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Stable carbon and nitrogen isotope discrimination in soft tissues of the leatherback turtle (*Dermochelys coriacea*): Insights for trophic studies of marine turtles

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ABSTRACT

The trophic ecology of marine vertebrates has been increasingly studied via stable isotope analysis of body tissues. However, the theoretical basis for using stable isotopes to elucidate consumer–prey relationships remains poorly validated for most taxa despite numerous studies using this technique in natural systems. In this study, we measured stable carbon and stable nitrogen diet-tissue discrimination (Δ_{dt}) in whole blood, red blood cells, blood plasma solutes, and skin of leatherback sea turtles (*Dermochelys coriacea*; $N=7$) maintained in captivity for up to 424 days and fed an isotopically consistent control diet with a mean C:N ratio of 2.94:1.00 and an energetic content of 20.16 ± 0.39 kJ g^{-1} Dry Mass. We used a random-effect repeated measure model to evaluate isotopic consistency among tissue samples collected on days 276, 348, and 424. Both δ^{13}C and δ^{15}N remained consistent among sampling events in all tissues (all 95% posterior intervals for the slopes of a linear model included zero), indicating that all tissues had fully integrated diet-derived stable isotope compositions. Mean tissue-specific δ^{13}C ranged from -18.30 ± 0.16‰ (plasma solutes) to -15.54 ± 0.14‰ (skin), whereas mean δ^{15}N was from 10.06 ± 0.22‰ (whole blood) to 11.46 ± 0.10‰ (plasma solutes). The computed Δ_{dt} factors for carbon ranged from -0.58‰ (plasma solutes) to $+2.25$‰ (skin), whereas Δ_{dt} for nitrogen was from $+1.49$ (red blood cells) to $+2.85$ (plasma solutes). As the only discrimination factors available for leatherback turtles, our data will be useful for future interpretations of field-derived stable isotope data for this species. The inherent variability in Δ_{dt} values among individuals was low, which supports the value of these data for dietary reconstructions. However, it is important to note that tissue-specific discrimination factors for leatherbacks contrast with the widely accepted values for endothermic species (0–1‰ for C, 3–5‰ for N), and are also different from values established for hard-shelled turtles. This underscores the need for species- and tissue-specific discrimination factors before interpreting trophic studies of wild animals, including marine turtles.

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1. Introduction

Stable carbon and nitrogen isotope analysis has become a common method for determining the trophic status and origins of nutrient resources for wildlife populations (Hobson, 1993; Caraveo-Patiño and Soto, 2005; Wallace et al., 2009). Dietary inferences based on stable isotopic profiles in animal tissues are possible because the isotope compositions of a consumer's body tissues are ultimately derived from those in its diet (Hobson and Clark, 1992; Michener and Schell, 1994; McCutchan et al., 2003). Thus, the utility of stable isotopes in diet studies is that the sources of carbon and nitrogen can be distinguished so that a consumer's diet can be inferred. Stable isotope analysis is particularly useful when an organism's diet is difficult to establish with conventional techniques (e.g. esophageal lavage, fecal analysis). By providing information on nutrients assimilated over extended periods, stable isotopes are much less affected by short-term temporal change in diet than other methods, which only provide dietary 'snapshots' of recently consumed food items (Peterson and Fry, 1987; Hobson et al., 1996). When examined for multiple individuals within a population, stable isotope analyses provide unique insights to trophic variability and niche width, or range of trophic levels at which a consumer feeds (Gu et al., 1997; Bearhop et al., 2004; Araújo et al., 2007).

Fundamental to the interpretation of stable isotope data is the need to understand the patterns by which diet isotopic values are reflected in consumer tissues. Prior studies have shown that stable carbon ($^{13}C/^{12}C=\delta^{13}C$) and stable nitrogen ($^{15}N/^{14}N=\delta^{15}N$) isotope ratios exhibit predictable differences (i.e., discriminate) between prey and consumer tissues, with most endothermic species showing an isotopic increase of 0‰ to 1‰ for δ^{13}C and 3‰ to 5‰ for δ^{15}N per trophic level (DeNiro and Epstein, 1978, 1981; Miniwaga and Wada, 1984; Post, 2002). The relatively small trophic shift for isotopic δ^{13}C

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makes this isotope ratio better suited for tracing carbon-based nutrient sources when potential inputs vary in their carbon isotopic signature (DeNiro and Epstein, 1978; Post, 2002), whereas the greater and more constant discrimination between consumer and prey $\delta^{15}N$ values enable nitrogen to be used for establishing trophic level of consumers when stable isotope data are available for primary producers or low-order consumers (Miniwaga and Wada, 1984; Vander Zanden and Rasmussen, 2001; Post, 2002). However, stable isotopic discrimination may vary widely, with some species ^{13}C-depleted by ~1‰ and ^{13}C-enriched by ~3‰ relative to their prey, and ^{15}N-enriched by less than 3‰ (Hesslein et al., 1993; Pinnegar and Polunin, 1999; McCutchan et al., 2003; Reich et al., 2008). The disparities in discrimination factors found among taxa underscore the need for additional feeding trials under controlled conditions for stable isotope ratios to be reliably used for addressing trophic linkages in unstudied groups.

An additional requirement for correct interpretation of field-derived stable isotope data is knowledge of the inherent (i.e., non-diet related) variability in tissue-specific stable isotope signatures among individuals within a population. Although the patterns of stable isotopic variation contribute to inferences about the individual diet specialization and trophic niche width of wild populations (Angerbjörn et al., 1994; Bearhop et al., 2004; Araújo et al., 2007), measurements of variation among individuals in populations maintained on constant diets are rare (Matthews and Mazumder, 2004; Sweeting et al., 2005; Barnes et al., 2008). Whereas Matthews and Mazumder (2004) have shown that greater variation in isotope signatures of primary producers manifests as greater stable isotope variance in consumer tissues, Barnes et al. (2008) found that inherent variability among individuals reared on a controlled diet is often equivalent to a large proportion of the observed variability among wild individuals. In such cases, inherent variability in isotopic values can be mistaken for variation in foraging strategy among individuals, which may result in an overestimation of the niche width or diet diversity of a population (e.g., Grey, 2001). With respect to studies of wild populations, this is exacerbated if food sources show spatial or temporal isotopic variation and consumers are sampled in different places or times (Dalerum and Angerbjörn, 2005; Araújo et al., 2007). Given that inherent variability is known to be dependent on species, life history stages, and the environment (Barnes et al., 2008), tissue-specific isotope variance should be quantified if stable isotope studies are intended to provide information on the trophic status and niche width of a population.

Knowledge of the patterns of stable isotope diet-tissue discrimination and inherent variance in wildlife populations is essential for the correct interpretation of field data. Within the Reptilia, several studies have described isotopic discrimination for hard-shelled turtles (Seminoff et al., 2006, 2007; Reich et al., 2008); however, to date there are no such data available for leatherback turtles (*Dermochelys coriacea*), the sole extant member of the family Dermochelyidae. Considering the unique metabolic status, osmoregulation, physiology, growth rates, and diet of leatherback turtles (Lutcavage and Lutz, 1986; Lutcavage et al., 1989; Wallace and Jones, 2008), and the substantial variability in stable isotope discrimination reported within other marine taxa (Hesslein et al., 1993; Hobson et al., 1996; Gorokhova and Hansson, 1999; Kurle, 2002), determining discrimination factors for leatherback turtles is required before stable isotopes can be used reliably for dietary reconstructions within this species. Understanding stable isotope discrimination in leatherbacks is of paramount importance considering that this species will continue to be the focus of isotopic evaluations (e.g. Caut et al., 2008), and individuals have, and may continue to be used as 'living platforms' to study oceanographic processes (Block et al., 2003; Wallace et al., 2006).

In this study, we measured carbon and nitrogen diet-tissue discrimination and modeled inherent stable isotope variability in whole blood, red blood cells, blood plasma solutes, and skin of leatherback turtles maintained in captivity and fed a controlled diet for 424 days. Leatherback turtles occur in tropical and temperate waters worldwide and have been shown to be dietary specialists, consuming scyphomedusae and other gelatinous prey (Lutcavage and Lutz, 1986; James and Herman, 2001; Salmon et al., 2004; Houghton et al., 2006; Witt et al., 2007). To our knowledge, this study represents the longest period that multiple leatherback turtles have been maintained in captivity (see Jones, 2009), and is the first controlled study examining stable isotope discrimination in this critically endangered species. Our goal was to facilitate accurate interpretations of data generated from future field-based studies, and to contribute to the general knowledge of species- and tissue-specific stable isotope discrimination within the Reptilia.

2. Materials and methods

2.1. Captive conditions

The present study was part of a larger effort to examine metabolism and energetics of leatherback turtles (Jones, 2009). Twenty hatchlings (emergence July 2nd, 2005) were transported from Tortola, British Virgin Islands (BVI) to the Animal Care Center, Department of Zoology, University of British Columbia. The complete husbandry protocols used in this study are given in Jones (2009). In short, the turtles were maintained in large oval tanks (5 m long × 1.5 m wide × 0.3 m deep) containing ~2500 l of re-circulated/filtered salt water. As the turtles grew in size, header tanks were added that doubled or tripled the active volume of filtered water per turtle. The water temperature was maintained at 24 ± 1 °C. Four fluorescent fixtures (40 W UVA/B; Repti-Glow® 8) suspended 0.5 m above each tank provided full spectrum radiation for 12 h per day; each tank was also exposed to ambient light. Water quality was maintained between the following levels: pH = 8.0–8.3; salinity = 28–33 ppt; and ammonia < 0.1 mg^{-1}.

Leatherbacks were maintained on a diet consisting of a pureed amalgamate of Pacific Ocean squid (*Todarodes pacificus*; mantle and tentacles only), vitamins (Reptavite™) and calcium (Rep-Cal™), that was blended with un-flavored gelatin and hot water, then chilled to create a gelatinous diet following protocols of Jones et al. (2000). The diet was 90% water and had an energetic content of 20.16 ± 0.39 kJ g^{-1} Dry Mass, with mean C:N ratio of 2.94:1. For diet with lipids, $\delta^{13}C$ and $\delta^{15}N$ (± SD) were −17.71 ± 0.37‰ ($n = 18$) and +8.64 ± 0.46‰ ($n = 19$), respectively, whereas for lipid-free diet, the respective $\delta^{13}C$ and $\delta^{15}N$ values were −17.76 ± 0.28‰ ($n = 18$) and +8.59 ± 0.73‰ ($n = 18$).

Turtles were fed three times daily to satiation. Food was weighed with an analytical balance (Ek-1200 A; Stites Scale Inc., Cincinnati, OH) prior to feeding, and individual food mass intake was recorded each day. Turtles were weighed and measured once each week to monitor growth. Straight carapace length (SCL), i.e., the distance from the center of the nuchal notch to the caudal peduncle (posterior of the carapace), was measured using a digital caliper to the nearest 0.1 mm. Body mass was measured using an electronic scale (± 0.02 kg; ADAM CPW-60, Dynamic Scales, Terre Haute, Indiana USA).

2.2. Sample collection and preparation

Blood and skin were collected from leatherbacks on days 276, 348, and 424 after hatching. This >1 year study duration was deemed necessary based on dietary assimilation rates observed in previous studies (Seminoff et al., 2006, 2007). Seven turtles were included in this study; however, we did not collect tissues from all turtles during each sampling period due to logistic constraints and/or poor body condition. During the initial sampling period, we sampled up to six individuals, whereas during sampling periods two and three fewer turtles were available due to deaths while in captivity, in which cases we sampled only four or five turtles (see

Fig. 1). Blood samples (~1.0 ml) were collected with a 21-gauge needle and syringe from the dorsal cervical sinus (Owens and Ruiz, 1980) and transferred to a non-heparinized container. Approximately one half of each blood sample was immediately separated into plasma and cellular components by centrifugation (5000 rpm for 5 min). Blood samples (whole blood, red blood cells, and blood plasma) were promptly dried at 60 °C for 24 h then powdered with a mortar and pestle. Skin samples (0.10–0.25 g wet mass) were collected with a 2 mm biopsy punch from the dorsal surface of the neck. The top layer of the skin (stratum corneum) was separated from the underlying tissue (stratum germinativum), rinsed with deionized water, finely diced with a scalpel blade, then dried at 60 °C for 24 h. Lipids were removed from skin samples and a portion of each diet sample using a Soxhlet apparatus with a 1:1 solvent mixture of petroleum ether and ethyl ether for at least two 10-h cycles. Lipids were not extracted from blood fractions due to the small size of tissue samples coupled with the anticipated loss of tissue during the lipid extraction process.

2.3. Sample analysis

Approximately 1.0 mg of each sample was loaded into sterilized tin capsules and analyzed by a continuous-flow isotope-ratio mass spectrometer in the Stable Isotope Laboratory at Scripps Institution of Oceanography, La Jolla, California, USA. We used a Costech ECS 4010 elemental combustion system interfaced via a ConFlo III device (Finnigan MAT, Bremen, Germany) to a Deltaplus gas isotope-ratio mass spectrometer (Finnigan MAT, Bremen, Germany). Sample stable isotope ratios relative to the isotope standard are expressed in the following conventional delta (δ) notation in parts per thousand (‰):

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

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 Baker Acetanilide (C_8H_9NO; $\delta^{13}C = -10.4$) calibrated monthly against the Peedee Belemnite (PDB) limestone formation international standard; $R_{standard}$ for ^{15}N was IAEA N1 Ammonium Sulfate ($(NH_4)_2SO_4$; $\delta^{15}N = +0.4$) calibrated against atmospheric N_2 and USGS Nitrogen standards. All analytical runs included samples of standard materials inserted every 6 to 7 samples to calibrate the system and compensate for any drift over time. Hundreds of replicate assays of standard materials indicated measurement errors of 0.06‰ and 0.12‰ for carbon and nitrogen, respectively. Samples were combusted in pure oxygen in the elemental analyzer. Resultant CO_2 and N_2 gasses were passed through a series of thermal conductivity detectors and element traps to determine percent compositions. Acetanilide standards (10.36% N, 71.09% C) were used for calibration.

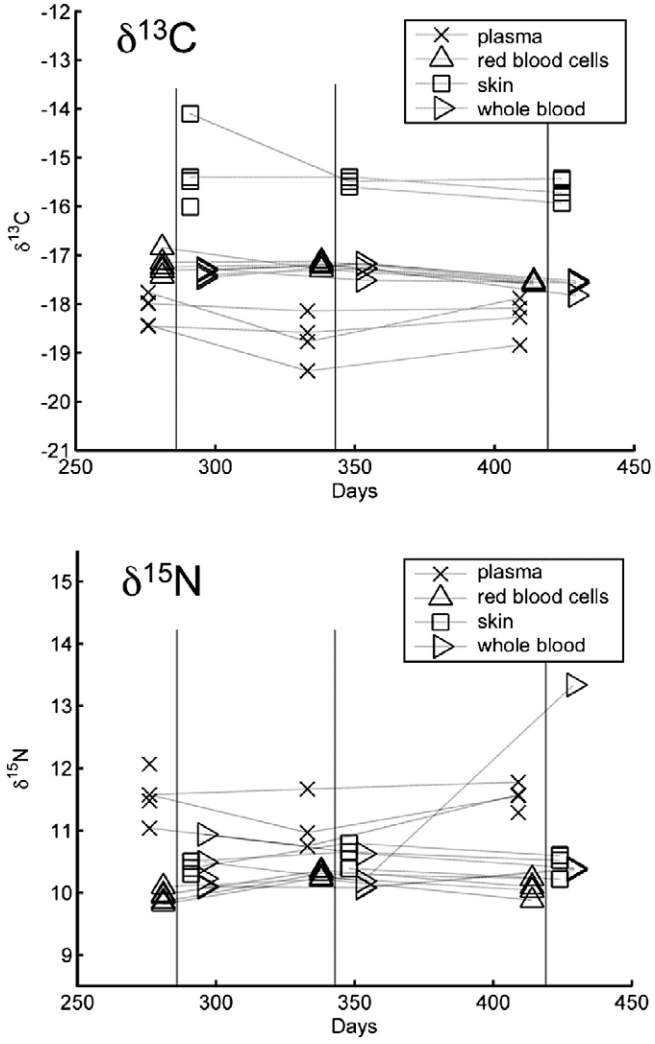

Fig. 1. Summary of $\delta^{13}C$ (top) and $\delta^{15}N$ (bottom) values for leatherback soft tissues sampled during this study. Vertical lines indicate sampling events. Values for each tissue type are offset from this date for easier viewing.

2.4. Discrimination modeling

Isotopic analyses were conducted on turtles that had been reared on the control diet for at least 276 days. This duration was more than three times longer than mean residence times (i.e., turnover rates) demonstrated for carbon and nitrogen in studies of isotopic turnover in chelonians (Seminoff et al., 2007; Reich et al., 2008), and presumably all maternal isotopic influences had been metabolized and the tissues reflected the isotopic influences of the control diet only. To establish that the stable isotope ratios ($\delta^{13}C$ or $\delta^{15}N$) within each tissue were in equilibrium with the diet (i.e. had achieved complete turnover from the maternal signal), we used a random-effect repeated measure model to estimate the slopes of isotope ratios over time. If an estimate of the slope was not significantly different from zero, we interpreted the isotope ratio in the tissue to be at equilibrium. The following model was used:

$$Y_{ij} \sim Normal(\alpha_i + \beta_i(x_j - \bar{x}), \sigma_Y^2)$$

where Y_{ij} is the stable isotope ratio of a tissue for the ith individual on the jth sample, x_j = the jth day of sampling (x_j = 276, 348, and 424), and $\bar{x}$ = the mean of x_js. The random effects are modeled via a hierarchical approach, where αs and βs are treated as random samples from a hyper-distribution:

$$\alpha_i \sim Normal(\mu_\alpha, \sigma_\alpha^2)$$

and

$$\beta_i \sim Normal(\mu_\beta, \sigma_\beta^2)$$

where μ and σ^2 are the means and variances of the hyper distributions (Gelman et al., 2004). Using the Bayesian approach, we made inferences on μ_βs, or the slopes of the linear model. If a 95% posterior interval of a μ_β included zero and the width of the interval was narrow, we concluded that the slope was near zero, indicating no change in the isotope ratio over the sampling period and equilibrium of the isotope in the tissue. Computations for this analysis were conducted using WinBugs (Lunn et al., 2000; http://www.mrc-bsu.cam.ac.uk/bugs/). We used vague prior distributions for all parameters

($\sigma_Y^2 \sim Gamma(0.001, 0.001)$, $\mu_\alpha \sim Normal(0, 1{,}000{,}000)$, $\sigma_\alpha \sim Unif(0, 100)$, $\sigma_\beta \sim Unif(0, 100)$). We used three independent chains of 100,000 steps, where every five steps were retained to avoid autocorrelations within the chain. Median and 95 percentiles were used to make inference on the slopes.

Leatherback diet-tissue discrimination estimates (Δ_{dt}; Cerling and Harris, 1999) were calculated as $\Delta_{dt} = \delta_{tissue} - \delta_{diet}$. To include the variability in measured stable isotope ratios among both diet and tissue samples, we used a parametric bootstrap method (Efron and Tibshirani, 1993). We used a bivariate normal distribution to represent the distribution of $\delta^{13}C$ and $\delta^{15}N$ values for diet and each tissue. To adequately incorporate the variability in the isotopic ratios of diet into the calculations of discrimination factors, we fitted a bivariate normal distribution to the data, where marginal distributions were used to compute element specific means and variances. To examine the relationship between tissue-specific variance in $\Delta_{dt}^{13}C$ and $\Delta_{dt}^{15}N$, we used the joint distributions. To compute the discrimination factors (Δ_{dt}) and model Δ_{dt} variance in the study group, 50,000 paired random samples were drawn from each distribution. This approach explicitly included the uncertainty in measurement of stable isotope ratios among individuals, which provided more realistic estimates of the discrimination factors and inherent variability than if only the means of stable isotope ratios were used. For skin samples, with lipids removed, we determined Δ_{dt} using $\delta_{diet\ without\ lipids}$, whereas for all other tissues we determined Δ_{dt} using $\delta_{diet\ with\ lipids}$. To make comparisons with other studies possible (e.g., Bearhop et al., 2004), we derived population level variance (σ^2) for $\Delta_{dt}^{13}C$ and $\Delta_{dt}^{15}N$ in each tissue. We also compared the stable carbon and nitrogen variance of the diet samples (with and without lipids removed) with each tissue to identify the preferred tissue types for examination in field studies. This was calculated as σ_{tissue}^2:σ_{diet}^2, whereby larger ratios (>1:1) reflect relatively higher isotope variance in tissues (i.e. are less preferable for field-based isotope studies). Statistical analyses were carried out using Matlab (The Mathworks, Inc., Natick, MA).

3. Results

3.1. Nutritional intake and growth

We observed feeding and measured growth by all seven turtles throughout the study (see Jones, 2009). Mean food intake during this study ranged from 4.5 ± 1.8 to 6.8 ± 0.6% of body mass per day. Between days 1 and 276, the mean SCL of turtles increased from 6.3 ± 0.1 to 25.8 ± 1.0 cm and mean body mass increased from 0.046 ± 0.001 to 2.4 ± 0.2 kg ($N = 7$). On day 424, mean SCL and body mass were 46.08 ± 1.7 cm and 11.89 ± 1.6 kg ($N = 4$), respectively.

3.2. Stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$)

Within leatherback tissues, mean $\delta^{13}C$ ranged from −18.35 ± 0.46‰ (plasma solutes) to −15.46 ± 0.49‰ (skin), and mean $\delta^{15}N$ from +10.08 ± 0.18‰ (red blood cells) to +11.45 ± 0.37‰ (plasma solutes) (Table 1). There was slightly greater variability for $\delta^{13}C$ in skin during the initial sampling period and a substantial outlier for $\delta^{15}N$ in whole blood recorded in the final sampling period (Fig. 1). Estimated slopes of the random-effect repeated measures linear model were not significantly different from zero for all tissues (all posterior intervals included zero, where the widths of 95% posterior intervals ranged from 0.005 to 0.04; Table 2). Consequently, we concluded that the carbon and nitrogen isotope ratios in the tissues were in equilibrium with the diet.

3.3. Diet-tissue discrimination (Δ_{dt})

To avoid potential overconfidence in precision of estimated Δ_{dt} factors, we kept the two outliers in stable isotope ratios when computing the discrimination factors. Mean $\Delta_{dt}^{13}C$ ranged from −0.58 ± 0.53‰ (plasma solutes) to +2.26 ± 0.61‰ (skin), with red blood cells, whole blood, and skin enriched in ^{13}C relative to diet, and blood plasma solutes ^{13}C-depleted (Table 1). All tissues were ^{15}N-enriched over the diet, with mean $\Delta_{dt}^{15}N$ ranging from +1.49 ± 0.76‰ (red blood cells) to +2.86 ± 0.82‰ (plasma solutes) (Table 1).

Table 1
Stable isotope values ($\delta^{13}C$, $\delta^{15}N$), discrimination values ($\Delta_{dt}^{13}C$, $\Delta_{dt}^{15}N$), and tissue-specific discrimination variance (σ_Δ^2) for leatherback soft tissues and the experimental diet.

	Mean stable isotope values		Mean diet-tissue discrimination	
	$\delta^{13}C$ (SD)	$\delta^{15}N$ (SD)	$\Delta_{dt}^{13}C$ (SD)	$\Delta_{dt}^{15}N$ (SD)
Plasma solutes	−18.35 (0.46)	+11.45 (0.37)	−0.58 (0.53)	+2.86 (0.82)
Red blood cells	−17.31 (0.22)	+10.08 (0.18)	+0.46 (0.35)	+1.49 (0.76)
Skin	−15.46 (0.49)	+10.50 (0.18)	+2.26 (0.61)	+1.85 (0.50)
Whole blood	−17.42 (0.18)	+10.57 (0.87)	+0.35 (0.33)	+1.98 (1.14)
Food-lipids	−17.71 (0.34)	+8.64 (0.47)		
Food-no lipids	−17.76 (0.28)	+8.59 (0.73)		

Discrimination values for skin samples (lipid-free) were determined $\delta_{diet\ without\ lipids}$, whereas discrimination for all other tissues was determined using $\delta_{diet\ with\ lipids}$. See Methods for analytical procedures.

Table 2
Median and 95% posterior limits (in square brackets) of the estimated slope of the mixed effects model.

	$\delta^{13}C$	$\delta^{15}N$
Plasma solutes	−0.0009 [−0.0085, 0.0062]	0.0003 [−0.0077, 0.0091]
Red blood cells	−0.0028 [−0.0063, 0.0214]	0.0012 [−0.0032, 0.0059]
Skin	−0.0038 [−0.0140, 0.0075]	0.0005 [−0.0043, 0.0235]
Whole blood	−0.0019 [−0.0048, 0.0022]	0.0055 [−0.0123, 0.0244]

3.4. Stable isotope variance (σ^2)

We examined stable isotopic variance in two ways: (1) comparing variance among $\delta^{13}C$ and $\delta^{15}N$ in the diet and leatherback tissues (σ_{tissue}^2:σ_{diet}^2; Fig. 2), and (2) modeling variance in $\Delta_{dt}^{13}C$ and $\Delta_{dt}^{15}N$ through parametric bootstrap techniques that generated a bivariate normal distribution for the Δ_{dt} for each isotope and tissue type (Fig. 3). The σ_{tissue}^2:σ_{diet}^2 ratios showed that skin, and, to a lesser extent plasma solutes, had the greatest $\delta^{13}C$ variability relative to diet, whereas whole blood by far had the largest $\delta^{15}N$ variability relative to diet.

The fitted bivariate normal density distributions showed differences in tissue-specific variance, with the relative degree of variance reflected by the width of the distributions (Fig. 3). Overall, these results were consistent with the ratio-based examination of variance. The variance heirarchy for $\Delta_{dt}^{13}C$ was: skin > plasma solutes > red blood cells > whole blood, whereas for $\Delta_{dt}^{15}N$ it was whole blood > plasma solutes > red blood cells > skin (Table 1). As would be expected, variance was greatest in tissues that had the outlying values (Fig. 1 and Table 1). Tissue-specific variance was not always consistent for both elements. For example, while skin showed the greatest level of variability for carbon, it had the least variability for nitrogen (Table 1; Fig. 3). The modeled Δ_{dt} distributions for carbon indicated a clear separation between skin and plasma, with these representing the minimum and maximum $\Delta_{dt}^{13}C$ values, respectively (Fig. 3, top). The modeled $\Delta_{dt}^{15}N$ distributions were more consistent, with substantial overlap among all four tissue types (Fig. 3, bottom).

4. Discussion

4.1. Stable isotope discrimination

It has been well established that diet-tissue discrimination varies among species and homologous tissues (e.g. Tieszen et al., 1983; Roth

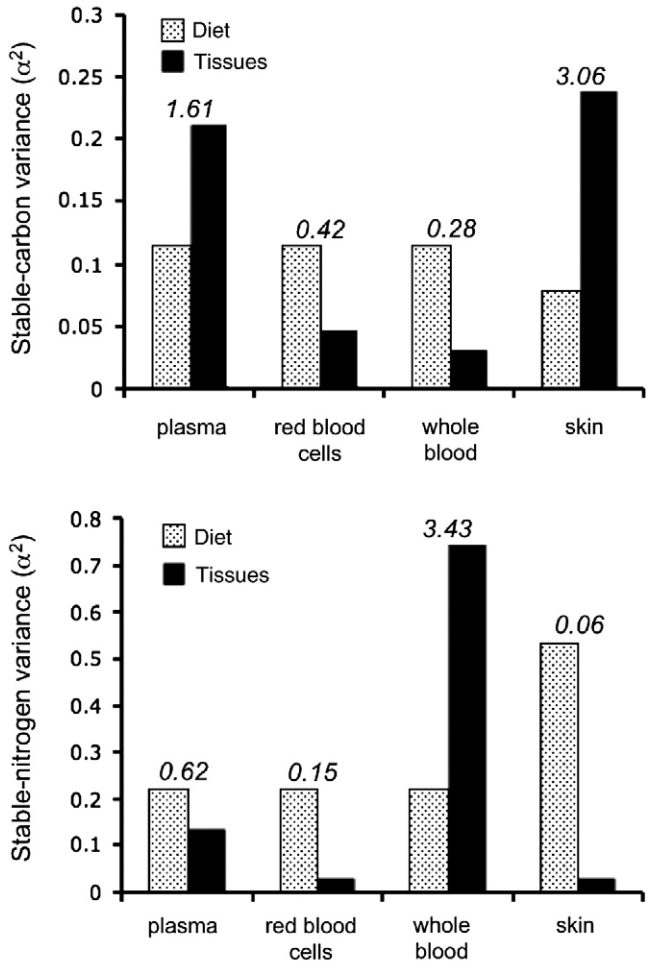

Fig. 2. Comparison of sample variance in the diet and soft tissues of leatherback turtles for $\delta^{13}C$ (top) and $\delta^{15}N$ (bottom). Diet variance was determined for diet with lipids (whole blood, red blood cells, plasma solutes) and diet without lipids (skin). Numeric values reflect $\sigma^2_{tissue}:\sigma^2_{diet}$, whereby larger ratios reflect relatively higher isotope variance in tissues versus diet (i.e. are less preferable for field-based isotope studies).

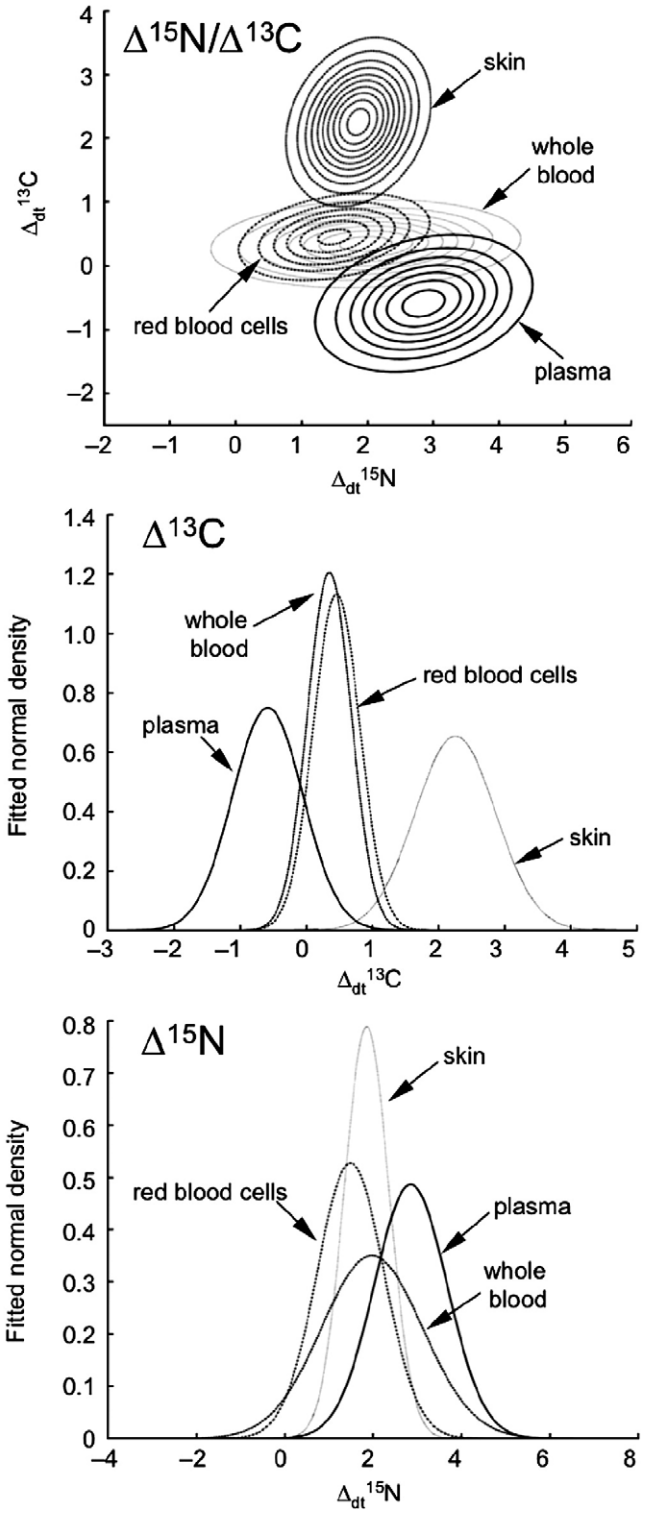

Fig. 3. Bivariate normal distributions for $\Delta^{13}_{dt}C$ and $\Delta^{15}_{dt}N$ of tissue samples from juvenile leatherback turtles kept captive for 419 days: joint distribution of $\Delta^{13}_{dt}C$ and $\Delta^{15}_{dt}N$ based on parametric bootstrap methods (top), and marginal distributions of $\Delta^{13}_{dt}C$ and $\Delta^{15}_{dt}N$ (middle, bottom).

and Hobson, 2000; Podlesak et al., 2005). Diet type may also affect discrimination due to varying protein, carbohydrate, and lipid concentrations and differential routing of these isotopically distinct dietary fractions to specific tissues or tissue components (Hobson and Clark, 1992; Tieszen and Fagre, 1993; Ayliffe et al., 2004). While understanding the fate of assimilated dietary components will be important to fully determine the biochemical mechanisms of discrimination (Gannes et al., 1997; Popp et al., 2007), an equally important step is to identify the patterns of isotopic discrimination in a variety of species with disparate, but well-studied physiologies and life histories. Conducting controlled stable isotopic discrimination and tissue-specific isotopic variance studies in a broad array of species will help elucidate the mechanisms responsible for disparities in discrimination. In the present study, we examined stable isotope discrimination in the highly migratory and wide ranging leatherback turtle, a species that is notoriously difficult to maintain in captivity (Birkenmeier, 1971; Jones et al., 2000; Jones, 2009). Hence, this study represents one of the few opportunities to examine this species in a controlled setting, and importantly, it provides discrimination information for one of the most unique reptiles in terms of size, physiology, thermoregulation, and diet (Lutcavage and Lutz, 1986; Lutcavage et al., 1989; Wallace and Jones, 2008).

The results of our study further establish that tissue-specific discrimination values for marine turtles do not fall within previously established discrimination values reported for vertebrates. Of the four tissues analyzed for stable carbon, only red blood cells (+0.46‰) and whole blood (+0.35‰) fell within the 0‰ to +1‰ range that has been widely considered to be the universal benchmark for stable carbon discrimination (DeNiro and Epstein, 1978; Miniwaga and Wada, 1984; Peterson and Fry, 1987). With respect to stable nitrogen discrimination, our results were consistently lower than the $\Delta^{15}_{dt}N$

range of +3.0‰ to +5.0‰ that has been commonly reported for endothermic taxa (DeNiro and Epstein, 1981; Miniwaga and Wada, 1984; Vanderklift and Ponsard, 2007). It is interesting to consider that hard-shell turtles (Fig. 4) and other ectothermic species such as broad whitefish (*Coregonus nasus*; Hesslein et al., 1993) and rainbow trout (*Oncorhynchus mykiss*; Pinnegar and Polunin, 1999; McCutchan et al., 2003) also show discrimination values that fall outside the assumed 'norm' for discrimination in vertebrate taxa. These discrepancies underscore the need for species- and tissue-specific Δ_{dt} values to facilitate accurate interpretation of field data.

The trend of lower nitrogen discrimination in leatherbacks versus previously studied terrestrial vertebrates may relate disparities in the nitrogenous waste strategies employed by these groups. Whereas leatherbacks and other marine turtles are largely ammonotelic (Jones, 2009), birds and terrestrial mammals — which constitute the vast majority of previous vertebrate discrimination studies (e.g. Hobson and Clark, 1992; Evans Ogden et al., 2004) — a largely ureotelic or uricotelic (Vanderklift and Ponsard, 2007). In an extensive literature review yielding 134 Δ_{dt}^{15}N estimates from controlled studies, Vanderklift and Ponsard (2007) found that there was a significant effect of the main biochemical form of nitrogenous waste, with ammonotelic organisms showing the lowest Δ_{dt}^{15}N among all animals studied. Thus, the disparity in Δ_{dt}^{15}N for leatherbacks versus other vertebrates may result from the number of biochemical steps related to the synthesis of different nitrogenous waste products. Whereas aquatic turtles eliminate nitrogenous waste largely in the form of ammonia (NH_4^+), most terrestrial vertebrates have developed a series of additional biochemical reactions to produce either uric acid or urea. If there are differential rates of reactions for ^{15}N and ^{14}N at each step, we would expect urea and uric acid to have proportionally more ^{14}N than ammonia (i.e. greater diet-tissue nitrogen fractionation). Alternatively, the low Δ_{dt}^{15}N may result from a high nitrogen diet, coupled with low nitrogen use efficiency, where leatherbacks' natural ability to assimilate nitrogen in such a high nitrogen diet might reach satiation, thus leading to relatively low assimilation of dietary N, and lower discrimination (see Wieser, 1985). Elucidating the potential role of nitrogenous waste strategies and nitrogen assimilation efficiency will require a greater understanding of leatherback nutritional ecology and physiology.

Notwithstanding the aforementioned similarity in Δ_{dt} among turtles (Fig. 4), our results showed that leatherbacks are unique among turtles in two ways. First, plasma solutes showed the greatest ^{13}C-depletion (−0.58‰) of any other turtle, and second, leatherback whole blood (+1.98‰) and red blood cells (+1.50‰) were substantially more ^{15}N-enriched than any hard-shelled marine turtle studied to date, and approached levels only seen in the freshwater turtle *Trachemys scripta* (Fig. 4). Because lipids are usually depleted in ^{13}C relative to protein and carbohydrates (DeNiro and Epstein, 1977), the ^{13}C depletion in these tissues may have been due to the presence of excessive lipids in the blood. Recall that we did not extract lipids from blood plasma — due to the small volume of blood samples and low yield of lipid-free plasma solutes during the lipid extraction process. If blood plasma serves as a reservoir for lipid accumulation (see Lehninger, 1982), higher concentrations of lipid in blood plasma relative to diet may result, causing a negative Δ_{dt}^{13}C. Depletion of ^{13}C in plasma solutes has also been found in green turtles (Fig. 4; Seminoff et al., 2006) and loggerhead turtles (Fig. 4; Reich et al., 2008), as well as in the lizard *Sceloporus undulatus* (−0.50‰; Barnes et al., unpubl. data). The ambiguity in potential effects of lipids on our δ^{13}C results underscores the importance of removing lipids when possible from all diets and tissues prior to stable isotope analyses.

Trophic enrichment of ^{15}N is generally attributed to fractionation during amino acid deamination and transamination, whereby ^{14}N amine groups are preferentially removed to produce isotopically light

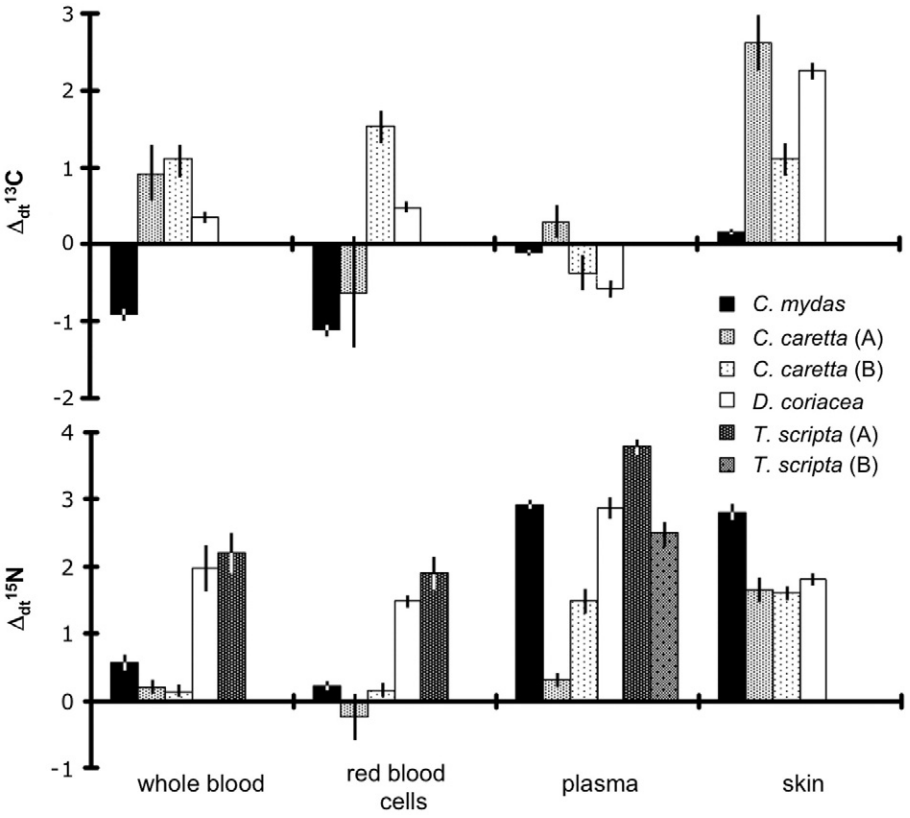

Fig. 4. Comparison of Δ_{dt}^{13}C and Δ_{dt}^{15}N among four turtle species. *Chelonia mydas* (green turtle) data from Seminoff et al., 2006; *Caretta caretta* (loggerhead turtle) data from Reich et al., 2008; *Trachemys scripta* (Pond slider) data from Seminoff et al., 2007; *Dermochelys coriacea* (Leatherback turtle) data from this study. (A) denotes soy protein diet; (B) denotes animal protein diet; Error bars represent standard deviation.

metabolites, leaving the remaining nitrogen pool enriched in ^{15}N (referred to as metabolic fractionation) (Macko et al., 1986; Gannes et al., 1997). Under this scenario, high tissue-specific metabolic activity should result in greater $\Delta^{15}_{dt}N$ owing to greater throughput of all nitrogen species and the resulting sequestration of ^{15}N. The increased $\Delta^{15}_{dt}N$ in leatherback whole blood and red blood cells relative to other turtles thus may relate to ionoregulation and its implications for red blood cell metabolism. Whereas hard-shelled marine turtles (Cheloniidae) have water turnover rates of 10–15% total body water (TBW) day^{-1} (Ortiz et al., 2000; Jones et al., in press), leatherbacks have water turnover rates of 24% TBW day^{-1} in adults (Wallace et al., 2005) and up to 40% TBW day^{-1} in juveniles (T. Jones, unpubl. data), exceeded only by freshwater turtles, that may have as high as 400% TBW day^{-1} (Booth, 2002). The red blood cells of reptiles are nucleated and with such high water turnover rates and extra-cellular fluid volume the red blood cells would have to use Na^+, K^+-ATPase pumps and Na^+,K^+, $2Cl^-$ co-transport or some other form of active transport to maintain cell volume. This would lead to increased cellular metabolic rate of species with higher TBW turnover, possibly leading to the increased $\Delta^{15}_{dt}N$. Thus, it is not surprising that $\Delta^{15}_{dt}N$ for leatherback whole blood and red blood cells is rivaled only by that for *T. scripta*, a freshwater turtle (Seminoff et al., 2007). However, to further substantiate this pattern we encourage studies quantifying TBW and Δ_{dt} for additional freshwater turtle species.

A critical assumption in our derivation of discrimination factors was that leatherback tissues were in equilibrium with the diet and did not significantly reflect any maternal stable isotopic influence. There are two processes that drive diet-tissue equilibration: (1) the 'dilution' of the maternal isotopic signature as a result of the formation of new tissue with nutrients mobilized from the control diet, and (2) the turnover of old tissue signatures with new dietary signatures during tissue repair and maintenance (Hesslein et al., 1993; Sweeting et al., 2005). The relative importance of each process varies with growth rate and body size, although dilution is more often the dominant process in growing animals (Hesslein et al., 1993; Klaassen et al., 2004; Sweeting et al., 2005). In the present study we infer that both dilution and turnover contribute to the diet-tissue isotopic equilibrium. First, leatherbacks grew by an average of >11 kg over the study period, which equates to a 250-fold increase in mass. Thus, in the unlikely absence of turnover, 99.6% of the turtles' body mass was added from nutrients garnered from the diet. Second, our study is more than three times longer than maximal stable carbon and nitrogen residence times reported by Reich et al. (2008) for tissues of loggerhead turtle hatchlings (83 days and 71 days, respectively). This suggests that nutrients from egg-constituents (i.e., maternal influence) had cycled out of the body well before the start of this study.

4.2. Application to field studies

The exorbitant growth of leatherbacks in this study raises a valid question relating to the role of somatic growth in isotopic discrimination. Namely, how would the results of our study of juvenile turtles compare to a study of adults? Although the latter would be nearly impossible due to husbandry challenges, insights to this question may be gained from the current understanding of animal nutrition. First, we believe that growth should exhibit maximal effects on isotopic discrimination of nitrogen versus carbon due to the fact that growth is dominated by protein synthesis and nitrogen is almost unique to protein. Indeed, higher rates of protein synthesis and catabolism have been suggested as a cause for significantly higher stable nitrogen enrichment in subadult (i.e. growing) red foxes (*Vulpes vulpes*) relative to adults, whereas there was no apparent effect of growth on carbon discrimination (Roth and Hobson, 2000). However, growing animals should show the same $\delta^{15}N$ values as those of adults fed the same diet if the total amount of nitrogen they assimilate during their growth is comparable to the total nitrogen content of their adult body (Ponsard and Averbuch, 1999). Considering the tremendous disparity in the mass of hatchlings (~40 g) versus adults (~800 kg), the total nitrogen assimilated over the course of somatic growth is almost equal to the nitrogen found within an adult. These considerations suggest that the discrimination factors obtained here for juvenile leatherback turtles are applicable to leatherbacks of a broad size range, perhaps including adults.

It has been well established that diet type will impact stable isotope discrimination within vertebrate species (Hobson and Clark, 1992; Tieszen and Fagre, 1993; Pearson et al., 2003; Ayliffe et al., 2004; Reich et al., 2008). Therefore, it is reasonable to consider the potential difference in Δ_{dt} for leatherbacks raised on our control diet versus leatherbacks foraging in the wild. In the present study, we used an amalgamate of gelatin (almost complete protein) and squid (high protein, low to no carbohydrates or lipids) with a C:N ratio of 2.94:1.00. In the wild, leatherbacks consume a specialized diet of schyphomedusae and other large gelatinous prey throughout their life-cycle (James and Herman, 2001; Salmon et al., 2004; Houghton et al., 2006; Witt et al., 2007) which are similar to our study diet in that they have very low lipid and carbohydrate concentrations (Doyle et al., 2007). Although it is not possible to conduct captive trials with all potential foods, these consistencies, coupled with the fact that leatherbacks generally eat the same types of food throughout their lives, bode well for applications of our results to wild turtles.

4.3. Stable isotopic variance

Fundamental to any use or investigation of stable isotopes in a wild predator population is knowledge of the level of inherent variance associated with individual physiology and diet-tissue fractionation. Our data indicate plasma solutes and skin have high inherent variance in $\delta^{13}C$ relative to diet, whereas whole blood has the highest inherent variance in $\delta^{15}N$ among all tissues (Fig. 2). Therefore any field studies that use these tissues and isotopes to determine leatherback-prey or leatherback trophic niche width should proceed cautiously. We acknowledge that large isotopic variance in these tissues relative to the study diet can result from two sources: (1) one or a few outliers in isotopic values within a subset of sample periods, and (2) consistently high isotopic variability among all sampling periods with no outliers. Whereas the former represents the situation with $\delta^{13}C$ in skin and $\delta^{15}N$ in whole blood, with single outliers present on day 276 and 424, respectively, the latter is reflective of $\delta^{13}C$ and $\delta^{15}N$ in plasma throughout all three sampling periods. Although we do not diminish the implications of high variance in tissue types, regardless of the causes, we believe that the former scenario, with occasional outlying isotope values, is of lesser concern when determining trophic niche width of wild populations (Bearhop et al., 2004). However, considering the apparent low tissue-specific variance relative to diet variance, we suggest that red blood cells is the ideal tissue for field studies using both stable carbon and nitrogen isotope analyses. This bodes well for isotopic examinations of threatened and endangered sea turtles since blood collection is among the least invasive sampling protocols and is on of the most commonly sampled tissues in monitoring and stranding programs (e.g. Fair and Hansen, 1998).

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