

DIET AND GROWTH INFLUENCE CARBON INCORPORATION RATES AND DISCRIMINATION FACTORS ($\Delta^{13}\text{C}$) IN DESERT BOX TURTLES, *TERRAPENE ORNATA LUTEOLA*

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Abstract.—Growth is a significant contributor to tissue stable isotope incorporation rates, particularly in ectotherms. However, few data exist describing how growth affects rates of carbon incorporation during discrete periods of growth and development within a species. Here we use a bi-directional diet switch experiment to estimate carbon incorporation rates and diet-to-tissue discrimination factors in two life-stages of Desert Box Turtles (*Terrapene ornata luteola*) growing at different rates. The younger more rapidly growing turtles had shorter carbon half lives in plasma (34 d) and red blood cells (RBCs; 69 d), relative to older, more slowly growing animals (61 d for plasma, while carbon isotope ratios in RBCs did not achieve equilibrium). Plasma carbon turnover was minimally influenced by growth in juvenile ($25\% \pm 7\%$) and sub-adult ($20\% \pm 7\%$) turtles. Growth was an important driver for RBC carbon incorporation rates, accounting for $50\% \pm 22\%$ in juvenile turtles. At equilibrium, diet-to-tissue discrimination factors varied in turtles feeding on a C₃ plant vs. a C₄ plant-based diet, a phenomenon probably related to differences in diet quality. We also found that the diet treatment affected the extent to which isotope ratios in the current diet influenced the stable isotope ratios in previously accrued growth rings (63–70% for turtles eating the C₃ diet vs. 32–37% for turtles eating the C₄ diet.). This study significantly adds to the comparative data available for reptiles, and increases our knowledge of how growth, developmental stage, and diet quality affect stable isotope dynamics in these organisms.

Key Words.—carbon retention time; diet-to-tissue discrimination factor; plasma; red blood cell; scute keratin

INTRODUCTION

Stable isotope methods provide an important tool for describing how animals interact with their environments and provide quantitative insight into consumer resource use. Using stable isotope approaches to understand trophic interactions, however, relies on specific knowledge about the rates at which carbon atoms are replaced in animal tissues and the faithfulness of their incorporation (discrimination factors). These rates and discrimination factors may vary greatly with organism growth rate, body size, diet, and taxonomic affinity. Because relatively small differences in these parameters may result in significantly different outputs from mixing models and other stable isotope analyses (Caut et al. 2009), it is critical to accurately quantify these parameters when using stable isotopes to study animal feeding ecology. As a consequence, there is a need for taxa-specific comparative data. Studies that define these factors are available for reptiles (e.g., Seminoff et al. 2006, 2007; Fisk et al. 2009; Murray and Wolf 2012; Vander Zanden 2012), as well as for a variety of other taxa such as mammals (e.g., Tieszen et al. 1983; DeMots et al. 2010), birds (e.g., Bauchinger and McWilliams 2009; Oppel and Powell 2010), fish (e.g., Hesslein et al. 1993; Nelson et al. 2011; Weidel et al. 2011) and

invertebrates (e.g., Fry and Arnold 1982; Spence and Rosenheim 2005). These studies typically use controlled feeding trials in a group of captive adult or juvenile animals to validate species-specific, diet-to-tissue discrimination ($\Delta^{13}\text{C}_{\text{tissue-diet}}$) and isotope incorporation rates. However, fewer groups have examined these processes concurrently within groups of animals in different developmental stages, growing at widely disparate rates (e.g., Trueman et al. 2005; Reich et al. 2008; McCue 2008; Carleton and Martínez del Rio 2010; Vander Zanden 2012).

Here we present carbon incorporation rates and $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values, or the isotopic spacing between animal tissue and diet, in rapidly growing juvenile (11% of adult mass) and more slowly growing sub-adult (45% of adult mass) Desert Box Turtles (*Terrapene ornata luteola*) fed two isotopically distinct diets. We explore the influences of growth and tissue catabolism on carbon incorporation rates in two age classes of turtles, and examine how dietary carbon stable isotope ratios (diets depleted in ¹³C vs. diets enriched in ¹³C) and diet quality determine $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values in turtles growing at different rates. These are some of the first data available for terrestrial emydid turtles, and contribute to the small, but growing body of comparative data on tissue turnover rates and discrimination factors in growing reptiles.

MATERIALS AND METHODS

We used 12 Desert Box Turtles from multiple clutches that hatched in captivity from a group of long term captive (> 10 y) adults in the care of the first author. Six of the turtles hatched in September 2006, and six hatched in September 2007 (from here on, referred to as the 2006 and 2007 cohorts). We housed turtles in groups of three within open-topped plastic storage bins (86 cm x 49 cm) in the University of New Mexico Biology Department. We kept turtles on a substrate of moist peat moss (10 cm thick) and provided them with clumps of silk plants for refuge. Simulated solar radiation/heat was provided by 60 watt heat lamps and ZooMed® UVB 10.0 fluorescent bulbs (San Luis Obispo, California, USA) that were arranged to maintain a diurnal temperature gradient of 26 to 34 °C within the enclosure. We maintained turtles on a photcycle that mimicked the natural photoperiod and allowed all turtles to experience normal winter dormancy where turtles were fasted and kept cool (14.7 ± 0.03 °C). During this period turtles were generally buried in the substrate and quiescent. We periodically soaked dormant animals in shallow water to ensure hydration. During the active season turtles were fed and watered daily. We weighed turtles on a digital scale (Ohaus model V31XH2 ± 0.1 g; Parsippany, New Jersey, USA) and recorded their straight carapace length ± 1.0 mm at intervals of every 30 to 60 days.

We knew the dietary history and initial isotopic value

of both turtle cohorts because they were hatched in captivity and did not have an opportunity to feed before the start of the experiment. We placed all turtles in brumation immediately after hatching in September and they subsequently did not take their first meals until the following May. After emerging from brumation, we fed all box turtles on an exclusive diet of Purina Aquamax® trout chow (Gray Summit, Missouri, USA; $\delta^{13}\text{C} = -19.9 \pm 0.3\text{‰}$ Vienna Pee Dee Belemnite; VPDB) until the start of the diet switch. (The captive adult turtles were also primarily fed the same diet.) During the active seasons of 2007 and 2008 (May - October), we provided the older (2006) cohort with fresh trout chow daily as we did with the younger (2007) cohort during its first active season (2008), thus the older cohort (2006) was fed on the trout chow diet for two growing seasons and the younger (2007) cohort for one season. On 28 September 2008 (day 0), after both cohorts had each grown a single scute ring during the 2008 growing season, we divided each turtle cohort into two groups, and started the animals on a bi-directional diet switch (Fig. 1). Both new diets were isotopically distinct from the initial diet. On day zero, we provided half of each cohort (2006 and 2007) a new diet that consisted of either mealworms (*Tenebrio molitor*) raised on a C₃ (wheat) based diet ($\delta^{13}\text{C} = -26.3 \pm 0.1\text{‰}$ VPDB), or mealworms raised on a C₄ (corn) based diet ($\delta^{13}\text{C} = -12.2 \pm 0.1\text{‰}$ VPDB). We placed all turtles in brumation approximately one month

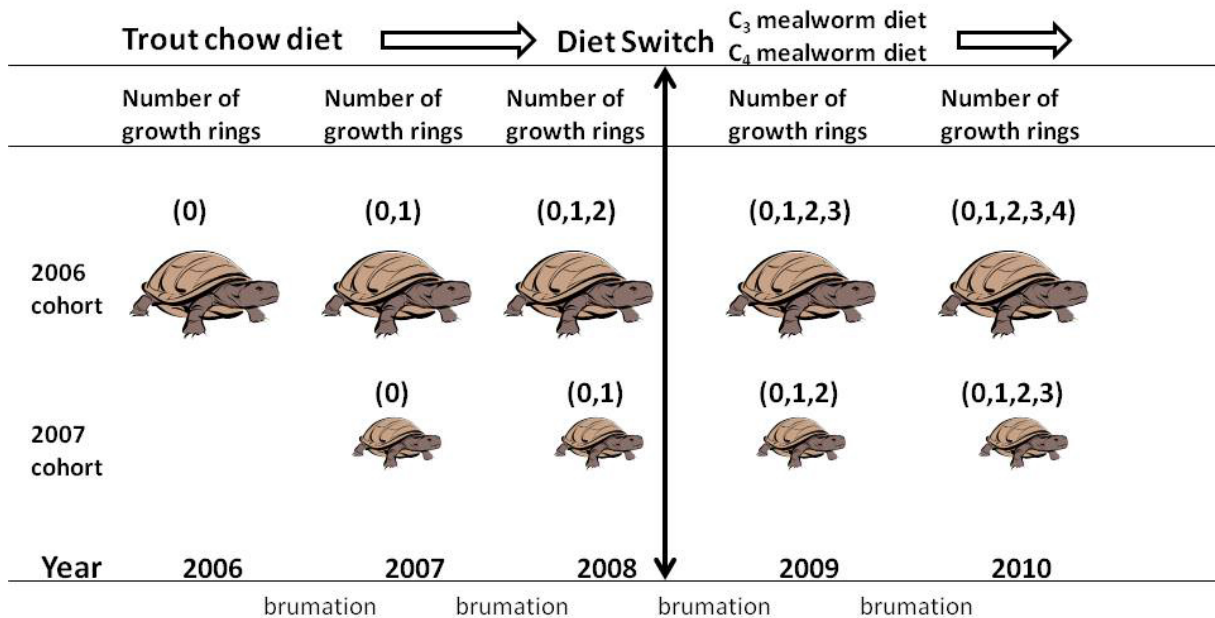


FIGURE 1. Schedule of scute ring growth and diet switching in the 2006 and 2007 cohort of Desert Box Turtles (*Terrapene ornata luteola*). Numbers in parentheses represent the growth rings present for each turtle in a particular year. Turtles in the 2006 cohort hatched in September of 2006, while turtles in the 2007 cohort hatched in September of 2007; events depicted by the first appearance of ring 0, the neonatal scute. All turtles were inactive and not feeding (in brumation) between October and May of each winter. A bi-directional diet switch (C₃ and C₄ based mealworm diets) was started in September 2008, after all turtles had accrued a complete growth ring during the 2008 activity season.

after the diet switch and did not feed them again until the following May. For approximately one month after turtles began feeding again, all of the animals in the C₄ group were mistakenly given a pelleted diet, in addition to their daily ration of C₄ mealworms, whereas all of the animals in the C₃ group were only given their daily allotment of C₃ mealworms throughout this time period. Consequently, carbon incorporation rates for blood tissue and representative growth rates for the turtles eating the C₄ diet cannot be determined, and are not included here. However, $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values and growth ring data taken at the end of the experiment were not likely to be compromised due to the slow nature of keratin growth and the extended length of time that the diet switch was maintained (after the feeding error was corrected, turtles doubled or tripled in body mass on the correct diet), and are thus included here.

Blood tissue dynamics experiment.—We measured the carbon turnover rates and carbon discrimination factors of red blood cells (RBCs), plasma solutes, and scute keratin. We sampled blood from between two to six turtles on days 0, 1, 3, 7, 15, 20, 221, 247, 285, 343, 595, 610, and 661 after the diet switch. Turtles undergoing winter dormancy have very low metabolic rates. Gatten (1974) found that at temperatures under 15 °C, ornate box turtles had a standard metabolic rate Q₁₀ (a metric for describing the rate of change of biological/chemical reactions over a span of 10 °C) of 54, a value highlighting the immense effect of increasing temperatures on metabolic rate at cool temperatures. Additionally, Penick et al. (2002) found that in the Eastern Box Turtle (*Terrapene carolina*), winter field metabolic rates were 23% those of active-season metabolic rates. Our turtles were not active and feeding during brumation and likely had minimal rates of carbon turnover, so we considered the gap between the previous fall, and the first spring sample (day 20 - day 221, and day 343 - day 595) as being one day for analyses (i.e., the sampling days became 0, 1, 3, 7, 15, 20, 21, 47, 85, 143, 144, 159, 210). We drew blood with a 12.5 mm long, 27 gauge needle and syringe from the dorsal cervical sinus that we transferred to a hematocrit tube and centrifuged down into plasma and RBC components. We followed blood preparation and analysis methods detailed in Warne et al. (2010). Blood typically contains relatively low amounts of lipid, and in sea kraits (*Laticauda* spp.), blood carbon stable isotope ratios were similar in samples with and without lipids (Bearhop et al. 2000; Brischox et al. 2011). Consequently we did not remove lipids from blood tissues in this study.

Scute growth ring experiment.—As turtles grow, the expanding bony carapace is protected by keratinized scutes, which enlarge via the addition of successive growth rings around their periphery (Cagle 1946;

Germano and Bury 1998; Wilson et al. 2003). Throughout the study, all box turtles grew and added one keratin ring during each activity season between 2007 and 2010. On the day of the diet switch (day 0), the 2006 cohort had two growth rings, and the 2007 cohort had one ring in addition to the natal scute. By day 210 after the diet switch, the 2006 cohort had produced two additional rings for a total of four rings, and the 2007 cohort had produced two additional rings for a total of three rings (Fig. 1). We sampled scute rings in October 2009 and in July 2010. We re-sampled the same rings during the second sampling period by sampling an adjacent strip of scute after all of the turtles had added one growth ring during the 2010 growing season. Growth ring sampling and preparation closely followed the procedures outlined in Murray and Wolf (2012). Each scute growth ring sample included all of the keratin extending from the bone tissue of the shell to the most superficial keratin layer. We measured the $\delta^{13}\text{C}$ values (‰ VPDB) of all tissue samples using a continuous flow isotope ratio mass spectrometer (Thermo-Finnigan IRMS Delta Plus; Waltham, Massachusetts, USA) in the University of New Mexico Earth and Planetary Sciences Mass Spectrometry lab. The precision of our measurements was $\pm 0.1\text{‰}$ SD using internal standards (soy $\delta^{13}\text{C} = -27.2\text{‰}$ VPDB) calibrated against international standards, and included in each sample run. We report results using standard delta notation (δ) in parts per thousand (‰) as $\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) * 1000$.

Statistical analyses.— We used the reaction–progress variable method (RPV) developed by Cerling et al. (2007) as a tool to determine whether tissue isotopic incorporation rates were best estimated using one-compartment or two-compartment models. The procedures used to inform whether one- or two-compartment models best fit the data, and how to interpret these models has been described elsewhere (Cerling et al. 2007; Wolf et al. 2009; Kurle 2009; Martínez del Rio and Carleton 2012). We then used the approach of Martínez del Rio and Anderson-Sprecher (2008) to quantitatively select the best-fit model using Akaike’s information criteria corrected for small sample sizes (AICc; Burnham and Anderson 2002). We estimated isotope incorporation rates with non-linear regression in SigmaPlot 8.0® (Chicago, Illinois, USA). If the one compartment model was the best fit for the data, we used the equation:

$$\delta_t = \delta_{\text{eq}} - (\delta_{\text{eq}} - \delta_{\text{init}})e^{(-T/\tau)} \quad (1),$$

if the two compartment model was the best fit, we used the equation:

$$\delta_t = \delta_{\text{eq}} - (\delta_{\text{eq}} - \delta_{\text{init}})[pe^{(-T/\tau_1)} + (1 - p)e^{(-T/\tau_2)}] \quad (2),$$

In both cases, δ_{eq} , δ_{init} , and δ_t are equilibrium, initial, and time 't' isotope ratios, T is time in days, τ is carbon residence time (tissue element half-life($t_{1/2}$) = $\tau \ln(2)$); fractional rates of incorporation (λ) = $1/\tau$, or the mean length of time that a carbon atom is retained in a particular tissue pool, and p is the fractional contribution of each compartment to the two compartment model (Hobson and Clark 1992; Carleton and Martínez del Rio 2005; Cerling et al. 2007). There are two processes (growth and catabolism) that may contribute to the fractional rate of incorporation of material into animal tissues. The models presented thus far only account for incorporation of materials into tissues due to catabolism (c; Hesslein et al. 1993) and thus fail to account for the second potential source of incorporated material associated with an animal's growth. Our turtles were immature at the start of the experiment and consequently turtles in both cohorts experienced significant growth over the course of the experimental treatment and thus growth (k) must be considered in our models of incorporation. Thus if we substitute λ for $1/\tau$, equation (2) becomes:

$$\delta_t = \delta_{eq} - (\delta_{eq} - \delta_{init})e^{-(k+c)T} \quad (3),$$

We can then use an exponential model ($y = ae^{(kt)}$) to estimate the fractional growth rate (k in $g \cdot day^{-1}$) of juvenile (2007 cohort) and sub-adult (2006 cohort) box turtles in SigmaPlot 8.0®. Because $\lambda = k + c$, our estimates of carbon incorporation rates (i.e. $\lambda = 1/\tau$) and fractional growth rates (k) allow us to quantitatively parse out the contributions of catabolism and growth to overall tissue turnover. Consequently if λ and k are the same, then growth can be assumed to be the only determinant of isotopic incorporation after the diet switch. If λ is higher than k, the difference is attributed to tissue catabolism. We reported diet-to-tissue discrimination ($\Delta^{13}C_{tissue-diet}$) values as the difference between turtle tissue $\delta^{13}C$ values at equilibrium and the $\delta^{13}C$ value of the diet. We evaluated any differences between tissue and diet $\delta^{13}C$ values, as well as among treatment differences in discrimination factors using t-tests and analysis of variance (ANOVA) with Tukey's HSD at $\alpha = 0.05$. We used paired t-tests to examine $\delta^{13}C$ values in growth rings sampled in 2009 versus the same rings re-sampled in 2010. Data were normally distributed (Anderson-Darling test for normality; $P > 0.05$). We list all $\delta^{13}C$ estimates as mean \pm SEM‰ VPDB.

RESULTS

At the start of the diet switch, turtles in both cohorts had similar plasma and RBC $\delta^{13}C$ values. Desert Box Turtles had mean plasma and RBC $\delta^{13}C$ values of $-17.6 \pm 0.2\text{‰}$ and $-19.3 \pm 0.1\text{‰}$, respectively, after feeding on

an exclusive diet of Purina Aquamax® trout chow ($-19.9 \pm 0.3\text{‰}$ VPDB) for two (2006 cohort) and one (2007 cohort) growing seasons. All of the turtles readily fed on the C_4 ($-12.2 \pm 0.1\text{‰}$) and C_3 mealworm diets ($-26.3 \pm 0.1\text{‰}$) starting at day 0 (the day of the diet switch), and running through day 210. The younger turtles in the 2007 cohort grew over twice as fast as the 2006 cohort, and this growth was well described by an exponential function (2007: $r^2 = 0.63$; 2006: $r^2 = 0.78$) with a fractional growth rate (k) of $0.005 \pm 0.0009 g \cdot day^{-1}$ and $0.002 \pm 0.0002 g \cdot day^{-1}$ for 2007 and 2006 turtles in the C_3 diet treatments, respectively (Fig. 2). The linearized output from the reaction progress variable (RPV) method supported the use of one-compartment models as the best fit for all tissues because the confidence intervals for all y-intercepts overlapped the origin (range = -0.5 – 0.2 ; Figs. 3, 4). Furthermore, the one-compartment model was supported by the AICc comparisons for all tissues due to the smaller value of AICc₁ relative to AICc₂ (plasma: AICc₁ = 10.3; AICc₂ = 16.4; RBC: AICc₁ = 11.2; AICc₂ = 18.1). Box turtle plasma and RBC (equilibrium was not reached for 2006 cohort RBCs, but final values are presented for comparative purposes) diet-to-tissue discrimination ($\Delta^{13}C_{tissue-diet}$) values for the 2006 cohort were significantly different across diet treatment (plasma: $F_{2,9} = 47.49$; $P < 0.001$; RBC: $F_{2,8} = 107.78$; $P < 0.001$; Table 1), and $\Delta^{13}C_{tissue-diet}$ values in turtles feeding on the C_3 mealworm diet were significantly greater relative to turtles eating the C_4 mealworm or trout chow diets (Tukey's HSD; $P < 0.05$). Plasma $\Delta^{13}C_{tissue-diet}$ values in the 2007 cohort were significantly greater for turtles eating the C_3 mealworm diet relative to the C_4 mealworm diet ($F_{2,7} = 13.71$; $P = 0.004$; Tukey's HSD; $P < 0.05$), but indistinguishable between turtles eating the C_3 mealworm and trout chow diets (Tukey's HSD; $P > 0.05$; Table 1). RBC $\Delta^{13}C_{tissue-diet}$ values in the 2007 cohort also varied significantly across diets ($F_{2,5} = 119.53$; $P < 0.001$) and RBC $\Delta^{13}C_{tissue-diet}$ values were greater for turtles eating C_3 mealworms compared to C_4 mealworms and trout chow (Tukey's HSD; $P < 0.05$; Table 1). Scute keratin $\Delta^{13}C_{tissue-diet}$ values were significantly greater for turtles in both cohorts eating the C_3 mealworm diet compared to the C_4 mealworm diet (2006 cohort: $4.0 \pm 0.9\text{‰}$ vs. $-0.9 \pm 0.1\text{‰}$; two-sample t-test, $t = 5.25$, $P = 0.03$; 2007 cohort: $5.1 \pm 0.4\text{‰}$ vs. $-0.2 \pm 0.7\text{‰}$; two-sample t-test, $t = 6.66$, $P = 0.007$; Table 1).

2006 Cohort – C_3 .—Mean (\pm SEM) carbon retention time (τ) in box turtle plasma was 87.4 ± 30.7 days (plasma = $-23.4 + 5.0e^{-T/87.4}$; $P = 0.01$). Carbon isotope equilibrium was not achieved in RBCs. Growth and catabolism both determined the rates of tissue carbon incorporation in blood plasma. Plasma ($0.01\text{‰} \cdot \text{day}^{-1}$) carbon incorporation rates (λ) were higher than the specific growth rate ($k = 0.002 g \cdot \text{day}^{-1}$). Tissue growth

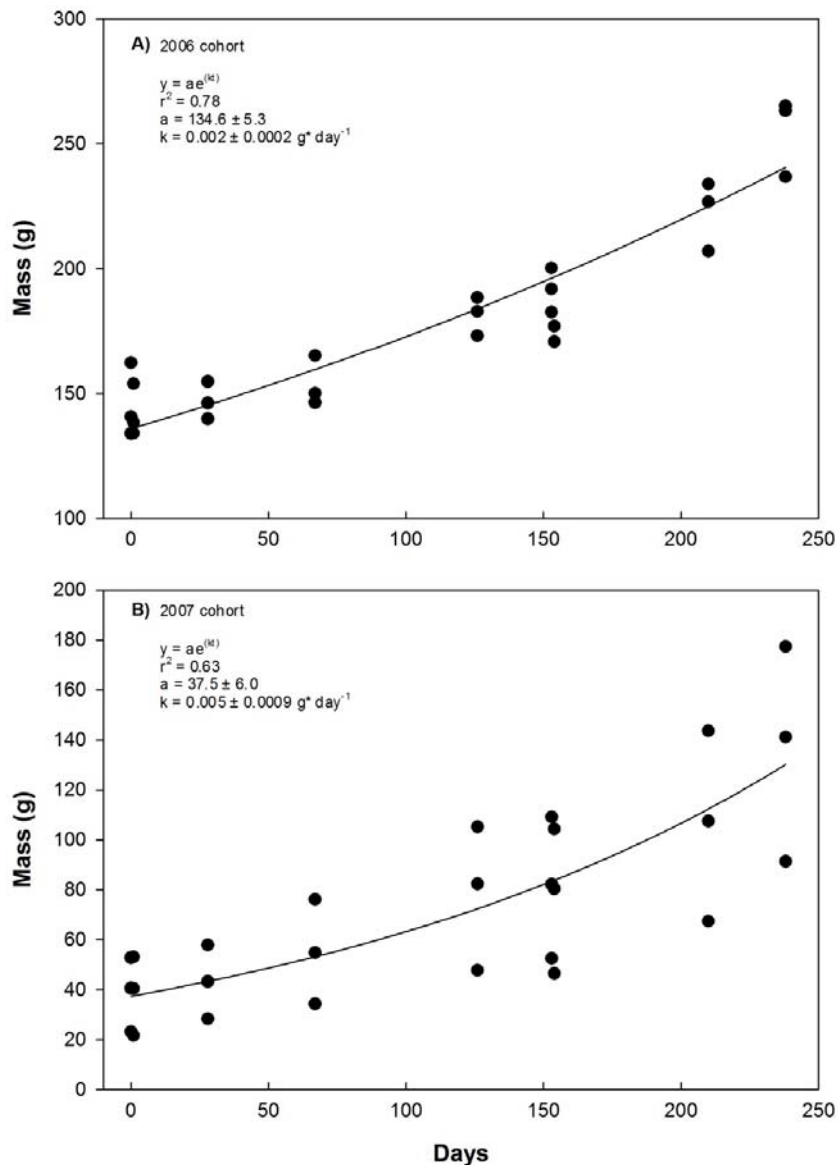


FIGURE 2. Growth in juvenile and sub-adult Desert Box Turtles (*Terrapene ornata luteola*), for 210 days following a diet switch. Each point represents the mass of an individual turtle. A) Sub-adult (2006 cohort) box turtle growth characterized by an exponential function ($y = ae^{(kt)}$; $r^2 = 0.78$; $k = 0.002 \text{ g} \cdot \text{day}^{-1}$). B) Juvenile (2007 cohort) box turtle growth characterized by an exponential function ($y = ae^{(kt)}$; $r^2 = 0.63$; $k = 0.005 \text{ g} \cdot \text{day}^{-1}$). Data are presented for turtles feeding on a C_3 mealworm based diet.

accounted for $20\% \pm 7\%$ of the carbon incorporation rate in blood plasma. Box turtle plasma $\Delta^{13}C_{\text{tissue-diet}}$ was $3.7 \pm 0.2\text{‰}$ and plasma carbon isotope ratios were significantly enriched in ^{13}C over that of diet ($-22.6 \pm 0.2\text{‰}$; one-sample t-test, $t = 17.12$, $P < 0.001$; Table 1). Using keratin sampled from each of the turtles' most recently accrued annulus after the diet switch (mean $\delta^{13}C = -22.4 \pm 0.9\text{‰}$), we observed a $\Delta^{13}C_{\text{tissue-diet}}$ value for scute keratin of $4.0 \pm 0.9\text{‰}$. Consequently, the scute keratin carbon isotope ratios were significantly enriched

in ^{13}C over diet (one-sample t-test, $t = 4.32$, $P = 0.05$). Desert Box Turtles feeding on the pre-switch diet for 10 mo (trout chow; $-19.9 \pm 0.3\text{‰}$ VPDB) had blood plasma ($1.9 \pm 0.3\text{‰}$) and RBC ($0.3 \pm 0.2\text{‰}$) $\Delta^{13}C_{\text{tissue-diet}}$ values that were significantly smaller than those measured after the diet switch to the C_3 mealworm diet (plasma: paired t-test, $t = 17.75$, $P = 0.003$; RBC: paired t-test, $t = 10.86$, $P = 0.008$; Table 1; Fig. 3).

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TABLE 1. Mean (\pm SEM) $\delta^{13}\text{C}$ values at equilibrium and diet-to-tissue discrimination for Desert Box Turtles (*Terrapene ornata luteola*) tissues. Tissue $\delta^{13}\text{C}$ equilibrium estimates were derived from fitted models. $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values found based on the model are compared with those in turtle tissues in equilibrium with an isotopically distinct diet fed before the diet switch. (Turtles eating the C_4 diet were fed the wrong diet for ca. one month, precluding the calculation of model estimates, but carbon isotope discrimination factors taken at the end of the experiment are presented.)

<i>Terrapene ornata</i>	Model $\delta^{13}\text{C}$ equilibrium	$\Delta^{13}\text{C}_{\text{tissue-diet}}$ (C_3 mealworm) (^{13}C depleted diet)	$\Delta^{13}\text{C}_{\text{tissue-diet}}$ (C_4 mealworm) (^{13}C enriched diet)	$\Delta^{13}\text{C}_{\text{tissue-diet}}$ (trout chow)* (initial diet)
Trout chow	-19.9 \pm 0.3	--	--	--
C_3 mealworm	-26.3 \pm 0.1	--	--	--
C_4 mealworm	-12.2 \pm 0.1	--	--	--
2006 cohort				
Plasma	-23.4 \pm 0.7	3.7 \pm 0.2 ^a	0.3 \pm 0.2 ^b	1.9 \pm 0.3 ^c
RBC	Equilibrium not reached	4.1 \pm 0.3 ^a	-2.5 \pm 0.5 ^b	0.3 \pm 0.1 ^c
Scute keratin	-22.3 \pm 0.9	4.0 \pm 0.9 ^a	-0.9 \pm 0.1 ^b	--
2007 cohort				
Plasma	-24.2 \pm 0.4	2.9 \pm 0.2 ^a	1.0 \pm 0.3 ^b	2.5 \pm 0.4 ^a
RBC	-23.9 \pm 0.9	3.2 \pm 0.2 ^a	-0.5 \pm 0.1 ^b	0.8 \pm 0.1 ^c
Scute keratin	-21.2 \pm 0.4	5.1 \pm 0.4 ^a	-0.2 \pm 0.7 ^b	--

Note: Different letters represent significant differences between $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values across the different diets (plasma and RBCs: Tukey's HSD, $P < 0.05$; scute keratin: two-sample t-test).

* The listed $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values for turtles eating the pre-switch diet (trout chow) are based on the mean tissue values after five and ten months of feeding on this diet for the 2007 and 2006 cohort, respectively.

2006 Cohort – C_4 .—Box turtles fed a C_4 diet after the switch showed blood plasma $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values of $0.3 \pm 0.2\text{‰}$, which were not significantly different over that of diet carbon isotope values ($-11.9 \pm 0.2\text{‰}$; one-sample t-test, $t = 1.53$, $P = 0.30$; Table 1). Turtle RBC carbon isotope values were significantly depleted in ^{13}C ($\Delta^{13}\text{C}_{\text{tissue-diet}} = -2.5 \pm 0.5\text{‰}$) relative to diet ($-14.7 \pm 0.5\text{‰}$; one-sample t-test, $t = -4.63$, $P = 0.04$). Scute keratin $\Delta^{13}\text{C}_{\text{tissue-diet}}$ as determined in the last rings grown was $-0.9 \pm 0.1\text{‰}$, and the scute keratin carbon isotope values were significantly lower than diet carbon isotope values ($-13.1 \pm 0.2\text{‰}$; one-sample t-test, $t = -9.52$, $P = 0.01$).

2007 Cohort – C_3 .—Mean (\pm SEM) blood plasma carbon retention times within the younger turtles in the 2007 cohort was 49.1 ± 11.7 days and 99.5 ± 40.2 days for RBCs (plasma = $-24.2 + 6.7e^{-T/49.1}$; $P = 0.001$; RBC = $-23.9 + 5.0e^{-T/99.5}$; $P = 0.026$; Fig. 4). The estimated values of carbon incorporation rates (λ) for plasma ($0.02\text{‰} \cdot \text{day}^{-1}$) and RBC ($0.01\text{‰} \cdot \text{day}^{-1}$) are both higher than the specific growth rate ($k = 0.005 \text{ g} \cdot \text{day}^{-1}$), and consequently growth and catabolism influenced carbon incorporation rates. Growth contributed $50\% \pm 22\%$ to RBC carbon incorporation rates, but only $25\% \pm 7\%$ to plasma incorporation rates. Box turtle plasma $\Delta^{13}\text{C}_{\text{tissue-diet}}$ was 2.9‰ , which means plasma was significantly enriched in ^{13}C over that of diet carbon isotope values ($-23.5 \pm 0.2\text{‰}$; one-sample t-test, $t = 12.3$, $P = 0.001$; Table 1). Turtle RBC carbon isotope ratios had higher $\delta^{13}\text{C}$ values ($\Delta^{13}\text{C}_{\text{tissue-diet}} = 3.2\text{‰}$) relative to diet $\delta^{13}\text{C}$ values ($-23.1 \pm 0.2 \text{‰}$; one-sample t-test, $t = 15.9$, $P = 0.004$). Using keratin sampled from each of the turtles' most recently accrued annulus after the diet switch (mean $\delta^{13}\text{C} = -21.2 \pm 0.4\text{‰}$), we observed a $\Delta^{13}\text{C}_{\text{tissue-diet}}$ for scute keratin of 5.1‰ , which means keratin was

significantly enriched in ^{13}C over diet (one-sample t-test, $t = 13.1$, $P = 0.006$). Desert Box Turtle plasma ($2.5 \pm 0.4\text{‰}$) and RBC ($0.8 \pm 0.1\text{‰}$) $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values after five months on the pre-switch, trout chow diet ($-19.6 \pm 0.0\text{‰}$ VPDB) were significantly smaller than those measured after the diet switch to the C_3 mealworm diet (plasma; paired t-test, $t = 5.24$, $P = 0.04$; RBC; paired t-test, $t = 9.75$, $P = 0.01$).

2007 Cohort – C_4 .—Box turtles eating a C_4 diet during the diet switch experiment had plasma $\delta^{13}\text{C}$ values ($\Delta^{13}\text{C}_{\text{tissue-diet}} = 1.0 \pm 0.3\text{‰}$) that were not significantly different from diet ($-11.2 \pm 0.3\text{‰}$; one-sample t-test, $t = 2.97$, $P = 0.10$; Table 1). Turtle RBC $\delta^{13}\text{C}$ values ($\Delta^{13}\text{C}_{\text{tissue-diet}} = -0.5 \pm 0.1\text{‰}$) were also indistinguishable from diet ($-12.7 \pm 0.1\text{‰}$; one-sample t-test, $t = -3.82$, $P = 0.20$). Scute keratin $\delta^{13}\text{C}$ values ($\Delta^{13}\text{C}_{\text{tissue-diet}} = -0.2 \pm 0.7\text{‰}$) in the 2007 cohort were not different from diet $\delta^{13}\text{C}$ values ($-12.4 \pm 0.7\text{‰}$; one-sample t-test, $t = -0.24$, $P = 0.80$).

Growth rings.—We sampled growth rings at the end of the 2009 activity season, when all animals had added one ring after the diet switch for a total of three rings in the 2006 cohort, and two rings in 2007 cohort. We additionally sampled growth rings in 2010, after all animals in each cohort had accrued an additional growth ring during the 2010 activity season (Fig. 1). Carbon isotope ratios in the growth rings of both cohorts of turtles in the C_4 diet group had mean $\delta^{13}\text{C}$ values that progressively changed from reflecting the pre-switch diet (trout chow) to more closely tracking that of the C_4 mealworm diet in each successively accrued growth ring. When we sampled the same growth rings in all of the C_4 diet animals in 2010, we saw non-significant increases (2006 cohort), or no change (2007 cohort) in

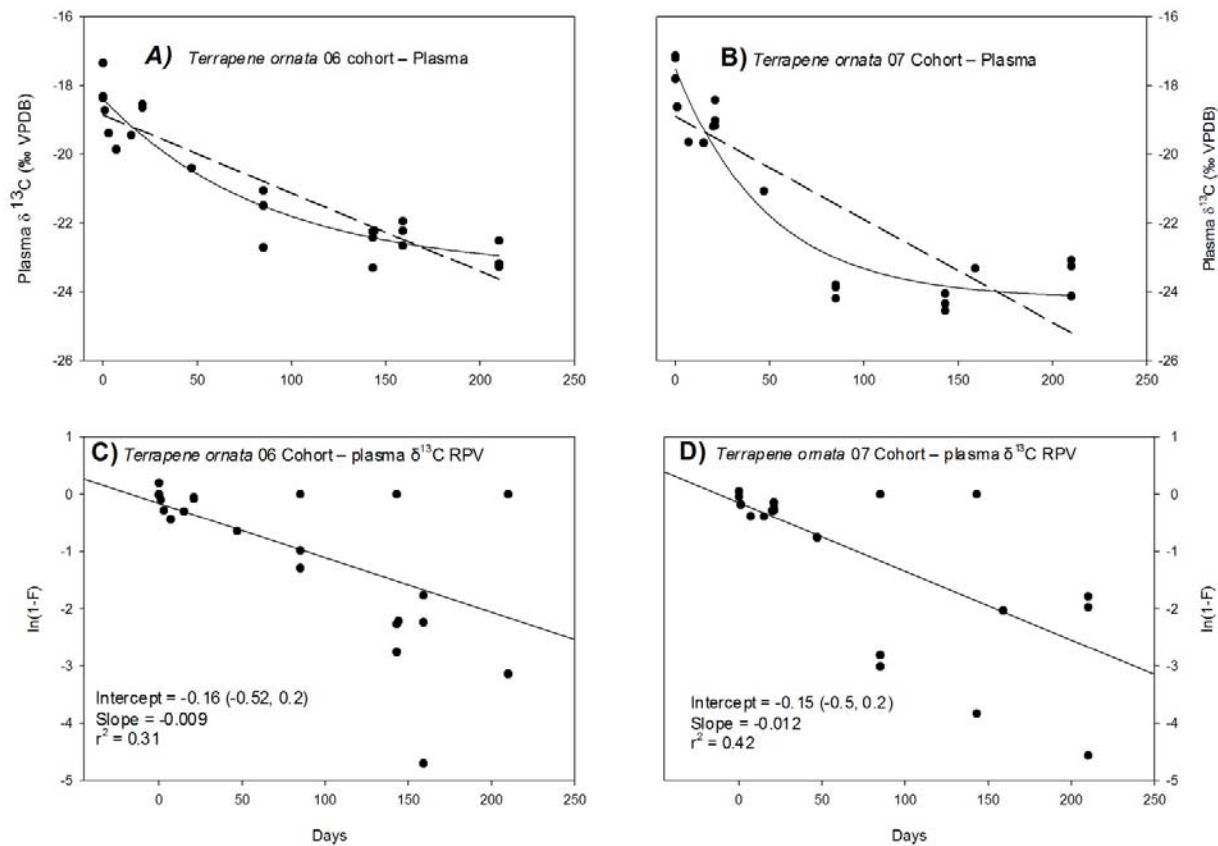


FIGURE 3. Changes in the $\delta^{13}\text{C}$ values of A) juvenile (2007 cohort) and B) sub-adult (2006 cohort) Desert Box Turtles (*Terrapene ornata luteola*) blood plasma during a 210 day diet switch experiment. Dashed lines represent the fit if growth is the sole determinant of carbon incorporation rates (juvenile: $k = 0.005 \text{ g}^*\text{day}^{-1}$; sub-adult: $k = 0.002 \text{ g}^*\text{day}^{-1}$). Data best suits a one-compartment model according to the RPV method for C) blood plasma in the 2006 cohort and D) blood plasma in the 2007 cohort where one tissue compartment contributes 100% to isotope exchange when the intercept overlaps the origin.

the carbon isotope ratios of growth rings, similar to the turtles in the C_3 diet group (Table 2; Figs. 5A,B). Because we did not sample growth rings at day 0, we do not have the initial $\delta^{13}\text{C}$ ring values at day 0, but we can examine shifts in carbon isotope ratios between the first and second seasons on the new diet (Table 2; Fig 5). Surprisingly, rings zero, one, and three in the 2006 C_3 cohort and all rings in the 2007 C_3 cohort showed non-significant trends towards enrichment in ^{13}C between 2009 and 2010 (Table 2; Fig. 5). Among the 2006 and 2007 C_4 diet treatment box turtles there was no significant difference in ^{13}C between rings sampled in 2009 and those sampled in 2010 (Table 2; Figs. 5).

DISCUSSION

There are few data describing how carbon isotope incorporation rates and diet-to-tissue discrimination ($\Delta^{13}\text{C}_{\text{tissue-diet}}$) values are affected by ectotherm growth rate. We report on the results of a long-term experiment

in which two cohorts of box turtles, growing at different rates and experiencing natural cycles of activity and dormancy, were put on a bi-directional diet shift with non-overlapping carbon isotope values. Tissue carbon retention times in blood plasma and RBCs were shorter in the younger, more rapidly growing turtles, relative to those animals growing half as fast. In both box turtle cohorts, metabolic turnover was the primary determinant of plasma incorporation rates, while in RBCs growth was also a significant contributor for turtles in the 2007 cohort. We further elaborate on a phenomenon whereby carbon from the current diet is layered into the pre-existing, keratinized growth rings deposited during past periods of growth (Murray and Wolf 2012). In the following sections we explore the tissue carbon isotope dynamics in two life-stages of box turtle, and how diet may impact diet-tissue discrimination.

Developmental stage influences carbon incorporation rates.—The available data for ectotherms

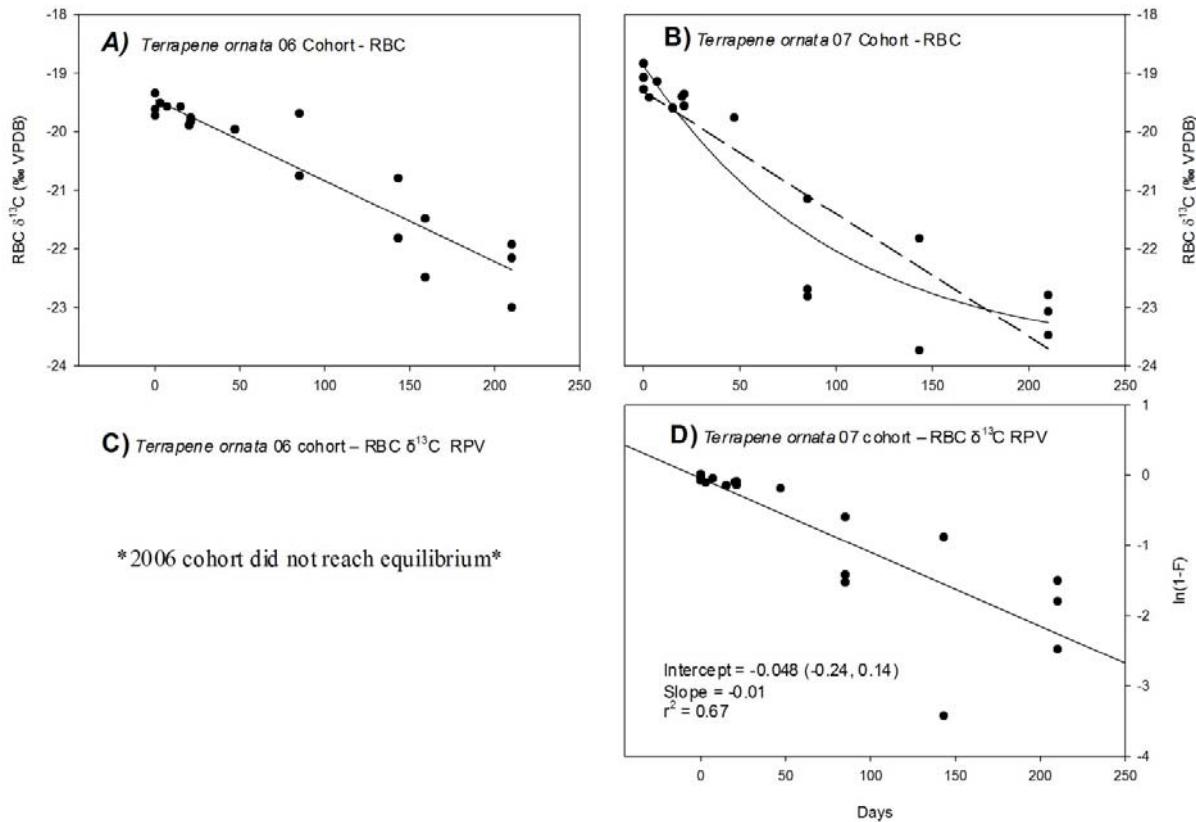


FIGURE 4. Changes in the $\delta^{13}\text{C}$ values of A) juvenile (2007 cohort) and B) sub-adult (2006 cohort) Desert Box Turtles (*Terrapene ornata luteola*) red blood cells (RBC) during a 210 day diet switch experiment. Dashed lines (B) represent the fit if growth is the sole determinant of carbon incorporation rates (juvenile: $k = 0.005 \text{ g}\cdot\text{day}^{-1}$). Turtles in the 2006 cohort (A, C) failed to attain equilibrium during the course of the experiment. Data best suits a one-compartment model according to the RPV method for D) red blood cells in the 2007 cohort where one tissue compartment contributes to 100% isotope exchange when the intercept overlaps the origin.

TABLE 2. Mean (\pm SEM) $\delta^{13}\text{C}$ values for scute growth rings of Desert Box Turtles (*Terrapene ornata luteola*) sampled one year (2009) and two years (2010) after the bi-directional diet switch for turtles in the 2006 and 2007 cohorts. Here we use ring 0 to denote the neonatal scute, and sequentially number the rings until the most recently added ring (positioned at the distal edge of the scute) is reached (ring 4 in the 2006 cohort turtles).

<i>Terrapene ornata</i>	Ring 0		Ring 1		Ring 2		Ring 3		Ring 4
Year:	2009	2010	2009	2010	2009	2010	2009	2010	2010
2006 cohort									
C ₃ diet	-19.9 \pm 0.1	-19.1 \pm 0.5	-20.0 \pm 0.1	-19.5 \pm 0.4	-20.0 \pm 0.4	-20.1 \pm 0.3	-22.1 \pm 0.1	-21.5 \pm 0.2	-21.5 \pm 0.1
C ₄ diet	-19.2 \pm 0.1	-18.0 \pm 0.9	-18.8 \pm 0.2	-18.0 \pm 0.3	-16.3 \pm 0.7	-15.3 \pm 0.9	-12.6 \pm 0.2	-12.3 \pm 0.2	-13.1 \pm 0.1
2007 cohort									
C ₃ diet	-20.4 \pm 0.2	-19.6 \pm 0.4	-21.0 \pm .08	-20.4 \pm 0.2	-22.6 \pm 0.1	-21.5 \pm 0.4	--	-21.6 \pm 0.2	--
C ₄ diet	-17.3 \pm 0.4	-17.6 \pm 0.8	-15.0 \pm 0.4	-14.5 \pm 0.3	-11.4 \pm 0.1	-11.4 \pm 0.1	--	-12.4 \pm 0.7	--

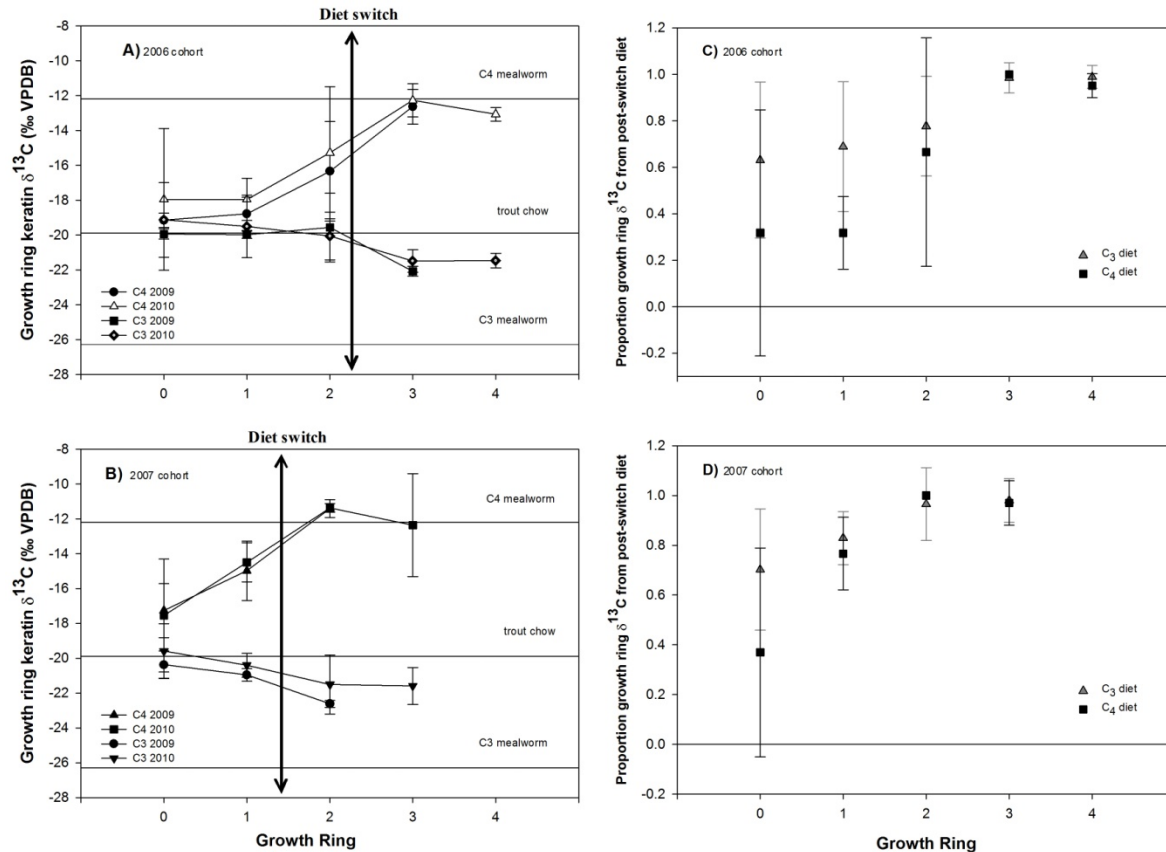


FIGURE 5. Growth ring keratin $\delta^{13}\text{C}$ values for growth rings of Desert Box Turtles (*Terrapene ornata luteola*) added while being fed two isotopically distinct diets. A) Sub-adult turtles (2006) accrued rings one and two on the pre-switch diet, and grew one ring during each of two activity seasons while on the experimental diet. $\delta^{13}\text{C}$ values for rings one through three sampled at the end of 2009, and the same rings plus a 4th one grown during 2010 are plotted for comparison. B) Juvenile box turtles (2007) grew the 1st ring on the pre-switch diet and one ring during the 2009 and 2010 activity periods while on the experimental diet. The $\delta^{13}\text{C}$ values for rings one and two sampled at the end of 2009, and the same rings plus a 3rd one grown during 2010 are plotted for comparison. C-D) Two source mixing model solving for the relative proportion of the experimental diet in growth ring keratin of rings sampled after 210 days on the new diet for the C) 2006 and D) 2007 cohorts on the C₃ and C₄ diets. (Mixing model; $\delta^{13}\text{C}_{(\text{keratin})} = p(\delta^{13}\text{C}_{(\text{mealworm})}) + (1-p)(\delta^{13}\text{C}_{(\text{troutchow})}) + \Delta$; Δ = apparent keratin discrimination factor (2006 *T. ornata* eating C₃ mealworms: 4.0‰; 2006 *T. ornata* eating C₄ mealworms: -0.9‰; 2007 *T. ornata* eating C₃ mealworms = 5.1‰; 2007 *T. ornata* eating C₄ mealworms = -0.2‰); N = 3 for all categories, error bars represent 95% confidence intervals.

and endotherms suggests that carbon incorporation rates (λ) decrease with increasing body size in an indirect way due to generally slower rates of protein turnover which scales in a similar fashion (but is not necessarily dependent upon) to the slower metabolic rates in larger organisms (Brown et al. 2004; Carleton and Martínez del Rio 2005; Warne et al. 2010). Turtles used in this study belonged to two different life-stages at the start of the diet switch and exhibited different body sizes and growth rates (and presumably different rates of protein turnover). Box turtles in the 2006 cohort were sub-adults that were growing more slowly at a relatively advanced stage in their development (45% of adult mass and 80% of straight carapace length [SCL]); minimum dimensions at maturity taken from Nieuwolt-Dacanay

1997). In contrast, the turtles in the 2007 cohort were growing rapidly at a much earlier point in their growth trajectory at 11% of mature mass and 16% of SCL. Not surprisingly, turtles in the 2006 cohort had lower plasma incorporation rates and larger carbon half-lives than the turtles in the more quickly growing 2007 cohort. We cannot empirically estimate the carbon incorporation rate and carbon half-lives in RBCs of the 2006 cohort because RBC carbon isotope ratios did not reach equilibrium in these sub-adult turtles. However, we note that the RBC carbon incorporation rate and carbon half-life in the 2007 cohort were almost identical to the corresponding estimates for blood plasma in the 2006 cohort. Given the well-established pattern that across all taxa RBCs have a slower carbon incorporation rate than

plasma solutes (Carleton et al. 2008), we can reliably assume that the carbon incorporation rates in the RBCs of the more slowly growing turtles in the 2006 cohort would be substantially smaller than the corresponding estimates in the rapidly growing 2007 cohort.

Tissue catabolism was the primary determinant of plasma solute incorporation rates in both groups of differentially growing box turtles. Growth contributed a similarly small fraction in both juvenile and sub-adult box turtles, and was close to the 13% reported in juvenile Desert Tortoises (*Gopherus agassizii*), but less than the 30-48% contribution in Loggerhead Sea Turtles (*Caretta caretta*) reported by Reich et al. (2008). The limited data available describing the importance of growth in chelonian RBC incorporation rates for juvenile tortoises (50%; Murray and Wolf 2012) and juvenile sea turtles (44%; Reich et al. 2008) are remarkably similar to the $50\% \pm 22\%$ (2007 cohort) contribution of growth in Desert Box Turtle RBCs. The nucleated RBCs in turtles turn over very slowly relative to plasma, and in Eastern Box Turtles, the lifespan of red blood cells may be up to 800 d (Altland and Brace 1962). As a result, the influence of growth during this lengthy period of turnover becomes more significant. Assuming the same contribution of growth (50%) to carbon incorporation rates in RBCs of the 2006 cohort, which did not reach equilibrium, the carbon incorporation rate for RBCs in these turtles would be an order of magnitude slower than the 2007 cohort, given an approximately two-fold difference in growth rates.

Diet-to-tissue discrimination factors depend on diet.

—Carbon isotope ratios of consumer tissues and diet are often not the same. This mismatch between tissue and diet is described by the diet-to-tissue discrimination factor ($\Delta^{13}\text{C}_{\text{tissue-diet}}$). The diet-tissue offset occurs upon nutrient incorporation and routing, and may be tissue, life stage, or species-specific (Tieszen et al. 1983; Schoeller 1999; reviewed by Caut et al. 2009; Vander Zanden et al. 2012), but the biochemical processes behind the sourcing and routing of carbon are not completely understood. The importance of characterizing these values in discrete tissues from different taxa as a necessary precursor to studying the movement of energy (and trophic level) in free-living animals is reflected in the growing number of studies describing taxa and tissue-specific stable isotope discrimination factors (Caut et al. 2009). Juvenile and sub-adult box turtle $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values were higher than the commonly accepted values of 0.4-1.0‰ in the case of the C_3 mealworm based diet (DeNiro and Epstein 1978; Caut et al. 2009). Several studies have found an apparent difference in the diet-to-tissue discrimination factors for consumers feeding on diets with different stable isotope ratios and nutritional qualities (Webb et al. 1998; Bearhop et al. 2002; Olive et al. 2003; Pilgrim

2005; Swink 2010). However, due to differences in macronutrient composition and the routing of nutrients from multiple dietary sources, comparing the stable isotope ratios of bulk diet with those of consumer tissues may not provide the most robust estimates of discrimination (Pearson et al. 2003; Podlesak and McWilliams 2006).

In this study, two groups of turtles were fed with isotopically distinct mealworm beetle larvae that were themselves raised on a C_3 or C_4 plant-based diet. Turtles eating mealworms raised on a C_3 -based diet had significantly larger separations between diet and tissue compared to the turtles eating mealworms raised on a C_4 -based diet. We assume here that the tissue isotope pools had completely turned over because turtles had either doubled (2006 cohort) or tripled (2007 cohort) in body mass over a diet switch encompassing multiple seasons of growth. However, we cannot rule out the possibility that one or more tissue compartments had not completely turned over, leading to the apparent differences in diet-to-tissue discrimination in the two diet treatments. In this scenario, one among multiple tissue pools could be turning over at such a slow rate that the contributions of this compartment to tissue growth would reflect the previous diet (trout chow), even though turtles would not have fed on this diet for months to years. We argue that this hypothesis is unlikely, and instead we speculate that differences in diet quality between mealworms grown on the C_3 and C_4 plant-based diets may have influenced the observed differences in tissue discrimination factors in turtles feeding on the two diet treatments. For example, the relative amounts of carbohydrates and proteins were significantly different in mealworms raised on a corn (C_4) or wheat (C_3) diet. C_4 -fed mealworms had a significantly higher C:N ratio compared to C_3 -fed mealworms. Moreover, C_4 mealworm colonies did not appear to grow as fast or reach the same large body sizes compared to C_3 mealworms, an observation similar to the larger body size reached by locusts feeding on wheat vs. corn based diets (Webb et al. 1998). Similarly, $\Delta^{13}\text{C}_{\text{tissue-diet}}$ in the chitin ($-0.2 \pm 0.1\text{‰}$) and muscle tissue ($0.7 \pm 0.2\text{‰}$) of adult locusts fed a corn-based diet was significantly lower than that seen in locusts fed a wheat-based diet (chitin = $2.2 \pm 0.2\text{‰}$; muscle = $3.7 \pm 0.3\text{‰}$; Webb et al. 1998). Additionally, Robbins et al. (2010) found that $\Delta^{13}\text{C}_{\text{tissue-diet}}$ in the blood plasma of laboratory rats was higher ($3.2 \pm 0.1\text{‰}$) when feeding on a wheat diet relative to a corn diet ($-1.3 \pm 0.2\text{‰}$) that was otherwise nutritionally balanced with the addition of vitamin and mineral supplements. Swink (2010) also noted large differences in $\Delta^{13}\text{C}_{\text{tissue-diet}}$ between the blood serum of two species of snakes fed mice raised on a C_4 -plant based diet vs. snakes fed mice raised on a C_3 plant based diet. Growth and reproduction in the C_4 plant fed mouse colony was also inferior to those in the C_3 plant fed

mice. Further, Pilgrim (2005) found that the protein, lipid, and energy density of mice raised on a C₃ plant vs. a C₄ plant diet were significantly different. Consequently, box turtles eating C₄ raised mealworms were ingesting less protein than those feeding on C₃ raised mealworms. Growth rates of turtles eating the C₄ plant based diet were lower than turtles feeding on the C₃ plant based diet in the 2007 cohort, but were the same in both diet treatments for the 2006 cohort. We did not analyze the amino acid profiles of the two mealworm diets, but given the different C:N ratios and the known deficiencies in essential amino acids characterizing corn it is possible that the incorporation of dietary carbon via metabolic routing occurred differently in the two diet groups because of a greater (C₃ diet) or lesser (C₄ diet) availability of amino acid skeletons (protein). In this case, turtles eating the protein-deficient C₄ mealworms may have used more carbon from carbohydrates and/or lipids for tissue synthesis and maintenance. When drawing carbon from carbohydrates and lipids, organisms metabolize this carbon with less discrimination (Webb 1997), explaining the smaller tissue-isotope discrimination seen in box turtles eating C₄ mealworms compared to turtles feeding on C₃ mealworms containing higher protein levels.

Growth rings.—Temporally separated periods of growth are separated by growth rings, or permanent indentations, on the keratinized scutes overlaying the bony shell of the turtle. Growth rings are layered structures where new ring growth can contribute layers of material to older rings because metabolically active tissue lies between the bony carapace and the keratin scutes, and becomes compressed into previously deposited keratin growth rings (Alibardi 2005; Alibardi 2006; Alibardi and Toni 2006). Growth ring counts may be a reliable method for aging turtles in some circumstances and species (i.e., Germano and Bury 1998; Wilson et al. 2003), as well as for examining how individual growth patterns may vary in a stochastic resource environment (Tucker et al. 1995). A logical extension of this scenario is that current diet $\delta^{13}\text{C}$ ratios may dilute the $\delta^{13}\text{C}$ values in previously accrued rings (Murray and Wolf 2012). The magnitude of this change in the $\delta^{13}\text{C}$ values of pre-existing rings of Desert Box Turtles after the addition of new growth rings varied with diet as well as ring position on the scute. Growth rings zero (neonatal scute) and one were provisioned and grown under similar maternal inputs/diets, but the mean percent of ^{13}C dilution was roughly $\frac{1}{2}$ in turtles eating the C₄ diet compared with the animals on the C₃ diet in the 2006 cohort. Box turtles eating the C₄ mealworm diet in the 2007 cohort also showed $\frac{1}{2}$ the ^{13}C dilution in ring zero compared to turtles eating the C₃ mealworms in the same cohort.

Mealworms raised on the C₄ diet were lower in protein content than mealworms raised on the C₃ diet, and corn is known to be deficient in several of the amino acids that cannot be synthesized by consumers (essential amino acids; Lewis et al. 1982). The beta-keratins that make up box turtle growth rings have high amounts of the amino acid leucine for reptiles in general, and a high proportion of proline and valine in the beta-pleated sheets in turtle beta-keratin in particular (Toni et al. 2007; Valle et al. 2009). The amino acids leucine and valine are lacking in corn, which means the lower amount of carbon dilution evident in the C₄ diet turtles may stem from deficiencies in several key amino acids necessary to beta-keratin formation, which may have ultimately led to less beta-keratin production and assimilation into the growth rings of the scute layer. In a case like this, the overall thickness of the scute would be thinner, but we are unable to test this hypothesis as we did not measure the thickness of the keratin rings. The previously described growth rings were not immediately adjacent to rings grown post-diet switch. Those rings that were adjacent to rings accrued while on the experimental diets (ring two for turtles in the 2006 cohort and ring one in the 2007 cohort) saw large inputs of material from current diet (66-83%), and these values were indistinguishable between diet shift groups and turtle cohort. Not surprisingly, all of the rings grown after the diet switch drew 100% of their carbon from the experimental diets in both cohorts of turtle on the two diets.

Box turtles in both cohorts feeding on the C₄ and C₃ mealworm diets grew a single growth ring per active season, but the magnitude of ^{13}C change in previously accrued, disparate growth rings of turtles feeding on a C₄-based diet was almost half that of animals eating a C₃-based diet. Previously, Murray and Wolf (2012) documented the existence of this ^{13}C dilution in Desert Tortoises, and here we find that the relative magnitude of ^{13}C change correlates with that of the isotopic nature (C₃ vs. C₄ based) of the diet, and may be a direct effect of differences in amino acid availability between the two diets. It is important to point out that both here, and in Murray and Wolf (2012), the study animals were all immature and only had relatively few growth rings. The magnitude of ^{13}C influx in wild animals feeding on a diverse diet, and having thicker scutes (due to more growth seasons of keratinocyte deposition) and many growth rings, may not be as large. However, at this time no comparative data exist that we know of where captive animals are studied from juvenile to asymptotic adult sizes.

This study adds to the limited dataset comparing the influence of organism developmental stage and growth rates on carbon incorporation rates. We also present comparative data for terrestrial ectotherms showing that tissue $\Delta^{13}\text{C}_{\text{tissue-diet}}$ values are variable and likely

dependent on differences in dietary nutritional quality. Our results underscore the importance that a thorough understanding of the nutritional components of bulk diet has in order to determine species and tissue specific diet-to-tissue discrimination factors, a necessary precursor for the stable isotope ecologist using carbon to study the movement of energy. Taken together, our data further the understanding of how carbon incorporation rates and isotopic discrimination influence the interpretation of ecological data that rely on stable isotope measurements of consumer tissues.

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