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ASSESSING THE USE OF NON-LETHAL TAIL CLIPS FOR MEASURING STABLE ISOTOPES OF PLETHODONTID SALAMANDERS

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Abstract.—Stable isotopes are increasingly used in ecology to study the diets, trophic position, and migratory patterns of wildlife including herpetofauna. When using stable isotopes, it is important to consider which tissues can or should be sampled, and how selecting tissues may affect the inferences drawn from stable isotope data. Amphibians offer fewer tissues than other larger organisms that can be harvested in sufficient quantity without killing the animal; however, many salamanders have tails that readily autotomize and regenerate. We used three species of plethodontid salamander (*Plethodon cinereus*, *P. metcalfi*, and *Desmognathus quadramaculatus*) to determine whether distal tail tissue had carbon and nitrogen stable isotope values comparable to commonly used tissues (liver and whole carcass [minus the liver and gonads]) that must be collected lethally. We found that variation in carbon values ($\delta^{13}\text{C}$) within and among tissues was negatively correlated with C:N (an indication of high lipid content). Nitrogen ($\delta^{15}\text{N}$) and Carbon ($\delta^{13}\text{C}$) values (once adjusted for C:N) of tail tissue were positively correlated with values for carcass tissue, and tail $\delta^{15}\text{N}$ values were positively correlated and biased with $\delta^{15}\text{N}$ values for liver tissue. Adjusted $\delta^{13}\text{C}$ values for tail tissue were unbiased and had weaker positive correlations with $\delta^{13}\text{C}$ values for liver tissue compared to whole carcass. The weak correlation between adjusted $\delta^{13}\text{C}$ values of tail and liver tissues may reflect differences in turnover rates between the two tissues. Our data show that, with calibration, non-lethal collection of tail clips is a suitable substitute to lethal tissue collection for measuring $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of plethodontid salamanders.

Key Words.—carbon; diet; nitrogen; Plethodontidae; salamanders; stable isotopes; trophic

INTRODUCTION

Stable isotopes are increasingly used in ecology to resolve patterns of energy flow and nutrient cycling, food chain lengths, food web organization, short- and long-term diet patterns, habitat use, and animal movements (e.g., Gannes et al. 1997, 1998; Post 2002). Stable isotopes are informative because they often exhibit predictable differences in the way they are routed through biochemical pathways and food chains. For example, isotope levels of ^{13}C shift little across trophic levels and can be used to estimate prey sources, while ^{15}N tends to enrich between consumers and prey, so this isotope can be used to estimate trophic position (Fry 2006). However, stable isotopes can behave differently among different tissues within a single organism for various reasons including isotopic routing, variation in tissue turnover rates, and variation in tissue composition (e.g., lipid content; Martinez del Rio and Wolf 2005; Post et al. 2007). Because the isotopic values of different tissues can vary within an organism, the selection of tissues sampled for stable isotope analysis can affect the inferences drawn from those analyses (Hobson et al. 1993; Doucett et al. 1999).

The choice to use a specific tissue versus whole organisms for measuring stable isotopes is made based on a number of factors specific to the question of interest. Tissues with high turnover rates, such as blood or the

liver, are expected to reflect a more recent diet history than tissues that are inert (e.g., hair or claws) or have slower turnover (e.g., bone; Tieszen et al. 1983; Hobson and Clark 1993; Post et al. 2007). An additional concern is whether an animal can or should be sacrificed. Liver and, in cases of small species, blood cannot be harvested non-lethally. This may preclude the use of isotopes in studies of rare or threatened species. Tissues such as claws, tail tips, limb lobes, and scales are potentially attractive for non-lethal tissue sampling; however, it is essential to know how these tissues compare to other commonly targeted tissues in order to guide inferences. Comparative reviews of tissues, such as claws or feathers, are available for some vertebrates (Dalerum and Angerbjorn 2005), including reptiles (McCue and Pollock 2008), and birds (Bearhop et al. 2003). Such comparative studies are absent for amphibians.

The objective of this paper was to determine whether tissue samples from the distal portion of salamander tails, which can be collected non-lethally and will regenerate, provide similar measures of carbon ($\delta^{13}\text{C}$, defined below) and nitrogen ($\delta^{15}\text{N}$, defined below) as carcass (body minus the distal tail, digestive tract, gonads, and liver) or liver samples. We hypothesized that $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of tail tissue would be positively correlated with isotope values for carcasses; however, because liver tissues have a higher lipid content and are generally assumed to turnover more rapidly than carcass

or tail tissues, we hypothesized that any correlation between tail and liver $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ would be weaker compared to correlations between tail and whole carcass tissues, and $\delta^{13}\text{C}$ levels in liver samples would be biased low (depleted) compared to tail tissue. We compared three species of plethodontid salamanders (*Plethodon cinereus*, *P. metcalfi*, and *Desmognathus quadramaculatus*) that represent numerically abundant species where they occur, are frequent focal species in ecological research, and are known to be influential in terrestrial and stream ecosystem processes (Wyman 1998; Watson et al. 2005; Walton et al. 2006; Peterman et al. 2008).

MATERIALS AND METHODS

All animals were collected by hand while searching ground cover, held live in containers containing moist toweling, and transported in coolers with ice until they were euthanized for tissue analysis (within five days). Between 26 October 2006 and 7 October 2007, we collected 61 *Plethodon cinereus* adults (mean snout-vent length [SVL] = 39 mm \pm 8.3, range = 20–51 mm) from Schuyler (51 animals collected; seven during October 2006, nine during May 2007, and 35 during October 2007) and Broome Counties (10 animals collected during October 2007), New York, U.S.A. Between 30 March and 14 May 2007, we collected 24 *Plethodon metcalfi* adults (mean SVL = 54.1 mm \pm 7.6, range = 40–70 mm) from Rabun Co., Georgia, U.S.A. (five animals collected during March and 19 animals collected during May 2007), and on 30 and 31 March 2007 we collected 23 *Desmognathus quadramaculatus* juveniles/adults (mean SVL = 55 mm \pm 15.3, range = 35–96 mm) from Stephens (11 animals collected) and Gilmer Counties (12 animals collected), Georgia, U.S.A.

Once in the laboratory, animals were euthanized by wrapping them in moist toweling saturated with a 1% solution of pH neutral-buffered MS-222 (ethyl *m*-amino-benzoate methanesulfonate). Immediately after the animal became non-responsive, we thoroughly rinsed the animal with distilled water to remove any residues of anesthetic from the carcass. Although we did not account for any effects of MS-222 on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, a number of studies have used MS-222 as an anesthetic and none reported any effects on stable isotope measurements (Herzka and Holt 2000; Harvey et al. 2002; Miller 2006) and we believe the rinsing of animals immediately after anesthesia reduces the potential for confounding effects. We used a clean scalpel to collect the distal 1-cm end of the tail, and then dissected the animal to remove the liver. These tissues were stored independently in clean microcentrifuge tubes. Finally, to prevent the presence of large, mature eggs from influencing our results, we removed the gonads from carcasses, and we removed the gut,

including the animal's stomach and intestines, to prevent fresh prey or feces from affecting isotopic composition.

We froze fresh tail, liver, and carcasses and then later oven dried them at 60° C to a constant mass. We milled tissues using a glass mortar and pestle. Generally, samples in studies such as these are ball-milled; however, we chose to hand mill samples because of the small amount of liver and tail tissue available. We determined relative abundance of stable isotopes of carbon (^{13}C) and nitrogen (^{15}N) in homogenized samples by continuous-flow isotope-ratio mass spectrometry at the University of Georgia Analytical Laboratory. Stable isotope results are presented as delta (δ). These values are not absolute isotope abundances, but the differences between the sample readings and a standard. In this study, we used standards of Pee Dee Belemnite for ^{13}C and atmospheric N_2 for ^{15}N .

We used general linear models (GLM; $\alpha = 0.05$) to examine whether species or $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of tail tissue was a significant predictor of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in either carcass or liver tissue. We included species as a categorical predictor and used $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ of tail tissue as the continuous predictor for the corresponding element in carcass and liver tissue. To correct for lipid content in $\delta^{13}\text{C}$ among tissue types, we plotted $\delta^{13}\text{C}$ against tissue C:N (a proxy for lipid content for each species [Post et al. 2007]) and then performed the $\delta^{13}\text{C}$ GLM with the residuals from the relationship between $\delta^{13}\text{C}$ of all tissues for each species and C:N value. We used linear regression ($\alpha = 0.05$) to examine relationships between all tissues and C:N ratios for each species. We used STATISTICA 6.0 (StatSoft, Inc., Tulsa, Oklahoma, USA) to conduct statistical analyses.

RESULTS

The model of species and tail stable isotope values as a function of liver or carcass stable isotope values showed significant correlations between $\delta^{13}\text{C}$ (‰) for carcass ($R^2 = 0.74$; $P \leq 0.001$) and liver ($R^2 = 0.42$; $P \leq 0.001$), respectively, and $\delta^{15}\text{N}$ (‰) for carcass ($R^2 = 0.83$; $P \leq 0.001$) and liver ($R^2 = 0.72$; $P \leq 0.001$). For all species, $\delta^{13}\text{C}$ varied consistently among the three tissues and was strongly negatively correlated with C:N (Fig. 1). Among all species, liver tissue (mean C:N \pm 1 SD = 5.85 \pm 1.68) had significantly greater mean C:N compared to tail tissue (3.77 \pm 0.55; $t = 12.234$, $P < 0.001$) or carcass tissue (3.81 \pm 0.39; $t = -12.31$, $P < 0.001$), which indicates the liver has higher lipid content. Liver $\delta^{13}\text{C}$ was generally lower than $\delta^{13}\text{C}$ of tail and carcass (i.e., depleted in liver relative to tail and carcass), which was explained largely by the higher C:N of liver tissue (Fig. 1). Mean C:N of carcass and tail tissue were not measurably different ($t = 0.584$, $P = 0.560$). Carbon ($\delta^{13}\text{C}$ ‰) was negatively related to C:N within and among tissue types (Fig. 1; *D. quadramaculatus*, $R^2 = 0.38$,

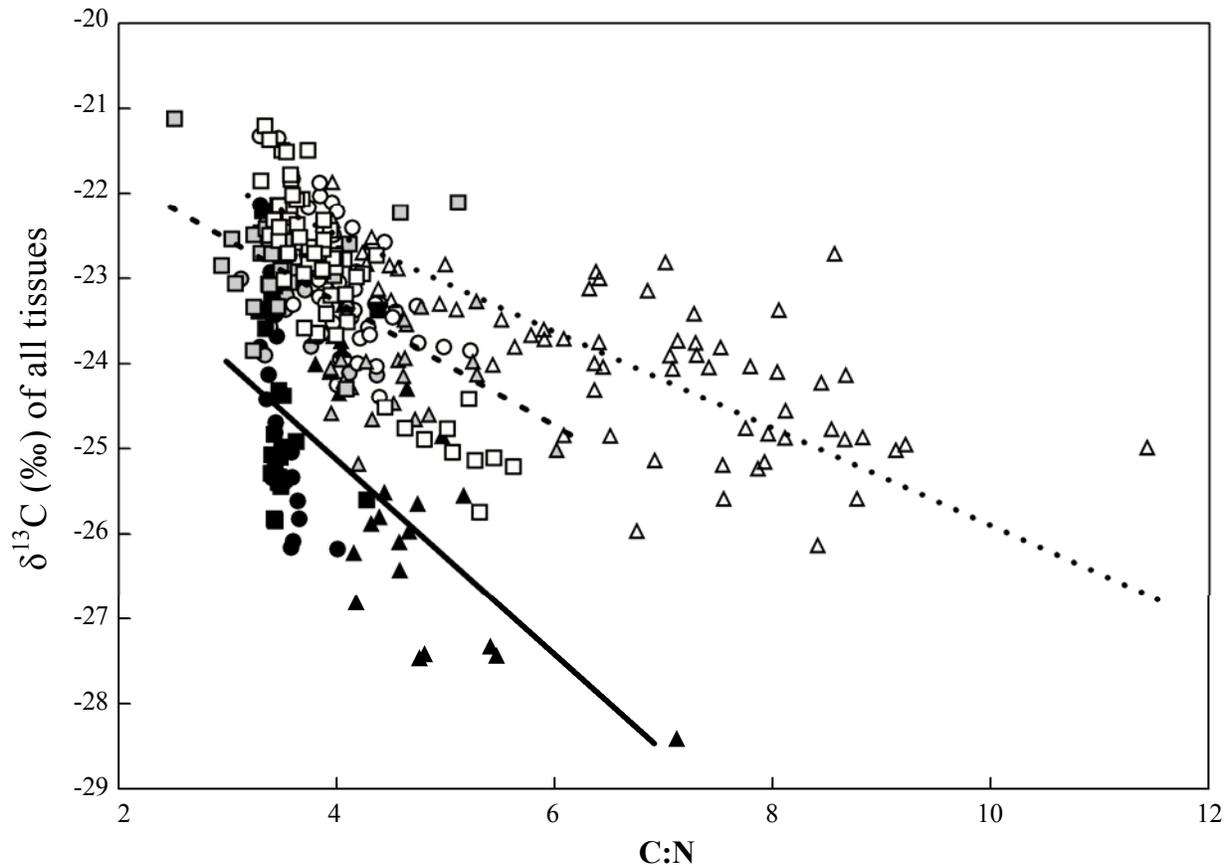


FIGURE 1. Linear regression of $\delta^{13}\text{C}$ and C:N for all tissues for each species. Shaded shapes with dashed line represent *Plethodon metcalfi*, open shapes with dotted line represent *P. cinereus*, and black shapes with solid line represent *Desmognathus quadramaculatus*. Triangles represent liver tissue, squares represent tail tissue, and circles represent carcass tissue. Regression equations are provided in text.

$P < 0.001$, $\delta^{13}\text{C} \text{‰} = -20.189 - 1.212 \text{ C:N}$; *P. metcalfi*, $R^2 = 0.30$, $P < 0.001$, $\delta^{13}\text{C} \text{‰} = -20.799 - 0.647 \text{ C:N}$; *P. cinereus*, $R^2 = 0.497$, $P < 0.001$, $\delta^{13}\text{C} \text{‰} = -21.176 - 0.432 \text{ C:N}$). For subsequent analyses, we used residual $\delta^{13}\text{C}$ values adjusted for C:N from these equations.

Tail tissue $\delta^{15}\text{N}$ was a significant predictor (positively correlated) of carcass and liver $\delta^{15}\text{N}$, and tail residual $\delta^{13}\text{C}$ value was a significant predictor (positively correlated) of carcass and liver residual $\delta^{13}\text{C}$ (Table 1). Consistent with our hypotheses, tail tissue $\delta^{15}\text{N}$ and residual $\delta^{13}\text{C}$ values were positively correlated with $\delta^{15}\text{N}$ and residual $\delta^{13}\text{C}$ carcass values, respectively. Values for $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were generally unbiased (i.e., generally distributed around the line of equality). Tail tissue $\delta^{15}\text{N}$ values were positively correlated with $\delta^{15}\text{N}$ values for liver tissue; however, these values were generally biased (i.e., generally distributed outside the line of equality). Residual $\delta^{13}\text{C}$ of tail tissue was positively correlated with residual $\delta^{13}\text{C}$ of liver tissue, but this correlation was weaker when compared to the correlation between $\delta^{13}\text{C}$ of tail and carcass tissue and was generally unbiased (Fig. 2).

DISCUSSION

Our study shows that tissue samples from plethodontid salamander tails, which can be collected non-lethally, provide highly comparable measures of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ to other commonly used tissues (liver and carcass) that require killing an animal to collect. The method of comparing non-destructive and destructive tissue in animals for stable isotope research has yielded successful results with fish (Shannon et al. 2001; Johnson et al. 2002; Jardine et al. 2005; Kelly et al. 2006; Sanderson et al. 2009), birds (Hobson and Clark 1993), and sea turtles (Seminoff et al. 2006), and this study shows this method can work well with other ectothermic vertebrates. Though our results suggest species-specific differences in stable isotope relationships among tissues, we caution that our study cannot resolve whether there were truly species-specific differences. Because we collected species from different locations and at different times, the relationships we found may be consequences of geographic or temporal differences in stable isotope levels. Thus, our results argue

TABLE 1. General linear model results for the effects of species and tail tissue values on predicting $\delta^{15}\text{N}$ and residual $\delta^{13}\text{C}$ ($\delta^{13}\text{C-r}$) in all species.

Dependent variable	Effect	df	MS	F	P
$\delta^{13}\text{C-r}$ carcass	Species	2	0.42	3.297	0.041
	$\delta^{13}\text{C}$ Tail	1	19.271	151.296	< 0.001
	Species \times $\delta^{13}\text{C-r}$ Tail	2	1.386	10.881	< 0.001
	Error	103	0.127	—	—
$\delta^{13}\text{C-r}$ liver	Species	2	2.389	6.322	0.003
	$\delta^{13}\text{C}$ Tail	1	14.147	37.441	< 0.001
	Species \times $\delta^{13}\text{C-r}$ Tail	2	0.414	1.096	0.338
	Error	103	0.378	—	—
$\delta^{15}\text{N}$ carcass	Species	2	0.651	7.713	0.001
	$\delta^{15}\text{N}$ Tail	1	22.349	264.638	< 0.001
	Species \times $\delta^{15}\text{N}$ Tail	2	0.649	7.687	0.001
	Error	103	0.084	—	—
$\delta^{15}\text{N}$ liver	Species	2	0.601	3.089	0.050
	$\delta^{15}\text{N}$ Tail	1	41.226	212.026	< 0.010
	Species \times $\delta^{15}\text{N}$ Tail	2	0.182	0.937	0.395
	Error	103	0.194	—	—

for caution when applying stable isotope relationships from one location to another or one species to another, even when those species are closely related and have similar physiologies.

Differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ among tissues are to be expected due to differences in isotopic routing and tissue turnover rates. In some cases, the differences among tissues may be informative (e.g., recent dietary shifts reflected in rapid turnover tissues that are not seen in slow turnover tissues). We did find that $\delta^{13}\text{C}$ was only weakly positively correlated between tail and liver tissues compared to the relatively strong correlation between tail and carcass. However, we note that the detection of any measurable positive correlation between tail and liver is interesting. One would expect that the liver, which is presumed to turnover at a faster rate, would be different from tissues that presumably turnover more slowly. The significant correlation of $\delta^{13}\text{C}$ values between tail and liver and between tail and carcass suggests that individual salamanders have relatively stable differences in $\delta^{13}\text{C}$ reflected in consistent values in faster and slower turnover tissues.

There are several potential interpretations for the apparent stability of $\delta^{13}\text{C}$ values across salamander species and the significant variation within species. First, the assumption there are large differences in liver

and tail (or carcass) tissue turnover rates may be flawed. Plethodontids are among the most metabolically efficient vertebrates known (Pough 1980; Pough 1983). While other vertebrate species show isotopic change as a result of short-term fasting or other physiological stressful events (Hobson et al. 1993; Kurle and Worthy 2001; Polischuk et al. 2001; Cherel et al. 2005; Lohuis et al. 2007), plethodontid salamanders do not show measurable stable isotope shifts in response to significant fasting up to 31 days (Milanovich and Maerz, unpubl. data). Second, individual variation in stable isotope levels may reflect localized differences in $\delta^{13}\text{C}$ signatures in basal resources. Salamanders, such as the species we studied, do have small, stable home ranges (e.g., Kleeberger and Werner 1982; Camp and Lee 1996; Lowe 2003; Peterman et al. 2008). However, since deciduous litter has a relatively consistent $\delta^{13}\text{C}$ ($\delta^{13}\text{C} \sim -27\text{‰}$) signature across much of the eastern deciduous forest range of North America (e.g., Walters et al. 2007; Taylor and Soucek 2010), we think that local spatial variation in the stable isotope levels of basal resources is unlikely to explain the individual variation we observed among salamanders. Third, individual salamanders may differ physiologically, affecting how stable isotopes fractionate within the individual or how isotopes are routed among tissues within the individual.

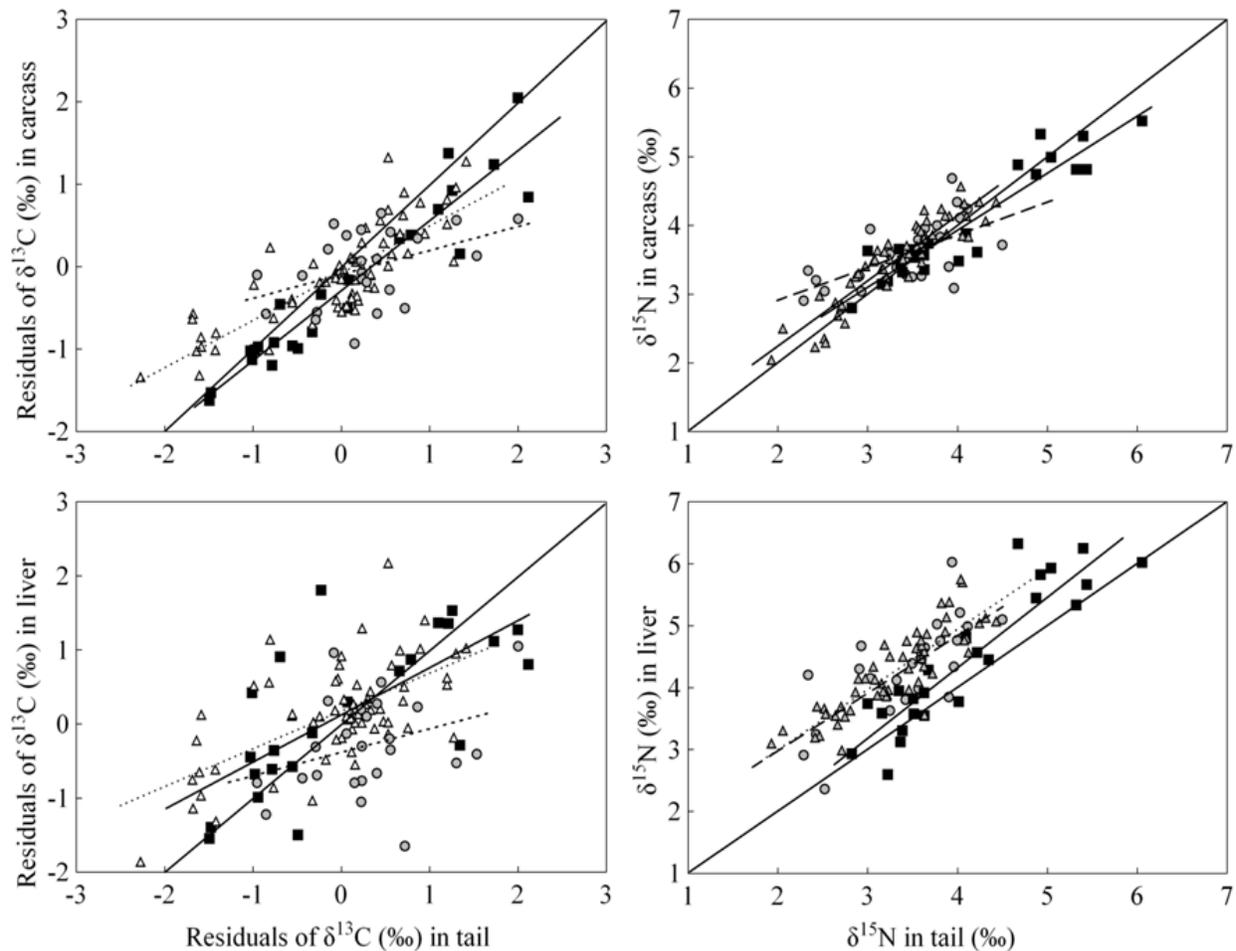


FIGURE 2. Linear relationships between stable isotope values for tail tissue and carcass or liver tissue for three species of plethodontid salamanders. Shaded circles with dashed line represent *Plethodon metcalfi*, open triangles with dotted line represent *P. cinereus*, and black squares with solid line represent *Desmognathus quadramaculatus*. Solid line connecting to margins of graph represents line of equality between variables. Linear equations for converting stable isotope $\delta^{15}\text{N}$ values from tail tissue samples (x) to carcass or liver tissue values are as follows: *P. cinereus* carcass, $R^2 = 0.84$, $P \leq 0.001$, $\delta^{15}\text{N}$ carcass tissue = $0.344 + 0.950 \times \delta^{15}\text{N}$ tail tissue; *P. metcalfi* carcass, $R^2 = 0.38$, $P = 0.001$, $\delta^{15}\text{N}$ carcass tissue = $1.96 + 0.476 \times \delta^{15}\text{N}$ tail tissue; *D. quadramaculatus* carcass, $R^2 = 0.88$, $P \leq 0.001$, $\delta^{15}\text{N}$ carcass tissue = $-0.607 + 0.831 \times \delta^{15}\text{N}$ tail tissue; *P. cinereus* liver, $R^2 = 0.73$, $P \leq 0.001$, $\delta^{15}\text{N}$ liver tissue = $1.032 + 0.979 \times \delta^{15}\text{N}$ tail tissue; *P. metcalfi* liver, $R^2 = 0.52$, $P \leq 0.001$, $\delta^{15}\text{N}$ liver tissue = $1.131 + 0.926 \times \delta^{15}\text{N}$ tail tissue; *D. quadramaculatus* liver, $R^2 = 0.83$, $P \leq 0.001$, $\delta^{15}\text{N}$ liver tissue = $-0.256 + 1.144 \times \delta^{15}\text{N}$ tail tissue. Linear equations for converting stable isotope $\delta^{13}\text{C}$ values from tail tissue samples (x) to carcass or liver tissue values are as follows: *P. cinereus* carcass, $R^2 = 0.62$, $P \leq 0.001$, $\delta^{13}\text{C}$ -r carcass tissue = $-0.073 + 0.571 \times \delta^{13}\text{C}$ -r tail tissue; *P. metcalfi* carcass, $R^2 = 0.19$, $P = 0.034$, $\delta^{13}\text{C}$ -r carcass tissue = $-0.099 + 0.289 \times \delta^{13}\text{C}$ -r tail tissue; *D. quadramaculatus* carcass, $R^2 = 0.90$, $P \leq 0.001$, $\delta^{13}\text{C}$ -r carcass tissue = $-0.291 + 0.847 \times \delta^{13}\text{C}$ -r tail tissue; *P. cinereus* liver, $R^2 = 0.38$, $P \leq 0.0001$, $\delta^{13}\text{C}$ -r liver tissue = $0.172 + 0.509 \times \delta^{13}\text{C}$ -r tail tissue; *P. metcalfi* liver, $R^2 = 0.11$, $P = 0.12$, $\delta^{13}\text{C}$ -r liver tissue = $-0.383 + 0.317 \times \delta^{13}\text{C}$ -r tail tissue; *D. quadramaculatus* liver, $R^2 = 0.50$, $P = 0.001$, $\delta^{13}\text{C}$ -r liver tissue = $0.123 + 0.636 \times \delta^{13}\text{C}$ -r tail tissue.

At this time, we have no data or information from the literature to address this possibility. Finally, our results may indicate stable differences in diet among individual salamanders within the same environment. Hatase et al. (2006) found that stable isotope differences among Green Sea Turtles (*Chelonia mydas*) were consistent with individual differences in feeding habits. At the population level, plethodontid salamanders consume a wide range of prey that fluctuate at fine spatial scales among habitats, seasons, and with climatic events such

as rain (Maerz et al. 2005). Maerz et al. (2006) showed that *P. cinereus* exhibit fine scale trophic polymorphisms related to differences in head morphology and diet within and among local habitats. Evidence of stable $\delta^{13}\text{C}$ differences among individuals collected in the same location is also consistent with diet differences among individuals. Combined, these results suggest that careful interpretation of stable isotope results could be a useful tool in studies of individual differences in diet and habitat use.

In summary, we show that non-lethal tail samples can provide a reliable and interpretable measure of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ levels in plethodontid salamanders. This should encourage the use of stable isotopes in studies of plethodontids including threatened or rare species where the non-lethal collection of tissue for stable isotope analysis might inform issues such as diet or habitat use. We demonstrate the importance for evaluating, when possible, potential biases among tissue types and for correcting for differences in lipid content among samples, even when lipid levels (or C:N) are low. Failure to address the effects of tissue selection may lead to erroneous or incorrect inferences drawn from stable isotope data.

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