican, L. Leka, and S. N. Meydani. 1997. Vitamin E status and immune function. Methods in Enzymology. 282:247-263.

Bjorndal, K. A. 1985. Nutritional ecology of sea turtles. Copeia. (3):736-751.

Bjorndal, K. A. 1997. Foraging ecology and nutrition of sea turtles. Pages 199-231 *in* The Biology of Sea Turtles. P. L. Lutz and J. A. Musick, eds. CRC Press, Inc., Boca Raton.

Bolten, A. B. and K. A. Bjorndal. 1992. Blood profiles for a wild population of green turtles (*Chelonia mydas*) in the southern Bahamas: size-specific and sex-specific relationships. Journal of Wildlife Diseases. 28(3):407-413.

Chytil, F. 1994. New horizons in retinoid research. Pages 11-28 *in* Retinoids: From Basic Science to Clinical Applications. M. A. Livrea and G. Vidali, eds. Birkhäuser Verlag, Basel.

Cosgrove, J. J., D. H. Beermann, W. A. House, B. D. Toddes, and E. S. Dierenfeld. 2002. Whole-body nutrient composition of various ages of captive-bred bearded dragons (*Pogona vitteceps*) and adult wild anoles (*Anolis carolinensis*). Zoo Biology. 21:489-497.

Cray, C., R. Varelia, G. Bossart, and P. L. Lutz. 2001. Altered in vitro immune responses in green turtles (*Chelonia mydas*) with fibropapillomatosis. Journal of Zoo and Wildlife Medicine. 32(4):436-440.

Dennert, G. 1984. Retinoids and the immune system: immunostimulation by vitamin A. Pages 373-390 *in* The Retinoids. Vol. 2. M. B. Sporn, A. B. Roberts, and D. S. Goodman, eds. Academic Press, Inc., Orlando.

Dierenfeld, E. S. 1994. Vitamin E in exotics: effects, evaluation and ecology. Journal of Nutrition. 124:2579S-2581S.

Dierenfeld, E. S., O. A. Leontyeva, W. B. Karesh, and P. P. Calle. 1999. Circulating α-tocopherol and retinol concentrations in free-ranging and zoo turtles and tortoises. Annual Conference of the American Association of Zoo Veterinarians, Columbus, Ohio:329-331.

Dierenfeld, E. S., E. B. Norkus, K. Carroll, and G. W. Ferguson. 2002. Carotenoids, vitamin A, and vitamin E concentrations during egg development in panther chameleons (*Furcifer pardalis*). Zoo Biology. 21:295-303.

Dierenfeld, E. S., C. E. Sandfort, and W. C. Satterfield. 1989. Influence of diet on plasma vitamin E in captive peregrine falcons. Journal of Wildlife Management. 53(1):160-164.

Diplock, A. T. 1985. Vitamin E. Page 319 *in* Fat-soluble vitamins: their biochemistry and applications. A. T. Diplock, ed. Technomic Publishing Co., Inc., Lancaster.

Draper, H. H. 1980. Nutrient interrelationships. Pages 272-288 *in* Vitamin E: A Comprehensive Treatise. L. J. Machlin, ed. Marcel Dekker, Inc., New York.

Ehrhart, L. M. 1985. Characterization of loggerhead and green turtle populations in the Indian River Lagoon System Florida USA with comments on use of the term juvenile and sub-adult. American Zoologist. 25(4):106A.

Ehrhart, L. M. and D. A. Bagley. 2002. In-water population studies of marine turtles on the east-central Florida coast; 1 January, 2001 through 31 December, 2001. University of Central Florida, Orlando.

Ehrhart, L. M., D. A. Bagley, W. E. Redfoot, S. A. Kubis, and S. Hirama. 2001. In-water population studies of marine turtles on the east-central Florida coast; September, 1999 through December, 2000. University of Central Florida, Orlando.

Ehrhart, L. M. and L. H. Ogren. 1999. Studies in foraging habitats: Capturing and handling turtles. Pages 61-71 *in* Research and Management Techniques for the Conservation of Sea Turtles. Vol. 4. K. L. Eckert, K. A. Bjorndal, A.-G. F. A., and M. Donnelly, eds. IUCN/SSC.

Frickel, F. 1984. Chemistry and physical properties of retinoids. Pages 7-145 *in* The Retinoids. Vol. 1. M. B. Sporn, A. B. Roberts, and D. S. Goodwin, eds. Academic Press, New York.

Galdieri, M. 1994. Retinoid modulation of sertoli cell function. Pages 127-136 *in* Retinoids: From Basic Science to Clinical Applications. M. A. Livrea and G. Vidali, eds. Birkhäuser Verlag, Basel.

Ghebremeskel, K., G. Williams, D. Spratt, and H. J. Samour. 1991. Plasma biochemistry of free-living giant tortoises (*Geochelone gigantea*) on Curieuse Island (Republic of Seychelles). Comparative Biochemistry and Physiology. 99A(1/2):65-67.

Hecht, H. and K. O. Honikel. 1995. Assessment of data sets containing considerable values below the detection limits. Zeitschrift fur Lebensmittel-Untersuchung und - Forschung. 201(6):592-597.

Herbst, L. H., E. C. Greiner, L. M. Ehrhart, D. A. Bagley, and P. A. Klein. 1998. Serological association between spirorchidiasis, herpesvirus infection, and fibropapillomatosis in green turtles from Florida. Journal of Wildlife Diseases. 34(3):496-507.

Hirama, S. 2001. Epizootiology of fibropapillomatosis in green turtle on the Atlantic coast of Florida. Masters Thesis, University of Central Florida, Orlando.

Holladay, S. D., J. C. Wolf, S. A. Smith, D. E. Jones, and J. L. Robertson. 2001. Aural abscesses in wild-caught box turtles (*Terrapene carolina*): Possible role of organochlorine-induced hypo-vitaminosis A. Ecotoxicology and Environmental Safety. 48:99-106.

Holloway-Adkins, K. G. 2001. A comparative study of the feeding ecology of *Chelonia mydas* (green turtle) and the incidental ingestion of *Prorocentrum spp*. Masters Thesis, University of Central Florida, Orlando.

Jacobson, E. R., J. M. Gaskin, M. B. Brown, R. K. Harris, C. H. Gardiner, J. L. LaPointe, H. P. Adams, and C. Reggiardo. 1991. Chronic upper respiratory tract disease of free-ranging desert tortoises (*Xerobates agassizii*). Journal of Wildlife Diseases. 27(2):296-316.

Jacobson, E. R., J. L. Mansell, J. P. Sundberg, L. Hajjar, M. E. Reichmann, L. M. Ehrhart, M. Walsh, and F. Murru. 1989. Cutaneous fibropapillomas of green turtles (*Chelonia mydas*). Journal of Comparative Pathology. 101(1):39-52.

Jennings, I. W. 1970. Vitamins in endocrine metabolism. Heinemann Medical, London.

Jensen, A. 1969. Tocopherol content of seaweed and seaweed meal: I.-Analytical methods and distribution of tocopherols in benthic algae. Journal of the Science of Food and Agriculture. 20:449-453.

Kasparek, S. 1980. Chemistry of tocopherols and tocotrienols. Pages 7-65 *in* Vitamin E: A Comprehensive Treatise. L. J. Machlin, ed, New York.

Lackovich, J. K., D. R. Brown, B. L. Homer, R. L. Garber, D. R. Mader, R. H. Moretti, A. D. Patterson, L. H. Herbst, J. Oros, E. R. Jacobson, S. S. Curry, and P. A. Klein. 1999. Association of herpesvirus with fibropapillomatosis of the green turtle, *Chelonia mydas* and the loggerhead turtle *Caretta caretta* in Florida. Diseases of Aquatic Organisms. 37:89-97.

Lance, V., T. Joanen, and L. McNease. 1983. Selenium, vitamin E, and trace elements in the plasma of wild and farm-reared alligators during the reproductive cycle. Canadian Journal of Zoology. 61:1744-1751.

Lance, V. A., L. A. Morici, R. M. Elsey, E. D. Lund, and A. R. Place. 2001. Hyperlipidemia and reproductive failure in captive-reared alligators: vitamin E, vitamin A, plasma lipids, fatty acids and steroid hormones. Comparative Biochemistry and Physiology Part B. 128:285-294.

Limpus, C. J. and D. J. Limpus. 2003. Biology of the loggerhead turtle in western South Pacific Ocean foraging habitats. Pages 93-113 *in* Loggerhead Sea Turtles. A. A. Bolten and B. E. Witherington, eds. Smithsonian Institution, Washington.

Lutz, P. L. 1998. Physiology and health assessment. NOAA Technical Memorandum NOS-NCCOS-CCEHBR-0003.

Lutz, P. L. and A. Dunbar-Cooper. 1987. Variations in the blood chemistry of the loggerhead sea turtle, *Caretta caretta*. Fishery Bulletin. 85(1):37-43.

Mason, K. E. and M. K. Horwitt. 1972. Effects of deficiency in animals. Pages 272-292 *in* The Vitamins: Chemistry, Physiology, Pathology, Methods. Vol. 5. second ed. W. H. Sebrell, Jr. and R. S. Harris, eds. Academic Press, New York.

Maynard, L. A. and J. K. Loosli. 1969. Animal Nutrition. 6th ed. McGraw-Hill, New York.

Meydani, S. N. and J. B. Blumberg. 1993. Vitamin E and the immune response. Pages 223-238 *in* Nutrient modulation of the immune response. S. Cunningham-Rundles, ed. Marcel Dekker, Inc., New York.

Mortimer, J. A. 1995. Feeding ecology of sea turtles. *in* Biology and Conservation of Sea Turtles. K. A. Bjorndal, ed. Smithsonian Institution Press, Washington, D.C.

Nelson, J. A. 1980. Pathology of vitamin E deficiency. Pages 397-428 *in* Vitamin E: A Comprehensive Treatise. L. J. Machlin, ed. Marcel Dekker, New York.

Osegovic, K. M. 2001. Hatching success, embryonic mortality, and infertility in loggerhead (*Caretta caretta*) and green (*Chelonia mydas*) sea turtles nesting in Brevard County, Florida. Masters Thesis, University of Central Florida, Orlando.

Owens, D. W. and G. J. Ruiz. 1980. New methods of obtaining blood and cerebrospinal fluid from marine turtles. Herpetologica. 36(1):17-20.

Packer, A. I. and D. J. Wolgemuth. 1999. Genetic and molecular approaches to understanding the role of retinoids in mammalian spermatogenesis. Pages 347-368 *in* Retinoids: The Biochemical and Molecular Basis of Vitamin A and Retinoid Action. H. Nau and W. S. Blaner, eds. Springer-Verlag, Berlin.

Patterson, J. I. 1974. Vitamin A requirement of the sea turtle, *Chelonia mydas*. Masters Thesis, University of Arizona.

Perrotta, S., B. Nobili, F. Rossi, D. Di Pinto, V. Cucciolla, A. Borriello, A. Oliva, and F. D. Ragione. 2003. Vitamin A and infancy: biochemical, functional, and clinical aspects. Pages 457-591 *in* Vitamins and Hormones: Advances in Research and Applications. Vol. 66. G. Litwack, ed. Academic Press, Amsterdam.

Pitt, G. A. J. 1985. Vitamin A. Page 319 *in* Fat Soluble Vitamins: Their Biochemistry and Applications. A. T. Diplock, ed. Technomic Publishing Co., Inc., Lancaster.

Quackenbush, S. L., R. N. Casey, R. J. Murcek, T. A. Paul, T. M. Work, C. J. Limpus, A. Chaves, L. duToit, J. V. Perez, A. A. Aguirre, T. R. Spraker, J. A. Horrocks, L. A. Vermeer, G. H. Balazs, and J. W. Casey. 2001. Quantitative analysis of herpesvirus sequences from normal tissue and fibropapillomas of marine turtles with real-time PCR. Virology. 287:105-111.

Raila, J., A. Schuhmacher, J. Gropp, and F. J. Schweigert. 2002. Selective absorption of carotenoids in the common green iguana (*Iguana iguana*). Comparative Biochemistry and Physiology Part A. 132:513-518.

Raphael, B. L., M. W. Klemens, P. Moehlman, E. S. Dierenfeld, and W. B. Karesh. 1994. Blood values in free-ranging pancake tortoises (*Malacochersus tornieri*). Journal of Zoo and Wildlife Medicine. 25(1):63-67.

Redfoot, W. E. 1997. Population structure and feeding ecology of green turtles utilizing the Trident Submarine Basin, Cape Canaveral, Florida as developmental habitat. Masters Thesis, University of Central Florida, Orlando.

Robbins, C. T. 1983. Wildlife Feeding and Nutrition. Academic Press, Orlando.

Ross, A. C. 2000. Vitamin A, retinoids and immune responses. Pages 83-95 *in* Vitamin A and Retinoids: An Update of Biological Aspects and Clinical Applications. M. A. Livrea, ed. Birkhäuser Verlag, Basel.

Sánchez-Machado, D. I., J. López-Hernández, and P. Paseiro-Losada. 2002. High-performance liquid chromatographic determination of a-tocopherol in macroalgae. Journal of Chromatography A. 976:277-284.

Scott, M. L. 1970. Studies on vitamin E and related factors in nutrition and metabolism. Pages 355-368 *in* The Fat-Soluble Vitamins. H. F. DeLuca and J. W. Suttie, eds. The University of Wisconsin Press, Madison.

Storer, G. B. 1974. Fluorometric determination of tocopherol in sheep plasma. Biochemical Medicine. 11:71-80.

Tanaka, H., K. Sato, Y. Matsuzawa, W. Sakamoto, Y. Naito, and K. Kuroyanagi. 1995. Analysis of possibility of feeding of loggerhead turtles during internesting periods based on stomach temperature measurements. Nippon Suisan Gakkaishi. 61(3):339-345.

Tengerdy, R. P. 1980. Effect of vitamin E on immune responses. Pages 429-444 *in* Vitamin E: A Comprehensive Treatise. L. J. Machlin, ed. Marcel Dekker, New York.

Tengerdy, R. P. 1989. Vitamin E, immune response, and disease resistance. Pages 335-344 *in* Vitamin E: Biochemistry and Health Implications. Vol. 570. A. T. Diplock, L. J. Machlin, L. Packer, and W. A. Pryor, eds. New York Academy of Sciences, New York.

Thompson, J. N. 1969. The role of vitamin A in reproduction. Pages 267-281 *in* The Fat-Soluble Vitamins. H. F. DeLuca and J. W. Suttie, eds. The University of Wisconsin Press, Madison.

Tucker, A. D. and M. A. Read. 2001. Frequency of foraging by gravid green turtles (*Chelonia mydas*) at Raine Island, Great Barrier Reef. Journal of Herpetology. 35(3):500-503.

Underwood, B. A. 1984. Vitamin A in animal and human nutrition. Pages 281-392 *in* The Retinoids. Vol. 1. A. B. Roberts and D. S. Goodwin, eds. Academic Press, New York.

Vogel, S., M. V. Gamble, and W. S. Blaner. 1999. Biosynthesis, absorption, metabolism and transport of retinoids. Pages 31-95 *in* Retinoids: The Biochemical and Molecular Basis of Vitamin A and Retinoid Action. Vol. 139. H. Nau and W. S. Blaner, eds. Springer-Verlag, Berlin.

Watson, R. R. and T. M. Petro. 1982. Cellular immune responses, corticosteroid levels, and resistance to *Listeria monocytogenes* and murine leukemia in mice fed a high vitamin E diet. Pages 205-208 *in* Vitamin E: Biochemical, Hematological, and Clinical Aspects. Vol. 393. B. Lubin and L. J. Machlin, eds. New York Academy of Sciences, New York.

Work, T. M., R. A. Rameyer, G. H. Balazs, C. Cray, and S. P. Chang. 2001. Immune status of free-ranging green turtles with fibropapillomatosis from Hawaii. Journal of Wildlife Diseases. 37(3):574-581.

Zile, M. H. 1999. Avian embryo as a model for retinoid function in early development. Pages 443-464 *in* Retinoids: The Biochemical and Molecular Basis of Vitamin A and Retinoid Action. H. Nau and W. S. Blaner, eds. Springer-Verlag, Berlin.