Research Article

Rapid Communications in Mass Spectrometry

Received: 23 June 2015 Revised: 4 September 2015 Accepted: 28 September 2015 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2016**, *30*, 9–21
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7410

Diet-tissue stable isotope ($\Delta^{13}C$ and $\Delta^{15}N$) discrimination factors for multiple tissues from terrestrial reptiles

Ronnie Steinitz[1]*, Jeffrey M. Lemm[2], Stesha A. Pasachnik[2] and Carolyn M. Kurle[1]

[1]Division of Biological Sciences, Ecology, Behavior, and Evolution Section, University of California, San Diego, La Jolla, CA 92093-0116, USA
[2]San Diego Zoo Institute for Conservation Research, Behavioral Ecology Division, Escondido, CA 92027-7000, USA

RATIONALE: Stable isotope analysis is a powerful tool for reconstructing trophic interactions to better understand drivers of community ecology. Taxon-specific stable isotope discrimination factors contribute to the best use of this tool. We determined the first $\Delta^{13}C$ and $\Delta^{15}N$ values for Rock Iguanas (*Cyclura* spp.) to better understand isotopic fractionation and estimate wild reptile foraging ecology.
METHODS: The $\Delta^{13}C$ and $\Delta^{15}N$ values between diet and skin, blood, and scat were determined from juvenile and adult iguanas held for 1 year on a known diet. We measured relationships between iguana discrimination factors and size/age and quantified effects of lipid extraction and acid treatment on stable isotope values from iguana tissues. Isotopic and elemental compositions were determined by Dumas combustion using an elemental analyzer coupled to an isotope ratio mass spectrometer using standards of known composition.
RESULTS: The $\Delta^{13}C$ and $\Delta^{15}N$ values ranged from –2.5 to +6.5‰ and +2.2 to +7.5‰, respectively, with some differences among tissues and between juveniles and adults. The $\Delta^{13}C$ values from blood and skin differed among species, but not the $\Delta^{15}N$ values. The $\Delta^{13}C$ values from blood and skin and $\Delta^{15}N$ values from blood were positively correlated with size/age. The $\Delta^{13}C$ values from scat were negatively correlated with size (not age). Treatment with HCl (scat) and lipid extraction (skin) did not affect the isotope values.
CONCLUSIONS: These results should aid in the understanding of processes driving stable carbon and nitrogen isotope discrimination factors in reptiles. We provide estimates of $\Delta^{13}C$ and $\Delta^{15}N$ values and linear relationships between iguana size/age and discrimination factors for the best interpretation of wild reptile foraging ecology. Copyright © 2015 John Wiley & Sons, Ltd.

The best practices for conservation and management of declining species and their habitats necessitate an understanding of the natural and anthropogenic processes driving the disappearance or persistence of a given species. Therefore, it is important to study species interactions such as foraging ecology and habitat use to assess their potential as drivers of community ecology and species decline.[1,2] There are many ways to study these, including long-term behavioral observations and fecal (scat) and stomach content analyses. However, these methods can be time and labor intensive,[3] resulting in small sample sizes that may not be representative of larger populations. In addition, stomach contents and fecal analyses only indicate an animal's most recent meal, precluding dietary estimations over longer temporal scales.[4]

Stable carbon ($^{13}C/^{12}C$ or $\delta^{13}C$ values) and nitrogen ($^{15}N/^{14}N$ or $\delta^{15}N$ values) isotope analyses of predator and prey tissues can provide a more comprehensive assessment of animal foraging over variable temporal scales with minimal disturbance, labor, and cost.[5,6] The $\delta^{13}C$ values from animal tissues indicate dietary carbon sources allowing for the distinction between ingestion of marine- or terrestrial-based primary production, plant or animal components, and C_3 or C_4 plants, among other things.[7] The $\delta^{15}N$ values from animal tissues largely reflect animal trophic position as the values from organisms increase predictably with increasing trophic levels and they can also indicate the nitrogen processes governing the base of a food web.[5,6,8]

Stable isotope analysis can also provide a wide range of temporal data as isotopic turnover varies depending upon the protein turnover of a particular tissue.[9] Therefore, the analysis of multiple tissues from a single individual can provide dietary insights from several time periods. In addition, stable isotope mixing models can be used to estimate the proportions that isotopically distinct dietary items contribute to an animal's total diet.[10,11] However, the best use of these models for estimating the diets of wild populations requires accurate model parameters, including reasonable estimates of the isotope discrimination or trophic enrichment factors.[6] These factors are the differences in isotope values between a consumer and its dietary items and are expressed as $\Delta^{13}C = \delta^{13}C_{PredatorTissue} - \delta^{13}C_{Prey}$ and

* *Correspondence to:* R. Steinitz, Division of Biological Sciences, Ecology, Behavior, and Evolution Section, University of California, San Diego, La Jolla, CA 92093-0116, USA.
E-mail: ronnie86@gmail.com

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similarly for nitrogen. Discrimination factors are typically obtained from studies using captive animals held on known, consistent diets for an adequate amount of time and they can aid in the interpretation of stable isotope data from wild animals (e.g.[12]).

It is frequently difficult to collect these data, as access to animal populations held on known diets is rare. Consequently, the determination of adequate discrimination factors is not common,[6] especially for terrestrial reptiles (but see Table 1). Therefore, the Δ^{13}C and Δ^{15}N values of ~1.0‰ and ~3.4‰, respectively, are generally used as discrimination factors for many studies.[7,8,13,14] However, given the large degree of variation in the Δ^{13}C and Δ^{15}N values observed among taxa and even within taxa among different tissues,[12,15–19] the use of these generalized numbers in stable isotope mixing models can be flawed and lead to erroneous interpretations.[20,21] A primary purpose of our study was to determine the stable isotope discrimination factors of captive Rock Iguanas (*Cyclura* spp.) for use in wild reptile studies and to better understand isotope fractionation patterns in reptiles.

Rock Iguanas are found exclusively in the West Indies. They are at risk due to multiple threats, including habitat destruction and invasive species, and their IUCN Red List status ranges from vulnerable to critically endangered.[22] They typically inhabit subtropical dry forests, require sandy or soil conditions in which to burrow and lay eggs, and depend heavily on the presence of rocky crevices for shelter as adults.[23] They are predominantly herbivorous, consuming foliage and fruits, but are also known to feed opportunistically on animal material (e.g.[25]). Because of their diet, Rock Iguanas play a key role in structuring their ecosystems. For example, Hartley *et al*.[26] demonstrated that seeds passing through iguana digestive tracts sprout earlier than those not ingested, and wide dispersal of the seeds by the iguanas[27] may lead to advantageous priority effects for these plant species.[28] Where they occur, Rock Iguanas are the largest native herbivore, making them essential for maintaining native plant communities in the highly endangered tropical dry forest ecosystems that they often inhabit.[23] Their threatened status combined with their important role as ecosystem engineers underscores the priority of the species-specific management efforts and conservation actions led by the International Union for Conservation of Nature (IUCN), Species Survival Commission (SSC), Iguana Specialist Group (ISG) and the International Iguana Foundation (IIF).

To better understand the foraging-related interactions between wild terrestrial reptiles in general, and *Cyclura* spp. in particular, and their communities, we determined the Δ^{13}C and Δ^{15}N values between diet and scat, blood, and skin components for females and males and across different age and size classes from three captive Rock Iguana species. We targeted tissue samples that were obtained both invasively (blood) and opportunistically (scat and skin) so as to maximize the applications of our data to studies of wild reptiles. In addition, different tissues have different protein turnover, and thus isotopic turnover times,[9] so sampling a variety of tissues from wild animals allows for reconstruction of foraging ecology at different time scales. Protein turnover is lower in ectotherms such as reptiles,[29] so the isotopic turnover is slower than in endotherms, but is probably on the order of days for scat,[30] 5 to 6 months for blood,[17] and greater than 6.5 months for skin.[17]

Rock Iguanas in the wild are assumed to shed (slough) their skin annually,[31] and this seemed to coincide with the resumption of rapid growth in the spring. Shedding is thus affected by growth rate, although accurate data is difficult to obtain for wild animals.[32] Street[33] also reported an annual shedding for *Cyclura nubila* in the wild, while, in captivity, *C. carinata* also shed their skin annually. However, captive individuals are typically fed more and higher quality food than wild individuals, and thus may exhibit higher growth rates and more frequent shedding.[31,34] Shedding may require up to several months for completion, and juveniles appear to shed more rapidly than adults, although this was reported as an observation and was not measured.[31,34]

In reptiles, tissue generation only occurs periodically in the inner skin layers, and when this happens the layers above them are replaced in their entirety. About 2 weeks before shedding, the inner layer begins active growth and a new layer of skin grows under the old one.[35–38] With the growth of this second, new skin, it follows that the old layer, composed of keratinized dead skin cells, would consist of the isotopic signatures incorporated when it was being generated while the previous layer was being keratinized and sloughed. Since this is reported to occur annually, the shed skin should reflect the isotopic signature accumulated during the previous year.

EXPERIMENTAL

Iguanas sampled

Body tissues and physical measurements (weight (g) and snout-vent length (mm), SVL) were obtained from 34 individuals of captive populations from three Rock Iguana species, *Cyclura collei*, *C. lewisi*, and *C. pinguis* (Table 2) held at the San Diego Zoo Institute for Conservation Research (ICR) facilities in Escondido (CA, USA). *Cyclura collei* historically ranged throughout Jamaica and, although the population was thought to be extinct in the wild until the 1990s when a trace population was found in the Hellshire Hills region of southeastern Jamaica. *Cyclura lewisi* are native to Grand Cayman Island, and *C. pinguis* are native to Anegada, Guana, and Norman Islands, in the British Virgin Islands.

The ICR held all individuals on a steady diet of 15 plant types (Table 3) for over 12 months, an adequate time period for full stable isotope equilibration of the iguanas' tissue to their experimental diet.[39] Individual iguanas were offered food once daily and had water available *ad libitum*. ICR staff chopped food according to the size of the individual being fed (adults: ~5 × 5 cm and juveniles: ~2.5 × 2.5 cm) and each animal was individually provided with all food types for each meal in the proportions detailed in Table 3. All iguanas were housed separately for most of the year, further ensuring that each animal ate the prescribed proportions of all diet items provided for the time periods represented in this study. All dietary items from the bulk pile of food that was prepared for the iguanas were sampled, collecting several replicates when possible, along with scat and shed skin from individual iguanas, over two sampling periods, spaced 6 months apart (February 2013 and August 2013) to account for potential seasonal variations in the stable isotope values from dietary

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Table 1. Stable carbon and nitrogen isotope discrimination factors (‰, ±SD when available) for different tissue types from reptiles

Species and diet	Tissue	$\Delta^{13}C$	$\Delta^{15}N$	Reference
Collared Lizards				
Crickets raised on C_4-based dog food	Plasma	+0.2 ± 0.3	–	Warne *et al.*[19]
Same as above	Red blood cells	+1.2 ± 0.6	–	Warne *et al.*[19]
Desert Box Turtle (younger cohort)				
Mealworms raised on a C_3 (wheat)-based diet	Plasma	+2.9 ± 0.2	–	Murray and Wolf[18]
Same as above	Red blood cells	+3.2 ± 0.2	–	Murray and Wolf[18]
Same as above	Scute keratin	+5.1 ± 0.4	–	Murray and Wolf[18]
Mealworms raised on a C_4 (corn)-based diet	Plasma	+1.0 ± 0.3	–	Murray and Wolf[18]
Same as above	Red blood cells	–0.5 ± 0.1	–	Murray and Wolf[18]
Same as above	Scute keratin	–0.2 ± 0.7	–	Murray and Wolf[18]
Desert Box Turtle (older cohort; +1 year)				
Mealworms raised on a C_3 (wheat)-based diet	Plasma	+3.7 ± 0.2	–	Murray and Wolf[18]
Same as above	Red blood cells	+4.1 ± 0.3	–	Murray and Wolf[18]
Same as above	Scute keratin	+4.0 ± 0.9	–	Murray and Wolf[18]
Mealworms raised on a C_4 (corn)-based diet	Plasma	+0.3 ± 0.2		Murray and Wolf[18]
Same as above	Red blood cells	–2.5 ± 0.5	–	Murray and Wolf[18]
Same as above	Scute keratin	–0.9 ± 0.1	–	Murray and Wolf[18])
Green Turtles (juveniles)				
Pelleted diet	Skin (epidermis[a])	+1.9 ± 0.6	+3.8 ± 0.4	Vander Zanden *et al.*[69]
Same as above	Dermis	+2.2 ± 0.6	+4.2 ± 0.5	Vander Zanden *et al.*[69]
Same as above	Plasma	+1.2 ± 0.6	+4.1 ± 0.4	Vander Zanden *et al.*[69]
Same as above	Red blood cells	+0.5 ± 0.6	+2.4 ± 0.4	Vander Zanden *et al.*[69]
Green Turtles (juveniles)				
Gelatin amalgamate with fixed portions of a pelleted diet	Skin (epidermis[a])	+0.2 ± 0.1	+2.8 ± 0.3	Seminoff *et al.*[70]
Same as above	Dermis	–	–	Seminoff *et al.*[70]
Same as above	Plasma	–0.1 ± 1.0	+2.9 ± 1.0	Seminoff *et al.*[70]
Same as above	Red blood cells	–1.1 ± 0.2	+0.2 ± 1.0	Seminoff *et al.*[70]
Green Turtles (adults)				
Pelleted diet	Skin (epidermis[a])	+1.6 ± 0.6	+4.0 ± 0.4	Vander Zanden *et al.*[69]
Same as above	Dermis	+2.6 ± 1.2	+4.9 ± 0.6	Vander Zanden *et al.*[69]
Same as above	Plasma	+0.2 ± 0.6	+4.2 ± 0.4	Vander Zanden *et al.*[69]
Same as above	Red blood cells	+0.3 ± 0.6	+2.5 ± 0.4	Vander Zanden *et al.*[69]
Leatherback Turtles (juveniles)				
Gelatin amalgamate of Pacific Ocean Squid	Skin (epidermis[a])	+2.3 ± 0.6	+1.9 ± 0.5	Seminoff *et al.*[71]
Same as above	Dermis	–	–	Seminoff *et al.*[71]
Same as above	Plasma	–0.6 ± 0.5	+2.9 ± 0.8	Seminoff *et al.*[71]
Same as above	Red blood cells	+0.5 ± 0.4	+1.5 ± 0.8	Seminoff *et al.*[71]
Loggerhead Sea Turtle (hatchlings)				
Pelleted, soy-protein-based diet	Skin (epidermis[a])	+2.6 ± 0.3	+1.7 ± 0.1	Reich *et al.*[39]
Same as above	Dermis	–	–	Reich *et al.*[39]
Same as above	Plasma	+3.0 ± 0.2	+0.3 ± 0.1	Reich *et al.*[39]
Same as above	Red blood cells	–0.6 ± 0.7	–0.3 ± 0.3	Reich *et al.*[39]

(*Continues*)

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Table 1. (Continued)

Species and diet	Tissue	$\Delta^{13}C$	$\Delta^{15}N$	Reference
Loggerhead Sea Turtle (juveniles)				
Pelleted, soy-protein-based diet	`Skin (epidermis[a])	+1.1 ± 0.2	+1.6 ± 0.1	Reich *et al.*[39]
Same as above	Dermis	–	–	Reich *et al.*[39]
Same as above	Plasma	–0.4 ± 0.2	+1.5 ± 0.2	Reich *et al.*[39]
Same as above	Red blood cells	+1.5 ± 0.2	+0.2 ± 0.1	Reich *et al.*[39]
Pond Sliders				
Soy-based commercial pellet food	Plasma	+3.8 ± 0.1	+2.5 ± 0.8	Seminoff *et al.*[17]
Same as above	Red blood cells	+1.9 ± 0.3	–	Seminoff *et al.*[17]
Same as above	Whole blood	+2.2 ± 0.2	–0.8 ± 0.8	Seminoff *et al.*[17]
Same as above	Liver	+3.0 ± 0.3	+0.4 ± 0.5	Seminoff *et al.*[17]
Same as above	Brain	+2.9 ± 0.3	–	Seminoff *et al.*[17]
Prairie Lizards				
Crickets raised on C_4-based dog food	Plasma	–0.5 ± 0.3	–	Warne *et al.*[19]
Same as above	Red blood cells	–1.1 ± 0.8	–	Warne *et al.*[19]
Same as above	Liver	–1.0 ± 0.2	–	Warne *et al.*[19]
Same as above	Skin	–0.8 ± 0.5	–	Warne *et al.*[19]
Same as above	Muscle	–1.9 ± 0.2	–	Warne *et al.*[19]
Rock Iguanas (juveniles)				
Various leafy greens, root vegetables and fruit. *See Table* 3	Scat	–0.6 ± 0.7	+2.7 ± 1.0	This study
Same as above	Whole blood	+1.7 ± 0.2	+2.9 ± 0.6	This study
Same as above	Skin	+2.3 ± 0.3	+4.2 ± 0.8	This study
Rock Iguanas (adults, overall)				
Various leafy greens, root vegetables and fruit. *See Table* 3	Scat	–1.3 ± 1.0	+3.4 ± 0.7	This study
Same as above	Whole blood	+2.5 ± 0.6	+4.1 ± 0.4	This study
Same as above	Skin	+4.5 ± 1.4	+6.0 ± 0.6	This study

[a]Tissue referred in cited literature as 'epidermis', and in this study as 'skin'.[72]

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Table 2. Species, mean weight (kg), mean snout-vent length (SVL; mm), mean age (years), and sex ratio (F/M) for all *Cyclura* species included in the study. Means are reported ± SD. All animals were adults except where otherwise noted

Species	N	Weight	SVL	Age	Sex
C. lewisi	5	1.4 ± 1.3[a]	293.2 ± 111.1[a]	5.8 ± 4.4[e]	1/4
C. lewisi (juvenile)	15	0.3 ± 0.4[b]	187.3 ± 51.8[b]	1.0 ± 0.0	5/10
C. collei	7	3.9 ± 1.0[c]	433.3 ± 367.7[c]	12.1 ± 6.4[e]	4/3
C. pinguis	7	5.5 ± 0.9[d]	484.0 ± 32.9[d]	23.0 ± 6.7[e]	2/5
Adult Total	19	3.5 ± 2.1	398.9 ± 112.6	14.5 ± 9.2	7/12
Total w/ juveniles	34	1.9 ± 2.2	293.1 ± 137.9	8.5 ± 9.6	12/22

Due to sampling constraints, weights and SVLs were obtained only for the following number of individuals of each species and these were used to calculate overall mean weights and SVLs:
[a]5 adults (age 2–11 years).
[b]13 juveniles (age 1 year).
[c]3 adults (age 7–19 years).
[d]5 adults (age 15–28 years).
[e]The age and sizes were statistically different among the adults of each species; ANOVA, $F_{2,16}$, $p \leq 0.001$.

Table 3. The mean (±SD) stable isotope ratio values (‰) and C:N ratios from dietary components offered weekly to captive Rock Iguanas (*Cyclura* spp.) in this study. The % source refers to the percentage that each dietary source contributed to the overall weekly diet budget. See text for the equation used to determine the δ^{13}C and δ^{15}N values for the overall diet

Diet item	Portion weight (g)	Times fed per week	Total weekly food budget (g)	% source	N	δ^{13}C	δ^{15}N	C:N
Dandelion greens	125	5	625	23.19	3	–28.8 ± 1.1	1.6 ± 1.4	8.6 ± 1.2
Collard greens	125	3	375	13.91	2	–28.3 ± 2.1	2.7 ± 1.2	8.1 ± 1.0
Green chard	125	3	375	13.91	2	**–29.9 ± 2.1**	1.7 ± 2.6	9.2 ± 2.2
Mustard greens	125	3	375	13.91	2	–30.5 ± 1.0	2.1 ± 1.9	7.6 ± 1.9
Bok choy	125	2	250	9.28	1	–28.5 ± 0.0	–0.7 ± 0.0	9.4 ± 0.0
Escarole	125	2	250	9.28	1	–27.6 ± 0.0	–2.4 ± 0.0	12.0 ± 0.0
Kale	125	2	250	9.28	1	–31.0 ± 0.0	2.8 ± 0.0	11.6 ± 0.0
Root vegetables[a]	15	5	75	2.78	4,4,4[a]	–27.2 ± 1.8	2.5 ± 0.7	31.8 ± 9.3
Fruit[b]	15	3	45	1.67	4,2,5[b]	–26.0 ± 0.6	1.7 ± 0.4	52.6 ± 37.8
Zucchini	15	3	45	1.67	2	–26.1 ± 1.1	1.3 ± 0.2	11.73 ± 2.1
Green beans	15	2	30	1.11	1	–25.6 ± 0.0	2.8 ± 0.0	14.4 ± 0.0
Total weekly diet			2695					
Overall diet						–29.0 ± 0.5	1.4 ± 0.6	

[a]Root vegetables: carrot, turnip, yam.
[b]Fruit: apple, honeydew, papaya.

items.[40] Blood was sampled only once in August 2013 due to animal husbandry constraints. We sampled tissues from adult males and females from all three species and from both juvenile and adult *C. lewisi* (Table 2).

Different species of Rock Iguanas mature at different ages, ranging from 2 to 7 years.[41] However, within species there are contradicting estimates of the age at maturity.[27,34,41] There are few long-term population studies of iguanas in the wild (but see[42]), so many observations come from captive individuals, and therefore the estimated age of sexual maturity may vary from the actual age in wild counterparts. It is generally accepted that *C. collei* and *C. pinguis* mature near the upper end of the 2–7 year age range, whereas *C. lewisi* can lay eggs as early as their second year.[34,43] The *C. collei* and *C. pinguis* individuals that were sampled were all adults (all ages ≥7 years), whereas the *C. lewisi* consisted of 15 juveniles (born Aug. 2011; age <2 years), and five adults (ages ≥2 years).

Stable isotope analysis

Scat samples were collected either directly from the iguanas if they defecated when handled, or from their captive enclosure if they defecated earlier in the day, and any external debris was removed either upon collection or later in the lab. We collected skin samples directly from individuals as it was naturally shed, washed the samples with several drops of dish soap diluted in ~500 mL deionized (DI) water to break down any surface oils and remove debris,[44] and rinsed them thoroughly with DI water. All scat and skin samples were frozen at –20 °C until they were processed for isotope analysis (see below). Approximately 200 μL of whole blood was collected from each individual from the ventral coccygeal vein using a butterfly needle during routine husbandry blood collection to minimize stress.[41] The blood was transferred onto pre-combusted Whatman GF/F glass microfiber filter papers (GE Healthcare, Little Chalfont, UK) that were dried

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in sterile scintillation vials in a drying oven at 32 °C for 48 h.[45] The blood samples were homogenized by hand using a mortar and pestle or metal spatula within a cryovial, and ~0.5 to 1.0 mg of these processed samples were weighed into 5 × 9 mm tin capsules. We did not extract lipids from blood as whole blood has a very low lipid content;[46] this was supported by the low (<4) C:N ratios observed from our stable isotope data.[47]

The scat and skin samples were freeze-dried for 48 h, and the scat samples were further dried at 120 °C for 48 h to kill any potentially remaining bacteria. We removed all undigested plant matter from the scats and sampled the remaining fecal matrix material by first dividing it into two subsamples. The samples targeted for δ^{13}C analysis were agitated with 0.5 M HCl for 3 h to remove any potential inorganic carbon, as we were interested in measuring the δ^{13}C values from the organic components only, then dried at 32 °C for 48 h. To test for the effects of HCl on the δ^{13}C and δ^{15}N values from scat, subsamples of untreated scat material were also analyzed. We homogenized all scat samples by hand using either a mortar and pestle or metal spatula within a cryovial, and packaged ~5 mg of each scat sample into 5 × 9 mm tin capsules.

The skin samples were cut into small pieces (<0.5 × 0.5 mm) using surgical scissors, then each sample was divided into two subsamples. Lipid extraction was performed on the samples targeted for δ^{13}C analysis via a method modified from Folch *et al*.[47–49] We placed skin samples in 15-mL glass centrifuge tubes, added 10 ml of petroleum ether,[50] capped the vials with perforated lids, and sonicated them for 10 min at 40 kHz in a 60 °C water bath. We then centrifuged the samples at 12,000 *g* for 5 min, pipetted off the petroleum ether, rinsed each sample with ultra-pure water, centrifuged them again at 12,000 *g* for 5 min, and removed the excess water with a pipette. All the samples were then dried in a drying oven at 45 °C for 72 h. The samples were re-homogenized before analysis. Subsamples from all skin samples were analyzed with their lipids intact to test for the effects of lipid extraction on the stable isotope values, and ~0.5 to 1.0 mg of all skin samples were packaged into 5 × 9 mm tin capsules.

Throughout the sampling periods, we collected all diet samples (Table 3), which were hand-washed, frozen at –20 °C for at least 48 h, then freeze-dried for 48 h. The samples were homogenized by hand and ~3 mg was packed into 5 × 9 mm tin capsules.

All stable isotope analyses were performed with a Carlo Erba CE1108 elemental analyzer (CE Elantech, Lakewood, NJ, USA) interfaced via a ConFlo III device (Thermo Fisher Scientific, San Jose, CA, USA) to a Delta Plus XP isotope ratio mass spectrometer (Thermo Fisher Scientific) at the Department of Earth and Marine Sciences, University of California, Santa Cruz. The average precision of these data was calculated as the standard deviation (SD) of the δ^{13}C and δ^{15}N values from a set of standards (acetanilide), and it was 0.1‰ for both.

Statistical analysis

All individuals were kept on a weekly diet schedule (Table 3) for which the weighted percentage of each diet item was calculated by comparing the weekly total weight of each diet item with the weekly total weight of all food consumed by each individual. Using this weighted percentage and the δ^{13}C and δ^{15}N values of each dietary item and the iguana tissues, the discrimination factors were calculated using the following equation:

$$\Delta X(‰) = (\delta X_{consumer}) - \Big[(\%_{source\ 1} \times \delta X_{source\ 1}) + (\%_{source\ 2} \times \delta X_{source\ 2}) + \ldots\Big],$$

where ΔX(‰) is the C or N isotope discrimination factor (Δ^{13}C or Δ^{15}N, respectively), $\delta X_{consumer}$ is the δ^{13}C or δ^{15}N value of the tissues from the iguanas, $\%_{source}$ is the contribution of a specific diet item to the animals' total, aggregate diet, and δX_{source} is the δ^{13}C or δ^{15}N value of that particular diet or source item.[15,16]

To determine the variance around the weighted average source δ^{13}C and δ^{15}N values, we squared the SD from the mean δ^{13}C and δ^{15}N values for each source item. We then divided the % source value by 100, squared each of those values, then multiplied these by the squared SD for each of the mean δ^{13}C and δ^{15}N values from the sources. We added these numbers to arrive at a weighted sum of variances, then took the square root of that to obtain the SD around the weighted mean δ^{13}C and δ^{15}N values for the overall diet sources. We used the weighted sum of variances to calculate a potential range of low to high δ^{13}C and δ^{15}N values for the diet source and used those to calculate a range of potential discrimination factors. The mean C and N discrimination factor ± SD for each tissue type was calculated using the mean source isotope values in the equation above, and the mean range of potential discrimination factors was calculated by subtracting the lowest and highest potential source stable isotope values from the stable isotope values from the iguana tissues and taking the averages of those values.

All statistical tests were performed with R,[51] using parametric methods as all the data met the assumptions for parametric tests. Paired t-tests were used to evaluate the effects of acid treatment and lipid extraction on the isotope values from scat and skin samples, respectively, and to test for differences in isotope values between the two collection periods. We used t-tests to determine the effects of sex, age-group, and sampling period on the stable isotope values from scat and skin, and of sex and age-group on the isotope values from blood samples.

To test for relationships between the stable isotope values from all tissues and iguana age, size (SVL; mm), weight (g), and the relationship between size and weight, linear regressions were conducted for all tissues using individuals for which age, SVL, and/or weight data were available for each respective analysis. For some, these measurements were not available and they were omitted from the regression analysis. Results were included from regression analyses for age, size, weight (although they are all related), so that the results will be useable by researchers with access to any of those variables as they work to best determine which Δ^{13}C or Δ^{15}N values would be most applicable to the reptile of interest. To test for differences in the stable isotope values among species for all tissues collected and among tissues for each species, we conducted analysis of variance (ANOVA) tests followed by Tukey's pairwise comparisons.[51] The values reported are means ± SD and significance was tested at the $\alpha = 0.05$ level.

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RESULTS

The δ^{13}C and δ^{15}N values from all tissues and dietary items collected during the two sampling periods were not different (paired t-tests, all p ≥0.07; see Supplementary Table S1, Supporting Information). Therefore, as we had slightly more data from the first sampling period, we used the isotope values collected during this period (February 2013) for the analysis of diet items, scat, and skin. As blood samples were only available from the second sampling period (August 2013), these were used for the isotope analysis of blood. The δ^{13}C and δ^{15}N values were the same between sexes within each tissue type (t-tests, all p ≥0.25; see Supplementary Table S1, Supporting Information), so females and males were grouped together for all comparisons.

The δ^{13}C and δ^{15}N values were different among tissue types (scat, blood, and skin) for each species (ANOVA, all p ≤0.01) and Tukey's post hoc tests confirmed that the isotope values from all tissues within each species were different except for the δ^{13}C values from blood and skin from *C. lewisi* (p = 0.26), and the δ^{15}N values from blood and skin for *C. pinguis* (p = 0.22) and from blood and scat for all three species (p = 0.10 to 0.56) (see Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1). The Δ^{13}C and Δ^{15}N values from all tissue types and across all species ranged from –2.5 to +6.5‰ and from +2.2 to +7.5‰, respectively, for adults, and from –1.6 to +2.8‰ and from +0.8 to +5.3‰, respectively, for juveniles (Table 4). We found that the relationship between weight (g) and size (SVL, mm) for all individuals (adults and juveniles) was curvilinear ($W = (1.2274 \times 10^{-5}) \times S^{3.216}$; log-transformed for regression: $R^2_{adj} = 1.0$, $F_{1,24} = 5301.0$, $p << 0.0001$), and juveniles had higher growth rates than the adults (Supplementary Table S1 and Fig. S1, Supporting Information).

Scat

There were no differences in the δ^{13}C values from scat samples processed with and without the HCl agitation to potentially remove inorganic carbon prior to isotope analysis (paired t-test, t = –1.0, df = 37.0, p = 0.4) (Supplementary Table S1, Supporting Information). We expected the acid treatment to have no effect on the δ^{15}N values; however, we found that scat samples processed with HCl exhibited slightly higher δ^{15}N values than those processed without HCl (5.3 ± 1.1‰ vs 4.8 ± 0.7‰, respectively; paired t-test, t = 3.5, df = 9.0, p < 0.01) (Supplementary Table S1, Supporting Information). Thus, we used the δ^{13}C and δ^{15}N values from the untreated scat samples for our analyses.

The Δ^{13}C and Δ^{15}N values from scats from all of the species had high degrees of variability, ranging from –2.5 to 0.7‰ and from 2.2‰ to 4.2 ± 0.6‰, respectively, but none of these values were statistically different among species (ANOVA, $F_{2,8} = 3.4$, p = 0.09 and $F_{2,8} = 1.5$, p = 0.28, respectively; Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1). There were no differences in the Δ^{13}C and Δ^{15}N values from scat between the two age groups sampled from *C. lewisi* (adults vs juveniles; t-tests, δ^{13}C: t = 0.2, df = 4.2, p = 0.85; δ^{15}N: t = 0.4, df = 6.6, p = 0.69) (Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1). Linear regression analysis demonstrated no correlation between age as a continuous variable and the Δ^{13}C and Δ^{15}N values from scat samples (δ^{13}C: $R^2_{adj} = 0.1$, $F_{1,9} = 2.2$, p = 0.17; δ^{15}N: $R^2_{adj} = –0.1$, $F_{1,9} = 0.1$, p = 0.82; Table 4 and Supplementary Table S1 and Figs. S2(a) and S2(b), Supporting Information). However, linear regression analysis exhibited a negative correlation between the Δ^{13}C values from scats and weight ($R^2_{adj} = 0.6$, $F_{1,5} = 9.1$, p = 0.03; Supplementary Table S1, Supporting Information, and Fig. 2(a)) and body size (SVL, $R^2_{adj} = 0.8$, $F_{1,5} = 20.6$, p = 0.01; Supplementary Table S1 and Fig. S3(a), Supporting Information) for adults of all three species. There were no significant relationships between weight, age, or size and the Δ^{15}N values from scats (Supplementary Table S1 and Fig. 2, and Supplementary Figs. S2(b) and S3(b) (Supporting Information)).

Blood

The Δ^{13}C values from blood differed among all three species (ANOVA, $F_{2,11} = 22.7$, p <0.01), but the Δ^{15}N values were not different ($F_{2,11} = 1.8$, p = 0.21) (Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1). Post hoc Tukey pairwise comparisons revealed that all three species had different mean Δ^{13}C values (all p ≤0.02), with *C. lewisi* as the lowest (1.5 ± 0.3‰), then *C. collei* (2.1 ± 0.4‰), and *C. pinguis* (2.8 ± 0.2‰) (Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1). In addition, the mean Δ^{15}N values from *C. lewisi* juveniles were significantly lower (2.9 ± 0.6‰) than those from the adults (4.1 ± 0.4‰; t-test, t = 2.9, df = 7.4, p = 0.02), but the Δ^{13}C values (1.4–3.5‰) did not differ between age groups (t-test, t = 1.0, df = 6.3, p = 0.38) (Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1).

We found positive correlations between age as a continuous variable and both the Δ^{13}C and the Δ^{15}N values from blood ($R^2_{adj} = 0.7$, $F_{1,12} = 26.8$, p <0.01 and $R^2_{adj} = 0.3$, $F_{1,12} = 7.8$, p = 0.02, respectively; Supplementary Table S1 and Figs. S2(a) and S2(b), Supporting Information). The Δ^{13}C and Δ^{15}N values from blood were also significantly, positively correlated with weight ($R^2_{adj} = 0.6$, $F_{1,11} = 17.5$, p <0.01 and $R^2_{adj} = 0.6$, $F_{1,11} = 19.1$, p <0.01, respectively; Supplementary Table S1, Supporting Information, and Fig. 2) and size ($R^2_{adj} = 0.3$, $F_{1,11} = 6.6$, p = 0.03 and $R^2_{adj} = 0.7$, $F_{1,11} = 32.0$, p <0.01, respectively; Supplementary Table S1 and Figs. S3(a) and S3(b), Supporting Information).

Skin

There were no differences in the δ^{13}C and δ^{15}N values from skin that had been lipid extracted or left intact (paired t-tests, t = –0.1, df = 7.0, p = 0.90 and t = 0.6, df = 9.0, p = 0.56, respectively). Therefore, we used isotope values from samples that were not lipid extracted to calculate the discrimination factors for skin. The Δ^{13}C values from skin varied among species (ANOVA, $F_{2,14} = 8.5$, p <0.01). The order of Δ^{13}C values from skin was the same as for blood: *C. lewisi* (2.4 ± 0.1‰), *C. collei* (3.7 ± 1.2‰), and *C. pinguis* (5.2 ± 0.9‰); however, Tukey's tests demonstrated that the δ^{13}C values from skin were not significantly different between *C. lewisi* and *C. collei* (p = 0.19). All other comparisons were significantly different (p ≤0.04; Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1). The Δ^{15}N values from skin did not differ among species (ANOVA, $F_{2,14} = 0.6$, p = 0.6, Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1). *C. lewisi* juveniles

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Table 4. Species, mean (±SD) stable isotope ratios and stable isotope discrimination values (‰), range of discrimination factors, and C:N ratios for tissues and diet items collected in 2013 for all *Cyclura* species in this study. All animals were adults except where otherwise noted. Stable isotope values from males and females were grouped for each tissue type within each species as there were no differences in isotope values between sexes. See Supplementary Table S1 (Supporting Information) for more details

			Animal		Diet		Mean discrimination factors		Range of discrimination factors		
Species	Tissue	N	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\Delta^{13}C$	$\Delta^{15}N$	$\Delta^{13}C$	$\Delta^{15}N$	C:N
C. lewisi[a]	Scat	4	–29.5 ± 0.9	4.3 ± 0.8	–29.0 ± 0.5	1.4 ± 0.6	–0.5 ± 0.9	2.9 ± 0.8	–1.5 to 0.7	2.2 to 3.8	9.6 ± 1.2
	Blood	5	–27.1 ± 0.3*	5.2 ± 0.6*	–29.0 ± 0.5	1.4 ± 0.6	1.9 ± 0.3*	3.8 ± 0.6*	1.5 to 2.1	3.2 to 4.6	3.6 ± 0.3
	Skin	3	–26.2 ± 0.1*	7.4 ± 0.7*	–29.0 ± 0.5	1.4 ± 0.6	2.8 ± 0.1*	6.0 ± 0.7*	2.7 to 2.8	5.4 to 6.8	3.5 ± 0.4
C. lewisi juvenile	Scat	10	–29.6 ± 0.7	4.1 ± 1.0	–29.0 ± 0.5	1.4 ± 0.6	–0.6 ± 0.7	2.7 ± 1.0	1.0 to -1.6	0.8 to 3.9	9.1 ± 0.8
	Blood	13	–27.3 ± 0.2	4.4 ± 0.6*	–29.0 ± 0.5	1.4 ± 0.6	1.7 ± 0.2	2.9 ± 0.6*	1.4 to 2.2	2.3 to 4.0	3.4 ± 0.1
	Skin	9	–26.8 ± 0.3*	5.6 ± 0.8*	–29.0 ± 0.5	1.4 ± 0.6	2.3 ± 0.3*	4.2 ± 0.8*	1.8 to 2.8	2.8 to 5.3	3.3 ± 0.1
C. collei[b]	Scat	4	–31.0 ± 0.4	5.3 ± 0.3	–29.0 ± 0.5	1.4 ± 0.6	–2.0 ± 0.4	3.4 ± 0.3	–2.5 to -1.7	3.6 to 4.2	9.7 ± 1.4
	Blood	4	–26.5 ± 0.4*	5.6 ± 0.2	–29.0 ± 0.5	1.4 ± 0.6	2.1 ± 0.4*	3.9 ± 0.3	2.2 to 3.0	4.0 to 4.5	4.3 ± 0.8
	Skin	7	–24.9 ± 1.2*	7.6 ± 0.8	–29.0 ± 0.5	1.4 ± 0.6	4.1 ± 1.2*	6.2 ± 0.8	2.8 to 6.5	5.0 to 7.5	3.0 ± 0.3
C. pinguis[c]	Scat	3	–30.1 ± 1.2	3.6 ± 2.3	–29.0 ± 0.5	1.4 ± 0.6	–1.6 ± 1.1	3.5 ± 0.6	–2.4 to -0.9	3.1 to 3.9	6.4 ± 4.7
	Blood	5	–25.9 ± 0.2*	5.7 ± 0.3	–29.0 ± 0.5	1.4 ± 0.6	3.1 ± 0.2*	4.3 ± 0.3	2.9 to 3.5	4.0 to 4.7	3.4 ± 0.1
	Skin	7	–23.5 ± 0.9*	7.2 ± 0.4	–29.0 ± 0.5	1.4 ± 0.6	5.5 ± 0.9*	5.8 ± 0.4	4.1 to 6.5	5.4 to 6.5	3.3 ± 0.1
Overall							$\Delta^{13}C$	$\Delta^{15}N$			
Scat							–1.3 ± 1.0	3.4 ± 0.7			
Blood							2.5 ± 0.6	4.1 ± 0.4			
Skin							4.5 ± 1.4	6.0 ± 0.6			

*Significant differences in stable isotope values and discrimination factors among species comparisons for each tissue type (ANOVAs, Tukey's pairwise comparisons). All statistical details are provided in Supplementary Table S1 (Supporting Information).
All among-tissue comparisons within species were statistically different (all $p \leq 0.02$), except:
[a]blood vs. skin $\Delta^{13}C$ values, $p = 0.26$; blood vs scat $\Delta^{15}N$ values, $p = 0.21$
[b]blood vs. scat $\Delta^{15}N$ values, $p = 0.56$
[c]blood vs. scat $\Delta^{13}C$ values, $p = 0.10$; blood vs skin $\Delta^{15}N$ values, $p = 0.20$

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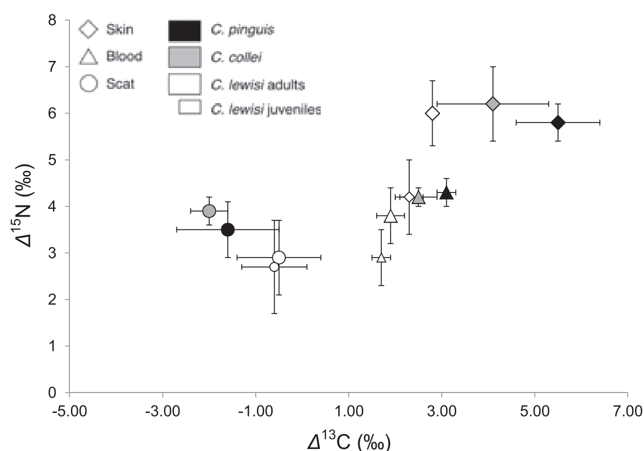

Figure 1. Mean (±SD) stable carbon (Δ^{13}C) and nitrogen (Δ^{15}N) isotope discrimination factors between diets and tissues (skin, blood and scat) from adult and juvenile captive Rock Iguanas (*Cyclura* spp.). See Table 2 for specific stable isotope and discrimination factor values, and Supplementary Table S1 (Supporting Information) for tests for differences among tissues, species and age groups.

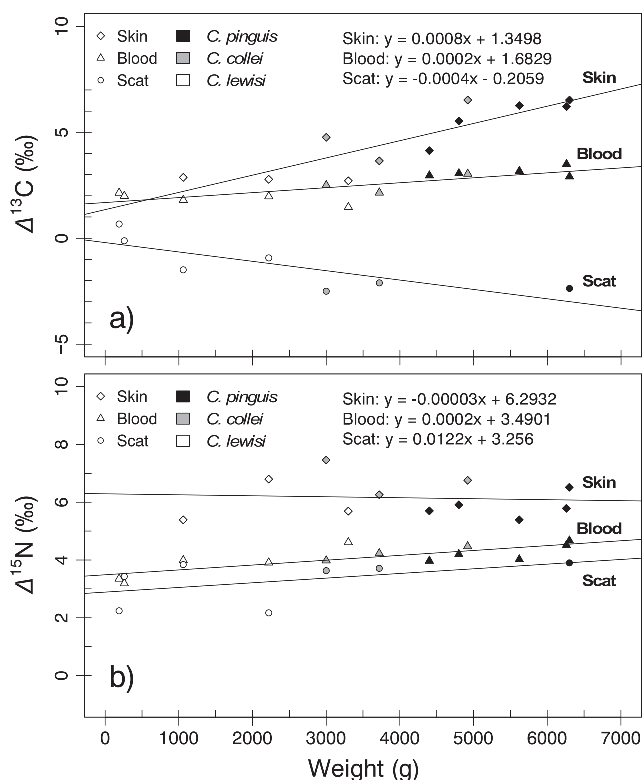

Figure 2. Linear relationships between (a) the stable carbon (Δ^{13}C) and (b) the nitrogen (Δ^{15}N) isotope discrimination factors and weight (g) from adult, captive Rock Iguanas (*Cyclura* spp.). There were significant linear relationships between the Δ^{13}C values from scat, blood, and skin and between the Δ^{15}N values from blood and weight from the three iguana species sampled. No other linear relationships were significant. See Supplementary Table S1 (Supporting Information) for all results and Supplementary Figs. S2 and S3 for linear regressions for age and size.

exhibited lower Δ^{13}C and Δ^{15}N values from skin (2.3 ± 0.3‰ and 4.2 ± 0.8‰, respectively) than *C. lewisi* adults (4.5 ± 0.1‰ and 6.0 ± 0.6‰, respectively) (t-test; Δ^{13}C: t = 4.6, df = 9.9, p < 0.01; Δ^{15}N: t = 3.6, df = 3.6, p = 0.03) (Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1).

The Δ^{13}C values from skin collected from adults of all species were positively correlated with age as a continuous variable (R^2_{adj} = 0.8, $F_{1,15}$ = 53.9, p << 0.01) (Supplementary Fig. S2(a), Supporting Information), but the Δ^{15}N values were not (R^2_{adj} = 0.0, $F_{1,15}$ = 0.8, p = 0.37) (Supplementary Table S1 and Fig. S2(b), Supporting Information). In addition, weight and size were positively correlated with the Δ^{13}C values from skin from adults (R^2_{adj} = 0.7, $F_{1,9}$ = 27.4, p < 0.01 and R^2_{adj} = 0.5, $F_{1,9}$ = 10.2, p = 0.01, respectively; Supplementary Figs. S2(a) and S3(a), Supporting Information), whereas there were no relationships between weight and size and the Δ^{15}N values from skin (R^2_{adj} = –0.1, $F_{1,9}$ = 0.1, p = 0.80 and R^2_{adj} = –0.1, $F_{1,9}$ = 0.1, p = 0.79, respectively; Supplementary Table S1 and Figs. S2(b) and S3(b), Supporting Information).

DISCUSSION

The range of Δ^{13}C and Δ^{15}N values from scat, blood, and skin from the captive rock iguanas was from –2.5 to +6.5‰ and from +2.2 to +7.5‰, respectively, for adults, and from –1.6 to 2.8‰ and from +0.8 to 5.3‰, respectively, for juveniles. These values are similar to those found in previous studies examining isotope discrimination factors in captive reptiles, although both Δ^{13}C and Δ^{15}N values from skin from adult Rock Iguanas are generally higher than those reported for skin from other adult reptiles, while values from skin from juvenile Rock Iguanas are similar to those from other species (Table 1). Of 24 comparisons of the Δ^{13}C and Δ^{15}N values among tissues for all species (blood vs skin vs scat for the adults of three species and for juvenile *C. lewisi*), only six were not different: the Δ^{13}C values from blood and skin in *C. lewisi* adults, the Δ^{15}N values from blood and skin in *C. pinguis* adults, and the Δ^{15}N values from blood and scat from adults of all three species and juvenile *C. lewisi* (Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1). Different tissues are composed of different amino acids, which vary in their δ^{13}C and δ^{15}N values.[52,53] Therefore, it is expected that different tissues from the same animal held on a constant diet could exhibit varying stable isotope values.[12]

The δ^{15}N (and Δ^{15}N) values from blood and skin among species were the same (Table 4, Fig. 1); however, they were significantly lower for juvenile *C. lewisi* than for the adult *C. lewisi*. In addition, all among-species comparisons for Δ^{13}C values from blood and skin were significantly different (Table 4 and Supplementary Table S1, Supporting Information). The Δ^{15}N values of tissues from vertebrates with higher growth rates, such as more rapidly growing hatchlings or juveniles, have been shown to be lower than those from animals that are not growing or have slowed growth.[12,39,54,55] This is because growing animals retain more ^{14}N via tissue deposition than they lose via excretion of waste than an animal that is not growing (but see[56]). This could explain the lower Δ^{15}N values that we observed in the juvenile *C. lewisi* than from the adult *C. lewisi*.

To our knowledge, this same phenomenon has not been shown to occur for δ^{13}C (and thus Δ^{13}C) values and, in fact, rapidly growing hatchling and juvenile sea turtles

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(loggerheads, *Caretta caretta*) demonstrated $\delta^{13}C$ values in line with other published values from adults.[39] However, the $\Delta^{13}C$ values from blood and skin from the adults in this study increased with increasing size and age (Tables 2, 4 and Supplementary Table S1, and Figs. S2(a) and S3(a), Supporting Information), which may be attributed to differential growth rates at different ages for reptiles. Supplementary Fig. S1 (Supporting Information) illustrates an increased growth rate for juveniles over adults in this study and the youngest, and physically smallest, species (*C. lewisi*) had the lowest $\delta^{13}C$ values, followed by the mid-size *C. collei*, then the oldest, largest *C. pinguis*. While little data exists on *Cyclura* growth rates, they are known to grow continuously throughout their lives,[57] but exhibit different growth rates at different life stages, including higher growth and metabolic rates at younger ages.[27,34,41,43]

Each species in this study includes individuals of significantly different mean ages (*C. pinguis*: 23.0 ± 6.7 years, *C. collei*: 12.1 ± 6.4 years, and *C. lewisi*: 2.2 ± 2.9 years (adults and juveniles); $p \leq 0.001$; Table 2) and they may be experiencing different growth and metabolic rates specific to these different ages (Supplementary Fig. S1, Supporting Information). Interestingly, only the $\Delta^{13}C$ values from skin (and not those from blood) were lower for the juveniles than the adult *C. lewisi*. At present, we do not know why the $\Delta^{13}C$ values correlated so strongly with age/size in the adults, whereas the $\Delta^{15}N$ values did not, and the potential role of differential sizes and growth rates in determining $\Delta^{13}C$ and $\Delta^{15}N$ values deserves further study in reptiles and other taxa.

The $\Delta^{13}C$ values from scats were all negative (excluding one adult with a $\Delta^{13}C$ value of +0.7‰). Consumer tissues generally contain more of the heavy isotopes of C and N than their food sources, thus leading to positive discrimination factors.[7,13,58] However, excreta generally have lower $\delta^{13}C$ values than their diets and their other tissues.[30,59,60] It is not known exactly why this happens (see[60]), but one hypothesis is that it is because the lighter isotope of carbon reacts more quickly than the heavier isotope, becoming more readily incorporated into waste products. This fractionation effect contributes to a predictable increase in both $\delta^{13}C$ and $\delta^{15}N$ values between trophic levels in a food web.[7,13,59] Thus, an accumulation of more of the light isotope of carbon in excreta could explain the negative $\Delta^{13}C$ values, and the somewhat lower $\Delta^{15}N$ values observed for iguana scats than for the other tissues. Finally, the $\Delta^{13}C$ values from scats were negatively correlated with iguana age, size, and weight. As growing animals tend to retain more of the lighter isotopes, it follows that their excreta would contain higher ratios of heavier to lighter isotopes than slower growing adults, leading to higher $\delta^{13}C$ (and thus $\Delta^{13}C$) values for their excreta. We might expect a similar pattern for the stable nitrogen isotope ratio values from scats, but there were no relationships between the $\Delta^{15}N$ values from iguana scats and their age, size, or weight.

In studies of wild reptile populations (Green Sea Turtles, *Chelonia mydas*, Loggerhead Turtles, *Caretta caretta*, and Leatherback Sea Turtles, *Dermochelys coriacea*), correlations of $\delta^{13}C$ and $\delta^{15}N$ values with body size, weight, or age are typically attributed to ontogenetic, or size- and age-related, shifts in diet preferences.[61–63] For example, such changes can occur as animals grow larger and are able to acquire larger prey. However, the San Diego Zoo ICR held individuals of all ages and species on a steady, identical diet throughout our study (12+ months). Therefore, diet shifts with increasing age or size would not account for the observed isotopic changes and this should be considered when examining stable isotope values from different age and size-class individuals in the wild. In addition, it is frequently difficult to obtain $\Delta^{13}C$ and $\Delta^{15}N$ values from captive animals for use in studies estimating wild animal foraging ecology. Our data indicate linear relationships between the $\Delta^{13}C$ values from blood, skin, and scat and iguana weight, size, and age, and the $\Delta^{15}N$ values from blood and iguana weight, size, and age. Therefore, it may be possible for others to use our regressions to estimate the best $\Delta^{13}C$ and $\Delta^{15}N$ values for these tissues for use in interpreting wild iguana foraging ecology if the weights, sizes, and/or ages of the iguana species of interest are known.

The captive populations at the San Diego Zoo ICR are kept on a constant, healthy diet, and are thus neither malnourished nor overweight.[34,41,43] Therefore, our weight-size curvilinear relationship (Supplementary Fig. S1, Supporting Information) represents that of healthy individuals. This model may be used for comparison of wild individuals for estimations of physical condition. This may be useful as an animal's physical condition can affect its tissue stable isotope values. For example, when resources are scarce, tissues undergo catabolism causing an increase in their $\delta^{15}N$ values ([64] and see review in[65]), an occurrence observed across taxa, including in reptiles.[65]

The treatment of scat samples with HCl was done to remove any potential inorganic carbon (Brown Reid, personal communication, Washington University, St. Louis, MO, USA) before stable isotope analysis. This is to ensure that organic carbon is targeted for analysis, as the $\delta^{13}C$ value from organic carbon is what reflects animal diet. Although we found that agitation of scat matrix in HCl did not significantly affect the $\delta^{13}C$ values in our samples, indicating that no inorganic carbon was present, those that were treated with HCl did exhibit higher $\delta^{15}N$ values than those that were not. The acidification process could cause leaching of organic nitrogen compounds (i.e. proteins or amino acids),[66] which could affect the $\delta^{15}N$ values in the scat samples. As the acidification treatment of scats did not affect their $\delta^{13}C$ values, but it did affect the $\delta^{15}N$ values, we recommend omitting this treatment from future protocols for preparing scats from iguanas for stable isotope analysis unless the samples in question clearly contain significant amounts of materials with inorganic carbon. In addition, we recommend that the use of stable isotope values from scat in future studies be considered with caution as scat proved to be the least reliable tissue in its consistency of stable isotope values across species and age groups (see Table 4 and Supplementary Table S1, Supporting Information, and Fig. 1). Studies using stable isotope analysis are often performed for the purpose of reconstructing diets of wild animals and scat would be a relatively easy, accessible, and noninvasive tissue to use for such analyses. However, Hwang *et al.*[67] found that the $\delta^{15}N$ and $\delta^{13}C$ values from scat did not consistently reflect the isotopic composition of the diet, and the $\Delta^{15}N$ and $\Delta^{13}C$ values from scat offered evidence of great variation in a study across several mammalian fore- and hindgut fermenters. These findings are in line with our scat analyses results. There may also be a representational bias as a scat sample will contain the highest proportion of remains of the least digestible dietary items, while the most digestible items will be least represented.

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The skin from the iguanas was treated with petroleum ether to remove any lipids that might be present as reptile skin can contain significant lipid concentrations that are thought to contribute to decreased cutaneous water loss[68] and the presence of lipids in animal tissues reduces $\delta^{13}C$ values.[47] However, lipid extraction had no significant effect on the $\delta^{13}C$ or $\delta^{15}N$ values from skin, indicating that it was unnecessary. This is probably because the lipid content of the iguana skin in our study was not high enough to affect the stable isotope values and this is supported by the C:N ratios from the skin samples which were less than 4.0, indicating a lipid content of less than 10%.[47]

In order to provide overall stable isotope discrimination factors across all three adult iguana species for use in studies employing other species, we have reported the mean $\Delta^{13}C$ and $\Delta^{15}N$ discrimination factors across all species for each tissue in Table 4 (and data are presented in Fig. 1). They were +4.5 ± 1.4‰ and +6.0 ± 0.6‰, respectively, for skin; +2.5 ± 0.6‰ and +4.1 ± 0.4‰, respectively, for blood; and –1.3 ± 1.0‰ and +3.4 ± 0.7‰, respectively, for scat (Table 4 and Supplementary Table S1, Supporting Information). However, since we found relationships between discrimination factors and size, we recommend using the $\Delta^{13}C$ and $\Delta^{15}N$ factors from the species in this study that are closest in size to the wild species of interest when applying stable isotope discrimination factors to known-size iguanas in the wild. In addition, where possible, we recommend utilizing our reported linear regression equations to estimate the appropriate discrimination factors for the size of the iguana species.

CONCLUSIONS

We found that age, size, and the growth rates associated with specific life stages probably play a role in affecting the $\delta^{13}C$ and $\delta^{15}N$ (and thus $\Delta^{13}C$ and $\Delta^{15}N$) values in Rock Iguanas, although the mechanisms causing these relationships require further study, especially for stable carbon isotopes. Tissues of varying molecular composition also varied isotopically, and the $\delta^{13}C$ values from scat exhibited inverse relationships with age and size to those from other tissues, possibly due to the digestion adaptations unique to hindgut fermenters.

Acknowledgements

The authors extend their thanks to Ron Swaisgood and the San Diego Zoo – Institute for Conservation Research, Applied Animal Ecology/Behavioral Ecology – for their collaboration; UCSD, Heart di Vite and the UCSD Undergraduate Academic Enrichment Program and David Marc Belkin Memorial Research Scholarship for providing funding that permitted this study; IUCN – Iguana Specialist Group for providing funding to present this research at the IUCN – ISG conference 2013; The UCSC Stable Isotope Lab and Dyke Andreasen; we thank Dr D. Holway, Dr E. Cleland, L. Bailey, K. Richardson, C. Turner Tomaszewicz, E. Hetherington, V. Hanna, S. Urata, C. Kelleher and members (two- and four-legged) of the Kurle Lab for laboratory help and comments that greatly improved this manuscript; and we thank the three anonymous reviewers for their comments that improved this manuscript.

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