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Effects of growth and tissue type on the kinetics of ^{13}C and ^{15}N incorporation in a rapidly growing ectotherm

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Abstract 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 and the factors that determine the magnitude of the difference in isotopic composition between the animal's diet and that of its tissues. We determined 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, *Caretta caretta*). We 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 $\Delta^{15}N = \delta^{15}N_{tissues} - \delta^{15}N_{diet}$) 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.

Keywords Diet shift · Growth · Isotopic discrimination · Isotopic turnover · *Caretta caretta*

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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 has 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. We 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).

Our 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. Our 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 we were 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 small 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. Both diets 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 $\delta^{13}C$ and $\delta^{15}N$ throughout the study to test for temporal variation in the isotopic composition of experimental diets. At the end of the trials, we released all turtles under Florida Wildlife Conservation Commission guidelines.

Tissues

We analyzed the isotopic composition of whole blood, red blood cells, plasma solutes, skin, and scute samples. We chose these tissues because they can be sampled non-invasively, and one of the goals of our study was to be able to release the turtles unharmed after its completion. In addition, we 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 0.02 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 (0.01 ml) of whole blood was removed and the remaining blood (0.01 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 mm^2 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 δ^{13}C $\pm$ SD and mean δ^{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, we grouped the turtles (2 hatchlings from each clutch per group) and sampled 1 of the 11 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, we began our 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 δ^{13}C $\pm$ SD and mean δ^{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 $\pm$ SD = 10.6 ± 1.35 cm) and from 105.0 to 385.7 g body mass (mean $\pm$ SD = 208.8 ± 97.5 g). We collected the same tissues from juvenile and hatchling turtles, using the same protocols except that we 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:

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

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 Vienna Pee Dee Belemnite (VPDB) limestone formation international standard. R_{standard} for ^{15}N was atmospheric N_2. IAEA CH-6 (δ^{13}C = -10.4) and IAEA N1 Ammonium Sulfate (δ^{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 accuracy of our measurements, measured as the SD of replicates of standards, was 0.11‰ for δ^{13}C and δ^{15}N ($N = 88$ and 91, respectively).

Statistical analyses

We estimated growth rate using both a linear and an exponential model in 45 individual hatchlings (Trial 1) and 8 individual juveniles (Trial 2). We 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, we compared their coefficients of determination using paired t tests and the difference in Akaike's Information Criteria (AIC) between the two models ($\Delta i = \text{AIC}_i - \text{AIC}_{\text{min}}$, where AIC_{min} 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 our 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) we chose the exponential over the linear growth model.

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

$$\delta X(t) = \delta X(\infty) + [\delta X(0) - \delta X(\infty)]e^{-k_{st}t}, \quad (1)$$

where $\delta X(t)$ is the isotopic composition at time t, $\delta X(\infty)$ is the asymptotic, equilibrium isotopic composition, $\delta X(0)$ 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). $\delta X(\infty)$ and $\delta X(0)$ 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 we 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 ($k_{dt} = k_{st} - k_{gt}$; see Hesslein et al. 1993). We 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. 1 was significantly different from k_g, we estimated k_{dt}, the contribution of catabolic turnover to the rate of isotopic incorporation, as $k_{st} - k_g$. We estimated isotopic discrimination (ΔX) as $\delta X(\infty)_{tissues} - \delta X_{diet}$. In addition, for juvenile turtles, we 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 our 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, we used k_g instead of k_{st} in Eq. 1. To assess the effect of variation in k_g in the pattern of incorporation curves, we 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 1 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 $1/k_{st}$. We used average residence time, rather than the more widely used "half-life" [Ln(2)/k_{st}] because we could estimate a standard error for $1/k_{st}$ as $SE(1/k_{st}) = SE(k_{st})/k_{st}^2$, where $SE(k_{st})$ is the asymptotic standard error of k_{st} (Stuart and Ord 1994). We 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 $r^2 \pm SD = 0.97 \pm 0.02$ and 0.98 ± 0.02, respectively, Fig. 1a). There was no significant difference between coefficients of determination of these models (paired $t = 0.91$, $P = 0.36$, $N = 45$). 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 equalled 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, we chose the exponential model. The exponential model estimated a fractional growth rate equal to 0.014 (±SD = 0.002) day^{-1}. Equation 1 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). For carbon, only plasma solutes and whole blood had rates of incorporation that differed significantly from the value expected from growth (Table 1). The estimated value of $k_{dt} = (k_{st} - k_g)$ for these two tissues equalled 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). 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 equalled 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. 3). Both $\Delta^{13}C$ and $\Delta^{15}N$ varied widely among tissues. $\Delta^{13}C$ ranged from −0.64 to 2.62‰ (Table 1) and $\Delta^{15}N$ ranged from −0.25 to 1.65‰ (Table 2).

Trial 2: juvenile turtles

Both linear and exponential models described the growth in mass of hatchlings relatively well (average $r^2 \pm SD = 0.96 \pm 0.02$ and 0.96 ± 0.02, respectively, Fig. 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, we assumed that turtles grew exponentially with a fractional growth rate (k_g) equal to 0.012 ± 0.001 day^{-1}). Equation 1

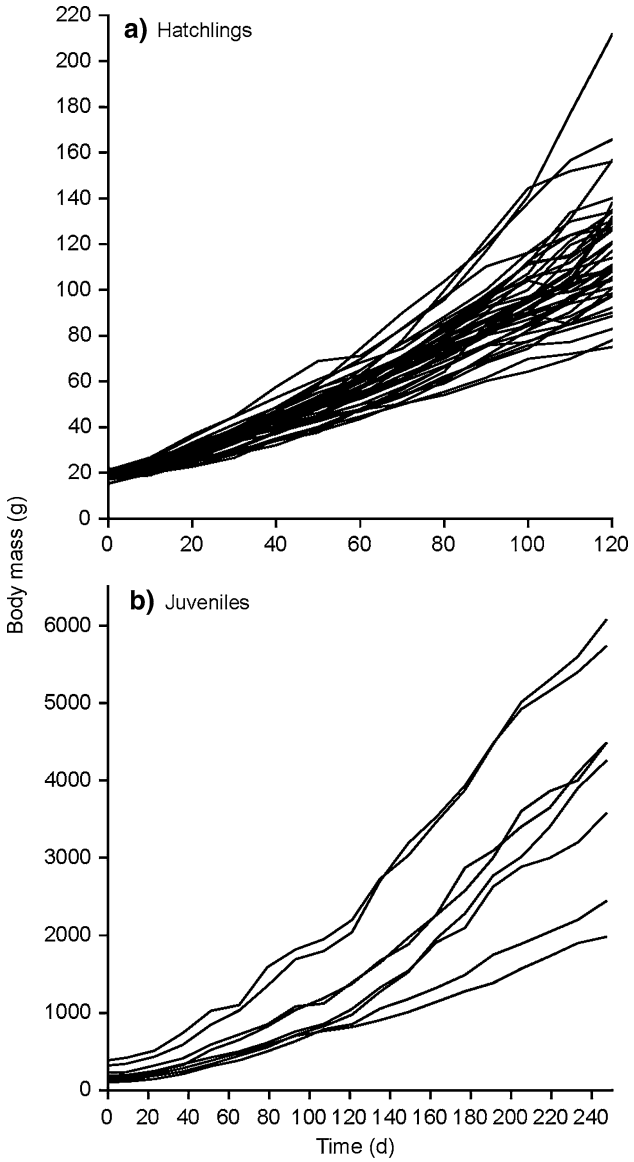

Fig. 1 Growth in **a** hatchling and **b** juvenile loggerhead turtles (*Caretta caretta*). Each *line* represents the growth trajectory of an individual. Growth trajectories are well described by exponential functions of the form $y = ae^{(bt)}$

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. 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. 4, Table 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 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 3). All tissues had significantly positive isotopic discrimination relative to bulk diet (Table 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. 4, Table 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 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 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. 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 our 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. Our 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) our results suggest that discrimination factors varied greatly among tissues, and perhaps among diets and/or developmental stages. Here we discuss each of these themes and consider their implications. Our discussion is limited by the absence of comparable data sets on other ectotherms, and

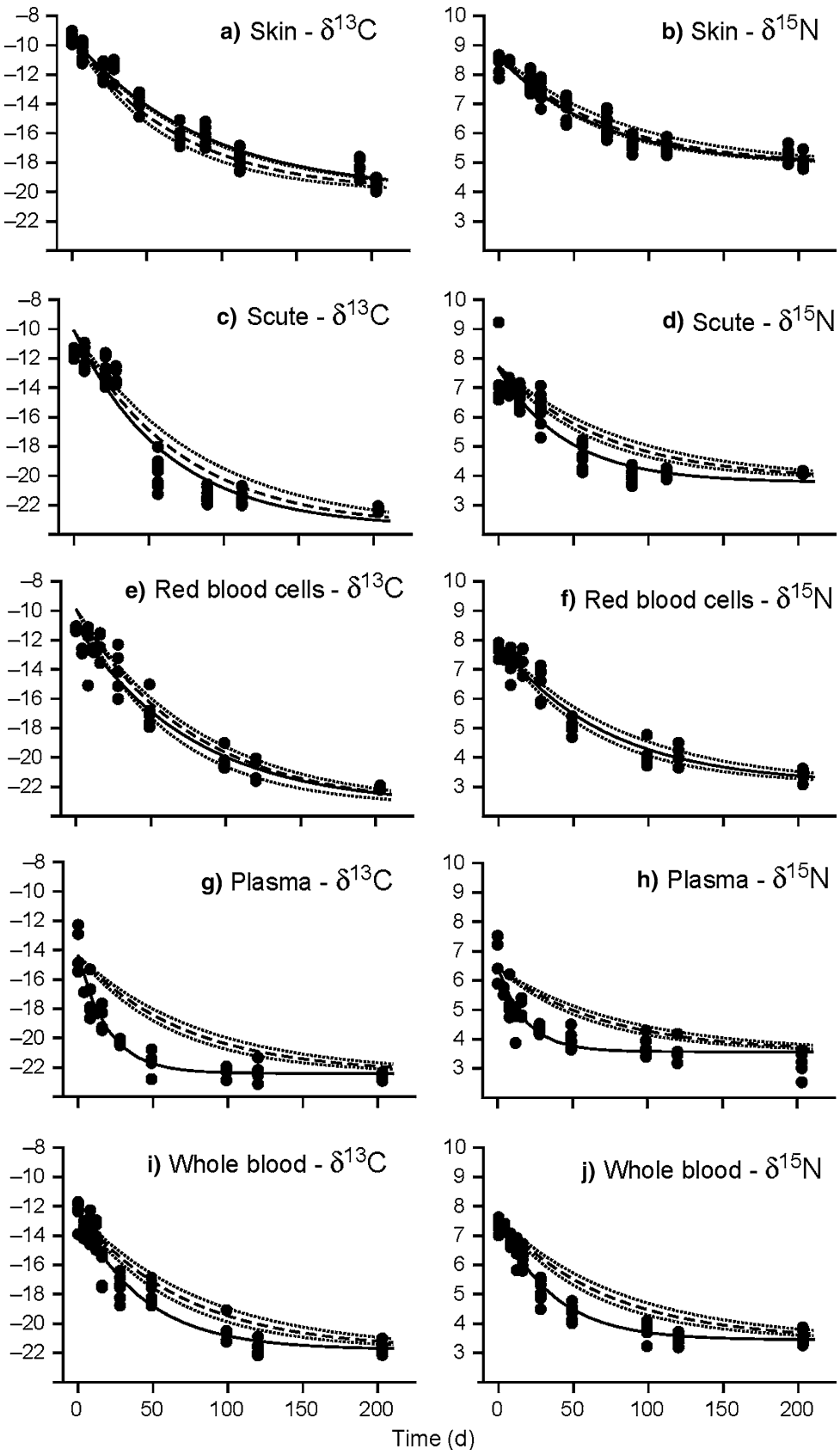

Fig. 2 Changes in $\delta^{13}C$ and $\delta^{15}N$ in loggerhead turtle hatchlings 0–203 days after a diet change (see Trial 1, Eq. 1); curves fitted by a non-linear routine (*line*). Expected levels of $\delta^{13}C$ and $\delta^{15}N$ if growth rate (k_g = 0.014 day^{-1}) was the sole determinant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

Table 1 In Trial 1, the isotopic incorporation of carbon from diet into tissues of hatchling loggerhead turtles was well described by the equation $\delta^{13}C(t) = \delta^{13}C(\infty) + [\delta^{13}C(0) - \delta^{13}C(\infty)]e^{-k_{st}t}$

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

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 $1/k_{st}$

Table 2 In Trial 1, the isotopic incorporation of nitrogen from diet into tissues of hatchling loggerhead turtles was well described by the equation $\delta^{15}N(t) = \delta^{15}N(\infty) + [\delta^{15}N(0) - \delta^{15}N(\infty)]e^{-k_{st}t}$

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

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 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 $1/k_{st}$

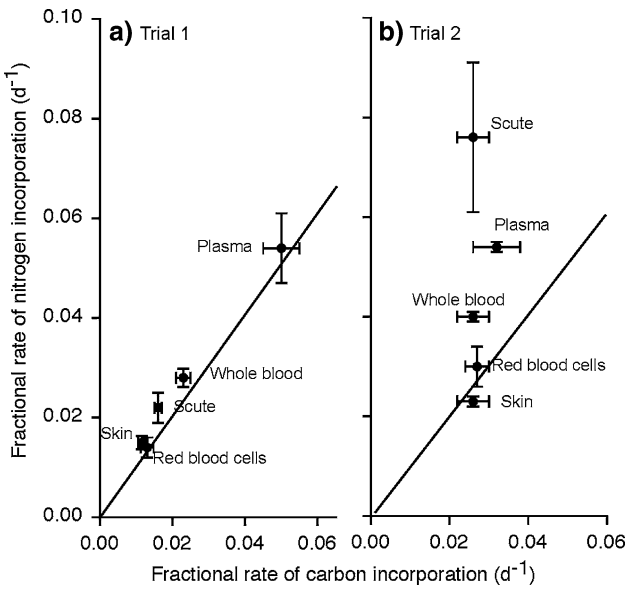

Fig. 3 Correlations of fractional incorporations of carbon and nitrogen into skin, scute, red blood cells, plasma, and whole blood of loggerhead turtles in **a** Trial 1 ($r = 0.99$, $P < 0.0006$) and **b** Trial 2 (NS), mean ± 1 SE. The *diagonal line* represents $y = x$

hence we framed some of the implications of our 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 1, 2, 3, 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

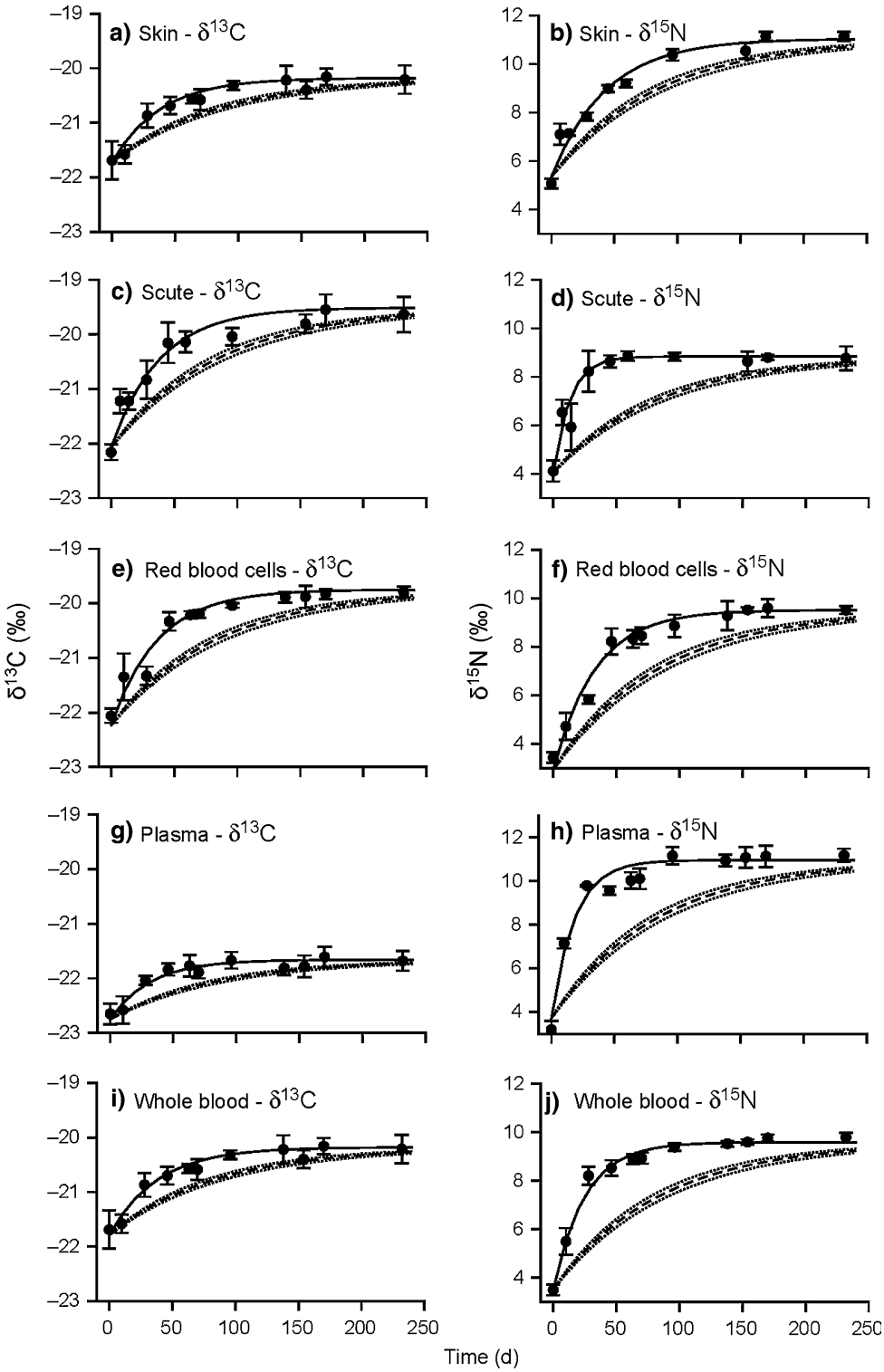

Fig. 4 Changes in $\delta^{13}C$ and $\delta^{15}N$ in loggerhead turtle juveniles 0–232 days after a diet change (see Trial 2, Eq. 1); curves fitted by a non-linear routine (*line*). Expected levels of $\delta^{13}C$ and $\delta^{15}N$ if growth rate (k_g = 0.012 day^{-1}) was the sole determinant of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

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.

We 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 1, 2).

Table 3 In Trial 2, isotopic incorporation of carbon from diet into tissues of juvenile loggerhead turtles was well described by the equation $\delta^{13}C(t) = \delta^{13}C(\infty) + [\delta^{13}C(0) - \delta^{13}C(\infty)]e^{-k_{st}t}$

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

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)

Table 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 $\delta^{15}N(t) = \delta^{15}N(\infty) + [\delta^{15}N(0) - \delta^{15}N(\infty)]e^{-k_{st}t}$

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

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)

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 our 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), we 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). Our 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. Our 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 1, 2, 3, 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

Our estimates of the relative contribution of growth rate and catabolic turnover must be qualified by the assumptions that we made. We 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 our study, turtle growth was very closely approximated by exponential functions (Fig. 1), and hence the first of Hesslein et al.'s (1993) assumptions was satisfied. Unfortunately, we have no growth data for the tissues used in our 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). Our experiments allowed us 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‰. Our 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. Our 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 our 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 we 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 non-invasive isotopic analyses demands that we begin understanding the variation in $\Delta^{15}N$ among tissues.

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