Zoology 120 (2017) 83–91

Contents lists available at ScienceDirect

Zoology

journal homepage: www.elsevier.com/locate/zool

ZOOLOGY

Ontogenetic shifts in the diet of plains hog-nosed snakes (*Heterodon nasicus*) revealed by stable isotope analysis

Andrew M. Durso*, Stephen J. Mullin[1]

Department of Biological Sciences, Eastern Illinois University, Charleston, IL 61920, USA

ARTICLE INFO

Article history:
Received 4 May 2016
Received in revised form 5 July 2016
Accepted 22 July 2016
Available online 25 July 2016

Keywords:
Community ecology
Prairies
Reptiles
Snake diet
Stable isotope analysis

ABSTRACT

Wild snake diets are difficult to study using traditional methods, but stable isotopes offer several advantages, including integrating dietary information over time, providing data from individuals that have not fed recently, and avoiding bias towards slowly-digesting prey items. We used stable isotope signatures of carbon and nitrogen from scale tissue, red blood cells, and blood plasma to assess the diet of wild plains hog-nosed snakes (*Heterodon nasicus*) in Illinois. We developed Bayesian mixing models which, taken together, predicted that *H. nasicus* shifted from a juvenile diet predominantly (31–63%) composed of six-lined racerunners (*Aspidoscelis sexlineatus*) and their eggs to an adult diet predominantly (44–56%) composed of eggs of the aquatic turtles *Chrysemys picta* and *Chelydra serpentina*, with a contribution from toads (*Anaxyrus* sp.; 6–27%) during their adolescent years. These results agreed with sparse data from gut contents. Combining traditional and isotopic techniques for studying the diets of wild snakes can increase the utility of both types of data.

© 2016 Elsevier GmbH. All rights reserved.

1. Introduction

Analysis of natural variation in the relative abundance of stable isotopes of carbon and nitrogen is enjoying increased use in ecological studies of wild animals (Gannes et al., 1998). By exploiting variation in resource isotopic signatures, ecologists are able to estimate the relative contribution of different resources to consumer diets, provided that sufficient variation exists among resources to permit differentiation. Strengths of using stable isotopes include minimally invasive non-lethal sampling, avoiding bias from differences in digestion speed among resources, integrating dietary information over long periods of time, collecting data from all captured individuals (instead of just those which have fed recently), and providing a quantitative measure of trophic niche width along continuous axes common to all species (Bearhop et al., 2004; Willson et al., 2010). Stable isotope ratios can offer insight into the range and trophic level of resources that are consumed, the evenness of prey components in the diet over time, foraging location, and variability in individual physiology and diet-tissue fractionation (Peterson and Fry, 1987; Criss, 1999; Olive et al., 2003).

Ontogenetic shifts in diet are the rule rather than the exception in snakes (reviewed in Shine and Wall, 2007), and sexual divergence in diet is also common (Shine, 1991). We still know very little about the diet of many snake species, however, especially those that occur at low densities, have cryptic habits, or occupy inaccessible microhabitats. We used natural abundance variation in stable isotopes of carbon and nitrogen to quantify the contribution of six prey types to the diets of plains hog-nosed snakes (*Heterodon nasicus*) in a sand prairie ecosystem in northwestern Illinois. We non-lethally sampled three different tissue types from *H. nasicus* to examine the effects of isotopic routing and differences in tissue turnover rate on dietary reconstruction, with particular attention to the utility of sampling multiple tissues for evaluating evidence of ontogenetic shifts in diet.

Stable isotopes have not been used extensively in studies of wild reptile diets, and terrestrial snakes in particular are poorly represented in the isotopic ecology literature (but see Rush et al., 2014). Snake diets in general are difficult to study because individual snakes feed infrequently and opportunities to directly observe predation by snakes are rare. Although many excellent studies on snake diets exist (e.g., Henderson et al., 1978; Rodríguez-Robles, 1998; Mociño-Deloya et al., 2015), generalizations about the composition of snake diets often come from museum collections, which generally integrate information from specimens collected

* Corresponding author. Present address: Department of Biology, Utah State University, Logan, UT 84321, USA.
E-mail addresses: amdurso@gmail.com, andrew.durso@aggiemail.usu.edu (A.M. Durso).
[1] Present address: Department of Biology, Stephen F. Austin State University, SFA Box 13003, Nacogdoches, TX 75962, USA.

http://dx.doi.org/10.1016/j.zool.2016.07.004
0944-2006/© 2016 Elsevier GmbH. All rights reserved.

without replication over large spatial and temporal scales. As a consequence, intra-population variation in diet is often poorly understood (Durso et al., 2013). Even studies that attempt to collect stomach content data from as many individual snakes as possible (e.g., Mushinsky and Hebrard, 1977; Seib, 1985; Hirai, 2004; Capula et al., 2015) are often hindered by the fact that usually ≥50% of stomachs are empty. Stable isotopes offer a remedy to several of these problems and have promise for future studies of snake ecology.

Carbon and nitrogen isotope ratios are used as quantitative measures of food web position and niche space; δ^{13}C values primarily reflect the photosynthetic fixation pathways of plants and are maintained in the tissues of consumers, whereas δ^{15}N values normally increase with trophic level (reviewed in chapter 8 of Karasov and Martínez del Rio, 2007). An analysis of changes in diet over an animal's lifetime is possible by comparing the isotopic signatures of animals of different sizes, as well as of different biological tissues, which grow, are renewed, and incorporate dietary information at different rates (Tieszen et al., 1983). Time scales measured in different tissues in reptiles range from a few weeks (for plasma and red blood cells; Rosenblatt and Heithaus, 2013) to several months (for scale tissue; Pilgrim, 2005). We primarily used data from scale tissue to draw conclusions about long-term diet, because it represents an integration of multiple tissue types (e.g., dermis, epidermis) and potentially integrates the effects of multiple fractionation factors over a long period of time (Pilgrim, 2005). We assessed whether plasma and red blood cells were more useful for making inferences about recent diet, particularly for juvenile snakes, which might show greater variance among tissues because they are still growing (Hertz et al., 2016) or have maternal isotopic signatures that have not yet "washed out" of tissues with longer turnover times (Pilgrim, 2007). By comparing snake isotopic values to those we sampled from the local prey community, we tested for variation in diet across years, between sexes, and over the range of body sizes. We predicted that adult and juvenile snakes would differ in diet, although we did not have specific predictions about when a shift would take place or what their diet might be, because there is very little information about wild snake diets.

2. Materials and methods

2.1. Study site and sample collection

We conducted our study at Thomson Sand Prairie in Carroll County, Illinois. This site is a relatively isolated sand prairie, ca. 100 ha in size, that supports a relict population of *H. nasicus* as well as other characteristic flora and fauna of the inland sand areas of Illinois (Gleason, 1910; Smith, 1961). The habitat is open and easy to survey, and *H. nasicus* is the most abundant species of snake present at the site (Kolbe, 1999). We captured 86 *H. nasicus* over six sampling occasions between 19 September 2009 and 20 June 2012, encompassing May–September, and collected samples of their ventral scale tissue as well as their blood, which we separated into a plasma supernatant and a red blood cell pellet, yielding three tissue samples per snake (Table 1). We only used the first set of tissue samples collected during a sampling occasion in our final analysis, but if we recaptured a snake more than three days after its most recent capture ($N = 12$), we collected an additional set of tissue samples, which we used for a separate analysis. All snakes were uniquely marked using a cautery unit (Winne et al., 2006), and RFID tags were inserted into snakes that were sufficiently large. Sex and body size (snout–vent length [SVL] and mass) were measured using standard methods. We also collected gut contents from the relatively few snakes that had them ($N = 13$; 13.5% of captures). Durso and Mullin (2014) reported additional sampling details.

Table 1
Mean ± one standard error of morphometrics and stable isotope ratios for blood plasma, red blood cells and scale carbon (δ^{13}C) and nitrogen (δ^{15}N) of 54 plains hog-nosed snakes (*Heterodon nasicus*) from the Thomson Sand Prairie (Carroll Co., Illinois), as measured from samples collected in 2010 and 2011. Sample sizes are shown in parentheses.

Age class	Snout–vent length (mm)		Mass (g)		Tissue type	Carbon isotope ratio (δ^{13}C)		Nitrogen isotope ratio (δ^{15}N)	
	2010	2011	2010	2011		2010	2011	2010	2011
Adults	541 ± 18 (18)	573 ± 21(8)	184 ± 17 (18)	231 ± 38 (8)	Plasma	−23.78 ± 0.32 (14)	−23.35 ± 0.55 (8)	11.53 ± 0.39 (14)	11.56 ± 0.53 (8)
					Red blood cells	−23.12 ± 0.34 (15)	−22.54 ± 0.44 (8)	11.11 ± 0.46 (15)	11.27 ± 0.90 (8)
					Scales	−22.91 ± 0.27 (18)	−22.34 ± 0.30 (8)	12.02 ± 0.45 (18)	11.39 ± 0.75 (8)
Large juveniles	219 ± 14 (11)	204 ± 3 (5)	13 ± 3 (11)	8 ± 1 (5)	Plasma	−23.30 ± 0.56 (8)	−23.00 ± 0.15 (5)	7.36 ± 0.51 (8)	7.11 ± 0.77 (5)
					Red blood cells	−22.93 ± 0.40 (8)	−22.88 ± 0.25 (5)	9.01 ± 0.75 (8)	8.54 ± 0.83 (5)
					Scales	−22.24 ± 0.45 (9)	−22.39 ± 0.27 (5)	8.86 ± 0.69 (9)	7.60 ± 0.72 (5)
Small juveniles	172 ± 3 (13)	180 ± 1 (2)	5.8 ± 0.2 (13)	5.5 ± 0.7 (2)	Plasma	−24.44 ± 0.10 (11)	−22.65 ± 0.04 (2)	9.87 ± 0.46 (11)	7.06 ± 0.17 (2)
					Red blood cells	−23.59 ± 0.09 (11)	−22.18 ± 0.05 (2)	11.51 ± 0.28 (11)	7.98 ± 1.21 (2)
					Scales	−23.53 ± 0.14 (3)	−21.68 ± 0.31 (2)	10.53 ± 0.68 (3)	7.56 ± 0.63 (2)

Table 2
Mean ± one standard error of stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) of five tissue types collected from 186 prey items in seven categories from Thomson Sand Prairie (Carroll Co., Illinois) in 2010 and 2011. Sample sizes are shown in parentheses. See text for explanations of how prey taxa were grouped in modeling analyses. We also collected 24 prey samples that are not included in this table or in the analyses for the following reasons: not collected in summer ($N = 10$); did not cluster tightly together (shrews; $N = 8$); only one or two individuals obtained ($N = 5$); outlier with suspected soil contamination ($N = 1$; box turtle toe, $\delta^{13}C = -26.62$).

Prey category	Tissue Type	Carbon isotope ratio ($\delta^{13}C$)		Nitrogen isotope ratio ($\delta^{15}N$)	
		2010	2011	2010	2011
Toads (*Anaxyrus americanus*)	Whole animal	−20.66 ± 1.01 (6)	−20.43 ± 1.48 (4)	3.94 ± 0.13 (6)	5.50 ± 1.02 (4)
Six-lined racerunners	Whole animal	−23.38 ± 0.10 (2)	−23.72 ± 0.39 (6)	2.09 ± 0.31 (2)	3.08 ± 0.20 (6)
(*Aspidoscelis*	Egg	−24.56 ± 0.26 (3)	−24.97 ± 0.68 (3)	3.58 ± 0.34 (3)	3.26 ± 0.40 (3)
sexlineatus)	Tail tip	–	−22.81 ± 0.13 (28)	–	3.45 ± 0.13 (28)
Mice (*Peromyscus* sp.)	Whole animal	−23.55 ± 0.25 (3)	−25.49 ± 0.18 (2)	2.23 ± 0.21 (3)	4.45 ± 0.14 (2)
Painted turtle (*Chrysemys picta*) eggs	Egg	−25.57 ± 0.28 (8)	−25.31 ± 0.45 (18)	9.99 ± 0.41 (8)	11.86 ± 0.42 (18)
Snapping turtle (*Chelydra serpentina*) eggs	Egg	−24.96 ± 0.29 (21)	−25.35 ± 0.26 (14)	10.11 ± 0.28 (21)	11.28 ± 0.24 (14)
Voles (*Microtus* sp.)	Whole animal	−27.31 ± 0.23 (7)	−26.16 ± 0.32 (2)	3.08 ± 1.08 (7)	3.05 ± 0.30 (2)
Leopard frogs (*Lithobates pipiens*)	Whole animal	−25.50 ± 0.53 (7)	−23.17 ± 1.14 (4)	7.11 ± 0.27 (7)	8.48 ± 0.64 (4)
Ornate box turtles	Blood	–	−22.80 ± 0.18 (23)	–	1.86 ± 0.18 (23)
(*Terrapene ornata*)	Toenail	–	−21.72 ± 0.17 (25)	–	1.69 ± 0.19 (25)

Although generalizations about the diet of all three species of *Heterodon* suggest that they eat mostly amphibians (Platt, 1969; Beane et al., 2014), these snakes are known to feed on a variety of other vertebrates, including other reptiles and small mammals. We therefore collected 133 whole vertebrates and their eggs from the site over the same time period (Table 2). We also collected non-lethal tissue samples from an additional 28 six-lined racerunners (*Aspidoscelis sexlineatus*; tail tips) and from 26 adult female ornate box turtles (*Terrapene ornata*; toenails [$N = 26$] and blood [$N = 23$]), for a total of 210 prey samples. We included samples from adult female *T. ornata* because we thought that adult female stable isotope ratios might reflect those of their eggs. *H. nasicus* have been observed feeding on *T. ornata* eggs at this site, but we could not destructively sample any because *T. ornata* is a threatened species in Illinois.

2.2. Sample processing

All snake and prey samples were stored in tinfoil and dried to a constant mass using a drying oven set to 60 °C. Because snakes consume their prey whole, we homogenized whole prey items using a combination of mortar and pestle, Wig-L-Bug Amalgamator (Dentsply International, York, PA, USA), and a Retsch ball grinder (Retsch Technology, Haan, Germany). We did not lipid extract prey prior to analysis or remove hair from mammals. Between 0.5 and 2.0 mg of each homogenized sample was placed into a tin capsule (5 mm x 9 mm; Costech Analytical, Valencia, CA, USA) and analyzed using an isotope-ratio mass spectrometer (Carlo Erba NA1500Elemental Analyzer; CE Instruments, Hindley Green, United Kingdom) coupled to a Delta V Isotope Ratio Mass Spectrometer (Thermo Finnigan LLC, San Jose, CA, USA) via the Conflo III Interface (Thermo Finnigan LLC) (University of Georgia Analytical Chemistry Laboratory). We measured stable carbon (^{13}C:^{12}C or $\delta^{13}C$) and nitrogen (^{15}N:^{14}N or $\delta^{15}N$) isotope ratios of snake scales, red blood cells, and blood plasma, and of whole or partial prey items. In addition to calibrations made using standard reference materials, a haphazardly chosen subset ($N = 29$) of our samples was run in duplicate to evaluate precision (mean coefficient of variation (CV) for $\delta^{15}N = 0.038‰$; mean CV for $\delta^{13}C = 0.007‰$).

2.3. Statistics

We used a type III ANCOVA (function 'Anova' in package 'car' in R; Fox and Weisberg, 2011) to test whether $\delta^{15}N$ or $\delta^{13}C$ of snake tissues varied with (i) year (2010 vs. 2011); (ii) sex; (iii) body size; and (iv) tissue type. Although neither response variable could be transformed to meet the assumption of normality, neither skewness (−0.30 for $\delta^{15}N$; 0.69 for $\delta^{13}C$) nor kurtosis (2.22 for $\delta^{15}N$; 2.89 for $\delta^{13}C$) was particularly extreme, so we proceeded because ANCOVA is robust to violation of this assumption (Schmider et al., 2010). We also tested for differences among $\delta^{15}N$ or $\delta^{13}C$ of prey species isotope signatures using a linear model with a post-hoc Tukey's test (function 'glht' in package 'multcomp' in R; Hothorn et al., 2008) and a similar rationale about robustness to violations of normality (skewness 0.57 for $\delta^{15}N$, 0.20 for $\delta^{13}C$; kurtosis 1.86 for $\delta^{15}N$, 3.64 for $\delta^{13}C$). We also used an ANOVA on the sparse isotopic data ($N = 26$ recaptures of 12 individuals within a season, and 15 recaptures of 7 individuals across seasons or years) from recaptured animals to assess the generality of ontogenetic shifts in diet. Finally, we used a piecewise linear regression (package 'segmented'; Muggeo, 2008) to test for non-linear effects of body size on stable isotope ratios of blood plasma, which we expected would be the first tissue type to show evidence of an ontogenetic shift in diet. All figures were made using ggplot2 (Wickham, 2009).

2.4. Diet modeling

We used the SIAR package (Parnell and Jackson, 2011) in R to produce Bayesian mixing models that estimate the percent contribution of each prey category to the diet of *H. nasicus*. We limited these models to data collected during the summers of 2010 and 2011, because too few samples were collected in the fall of those years or in 2009 and 2012 to be useful. The data and code are available on figshare (data: http://bit.ly/durso16data; code: http://bit.ly/durso16code).

We categorized prey into the following groups (Table 2): (1) toads (*Anaxyrus* sp.), (2) mice (*Peromyscus* sp.) and six-lined racerunners (*Aspidoscelis sexlineatus*; whole animals, eggs, and tail tips), (3) aquatic turtle eggs (*Chelydra serpentina* and *Chrysemys picta*), (4) voles (*Microtus* sp.), (5) northern leopard frogs (*Lithobates pipiens*), and (6) ornate box turtles (*Terrapene ornata*; toenails and blood). We combined taxa for groups 2 and 5 because they clustered together visually (Fig. 1; Phillips et al., 2005) and were the only taxa that were similar in both $\delta^{15}N$ (Tukey HSD $p > 0.39$) or $\delta^{13}C$ (Tukey HSD $p > 0.60$). We did not include data from a few potential prey for which we obtained only one or two individuals (e.g., *Hyla chrysoscelis*, *Lithobates catesbeianus*, *Spermophilus tridecemlineatus*). We also omitted shrews (*Sorex* sp. and *Blarina* sp.; $N = 7$) because they did not cluster tightly together and overlapped broadly with the other prey.

For our mixing models, we used natural breaks in the data to create three body size categories, about which we made rough

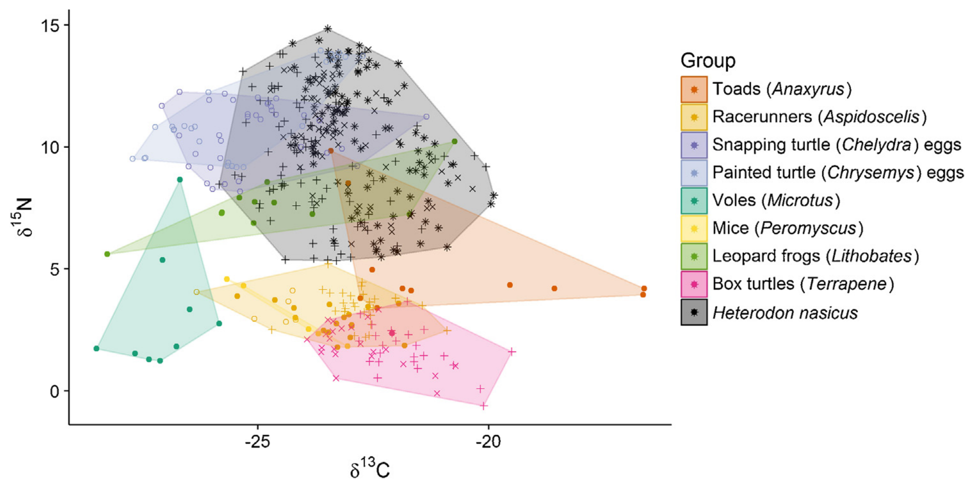

Fig. 1. Stable δ^{13}C and δ^{15}N isotope structure of *Heterodon nasicus* and the prey community in 2010 and 2011 at Thomson Sand Prairie (Carroll County, Illinois). One outlier, a box turtle toe (δ^{15}N = 7.31, δ^{13}C = –26.62) is shown but is not included in the polygon and was omitted from analyses. Filled circles represent whole animals, open circles represent eggs, crosses represent subsamples (tail tips for *Aspidoscelis sexlineatus*; toenail [+] or blood [X] for *Terrapene ornata*; plasma [+], red blood cells [X], or scale [*] for *Heterodon nasicus*).

assumptions about age following Kolbe (1999). We called our three categories adults (SVL > 400 mm; all of which were sexually mature and probably at least two years old), large juveniles (SVL = 190–210 mm; between about one month and one year old), and small juveniles (SVL < 190 mm; probably hatched within the past month), and we modeled the diet of each group separately (Table 1). Two individuals were intermediate in size and probably of an age between the adult and large juvenile categories (SVL = 285 and 330 mm; perhaps between one and two years old), and we modeled these individually using the SIAR function siarsolomcmcv4. Each model used 200,000 iterations with a burn-in of 50,000 iterations, except for the solo models, which used 500,000 iterations with a burn-in of 50,000 iterations.

We used gut content data from the 13 snakes that had any to generate informative priors, and assessed the influence of the priors on the model output for all models (Moore and Semmens, 2008). We could find no literature precedent for incorporating data from the empty stomachs of 86.5% of our captures, a proportion that is typical of wild snakes. Instead, we used a minimum value of 1 as a placeholder when a prey category was never found among gut contents, and used the ratio of our gut content sample size (N = 7 for adults, N = 6 for juveniles) to our stable isotope sample size (N = 26 for adults, N = 19 for juveniles) as a multiplier of the vector of prior proportions. In this way, we represented the relative amount of information contained by the two data types; in our models, our isotope data were 3–4 times more informative than our gut content data because our sample size was higher by that amount. Neither of the two intermediate-sized snakes that were modeled individually had gut contents, so no priors were used in these models.

Once we arrived at a final model, we varied trophic fractionation means and variances around the literature averages (3.0 ± 0.5‰ for δ^{15}N and 1.0 ± 0.05‰ for δ^{13}C), using the meta-analysis of Post (2002) to select wide limits that encompass empirical values from other studies on snakes (Pilgrim, 2005; Fisk et al., 2009), to assess their effect on the model output (see Figs. S1–S3 in the supplementary online Appendix). In contrast to other such simulations (Bond and Diamond, 2011), the effect was minimal (14.8% of simulations predicted a different prey group as most important), particularly when priors were used (0% of simulations predicted a different prey group as most important). As a result, we adhered to the common practice of using literature average values across taxa (Post, 2002).

3. Results

We analyzed data from 54 *H. nasicus* (Table 1) and 186 prey samples (Table 2) collected during the summers of 2010 and 2011. A minority (N = 24) of prey samples were not used in the final analysis (see Section 2.4 and Table 2 for details). Examination of plots of stable isotope ratios of carbon and nitrogen (Fig. 1) showed that (i) the isotopic signatures of most prey groups had limited overlap, (ii) nitrogen isotope ratios (usually interpreted as a proxy for trophic level) of *H. nasicus* were higher than those of most of their potential prey items, and (iii) annual variation in prey and *H. nasicus* isotope ratios was minimal. Sparse data from fall 2009 (N = 6 for *H. nasicus* scales and N = 7 for *A. sexlineatus*) and summer 2012 (N = 15 for *H. nasicus* scales) also supported the conclusion that annual variation was minor, at least over the course of the study. Although we detected statistically significant differences in δ^{15}N between years in *H. nasicus* ($F_{1,118}$ = 26.7, p = 0.004) and some prey categories ($F_{1,124}$ = 46.57, p < 0.0001), we ultimately modeled the two years together for simplicity, because results from the two separate models (not presented here) were not qualitatively different and examination of plots of the data suggests that there is little inter-annual variation of biological significance. We did not detect any statistically significant differences among years in δ^{13}C of snake tissues or prey ($F_{1,118}$ = 4.2, p = 0.07 for snakes; $F_{1,179}$ = 1.79, p = 0.18 for prey). There was also little evidence for differences in either δ^{15}N or δ^{13}C between sexes ($F_{1,118}$ < 3.4; p > 0.07) or among the three tissue types ($F_{1,118}$ < 10.68, p = 0.18). Finally, there was an effect of snake SVL on δ^{15}N ($F_{1,118}$ = 208.13, p < 0.0001), but not δ^{13}C ($F_{1,118}$ = 1.3, p = 0.32).

Data from recaptures suggest that there is a positive relationship between change in body size and change in δ^{15}N and δ^{13}C of all tissue types over time for juvenile snakes, but not for adult snakes (Fig. 2A–C; interaction term of combined analysis $F_{1,30}$ = 7.7, p = 0.009). This relationship was also evident in some analyses where tissue types and elements were kept separate ($F_{1,11}$ > 7.48, p < 0.02 for scales; $F_{1,11}$ = 9.62, p = 0.01 for red blood cells and nitrogen, $F_{1,11}$ = 0.19, p = 0.67 for red blood cells and carbon; $F_{1,11}$ < 0.80, p > 0.39 for plasma).

The plasma δ^{15}N signature in young *H. nasicus* falls from 12‰ to 5‰, until they reach ≈200 mm SVL, at which point it reverses direction and climbs to a maximum of 14‰ at adulthood (Fig. 3A). Likewise, the plasma δ^{13}C signature of young *H. nasicus* rises

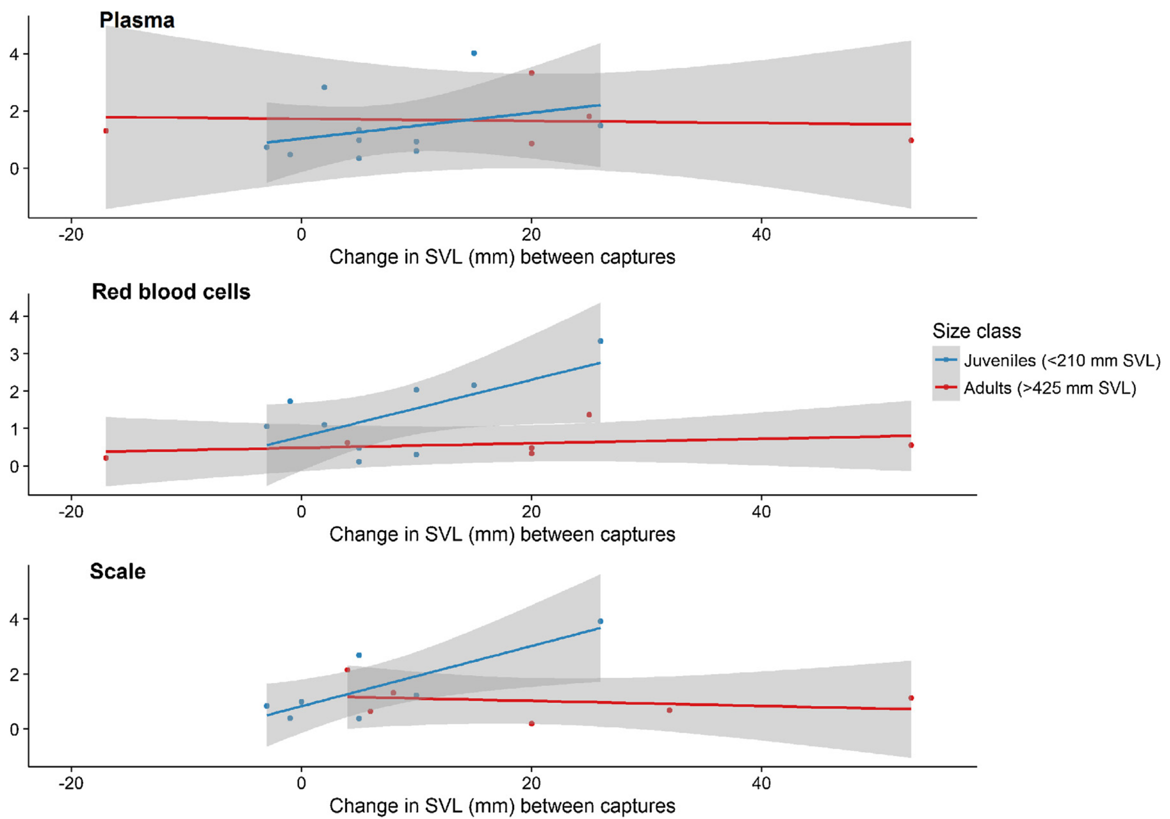

Fig. 2. Relationship between change in snout–vent length (SVL) and change in isotope values of plasma (A), red blood cells (B), and scale (C) from recaptured adult (>400 mm SVL; red) and juvenile (<210 mm SVL; blue) snakes. There is a significant interaction between the size class (adult or juvenile) and the slope of the relationship between the change in body size and length of the vector connecting the stable isotope signatures of C and N measured at the two captures. This relationship is positive for juvenile snakes, but not for adult snakes, and is evident in analyses where tissue types were combined and kept separate. Gray shading represents confidence interval as specified by "se = T" option in geom_smooth.

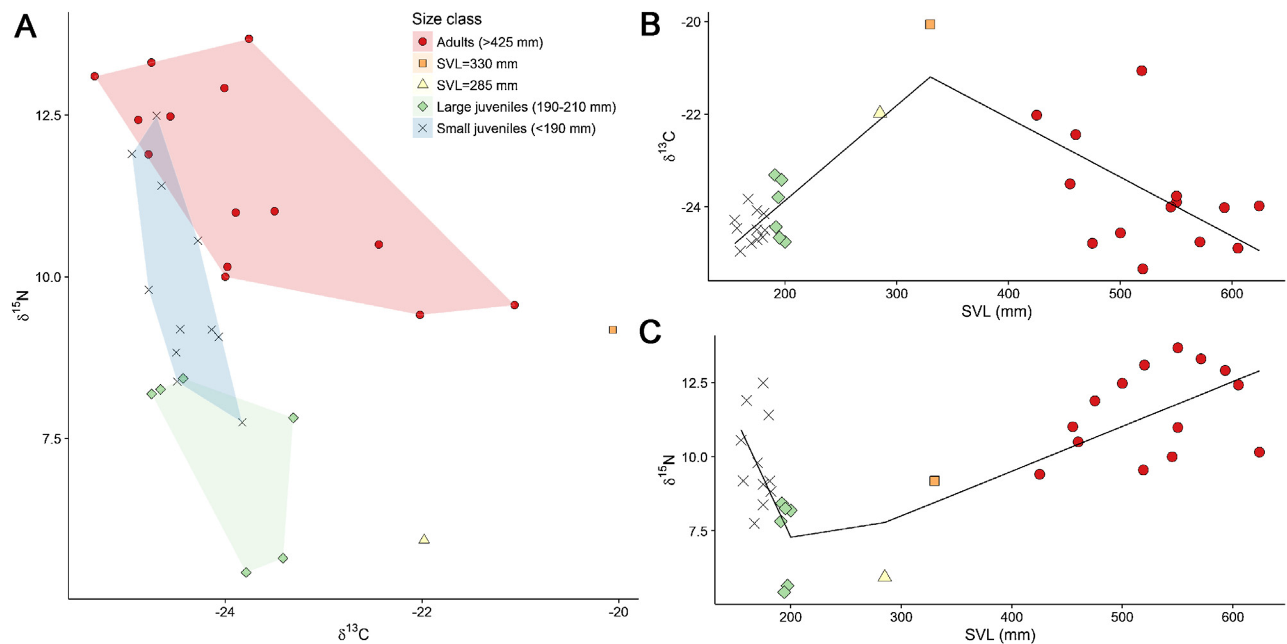

Fig. 3. (A) Ontogenetic shift in stable isotope ratios for plasma carbon (δ^{13}C) and nitrogen (δ^{15}N) of 33 *Heterodon nasicus* collected in 2010. (B) Segmented linear relationship between carbon isotope ratios of plasma and body size (break point = 330.38 ± 31.76 mm SVL, p = 0.008, R^2 = 0.51). (C) Segmented linear relationship between nitrogen isotope ratios of plasma and body size (break point = 208.27 ± 16.24 mm SVL, p = 0.006, R^2 = 0.56).

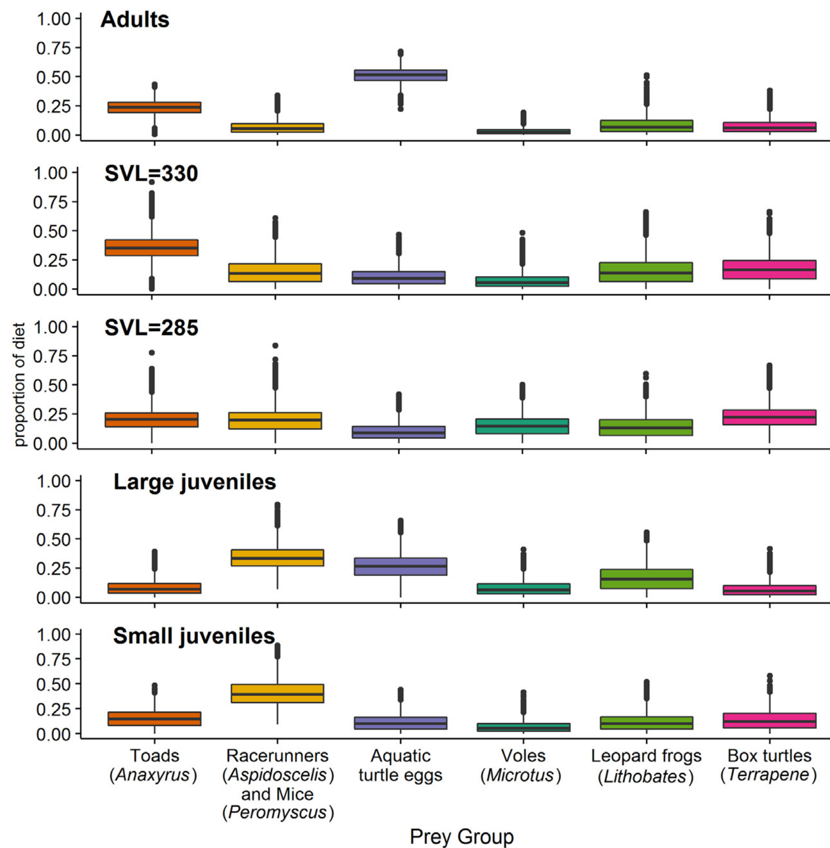

Fig. 4. Proportion of *Heterodon nasicus* diet made up of six prey categories, estimated by SIAR using δ^{13}C and δ^{15}N stable isotope data from adults (>400 mm SVL), a single individual measuring 330 mm SVL, a single individual measuring 285 mm SVL, large juveniles (190–210 mm SVL), and small juveniles (<190 mm SVL). Informative priors were used for adults, large juveniles, and small juveniles. Error bars represent 95% credible intervals. See Table 1 for sample sizes.

from −25‰ to −20‰ until they reach ≈330 mm SVL, at which point it reverses direction and falls to a minimum of −25.5‰ at adulthood (Fig. 3A). Isotope signatures of the other two tissues show a similar pattern. Piecewise linear regression (package 'segmented'; Muggeo, 2008) indicated that the relationship between δ^{13}C and body size changes direction at 330 ± 32 mm SVL ($p = 0.008$, $R^2 = 0.51$; Fig. 3B), whereas the relationship between δ^{15}N and body size changes direction at 208 ± 16 mm SVL ($p = 0.006$, $R^2 = 0.56$; Fig. 3C).

Gut contents of juveniles included *A. sexlineatus* ($N = 2$) and their eggs ($N = 4$), whereas gut contents of adults included the eggs of common snapping turtles (*Chelydra serpentina*; $N = 2$), painted turtle eggs (*Chrysemys picta*; $N = 3$), and turtle eggs that were too digested to identify to species ($N = 2$). Incorporating priors from gut contents (which included *A. sexlineatus* but not *Peromyscus*) into mixing models increased our confidence in, but did not substantially alter, the following results.

The results of mixing models carried out using data from each of the three tissue types were very similar within size classes. Mixing models with priors using scale tissue (Fig. 4) showed that adult *H. nasicus* at our site eat mostly aquatic turtle eggs (median proportion of diet = 51%, 95% credible interval range 37–62%), with a lesser contribution from toads (median = 23%, 95% credible interval range 8–35%). There was no direct evidence and limited indirect evidence that adult *H. nasicus* consumed other prey (lizards, mice, voles, *Lithobates pipiens*, or *T. ornata*) at our site. The single-data-point mixing model from the larger of the two intermediate-sized individuals suggested that toads made up a higher percentage of its diet than of any other snake in the study (median = 35%, 95% credible interval range 18–58%). The single-data-point mixing model from the smaller of the two intermediate-sized individuals was more equivocal (five of the six prey category means between 13 and 22%, no upper 95% credible interval above 41%). In contrast, mixing models showed that juvenile *H. nasicus* at our site eat mostly racerunners and their eggs (for large juveniles, median = 33%, 95% credible interval range 18–56%; for small juveniles, median = 39%, 95% credible interval range 20–70%). For large juveniles, the model also predicted a substantial contribution of aquatic turtle eggs (median = 26%, 95% credible interval range 5–47%).

4. Discussion

Ontogenetic shifts and other intraspecific variation in the diets of wild animals are important components of ecology, with implications for population dynamics, conservation, and theoretical ecology (e.g., Catry et al., 2016). The most significant aspect of our study is the fact that we were able to collect data on diet from every individual snake we encountered, despite the fact that most of these snakes had empty stomachs. Our examination of gut contents yielded a relatively small data set providing only a snapshot of the diet that overlooked the importance of amphibians to the diet of intermediate-sized and probably adult *H. nasicus*. By using stable isotopes alongside gut content surveys, we were able to increase the size of our dataset more than four-fold. Combining traditional and isotopic techniques to studying diet is more laborious, but where the results of the techniques agree, our certainty improves (e.g., Meckstroth et al., 2007; Resano-Mayor et al., 2014). Over 43% of snake species reviewed by Shine and Wall (2007) showed ontogenetic shifts in diet, although they used data from Platt (1969) to characterize *H. nasicus* as feeding on amphibians throughout its life, whereas our results indicate that, at least at our site, they shift

their diets from lizards to toads to turtle eggs over the course of their lifetime.

Our results corroborate what has been learned from decades of field and museum studies on *H. nasicus* and illuminate the importance of two diet items, lizards and turtle eggs, to different size classes of this population during the active season. Although our study population is isolated from the main portion of the range of *H. nasicus* (Walley and Eckerman, 1999), reports from our site in Illinois (Barten, 1980), from other isolated populations (Hoaglund and Smith, 2012), and from the center of the range (Iverson, 1990) indicate some level of nutritional reliance by breeding adult *H. nasicus* on turtle eggs. Previous records of predation on lizards and their eggs (Durso et al., 2011; Kolbe et al., 1999) and bird eggs (Murphy and Dloogatch, 1980) also exist from our study site. In contrast, eggs were relatively rare in Platt's (1969) comprehensive studies in Kansas and Nebraska (not more than 11.1% of the diet), whereas we estimate that >50% of the adult diet is made up of turtle eggs at our site. Taken together with evidence that these snakes repeatedly visit and depredate reptilian nests (Durso et al., 2011; Hoaglund and Smith, 2012), such consistent trophic interactions have implications for reptile population dynamics (Campbell et al., 2012), behavior (including nest site choice; e.g., Spencer and Thompson, 2003; and parental care; e.g., Iverson, 1990; Pike et al., 2016), and conservation. Furthermore, this pattern is an example of an aquatic–terrestrial linkage that sheds light on the role of reptiles in nutrient cycling (Sterrett et al., 2015). At our site and in many places throughout its range, adult *H. nasicus* probably take advantage of aquatic turtle eggs, a seasonally abundant resource, to store energy for use throughout the rest of the active season (Willson et al., 2010; Pike et al., 2016).

Prey other than lizards, lizard eggs, and turtle eggs were not represented in gut contents. Mixing models did not predict reliance on any of these prey types, with the exception of toads. This does not necessarily mean that *H. nasicus* never consume these prey items, but they are probably rare components of the snakes' diet at this site. Solo mixing models indicated that, at intermediate sizes (≈210–400 mm), *H. nasicus* might shift their diets to primarily toads (up to 35%). Unfortunately, only two individual snakes in this size range were captured in the entire study, and both of their stomachs were empty. If they are feeding on amphibians, it is possible that *H. nasicus* in this size class are mostly foraging in the riparian habitat adjacent to the sand prairie, which we could not sample because it is made up of very dense vegetation, which would explain why we did not capture more of them.

The result that aquatic turtle eggs make a substantial contribution to the diet of juvenile *H. nasicus* is clearly spurious, because these young snakes are too small to consume such large prey. Instead, we interpret this as a maternal effect, wherein some portion of the scale tissue was composed of maternally-derived nutrients, lasting at least several weeks (Pilgrim, 2007). In the mixing model output where tissues were separated, additional evidence for a shift away from maternal diet was most evident in the model using plasma data from large juveniles in 2011 (see Figs. S4–S6 in the supplementary online Appendix). This difference notwithstanding, the absence of differences between stable isotope signatures of scales, red blood cells, and plasma is somewhat surprising; although in several cases particular patterns are clearest using data from a particular tissue, in almost every case the same pattern is visible as in the other two tissues.

It is difficult to comment on whether *T. ornata* eggs play a substantial role in the diet of *H. nasicus* at our site. No models predicted a large contribution of this prey category to the diet. This could either be because adult female *T. ornata* stable isotope ratios did not reflect those of their eggs, or because predation on *T. ornata* eggs is relatively rare. We suggest that the former is probably true. Detection of predation events at our site is biased upwards because of biologists using radio-telemetry to locate and monitor *T. ornata* nests at this site (Tucker et al., 2014), but five out of five *T. ornata* nests laid by reintroduced turtles at the similar Lost Mound Unit of the Upper Mississippi River National Wildlife & Fish Refuge/Savanna Army Depot (Jo Daviess County, Illinois) were depredated by *H. nasicus*. The extent to which the stable isotope signatures of *T. ornata* eggs are similar to those of aquatic turtles is unknown, but given the vastly different ecology of adults, there is no reason to suspect they are similar.

Although incorporating data from stable isotopes substantially improved our ability to characterize snake diets, we were fortunate that the ecological signal was so strong that it overwhelmed any unmeasured inexactness in the physiological fractionation signal (see the supplementary online Appendix; Griffiths, 1991). We consider this to be the best-case scenario and do not necessarily expect that it is the general case for snakes or other wild animals. Many authors have challenged the numerous simplifying assumptions of mixing models and cautioned against yielding to their temptation (Gannes et al., 1997, 1998; Pilgrim, 2005; Karasov and Martínez del Rio, 2007). We made efforts to address some of these limitations, including measuring multiple tissues from animals of different ages at a single site, measuring prey from the same time and place as our predators, measuring isotopes from recaptures, and collecting and incorporating gut content data. We were also fortunate that temporal variation in isotope signals was minimal, that prey isotope signatures were more variable among groups than within groups, and that source variation dominated our system. Additionally, snakes are strict carnivores, using protein both for energy and tissue synthesis, so differences in isotopic routing among tissues are likely minimized compared with those observed in omnivores (Ambrose and Norr, 1993; Martinez del Rio and Wolf, 2005; Karasov and Martínez del Rio, 2007), although snakes do exhibit some degree of nutrient routing (McCue et al., 2015). Depletion of lipid reserves and protein catabolism during long periods of fasting might cause isotopic enrichment of reptile tissues (McCue and Pollock, 2008; Hatch, 2012), but the measured magnitudes of these effects (1‰ for carbon; 3‰ for nitrogen) are smaller than what we interpreted as an ontogenetic diet shift over a similar time scale (5‰ for carbon, −7‰ for nitrogen), and in the wrong direction for nitrogen. However, future studies should aim to quantify the effects of fasting and hibernation on the isotope ratios of wild snakes.

Our data expand the current knowledge of the diet of *H. nasicus*, highlighting the heretofore unappreciated importance of aquatic turtle eggs, and our methods expand the strategies used to examine such questions. Using stable isotopes allowed us to augment sparse gut content data to describe an ontogenetic shift in the diet of a wild reptile. We encourage other investigators to explore using stable isotopes in conjunction with gut content surveys for studies of wild snake diets.

Acknowledgements

We thank the students and mentors of Dr. F. Janzen's Iowa State University Turtle Camp Research and Education in Ecology program, especially R. Alverio, B. Bodensteiner, D. Francis, C. Hinsley, S. Jahen, F. Janzen, A. Jergenson, S. Kinsey, J. Maciel, T. Mitchell, L. Neuman-Lee, M. Piñon, A. Reedy, J. Refsnider, J. Reneker, J. Ward, T. Ward, and D. Warner, for assistance with capturing study animals and for sharing the study site. United States Fish & Wildlife Service personnel E. Britton, J. Strickland, and C. Tucker provided logistical support. M. Pilgrim provided us with invaluable advice regarding study design. T. Maddox and P. Smith oversaw and assisted with sample processing through the University of Georgia Stable Isotope Laboratory. Partial funding for this research came from the

Illinois Department of Natural Resources (IDNR), the North Carolina Herpetological Society, and the Graduate School and the Department of Biological Sciences at Eastern Illinois University. Specimens were collected under the authority of IDNR permits #NH10-0946 and #09-39S and EIU IACUC #10-001. Comments from E. Brodie, L. Neuman-Lee, and members of the herpetology lab group at Stephen F. Austin State University helped improve this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.zool.2016.07.004.

References

Ambrose, S.H., Norr, L., 1993. Carbon isotope evidence for routing of dietary protein to bone collagen, and whole diet to bone apatite carbonite: purified diet growth experiments. In: Lambert, J., Groupe, G. (Eds.), Molecular Archaeology of Prehistoric Human Bone. Springer, Berlin, pp. 1–37.

Barten, S.L., 1980. The consumption of turtle eggs by a western hognose snake, *Heterodon nasicus*: a field observation. Bull. Chicago Herpetol. Soc. 15, 97–98.

Beane, J.C., Graham, S.P., Thorp, T.J., Pusser, L.T., 2014. Natural history of the southern hognose snake (*Heterodon simus*) in North Carolina, USA. Copeia 2014, 168–175.

Bearhop, S., Adams, C.E., Waldron, S., Fuller, R.A., Macleod, H., 2004. Determining trophic niche width: a novel approach using stable isotope analysis. J. Anim. Ecol. 73, 1007–1012.

Bond, A.L., Diamond, A.W., 2011. Recent Bayesian stable-isotope mixing models are highly sensitive to variation in discrimination factors. Ecol. Appl. 21, 1017–1023.

Campbell, I., Yackel, E.W., Adams, A.A., Converse, S.J., Fritts, T.H., Rodda, G.H., 2012. Do predators control prey species abundance? An experimental test with brown treesnakes on Guam. Ecology 93, 1194–1203.

Capula, M., Rugiero, L., Capizzi, D., Franco, D., Milana, G., Luiselli, L., 2015. Long-term, climate-change-related shifts in feeding frequencies of a Mediterranean snake population. Ecol. Res. 31, 49–55.

Catry, I., Catry, T., Alho, M., Franco, A.M.A., Moreira, F., 2016. Sexual and parent-offspring dietary segregation in a colonial raptor as revealed by stable isotopes. J. Zool. (Lond.) 299, 58–67.

Criss, R.E., 1999. Abundance and measurement of stable isotopes. In: Criss, R.E. (Ed.), Principles of Stable Isotope Distribution. Oxford University Press, New York, pp. 1–39.

Durso, A.M., Mullin, S.J., 2014. Intrinsic and extrinsic factors influence the expression of defensive behaviors in plains hog-nosed snakes (Colubridae: *Heterodon*). Ethology 120, 140–148.

Durso, A.M., Warner, D.A., Mitchell, T.S., Reedy, A.M., 2011. *Heterodon nasicus* (western hog-nosed snake): diet. Herpetol. Rev. 42, 439–440.

Durso, A.M., Willson, J.D., Winne, C.T., 2013. Habitat influences diet overlap in aquatic snake assemblages. J. Zool. (Lond.) 291, 185–193.

Fisk, A.T., Sash, K., Maerz, J., Palmer, W., Carroll, J.P., MacNeil, M.A., 2009. Metabolic turnover rates of carbon and nitrogen stable isotopes in captive juvenile snakes. Rapid Commun. Mass Spectrom. 23, 319–326.

Fox, J., Weisberg, S., 2011. An {R} Companion to Applied Regression, 2nd ed. Sage, Thousand Oaks, CA, USA.

Gannes, L.Z., O'Brien, D.M., Martinez del Rio, C., 1997. Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. Ecology 78, 1271–1276.

Gannes, L.Z., Martinez del Rio, C., Koch, P., 1998. Natural abundance variations in stable isotopes and their potential uses in animal physiological ecology. Comp. Biochem. Physiol. A 119, 725–737.

Gleason, H.A., 1910. The vegetation of the inland sand deposits of Illinois. Bull. Ill. State Lab. Nat. Hist. 9, 5–176.

Griffiths, H., 1991. Applications of stable isotope technology in physiological ecology. Funct. Ecol. 5, 254–269.

Hatch, K.A., 2012. The use and application of stable isotope analysis to the study of starvation, fasting, and nutritional stress in animals. In: McCue, M.D. (Ed.), Comparative Physiology of Fasting, Starvation, and Food Limitation. Springer, Berlin, pp. 337–364.

Henderson, R.W., Dixon, J.R., Soini, P., 1978. Resource partitioning in Amazonian snake communities. Milw. Public. Mus. Contrib. Biol. Geol. 22, 1–11.

Hertz, E., Trudel, M., El-Sabaawi, R., Tucker, S., Dower, J.F., Beacham, T.D., Edwards, A.M., Mazumder, A., 2016. Hitting the moving target: modelling ontogenetic shifts with stable isotopes reveals the importance of isotopic turnover. J. Anim. Ecol. 85, 681–691.

Hirai, T., 2004. Dietary shifts of frog eating snakes in response to seasonal changes in prey availability. J. Herpetol. 38, 455–460.

Hoaglund, E.P., Smith, C.E., 2012. Lessons learned: notes on the natural history of the plains hognose snake (*Heterodon nasicus*) in Minnesota. Reptiles and Amphibians 19, 163–169.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biometr. J. 50, 346–363.

Iverson, J.B., 1990. Nesting and parental care in the mud turtle, *Kinosternon flavescens*. Can. J. Zool. 68, 230–233.

Karasov, W.H., Martínez del Rio, C., 2007. Physiological Ecology: How Animals Process Energy, Nutrients, and Toxins. Princeton University Press, Princeton, NJ, USA.

Kolbe, J.J., 1999. Size and demographic structure of an isolated population of western hognose snakes, *Heterodon nasicus*, in northwestern Illinois. Bull. Chicago Herpetol. Soc. 34, 149–152.

Kolbe, J., Harmon, L., Warner, D., 1999. New state record lengths and associated natural history notes for some Illinois snakes. Trans. Ill. State Acad. Sci. 91, 133–135.

Martinez del Rio, C., Wolf, B., 2005. Mass-balance models for animal isotopic ecology. In: Starck, J.M., Wang, T. (Eds.), Physiological and Ecological Adaptations to Feeding in Vertebrates. Science Publishers, Enfield, pp. 141–174.

McCue, M.D., Pollock, E.D., 2008. Stable isotopes may provide evidence for starvation in reptiles. Rapid Commun. Mass Spectrom. 22, 2307–2314.

McCue, M.D., Guzman, R.M., Passement, C.A., 2015. Digesting pythons quickly oxidize the proteins in their meals and save the lipids for later. J. Exp. Biol. 215, 2089–2096.

Meckstroth, A.M., Miles, A.K., Chandra, S., 2007. Diets of introduced predators using stable isotopes and stomach contents. J. Wildl. Manag. 71, 2387–2392.

Mociño-Deloya, E., Setser, K., Heacker, M., Peurach, S., 2015. Diet of New Mexico ridge-nosed rattlesnake (*Crotalus willardi obscurus*) in the Sierra San Luis and Sierra Pan Duro, México. J. Herpetol. 49, 104–107.

Moore, J.W., Semmens, B.X., 2008. Incorporating uncertainty and prior information into stable isotope mixing models. Ecol. Lett. 11, 470–480.

Muggeo, V.M.R., 2008. Segmented: an R package to fit regression models with broken-line relationships. R News 8, 20–25.

Murphy, J.C., Dloogatch, M., 1980. An additional note on the egg-eating habits of the western hognose snake in Illinois. Bull. Chicago Herpetol. Soc. 15, 98.

Mushinsky, H.R., Hebrard, J.J., 1977. Food partitioning by five species of water snakes in Louisiana. Herpetologica 33, 162–166.

Olive, P.J., Pinnegar, J.K., Polunin, N.V.C., Richards, G., Welch, R., 2003. Isotope trophic-step fractionation: a dynamic equilibrium model. J. Anim. Ecol. 72, 608–617.

Parnell, A., Jackson, A., 2011. SIAR: Stable Isotope Analysis in R. R package version 4.2. http://mathsci.ucd.ie/∼parnell_a/.

Peterson, B.J., Fry, B., 1987. Stable isotopes in ecosystem studies. Annu. Rev. Ecol. Syst. 18, 293–320.

Phillips, D.L., Newsome, S.D., Gregg, J.W., 2005. Combining sources in stable isotope mixing models: alternative methods. Oecologia 144, 520–527.

Pike, D.A., Clark, R.W., Manica, A., Tseng, H.-Y., Hsu, J.-Y., Huang, W.-S., 2016. Surf and turf: predation by egg-eating snakes has led to the evolution of parental care in a terrestrial lizard. Sci. Rep. 6, 22207.

Pilgrim, M., 2005. Linking Microgeographic Variation in Pigmy Rattlesnake (*Sistrurus miliarius*) Life History and Demography with Diet Composition: A Stable Isotope Approach. Ph.D. Thesis. University of Arkansas, Fayetteville.

Pilgrim, M., 2007. Expression of maternal isotopes in offspring: implications for interpreting ontogenetic shifts in isotopic composition of consumer tissues. Isotopes Environ. Health Stud. 43, 155–163.

Platt, D.R., 1969. Natural history of the hognose snakes *Heterodon platyrhinos* and *Heterodon nasicus*. Univ. Kansas Publ. Mus. Nat. Hist. 18, 253–420.

Post, D.M., 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 83, 703–718.

Resano-Mayor, J., Hernández-Matías, A., Real, J., Parés, F., Inger, R., Bearhop, S., 2014. Comparing pellet and stable isotope analyses of nestling Bonelli's eagle *Aquila fasciata* diet. Ibis 156, 176–188.

Rodríguez-Robles, J.A., 1998. Alternative perspectives on the diet of gopher snakes (*Pituophis catenifer*, Colubridae): literature records versus stomach contents of wild and museum specimens. Copeia 1998, 463–466.

Rosenblatt, A.E., Heithaus, M.R., 2013. Slow isotope turnover rates and low discrimination values in the American alligator: implications for interpretation of ectotherm stable isotope data. Physiol. Biochem. Zool. 86, 137–148.

Rush, S.A., Sash, K., Carroll, J., Palmer, B., Fisk, A.T., 2014. Feeding ecology of the snake community of the Red Hills region relative to management for Northern Bobwhite: assessing the diet of snakes using stable isotopes. Copeia 2014, 288–296.

Schmider, E., Ziegler, M., Danay, E., Beyer, L., Bühner, M., 2010. Is it really robust? Reinvestigating the robustness of ANOVA against violations of the normal distribution assumption. Methodology 6, 147–151.

Seib, R.L., 1985. Euryphagy in a tropical snake, *Coniophanes fissidens*. Biotropica 17, 57–64.

Shine, R., 1991. Intersexual dietary divergence and the evolution of sexual dimorphism in snakes. Am. Nat. 138, 103–122.

Shine, R., Wall, M., 2007. Why is intraspecific niche partitioning more common in snakes than in lizards? In: Reilly, S.M., McBrayer, L.D., Miles, D.B. (Eds.), Lizard Ecology: The Evolutionary Consequences of Foraging Mode. Cambridge University Press, Cambridge, pp. 173–208.

Smith, P.W., 1961. The Amphibians and Reptiles of Illinois. Illinois Natural History Survey, Urbana.

Spencer, R.J., Thompson, M.B., 2003. The significance of predation in nest site selection of turtles: an experimental consideration of macro-and microhabitat preferences. Oikos 102, 592–600.

Sterrett, S.C., Maerz, J.C., Katz, R.A., 2015. What can turtles teach us about the theory of ecological stoichiometry? Freshw. Biol. 60, 443–455.

Tieszen, L.L., Boutton, T.W., Tesdahl, K., Slade, N.A., 1983. Fractionation and turnover of stable carbon isotopes in animal tissues: implications for $\delta^{13}C$ analysis of diet. Oecologia 57, 32–37.
Tucker, C.R., Radzio, T.A., Strickland, J.T., Britton, E., Delaney, D.K., Ligon, D.B., 2014. Use of automated radio telemetry to detect nesting activity in ornate box turtles, *Terrapene ornata*. Am. Midl. Nat. 171, 78–89.
Walley, H., Eckerman, C., 1999. *Heterodon nasicus*. Cat. Am. Amphib. Reptil. 698, 1–10.
Wickham, H., 2009. ggplot2: Elegant Graphics for Data Analysis. Springer, New York.
Willson, J.D., Winne, C.T., Pilgrim, M.A., Romanek, C.S., Gibbons, J.W., 2010. Seasonal variation in terrestrial resource subsidies influences trophic niche width and overlap in two aquatic snake species: a stable isotope approach. Oikos 119, 1161–1171.
Winne, C.T., Willson, J.D., Andrews, K.M., Reed, R.N., 2006. Efficacy of marking snakes with disposable medical cautery units. Herpetol. Rev. 37, 52–54.