SEA TURTLE LIFE HISTORY PATTERNS REVEALED THROUGH STABLE ISOTOPE ANALYSES

By

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To my Mom

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SEA TURTLE LIFE HISTORY PATTERNS REVEALED THROUGH STABLE ISOTOPE ANALYSES

By

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For my doctoral research, I used stable isotope analyses to explore aspects of sea turtle life history that had not been studied previously. I determined isotopic discrimination factors and the contribution of growth and catabolic turnover to the rate of ^{13}C and ^{15}N incorporation into skin, scute, whole blood, red blood cells, and plasma solutes in two age classes of rapidly growing loggerheads. The isotopic discrimination factors of nitrogen ranged from -0.64 - 1.77‰. These values are lower than the commonly assumed 3.4‰ discrimination factors reported for whole body and muscle isotopic analysis. Growth explained from 26 - 100% and 15 - 52% of the total rate of incorporation in hatchling and juvenile turtles, respectively. To my knowledge, this is the first study to determine isotopic discrimination and incorporation in a reptile.

I used stable isotopes of carbon and nitrogen retained in scute (the top keratin layer of a turtle's shell) to investigate the habitats, diets and duration of a "missing" life stage: the early juvenile stage of the green turtle, *Chelonia mydas*. I developed a technique to micro-sample successive layers (50µ) of keratin from small green turtles that had recently recruited to neritic waters. The oldest or outermost layer of scute contains the oldest retained isotopic "history" of diet and habitat available to scientists from a living turtle. Analyses revealed that small green

turtles spend 3-5 years as carnivores in oceanic habitats before undergoing a rapid shift to an herbivorous diet in neritic habitats.

To investigate diet and habitat of Florida's nesting loggerhead population prior to their recruitment to nesting grounds I collected skin at four locations in Florida in 2003 and 2004. Cluster analysis based on stable isotope signatures revealed a previously undocumented bimodal foraging pattern with females almost equally divided between oceanic/pelagic and neritic/benthic foraging. Oceanic foraging females were significantly smaller (mean CCLmin = 97.6cm) than neritic foraging females (mean CCLmin = 100.2cm) though there was considerable overlap between the two groups. The distribution of 35 species of epibionts collected from 52 loggerheads are consistent with the foraging habitats assigned to the turtles by cluster analysis.

CHAPTER1
INTRODUCTION

Species with cryptic lifestages—in unknown or inaccessible locations—pose a special challenge to scientists and conservationists. Sea turtles undergo multiple ontogenetic shifts in habitat and foraging strategies through several lifestages. Access to adult sea turtles is limited, due in part to the fact that, with the exception of brief periods when reproductive females return to the beach to deposit their eggs, adult turtles spend their lives at sea. Upon hatching, neonate turtles leave the beach and disappear into the ocean. Despite intensive effort by scientists, sightings of hatchlings of most species are rare. Juvenile turtles are also elusive, with few sightings reported prior to recruitment to known juvenile foraging locations. In my dissertation, I demonstrate how stable isotopes can be used to evaluate the ecology of unknown or inaccessible lifestages of sea turtles.

Stable isotopes of carbon and nitrogen in the marine environment provide a tool to investigate habitat use as well as trophic level (Lathja and Michener 1994; Hobson and Schell 1998). The use of stable isotope analysis of carbon and nitrogen to investigate movement, trophic level, and foraging habits of free ranging animals in the marine environment has increased steadily in the last decade, including studies of seabirds, marine mammals, and marine turtles (Best & Schell 1996; Cherel et al. 2000, Godley et al. 1998).

A naturally occurring gradient exists for $\delta^{13}C$ values, in which $\delta^{13}C$ values are depleted in oceanic or pelagic habitats relative to neritic or benthic habitats (Lorian et al. 1992; Hobson et al. 1994; France 1995). A trophic gradient also exists for $\delta^{15}N$ values with values increasing at higher trophic levels (Minagawa and Wada 1984; Macko et al. 1986). These naturally occurring gradients provide powerful tools for addressing questions of foraging dynamics in all lifestages and species of sea turtles.

Our understanding of the ecology of sea turtles depends in part on our ability to identify geographic regions used by animals for migrating, breeding, and feeding. In the field of ecology, stable isotope analyses are being used increasingly to investigate feeding habits, migratory patterns, and even geographic origins in migratory species that are difficult to study using conventional methods (Chamberlain et al. 1997; Rooker et al. 2008; Wunder and Norris 2008).

Conventional methods used to investigate dietary habits include direct observation, stomach content analyses, esophageal lavage, and fecal analysis (Bjorndal 1997). These methods provide information on recent feeding events, but they cannot provide a history of feeding habits. Similarly, satellite telemetry and mark-recapture methods used commonly for tracking migratory species, including larger sea turtles, have provided information on animal movement, but these techniques cannot provide any history of earlier movements (Addison et al. 2002; Godley et al. 2002; Hayes et al. 2001; Hatase 2002b; Polovina et al. 2000; Seminoff et al. 2002). In addition, telemetry is often inappropriate to study movements of small animals such as hatchling sea turtles because of the size of the instrumentation.

Stable isotope studies can be especially useful to determine diets of animals that are difficult to observe in the wild (DeNiro and Epstein 1978, Peterson and Fry 1987, Hobson 1999) and to investigate movement patterns of animals that are difficult to follow (Gannes et al. 1998). Isotopic ratios can be useful to study diet, trophic interactions, and movements of a wide range of species (Lajtha and Michener 1994). Through comparative studies of natural isotope abundance in diet and the isotope signatures in the tissues of the animal under investigation, one can begin to assemble a picture of where that animal has been and what it has consumed.

In 1983, Killingley and Lutcavage used oxygen and carbon isotopes in barnacles removed from the shells of six large sub-adult loggerhead turtles (*Caretta caretta*) to evaluate movements

of loggerheads between nearshore and offshore habitats. Godley et al. (1998) successfully applied the stable isotope technique to predict known diets of loggerheads, green turtles (*Chelonia mydas*), and leatherbacks (*Dermochelys coriacea*) from shallow water habitats in the Mediterranean, and Barrick et al. (1999) demonstrated that oxygen isotopes in bones of loggerhead and leatherback sea turtles could be used to identify the geographic regions that the turtles have occupied. Stable isotopes provide a powerful tool that can be used with minimally invasive sampling techniques to address questions of sea turtle migration and foraging dynamics.

One factor hindering the interpretation of stable isotope data in studies of sea turtles has been the lack of data on the rate of isotopic incorporation and discrimination factors in sea turtles. The use of stable isotopes to investigate sea turtle foraging dynamics requires knowledge of the rate at which animals incorporate the carbon and nitrogen from their diets and the magnitude of the difference in isotopic composition between the animal's diet and that of its tissues (discrimination factor).

The mechanism of isotopic discrimination (change in isotope ratios in the animal's tissues relative to the food source) and turnover rate (the time required for the existing isotopes in the tissue to be replaced) is generally poorly understood. Isotopes present in the diet discriminate or are differentially assimilated into the tissue of the consumer and isotope ratios can vary among tissues within an individual (Tieszen et al. 1983). Differences in diet-tissue discrimination factors and isotopic incorporation rate may result from body size, age, diet, and metabolism (Tieszen et al. 1983) or growth rate (Chapter 2). Diet-tissue discrimination factors and isotopic incorporation rate can be assessed by maintaining animals on a controlled diet of known isotopic value until isotopic turnover is achieved in all tissues of interest. In my dissertation, I have applied this technique to sea turtles and revealed novel information.

First, in Chapter 2, I conducted a 12 month diet study in which I investigated both the dynamics and consistency of carbon and nitrogen incorporation and discrimination in the tissues of young loggerhead turtles after a diet shift. I conducted feeding trials on two age classes of loggerhead turtles. In the first trial, 108 loggerhead hatchlings were fed a pelleted diet that was significantly different in C and N values from the initial values of the tissues of interest (blood, skin, and scute). Over a period of 120 days, measurements of stable isotopes of carbon and nitrogen in blood, epidermis and scute were obtained at regular intervals. In trial two, eight juvenile loggerheads were switched to a diet with a different isotopic composition for an additional 232 days. I collected samples from the same tissues for stable isotope analyses in both trials. This is the first study in which both the isotopic incorporation and the isotopic discrimination factor in a variety of tissues is reported for a reptile.

This study was essential to identify turnover and discrimination values. Results of this study demonstrate that 1) in both hatchling and juvenile turtles, growth contributes significantly to the rate of isotopic incorporation, and 2) this contribution differed among tissues. In addition, isotopic discrimination values differ significantly among tissues in both hatchling and juvenile turtles, and 2) isotopic discrimination values of the same tissues from the two age classes (hatchling and juvenile) also show significant differences. These results suggest that discrimination factors may vary among diets and developmental stages. These data provide a baseline by which, for the first time, stable isotope analyses of sea turtle tissues can be interpreted using known values for diet tissue discrimination, and tissue turnover rates.

Chapter 2 has been published. The citation is Reich K .J., K.A. Bjorndal, and C. Martínez del Rio (2008) Effects of growth and tissue type on the kinetics of ^{13}C and ^{15}N incorporation in a rapidly growing ectotherm. Oecologia 155:651-663.

In Chapter 3, I employed stable isotope analysis to explore the "lost year" of green turtles. One of the greatest mysteries remaining in sea turtle conservation is how do endangered green turtles spend their first years of life? Finding where hatchling and post-hatchling turtles go and what they do during their “lost” years was identified by Carr (1952) as critical for the recovery of green turtle populations. The location of this lifestage remains a mystery for most species of sea turtles (Hughes 1974, Bolten et al. 1993, Bolten and Balazs 1995). It has been assumed that post-hatchling green turtles are in oceanic habitats with carnivorous or omnivorous diets, but there are only a few anecdotal data available for this age class (Bjorndal 1997).

Oceanic sightings of small green turtles are rare and intensive efforts to locate post-hatchling green turtles in the epipelagic environment of the open ocean have turned up relatively few clues as to where they are spending the early years of their lives. I used ^{13}C and ^{15}N isotope analyses of scute--the top, keratin layer of a turtle shell--to investigate diet and trophic position of North Atlantic green turtles prior to their recruitment to neritic waters in Florida and the Bahamas.

My studies confirmed that scute carries a record of previous diet and habitat use by comparing samples of old and new scute from green turtles that had recently recruited to seagrass meadows in the Bahamas. Isotope analyses of serial samples of scute illustrate changes in stable nitrogen and carbon values with increasing depth from the scute surface. The oldest dietary record is retained in the outermost layer of scute and each successive layer (0.05mm) reveals more recent diet and habitat use.

To interpret the nitrogen and carbon values of these samples, I used discrimination values from a previous study of captive loggerheads (Chapter 2) and results of stable isotope analysis of juvenile loggerheads in developmental foraging grounds in oceanic habitats near the Azores

(Chapter 3). Analyses of ^{13}C and ^{15}N signatures of green turtle scute provide evidence of a shift from a primarily carnivorous diet in the pelagic zone of oceanic habitats to an herbivorous diet in neritic habitats.

A publication resulted from the study reported in Chapter 3. The citation is Reich, K.J., K.A. Bjorndal, and A.B. Bolten (2007) The "lost years" of green turtles: using stable isotopes to study cryptic lifestages. Biology Letters 3:712-714.

In Chapter 4, I used stable isotope analysis of carbon and nitrogen from the skin of nesting loggerhead turtles to determine the foraging strategies of female loggerheads nesting in Florida. Florida has the largest nesting aggregation of loggerheads in the Atlantic and is one of only two populations worldwide with more than 10,000 loggerhead females nesting each year (Baldwin et al. 2003; Ehrhart et al. 2003). This population was assumed to have a totally neritic lifestyle, based on tag returns that are largely fishery dependent. Fishery-dependent data can be misleading because tagged turtles will only be captured at fishing grounds rather than in all areas occupied by tagged turtles. As a result, the numbers of tag returns from neritic fishing grounds far outnumbered tag returns from other habitats (National Marine Fisheries Service and U.S. Fish and Wildlife Service 1991). These results led scientists to conclude that adult loggerheads occupy neritic habitats. Using stable isotopes of carbon and nitrogen in the skin of nesting loggerheads on Florida nesting beaches allowed me to avoid the problem of fishery-dependent data.

I evaluated stable isotopes of carbon and nitrogen in samples of skin collected from 310 loggerheads nesting at four locations on the east coast of Florida. The stable isotope signature in skin represents a temporal integration of the isotopes assimilated during the synthesis of the tissue before the nesting season.

I also collected epibionts from 48 of the 310 loggerheads sampled for stable isotope analyses. Loggerheads serve as a substrate for a diverse array of epibionts (Caine 1986), and my hypothesis was that these epibiont communities would reflect the pre-nesting habitat of the host turtle. Analyses of stable isotopes and epibionts revealed that loggerheads nesting in Florida have a bimodal foraging strategy and are divided almost equally between oceanic and neritic foraging groups.

Chapter 4 has been submitted for publication. The citation will be: Reich, K.J., K.A. Bjorndal, M.G. Frick, B.E. Witherington, C. Johnson, and A.B. Bolten. Bimodal foraging in adult loggerheads (*Caretta caretta*): changes to life history models. In my final chapter, I review how my studies have advanced the field of sea turtle biology and discuss where we should go from here.

CHAPTER 2
EFFECTS OF GROWTH AND TISSUE TYPE ON THE KINETICS OF 13C AND 15N INCORPORATION IN A RAPIDLY GROWING ECTOTHERM

Introduction

The use of stable isotopes in animal ecology depends on the observation that, isotopically speaking, animals are what they eat plus or minus a small difference (called isotopic discrimination factor, $\Delta X = \delta X$ tissues $- \delta X$ diet). This observation has two components: (1) the tissues of animals resemble the isotopic composition of their diets (DeNiro and Epstein 1978, 1981; Hobson and Clark 1992; Michener and Schell 1994), and (2) the match between the isotopic composition of an animal's tissues and that of its diet is not perfect (Schoeller 1999). Both of these components are useful. The former allows us to determine the sources of the nutrients that animals assimilate, whereas the latter allows us to diagnose trophic position (Peterson and Fry 1987; Post 2002). Using stable isotopes in animal ecology judiciously demands that we understand why there are often differences between the isotopic composition of an animal and that of its diet.

The differences in the isotopic composition between an animal's tissues and its diet can be due to three factors: (1) isotopic "memory", (2) metabolic fractionation (defined as the difference in isotopic composition between reactants and products in biochemical reactions), and (3) isotopic routing (Martínez del Rio and Wolf 2005). The first of these factors is the best studied and the main focus of this study. The term isotopic memory refers to the observation that when animals change diets, the isotopic composition of their tissues does not change immediately to reflect that of their diet. Instead, tissues incorporate the diet's isotopic composition with characteristic temporal dynamics (Fry and Arnold 1982; Phillips and Eldrige 2006). The dynamics of incorporation depend on a variety of factors including animal size (Carleton and Martínez del Rio 2005), nutrient composition of the diet (Gaye-Seisseggar et al. 2003, 2004), the catabolic turnover

of the tissue type (Tieszen et al. 1983; Hobson and Clark 1992; Martínez del Rio and Wolf 2005), and the animal's growth rate (Fry and Arnold 1982; Hesslein et al. 1993; MacAvoy et al. 2001; Martínez del Rio and Wolf 2005).

Although it is well established that the rate of isotopic incorporation into an animal's tissues depends on both the rates of tissue growth and of catabolic turnover (Fry and Arnold 1982; Hesslein et al. 1993), only a handful of studies have used stable isotopes to partition the contribution of growth and catabolic turnover to the rate of isotopic incorporation (reviewed by MacAvoy et al. 2001). These studies have revealed that in rapidly growing animals, net growth rate is an important determinant of the rate at which the isotopic signal of diet is incorporated into an animal's tissues. I investigated both the dynamics and consistency of ^{13}C and ^{15}N incorporation into the tissues of two age classes of a rapidly growing ectotherm, the loggerhead sea turtle (*Caretta caretta*), after a diet shift. Ectotherms such as sea turtles have relatively low protein turnover (Houlihan et al. 1995) and hence, presumably, low rates of tissue catabolic turnover (Hesslein et al. 1993; MacAvoy et al. 2001; Tominga et al. 2003).

My research was guided by two hypotheses: (1) that growth would be the major factor determining the rate of isotopic incorporation, and (2) that the dominant effect of growth would erase the differences in isotope incorporation rates often observed among tissues (Fry and Arnold 1982; Hesslein et al. 1993; MacAvoy et al. 2001; Martínez del Rio and Wolf 2005). Although differences in isotopic incorporation among tissues have been relatively well documented in birds and mammals (Dalerum and Angerbjorn 2005), they have not been well studied in fish, amphibians, and reptiles. Differences in incorporation rates among tissues are useful because they permit identifying dietary changes at contrasting time scales (reviewed by Dalerum and Angerbjorn 2005). Phillips and Eldrige (2006) have proposed that differences in isotopic

incorporation among tissues may allow constructing an "isotopic clock" to date the time of a diet shift. My conjecture that rapid growth may homogenize the incorporation rates among tissues would limit the use of these two applications. By measuring body growth concurrently with the rate of isotopic incorporation of carbon and nitrogen in multiple tissues I was able to (1) partition the contribution of growth and catabolic turnover to the rate of isotopic incorporation in several tissues, and (2) determine whether rate of isotopic incorporation varied among tissues.

Methods

Loggerhead hatchlings from hatcheries in Broward County, Florida, were transported to the animal vivaria at the Department of Zoology, University of Florida (Gainesville, FL, USA) in June 2002. Hatchlings (n = 120; 20 hatchlings from each of 6 clutches) ranged in size from 4.3 to 4.9 cm in straight carapace length (SCL; mean ± SD = 4.6 ± 0.11) and from 15.3 to 22.4 g in body mass (mean ± SD = 19.8 ± 1.33). Turtles were marked for identification with 2-mm plastic discs glued to the carapace and housed in indoor tanks at 26.5°C (±1°) on a 12:12 light:dark cycle with 20-W full spectrum fluorescent bulbs (vita-light) and 60-W outdoor flood lights. Each turtle was measured (SCL) and weighed every 10 days for the duration of the study. Hatchling and juvenile turtles were fed daily (~3% of body mass). Food remaining after 45 min was removed from the tank. Diets for both phases of feeding trials were purchased in single batches from Mellick Aquafeed (Catawissa, PA) and stored at –4°C. Food sub -samples (n = 9) were collected and analyzed for δ13C and δ15N throughout the study to test for temporal variation in the isotopic composition of experimental diets. At the end of the trials, I released all turtles under Florida Wildlife Conservation Commission guidelines.

Tissues

I analyzed the isotopic composition of whole blood, red blood cells, plasma solutes, skin, and scute samples. I chose these tissues because they can be sampled non-invasively, and one of the goals of my study was to be able to release the turtles unharmed after its completion. In addition, I used blood and its components because they are widely used in stable isotope analyses in vertebrates (with the exception of fish) and are the tissues most widely used in isotopic incorporation studies (Dalerum and Angerbjorn 2005). Approximately 0.2 ml of blood was collected with a 25-gauge needle and syringe from the dorsal cervical sinus and transferred to a non-heparinized container. A sub-sample (0.1 ml) of whole blood was removed and the remaining blood (0.1 ml) was separated into plasma solutes, red blood cells, and white blood cells by centrifugation. After the tissues were separated white blood cells were discarded. Skin samples were collected from the dorsal surface of the neck region using a 2-mm sterile biopsy punch. Scute samples were collected from the newly grown, anterior edge of the second caudal scute by scoring ~6 mm2 with a #21 scalpel blade and peeling the scute from the carapace with forceps.

Trial 1: Hatchling Turtles

Hatchling turtles were fed for 203 days on a pelleted diet in which the main protein source was soy protein isolate (mean $\delta^{13}C \pm$ SD and mean $\delta^{15}N \pm$ SD of bulk diet equaled −22.27 ± 0.26‰, and 3.25 ± 0.47‰, respectively). This diet contained 3% lipids and 30% crude protein. Blood, skin, and scute samples were collected from 108 loggerheads. Because small body size precluded repeated sampling of individuals, I grouped the turtles (2 hatchlings from each clutch per group) and sampled 1 of the 9 experimental groups (12 hatchlings) at each sampling period. Because hatchling turtles assimilate nutrients from the remaining yolk sac for a period of up to two weeks after leaving the egg (Kraemer and Bennett 1981), and due to the acclimation period

needed for hatchlings to begin feeding regularly on the prepared diet, I began my analysis of isotopic change eight days after the turtles were first offered food.

Trial 2: Juvenile Turtles

One group of twelve hatchlings was maintained throughout the hatchling turtle trial under identical environmental conditions but was not sampled. These turtles were the subjects of Trial 2. At the conclusion of the hatchling trial, the remaining turtles in this group (n = 8) were switched to a diet with an animal based protein source (40% protein and 12% lipid) and a different isotopic composition (mean $\delta^{13}C \pm SD$ and mean $\delta^{15}N \pm SD$ of bulk diet equaled −21.29 ± 0.29‰ and 9.45 ± 0.37‰, respectively) for an additional 232 days. At the start of Trial 2, the eight juvenile turtles ranged from 9.0 to 13.1 cm SCL (mean ± SD = 10.6 ± 1.35 cm) and from 105.0 to 385.7 g body mass (mean ± SD = 208.8 ± 97.5 g). I collected the same tissues from juvenile and hatchling turtles, using the same protocols except that I sampled tissues of each juvenile turtle repeatedly.

Sample Preparation and Mass Spectrometry

Skin and scute samples were rinsed in distilled water, finely diced with a scalpel blade and dried to constant weight for 24–48 h at 60°C. Blood samples (whole blood, plasma solutes, and red blood cells) were dried for 24–48 h at 60°C and homogenized with a glass cell homogenizer. Lipids were extracted from dry skin and scute samples with petroleum ether in a Dionex Accelerated Solvent Extractor (ASE®, Dodds et al. 2004). Lipid extraction was not performed on blood components due to the small amount of blood collected. Approximately 450 µg of diet and tissue samples were loaded into pre-cleaned tin capsules, combusted in a COSTECH ECS 4010 elemental analyzer interfaced via a Finnigan-MAT ConFlow III device (Finnigan MAT, Breman, Germany) to a Finnigan-MAT DeltaPlus XL (Breman, Germany) isotope ratio mass spectrometer in the light stable isotope lab at the University of Florida, Gainesville, FL, USA. Stable isotope

abundances are expressed in delta (δ) notation, defined as parts per thousand (‰) relative to the standard as follows:

$$\delta = ([R_{sample}/R_{standard}] - 1)\ (1000) \quad (2\text{-}1)$$

where R sample and R standard are the corresponding ratios of heavy to light isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample and standard, respectively. R standard for ^{13}C was Vienna Pee Dee Belemnite (VPDB) limestone formation international standard. R standard for ^{15}N was atmospheric N_2. IAEA CH-6 ($\delta^{13}C = -10.4$) and IAEA N1 Ammonium Sulfate ($\delta^{15}N = +0.4$), calibrated monthly to VPDP and atmospheric N_2, respectively, were inserted in all runs at regular intervals to calibrate the system and assess drift over time. The analytical precision of my measurements, measured as the SD of replicates of standards, was 0.11‰ for $\delta^{13}C$ and $\delta^{15}N$ (N = 88 and 91, respectively).

Statistical Analyses

I estimated growth rate using both a linear and an exponential model in 45 individual hatchlings (Trial 1) and 8 individual juveniles (Trial 2). I fitted the parameters of linear growth with standard least squares procedures and estimated the fractional growth rate of the exponential model (k g in days−1) using a non -linear fitting procedure (JMP®). To assess whether hatchling and juvenile growth was better described by linear or by exponential models, I compared their coefficients of determination using paired t tests and the difference in Akaike's Information Criteria (AIC) between the two models (Δi = AIC i − AICmin, where AICmin is the smallest value in a comparison and AIC i is the value of the alternative model, Burnham and Anderson 2002). The comparison of r 2 and AIC gave the same results. Both models described my data equally well. Because ontogenetic growth in most animals is well described by sigmoidal functions with an exponential phase during the early stages of development (West et al. 2001;

Zimmerman et al. 2001; Swingle et al. 2005) I chose the exponential over the linear growth model.

I estimated the fractional rate of isotopic incorporation k st (in day^{-1}), using a non-linear fitting procedure (JMP®) using the equation

$$d X(t)=d X(8) +(d X(0) -d X(8))e-kst t \quad (2\text{-}2)$$

where δX(t) is the isotopic composition at time t, δX(∞) is the asymptotic, equilibrium isotopic composition, δX(0) is the initial isotopic composition, and k st is the fractional rate of isotope incorporation of a tissue (O'Brien et al. 2000; Martínez del Rio and Wolf 2005). δX(∞) and δX(0) were estimated by the same non-linear procedure. Hesslein et al. (1993) demonstrated that for tissues growing exponentially k st equals the sum of net growth k gt of a tissue and catabolic turnover k dt (k st = k gt + k dt). If the tissues are at steady state, then growth equals catabolic degradation (k st = k dt). If the tissue is growing exponentially, then I can measure growth and partition the contribution of net growth and catabolic turnover to k st. The term k gt can be measured as the mass specific rate of change in the size of the tissue (k gt), and k dt can be estimated by difference (k dt = k st − k g t; see Hesslein et al. 1993). I assumed that the fractional rate of growth of tissues was the same as that of the whole hatchling (k g) and compared k st with k g using t tests. If k st estimated with Eq. 2-2 was significantly different from k g, I estimated k dt, the contribution of catabolic turnover to the rate of isotopic incorporation, as k st − k g. I estimated isotopic discrimination (ΔX) as δX(∞)tissues − δX diet. In addition, for juvenile turtles, I compared the parameters of isotopic incorporation (k st, $\Delta^{13}C$ and $\Delta^{15}N$) among tissues using univariate repeated measures ANOVA, after checking whether my data set satisfied sphericity assumptions, followed by Tukey's HSD. These analyses were not conducted for hatchling turtles (Trial 1) because their small size precluded repeated samples. To compare visually the

incorporation pattern that would result if accretion was the only process contributing to changes in the isotopic composition of tissues, I used k g instead of k st in Eq. 2-2. To assess the effect of variation in k g in the pattern of incorporation curves, I plotted isotopic incorporation curves using both the average value of k g and k g + SD and k g– SD. Using a symmetrical estimate of variation for k g in this exercise is justified because the distribution of k g values was close to normal [Shapiro–Wilks W = 0.85 (N = 45) and 0.91 (N = 8), P > 0.2, for Trials 1 and 2, respectively]. Equation 2-2 assumes that the time that a C or N molecule stays in a tissue is distributed as a negative exponential with average residence time equal to 1/k st. I used average residence time, rather than the more widely used "half-life" [Ln(2)/k st] because I could estimate a standard error for 1/k st as SE(1/k st) = SE(k st)/k st2, where SE(k st) is the asymptotic standard error of k st (Stuart and Ord 1994). I estimated SE(k st) using the non-linear procedure described above.

Results

Trial 1: Hatchling Turtles

Both linear and exponential models described the growth in mass of hatchlings relatively well (average r^2 ± SD = 0.97 ± 0.02 and 0.98 ± 0.02, respectively, Fig. 2-1a). There was no significant difference between coefficients of determination of these models (paired = 0.91, P = 0.36, N = 45). In 20 comparisons, the exponential model had a higher Δ_i value (Δ_i ranged from 5.3 to 29.9), in 20 the linear models had a higher value (Δ_i ranged from 3.7 to 25.3), and in 5 cases Δ_i equaled 0 (these were the cases in which the coefficient of determination of the two models was identical). Because both models fitted the data set equally well, I chose the exponential model. The exponential model estimated a fractional growth rate equal to 0.014g (±SD = 0.002) day^{-1}. Equation 2-2 described the changes in $\delta^{13}C$ and $\delta^{15}N$ through time after a diet change adequately well in all tissues (r^2 ranged from 0.88 to 0.95; Fig. 2-2). For carbon, only

plasma solutes and whole blood had rates of incorporation that differed significantly from the value expected from growth (Table 2-1). The estimated value of $k_{dt} = (k_{st} - k_g)$ for these two tissues equaled 0.036 and 0.009 day^{-1}, respectively. For nitrogen, the rate of isotopic incorporation into skin and red blood cells was indistinguishable from that expected by growth alone (Table 2-2). However, the incorporation into scute, plasma solutes, and whole blood was higher than that expected from growth. The value of k_{dt} for these tissues equaled 0.008, 0.040, and 0.014 day^{-1}, respectively. The fractional rates of incorporation of $\delta^{13}C$ and $\delta^{15}N$ were tightly and linearly correlated ($r = 0.99$, $P < 0.0006$, Fig.2-3). Both $\Delta^{13}C$ and $\Delta^{15}N$ varied widely among tissues. $\Delta^{13}C$ ranged from−0.64 to 2.62‰ (Ta ble 2-1) and $\Delta^{15}N$ ranged from−0.25 to 1.65‰ (Table 2-2).

Trial 2: Juvenile Turtles

Both linear and exponential models described the growth in mass of hatchlings relatively well (average $r^2 \pm$ SD = 0.96 ± 0.02 and 0.96 ± 0.02, respectively, Fig. 2-1b). In four comparisons, the exponential model had a higher Δ_i value (Δ_i ranged from 5.5 to 17.5) and in four the linear models had a higher value (Δ_i ranged from 4.3 to 26.3). Because both models described the data equally well, I assumed that turtles grew exponentially with a fractional growth rate (k_g) equal to 0.012 ± 0.001g day^{-1}). Equation 2-2 described the changes in $\delta^{13}C$ and $\delta^{15}N$ after a diet change adequately (r^2 ranged from 0.92 to 0.96, Fig.2-4). The rate of fractional incorporation (mean ± SD = 0.027 ± 0.010 day^{-1}) and residence time ($1/k_{st}$, mean ± SD = 44.7 ± 25.0 days) of carbon did not differ significantly among tissues [RM ANOVA, $F_{4,28}$ (tissue) = 1.02, $P = 0.41$ and $F_{4,24} = 0.37$, $P = 0.82$, respectively, Fig. 2-4, Table 2-3]. The value of k_{st} was significantly different from that estimated assuming that growth was the sole determinant of isotopic incorporation (i.e., $k_g = 0.012$ day^{-1}) in all tissues. Thus, replacement of carbon lost through catabolic turnover ($k_{dt} = k_{st} - k_g$) contributed significantly to the rate of isotopic incorporation

(Table 2-3). The rate of catabolic turnover for carbon did not differ among tissues (mean ± SD = 0.015 ± 0.011, RM ANOVA $F_{4,24} = 1.02$, $P = 0.41$) and was significantly different from 0 in all tissues (one-sample t ranged from 3.16 to 4.67, $P < 0.02$). The isotopic discrimination ($\Delta^{13}C = \delta^{13}C_{tissue} - \delta^{13}C_{diet}$) differed significantly among tissues (RM ANOVA $F_{4,24} = 48.40$, $P < 0.001$, Table 2-3). All tissues had significantly positive isotopic discrimination relative to bulk diet (Table 2-3) except that of plasma solutes which was statistically indistinguishable from 0.

The rate of nitrogen fractional incorporation and its residence time differed significantly among tissues [RM ANOVA, $F_{4,28}$ (tissues) = 7.0, $P = 0.0007$ and $F_{4,28}$ (tissues) = 10.39, $P = 0.0001$, respectively, Fig. 2-4, Table 2-4]. The value of k_{st} was significantly different from that estimated assuming that growth was the sole determinant of isotopic incorporation (i.e., $k_g = 0.012$) in all tissues. Thus replacement of nitrogen lost through catabolic turnover contributed significantly to the rate of isotopic incorporation. The rate of catabolic turnover (k_{dt}) of nitrogen also differed significantly among tissues [RM ANOVA, $F_{4,28}$ (tissues) = 7.10, $P = 0.0006$] and was significantly different from 0 in all tissues (t ranged from 4.3 to 11.0, $P < 0.01$, Table 2-4). The nitrogen isotopic discrimination ($\Delta^{15}N$) differed significantly among tissues [RM ANOVA, $F_{4,28}$ (tissues) = 85.82, $P < 0.001$] and was significantly positive only for skin and plasma solutes. Red blood cells and whole blood had $\Delta^{15}N$ values that did not differ from 0, and scute tissue was significantly depleted in ^{15}N relative to diet (Table 2-4). The rate of fractional incorporation of nitrogen was more variable among tissues than that of carbon ($F_{5,5} = 65.34$, $P < 0.001$, Fig. 2-3), and unlike in Trial 1, these rates were not correlated (mean r ± SD = −0.069 ± 0.65, $P = 0.78$, $N = 8$).

Discussion

To my knowledge, this is the first study in which both the isotopic incorporation and the isotopic discrimination factor in a variety of tissues is reported for a reptile. Indeed, there is a

paucity of studies on the differences in isotopic incorporation and discrimination factors among tissues in ectothermic vertebrates. My results demonstrate that (1) in both hatchling and juvenile turtles growth contributes significantly to the rate of isotopic incorporation, and (2) this contribution differed among tissues. In addition, (3) my results suggest that discrimination factors varied greatly among tissues, and perhaps among diets and/or developmental stages. Here I discuss each of these themes and consider their implications. My discussion is limited by the absence of comparable data sets on other ectotherms, and hence I framed some of the implications of my study as hypotheses to be tested rather than as conclusive patterns.

Contributions of Growth and Catabolic Turnover to the Rate of Isotopic Incorporation

The rates of incorporation of dietary C and N differed among tissues in both hatchling and juvenile turtles, but the variation among tissues was considerably smaller than that found in other studies. In gerbils, half-lives of carbon in different tissues varied from 6.4 to 47.5 days (Tieszen et al. 1983); in Japanese quail, half-lives of carbon varied from 2.6 to 173.3 days (Hobson and Clark 1992). In juvenile turtles the half-life, or median residence time, of carbon [estimated by multiplying the average residence times in Tables 2-1, 2-2, 2-3, 2- 4 by Ln(2) = 0.69] ranged from 27 to 35 days and that of nitrogen ranged from 11 to 31 days. Variation among tissues was slightly higher for hatchling turtles, but it still was lower than that found in previous studies (Tieszen et al. 1983; Hobson and Clark 1992). The median residence time of carbon in hatchlings ranged from 14 to 57 days and that of nitrogen ranged from 13 to 49 days. In agreement with other studies (summarized by Dalerum and Angerbjorn 2005), plasma solutes had relatively high incorporation rates of C and N in both trials.

I hypothesize that the relative homogeneity in rates of isotopic incorporation among tissues is probably due to the rapid growth that masked potential differences in catabolism among tissues. In hatchling turtles, several tissues had rates of incorporation that were indistinguishable from

whole body growth rate. In the tissues that differed, the contribution of growth rate to incorporation ranged from ≈30% (in plasma solutes) to 60% (in scute, Tables 2-1, 2-2). In juveniles, the contribution of growth rate to isotopic incorporation was high as well, and ranged from 31 to 46% for carbon, and from 15 to 52% for nitrogen. High contributions of growth to isotopic incorporation have been reported in several species of fish, tadpoles, and two species of snails (McIntyre and Flecker 2006). Indeed, as in my study, McIntyre and Flecker (2006) reported that incorporation rates were very similar to growth rates in catfish and tadpoles. The contribution of growth to the rate of isotopic incorporation in the tissues of these ectotherms is high relative to that reported by MacAvoy et al. (2005) for adult mice in which growth accounted for only ≈10% of the rate of incorporation of carbon and nitrogen.

These observations could lead one to hypothesize that there is a difference in the relative contribution of growth and catabolic turnover to the rate of isotopic incorporation between endotherms (mice) and ectotherms (fish, amphibians, and reptiles). Although this hypothesis has merit, it must be qualified by differences in the developmental stages of the endotherms and ectotherms that have been investigated. The mice studied by MacAvoy et al. (2005) were close to their asymptotic, maximal size, whereas most of the studies on ectotherms have been conducted in rapidly growing animals. West et al. (2001) have hypothesized that the fraction of energy and nutrients used for growth, relative to other functions, is roughly the same for all species at the same stage of development, as measured relative to their asymptotic mass. Thus a newborn calf and a 6-year-old cod are at the same developmental stage (1/15th of their asymptotic mass) and should devote roughly the same fraction of their energy/nutrients to growth (Kohler 1964; West et al. 2001). Following West et al. (2001), I hypothesize that the relative contribution of growth to isotopic incorporation will be roughly the same in ectotherms and endotherms, provided that the

animals are measured at comparable developmental stages (as defined above). This hypothesis implies that, in general, growth rates will be more important determinants of isotopic incorporation in ectotherms than in endotherms. Among vertebrates, endotherms reach their asymptotic mass in a relatively short time and then stop growing (they are "determinate growers"), whereas many (albeit not all) ectotherms continue growing for most of their lives (they are "indeterminate growers", Sebens 1987).

The effect of growth on the rate of isotopic incorporation has several consequences for the interpretation of isotopic measurements in the field. The first one was recognized by Perga and Gerdeaux (2005). These authors found that the isotopic composition of muscle in whitefish reflected the isotopic composition of prey consumed only in the spring and summer, when the somatic tissues of fish were growing. In contrast, the isotopic composition of liver, which had a higher contribution of catabolic turnover, tracked the isotopic composition of the diet closely throughout the year. Perga and Gerdeaux (2005) concluded that stable isotope analyses may be deceptive if the tissue measured reflects only the isotopic composition of food ingested during the time when the tissue is growing. Because many ectothermic vertebrates grow seasonally (Castanet 1994; Youngson et al. 2005), the confounding effects of seasonal growth on stable isotope analyses are probably a prevalent, albeit so far relatively unstudied, potentially confounding factor in stable isotope field studies. In seasonal environments, the isotopic composition of "slow" tissues, such as muscle may reflect the integration of dietary inputs over the growing season.

Stable isotopes can provide an integrated view of animal diets (Araujo et al. 2007). However, the time window of integration depends on the rate at which animals incorporate the isotopic composition of their diets (Newsome et al. 2007). My study demonstrates that growth rate is an important determinant of isotopic incorporation rate, and thus of the time window of

integration of diet's composition. Carleton and Martínez del Rio (2005) demonstrated an allometric relationship between the rate of isotopic incorporation and body size in full-grown birds. Because growth rate is an allometric function of size (West et al. 2001), it is likely that the window of isotopic integration of diets is size-dependent in animals with "indeterminate" growth.

A second consequence of the effect of growth on the rate of isotopic incorporation is that growth can reduce the differences in the isotopic incorporation rates among tissues, and thus limit the usefulness of measuring the isotopic composition of different tissues to investigate diet at different time scales (Dalerum and Angerbjorn 2005). The homogenizing effect of growth may also reduce the application of the isotopic clock proposed by Phillips and Eldrige (2006). Phillips and Eldrige (2006) demonstrated that confidence in the isotopic clock increases as the difference in incorporation rates between tissues increases. My results suggest that growth reduces the differences in isotopic incorporation among tissues, but it does not eliminate them. In both hatchling and juvenile loggerheads, plasma solutes had consistently high incorporation rates that, in all cases, were the result of a significant contribution of catabolic turnover (Tables 2-1, 2-2, 2-3, 2-4). Significantly the incorporation rate of plasma was higher, and thus the average residence time was shorter, than that of red blood cells. Plasma proteins are primarily synthesized in the liver (Turner and Hulme 1970; Adkins et al. 2002), a tissue with high rates of protein turnover and hence with high rates of isotopic incorporation (Haschemeyer and Smith 1979; Dalerum and Angerbjorn 2005). It is likely that liver and plasma proteins are in isotopic equilibrium (Tsudaka et al. 1971). The observation of a consistent difference in the rate of incorporation of blood cells and plasma proteins is significant because blood is one of the easiest tissues to sample non-invasively in vertebrates and a single blood sample yields two "tissues" with different rates of isotopic incorporation.

Assumption and Caveats in the Estimation of the Effect of Growth Rate on Isotopic Incorporation

My estimates of the relative contribution of growth rate and catabolic turnover must be qualified by the assumptions that were made. I used the approach of Hesslein et al. (1993) to partition the contributions of growth and catabolism to the rate of isotopic incorporation. Using this approach requires that the animals are growing exponentially (Hesslein et al. 1993) and that growth rates do not differ among tissues. In my study, turtle growth was very closely approximated by exponential functions (Fig. 2-1), and hence the first of Hesslein et al.'s (1993) assumptions was satisfied. Unfortunately, I have no growth data for the tissues used in my study and cannot confirm the second assumption. However, tissue mass usually scales isometrically with body mass (Brown et al. 2000; Carleton and Martínez del Rio 2005) and hence the fractional rate of tissue growth can probably be estimated by that of the whole body (Iverson 1984; Miller and Birchard 2005).

Differences in Isotopic Discrimination Among Tissues and Between Age Classes

The isotopic discrimination of nitrogen, defined as $\Delta^{15}N = \delta^{15}N_{tissues} - \delta^{15}N_{diet}$ when the animal's tissues and diet are in equilibrium (Cerling and Harris 1999), is at the heart of the isotopic approach used to diagnose an animal's trophic position. Most, albeit not all, studies that aim to diagnose an animal's trophic position, use isotopic measurements of muscle or of the animal's homogenized whole bodies (Post 2002; McCutchan et al. 2003; but see Bósl et al. 2006 and Wallace et al. 2006 as examples of studies using other tissues). However, one of the virtues of isotopic measurements is that they allow studying important aspects of an animal's ecology non-invasively (Gustafson et al. 2007). My experiments allowed me to assess the variation in isotopic discrimination among tissues, and thus the feasibility of using tissues that can be collected non-invasively in food web studies.

Isotopic discrimination of $\delta^{13}C$ differed significantly among tissues between age classes. In hatchling turtles only skin and whole blood showed positive isotopic discrimination. In juvenile turtles, with the exception of plasma, all tissues showed small, though significant, positive isotopic discrimination. The carbon isotopic composition of plasma solutes was statistically indistinguishable from that of the diet. The isotopic discrimination of tissues ranged from −0.38‰ in plasma solutes to 1.77‰ in scute, a difference of 2.15‰. My results are consistent with the values reported for isotopic discrimination of carbon (from −1.5 to 3.4‰; Hesslein et al. 1993; Hobson et al. 1993; Pinnegar and Poulin 1999; Roth and Hobson 2000; Lesage et al. 2002; Pearson et al. 2003; McCutchan et al. 2003; Seminoff et al. 2006), but several of the values reported here (1.77 and 2.62‰) are higher than the commonly accepted carbon discrimination of from 0 to 1‰ (DeNiro and Epstein 1978; Peterson and Fry 1987).

Isotopic discrimination of $\delta^{15}N$ also differed significantly among tissues and between age classes. Isotopic composition of $\delta^{15}N$ in hatchling tissues relative to that of the diet was positive for skin, scute, whole blood, and plasma solutes but negative for red blood cells. Isotopic discrimination of nitrogen ranged from 1.65‰ in skin to −0.25‰ (a value that did not differ significantly from 0) in red blood cells. Juvenile turtle skin and plasma solutes had $\delta^{15}N$ values that were significantly positive, red blood cells and whole blood had values that did not differ from 0, whereas scute tissue was significantly depleted in $\delta^{15}N$ relative to diet. Isotopic discrimination ranged from −0.64 to 1.60‰.

Why did the turtles' tissues have low $\Delta^{15}N$ values? A mathematical model crafted by Martínez del Rio and Wolf (2005) predicts that $\Delta^{15}N$ decreases as the ratio of nitrogen incorporation in tissues exceeds the ratio of nitrogen loss. Because this ratio is higher in growing young animals than in non-growing adults, Martínez del Rio and Wolf (2005) predicted a lower

$\Delta^{15}N$ in growing animals than in non-growing ones. My results support this prediction. In a meta-analysis Vanderklift and Ponsard (2003) found significant variation in $\Delta^{15}N$ among the tissues of birds and mammals. The wide inter-tissue variation in $\Delta^{15}N$ in loggerhead turtles described here, suggests that this phenomenon may be common among vertebrates. The differences in $\Delta^{15}N$ among tissues in loggerhead turtles and those reported by Vanderklift and Ponsard (2003) have not been explained adequately, but have consequences for the interpretation of results of field studies that increasingly rely on tissues that can be sampled non-invasively (Sullivan et al. 2006). A factor that may explain differences in $\Delta^{15}N$, and perhaps $\Delta^{13}C$, among tissues is variation in amino acid profiles. The $\delta^{13}C$ and $\delta^{15}N$ of individual amino acids can vary significantly (McClelland and Montoya 2002; Fogel and Tuross 2003), and thus differences in the amino acid composition of a tissue can lead to differences in isotopic discrimination among tissues (Howland et al. 2003). Pinnegar and Polunin (1999) postulated that amino acid profiles could influence the discrimination factor of different tissues. However, to my knowledge, this effect has not been investigated systematically.

The nitrogen isotopic composition of an animal's tissues is widely used to diagnose trophic position in food webs (reviewed by Post 2002; Vanderklift and Ponsard 2003; McCutchan et al. 2003). The $\Delta^{15}N$ values in this study were lower than the $\Delta^{15}N$ = 3.4‰ and the 2.3‰ values reported as the average discrimination factor for muscle and whole animal isotopic measurements by Post (2002) and McCutchan et al. (2003), respectively (see also DeNiro and Epstein 1978; Peterson and Fry 1987; Kelly 1999). Seminoff et al. (2006) also reported low $\Delta^{13}C$ and $\Delta^{15}N$ values that differed greatly among several soft tissues (red blood cells, plasma solutes and skin) of a sea turtle (*Chelonia mydas*). The comparison between the values reported in this study and those reviewed by Post (2002), McCutchan et al. (2003), and Vanderklift and Ponsard (2003) must be

qualified by the observation that I did not use the tissues used in these reviews: muscle and whole animal homogenates. However, because the estimation of trophic level is sensitive to variation in $\Delta^{15}N$ (Post 2002), studies that aim to estimate trophic position using stable isotopes may have to account for the type of tissue used (McCutchan et al. 2003). The increased reliance of researchers on minimally-invasive isotopic analyses demands that we begin understanding the variation in $\Delta^{15}N$ among tissues.

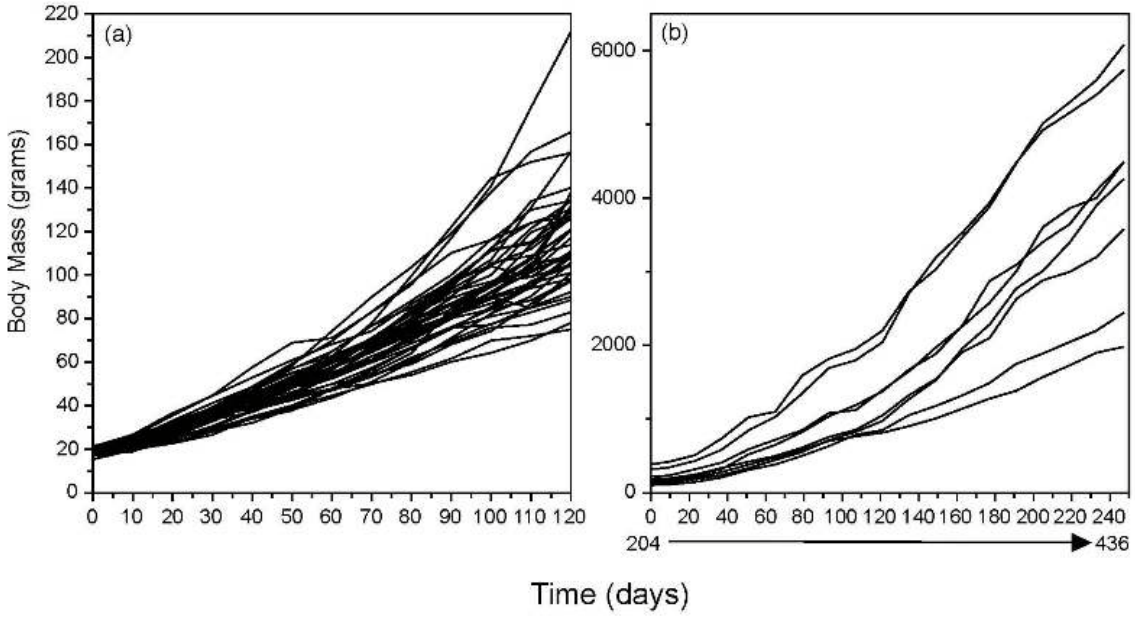


Figure 2-1. Growth in a hatchling and b juvenile loggerhead turtles (Caretta caretta). Each line represents the growth trajectory of an individual. Growth trajectories are well described by exponential functions of the form y = ae(bt).

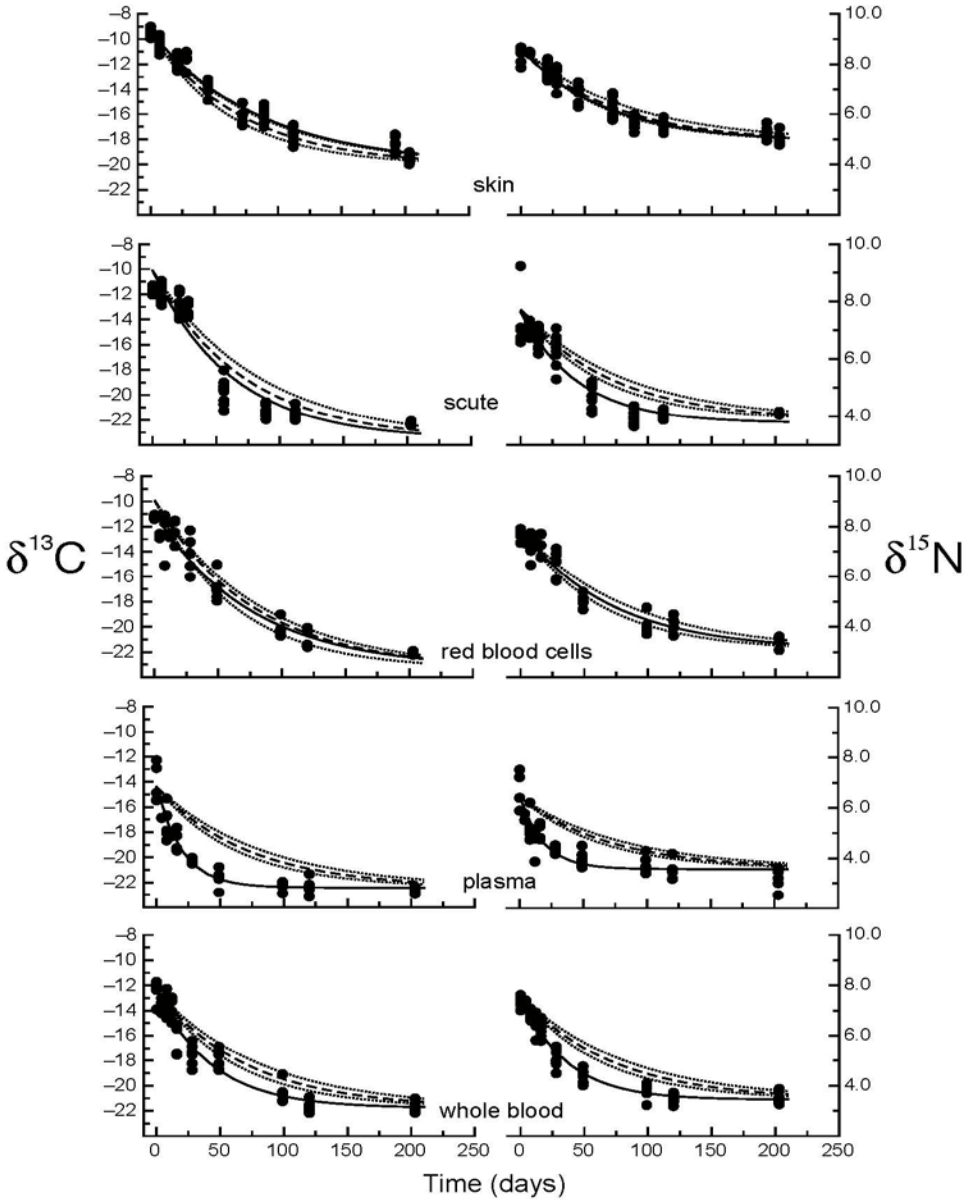


Figure 2-2. Changes in $\delta^{13}C$ and $\delta^{15}N$ in loggerhead turtle hatchlings 0–203 days after a diet change (see Trial 1, Eq. 2-2); curves fitted by a non-linear routine (line). Expected levels of $\delta^{13}C$ and $\delta^{15}N$ if growth rate (k g = 0.014 day−1) was the sole determinant of the rate of isotopic incorporation shown (dashed line) with k g ± 1 SD (dotted line).

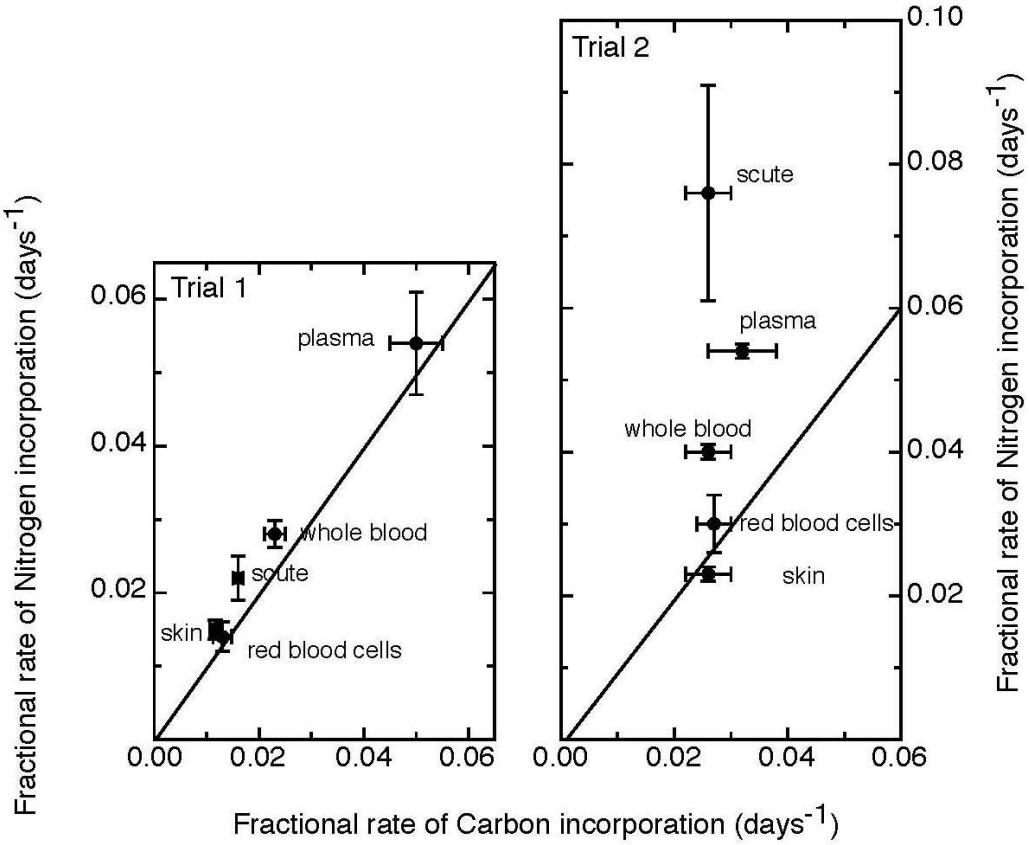


Figure 2-3. Correlations of fractional incorporations of carbon and nitrogen into skin, scute, red blood cells, plasma, and whole blood of loggerhead turtles in a Trial 1 ($r = 0.99$, $P < 0.0006$) and b Trial 2 (NS), mean ± 1 SE. The diagonal line represents $y = x$.

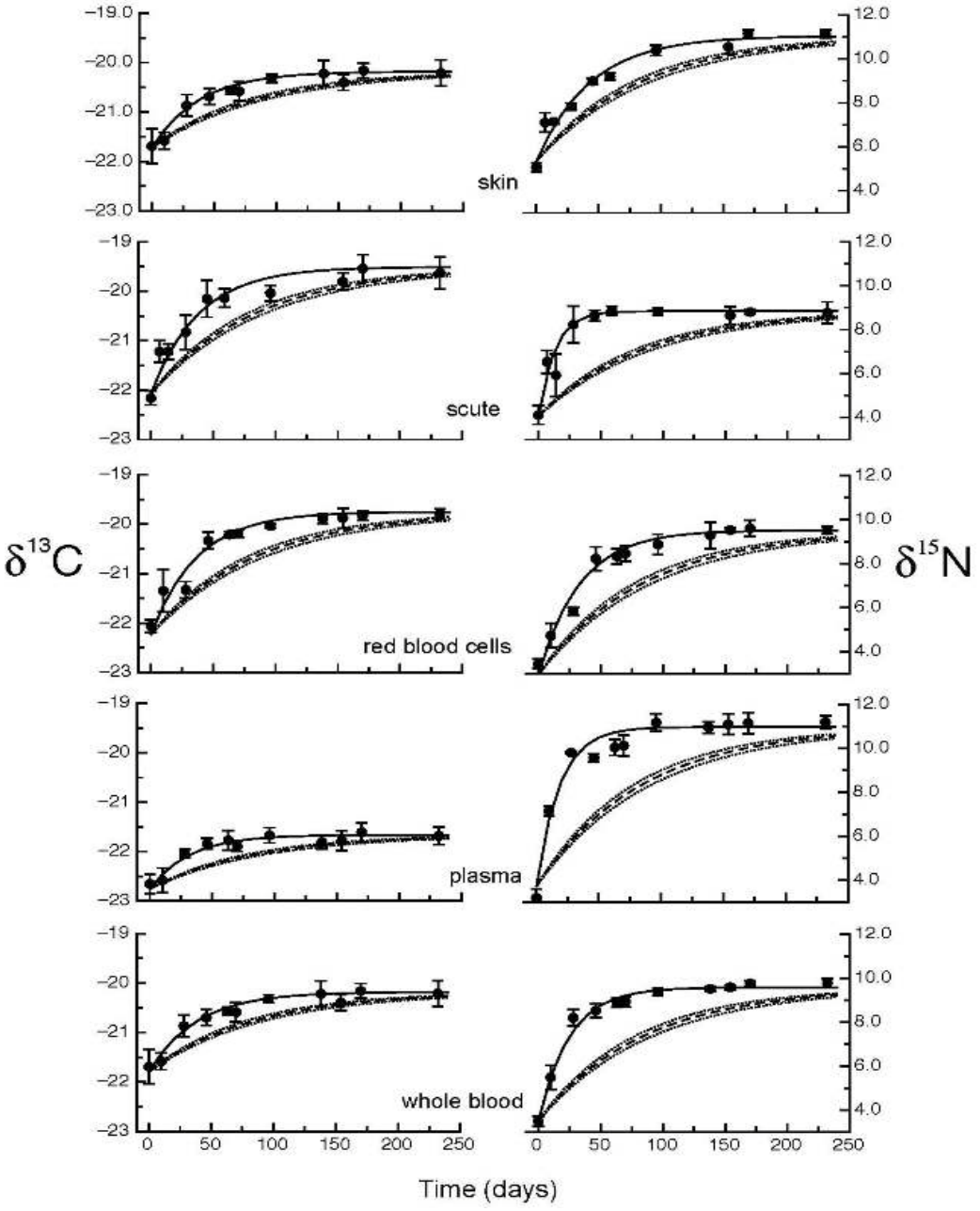


Figure 2-4. Changes in $\delta^{13}C$ and $\delta^{15}N$ in loggerhead turtle juveniles 0–232 days after a diet change (see Trial 2, Eq. 2-2); curves fitted by a non-linear routine (line). Expected levels of $\delta^{13}C$ and $\delta^{15}N$ if growth rate (k g = 0.012 day−1) was the sole determinant of the rate of isotopic incorporation shown (dashed line) with k g ± 1 SD (dotted line).

Table 2-1. In Trial 1, the isotopic incorporation of carbon from diet into tissues of hatchling loggerhead turtles was well described by the equation $\delta^{13}C(t)=\delta^{13}C(\infty)+(\delta^{13}C(0)-\delta^{13}C(\infty))e^{-k_{st}t}$. The value of k_{st} was not significantly different from that estimated assuming that growth was the sole determinant of isotopic incorporation (i.e., k_g = 0.014) in skin, scute, and red blood cells (t-value; ns denotes not significant). However, plasma solutes and whole blood had higher rates of isotopic incorporation, than those expected from growth (** indicates $p < 0.01$). $\Delta^{13}C$ = diet-tissue discrimination (one-sample t test, * and ** indicate significant difference from 0 with $p < 0.05$ and 0.01, respectively; ns = not significantly different from 0). Average residence time was estimated as $1/k_{st}$.

Tissue	*Equation*	*t-value*	$\Delta^{13}C$	*Average residence time (days)*
Skin	$-20.08+10.65e^{-0.012(time)}$	1.2(ns)	2.62 ± 0.34(**)	83.0 ± 7.02
Scute	$-23.56+13.44e^{-0.016(time)}$	1(ns)	-0.86 ± 0.57(ns)	62.5 ± 7.31
Red blood cells	$-23.34+13.44e^{-0.013(time)}$	0.5(ns)	-0.64 ± 0.73(ns)	76.9 ± 11.34
Plasma solutes	$-22.41+8.00e^{-0.050(time)}$	18(**)	0.29 ± 0.20(ns)	20.0 ± 6.34
Whole blood	$-21.78+9.32e^{-0.023(time)}$	3.75(**)	0.92 ± 0.34(*)	43.5 ± 2.34

Table 2-2. In Trial 1, the isotopic incorporation of nitrogen from diet into tissues of hatchling loggerhead turtles was well described by the equation $\delta^{15}N(t)=\delta^{15}N(\infty)+(\delta^{15}N(0)-\delta^{15}N(\infty))e\text{-}kstt$. The value of kst was not significantly different from that estimated assuming that growth was the sole determinant of isotopic incorporation (i.e., kg = 0.014) in skin and red blood cells (t-value, ns denotes not significant). However, scute, plasma solutes and whole blood had higher rates of isotopic incorporation than those expected from growth (** $p < 0.01$). $\Delta^{15}N$ = diet-tissue discrimination (one-sample t test, * and ** indicate significant difference from 0 with $p < 0.05$ and 0.01, respectively; ns = not significantly different from 0). Average residence time was estimated as 1/kst.

Tissue	*Equation*	*t-value*	$\Delta^{15}N$	*Average residence time (days)*
Skin	$4.91+3.77e^{-0.015(time)}$	0.5 ns	1.65 ± 0.12(**)	66.7 ± 7.36
Scute	$3.86+3.86e^{-0.022(time)}$	4.0(**)	0.61 ± 0.16(**)	45.5 ± 5.48
Red blood cells	$3.08+4.82e^{-0.014(time)}$	0 (ns)	-0.25 ± 0.30(ns)	71.4 ± 10.66
Plasma solutes	$3.57+2.86e^{-0.054(time)}$	20(**)	0.32 ± 0.09(ns)	18.5 ± 4.25
Whole blood	$3.44+4.15e^{-0.028(time)}$	7(**)	0.19 ± 0.08(*)	35.7 ± 2.73

Table 2-3. In Trial 2, isotopic incorporation of carbon from diet into tissues of juvenile loggerhead turtles was well described by the equation $\delta^{13}C(t)=\delta^{13}C(\infty)+(\delta^{13}C(0)-\delta^{13}C(\infty))e^{-k_{st}t}$. The rate of fractional incorporation (k_{st}) did not differ significantly among tissues (RM ANOVA; values in Equation column). k_{dt} is rate of catabolic turnover of carbon; $\Delta^{13}C$ = diet-tissue discrimination; average residence time was estimated as $1/k_{st}$. * and ** indicate when a one-sample t test revealed that k_{dt} or $\Delta^{13}C$ was significantly different from 0 with $p < 0.05$ and 0.01, respectively; ns = not significant. Means labeled by the same letter are not different from each other (RM ANOVAs).

Tissue	*Equation*	k_{dt}	$\Delta^{13}C$	*Average residence time (days)*
Skin	$-20.18-1.54e^{-0.026(time)}$ a	0.014 ± 0.004(**) a	1.11 ± 0.17(**) b	46.1 ± 8.9 a
Scute	$-19.51-2.56e^{-0.026(time)}$ a	0.013 ± 0.003(**) a	1.77 ± 0.58(*) a	50.9 ± 13.14 a
Red blood cells	$-19.75-2.48e^{-0.027(time)}$ a	0.014 ± 0.003(**) a	1.53 ± 0.17(**) ab	40.1 ± 3.4 a
Plasma solutes	$-21.66-1.09e^{-0.031(time)}$ a	0.019 ± 0.006(**) a	-0.38 ± 0.21(ns) c	39.6 ± 9.1 a
Whole blood	$-20.18-1.61e^{-0.026(time)}$ a	0.014 ± 0.004(**) a	1.11 ± 0.18(**) b	46.1 ± 8.9 a

Table 2-4. In Trial 2, the incorporation of the nitrogen isotopic composition of diet into the tissues of juvenile loggerhead turtles was well described by the equation $\delta^{15}N(t)=\delta^{15}N(\infty)+(\delta^{15}N(0)-\delta^{15}N(\infty))e^{-k_{st}t}$. The rate of fractional incorporation (k_{st}) differed significantly among tissues (RM ANOVA; values in Equation column). k_{dt} is rate of catabolic turnover of carbon; $\Delta^{15}N$ = diet-tissue discrimination; average residence time was estimated as $1/k_{st}$. * and ** indicate when a one-sample t test revealed that k_{dt} or $\Delta^{15}N$ was significantly different from 0 with $p < 0.05$ and 0.01, respectively; ns = not significant. Means labeled by the same letter are not different from each other (RM ANOVAs).

Tissue	*Equation*	k_{dt}	$\Delta^{15}N$	*Average residence time (days)*
Skin	$11.04-5.69e^{-0.023(time)}$ b	0.011 ± 0.001(**) b	1.60 ± 0.07(**) a	44.9 ± 3.1 a
Scute	$8.84-4.78e^{-0.076(time)}$ a	0.064 ± 0.015(**) a	-0.64 ± 0.09(**) c	16.2 ± 2.3 c
Red blood cells	$9.51-6.57e^{-0.030(time)}$ b	0.017 ± 0.004(**) b	0.16 ± 0.08(ns) b	36.3 ± 3.4 ab
Plasma solutes	$10.96-7.24e^{-0.054(time)}$ b	0.042 ± 0.008(**) ab	1.50 ± 0.17(**) a	22.5 ± 5.1 bc
Whole blood	$9.59-6.06e^{-0.040(time)}$ b	0.027 ± 0.004(**) b	0.14 ± 0.06(ns) b	27.7 ± 3.5 bc

CHAPTER 3
THE "LOST YEARS" OF GREEN TURTLES: USING STABLE ISOTOPES TO STUDY CRYPTIC LIFESTAGES

Introduction

Species with cryptic lifestages – lifestages in unknown or inaccessible locations – pose a special challenge to scientists and conservationists. My study demonstrates how stable isotopes can be used to evaluate the ecology of an unknown or inaccessible lifestage of an organism. I used stable isotopes to study the early juvenile stage of green turtles, *Chelonia mydas*, a lifestage of unknown location. I solved a 50-year mystery in the biology of marine turtles posed by Archie Carr in 1952: where do green turtles spend their first years of life?

After leaving the nesting beach as 5-cm hatchlings, green turtles "disappear" until they recruit to neritic habitats as > 20 cm juveniles and feed primarily on seagrasses and algae. Archie Carr (1952) identified finding where "hatchling and post hatchling turtles go and what do they do" during their "lost" years as critical for the restoration of green turtle populations.

In 1986, Carr postulated that the early juvenile stage of all sea turtle species was spent in the surface waters of oceanic habitats (Carr 1986; 1987). Since that time, we have learned that North Atlantic loggerheads, *Caretta caretta*, conform to Carr's hypothesis, and spend their first 10 years in oceanic habitats feeding primarily on sea jellies and salps (Bolten et al. 1998; Bjorndal et al. 2003; Bolten 2003a). Carr's hypothesis has been generally accepted as the working hypothesis for other sea turtle species (Musick and Limpus 1997). However, extensive searching in the North Atlantic have yielded thousands of sightings of loggerheads, but green turtles are rarely seen (Witherington 2002; Bolten 2003a,b). Therefore, whether green turtles undergo an ontogenetic shift from oceanic to neritic habitats remains a question.

Stable isotopes of nitrogen and carbon have been used to study migration, feeding ecology, and trophic structure in marine and terrestrial ecosystems (Hobson and Welch 1992; Post 2002;

Cerling et al. 2006). Levels of ^{15}N are used to determine trophic position. In the marine environment, carbon isotopes can distinguish between oceanic and neritic habitat use. Stable isotope values in keratinized tissues have been used to track changes in diet and habitat in baleen whales (Hobson and Schell 1998).

I tested Carr's hypothesis with the stable isotope record stored in green turtle scute tissue – the hard, keratinized tissue covering the boney shell of most chelonians. Scute is continually produced over the entire surface, so as a turtle grows and the boney shell increases in area, scute accumulates and becomes thicker over the older areas, while areas of recent growth expansion are covered by only thin, young scute tissue. Once produced, scute is inert and, although it is susceptible to wear, retains a history of diet and habitat. I used stable isotope values from young loggerheads in oceanic habitats to evaluate the diets and habitats of "lost year" green turtles. If Carr is correct, the oldest scute removed from green turtles newly recruited to neritic foraging grounds should contain a stable isotope signature similar to that of the oceanic-stage loggerheads and the signature of the youngest tissue should approach that of resident green turtles in neritic habitats (Fig. 3-1a).

Methods

Scute samples were collected between 2001 and 2005 from two regions. At a long-term study site off Great Inagua, Bahamas (Bjorndal et al. 2005), samples were collected from 16 previously untagged green turtles and 2 previously untagged hawksbills < 36 cm straight carapace length (SCL). These turtles (recruits) were assumed to have recruited to the study area in the previous year because a saturation mark-recapture study has been conducted at this site for over 30 years. Samples were also collected from 28 green turtles tagged in previous years and thus known to have been resident for at least 1 year (residents). In Florida, samples were collected opportunistically from 11 green turtles, 2 hawksbills, and 1 Kemp's ridley (all < 36 cm SCL) that

stranded dead on the east coast. To minimize the possibility of stable isotope values being affected by body condition (Hobson et al. 1993), samples were only collected from turtles in apparent good health prior to death (e.g., turtles killed by boat strikes or drowning in fishing nets). Only isotope values from the oldest tissues were determined for Florida turtles; because turtle carcasses can float long distances before stranding, the habitat at time of death could not be determined.

Sample Collection

I used sterile biopsy punches, with 6 mm diameter to remove scute samples encompassing the full depth of the scute from the surface (oldest scute) to the origin (newest scute). Samples were collected from the posterior and anterior sites of the second lateral scute (Fig. 2-2). Samples from the Bahamas were stored in 70% ethanol and samples from stranded turtles (previously frozen) were kept frozen until preparation for stable isotope analysis.

Method of Collection of Scute Layers: Each scute sample was cleaned with isopropyl alcohol, rinsed in distilled water, and dried at 60°C for at least 24 hr. Lipids were then removed from all samples using an Accelerated Solvent Extractor with petroleum ether as the solvent. Posterior scute was ground to a depth of 50 µm (yielding ~ 500 µg) from the dorsal side of each sample using a carbide end mill. I collected successive layers of scute by repeating this procedure on samples collected in 2005 from 8 green turtle recruits. The depth of each layer was dictated by the minimum quantity needed (~500 µg) for analyses. Anterior scute samples were too thin to collect multiple layer sub-samples; anterior scute samples were homogenized with a razor blade.

Stable Isotope Analysis

All samples were combusted in a COSTECH ECS 4010 elemental analyzer interfaced via a Finnigan-MAT ConFlow III device (Finnigan MAT, Breman, Germany) to a Finnigan-MAT DeltaPlus XL (Breman, Germany) isotope ratio mass spectrometer in the light stable isotope lab

at the University of Florida, Gainesville, Florida, USA. Stable isotope abundances were expressed in delta (δ) notation, defined as parts per thousand (‰) relative to the standard as follows:

$$\delta = ([R_{sample}/R_{standard}] - 1)\ (1000) \quad (3\text{-}1)$$

where R_{sample} and $R_{standard}$ are the corresponding ratios of heavy to light isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample and international standard, respectively. $R_{standard}$ for ^{13}C was Vienna Pee Dee Belemnite (VPDB) and for ^{15}N was atmospheric N_2. Internal standards were inserted in all runs at regular intervals to calibrate the system and assess drift over time. The analytical accuracy of measurements, measured as the SD of replicates of standards, was 0.11‰ for both $\delta^{13}C$ and $\delta^{15}N$ (*n* = 88 and 91, respectively). Statistical analyses were performed with S-Plus software (v. 7.03; Insightful Corporation).

Results

Signatures of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were significantly different (Wilcoxon rank sum tests, $P < 0.0001$, n = 16, 16) between the oldest and youngest scute tissues from green turtles that had recruited within the previous year to neritic seagrass habitats (recruits; n = 16; Fig. 2-1b). Isotope signatures were not significantly different between Azores loggerheads and the oldest tissues from green turtle recruits (Wilcoxon rank sum tests, n = 12, 16; $P = 0.423$ for $\delta^{13}C$ and $P = 0.593$ for $\delta^{15}N$). The youngest scute tissues from green turtle recruits and those of green turtles resident in the same neritic seagrass habitat for at least 1 year (residents), did not differ significantly in $\delta^{15}N$ values (Wilcoxon rank sum test, n = 16, 28; $P = 0.150$), but differed in $\delta^{13}C$ ($P = 0.0003$).

Discussion

My data support Archie Carr's hypothesis. Stable isotopes in scute tissue reveal that, before recruiting to neritic habitats, juvenile green turtles occupy similar habitats and feed at the same

trophic level as do oceanic-stage loggerheads. As predicted, the isotope values of youngest scute tissue from recent recruits approach those of residents on neritic foraging grounds. The $\delta^{15}N$ values are not significantly different between these two groups, but the $\delta^{13}C$ values are significantly lower in recruits, indicating that incorporation of the new nitrogen signature into scute tissue is more rapid than that of carbon. This pattern matches the relative rates of N and C incorporation into scute in captive juvenile loggerheads (Reich et al.2008) and provides further support that N and C incorporations can be uncoupled and cannot be assumed to be equal (Hobson and Stirling 1997; Hobson and Bairlein 2003; Carleton and Martínez del Rio 2005).

A few data points in Fig. 3-1b do not conform to the general pattern. The youngest scute point (square) in the midst of the oldest scute points (triangles) and the oldest scute point that falls within the youngest scute points probably represent, respectively, an individual that had just recruited and had not yet incorporated a neritic signature and an individual that had recruited earlier but had escaped tagging in the previous year. The two points for oldest tissue that fall between the two clusters represent sampling layers that combined tissues with the oceanic and neritic signatures, a transition habitat and diet between the oceanic and neritic signatures, or a different habitat and diet in the early life stage of these two individuals.

Successive layers of scute store a chronological record of diets and habitats. I can draw conclusions about rates of change if rate of scute deposition is used as a proxy for time. These conclusions must be considered with caution because my 50-µm sampling layers were based on the minimum amount of sample needed for analysis; I do not know the biological significance of this depth. A relatively rapid and direct transition from oceanic to neritic habitats is indicated by the paucity of values between the primary oceanic and neritic signatures (Fig. 3-1c) and the oceanic signature still present in the youngest scute tissue of one turtle caught on neritic foraging

grounds (Fig. 3-1b). The oldest 2 to 3 layers in most turtles had the same oceanic foraging signature (Fig. 3-1c) suggesting that these isotopic values represent either the entire, or a major portion of, the lifestage between hatching and recruitment to neritic habitats.

The similarity of diets between oceanic-stage green turtles and loggerheads suggests that growth rates of young green turtles may be similar to those of loggerheads. If so, we can estimate the duration of the oceanic stage of green turtles as the time required for loggerheads to grow to 25-35 cm (sizes at which green turtles recruit to neritic habitats). Because oceanic-stage loggerheads in the eastern Atlantic reach 25 and 35 cm in approximately 2.8 and 4.6 yr, respectively (Bjorndal et al. 2003), I estimate the duration of the green turtle oceanic stage is approximately 2.8 to 4.6 yr, as well. This range is similar to an estimate for green turtles based on skeletochronology of 3 to 6 yr (Zug and Glor 1999). Of course, variation in temperature, diet quality, and food availability would affect growth rates of green turtles.

Preliminary scute samples from green turtles stranded dead in Florida (n = 11), hawksbills (*Eretmochelys imbricata*, n = 4), and a Kemp's ridley (*Lepidochelys kempi*, n = 1) indicate that all have a similar oceanic signature (Fig 3-3). Other populations of green turtles and, apparently, other species of sea turtles share similar oceanic habitats and diets in early juvenile stages. More extensive sampling is needed.

Stable isotopes of scute provided insights into the early juvenile stage of green turtles, a lifestage whose geographic location remains unknown. Tissues such as scute of marine turtles and baleen in whales that retain a stable isotope record provide a powerful tool for studying inaccessible lifestages.

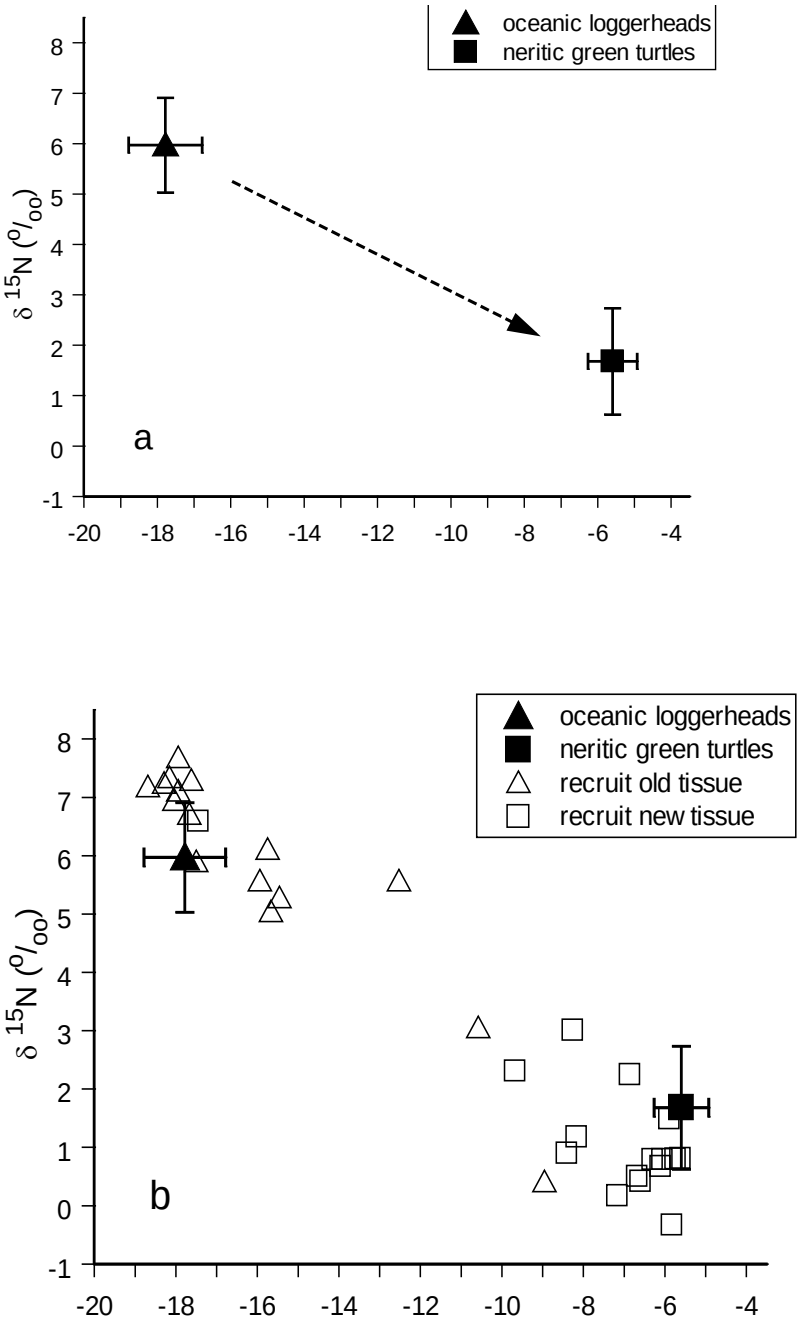


Figure 3-1. Mean values (± 1 SD) of δ ^{13}C and δ ^{15}N (‰) from oceanic-stage loggerheads (a) Mean values (± 1 SD) of δ ^{13}C and δ ^{15}N (‰) from oceanic-stage loggerheads (n = 12) and neritic green turtles resident in seagrass habitat (n = 28). If Carr's conjecture is correct, these values should be equivalent to the shift in stable isotope values (indicated by arrow) from oldest to youngest scute tissues from green turtles recently recruited to neritic habitats. (b) Values of δ ^{13}C and δ ^{15}N (‰) from 16 green turtle recruits, added to (a). (c) Values of δ ^{13}C and δ ^{15}N for successive scute layers from 8 green turtles. Each line is an individual; each point is a different layer.

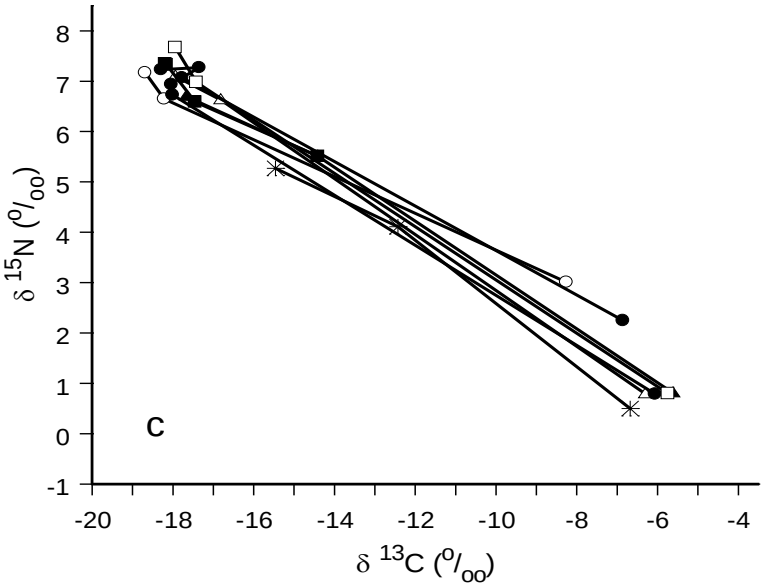


Figure 3-1 Continued.

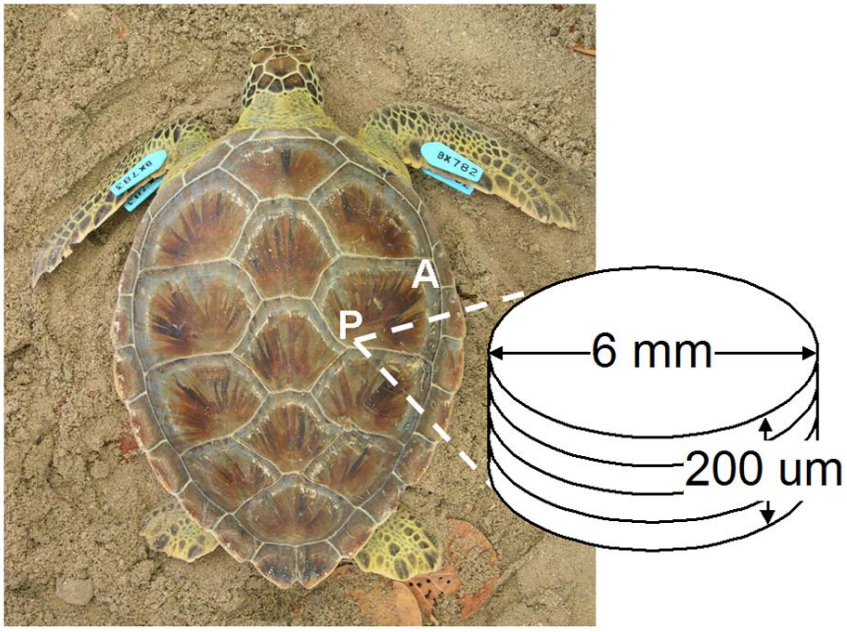


Figure 3-2. Green turtle showing the 2 sampling sites anterior (A) and posterior (P). Diagram illustrates the sequential sample layers from posterior scute samples. Grey tissue around the anterior and lateral sides of each scute is new tissue.

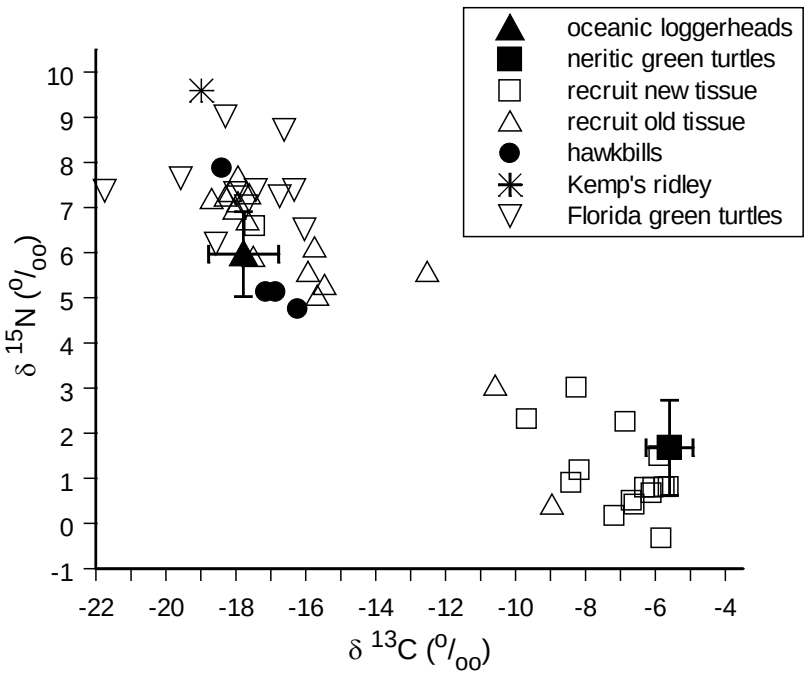


Figure 3-3. Mean values (± 1 SD) of $\delta^{13}C$ and $\delta^{15}N$ (‰) from oceanic-stage loggerheads (n = 12) and neritic green turtles resident in seagrass habitat (n = 28), individual values of $\delta^{13}C$ and $\delta^{15}N$ (‰) for the oldest scute tissue and youngest scute tissue from 16 green turtles that had recruited to seagrass habitat in the Bahamas within the previous year (recruits), and individual values of $\delta^{13}C$ and $\delta^{15}N$ (‰) for the oldest scute tissue from small (< 36 cm) Florida green turtles (n = 11), hawksbills (n = 4) and Kemp's ridley (n = 1).

CHAPTER 4
BIMODAL FORAGING IN ADULT LOGGERHEADS (*CARETTA CARETTA*): CHANGES TO LIFE HISTORY MODELS

Introduction

Loggerhead hatchlings (*Caretta caretta*) emerge from nests on beaches along the southeastern US coast from Florida to North Carolina and enter the North Atlantic. They are incorporated into long-shore currents and are carried to oceanic foraging areas (Bolten 2003a) where they feed primarily on sea jellies (Bjorndal 1997). Juvenile loggerheads recruit to neritic foraging areas at sizes between 46 to 64 cm carapace length, after about 7 to 12 years in oceanic habitats (Bjorndal et al. 2000, 2003). In neritic habitats, loggerheads shift to a diet primarily composed of hard-shelled, benthic invertebrates (Bjorndal 1997; Seney and Musick 2007). The original hypothesis was that this shift from the oceanic to the neritic environment is a unidirectional ontogenetic niche shift (Carr 1986). However, anecdotal reports (e.g., Eckert and Martins 1989) began to accumulate indicating that some individuals in neritic habitats may return to oceanic habitats and some may never leave oceanic habitats except to reproduce. Thus, the life history model developed by Bolten (2003a) included these possibilities as speculative connections.

Hatase et al. (2002a) were the first to confirm that some adult loggerheads utilize oceanic habitats between nesting seasons. Using stable isotopes (n = 149) and satellite telemetry (n = 5), they discovered nesting loggerheads from two different nesting beaches in Japan had been foraging in either oceanic or neritic waters. Through the use of satellite telemetry, Hawkes et al. (2006) documented the same foraging dichotomy for loggerheads (n = 10) from the population nesting in the Cape Verde Islands. In both studies, females that foraged in oceanic habitats were significantly smaller than those foraging in neritic habitats, although there was overlap in body size in Japan (Hatase et al. 2002a). In a study of large juvenile loggerheads captured in estuaries

of North Carolina, USA, satellite telemetry revealed that 10 loggerheads moved off the continental shelf and into oceanic habitats to forage, while 13 remained in neritic habitats (McClellan and Read 2007).

Stable isotopes of carbon and nitrogen in the marine environment provide a tool to investigate oceanic/pelagic vs coastal/benthic habitat use as well as trophic level (Lathja and Michener 1994; Hobson and Schell 1998). Studies have identified a naturally occurring gradient in $\delta^{13}C$ values, in which oceanic regions have more negative $\delta^{13}C$ values than do coastal areas (Lorian et al. 1992; Hobson et al. 1994; France 1995). A trophic gradient exists with $\delta^{15}N$ values increasing at higher trophic levels (Minagawa and Wada 1984; Macko et al. 1986).

The Atlantic USA nesting population of loggerheads is the largest loggerhead population in the Atlantic system. The loggerheads nesting in Florida are a large proportion of this population, but Florida's nesting population has declined dramatically by 49% between 1998 and 2006 (Witherington et al. 2009). Information on the locations of foraging grounds for Florida loggerheads between reproductive seasons is incomplete but necessary to develop appropriate management strategies. Loggerheads are listed as Endangered on the IUCN Red List and as Threatened under the U.S. Endangered Species Act.

Do loggerheads nesting in Florida exhibit a polymorphism in foraging strategies? I used two approaches to answer this question and evaluate the foraging strategies of loggerheads before they arrived in Florida to nest. First, I evaluated stable isotopes of carbon and nitrogen in samples of skin collected from 310 loggerheads nesting at four locations on the east coast of Florida. The stable isotope signature in skin represents a temporal integration of the isotopes assimilated during the synthesis of the tissue before the nesting season. Second, I analyzed epibionts from 52 of the 310 loggerheads. Loggerheads serve as a substrate for a diverse array of

epibionts (Caine 1986), and these epibiont communities should reflect the pre-nesting habitat of the host turtle.

Methods

Sample Collection

Skin samples were collected from 310 loggerhead turtles nesting on beaches at Canaveral National Seashore (CNS), Melbourne Beach (MEL), and Juno Beach (JUN) during the first six weeks (2 May – 15 June) of the 2003 and 2004 nesting seasons and, in 2003 only, from Pompano and Ft. Lauderdale beaches in Broward County (BRO) (Table 4-1; Fig.4-1). Stable isotopes of carbon and nitrogen assimilated from the diet into the skin of juvenile loggerheads have a mean residence time of 44.9 (± 3.1) days (Reich et al. 2008). Because residence times of isotopes in tissues decrease with increasing growth rates and because adult turtles grow more slowly than the juveniles used to calculate average residence time in loggerhead skin, I am confident that my sample period (45 days) is appropriate for assessing foraging location and trophic level of the turtles prior to their migration to the nesting grounds.

I used a sterile 6-mm biopsy punch (designed for collecting epidermis samples from humans) to collect samples of non-keratinized skin from the "shoulder" area of each turtle after cleaning the area with alcohol. Skin samples were stored in 70% ethanol at room temperature. Minimum curved carapace length (CCL) was measured from anterior notch to posterior notch; standard flipper tags were applied to both front flippers of untagged turtles to avoid re-sampling individuals.

In 2003 and 2004, epibionts were collected from the carapace of 52 loggerhead turtles (also sampled for stable isotopes) nesting at Canaveral National Seashore. All epibionts present in an area of 20 cm2 on the posterior right quadrant of the carapace were collected and preserved

in 70% ethanol (Frick et al. 1998; Pfaller et al. 2006). Samples were later sorted and identified to the lowest taxonomic level possible under light microscopy (magnification up to 1000x).

Stable Isotope Analysis

Skin biopsy samples were rinsed in distilled water to remove any epibionts or other organic material and cleaned with isopropyl alcohol swabs. The surface epidermis was removed and homogenized with a scalpel blade. The homogenized sample was dried at 60°C for a minimum of 24 hours. After drying, lipids were removed from all samples using an Accelerated Solvent Extractor (ASE) with petroleum ether as the solvent. Approximately 550 µg of each dried, lipid-free sample was loaded into a pre-cleaned 4x6mm tin capsule.

All samples were combusted in a COSTECH ECS 4010 elemental analyzer interfaced via a Finnigan-MAT ConFlow III device (Finnigan MAT, Breman, Germany) to a Finnigan-MAT DeltaPlus XL (Breman, Germany) isotope ratio mass spectrometer in the light stable isotope lab at the University of Florida, Gainesville, Florida, USA. Stable isotope abundances were expressed in delta (δ) notation, defined as parts per thousand (‰) relative to the standard as follows:

$$\delta = ([R_{sample}/R_{standard}] - 1)\ (1000) \qquad (4\text{-}1)$$

where R_{sample} and $R_{standard}$ are the corresponding ratios of heavy to light isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample and international standard, respectively. $R_{standard}$ for ^{13}C was Vienna Pee Dee Belemnite (VPDB) and for ^{15}N was atmospheric N_2. Internal standards were inserted in all runs at regular intervals to calibrate the system and assess drift over time. The analytical accuracy of my measurements, measured as the SD of replicates of standards, was 0.09 for both $\delta^{13}C$ and $\delta^{15}N$ (n = 88 and 91, respectively).

Statistical Analyses

I assigned each female to oceanic habitat or neritic habitat based on their $\delta^{13}C$ and $\delta^{15}N$ signatures with cluster analysis (kmeans function, S-PLUS v. 7.0.3; 1000 iterations). The kmeans function minimizes the within-cluster sum of squares from the cluster centers based on Euclidian distance (Crawley 2002).

For each location for which I had 2003 and 2004 data (CNS, MEL, JUN), I tested for a year effect on the distribution of turtles between oceanic and neritic foraging grounds using chi square. I also used chi-square tests to evaluate differences in the proportions of oceanic and neritic turtles among the four nesting locations. To compare size differences, I compared CCL of the oceanic and neritic foraging groups with a t-test.

I analyzed epibiont data to evaluate whether habitat-specific epibionts were consistent with my habitat (cluster) assignments. Epibiont species were characterized as either oceanic, neritic, or occurring in both habitats (Akoi 1997; Chace 1951; Foster et al. 2004; Frick et al 2003, 2004, 2006; McCain 1995; Williams 1984). I used chi-square tests to evaluate if the occurrences of neritic epibionts or oceanic epibionts were significantly different between the two foraging groups of loggerheads. I conducted chi-square tests using the computer program CHIRXC (Zaykin and Pudovkin 1993), which calculates probabilities of independence using a Monte Carlo randomization method (1000 iterations).

Statistical analyses were conducted in S-PLUS (v. 7.0.3) except for CHIRXC. Unless otherwise noted, alpha = 0.05.

Results

The $\delta^{13}C$ and $\delta^{15}N$ signatures of the 310 nesting females fell into two clusters (Fig. 4-2). Cluster analysis assigned 158 females to oceanic foraging habitats and 152 females to neritic foraging habitats. The center for the oceanic cluster was at $\delta^{13}C$ = -14.907, $\delta^{15}N$ = 11.213 and for the

neritic cluster was at $\delta^{13}C$ = -10.042, $\delta^{15}N$ = 7.488. Within-cluster sum of squares was 788.6 for oceanic and 567.3 for neritic.

Comparing 2003 and 2004 distributions of turtles between oceanic and neritic foraging grounds within each nesting location, I found no significant difference for any nesting location (chi-square tests, df = 1, P >0.05). Therefore, for each nesting location, I combined data from the two years for CNS, MEL and JUN. The distributions between oceanic and neritic foraging grounds are significantly different among the four nesting locations (chi-square test, df = 3, χ = 17.03, P = 0.0007). The proportion of oceanic foragers declines from north to south (Fig. 4-3).

Oceanic-foraging females (CCL mean (SD) = 97.6 cm (6.0)) were significantly smaller than neritic-foraging females (CCL mean (SD) = 100.2 cm (5.7); t-test, df = 307, t = -3.903, P = 0.0001). There was, however, substantial overlap in CCL between the two groups (Fig. 4-4).

The distributions of 35 species of epibionts (Table 4-2) on 33 oceanic loggerheads and 15 neritic loggerheads are consistent with the foraging habitats assigned by cluster analysis based on stable isotope signatures. The occurrence of neritic epibionts was significantly higher on neritic foraging females than on oceanic foraging females (chi-square, df = 1, χ = 10.909, P < 0.0001). The occurrence of oceanic epibionts was significantly higher on oceanic foraging females (chi-square, df = 1, χ = 16.329, P < 0.0001).

Discussion

Only two loggerhead nesting aggregations in the world have more than 10,000 females nesting each year: Florida, USA, and Masirah, Oman (Baldwin et al. 2003; Ehrhart et al. 2003). Analyses of stable isotopes and epibionts reveal that loggerheads nesting in Florida have a bimodal foraging strategy and are divided almost equally between oceanic and neritic foraging groups. Because of the large size of this loggerhead population and the large proportion of oceanic foragers, these results cause a major paradigm shift in the perceived roles of loggerheads

in marine ecosystems and in the appropriate management plans for the conservation of this endangered species. Loggerheads are major marine predators (Bjorndal 2003), but we now know that, as large juveniles and adults, these predators are not supported entirely within neritic foodwebs, as previously supposed. Protecting large juvenile and adult loggerheads in oceanic habitats must now be added to the requirement of protecting them in neritic habitats, as has been the focus up to this time (National Marine Fisheries Service and U.S. Fish and Wildlife Service 1991).

The differential distribution of oceanic and neritic epibionts on loggerheads with oceanic and neritic stable isotope signatures confirms the bimodal foraging pattern. My study is the first to use epibionts to evaluate the bimodal foraging strategy in loggerheads. Earlier, Caine (1986) examined epibionts from loggerheads nesting at one site in South Carolina and 5 sites in Florida. He concluded that the epibionts revealed a separation between northern and southern loggerhead populations with the dividing point between Daytona Beach and Cape Canaveral, Florida.

A significant trend exists for a decreasing proportion of nesting turtles to recruit from oceanic habitats from north to south along the Florida coast (Fig. 3). Reasons for this north to south trend are not known, but may involve the proximity of the Gulf Stream current and the width of the continental shelf, both of which vary substantially among my sampling areas. Also the shorter distances between the extensive neritic habitats on the Great Bahamas Bank and my southernmost sampling areas may help account for the higher proportion of neritic-foraging turtles from my southern sampling areas. A study is underway to determine if this north to south trend continues northward for loggerheads nesting in Georgia, South Carolina, and North Carolina, and to integrate stable isotope results with tracks from satellite telemetry for loggerheads nesting in Georgia and North Carolina.

The mean size of turtles foraging in oceanic waters is significantly smaller than the mean size of turtles foraging in neritic waters (Fig. 4-4). This size difference is consistent with the findings of Hatase et al. (2002a) and Hawkes et al. (2006). Again, I do not know the source of this difference. Oceanic turtles may be younger animals that may later change to neritic foraging. The relative food (either quantity or quality) and temperature regimes of the two habitats may result in slower growth rates in oceanic turtles, although Hatase et al. (2004) found no relationship between growth rates and body size in loggerheads nesting in Japan, in a study in which they assumed small size would reflect an oceanic foraging strategy, based on their earlier study (Hatase et al. 2002a). However, they found a non-significant trend for smaller turtles to have longer intervals between nesting seasons, which they suggested may mean that loggerheads in oceanic habitats require more time to replenish nutrient stores necessary for reproduction due to a poorer diet.

The occurrence of this bimodal foraging has not been evaluated in males. Two adult male loggerheads captured in neritic waters during the non-reproductive season in Japan were tracked by satellite telemetry (Sakamoto et al. 1998; Hatase et al. 2002b) into oceanic foraging grounds. Because both males were of relatively small size, Hatase et al. (2002b) suggested males may exhibit the same size-related foraging dichotomy as in female loggerheads in Japan.

Our understanding of the life cycle of loggerheads has been advanced substantially since last reviewed by Bolten (2003a,b). Fig. 4-5a (modified from Bolten 2003a,b) shows the basic concept of a linear ontogenetic sequence of lifestages that pass through different marine zones with two speculative connections that would disrupt the linear sequence. Bolten (2003a) suggested that juvenile neritic loggerheads may return to the oceanic zone. This suggestion has now been confirmed through satellite telemetry of neritic juvenile loggerheads moving into

oceanic habitats (McClellan and Read 2007) and is represented in Fig. 5b as the double-headed arrow marked as connection #2. Bolten (2003a) also suggested that some juvenile loggerheads may never enter the oceanic zone and thus complete their life cycle within neritic habitats. This suggestion has yet to be confirmed and is represented by connection #5 in Fig. 4-5c.

A major change in our conception of the life cycle of loggerheads is that some females that arrive at nesting beaches have come from oceanic foraging habitats and some return to oceanic habitats. This new link, represented by connection #1 in Fig. 4-5b, has been verified in both directions. The connection from oceanic to nesting beaches has been documented through stable isotopes (Hatase et al. 2002a; this study) and epibionts (this study). The connection from nesting beaches to the oceanic zone has been documented through satellite telemetry (Hatase et al. 2002a; Hawkes et al. 2006).

An additional question is whether adults in the oceanic zone derive from juveniles in the oceanic zone (speculative connection #3 in Fig. 4-5c). If so, some loggerheads may have an entirely oceanic existence, other than the forays to the nesting beaches. Or do oceanic adults derive from neritic adults shifting habitats (speculative connection #4 in Fig. 4-5c). As more is learned about the movements of juvenile and adult loggerheads, we may find that loggerheads in all lifestages shift repeatedly between oceanic and neritic foraging habitats.

My result of bimodal foraging strategies in female loggerheads nesting in Florida is consistent with the results for female loggerheads nesting in Japan (Hatase et al. 2002a) and Cape Verde Islands (Hawkes et al. 2006). Given the wide distribution of studies that have revealed bimodal foraging strategies in nesting loggerheads, it may well be that loggerheads throughout the world exhibit this bimodal pattern. Of course, other populations must be studied; high priority should be given to the nesting populations in Brazil, Oman, Australia, and the

Mediterranean. Adult males in all of these breeding areas should also be sampled to determine if they exhibit the same bimodal strategy, and in similar proportions, as the females. Where possible, results from stable isotope analyses, tracks from satellite telemetry, and epibionts should be integrated to provide the most robust conclusions.

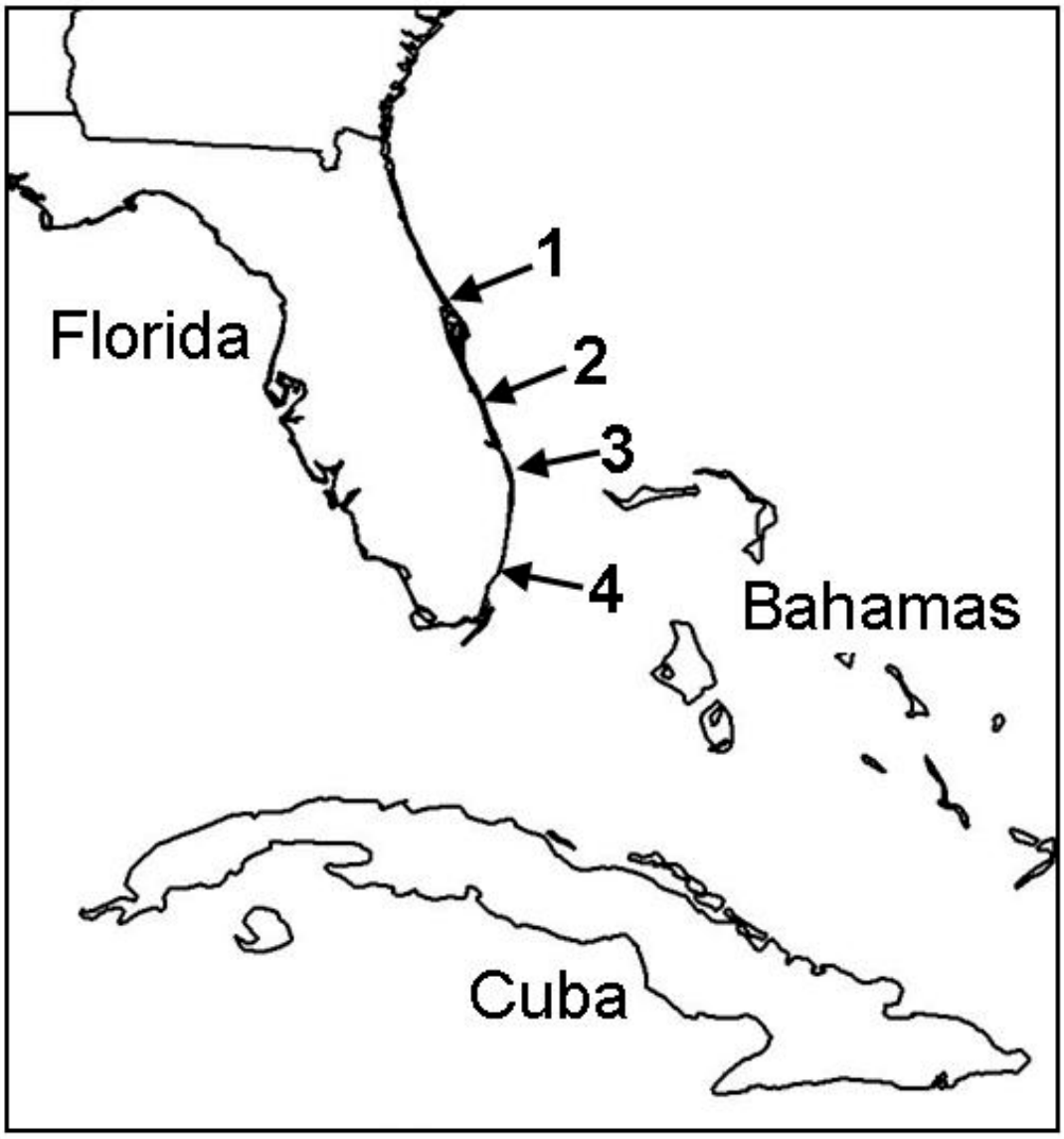


Figure 4-1. Locations of the four sampling sites. (1) Canaveral National Seashore (CNS); (2) Melbourne Beach (MEL); (3) Juno Beach (JUN); and (4) Broward County (BRO).

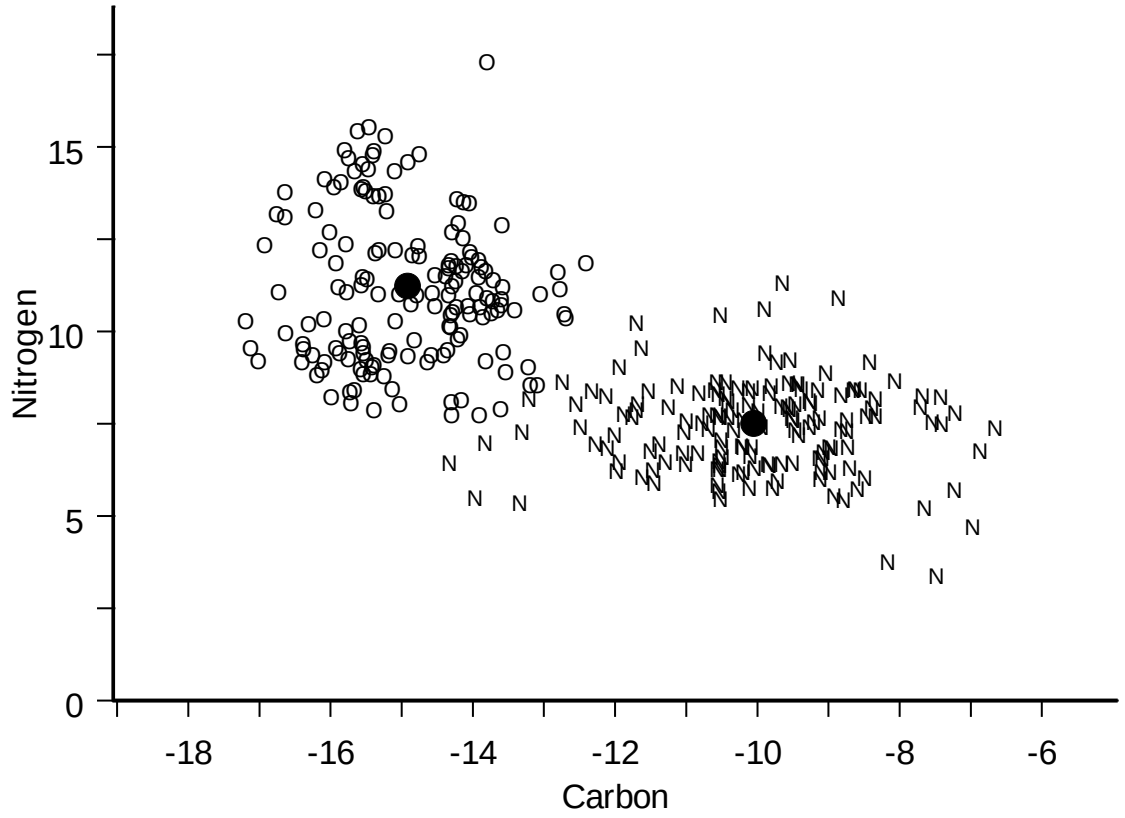


Figure 4-2. Distribution of stable isotope values from nesting loggerheads (n = 310) at four sites in Florida as determined by cluster analysis [O = oceanic forager (*n* = 158), N = neritic forager (*n* = 152)]. Solid circles indicate the centers of the two clusters derived from the cluster analysis (see text).

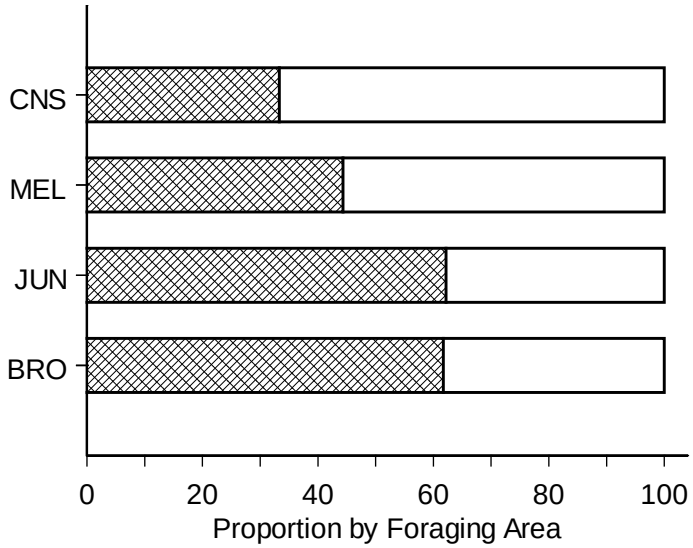


Figure 4-3. The proportions of oceanic/pelagic foragers (open bars) and neritic/benthic foragers (hatched bars) are significantly different among the four nesting locations (chi-square test, df = 3, χ = 17.03, P = 0.0007). Locations are arranged from north to south; see Fig. 4-1 for abbreviations.

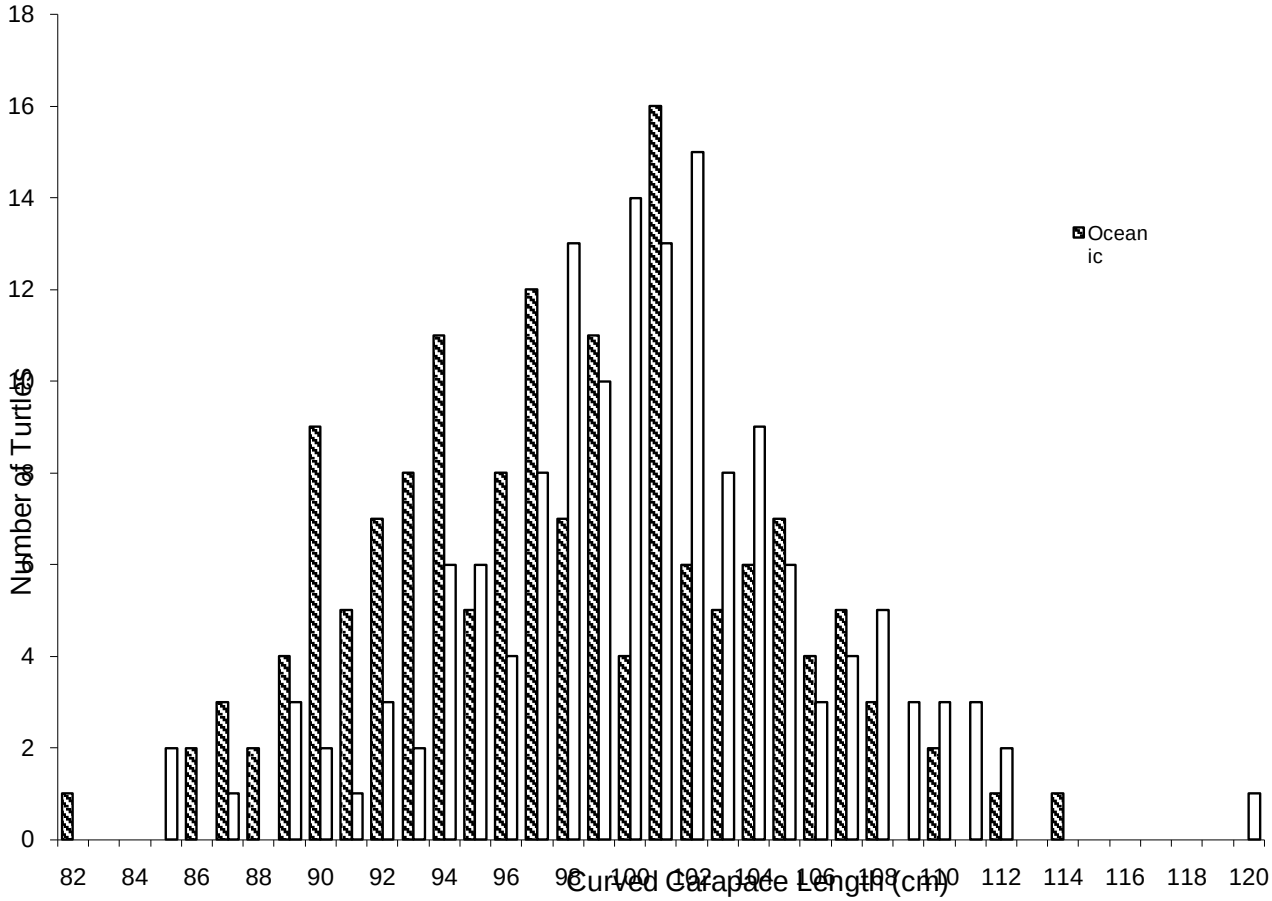


Figure 4-4. Size distributions of oceanic foragers (*n* = 158; diagonal hatching) and neritic foragers (*n* = 152; open bars) among nesting loggerheads in Florida. Mean size of neritic foragers is significantly larger than the mean size of oceanic foragers (see text).

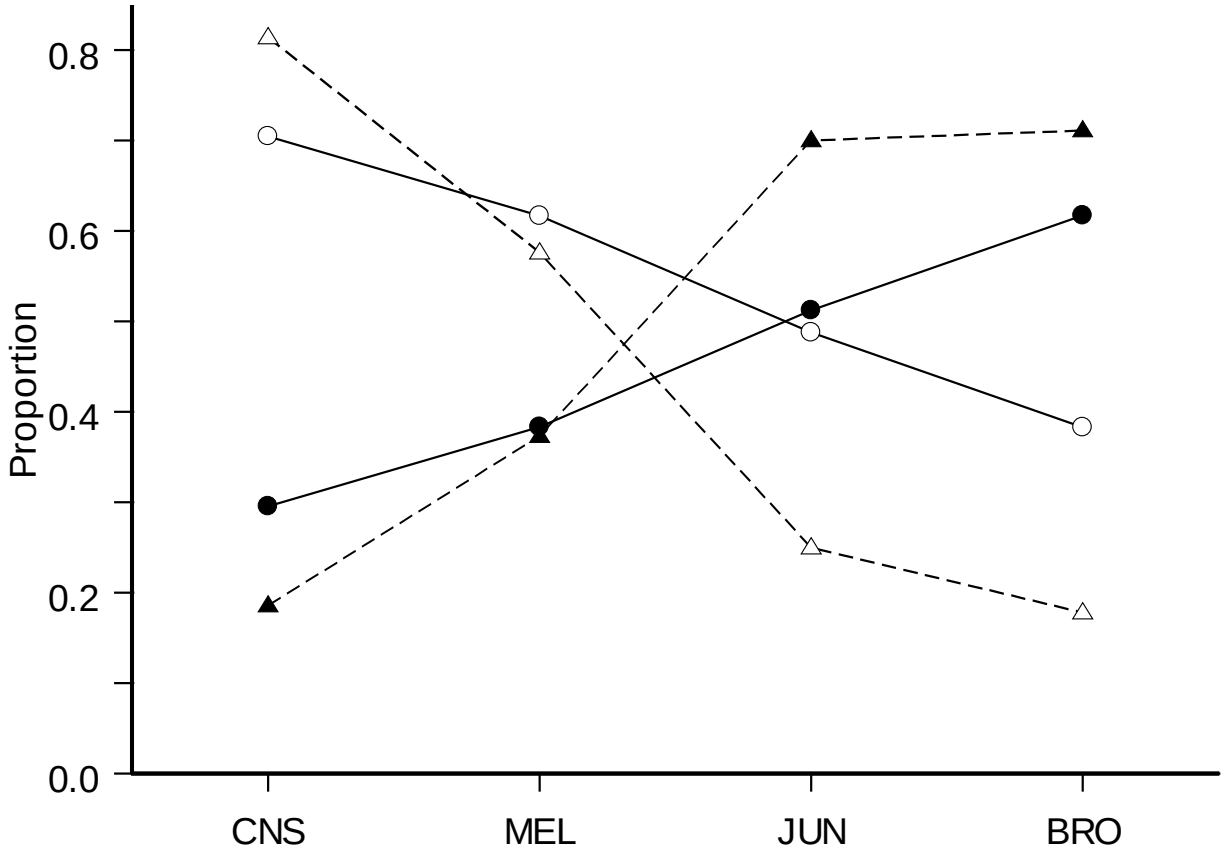
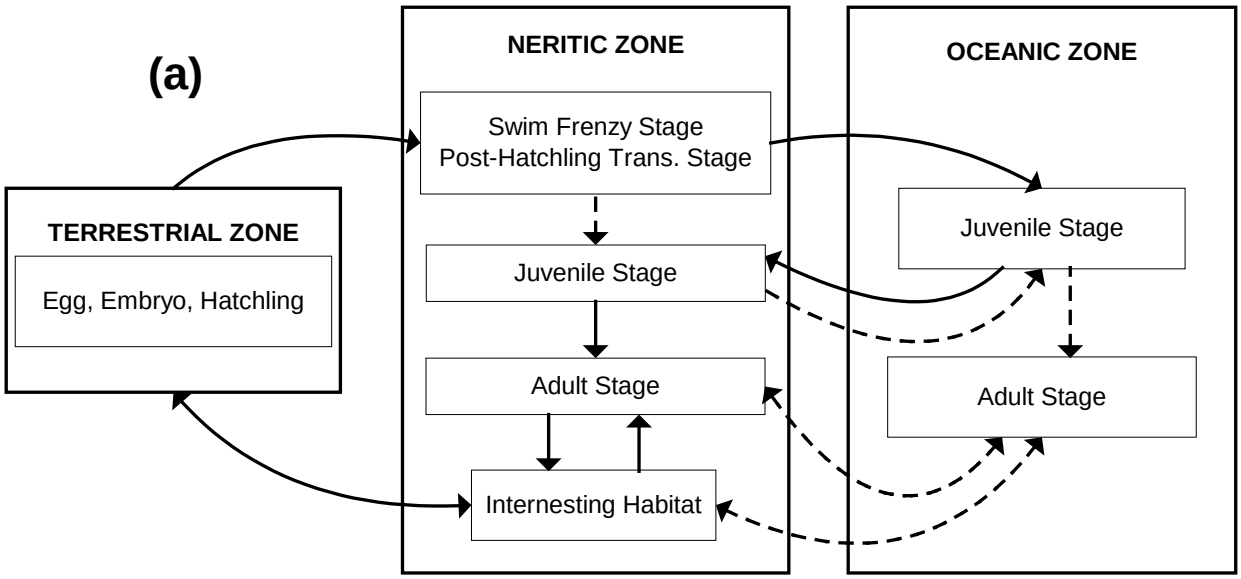


Figure 4-5. Clinal changes in haplotype frequencies and foraging strategies for female loggerheads nesting at four locations in Florida. Open triangles = haplotype CC-A1, closed triangles = haplotype CC-A2, open circles = oceanic/pelagic foragers, and closed circles = neritic/benthic foragers. These clines are independent (see text). For location abbreviations see Fig. 4-1.

Figure 4-6 Life history pattern for loggerhead sea turtles showing the sequence of lifestages that pass through different marine zones a) Basic Type 2 life history pattern for loggerhead sea turtles showing the sequence of lifestages that pass through different marine zones. Solid lines are confirmed connections; dashed lines are speculative connections (modified from Bolten 2003a, b, 2007; and Harrison and Bjorndal 2006).(b) Variations to the basic Type 2 life history pattern that have now been documented for loggerhead sea turtles. Connection (1) between oceanic and neritic juveniles is now known to be a 2-way connection; McClellan and Read (2007) confirmed speculative connection of Bolten (2003a). Connection (2) is between the oceanic and internesting habitats in the neritic zone for adult nesting females (Hatase et al. 2002a; Hawkes et al. 2006; this study). Connections (3), (4), and (5) are still speculative. Connection (3) indicates that oceanic juveniles may attain maturity in the oceanic zone; a portion of the loggerhead population may never inhabit neritic foraging grounds. Connection (4) indicates that adult loggerheads may move between the neritic and oceanic foraging zones. Connection (5) suggests that some juveniles may not have an early oceanic juvenile stage and may complete their development in the neritic zone.


(a)
NERITIC ZONE
OCEANIC ZONE
TERRESTRIAL ZONE
Egg, Embryo, Hatchling
Swim Frenzy Stage
Post-Hatchling Trans. Stage
Juvenile Stage
Adult Stage
Internesting Habitat
Juvenile Stage
Adult Stage

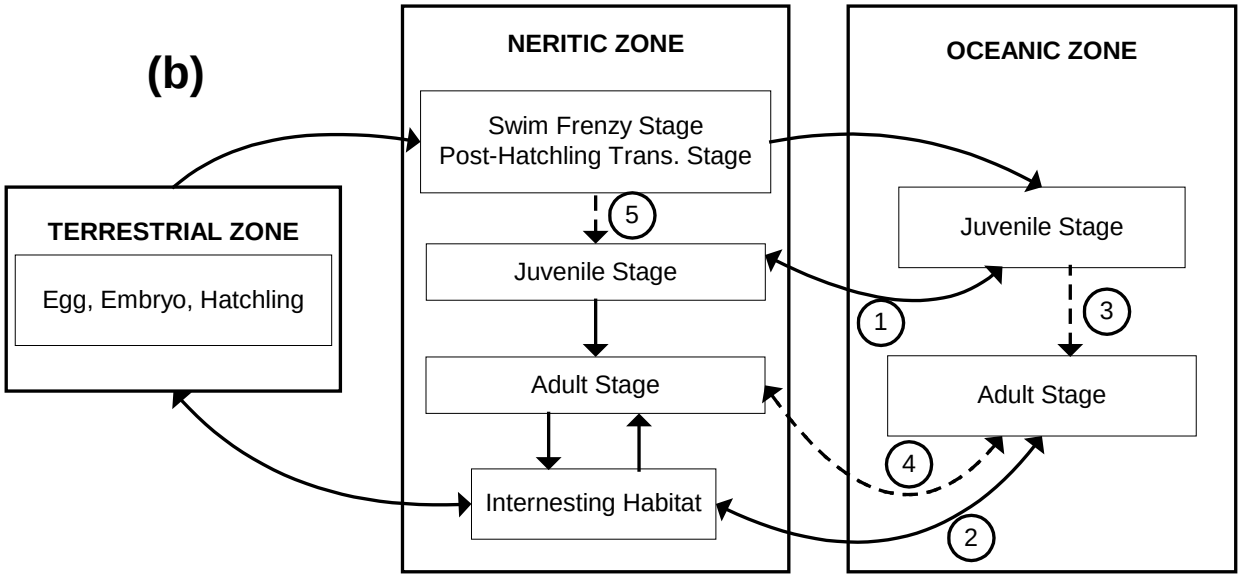

(b)
NERITIC ZONE
OCEANIC ZONE
TERRESTRIAL ZONE
Egg, Embryo, Hatchling
Swim Frenzy Stage
Post-Hatchling Trans. Stage
5
Juvenile Stage
Adult Stage
Internesting Habitat
Juvenile Stage
1
3
Adult Stage
4
2

Table 4-1. Number of skin samples collected from nesting loggerheads each year by location: Canaveral National Seashore (CNS), Melbourne Beach (MEL), Juno Beach (JUN) and Pompano and Ft. Lauderdale beaches in Broward County (BRO).

	CNS	MEL	JUN	BRO	*total*
2003	44	60	41	47	192
2004	31	46	41	0	118
total	73	106	82	47	*310*

Table 4-2. Epibiont species characteristic of either oceanic/pelagic or neritic/benthic habitats identified on loggerheads nesting at Canaveral National Seashore, habitat where each epibiont species is typically found, and the number of turtles (oceanic or neritic) on which the epibiont was identified. Higher taxonomic designations are given in Pfaller et al. (2008).

	Typical habitat of epibiont		Occurrence of epibiont	
Epibiont	Oceanic / pelagic	Neritic / benthic	Oceanic/pelagic turtle (n=33)	Neritic/benthic turtle (n=15)
Lepas pectinata	x		25	2
Membranipora tuberculata	x		2	0
Anadara transversa		x	0	2
Arbacia punctulata		x	0	2
Bugula fulva		x	0	4
Caprella equilibra		x	0	4
Caprella penantis		x	2	13
Caprella scaura		x	2	2
Conopea galeata		x	0	2
Leptogorgia virgulata		x	1	6
Lytechinus variegatus		x	0	1
Membranipora arborescens		x	1	11
Mitrella lunata		x	16	14
Molgula occidentalis		x	2	14
Obelia dichotoma		x	0	9
Ostrea equestris		x	1	0
Podarke obscura		x	0	3
Ricordia florida		x	0	3
Strombus alatus		x	0	2
Strombus gigas		x	0	1
Thalamoporella floridana		x	0	2

CHAPTER 5
CONCLUSIONS

Stable Isotopes and Sea Turtle Ecology

Advancing the Field

My work has advanced both our ability to use stable isotopes to study sea turtles and our knowledge of sea turtle biology through the use of stable isotopes. Before I began my doctoral studies, there was no information on isotopic discrimination or incorporation in sea turtles. As a result, studies in sea turtles were limited to using associated organisms (Killingly and Lutcavage 1983) to predict sea turtle movements or dependent upon isotopic discrimination factors and incorporation rates generated by studies of other species (i.e., birds and terrestrial mammals) to predict the diet of sea turtles (Godley et al. 1998, Hatase 2002a).

The use of stable isotopes to investigate animal diets, habitat use, and trophic level requires understanding the rate at which animals incorporate the ^{13}C and ^{15}N from their diets (average residence time) and the factors that determine the magnitude of the difference (isotopic discrimination) in isotopic composition between the animal's diet and that of its tissues. Results of my isotopic incorporation and discrimination study facilitate accurate interpretation of stable isotope data from multiple tissues of sea turtles, thus providing a tool for data analyses in future studies incorporating stable isotope analysis.

Three factors can influence isotopic discrimination between an animal's tissue and its diet: (1) isotopic ‘‘memory’’, (2) metabolic fractionation (defined as the difference in isotopic composition between reactants and products in biochemical reactions), and (3) isotopic routing (Martínez del Rio and Wolf 2005).

The first of these factors, isotopic "memory", is the primary focus of the research reported in Chapter 2 and refers to the finding that when animals change diets or habitats, the isotopic

signature of their tissues do not immediately reflect the isotopic signature of their new diet and/or habitat. Fry and Arnold (1982) and Phillips and Eldrige (2006) established that tissues incorporate isotopic composition of new diet and/or change in habitat with characteristic temporal dynamics. The dynamics of incorporation depend on several factors including animal size (Carleton and Martínez del Rio 2005), nutrient composition of the diet (Gaye-Seisseggar et al. 2003; 2004), the catabolic turnover of the tissue type (Tieszen et al. 1983; Hobson and Clark 1992; Martínez del Rio and Wolf 2005), and the animal's growth rate (Fry and Arnold 1982; Hesslein et al. 1993; MacAvoy et al. 2001; Martínez del Rio and Wolf 2005).

Although it has been well established that the rate of isotopic incorporation into an animal's tissues depends on both the rates of tissue growth and of catabolic turnover (Fry and Arnold 1982; Hesslein et al. 1993), there are few studies that use stable isotopes to partition the contribution of growth and catabolic turnover to the rate of isotopic incorporation (reviewed by MacAvoy et al. 2001). I measured both the dynamics and consistency of ^{13}C and ^{15}N incorporation into the tissues of two age classes of a rapidly growing ectotherm, the loggerhead sea turtle (*Caretta caretta*), after a diet shift. I report isotopic discrimination factors and incorporation rates (allowing me to predict the average residence time of C and N) of isotope ^{13}C and ^{15}N in whole blood, red blood cells, plasma, skin, and scute of both hatchling and juvenile loggerhead turtles.

Knowing the isotopic discrimination factor of individual tissues allows for interpretation of carbon and nitrogen values in predator - prey isotope analyses. Knowing the average time (average residence time) that a tissue retains the signature of the assimilated diet allows scientists to select the tissue that will most accurately assess the diet and habitat use for the period of interest, past or present. Skin from juvenile loggerheads, for example, has an average residence

time (retains the isotopic signature of carbon and nitrogen from the turtles diet) of 45 days (Chapter 2). Thus, skin collected from females early (first 45 days) in the nesting season, is an appropriate tissue to identify the diet and habitat of female loggerheads prior to their arrival at the breeding and nesting grounds. Plasma, with an average residence time of days (Chapter 2) would be an appropriate tissue for looking at current diet or by analyzing a series of plasma samples from the same turtle over a period of months, identifying short term diet changes.

Growth, Isotopic Discrimination, and Isotopic Incorporation in Loggerheads

In Chapter 2, I present the contribution of growth and catabolic turnover to the rate of ^{13}C and ^{15}N incorporation into several tissues that can be sampled non-invasively (skin, scute, whole blood, red blood cells, and plasma solutes) in two age classes of a rapidly growing ectotherm loggerhead turtles. I found significant differences in C and N incorporation rates and isotopic discrimination factors ($\Delta^{13}C = \delta^{13}C_{tissues} - \delta^{13}C_{diet}$ and $\Delta 15N = \delta^{15}N_{tissues} - \delta^{15}N_{diet}$) among tissues and between age classes. Growth explained from 26 to 100% of the total rate of incorporation in hatchling turtles and from 15 to 52% of the total rate of incorporation in juvenile turtles. Because growth contributed significantly to the rate of isotopic incorporation, variation in rates among tissues was lower than reported in previous studies. The contribution of growth can homogenize the rate of isotopic incorporation and limit the application of stable isotopes to identify dietary changes at contrasting time scales and to determine the timing of diet shifts. The isotopic discrimination factor of nitrogen ranged from –0.64‰ to 1.77‰ in the turtles' tissues. These values are lower than the commonly assumed average 3.4‰ discrimination factors reported for whole body and muscle isotopic analyses. The increasing reliance on non-invasive and non-destructive sampling in animal isotopic ecology requires that we recognize and understand why different tissues differ in isotopic discrimination factors.

Results of my study of isotopic discrimination and incorporation in hatchling and juvenile turtles provide a baseline for interpreting data from studies of sea turtles that incorporate stable isotope analysis. These data also provide a tool for experimental design, allowing scientists to identify and subsequently collect the tissue that will provide diet and habitat data for the time frame of interest.

Solving a Mystery – "Lost Years of Small Green Turtles"

The tissue with the greatest potential to provide scientists with data of historical diet and habitat use is scute, the keratinized epidermal tissue that forms the outer layer of the carapace. Each scute is comprised of multiple layers that are deposited over time. Because keratin is inert once deposited, there is no change in isotopic signature of the tissue once it has been formed. Because scute is continuously growing it can be used to evaluate the ecology of inaccessible lifestages. In Chapter 3, I report how through the use of stable isotopes and the development of a new technique, I have answered a question that has plagued sea turtle biologists for decades. Archie Carr's quest to locate the developmental habitat of small juvenile green turtles, *Chelonia mydas*, began more than 50 years ago (Carr 1952). After extensive surveys in neritic waters yielded no sightings, Carr (1987) hypothesized that green turtle post-hatchlings spend their first years in oceanic habitats feeding primarily on invertebrates such as sea jellies and salps before shifting to feed on seagrasses and algae in neritic waters. I used stable isotopes to test Carr's conjecture. Analyses of stable carbon and nitrogen isotopes in successive layers of scute from small, neritic green turtles documented a clear ontogenetic shift in diet and habitat. Turtles were primarily carnivorous and oceanic early in life and changed to an herbivorous diet in neritic habitats as they grew. My data provide strong support for Carr's long-standing conjecture.

Loggerhead Life History – A New Perspective

In Chapter 4, I used stable isotopes of carbon and nitrogen to investigate the diet of Florida's nesting loggerhead population; this study yielded unexpected results. Analyses of skin samples for stable carbon and nitrogen isotopes were used to evaluate diet and habitat use of female loggerhead turtles (n=310) prior to their arrival at nesting beaches in Florida. Samples were collected in 2003 and 2004 from turtles nesting at four locations on the east coast of Florida. I simultaneously collected samples of invertebrates on the carapaces of nesting turtles (n=52) from one of the sites, Canaveral National Seashore. My results indicate that prior to the breeding season, loggerheads nesting in Florida use two different foraging strategies, with females nearly equally distributed between oceanic and neritic foraging groups. These results are further supported by analysis of the epibiont communities collected from the carapace of 52 of the 310 turtles sampled. The differential distribution of oceanic and neritic epibionts on loggerheads with oceanic and neritic stable isotope signatures confirms the bimodal foraging pattern. My study is the first to use epibionts to evaluate the bimodal foraging strategy in loggerheads. Because of the large size of this loggerhead population and the large proportion of oceanic foragers, these findings imply a major paradigm shift in the perceived roles of loggerheads in marine ecosystems and in the appropriate management plans for the conservation of this endangered species.

Future Research Needed

Studies to Improve Our Ability to Use Stable Isotope Analyses in Sea Turtle Biology

A global effort is needed to standardize the methodology of sample collection, preservation, preparation and analysis use of stable isotope analysis to study sea turtles. We need to know the effect of commonly used preservation and lipid extraction methods on specific tissues. Another gap in our knowledge is the degree of homogeneity within a given tissue; i.e.,

we need to determine if skin from the shoulder area of a turtle is homogeneous within the shoulder area, or with skin from other locations such as the flippers. These data would contribute to the establishment of a global protocol, both in determining an optimum site for collection of skin samples and in the method of homogenizing the sample in preparation for analysis. To interpret more accurately the isotopic discrimination factors and isotopic turnover in each species of sea turtle, isotopic incorporation and discrimination studies are needed for the six remaining species.

Studies to Advance Our Knowledge of Sea Turtles and Our Ability to Conserve Them

Stable isotope analyses when used in conjunction with existing methods of tracking sea turtles (satellite and radio telemetry, mark- recapture tagging programs) can aid scientists by providing a dietary history to accompany the geographical data collected via satellite or recovery of a previously tagged turtle. For example, satellite telemetry is capable of reporting the number, depth and duration of dives made by a turtle. What it cannot provide are data on what the turtle was doing during and between dives—is the dive made for the purpose of foraging? Or is the turtle foraging while at the surface? Stable isotope analysis can provide data on what habitat the turtle is using to forage, benthic or pelagic. Another long standing question about sea turtle biology is "do nesting females forage during the inter-nesting period?" Analyses of serial samples of plasma collected from individual females across a nesting season may provide the answer to that question. We also need to be able to compare stable isotope analysis of diet and trophic level of populations from different regions. Because the carbon and nitrogen values of organisms that form the base of the food web (i.e., plankton, POM-particulate organic matter) differ between ocean basins (Wallace et al. 2006), it is important that we include analyses of turtles potential prey, plankton, and POM samples in our studies of sea turtle diet and habitat use. These data will contribute to the construction of an isoscape (map of baseline carbon and

nitrogen values) of marine study sites and facilitate accurate diet, habitat and trophic level comparisons of sea turtle populations across the globe.

Development of management and conservation policy relies on research to identify critical habitats, and to identify the role of these threatened and endangered animals in marine systems. We need to know what habitat each lifestage occupies, how long they stay, and what they do there. My research has shown that stable isotopes can aid scientists in attaining this goal.

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BIOGRAPHICAL SKETCH

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