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ARTICLE

Methods, Tools, and Technologies

Greater than the sum of your parts: Nonlethal stable isotope sampling methods in spiders

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Abstract

As top consumers and generalist predators, spiders are ideal organisms to study food webs and complex ecological functions using stable isotopes. Most researchers use whole-body samples to analyze stable isotope ratios in spiders. Spiders can regrow lost legs and produce multiple molts during a life cycle, and nonlethal sampling utilizing legs and molts may provide a useful alternative to whole-body sampling especially in larger bodied or threatened species. Furthermore, removing spider abdomens and thus leftover prey in the gut contents may provide a more accurate isotopic value. We tested the hypothesis that the δ^{15}N, δ^{13}C, or δ^{2}H isotopic values in spider legs are reliable proxies for spider prosomas, abdomens, or whole bodies. We used laboratory-reared large-bodied spiders (*Pterinochilus murinus*) and field-collected Lycosidae to compare lethal and nonlethal tissue isotopic values. We found that nonlethal samples of spider legs and molts are acceptable alternatives to lethal whole-body samples to determine δ^{13}C and δ^{15}N stable isotope signatures. Nonlethal samples are not suitable proxies for whole-body samples to determine δ^{2}H isotopic values. Using nonlethal leg or molts samples in stable isotope investigations of spiders will allow researchers to promote conservation efforts and study threatened species while ensuring accurate and repeatable results.

KEYWORDS
discrimination values, hydrogen, Lycosidae, nonlethal sampling, Theraphosidae, trophic shift

INTRODUCTION

Understanding food web structure within an ecological community is essential to track the effects of natural or anthropogenic disturbances on an ecosystem. Ecologists have readily accepted the use of stable isotopes to investigate the food webs (i.e., trophic position and diet composition) of different organisms in their natural environments and in the laboratory (Boecklen et al., 2011; Wolf et al., 2009). In many cases, sampling for stable isotope analysis (SIA) in smaller animals such as juvenile fishes, spiders, or other arthropods requires sacrificing the organism to obtain sufficient sample tissue. This sampling technique limits the inclusion of threatened or endangered species in isotopic studies, thus hindering critical research. Furthermore, whole-body sampling in arthropods can be problematic as prey remains in guts and tissue turnover rates between different body parts may skew or

misrepresent the true isotopic values of the target organism (Belivanov & Hambäck, 2015; Gratton & Forbes, 2005; Hill & McQuaid, 2011). The problems associated with lethal sampling have led many researchers to pursue nonlethal alternatives. Nonlethal sampling in SIA is very common in mammals (Crawford et al., 2008) and fish (Cano-Rocabayera et al., 2014; Hayden et al., 2017; Valladares & Planas, 2012; Willis et al., 2013) but has also been applied in salamanders (Gillespie, 2013) and amphipods (Wilhelm & Nelson, 2012), and such a method would be especially valuable to study organisms where, in current research, the whole animal is typically sacrificed.

The use of stable isotopes is now more commonplace, but the complexity and variables that affect discrimination values (differences in δ values between an animal and its prey) through a food web may create flawed SIA models and lead to misleading ecological interpretations (Phillips et al., 2014; Vander Zanden et al., 2016). For example, a spider's isotopic value varies depending on the base diet of their prey (i.e., aquatic algae vs. terrestrial leaf litter) and, typically, the isotopic values in spiders will reflect a mixture of prey basal food sources (Collier et al., 2002; Herwig et al., 2007; Kato et al., 2004). Additionally, discrimination values will vary depending on diet quality, water source, and type of organism (Caut et al., 2009; Vanderklift & Ponsard, 2003; Webb et al., 1998). Furthermore, many of the discrimination values used in SIA models are derived from meta-analyses combining isotopic values from different animal groups often tested under significantly different conditions (Caut et al., 2009; Oelbermann & Scheu, 2002; Post, 2002) and thus may not be suitable to accurately model a specific animal's diet. SIA is a powerful tool, but accurate calculation of trophic positons, food source, and the analysis of isotopic values may be reduced or misinterpreted in investigations where the discrimination values of the research organism are understudied.

In most environments, arthropods are abundant, widespread, easy to sample, and represent an extensive range of resource consumption and predation strategies. For these reasons, insects and spiders are ideal organisms to study diet assimilation, dispersal methods, population, and feeding behavior using SIA (Hood-Nowotny & Knols, 2007). As ubiquitous and generalist predators, spiders are used in SIA investigations to track energy flow and subsidy sources in a range of environments such as riparian zones, agroecosystems, and urban areas (Akamatsu et al., 2004; Krell et al., 2015; Sullivan et al., 2019). Spiders are easy to sample and certain families can be targeted depending on the environment and collection method. Like most arthropods, the small size of spiders usually leads a researcher to utilize whole bodies or pool several individuals in one sample for SIA. Fortunately, spiders have two biological strategies making them ideal for nonlethal sampling. First, spiders can lose multiple legs (autotomy) and regrow them between molts, while still experiencing full lives with little loss in overall fitness (Brueseke et al., 2001). Second, spiders go through five or more molts as they mature from juveniles to adulthood (Foelix, 2010) and the molted remains may be especially suitable for SIA (Belivanov & Hambäck, 2015). Using spider molts in a laboratory or field setting may provide researchers isotopic values over a longer period, assist in calculating the timing of a diet shift, or indicate an ecosystem disturbance.

Although arthropods are used in SIA, when compared to their enormous group size and range of environments they inhabit, they remain an underutilized resource for isotopic investigations (Quinby et al., 2020). Spiders can signal trophic shifts from ecosystem disturbances (Krell et al., 2015; Stenroth et al., 2015; Sullivan et al., 2019) and indicate the effects of ecosystem restoration (Kupilas et al., 2020). Conversely, ecosystem disturbances and changes in ecosystem structure have a direct effect on spider diversity, community composition, and dispersal ability (Lafage et al., 2019; Öberg et al., 2007; Prieto-Benítez & Méndez, 2011). As top terrestrial predators and critical links in complex ecosystems, the more frequent use of spiders in SIA and food web studies is necessary to understand the effects of natural and anthropogenic disturbances on both ecosystems and spiders themselves. As such, researchers should make an effort toward ecological stewardship and conservation, the first steps of which can be accomplished with increased nonlethal sampling in SIA studies.

Our study experimentally tested whether nonlethal spider tissues accurately reflect the isotopic values of other spider body tissues. We assumed spider legs and molts represented nonlethal samples compared to the lethal samples of other tissues. We performed a controlled laboratory study by feeding spiders with prey representing two distinct basal food sources. Using these spiders, we compared the isotopic relationship between spider legs or molts and spider prosomas, abdomens, and whole bodies as well as the basal food source. In addition, we examined the isotopic relationship between legs and abdomens of field-collected spiders with an unknown prey source. We hypothesized that the isotopic values of laboratory-reared spider legs were comparable to isotopic values in the same spider abdomens, prosomas, and whole spiders. We hypothesized that the isotopic values of the field-collected spider legs were comparable to the isotopic values in their respective abdomens. Finally, we investigated the discrimination values of spider body tissues to examine how they may effect stable isotope investigations.

METHODS

Study species and rearing conditions

To trace food source stable isotope signatures, we chose the spider *Pterinochilus murinus* (Araneae: Theraphosidae) as it is easily kept in captivity, rarely rejects prey, and is fast growing providing substantial material for analysis (Charpentier, 1993). All spiders came from the same egg sac ensuring uniform initial stable isotope signatures and developmental stage. Spiders were individually kept in separate 12 cm high, 520-ml plastic containers containing a 3:1 mixture of coconut fiber and vermiculite substrate filled to 6 cm and sprayed with water once a week. Spiders were acclimated to containers and environment without disturbance for 1 week. Growth data and spider size were not recorded to minimize disturbance and as a safety precaution for laboratory technicians.

For prey, we used a single colony of Turkestan cockroaches (*Shelfordella lateralis*) as they are easily kept in captivity and will accept a wide range of food. The roach colony was separated into two 40-L plastic containers (40 × 60 × 25 cm) with polyethylene tubing substrate. We chose the roach prey's basal food sources to represent two extremes (terrestrial vs. aquatic) of potential carbon resources that spiders may feed on in a field setting. One group was fed organic kale (*Brassica oleracea*) ad libitum (hereafter, terrestrial prey) and the other group was fed 100% organic flaked *Chlorella* spp. (Superfruit Scandinavia AB) ad libitum (hereafter, aquatic prey). Dried *Chlorella* powder was formed into flakes by adding equal parts by volume water and *Chlorella*, spreading the mixture out on a flat surface, and allowing it to dry out completely. Both cockroach groups were provided water using superabsorbent water gel to prevent drowning. Roaches were acclimated to the environment and were allowed to feed on each food source for 2 weeks prior to the start of the feeding trials. Spiders and roaches were kept in a controlled environment at a constant 26°C with a light regime of 16:8 light : dark. Stable isotope values differed among feeding groups in the cockroach prey. The cockroaches showed a large difference for both $\delta^{13}C$ (aquatic vs. terrestrial group: $\bar{x} = -19.7 \pm 0.31$ vs. -27.3 ± 0.37; $t(8) = -35.5$, $p < 0.0001$) and $\delta^{15}N$ (aquatic vs. terrestrial group: $\bar{x} = 1.5 \pm 0.61$ vs. 4.8 ± 0.64; $t(8) = 8.3$, $p < 0.0001$). There was no difference in $\delta^{2}H$ for cockroaches (aquatic vs. terrestrial group: -94.0 ± 10.3 vs. -106.4 ± 27.1; $t(8) = -1.0$, $p = 0.37$).

Feeding trials

Spiders ($n = 28$) were randomly separated into two equal groups: (1) terrestrial and (2) aquatic. Each group of spiders was fed from their respective prey group as often as they would accept prey. We watched each spider to ensure that it captured the offered prey. If a spider captured and pulled a roach into a burrow, we assumed it was eaten. We removed all accessible prey remains after feeding and saved all accessible spider molts. We recorded the assumed molt sequence number and estimated date of molt.

The experiment was ended after maintaining and feeding spiders for 220 days. All spiders were sacrificed 7 days after the final offer of prey to minimize the effects of any prey remains in the gut. Three spiders died during the feeding trial leaving a total of 25 for SIA. In addition, we sampled terrestrial and aquatic prey for SIA. Substrate in each spider container was searched for any overlooked molts, and if molts were found, we estimated the molting sequence based on size comparison with those previously removed. Each spider in the aquatic feeding group completed a maximum of two molting events resulting in 18 total molts and the terrestrial feeding group completed a maximum of four molting events resulting in 27 total molts.

Field-collected spiders

Lycosidae spiders were sampled using a suction sampler and identified to species at three riparian sites along the Klarälven River (Sweden) during spring 2018 (Lafage et al., 2020). We collected 378 spiders representing 16 species. Species with less than 10 individuals were removed leaving five species and 339 individuals for analysis. Prior to SIA, field-collected spiders were dissected into abdomens and legs. For comparisons in this study, abdomens and legs were assumed to represent lethal and nonlethal tissue, respectively.

Stable isotope analysis

All spiders and prey were placed in 70% ethanol in individual vials until SIA preparation. Laboratory spiders were randomly selected for whole-body preparation or dissection into parts. Dissected laboratory spiders were separated into legs, prosoma, and abdomen. Whole spiders, dissected spiders parts, and prey were individually placed in sterile glass vials and dried at 60°C for 48 h. Each dried sample was completely pulverized to a homogeneous powder, sealed in clean glass vials, and held in a desiccator until sent for analysis. SIA was performed by Iso-Analytical (Crewe, UK) using elemental analysis isotope ration mass spectrometry. Analysis for each element (carbon, nitrogen, and deuterium) included the δ isotope ratios expressed per mil (‰).

Statistical analysis

To determine whether the $\delta^{13}C$, $\delta^{15}N$, or $\delta^{2}H$ isotopic values of whole bodies, prosomas, or abdomens of laboratory-fed spiders could be predicted as a function of the $\delta^{13}C$, $\delta^{15}N$, or $\delta^{2}H$ values of spider legs, we used a linear mixed model. For this analysis, we created a pseudo-whole-body spider for each isotope by averaging and combining the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ isotopic values of each tissue (legs, abdomen, and prosoma). We determined that the pseudo-whole body ($n = 12$, for each isotope) was a valid representation for modeling by comparing it against our whole-body samples ($n = 13$, for each isotope) ($\delta^{13}C$: $t(23) = 0.15$, $p = 0.88$; $\delta^{15}N$: $t(23) = -0.27$, $p = 0.79$; $\delta^{2}H$: $t(23) = 2.02$, $p = 0.06$). Pseudo-whole body, abdomen, and prosoma isotope values were the response variables; leg tissue isotope values were the predictor variables, and feeding group (aquatic or terrestrial) was a fixed factor. Abdomens and prosomas were included in the analysis to examine the isotopic relationship between lethal and nonlethal tissues. This relationship is especially important when a whole body of a single specimen is divided up for isotopic or genetic analysis. We combined isotopic values from the aquatic and terrestrial feeding groups to represent an average range of isotopic values for tissue comparison analysis. To further explore the laboratory spider data, we tested each tissue–leg combination against a hypothetical 1:1 fit line ($x = y$) using one-way ANOVA.

To compare $\delta^{13}C$ or $\delta^{15}N$ isotopic values of molts with prey and spider tissue, we used a linear mixed model where either $\delta^{13}C$ or $\delta^{15}N$ isotopic value was the response variable, tissue type was the predictor variable, and individual spiders was a random factor. We separated molts, prey, and spider tissues into feeding groups for the analysis. To compare molt, prey, and spider tissue values, we performed post hoc analysis using pairwise comparisons of estimated marginal means for each molt sequence with prey and other tissues adjusting for multiple comparisons.

To determine whether $\delta^{13}C$ or $\delta^{15}N$ leg isotopic values are suitable predictors of $\delta^{13}C$ or $\delta^{15}N$ abdomen isotopic values in field-collected spiders, we used a least squares linear model with abdomen isotope values as the response variable and leg isotope values as the predictor variable. To further explore the field-collected spider data, we tested each regression model against a hypothetical 1:1 fit line ($x = y$) using one-way ANOVA.

Finally, to calculate discrimination factors, we separated laboratory spider tissues and prey into feeding groups for isotopic discrimination analysis. Discrimination factors were calculated for each tissue–prey combination by averaging the isotopic values of the prey and subtracting that value from all isotopic values of each tissue and averaging the result. We then compared $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ isotope values for prey and all spider tissues using separate one-way ANOVAs for each isotope. To confirm significant discrimination values, we performed post hoc analysis using pairwise comparisons of estimated marginal means between each tissue and the prey in the respective feeding groups adjusting for multiple comparisons.

All analyses were performed in R Studio v.1.2.5033 (RStudio Team, 2021) with R v.3.6.3 (R Core Team, 2020) using the libraries "nlme" (Pinheiro et al., 2020) and "emmeans" (Lenth, 2020) and assuming the alternative hypothesis at $p < 0.05$.

RESULTS

Laboratory experiment

Our results (Tables 1 and 2) showed a significant relationship for the $\delta^{13}C$ and $\delta^{15}N$ isotopic values of prosomas, abdomens, and pseudo-whole bodies as a function of the leg tissue (Figure 1 and Table 3). There were no significant relationships for $\delta^{2}H$ between all tissues and leg tissue (Figure 1 and Table 3). $\delta^{13}C$ and $\delta^{15}N$ regression analysis showed very strong relationships (all models: conditional $R^2 > 0.79$). In all tissues, $\delta^{13}C$ and $\delta^{15}N$ model regression slopes were not significantly different from the slope of the hypothetical 1:1 fit line (Table 3).

$\delta^{15}N$ values of molts were significantly different compared to prey and other tissues in the terrestrial group, while no $\delta^{13}C$ values were different from any other tissues tested in the same group (model root mean squared error [RMSE]; N molts = 0.59; C molts = 0.92). Both $\delta^{15}N$ and $\delta^{13}C$ values of molts were significantly different within the aquatic group (model RMSE; N molts = 0.23; C molts = 1.85). Post hoc analysis of $\delta^{15}N$ in both feeding groups showed a distinct trend in increasing isotopic value from prey through each subsequent molt to final spider tissues (Figures 2 and 3). One molt with a $\delta^{15}N$ outlier value, likely because of analysis error, was removed from the analysis.

Discrimination values of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ showed significant differences between tissues and prey within both feeding groups (terrestrial, C: $F_{5,29} = 19.9$, $p < 0.001$; N: $F_{5,29} = 34.6$, $p < 0.001$; H: $F_{5,29} = 26.94$, $p < 0.001$; aquatic, C: $F_{5,30} = 8.04$, $p < 0.001$; N: $F_{5,30} = 114.2$, $p < 0.001$; H: $F_{5,30} = 73.9$, $p < 0.001$). Post hoc testing showed a significant increase in $\delta^{15}N$ values in all tissues in both feeding groups (Table 4). $\delta^{13}C$ values

increased in prosomas, legs, and pseudo-whole tissues, and prosomas and legs in the aquatic and terrestrial groups, respectively (Table 4). δ^2H values showed a significant decrease in abdomens in both feeding groups, an increase in leg tissue values in both feeding groups, and an increase in prosoma values in the aquatic feeding group (Table 5).

TABLE 1 Summary statistics for ^{13}C, ^{15}N, and ^{2}H for whole spider, pseudo-whole spiders, each tissue analyzed, and prey

Isotope	Tissue	*n*	Mean ± SD	Min	Max
^{13}C	Whole	13	−22.90 ± 4.32	−27.76	−18.42
	Pseudo-whole	12	−22.64 ± 4.10	−27.19	−18.28
	Leg	12	−21.96 ± 3.92	−26.28	−17.87
	Prosoma	12	−22.28 ± 3.86	−26.83	−18.21
	Opisthosoma	12	−23.70 ± 4.53	−28.62	−18.73
	Prey	10	−23.48 ± 4.05	−27.80	−19.31
^{15}N	Whole	13	8.94 ± 1.11	6.63	10.25
	Pseudo-whole	12	8.84 ± 0.78	7.75	10.10
	Leg	12	9.05 ± 0.75	8.11	10.26
	Prosoma	12	9.15 ± 0.65	8.24	10.33
	Opisthosoma	12	8.32 ± 1.09	6.33	9.98
	Prey	10	3.13 ± 1.84	0.77	5.60
^{2}H	Whole	13	−99.91 ± 11.15	−123.68	−84.86
	Pseudo-whole	12	−91.40 ± 9.93	−111.31	−77.34
	Leg	12	−55.32 ± 7.38	−66.70	−42.84
	Prosoma	12	−75.31 ± 10.19	−99.57	−63.32
	Opisthosoma	12	−143.58 ± 20.78	−190.52	−122.99
	Prey	10	−100.2 ± 20.41	−140.76	−83.51

Note: Spiders from both feeding groups (aquatic and terrestrial) were combined to calculate summary statistics. Min and Max for pseudo-whole spiders represent an average of the combined minimum and maximum values from individual tissues.

Field spiders

Field-collected spiders (Table 6) regression analysis showed significant relationships between the δ^{13}C and δ^{15}N isotope signatures of abdomens as a function of the leg tissue for all field-collected spiders combined and each species except δ^{13}C in *Piratula hygrophila* ($p = 0.29$) (Table 7 and Figure 4). Regression models reflected weak-to-moderate relationships between legs and abdomens in all significant models (all models: R^2 0.06–0.59, RMSE 0.62–0.96). δ^{13}C model regression slopes were not significantly different from the slope of the hypothetical 1:1 fit line for all tested species except *Pardosa* spp. and *P. hygrophila* (Table 5). δ^{15}N model regression slopes were not significantly different from the slope of the hypothetical 1:1 fit line for all species except *Pardosa lugubris* and *Pardosa* spp. (Table 7).

DISCUSSION

Nonlethal sampling as a viable method

Our study highlights the use of nonlethal sampling methods with large-bodied spiders for future stable isotope studies. In general, our results indicate that nonlethal samples of spider legs or molts are acceptable alternatives to lethal whole-body sampling to determine

TABLE 2 Summary statistics of C, N, and H% content and C:N ratio for food source, prey, whole spiders, and each tissue analyzed separated by feeding group

Group	Food source/tissue	*n*	%C ± SD	%N ± SD	C:N	%H ± SD
Terrestrial	Kale	4	42.15 ± 0.64	3.39 ± 0.71	12.43	-
	Whole	6	51.32 ± 1.68	11.55 ± 0.51	4.44	7.70 ± 0.28
	Prosoma	6	46.98 ± 1.26	12.22 ± 0.83	3.84	7.18 ± 0.36
	Abdomen	6	56.08 ± 3.75	10.55 ± 10.59	5.32	9.04 ± 0.55
	Leg	6	47.17 ± 1.12	13.05 ± 8.61	3.61	6.74 ± 0.23
	Prey	4	48.02 ± 5.29	15.13 ± 1.24	3.17	6.29 ± 1.41
Aquatic	Algae	5	50.10 ± 1.26	10.28 ± 1.28	4.87	-
	Whole	7	51.41 ± 2.42	13.31 ± 3.18	3.86	7.50 ± 0.54
	Prosoma	6	49.26 ± 1.88	12.64 ± 3.56	3.90	6.89 ± 0.30
	Abdomen	6	57.67 ± 2.05	9.26 ± 5.07	6.23	9.18 ± 0.40
	Leg	6	47.43 ± 2.43	12.92 ± 4.48	3.67	6.80 ± 0.24
	Prey	5	40.96 ± 1.31	22.65 ± 3.73	1.81	3.91 ± 0.33

Note: H% content was not analyzed for food sources.

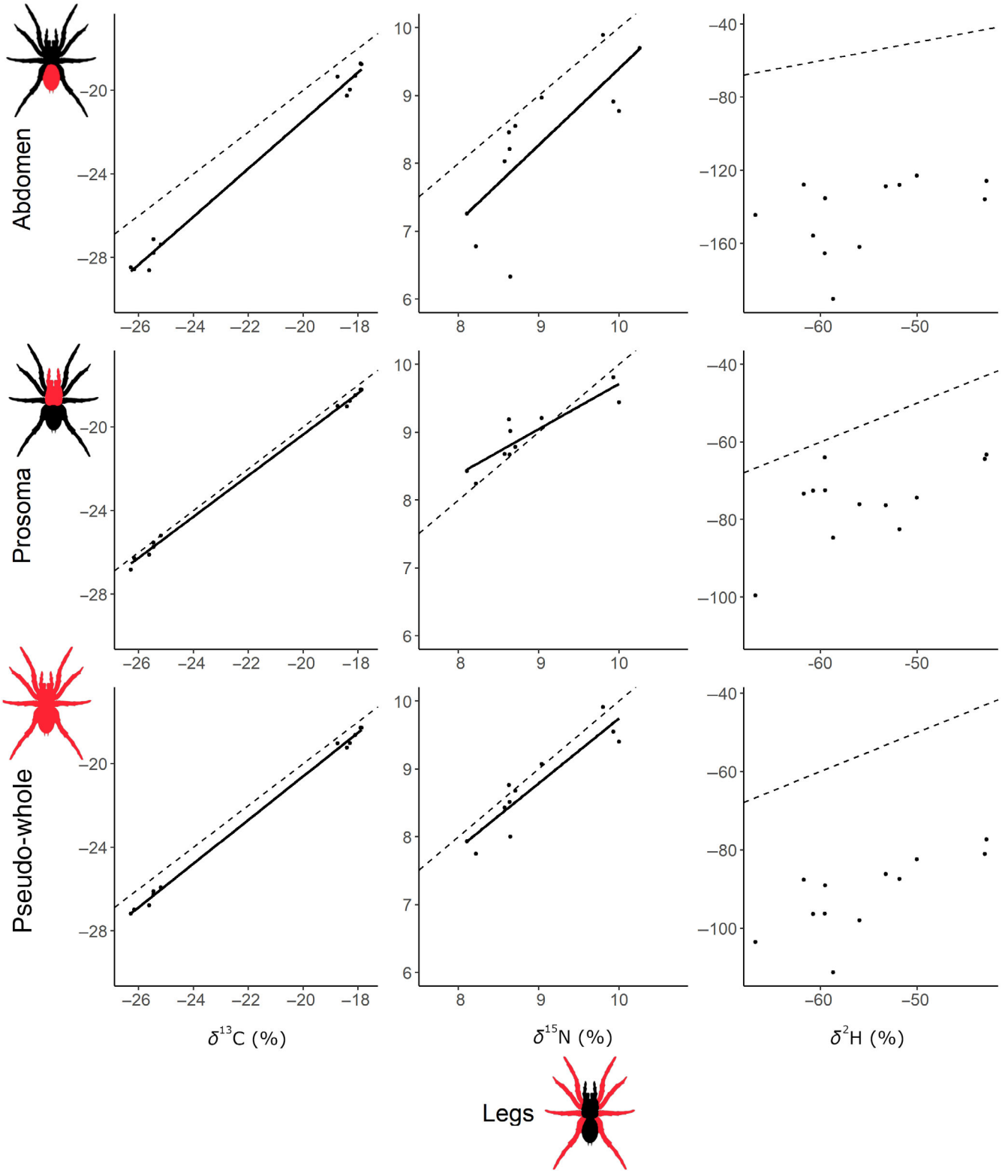

FIGURE 1 $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$ isotopic values of abdomen, prosoma, and pseudo-whole tissues as a function of leg tissue in laboratory-reared *Pterinochilus murinus* spiders. Isotopic values from both feeding groups were combined for regression analysis. Each plot shows a 1:1 line for comparison (dashed line). Note that the fitted line is shown only in significant relationships (see Table 3). Each tissue tested is represented by red-highlighted body parts

TABLE 3 Linear mixed model regression equations to estimate laboratory-reared spider abdomen, prosoma, or pseudo-whole-body $\delta^{13}C$, $\delta^{15}N$, or $\delta^{2}H$ isotope values based on the respective isotope values in the legs

Isotope	Model	Regression equation	H_0: $a = 0$, p[a]	H_0: $a = 1$, p[a]	H_0: $b = 0$, p[a]	R^2[b]
^{13}C	Abd ~ Leg	Abd = 1.12(Leg) + 1.00	**0.021**	0.774	0.895	0.99
	Pro ~ Leg	Pro = 1.17(Leg) + 2.68	**<0.001**	0.291	0.353	0.99
	PW ~ Leg	PW = 1.10(Leg) + 1.26	**<0.001**	0.571	0.687	0.99
^{15}N	Abd ~ Leg	Abd = 1.72(Leg) − 6.71	**0.003**	0.133	0.104	0.79
	Pro ~ Leg	Pro = 0.72(Leg) + 2.53	**0.002**	0.143	0.119	0.85
	PW ~ Leg	PW = 1.15(Leg) − 1.41	**<0.001**	0.425	0.379	0.89
^{2}H	Abd ~ Leg	Abd = −0.65(Leg) − 161.86	0.519	-	0.009	NA
	Pro ~ Leg	Pro = 1.08(Leg) − 18.21	0.079	-	0.524	NA
	PW ~ Leg	PW = 0.48(Leg) − 60.02	0.243	-	0.013	NA

Note: Bold indicates a significance at $p < 0.05$.

Abbreviations: Abd, abdomen; Pro, prosoma; PW, pseudo-whole.

[a]Models were tested for significance against a null hypothesis (H_0) of slope (a) = 0. If model was significant, we tested it against a 1:1 fit line of slope = 1. Intercept (b) coefficient tested against the null hypothesis of intercept = 0.

[b]R^2 is calculated as the *conditional* R^2, which describes the proportion of variance explained by both the fixed and random factors (see Nakagawa & Schielzeth, 2013). Conditional R^2 is not relevant for nonsignificant models.

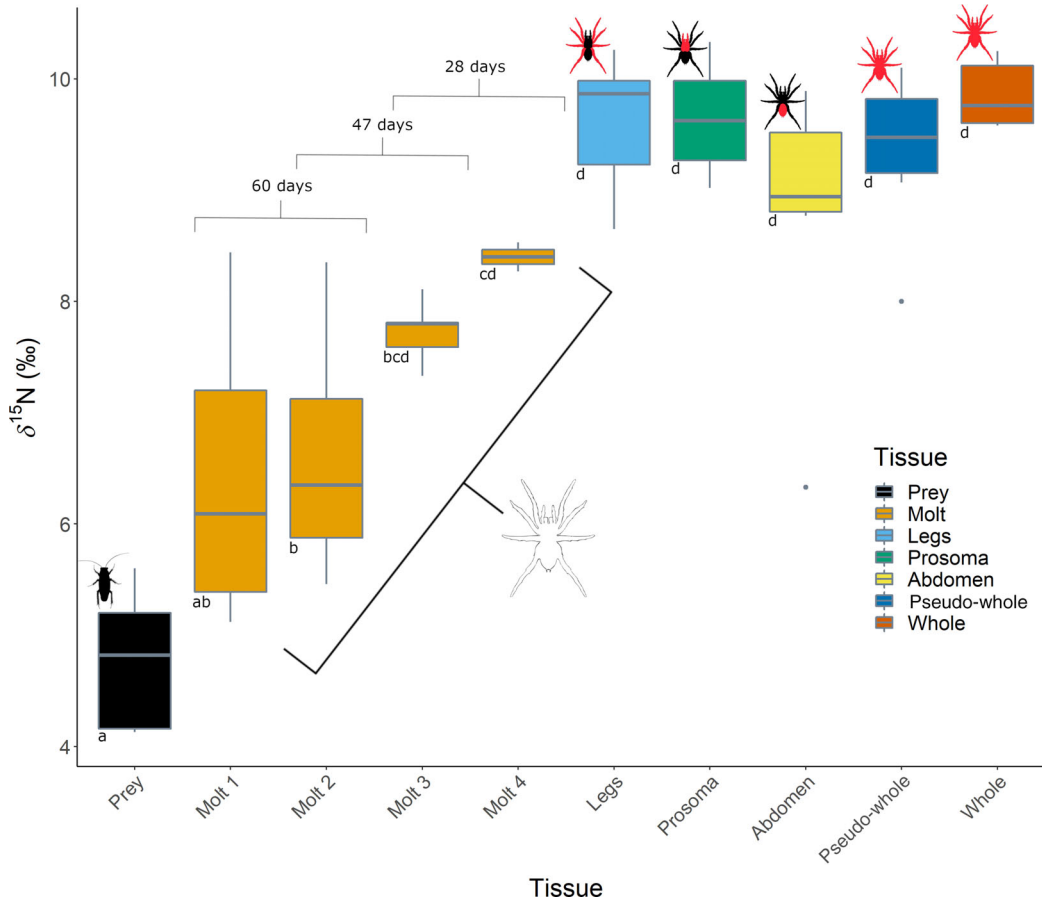

FIGURE 2 Boxplot of $\delta^{15}N$ isotopic values of prey, molts, tissue, and whole spiders in the laboratory terrestrial feeding group. Average days between molting events are listed above subsequent molt boxplots. Note that spiders in the terrestrial group underwent a maximum of four molting events. Letters under each boxplot indicate significant differences between tissues based on calculation of estimated marginal means post hoc testing with Tukey adjustment for multiple comparisons ($p < 0.05$). Each tissue tested is represented by red-highlighted body parts and molts are uncolored

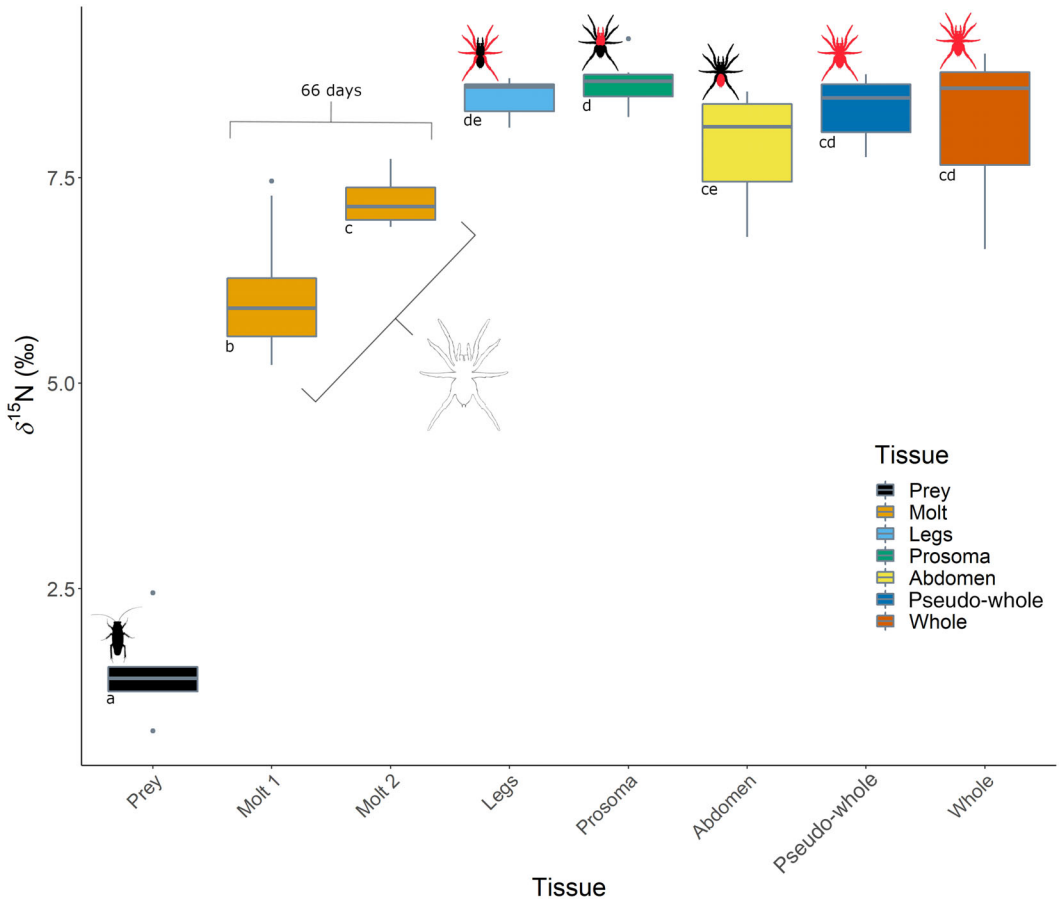

FIGURE 3 Boxplot of δ^{15}N isotopic values of prey, molts, tissue, and whole spiders in the laboratory aquatic feeding group. Average days between both molting events are listed above subsequent molt boxplots. Note that spiders in the aquatic group underwent a maximum two molting events. Letters under each boxplot indicate significant differences between tissues based on estimated marginal means post hoc testing with Tukey adjustment for multiple comparisons ($p < 0.05$). Each tissue tested is represented by red-highlighted body parts and molts are uncolored

δ^{13}C and δ^{15}N stable isotope signatures in laboratory and field experiments. In our laboratory study, spider legs and prosomas had similar isotopic values in both δ^{13}C and δ^{15}N, which agree with past studies measuring δ^{13}C and δ^{15}N values in field-collected spiders (Beaubien et al., 2019; Collier et al., 2002). In some situations, it may be unrealistic to remove only spider legs for SIA due to the size of the target family or species. This has often motivated other researchers to use the entire spider body to calculate stable isotope values (Akamatsu et al., 2004; González Macé et al., 2019; Kennedy et al., 2018). However, this approach can be problematic as prey remains may be present in specimen guts during preparation for SIA and can skew isotopic values (Hill & McQuaid, 2011). Our results show that legs alone, for larger spider species, or both spider legs and prosomas, for small spider species, can be combined to determine δ^{13}C and δ^{15}N isotopic values.

Despite the fact that we tested four different species, collected from three different sites, >35 days (Lafage et al., 2020), in a variety of conditions, and with all species presumed to be feeding on different food sources, we found strong correlations between legs and abdomens in our field-collected spiders. We found strong correlations for all species for δ^{15}N and for all but *P. hygrophila* for δ^{13}C. We believe that the absence of correlation in *P. hygrophila* is connected to its small sample size ($n = 14$). In fact, other species that were removed from this analysis due to small sample sizes failed to achieve significance in δ^{13}C (Table S1). We believe testing with larger sample sizes would reveal a similar pattern of isotopic predictive capacity of abdomens as a function of legs. Deviations of fitted regression lines from the 1:1 hypothetical fit line for some field species is likely associated with diets and the different tissue turnover rates between the tested tissues (Willis et al., 2013). Spider leg tissue has an average turnover time 2–3 times longer than abdomens (Belivanov & Hambäck, 2015). Leg and abdomen tissues in the field-collected spiders were likely reflecting an isotopic value corresponding to different

TABLE 4 Average (±SD) δ^{13}C and δ^{15}N isotopic values and C and N discrimination values for each tissue and prey separated by feeding group

Group	Tissue	*n*	δ^{13}C ± SD	Δ^{13}C[a]	δ^{15}N ± SD	Δ^{15}N[a]
Terrestrial	Whole	6	−27.37 ± 0.29	−0.05 ± 0.29	9.86 ± 0.31	**5.07** ± 0.31
	Pseudo-whole	6	−26.55 ± 0.52	0.77 ± 0.52	9.34 ± 0.75	**4.56** ± 0.75
	Prosoma	6	−25.95 ± 0.58	**1.36** ± 0.58	9.64 ± 0.50	**4.86** ± 0.5
	Abdomen	6	−28.00 ± 0.65	−0.68 ± 0.65	8.76 ± 1.28	**3.98** ± 1.28
	Leg	6	−25.69 ± 0.43	**1.62** ± 0.43	9.61 ± 0.63	**4.83** ± 0.63
	Prey	4	−27.31 ± 0.37	-	4.78 ± 0.64	-
Aquatic	Whole	7	−19.07 ± 0.50	0.59 ± 0.50	8.16 ± 0.91	**6.67** ± 0.91
	Pseudo-whole	6	−18.74 ± 0.41	**0.91** ± 0.41	8.34 ± 0.41	**6.86** ± 0.41
	Prosoma	6	−18.61 ± 0.36	**1.04** ± 0.36	8.67 ± 0.32	**7.18** ± 0.32
	Abdomen	6	−19.40 ± 0.62	0.26 ± 0.62	7.88 ± 0.71	**6.40** ± 0.71
	Leg	6	−18.22 ± 0.33	**1.43** ± 0.33	8.48 ± 0.25	**7.00** ± 0.25
	Prey	5	−19.65 ± 0.31	-	1.49 ± 0.61	-

Note: Bold values indicate a significant difference between prey δ values and tissue δ values at $p < 0.05$.
[a]Δ^{13}C and Δ^{15}N are discrimination values calculated by subtracting the average δ value of prey from each individual tissue sample δ value and averaging the result.

TABLE 5 Average (±SD) δ^{2}H isotopic values and H discrimination values for each tissue and prey separated by feeding group

Group	Tissue	*n*	δ^{2}H ± SD	Δ^{2}H[a]
Terrestrial	Whole	6	−104.46 ± 11.57	1.96 ± 11.57
	Pseudo-whole	6	−98.87 ± 7.96	7.54 ± 7.96
	Prosoma	6	−78.37 ± 12.34	28.04 ± 12.34
	Abdomen	6	−157.69 ± 21.10	**−51.27** ± 21.10
	Leg	6	−60.56 ± 3.60	**45.86** ± 3.60
	Prey	4	−106.42 ± 27.10	-
Aquatic	Whole	7	−96.02 ± 9.94	−2.04 ± 9.94
	Pseudo-whole	6	−83.93 ± 4.42	10.05 ± 4.42
	Prosoma	6	−72.26 ± 7.33	**21.73** ± 7.33
	Abdomen	6	−129.47 ± 5.15	**−35.49** ± 5.15
	Leg	6	−50.08 ± 6.39	**43.91** ± 6.39
	Prey	5	−93.98 ± 10.30	-

Note: Bold values indicate a significant difference between prey δ values and tissue δ values at $p < 0.05$.
[a]Δ^{2}H are discrimination values calculated by subtracting the average δ value of prey from each individual tissue sample δ value and averaging the result.

times and thus different predation events where unique prey were consumed. However, our results from field-collected spiders provide strong evidence, in addition to the laboratory results, that legs can be used as nonlethal alternatives to determine δ^{13}C and δ^{15}N isotopic values or can be used in cases where abdomens are required for other analyses.

Distinct differences over time were much more evident in δ^{15}N isotopic values in molts compared to δ^{13}C. The single prey source in our study revealed a lack of a shift in δ^{13}C values in molt remains suggesting that molts may be used to track or expose diet shifts when sampled over time in the field where a spider diet is expected to fluctuate. These results highlight a need for further exploration of δ^{13}C shifts in spider molts. Belivanov and Hambäck (2015) also used spider molts to track isotopic shifts over time focusing on a diet switch and using spiders similar to ours (*Lasiodora parahybana*). They found a significant and traceable shift in δ^{13}C values, but their δ^{15}N values were too small to detect any meaningful change. In our study, δ^{15}N isotopic values show a significant trend of shifting tropic position through each molt stage to the final tissue samples suggesting that spider molts can be used to track distinct and significant shifts in δ^{15}N isotopic values. However, further investigation is needed to understand the exact mechanism of the shifting δ^{15}N values of spider molts. Previous studies of trophic position point out that isotopic enrichment in tissues is related to a combination of tissue assimilation rate, excretion, and prey source (Olive et al., 2003), each of which may have affected the δ^{15}N values in our results. Furthermore, the isotopic signature of molts should be interpreted as a past diet, not the current spider diet, and multiple molts are necessary to establish diet trends or trophic shifts. Regardless, spider molts have strong potential to investigate δ^{15}N values and trophic position in field and laboratory studies. However, the authors recognize the difficulty in molt sampling in

TABLE 6 Isotopic values (mean ± SD) for ^{13}C and ^{15}N for individual species and all species combined of field-collected spiders

			$\delta^{13}C$			$\delta^{15}N$		
Species	***n***	**Tissue**	**Mean ± SD**	**Min**	**Max**	**Mean ± SD**	**Min**	**Max**
Pardosa amentata	124	Abd	−27.23 ± 1.02	−30.57	−25.33	6.00 ± 1.33	2.90	9.64
		Leg	−26.87 ± 0.50	−28.17	−25.88	5.54 ± 0.79	3.74	7.45
Pardosa lugubris	105	Abd	−26.96 ± 0.92	−28.63	−23.32	4.25 ± 1.13	1.75	7.50
		Leg	−26.34 ± 0.39	−27.41	−25.39	3.82 ± 1.19	1.33	6.71
Pardosa prativaga	35	Abd	−27.42 ± 0.96	−30.40	−25.91	5.15 ± 1.40	2.03	7.73
		Leg	−26.80 ± 0.68	−27.81	−24.86	5.07 ± 1.08	2.89	7.12
Pardosa spp.	59	Abd	−27.30 ± 0.90	−29.84	−24.19	4.12 ± 1.38	1.17	6.55
		Leg	−26.54 ± 0.93	−30.77	−24.64	4.55 ± 1.37	1.95	7.41
Piratula hygrophila	16	Abd	−27.39 ± 1.12	−30.47	−26.16	4.12 ± 0.81	2.92	5.78
		Leg	−26.38 ± 0.34	−26.85	−25.74	4.49 ± 0.60	3.62	5.60
All species	339	Abd	−27.19 ± 0.98	−30.57	−23.32	4.97 ± 1.52	1.17	9.64
		Leg	−26.62 ± 0.62	−30.77	−24.64	4.75 ± 1.28	1.33	7.45

Note: *Pardosa* spp. group represent juvenile and subadults in the *Pardosa* genus.
Abbreviation: Abd, abdomen.

TABLE 7 Slope and intercept coefficients for linear mixed model regression of abdomen $\delta^{13}C$ and $\delta^{15}N$ isotope values as a function of leg isotope values of individual field-collected Lycosid spiders and all species combined

Species	***n***	**Isotope**	**Slope**	**H_0: $a = 0$, p^a**	**H_0: $a = 1$, p^a**	**Intercept**	**H_0: $b = 0$, p^a**	**R^2**
Pardosa amentata	122	C	0.96 ± 0.17	**<0.001**	0.83	−1.31 ± 4.45	0.768	0.21
	122	N	1.17 ± 0.11	**<0.001**	0.14	−0.46 ± 0.63	0.464	0.47
Pardosa lugubris	100	C	0.62 ± 0.23	**0.009**	0.10	−10.76 ± 6.0	0.078	0.06
	100	N	0.63 ± 0.07	**<0.001**	**<0.01**	1.84 ± 0.3	**<0.001**	0.41
Pardosa prativaga	31	C	0.95 ± 0.21	**<0.001**	0.81	−1.97 ± 5.54	0.724	0.40
	33	N	1.03 ± 0.13	**<0.001**	0.99	−0.05 ± 0.68	0.940	0.59
Pardosa spp.	54	C	0.46 ± 0.12	**<0.001**	**<0.01**	−15.16 ± 3.1	**<0.001**	0.21
	54	N	0.7 ± 0.1	**<0.001**	**<0.01**	0.99 ± 0.45	**0.032**	0.50
Piratula hygrophila	14	C	1.01 ± 0.92	0.290	-	−0.76 ± 24.2	0.975	NA
	15	N	0.82 ± 0.29	**0.014**	0.56	0.41 ± 1.3	0.700	0.32
All species	345	C	0.66 ± 0.08	**<0.001**	**<0.01**	−9.7 ± 2.13	**<0.001**	0.17
	350	N	0.9 ± 0.04	**<0.001**	**0.02**	0.74 ± 0.21	**<0.001**	0.57

Note: Bold indicates a significance at $p < 0.05$.
[a]Models were tested for significance against a null hypothesis (H_0) of slope (a) = 0. If model was significant we tested it against a 1:1 fit line of slope = 1. Intercept (b) coefficient tested against the null hypothesis of intercept = 0.

the field and the limited ability to identify molts to lower taxonomic levels, especially with cursorial spiders. With proper experimental set up, SIA of field-collected spider molts can give a high taxonomic level (Araneae) or a specific community level (i.e., web-building or cursorial species) snapshot of spider trophic position, for example, before a common seasonal event (i.e., flooding) or after a large-scale disturbance (i.e., recolonization after a fire). This type of investigation is well suited for web-building or burrowing spiders that often discard molts on the web periphery or just outside of a burrow. Additionally, laboratory studies can use spider molts to create robust feeding trials focusing on isotopic changes over long life cycles while requiring fewer live specimens.

Discrimination values using ^{13}C and ^{15}N

Our results show that discrimination values of spiders for ^{15}N are considerably higher than previously reported.

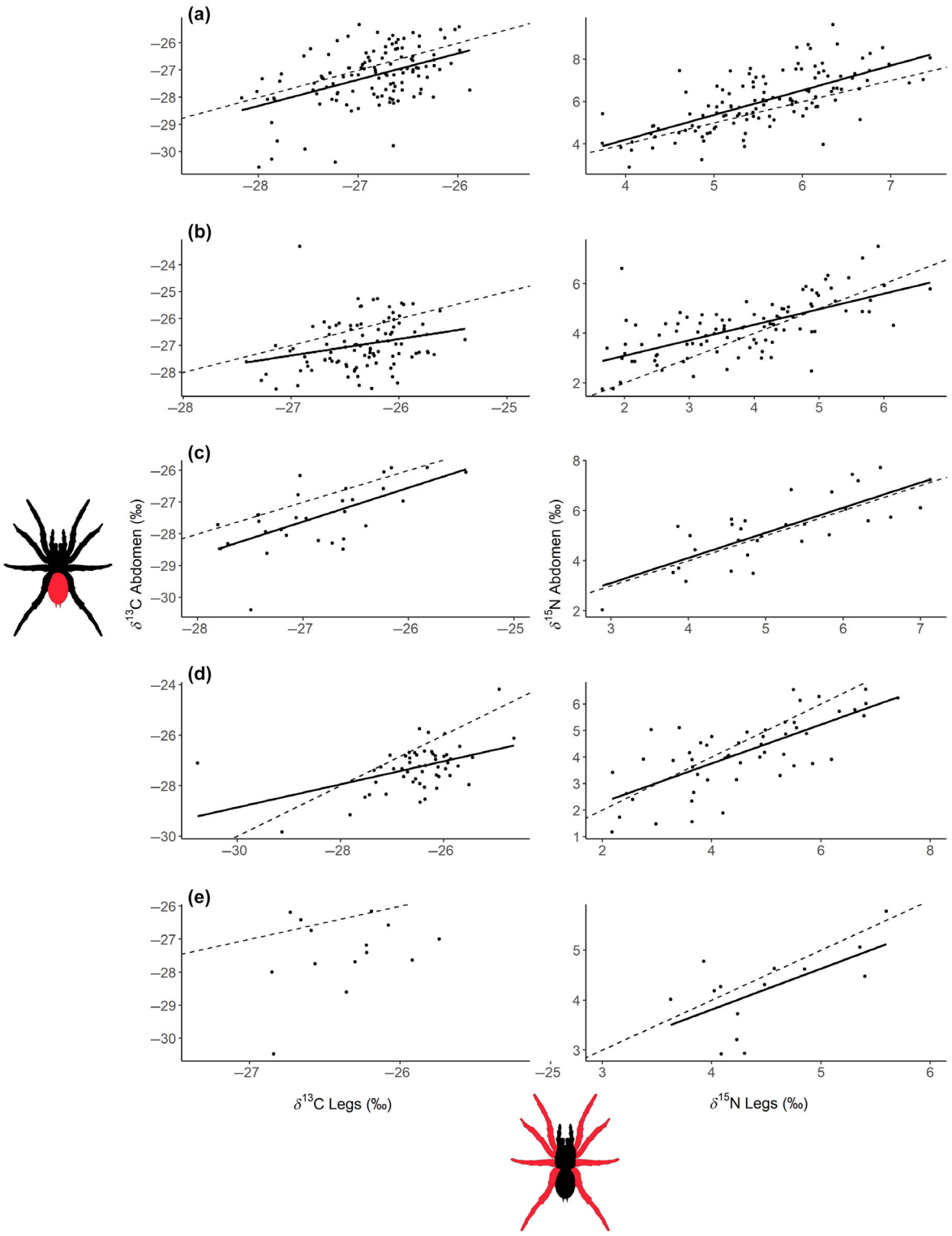

FIGURE 4 δ^{13}C and δ^{15}N isotopic values of abdomens as a function of leg tissue in: (a) *Pardosa amentata*, (b) *P. lugubris*, (c) *P. prativaga*, (d) *Pardosa* spp., and (e) *Piratula hygrophila*. Solid lines represent the fitted regression line. Each plot shows a 1:1 line for comparison (dashed line). Fitted line is shown only in significant relationships (see Table 7). Each tissue tested is represented by red-highlighted body parts

However, these values are within a common range of other studied arthropods such as locust (5.1‰), woodlice (5.7‰), and mites feeding on *Chlorella* spp. (9.8‰) (Heethoff & Scheu, 2016; Post, 2002; Rothe & Gleixner, 2000; Webb et al., 1998). Discrimination values of ^{15}N averaged 4.66‰ in the terrestrial group and 6.82‰

in the aquatic group and were different from prey in all tissues. ^{15}N discrimination values were not significantly different between spider legs and whole bodies suggesting that nonlethal samples of spider legs can be used to determine ^{15}N discrimination values without using an offset. Discrimination values of ^{13}C were only significantly different from prey in legs and prosomas averaging 1.49‰ and 1.24‰ in the terrestrial and aquatic feeding groups, respectively. ^{13}C discrimination values in legs were significantly different from whole bodies in both feeding groups by an average of 1.67‰ and 0.84‰ in the terrestrial and aquatic feeding groups, respectively. The discrimination values between spider legs and prey support other studies showing that ^{13}C can be used to show trophic shifts in arthropods (Heethoff & Scheu, 2016; Oelbermann & Scheu, 2002; Rothe & Gleixner, 2000; Webb et al., 1998). We believe that the exclusion of the abdomen from analysis removes the possibility of isotopic signatures partially reflecting leftover prey thus revealing the trophic shift using ^{13}C discrimination values. Nonlethal samples of spider legs can be used to determine ^{13}C discrimination, but it may require a discrimination value offset of ~1.5‰ to estimate the whole-body values.

δ^2H in spiders

In contrast to $\delta^{13}C$ and $\delta^{15}N$ in laboratory-tested spiders, δ^2H signatures and discrimination values in spider legs are not reliable substitutes for whole-body spider δ^2H isotopic values. Our results showed no linear relationship and consequently no predictive power of spider legs to proxy other spider body tissues. In addition, the δ^2H values in our study revealed no difference between the aquatic and terrestrial cockroach groups nor any significant indication of a shift in trophic position between cockroaches and spider whole bodies. Other studies suggest that δ^2H signatures can be used to indicate trophic position or differentiate between autochthonous and allochthonous primary food sources (Doucett et al., 2007; Finlay et al., 2010; Vander Zanden et al., 2016). The difference between our results and previous studies may be linked to water source. In our experiment, all cockroaches and spiders were provided the same water source, which is a primary driver of δ^2H values (Hobson et al., 1999; Solomon et al., 2009). Furthermore, past investigations showing significant separation in basal food sources using δ^2H (Doucett et al., 2007; Finlay et al., 2010) were carried out in arid locations that have significant effects on plant evaporation and transpiration, two metabolic processes that greatly influence δ^2H isotopic values and isotopic fractionation (Vander Zanden et al., 2016). If δ^2H isotope signatures are necessary or if water source is not accounted for in an analysis, we suggest using the entire spider body to help control for the high variation in isotopic values between tissues.

Conclusions

Our study shows that spider legs and molts are reliable proxies of whole-body $\delta^{13}C$ and $\delta^{15}N$ stable isotope values and should be considered as an alternative to lethal whole-body sampling in practical situations in both laboratory and field settings. However, future researchers using nonlethal tissues need to have clear understanding of the isotopic time reflected by each tissue. We also found that nonlethal spider leg samples are not reliable substitutes for δ^2H isotopic value in whole bodies, although the drivers of δ^2H values require much more research. Our study has also highlighted the varying discrimination values found in spiders and spider tissues and how they can affect nonlethal sampling in stable isotope investigations. The demonstrated nonlethal sampling technique is a critical step to study food webs or diet sources of rare or endangered spider species (Wilhelm & Nelson, 2012). Nonlethal sampling is especially well suited for threatened species such as *Dolomedes plantarius* (Monsimet et al., 2020), which has a body size and life history suitable for such a method. Appendage loss (autotomy) in spiders may influence mating, foraging, or locomotion (Fraser et al., 2020), and concerns about removing legs of live specimens are warranted. However, juvenile and subadult spiders regrow legs between molts and most spiders can generally live full lives with fewer than eight legs (Brueseke et al., 2001; Foelix, 2010). Using legs in laboratory settings may be even more valuable than whole bodies as spider leg regeneration allows for robust stable isotope feeding studies with fewer spiders needed for time series analysis. Additionally, using only spider legs for isotope analysis may be more advantageous as the longer tissue turnover time in spider legs (Belivanov & Hambäck, 2015) reflects the diet over a longer period compared to the abdomen. When coupled with other methods to study spider diet, such as DNA barcoding, an investigator can produce a conclusive model of spider diets over longer periods (Hambäck et al., 2016). Stable isotope research using spiders should continue to focus on nonlethal sampling techniques and understanding the factors that influence isotopic discrimination values.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available from Zenodo (http://doi.org/10.5281/zenodo.5075207).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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