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Realistic Fasting Does Not Affect Stable Isotope Levels of a Metabolically Efficient Salamander

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ABSTRACT.—**Stable isotopes are commonly used to examine various aspects of animal ecology. The use of stable isotopes generally proceeds under the implicit assumption that resource use is the only factor driving variation in stable isotope levels; however, a wealth of studies demonstrate that a range of common ecological factors can affect the behavior of stable isotopes in animal tissues and potentially confound inferences. For example, studies of some invertebrates and endothermic vertebrates show that animals fasted for ecologically realistic time periods have higher nitrogen ($\delta^{15}N$) or lower carbon ($\delta^{13}C$). We examined whether realistic fasting would influence the stable isotope composition of one of the most metabolically efficient ectothermic vertebrates, the Eastern Red-backed Salamander, *Plethodon cinereus*. We fasted salamanders for 7, 14, 21, 28, or 35-day intervals and examined whether $\delta^{15}N$ or $\delta^{13}C$ levels of tissues changed between fasted and fed animals. We investigated whether body condition (body mass to length and C:N [an index of lipid levels]) declined in fasted animals and whether there was a relationship between C:N and $\delta^{15}N$ or $\delta^{13}C$. Body mass to length index and C:N, $\delta^{13}C$, and $\delta^{15}N$ of tail and liver tissues did not differ between fasted and fed animals between 7 and 35 days. Because of their extreme metabolic efficiency, vertebrate ectotherms such as lungless salamanders (Plethodontidae) may not show the effects of fasting on stable isotopes observed in endothermic vertebrates and some invertebrates. This difference should lead to simpler interpretation of stable isotope results from field studies of these animals.**

The use of stable isotopes is an increasingly common tool in animal ecology to infer animal diets, trophic position, movements, habitat use, and food web structure (Gannes et al., 1998; Hobson, 1999; Post, 2002). A variety of factors besides diet differences, such as metabolism and isotopic routing, contribute to variation in stable isotope values. Failure to account for those factors can lead to the misinterpretation of stable isotope data (Gannes et al., 1997, 1998; Rubenstein and Hobson, 2004).

Fasting is common among wild animals, and fasting may routinely influence stable isotope composition (Mrosovsky and Sherry, 1980). Fasting occurs when conditions prohibit foraging as well as during periods of hibernation, egg incubation, and migration. When animals fast, they have a negative nitrogen balance which causes them to metabolize their own proteins. Through metabolism, the heavier nitrogen isotope (^{15}N) becomes retained while the lighter isotope (^{14}N) is eliminated (Minigwa and Wada, 1984; Macko et al., 1986; Cherel et al., 2005). As a result, $\delta^{15}N$ in fasting animals can become higher (Hobson et al., 1993; Gaye-Siessegger et al., 2007; Lohuis et al., 2007; Ciancio et al., 2008). Positive shifts in $\delta^{15}N$ would lead to the over-estimation of trophic position among animals where fasting is relatively common.

Shifting of $\delta^{15}N$ (and decrease of $\delta^{13}C$, or both) as a result of fasting has been reported for a variety of vertebrate and invertebrate taxa. Endothermic vertebrates (birds and mammals) respond to food deprivation using identical metabolic processes, and both have been shown to positively shift $\delta^{15}N$ during fasting (Hobson et al., 1993; Kurle and Worthy, 2001; Polischuk et al., 2001; Cherel et al., 2005; Lohuis et al., 2007). Despite more-variable responses to fasting, some ectothermic vertebrates (fishes; Doucett et al., 1999; Gaye-Siessegger et al., 2007; Ciancio et al., 2008) and invertebrates (Oelbermann and Scheu, 2002) show positive shifting of $\delta^{15}N$ (and depletion of $\delta^{13}C$) following fasting or food deprivation; however, recent work on reptiles suggests that $\delta^{15}N$ shifting may be different among more-metabolically efficient species adapted to frequent or prolonged periods of fasting or starvation (Castillo and Hatch, 2007; McCue, 2007; McCue and Pollock, 2008).

Most amphibians experience frequent periods of fasting or negative energy balance. Amphibian activity is often regulated by weather, resulting in seasonal and stochastic foraging bouts. For example, dehydration risk limits foraging by the Red-backed Salamander, *Plethodon cinereus* (Green, 1818) such that foraging is maximized during cooler, wetter months and episodically during rain events (Jaeger, 1980; Petranka, 1998; Casper, 2005; Maerz et al., 2005). Jaeger (1980) estimated that *P. cinereus* are routinely under negative energy balance throughout the warmer summer months. Female *P. cinereus* fast for up to 60 days during egg-brooding (Ng and Wilbur, 1995). Because plethodontid salamanders, including *P. cinereus*, are ectothermic and lungless (relying on passive cutaneous respiration), they are adapted to frequent and prolonged fasting. Furthermore, their resting metabolic rates are nearly an order of magnitude lower than those of birds or mammals (Feder, 1983; Pough, 1983). For example, *P. cinereus* converts up to 60% of new energy into new tissue (Burton and Likens, 1975) and have digestion efficiencies up to 90% (Crump, 1979).

Our objective was to quantify the effect of realistic fasting, or the fasting that individuals undergo under typical conditions, on body condition and $\delta^{13}C$ and $\delta^{15}N$ of tail and liver tissues of *P. cinereus*. We focused on tail tissue because it can be sampled nonsacrificially, and liver is a common target tissue for stable isotope studies (Milanovich and Maerz, 2012). Furthermore, liver tissue is known to have a more-rapid turnover than other tissue commonly used such as bone or muscle (Tieszen et al., 1983; Hobson and Clark, 1992; Perga and Gerdeaux, 2005) and is rich in lipid content.

MATERIALS AND METHODS

Experiment.—On 7 October 2006, 27 salamanders were collected from the Binghamton Nature Preserve (Broome Co., New York) and transported live to the University of Georgia in containers with moist paper towel and stored in a cooler with

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ice (for 3 days). Upon arrival, animals were held individually in 10-cm diameter Petri dishes with a moist paper towel at 19°C and an ambient photoperiod. All animals were fed daily on a diet of commercial crickets and wild-caught termites for 14 days. Termites were collected under oak (*Quercus* spp.) cover boards within the Whitehall Experimental Forest, Clarke County, Georgia. On 24 October 2006 we initiated fasting of 15 randomly selected salamanders while the remaining 12 "control" salamanders were fed 10 wild-caught termites once per week. Therefore, control salamanders experienced 6 days of fasting weekly followed by a large meal. We believe this episodic feeding emulated natural foraging patterns among *P. cinereus* and was sufficient to maintain a stable body mass among the control salamanders. Paper towels were rewetted every other day and changed once a week during the experiment. Three randomly selected "fasted" salamanders and three randomly selected control salamanders were sacrificed after 14, 21, and 28 days. Three randomly selected control salamanders were sampled on day 7 and six randomly selected fasted salamanders were sampled on day 35 (Table 1).

Animals were euthanized by wrapping them in a paper towel saturated with a 1.0% solution of pH neutral buffered MS-222 (ethyl *m*-amino-benzoate methanesulfonate) solution and then the animals were rinsed and frozen. Although we did not account for any potential effects of MS-222 on $\delta^{13}C$ and $\delta^{15}N$ values, a number of studies have used MS-222 as an anesthesia and none report any effects on stable isotope measurements (Herzka and Holt, 2000; Harvey et al., 2002; Miller, 2006; Milanovich and Maerz, 2012). The liver and distal portion of the tail (approximately 1 cm) were removed using a clean scalpel; these tissues were stored independently in clean microcentrifuge tubes. Tissues were dried at 60°C for 5 days, ground to a talcum powder consistency using a glass mortar and pestle, and prepared for stable isotope analysis. Relative abundance of stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in homogenized samples were determined by continuous-flow isotope-ratio mass spectrometry using a Thermo-Finnigan Delta V Isotope Ratio Mass Spectrometer (Bremen, Germany) coupled to a Carlo Erba CHN Combustion Analyzer via Thermo-Finnigan Conflo III Interface (Milan, Italy) at the University of Georgia Analytical Laboratory. Results are presented relative to Pee Dee belemnite ($\delta^{13}C$) and atmospheric N_2 ($\delta^{15}N$) in conventional delta notation (Ehleringer and Rundel, 1989).

Statistical Analysis.—To determine whether fasting had an influence on salamander mass, we utilized a general linear model with final dry mass of subsamples as dependent variables, fed (control)/fasted as categorical predictors, and snout–vent length (SVL, mm) as the continuous variable. We compared the C:N ratio of tail and liver tissue, respectively, between fasted and fed (control) animals using a *t*-test to determine if lipid levels decreased following fasting. Finally, to examine the influence of fasting and number of days fasted on the isotopic composition of liver and tail tissue, we utilized a general linear model with tail and liver $\delta^{13}C$ or $\delta^{15}N$ as dependent variables, fed (control)/fasted as categorical predictors, and days to harvest since start of experiment as the continuous variable. To correct for lipid content in $\delta^{13}C$ among tissue types, we plotted $\delta^{13}C$ against tissue C:N (a proxy for lipid content for each species; Post et al., 2007) and then performed subsequent analyses with the residuals from the relationship between $\delta^{13}C$ and C:N value. Data were analyzed using STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK).

TABLE 1. Summary data of *P. cinereus* fasted or fed (control) throughout the experiment. Mean snout–vent length (SVL) measured from the tip of the snout to the anterior portion of the tail (i.e., vent). Each row is the mean (±1 SD) of three individuals.

Treatment	No. days fasted	No. days since start of experiment	Mean SVL (mm)	Mean dry mass (mg)	Mean stable isotope				Mean C:N	
					$\delta^{13}C$ Tail	$\delta^{13}C$ Liver	$\delta^{15}N$ Tail	$\delta^{15}N$ Liver	Tail	Liver
Fed (control)	6	7	40.3 ± 3.8	143.8 ± 22.9	−22.2 ± 0.1	−25.4 ± 0.9	3.5 ± 0.2	3.9 ± 0.2	3.4 ± 0.03	5.1 ± 1.3
Fed (control)	6	14	38.3 ± 1.2	126.1 ± 14.8	−22.6 ± 0.3	−24.1 ± 0.2	3.8 ± 0.5	3.8 ± 0.1	3.4 ± 0.03	4.9 ± 0.6
Fed (control)	6	21	37.7 ± 3.5	125.4 ± 27.3	−22.5 ± 0.6	−22.6 ± 0.5	3.6 ± 0.4	3.8 ± 0.3	3.4 ± 0.04	4.9 ± 0.7
Fed (control)	6	28	39.0 ± 2.0	129.1 ± 15.7	−22.4 ± 0.3	−24.8 ± 1.0	3.5 ± 0.6	4.1 ± 0.7	3.4 ± 0.01	5.9 ± 1.3
Fasted	14	14	35.3 ± 7.5	159.3 ± 74.3	−22.2 ± 0.4	−24.1 ± 0.5	4.1 ± 0.8	4.3 ± 0.9	3.4 ± 0.01	5.2 ± 1.6
Fasted	21	21	39.7 ± 3.2	139.1 ± 17.5	−23.3 ± 0.6	−25.0 ± 0.5	3.9 ± 0.3	4.3 ± 0.8	3.6 ± 0.06	5.0 ± 0.4
Fasted	28	28	39.3 ± 5.0	107.9 ± 9.7	−22.5 ± 0.6	−24.9 ± 0.8	3.7 ± 0.5	4.3 ± 0.5	3.4 ± 0.05	4.9 ± 1.5
Fasted	35	35	36.2 ± 5.6	104.9 ± 47.2	−22.7 ± 0.4	−23.9 ± 0.8	3.5 ± 0.5	4.0 ± 0.5	3.4 ± 0.03	4.8 ± 1.0

TABLE 2. Results of general linear model examining the effects of fasting on the final dry mass of fasted and fed (control) animals for tail and liver tissue.

Source	df	*MS*	*F*	*P*
Fed (control)/fasted	1	457.638	2.976	0.098
SVL	1	9,153.906	0.716	0.406
Fed (control)/fasted * SVL	1	418.919	14.322	0.001
Error	23	639.160	0.655	0.426

RESULTS

Measures of salamander body condition and stable isotope levels remained stable and did not differ in response to fasting throughout the duration of the experiment. Final dry mass did not differ between fasted and fed (control) salamanders (Table 2; Fig. 1). There was no measurable difference between fasted and fed (control) salamanders in the C:N ratios of tail or liver tissue for tail ($t = 0.11$; $P = 0.91$) and liver tissue ($t = -0.66$; $P = 0.51$; Fig. 2). Overall, we found no significant difference in $\delta^{13}C$ or $\delta^{15}N$ of liver or tail tissues between fasted and fed (control) salamanders up to 35 days (Table 3; Fig. 3).

DISCUSSION

We found no shifting of $\delta^{15}N$ or $\delta^{13}C$ in either liver or tail tissues of salamanders fasted up to 35 days. This is inconsistent with the reaction of stable isotopes in endothermic vertebrates such as birds fasted for 18 (Hobson et al., 1993) and 25 (Cherel et al., 2005) days and in some ectothermic taxa such as fish fasted for 35 days (Gaye-Siessegger et al., 2007) and invertebrates fasted between 5–243 days (see review in Martinez del Rio et al., 2009). However, our results are consistent with other studies of metabolically efficient ectothermic vertebrates that are adapted to frequent or prolonged periods of starvation, such as lizards fasted for 14 days (Castillo and Hatch, 2007) and rattlesnakes fasted for 24 weeks (McCue, 2007).

One potential criticism of our study design was the decision to provide control salamanders with a single weekly feeding instead of an ad libitum food supply. We believe our control-feeding regime emulates the natural episodic feeding of salamanders, and the fact that body mass, C:N, and stable isotope values of control animals were stable throughout the study period suggests that our control-feeding regime was adequate. We suggest the failure of fasted salamanders to exhibit shifts in $\delta^{15}N$ or $\delta^{13}C$ after 35 days of fasting reflects their extreme metabolic efficiency and adaptation to frequent prolonged periods without foraging. In light of the recent work on reptiles (Castillo and Hatch, 2007; McCue, 2007), we suggest that stable isotope levels of reptiles and amphibians may be less sensitive to short and moderate periods of fasting than is expected of birds, mammals, fishes, and invertebrates. This lack of sensitivity is corroborated by our results indicating that fasting did not enrich liver tissue, which retains high rates of protein synthesis and which would be expected to become higher during a fast (Martinez del Rio et al., 2009). Castillo and Hatch (2007) and McCue (2007) did not analyze reptile tissues with high protein synthesis. However, those studies did report enrichment in excreta; this suggests some reptile organs may have higher $\delta^{15}N$ during a fast, which results in a loss of

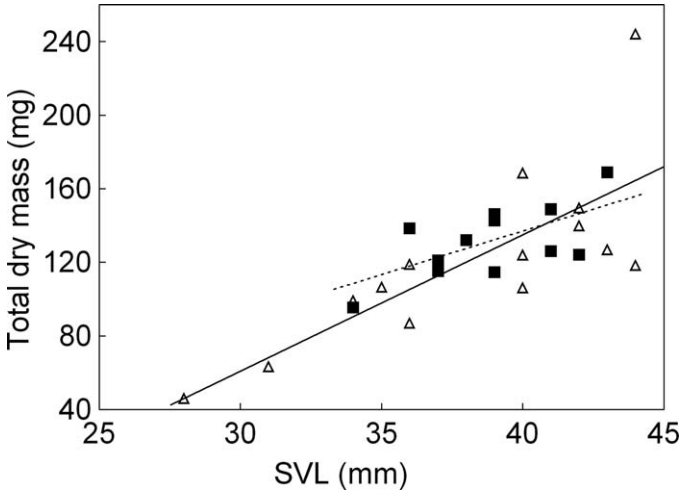

FIG. 1. Relationship between snout–vent length (mm) and final dry body mass (mg) for fasted ($r^2 = 0.59$; $P < 0.001$; dry mass = 161.1 + 7.4*SVL) and fed (control) animals ($r^2 = 0.42$; $P = 0.023$; dry mass = −55.05 + 4.79*SVL). Filled squares (dotted line) represent fed (control) animals while open triangles (solid line) represent fasted animals. Mean final dry mass of fasted (123.2 ± 46.6 mg) and fed (control; 131.1 ± 19.4 mg) animals was not significantly different.

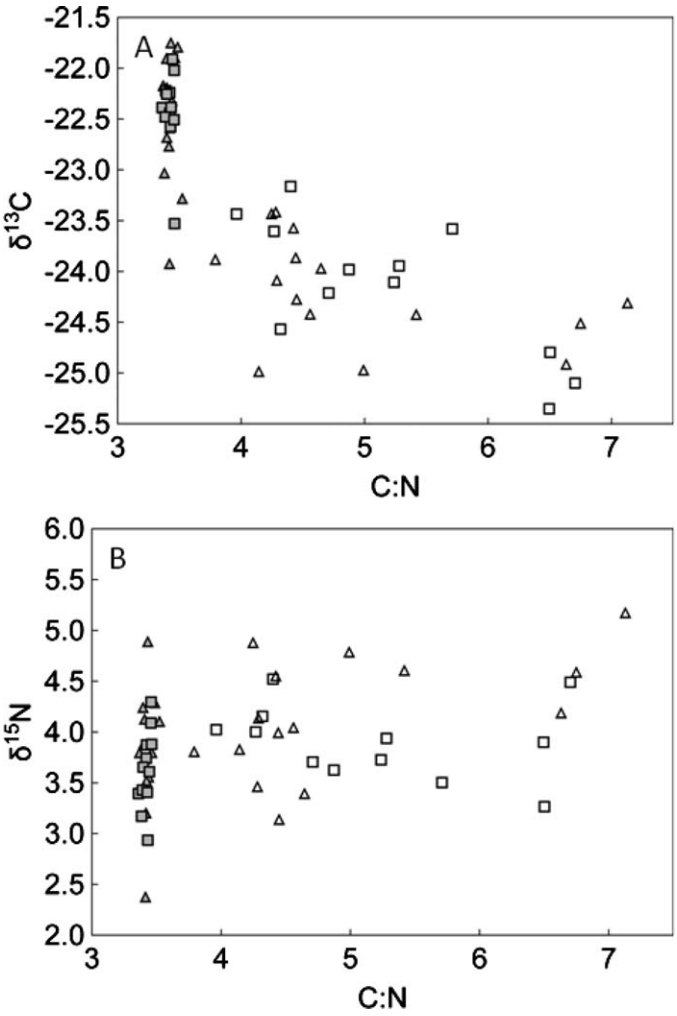

FIG. 2. The relationship between C:N ratio and (A) $\delta^{13}C$ and (B) $\delta^{15}N$ for tail and liver tissue in fasted and fed (control) animals used to show differences in lipid content between the two tissues. Filled squares represent tail tissue from fed (control) animals, filled triangles represent tail tissue from fasted animals, open squares represent liver tissue in fed (control) animals, and open triangles represent liver tissue in fasted animals.

TABLE 3. Results of general linear model examining the effects of fasting on the $\delta^{15}N$ and $\delta^{13}C$ of fasted and fed (control) animals for tail and liver tissue.

Response						
Isotope	Tissue	Source	df	*MS*	*F*	*P*
$\delta^{15}N$	Tail	Fed (control)/fasted	1	0.576	2.432	0.221
		Days to harvest	1	0.411	1.739	0.200
		Fed (control)/fasted * days to harvest	1	0.282	1.193	0.286
		Error	23	0.237	–	–
$\delta^{15}N$	Liver	Fed (control)/fasted	1	0.426	1.582	0.221
		Days to harvest	1	0.003	0.012	0.914
		Fed (control)/fasted * days to harvest	1	0.186	0.669	0.422
		Error	23	0.269	–	–
$\delta^{13}C$	Tail	Fed (control)/fasted	1	0.049	0.170	0.684
		Days to harvest	1	0.170	0.592	0.449
		Fed (control)/fasted * days to harvest	1	0.039	0.135	0.717
		Error	23	0.287	–	–
$\delta^{13}C$	Liver	Fed (control)/fasted	1	0.373	0.908	0.351
		Days to harvest	1	0.020	0.049	0.827
		Fed (control)/fasted * days to harvest	1	0.705	1.719	0.203
		Error	23	0.410	–	–

protein-nitrogen, but are able to maintain protein synthesis (Martinez del Rio et al., 2009).

Due to this lack of significant difference, our results suggest short-term nutritional status is not a significant concern when utilizing isotopic composition for ecological studies using *P. cinereus*. This conclusion may extend beyond *P. cinereus*, as the physiology and life history of most terrestrial plethodontids are similar. Given that our results contradict other studies showing effects from short-term fasting, there may be an absence of a general rule regarding interactions between physiological and

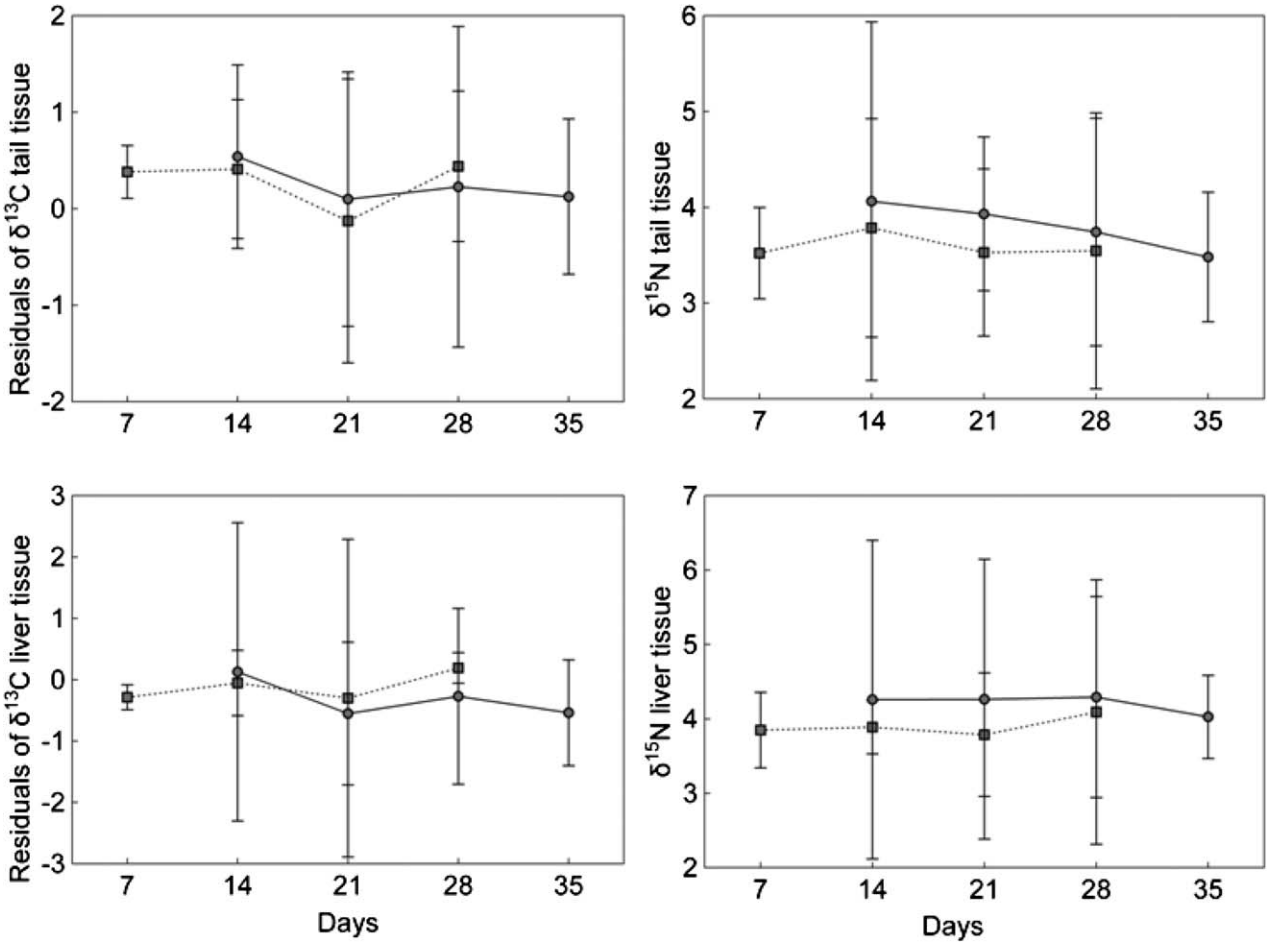

FIG. 3. Results of general linear model examining the effects of the number of days fasted on the $\delta^{15}N$ and $\delta^{13}C$ of fasted and fed (control) animals for tail and liver tissue. Filled squares (dashed line) represent mean residual $\delta^{13}C$ or raw $\delta^{15}N$ values for animals fed every 7 days (fed [control]) while open circles (solid line) represent mean residual $\delta^{13}C$ or raw $\delta^{15}N$ values for animals fasted since day zero. Confidence bands represent SE bars (± 1).

metabolic processes and stable isotope composition—especially in metabolically efficient organisms. The dichotomy between fasting effects on stable isotope compositions between ecto- and endothermic vertebrates needs further exploration.

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