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Using δ^{13}C stable isotopes to quantify individual-level diet variation

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Abstract Individual-level diet variation can be easily quantified by gut-content analysis. However, because gut contents are a 'snapshot' of individuals' feeding habits, such cross-sectional data can be subject to sampling error and lead one to overestimate levels of diet variation. In contrast, stable isotopes reflect an individual's long-term diet, so isotope variation among individuals can be interpreted as diet variation. Nevertheless, population isotope variances alone cannot be directly compared among populations, because they depend on both the level of diet variation and the variance of prey isotope ratios. We developed a method to convert population isotope variances into a standardized index of individual specialization (WIC/TNW) that can be compared among populations, or to gut-content variation. We applied this method to diet and carbon isotope data of four species of frogs of the Brazilian savannah. Isotopes showed that gut contents provided a reliable measure of diet variation in three populations, but greatly overestimated diet variation in another population. Our method is sensitive to incomplete sampling of the prey and to among-individual variance in fractionation. Therefore, thorough sampling of prey and estimates of fractionation variance are desirable. Otherwise, the method is straightforward and provides a new tool for quantifying individual-level diet variation in natural populations that combines both gut-content and isotope data.

Keywords Carbon stable isotopes · *Cerrado* · Gut contents · Individual specialization · Fractionation

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Introduction

Many natural populations are composed of ecologically heterogeneous individuals that use different subsets of the available resources (Heinrich 1979; Price 1987; Roughgarden 1972; Svanbäck and Bolnick 2005; Van Valen 1965; Werner and Sherry 1987). Individuals within a population may use different resources because they inhabit different microhabitats (Durell 2000), are different sexes (Slatkin 1984), or different ages (Polis 1984). However, individuals can also exhibit niche variation within sex or age class, and within a single site or time. This indi-

vidual-level variation is called ''individual specialization,'' in which individuals use a significantly narrower set of resources than the population as a whole (Bolnick et al. 2003). This variation may have important ecological implications, such as a reduction of intraspecific competition (Swanson et al. 2003) or the differential response of individuals to both intra- and interspecific competition (Taper and Case 1985) or predation, which can ultimately affect population dynamics (Lomnicki 1992). Moreover, this variation permits frequency-dependent interactions that can drive disruptive selection and evolutionary divergence (Bolnick 2004; Dieckmann and Doebeli 1999).

Many studies focusing on individual specialization have relied on gut contents as a source of diet information (Bryan and Larkin 1972; Fermon and Cibert 1998; Robinson et al. 1993; Roughgarden 1974; Schindler 1997; Warburton et al. 1998; Svanback and Bolnick manuscript). An important underlying assumption in these studies is that the prey found in the stomach actually represents the long-term resource use of individuals. However, this assumption may not hold if prey are patchily distributed, their abundances vary over time, or stomachs can only contain a few items at a time, because individuals' gut contents reflect their recent encounters rather than long-term preferences. For instance, Warburton et al. (1998) analyzed the gut contents of the silver perch (*Bidyanus bidyanus*) and observed that individuals were highly specialized on different resources, but only over periods of time of 2–4 weeks, after which they changed their diets in response to prey abundance variation. These sampling problems will lead one to believe individuals are more specialized than they really are, overestimating the degree of diet variation (Bolnick et al. 2002, 2003).

There are cases, however, in which gut contents are a fairly good indicator of individual long-term resource use. The most compelling examples come from studies on fishes, in which researchers repeatedly sampled stomachs of the same individuals and observed high temporal consistency of individual diets (e.g., Bryan and Larkin 1972; Schindler 1997). Other studies showed that morphological variation among consumers explained some of the variation in stomach contents (Fermon and Cibert 1998; Robinson et al. 1993; Svanback and Bolnick manuscript). Such morphology–diet correlations are strong evidence that some of the stomach content variation represents consistent diet variation among foragers. Finally, several studies have relied on the quantification of stable isotopes (Fry et al. 1978; Gu et al. 1997) to infer temporal consistency in the diets of individuals.

The utility of stable isotopes in diet studies is that the sources of, for example, carbon and nitrogen can be distinguished so that a consumer's diet can be inferred. Since stable isotopes have relatively slow turnover rates compared to feeding episodes, varying from days to years depending on the tissue analyzed (several months in the case of muscle), they can be used to infer dietary carbon and nitrogen intake over long time periods (Dalerum and Angerbjörn 2005; Tieszen et al. 1983). Due to their slow turnover (Tieszen et al. 1983), isotopes will not be subject to the same stochastic sampling effects as gut contents and can be a more reliable way to infer individual temporal consistency in food-resource use. In fact, carbon stable isotopes have been used as a measure of intra-population diet variation (Angerbjörn et al. 1994; Fry et al. 1978; Gu et al. 1997; Sweeting et al. 2005). For example, Fry et al. (1978) measured the standard deviation (SD) of individual carbon isotope ratios in different species of grasshoppers and observed that species that fed on both C_3 and C_4 plants had higher SD values than those specialized on either C_3 or C_4 plants. Isotope variation thus offers a method of testing for diet variation that is complementary to gut-content analysis, and can be used to evaluate the reliability of gut contents.

However, using isotope variance to test for individual specialization has some important caveats. If there are more food sources than we can discriminate with isotopes (Phillips and Gregg 2003), isotope variation may underestimate diet variation among individuals (Matthews and Mazumder 2004). On the other hand, if food sources show isotopic variation in space and/or time and consumers were sampled in different places or times, one will observe isotopic variation that is not necessarily related to diet variation (Dalerum and Angerbjörn 2005; Matthews and Mazumder 2005). Moreover, for a given level of diet variation, populations using more isotopically variable prey will themselves show higher isotope variances (Matthews and Mazumder 2004). Consequently, measures of population isotopic variance can be a misleading guide diet variation if the prey isotopic variance is not taken into account. Matthews and Mazumder (2004) proposed null models that allow one to test for significant individual specialization in populations, provided that the isotope ratios of prey are known. Their method, therefore, allows one to test the null hypothesis that individuals in a population sample randomly from the population distribution (individual generalists), and as a consequence to detect cases of individual specialization. However, they do not allow us to use isotope variances to quantify the degree of individual specialization or compare the amount of diet variation among populations. Therefore, it would be useful to be able to scale isotopic variance to a measure of individual specialization that can be compared across different populations.

In this paper, we present a method that allows the use of $\delta^{13}C$ variance to estimate a standardized index of individual specialization (Bolnick et al. 2002) that can be

compared across different populations. We apply this method to isotope and gut-content data of four populations of leptodactylid frogs (*Adenomera* sp., *Eleutherodactylus* sp., *Leptodactylus fuscus*, and *Proceratophrys* sp.) that inhabit an area of savannah in southeastern Brazil. These are terrestrial, relatively sedentary animals, feeding in a potentially patchy environment, in which we would expect gut contents to overestimate diet variation due to stochasticity in food consumption. By comparing the estimates of the degree of individual specialization resulting from our method to those derived from gut contents, we were able to evaluate the utility of cross-sectional data in studies of diet variation.

Materials and methods

Study area

We analyzed the stomach contents and stable carbon isotopes of muscle tissue of four species of frogs from a savannah formation in southeastern Brazil locally known as *cerrado* (Oliveira and Marquis 2002). There is marked seasonality in the area, with a wet/warm season (henceforth ''wet season'') from September to March and a dry/mild season (henceforth ''dry season'') from April to August (Rosa et al. 1991). Specimens of four species (*Adenomera* sp., *Eleutherodactylus* sp., *Leptodactylus fuscus*, and *Proceratoprhys* sp.; $N = 104$, 115, 86, and 55 individuals, respectively) were obtained from the collection of the Museu de Biodiversidade do Cerrado of the Universidade Federal de Uberlândia (MBC-UFU). Specimens were collected in the municipality of Uberlândia (18° 55′S–48° 17′W, 850 m), in the state of Minas Gerais, southeastern Brazil, in five sites within each of two of the remnants of *cerrado* still present in the municipality (Goodland and Ferri 1979). Frogs were collected weekly in the wet season and once every 2 weeks in the dry season, for a period of 2 years. Frogs were immediately killed upon collection, fixed in 5% formalin and later preserved in 70% ethanol.

Data collection

Diet data

Preserved specimens were dissected under a microscope to obtain stomach contents. Upon dissection individuals were sexed by examination of gonads. Prey items were counted, measured for total length using an eyepiece coupled with a stereomicroscope, and identified to order or more commonly family level (following Borror and DeLong 1988).

Stable isotopes

We measured stable isotopes from the frogs and the prey found in gut contents. Carbon isotopic signatures of animal tissues can be altered by ethanol and formalin preservation (Kaehler and Pakhomov 2001; Sweeting et al. 2004). However, since we are interested in estimating the variance among individual isotopic ratios and all our samples were subject to the same preservation conditions, preservation should not be a problem in our study. To quantify $\delta^{13}C$ in the frogs, a piece of muscle from the thigh was collected from a subsample of 60 specimens chosen randomly from the larger sample of available specimens (*Adenomera* sp., *Eleutherodactylus* sp., and *L. fuscus*); in the case of *Proceratophrys* sp. all the 55 individuals were analyzed. To quantify $\delta^{13}C$ in the prey, we analyzed whole prey items obtained from 47 gut contents across the four species. Some prey taxa were not abundant or large enough to measure isotopic ratios. Those prey were all very rare in the samples, each one representing no more than 1% of the number of prey consumed, and were lumped under the category 'others' (see Electronic Supplementary Material 1 for details). Samples were rinsed for 1 min in distilled deionized water (Sweeting et al. 2004), oven-dried to constant mass at 50°C (Magnusson et al. 1999), ground, and weighed (c. 1 mg) into 4 × 3.2-mm tin capsules. Prey items were grouped by taxon, generally order, though we split Coleoptera and Heteroptera into finer categories based on feeding habits (according to Borror and DeLong 1988). We did this to minimize isotope variation within taxa. A list of the taxa comprising each of these feeding-habit groups is provided in Table S1 (Electronic Supplementary Material 1). We analyzed the isotopes of a total of 23 prey categories (Table S1). Prey items belonging to the same categories were dried and ground together. The abundances of ^{13}C and ^{12}C were determined at the University of California at Davis Stable Isotope Facility using a continuous flow, isotope ratio mass spectrometer (CF-IRMS, Europa Scientific, Crewe, UK), interfaced with a CN sample converter. Two samples of an internal reference material were analyzed after every 12 samples, to calibrate the system and to compensate for drift with time. The $^{13}C/^{12}C$ compositions are reported using conventional delta notation, showing differences between the observed concentration and that of Pee Dee Belemnite. Experimental precision was estimated as the standard deviation of replicates of the internal reference material, and was 0.03%.

Prey dry masses

We estimated the dry masses of prey categories by weighing all the remaining intact items found in the stomach contents. Items belonging to each category were

oven-dried and weighed in a high-precision balance (0.01 mg). The final dry mass of each category was divided by the number of items weighed, which varied from 2 to 202 ($\bar{x} = 37.2$; SD = 51.5). There were four categories (Coleoptera dead-wood consumers, Heteroptera granivores, Heteroptera predators, and Hymenoptera non-formicidae) for which we were not able to directly estimate dry masses due to insufficient material. In those cases, we used published regression equations (Hódar 1996; Sample et al. 1992) to estimate insect biomasses from length measures.

Data analyses

Analysis of diet data

Diet variation can be a function of sex or age. Moreover, both diet and isotopic variation are subject to spatial and temporal effects. As a consequence, we had to rule out these confounding effects before quantifying diet and isotopic variation in our samples. We therefore tested if the diets and isotopic signatures among individuals varied as a function of sex, age class, collection site, and season (wet and dry). In the case of the diet data, we first did a Principal Component Analysis on the arcsine-square root transformed proportions of prey use by individuals. We then took the PC scores of the major axes (axes that explained >5% of the variation) and did a multi-way MANOVA, with PC scores as dependent variables and sex, age class, collection site, and season as factors. In addition, we used a multi-way ANOVA to test for those same effects on $\delta^{13}C$ ratios within each frog species. By doing this, we ensure that any diet or isotope variation is not due to either sex/age effects or spatial/temporal variation in prey availability or signatures. This in turn allows us to interpret the results in terms of individual specialization. The MANOVAs and ANOVAs were performed in SYSTAT11.

We next calculated a measure of the degree of individual specialization using frequencies of prey categories in individuals' guts. We used Roughgarden's (1979) measure of individual specialization WIC/TNW, in which TNW is the total niche width of a population, WIC is the within-individual component of niche width (average of individual niche widths), and BIC is the between-individual component (the variance among individuals' niches). Traditionally, the degree of diet variation is described by calculating the percent of total niche variation ascribed to individual niche widths (WIC/TNW). The higher the value of WIC relative to TNW, the lesser variable individuals are, and vice-versa. Therefore, WIC/TNW varies from 0 (maximum variation among individuals) to 1 (no variation among individuals). For comparison, we also estimated a second measure of individual specialization (IS) based on distribution overlap (Bolnick et al. 2002). Since results were qualitatively the same, we focus on the former measure. Readers are referred to Bolnick et al. (2002) for the formulas of the indices and details on their calculation. All the analyses of individual specialization were performed in IndSpec1, a program to calculate indices of individual specialization (Bolnick et al. 2002).

We used a non-parametric Monte Carlo procedure to test the null hypothesis that any observed diet variation arose from individuals sampling stochastically from a shared distribution. Each individual was randomly reassigned a new diet via multinomial sampling from the observed population resource distribution, and WIC/TNW was recalculated for the resulting population diet variation. IndSpec1 generated 1,000 such populations, and the null hypothesis can be rejected if the observed value of WIC/TNW is less than 95% of the null WIC/TNW values. This Monte Carlo procedure assumes that every prey item observed in a stomach represents an independent feeding event. We acknowledge, however, that this assumption may be violated for prey such as ants and termites that are found in tightly clustered groups.

Comparing isotope variation to gut-content variation

We developed a method that allows us to quantify the degree of individual specialization based on among-individual isotope variation (Fig. 1). This method uses the observed population diet to generate a large number of simulated populations with varying degrees of individual specialization ($0 < \text{WIC/TNW} < 1$). Empirical prey isotope ratios and dry masses are then used to calculate the isotopic variance $\text{Var}\delta_i$ for each simulated population. The simulations thus establish a relationship between $\text{Var}\delta_i$ and WIC/TNW. Finally, we use this relationship to convert an empirical $\text{Var}\delta_i$ into an estimate of WIC/TNW (Fig. 1). In our model, we are making three important simplifications: first that individuals do not selectively assimilate different isotopic components of a food source (differential assimilation); second that there is no fractionation between a consumer and its diet; third that there is no isotopic routing (Gannes et al. 1997). We acknowledge, however, that these processes can potentially affect the estimates of a population's isotopic variance and consequently the interpretations of our model. We address this problem further in the ''Discussion.'' In the following paragraphs, we explain in detail how population diets and $\text{Var}\delta_i$ were simulated.

Each simulated population was composed of the empirically observed number of individuals, *N*. Each individual's resource distribution was assigned by a multinomial sample from the empirical population's resource distribution. We could control the level of diet variation among individuals by setting the number of multinomial

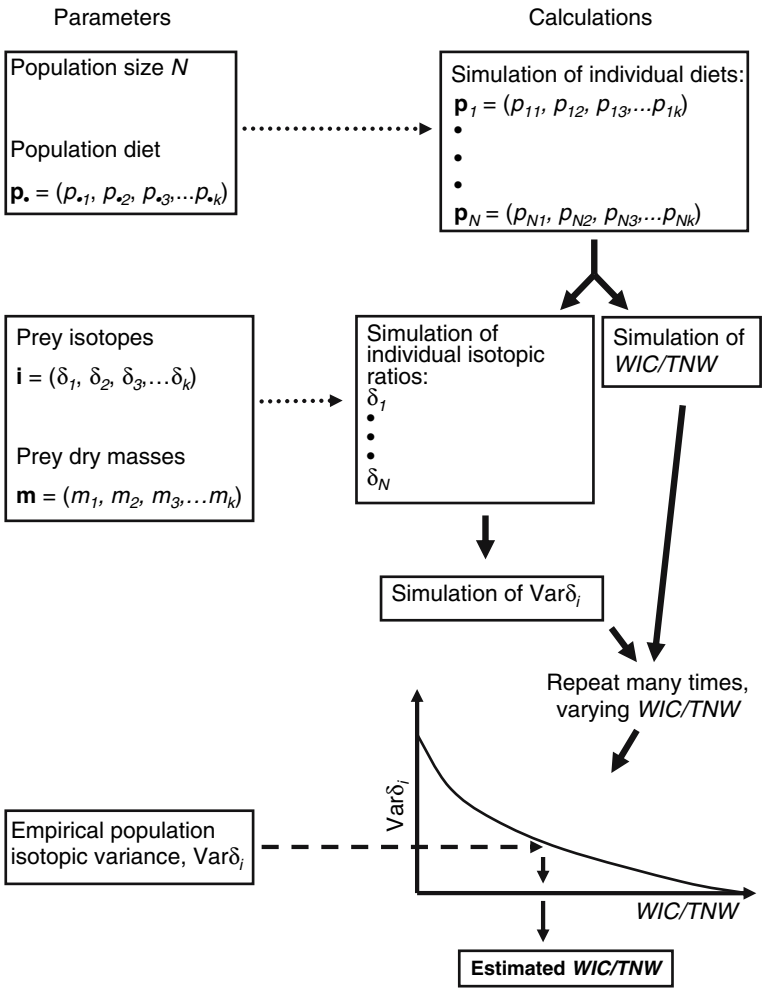

Fig. 1 Flow chart of the model used to generate measures of individual diet specialization (WIC/TNW) and among-individual variance in isotopic ratios (Varδ_i) of simulated populations. The chart outlines the procedure to generate simulated populations, showing parameters on the *left* and calculations on the *right*, composed of N individuals feeding on k prey categories with $\delta^{13}C$ signatures δ_k and dry mass m_k, and calculations to interpolate an estimated WIC/TNW from the empirical prey isotope variance. See text for details

draws that each individual took from the population's distribution. Due to the Law of Large Numbers, individuals given few draws had narrower and, as a consequence, more variable diets than when individuals had many draws. We would like to emphasize that this approach is merely a technique to generate different levels of among-individual diet variation and does not assume any underlying biological mechanism.

The first step in our simulation is to sum across the stomach contents of all N consumers in our empirical sample and calculate the frequency $p_{\bullet j}$ of each diet type j in the overall population's resource distribution. The resulting population diet vector is $\mathbf{p}_\bullet = (p_{\bullet 1}, p_{\bullet 2}, p_{\bullet 3},... p_{\bullet k})$. Then, each simulated individual is given s random draws (with replacement) from this multinomial probability distribution. The goal is to use the resulting number of draws (n_{ij}) of each prey type j to represent a long-term diet vector $\mathbf{p}_i$ for the simulated individual (Fig. 1). Although we acquired this vector by a sampling process, we use it to represent the vector of individual diet preferences. If an individual is given only a single draw ($s = 1$), it will persistently specialize on a single type of prey resource, e.g., $\mathbf{p_i} = (1.0, 0, 0,...0)$. Since different individuals will eventually draw different prey from the population vector, $s = 1$ yields the maximum level of among-individual variation. As s increases, individuals' diet vectors $\mathbf{p_i}$ converge towards $\mathbf{p}_\bullet$ (Law of Large Numbers) and diet variation declines.

After calculating the $\mathbf{p}_i$ vectors, our simulation uses the empirically obtained prey masses and isotope signatures to calculate each simulated individual's isotope signature

$$E(\delta_i) = \sum_j \frac{p_{ij} m_j}{\sum_j p_{ij} m_j} \delta_j.$$

The program then calculates WIC/TNW and the population isotopic variance Varδ_i, which are the outputs for the simulated population (Fig. 1). The model repeats this procedure for n replicate populations for each of 57 values of s (ranging from 1 to 1,000 in increasing increments). In our simulations, n was set at 100. A PC-compatible pro-

gram, VarIso1, to perform these simulations was written in C language and is available for public use at http://www.webspace.utexas.edu/dib73/Bolnicklab/links.htm.

We used quadratic regressions to establish the relationship between simulated WIC/TNW and Varδ_i. We used the resulting equation, and the empirical value of Varδ_i, to solve for an estimated value of WIC/TNW (Fig. 1). Confidence intervals were obtained using a prediction interval (the limits within which a new observation would lie if added to the regression model, with a probability of 95%), obtained in STATISTICA6.0. Finally, we tested whether WIC/TNW values from stomach contents fell outside the confidence interval for the isotope-derived value, which would indicate that stomach contents are a poor guide to long-term diet variation.

Results

Diet and stable isotopes data

All four frog species are generalist, feeding on a wide range of prey categories (Table S1). However, any given individual's stomach contained only a subset of its population's resource distribution, so that WIC/TNW < 0.5 for all four species (Table 1). This means that within-individual variation only accounted for approximately 40–50% of the total niche width, ranking among the strongest measures of individual specialization in the published literature (Bolnick et al. 2003). This diet variation is greater than would be expected under random independent sampling of prey from a common distribution (Monte Carlo bootstraps; Table 1). However, as discussed above, gut contents may not be a reliable measure of diet variation. Turning instead to isotope data, we found that isotope variances ranged from 1.38 (*Eleutherodactylus* sp.; Table 1; Fig. 2b) to 8.35 (*Proceratophrys* sp.; Table 1; Fig. 2c). Prey isotopic signatures were also variable, spanning from –24.57 to –13.32‰ (Table S1; Fig. 2).

Sex, age, and season had no significant effects on gut contents or isotopes (Table S2). This indicates that resource use differences were not an artifact of collection season, and that diet variation occurred at the individual level. We therefore pooled samples by sex, age, and date in later analyses. However, we did observe an effect of collection site on isotope ratios (*Adenomera* sp. and *L. fuscus*; Table S2) and gut contents (*Proceratophrys* sp.; Table S2). In those cases, we did additional post-hoc tests (Tukey) in order to identify those sites that differed from each other. Based on these results (not shown), we split samples of *Adenomera* sp. and *L. fuscus* into two subsamples (henceforth ss1 and ss2). In the case of *Proceratophrys* sp., we removed the sparsely collected site 1 ($N = 3$ frogs) from the analyses.

Comparing isotope variation to gut-content variation

Our simulations provided an expected relationship between WIC/TNW and Varδ_i (Fig. 3). Using the empirical $\delta^{13}C$ variances and this curvilinear relationship, we estimated values of WIC/TNW (Fig. 3). The values of WIC/TNW obtained from gut contents consistently fell within the isotope-derived confidence intervals in *Adenomera* sp. and in *L. fuscus*-ss1, but outside the confidence intervals in *Eleutherodactylus* sp., *L. fuscus*-ss2, and *Proceratophrys* sp. (Fig. 3). Contrary to our expectations, stable isotopes indicated that individual specialization was actually stronger than we inferred from gut contents in *Proceratophrys* sp. (Fig. 3d). In *L. fuscus*-ss2 (Fig. 3f), gut contents revealed a higher level of individual specialization than did the isotopes. Isotopes revealed negligible diet variation in *Eleutherodactylus* sp. (Fig. 3c), in stark contrast to the gut-content results. Results using the IS index of individual specialization were qualitatively similar (Table S3, Fig. S1).

Discussion

Our results show that there is evidence of individual specialization in the studied populations and that species vary

Table 1 Measures of intra-population variation in food-resource use in four species of Brazilian frogs

Species	WIC/TNW$_{obs}$	Var$\delta^{13}C$	WIC/TNW$_{exp}$
Adenomera sp.			
ss1 (39)	0.4738***	5.38	0.3967
ss2 (35)	0.4266***	4.90	0.4155
Eleutherodactylus sp. (56)	0.4573**	1.38	0.8636
Proceratophrys sp. (49)	0.3700***	8.35	0.1585
L. fuscus			
ss1 (38)	0.4873***	2.87	0.4965
ss2 (29)	0.4127***	2.01	0.6419

Numbers in parenthesis are sample sizes. Empirical WIC/TNW values were tested against null distributions generated with Monte Carlo bootstraps (1,000 simulations)

WIC/TNW$_{obs}$ Roughgarden's (1979) index of individual specialization based on gut-content data, *Var$\delta^{13}C$* empirically estimated isotopic variances of frog samples, *WIC/TNW$_{exp}$* expected value of the index based on isotope data. *L. fuscus Leptodactylus fuscus*, *ss1* and *ss2* subsamples 1 and 2, respectively (see text for details)

**$P = 0.01$, ***$P < 0.001$

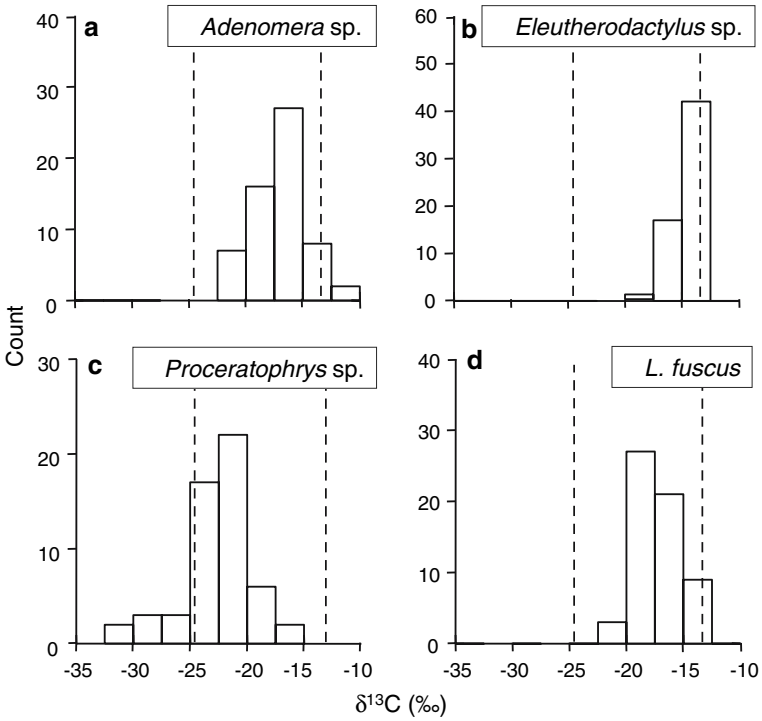

Fig. 2 Histograms of the empirically measured individual $\delta^{13}C$ signatures in four species of Brazilian frogs: **a** *Adenomera* sp. ($N = 60$), **b** *Eleutherodactylus* sp. ($N = 60$), **c** *Proceratophrys* sp. ($N = 55$), **d** *Leptodactylus fuscus* ($N = 60$). *Dashed lines* indicate the range of $\delta^{13}C$ of consumed prey

in the degree of individual specialization. Surprisingly, gut-content variation provided fairly good estimates of overall levels of individual specialization in *Adenomera* sp. and *L. fuscus*. On the other hand, gut contents greatly overestimated individual specialization in *Eleutherodactylus* sp. and greatly underestimated it in *Proceratophrys* sp. (Fig. 3). In the following discussion, we comment on: (1) why gut contents and isotopes may over- or underestimate individual specialization; (2) the impact of missing prey categories; (3) the impact of the variance in fractionation among individuals on our method.

Value of gut contents and isotopes in measuring diet variation

Had we only analyzed gut contents, we would have concluded that the four species had roughly similar degrees of individual specialization (approximately 0.45). Double-checking these estimates with comparable isotope-derived measures of diet variation, we found a moderately close agreement between gut content and isotope-based measures of individual specialization in *Adenomera* sp. and *L. fuscus*. This supports the idea that gut-content variation may be a reasonable measure of diet variation in some systems, even in the case of terrestrial, relatively sedentary animals like frogs. However, in two other species gut contents appear to have yielded misleading measures of diet variation. For instance, isotopes suggest that *Eleutherodactylus* sp. has a much lower degree of individual specialization than the other species (expected WIC/TNW = 0.86). Since there is no a priori way of knowing how well gut contents will perform, we do not recommend the use of gut contents alone in studies of individual specialization, unless individuals are repeatedly sampled over time. In the case of 'snapshot' samples, other measures of temporal consistency (e.g., morphology and stable isotopes) should be used as a complementary approach.

It is reasonably easy to understand how gut contents would lead one to overestimate levels of individual specialization ('false specialists;' Warburton et al. 1998). If all individuals had similar preferences (low individual specialization), one may nevertheless see substantial variation among stomachs due to stochastic effects associated with patchy prey distributions, or limited stomach volume so that each consumer holds only a few prey at a time (Bolnick et al. 2002). This appears to be the case in *Eleutherodactylus* sp., since isotopes indicated that there was far less diet variation than we observed in gut contents (WIC/TNW = 0.86 and 0.46, respectively). *Eleutherodactylus* sp. is a small-sized frog (mean ± SD SVL = 14.5 ± 2.65 mm; $N = 124$) with small stomach capacity (mean ± SD number of prey items per stomach = 4.0 ± 2.42) that is found both on the ground and on the vegetation (up to 1 m high; A.A. Giaretta personal observation). These two microhabitats may constitute different 'patches' in terms of prey availability, which combined with the low stomach capacity of individuals' generated false specialists.

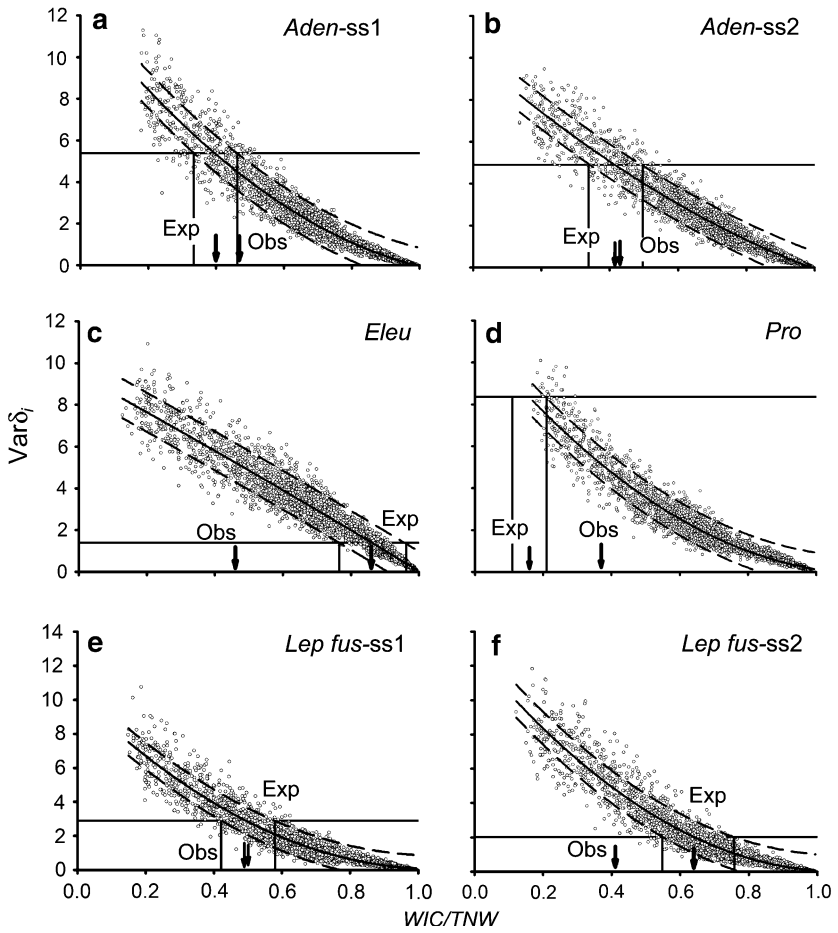

Fig. 3 Interpolation of WIC/TNW from isotope variances: the values of $\delta^{13}C$ variances ($Var\delta_i$) were regressed onto measures of individual specialization (WIC/TNW) of simulated populations (see text for details). *Solid curves* indicate quadratic fitted regressions; *dashed curves* are the prediction bands of the regressions; *horizontal solid lines* indicate the empirically estimated $Var\delta_i$; *vertical solid lines* define the confidence limits (95%) around the expected WIC/TNW. *Arrows* indicate the expected (*Exp*) WIC/TNW interpolated from the empirical $Var\delta_i$ using the regression equations and the observed (*Obs*) WIC/TNW from gut contents of four Brazilian frogs. **a** *Adenomera* sp.-ss1 ($N = 39$), **b** *Adenomera* sp.-ss2 ($N = 35$), **c** *Eleutherodactylus* sp. ($N = 56$), **d** *Proceratophrys* sp. ($N = 55$), **e** *Leptodactylus fuscus*-ss1 ($N = 38$), **f** *Leptodactylus fuscus*-ss2 ($N = 29$). *ss1* and *ss2* subsamples 1 and 2, respectively (see text)

It is more difficult to see why stomach contents would underestimate diet variation as compared to isotope variance, as in *Proceratophrys* sp. We propose three possible explanations for this conflict. First, if prey isotopic signatures vary temporally or spatially, individuals feeding on the same prey taxa, but collected in different times or places will show variation in signatures, so that one will observe isotopic variance that is not actually related to diet variation (Matthews and Mazumder 2004; Matthews and Mazumder 2005). In our study, we tried to mitigate this problem by testing for seasonal and spatial effects on the consumers' isotopic ratios, but we acknowledge we cannot rule out those effects entirely. Ideally, future studies should strive to assess the seasonal and spatial patterns of variation in the prey isotopic landscape by sampling prey isotopes in the field over the seasons and over space. Bearing those caveats in mind, we did not find any among-site or seasonal differences in the isotopes of *Proceratophrys* sp. (Table S2), indicating that spatial and seasonal variation in prey isotopes seems an unlikely explanation for the apparent conflict between gut contents and isotopes in this species.

Second, the preservation times before the isotopic analysis differed among individuals. If there are consistent shifts in isotopic signatures related to the time of preservation, this could have increased the isotopic variances of our samples. We tested isotopic signatures of our samples against time of preservation and found a positive relationship in *Proceratophrys* sp. ($r^2 = 0.133$; $F_{1,53} = 8.152$; $P = 0.006$; Fig. S2), but not in the other species (all P-values > 0.56; Fig. S2). This significant relationship, albeit weak, may indicate either an effect of preservation time or a temporal trend in the prey isotopic ratios. We see the former as an unlikely explanation for two reasons. First, Kaehler and Pakhomov (2001) and Sweeting et al. (2004) observed that after an initial period of isotopic shifts (4 weeks in the former and 1 day in the latter study) due to formalin or ethanol preservation of animal tissues, isotopic signatures remained stable for the whole duration of experiments (12 weeks in the former and 21 months in the latter). Since all our samples were analyzed after at least 45 months of preservation, we would not expect to see such a trend in the isotopic ratios of our samples due to the effect of preservatives. Second, if preservatives were

causing this shift, we would expect to see it in all the four species, because all samples were subject to the same type of preservation. We therefore believe it is more likely that this pattern reflects a temporal trend in one or a few food sources that were more consumed by *Proceratophrys* sp. than the other species (e.g., seeds; Table S1). In either case, future studies would benefit from standardizing the time of preservation of samples so that biases in the isotopic variance due to preservative-induced isotopic shifts will be avoided.

Finally, the very low isotope-derived value of WIC/TNW in *Proceratophrys* sp. might be a result of undersampling prey isotope variation (see below). It is likely that some individuals in our sample fed on some unknown prey with isotope signatures outside the range of what we observed in the most common prey. This is because some individual frogs had isotope signatures outside the range of prey isotopes (Fig. 2), and in *Proceratophrys* sp. the number of isotopic outliers was higher than in the other species (Fig. 2).

The mismatch between frogs' and prey signatures in our samples may have several reasons. First, consumers may show shifts in δ^{13}C in relation to their food sources due to fractionation (Vander Zanden and Rasmussen 2001). Second, we may have lacked the taxonomic resolution that would have allowed us to measure all the isotopic range of the consumed prey. While we made an effort in trying to avoid lumping ecologically divergent prey types into a single category, our taxonomic resolution (23 prey taxonomic categories, some including many families) almost certainly mixed prey with different signatures. The estimated average values for each category therefore masks a potentially higher variation among member taxa. It is possible that the prey signatures that would encompass all frog signatures in our samples are among these lumped taxa. Third, we may have actually missed some prey taxa in our sample. In principle, this should not be a likely explanation, given our large sample sizes. Moreover, we would not expect those missing prey types to be used frequently enough to strongly influence the frogs' isotopic ratios. This argument also holds for the taxa lumped under the category 'others,' which were not included in the isotope analyses for lack of material. None of these taxa accounted for more than 1% of prey items within any species' diet. Interestingly, however, in the case of *Proceratophrys* sp., all isotopic outliers are small juveniles (all below the 25th percentile of SVL). Differently from the other studied species, *Proceratophrys* sp. reproduces in permanent streams and has a long (many weeks; Giaretta A.A. unpublished results) aquatic larval development, whereas *Adenomera* sp. and *Eleutherodactylus* sp. have totally terrestrial development, and *L. fuscus* has a very short (2 weeks maximum) aquatic larval phase (Kokubum and Giaretta 2005; Giaretta A.A. unpublished results). The feeding habits of the larvae of *Proceratophrys* sp. are unknown, but the diet of tadpoles may well include isotopically depleted sources found in the aquatic environment (Matthews and Mazumder 2005; Paterson et al. 2006). Since we only sampled prey consumed in the terrestrial environment, this would explain why the isotopic range in this species was much larger than that of the sampled prey.

Impact of missing prey categories

Our simulation model is based on the assumption that we have a sufficient sample of prey taxa to generate a realistic relationship between WIC/TNW and Varδ_i. Underestimating the true variance in prey isotopes could lead to spurious estimates of WIC/TNW. To understand why this is the case, consider the *y*-intercept of the simulated curves (Fig. 3). This is the isotopic variance when each individual uses a single prey type (hence WIC/TNW = 0), and will be equal to the variance in the empirically determined prey isotopes (weighted by prey frequency in the population diet). Consequently, greater variances in the estimated prey isotopes will generate steeper regression curves. If one underestimates the true prey isotope variance (due to the problems discussed above), the simulated curve will be lower than it should actually be (Fig. 4a). As a result, a given empirical value of Varδ_i will lead to an interpolated WIC/TNW that is too low (overestimating individual specialization; Fig. 4a). This observation is of utmost importance for our results, because we used empirically estimated δ^{13}C variances to generate expected values of WIC/TNW. If we underestimated the variance in prey isotopes, as suggested by the isotopic outliers in *Proceratophrys* sp., then our regression curves are less steep than they should be, and we might have overestimated the degree of individual specialization. Conversely, overestimating prey variances, for instance by missing isotopically intermediate prey, will lead to an underestimate of individual specialization (higher WIC/TNW).

In light of these biases, our interpolation technique is most appropriate when isotope data are available for all prey taxa. This was not possible in this study due to the coarse taxonomic resolution for observed prey and/or our inability to ensure that all prey taxa were accounted for. Since we are unable to determine which prey isotope values we are missing, we took another approach to evaluating the impact of missing prey on our results. We redid our analysis of *Proceratophrys* sp., eliminating the individual frogs whose isotope signatures could not be explained by the observed prey isotopes (see Electronic Supplementary Material 5 for details). Eliminating the ten isotopic outliers reduced the δ^{13}C variance from 8.35 to 3.32, but did not

effectively change the gut-content estimates of WIC/TNW (0.37 vs. 0.38). In contrast, the isotope-derived estimates of WIC/TNW increased from 0.16 to 0.52, coming closer in line with the gut-content estimates and the values for two of the other species (Table S4, Fig. S3). This supports our view that the low isotope-derived value of WIC/TNW observed in *Proceratophrys* sp. may be a result of insufficient data on prey isotopes. Similar reanalysis increased the estimates of WIC/TNW of *Adenomera* sp. from approximately 0.40 to approximately 0.55 and had negligible effects on results for the other species (Electronic Supplementary Material 5), which had fewer isotopic outliers.

Impact of the variation in fractionation among individuals

As mentioned earlier, differential assimilation, fractionation, and isotopic routing may all cause a mismatch between signatures of food sources and those of consumers. More important, if there is variation among individuals in, e.g., fractionation, the population isotopic variance will be higher than would be expected based solely on diet variation (Matthews and Mazumder 2005). As a way of assessing the impact of fractionation on our model, we did simulations incorporating among-individual variance in fractionation (Var_Δ). We computed from the literature empirical measures of variation in fractionation among individuals fed on the same diet. We used an average $Var_\Delta = 0.73$ based on 12 such variances, 9 from the gerbil *Meriones unguienlatus* (Tieszen et al. 1983) and one from each of three bird species, the quail *Coturnix japonica*, the chicken *Gallus gallus*, and the gull *Larus delawarensis* (Hobson and Clark 1992). We ran simulations in which a fractionation value drawn randomly from a uniform distribution with variance 0.73 (range 0–2.96) was added to an individual's isotopic signature. In these simulations, sample size was set at $N = 30$, population diet was (0.2, 0.2, 0.2, 0.2, 0.2) and prey isotopes were (–31, –30, –29, –28, –27). The incorporation of Var_Δ in our model caused an upward shift in the resulting curve, so that even in the absence of diet variation (WIC/TNW = 1), there was a baseline isotopic variation (Fig. 4b). Interestingly, the difference between the *y*-values of this curve and that of a control curve generated with the same set of parameters, but no fractionation, corresponds to approximately Var_Δ (Fig. 4b). Additional simulations changing the value of Var_Δ (0.5 and 1.0) and the prey isotope range (–34, –32, –30, –28, –26) did not change this pattern. Therefore, if one has an estimate of Var_Δ for the studied organism, it is possible to correct its effect on the results of the model by subtracting Var_Δ from the empirical $Var\delta_i$ before interpolating the expected value of WIC/TNW (Fig. 4b). Using $Var_\Delta = 0.73$ as a correction for our samples, the expected WIC/TNW values increased by 0.05–0.1, indicating less diet variation. The change was not substantial, though, and there is still evidence of diet variation in three of the four species. It is worth mentioning that empirical estimates of Var_Δ may vary considerably among different taxonomic groups (e.g., gerbils = 1.01; birds = 0.4; average values). A more realistic estimate in the case of frogs might be quite different from 0.73.

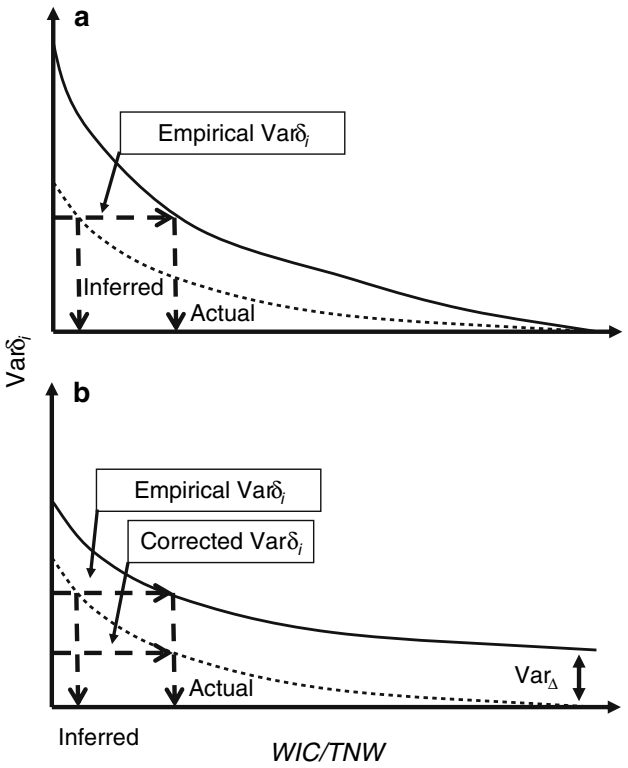

Fig. 4 Illustration of the effect of **a** incomplete sampling of prey on our model, and **b** variation in fractionation among individuals, based on simulations. **a** The *solid curve* represents the ''true'' relationship for a hypothetical prey community, while the *dotted curve* represents the relationship that is inferred from an incomplete sample of prey that missed isotopically extreme taxa and so underestimates the prey isotope variance. Using this incomplete dataset, one would infer an excessively low value of WIC/TNW. **b** *Solid curve* as in **a**, but now assuming that individuals vary in fractionation, while in the *dotted curve* no fractionation is assumed. By denying the among-individual variance in fractionation (Var_Δ), one would also underestimate WIC/TNW. However, if an empirical estimate of Var_Δ is available, it is possible to correct the estimate of WIC/TNW by using a ''corrected'' $Var\delta_i$, where corrected $Var\delta_i$ = empirical Var δ_i – Var_Δ

Conclusions

Gut contents may be a useful source of information on individual-level diet variation, especially if coupled with

data on stable isotopes. Information on the population $\delta^{13}C$ variance, combined with information on prey isotopes, can be a useful tool to test for the presence of individual specialization (Matthews and Mazumder 2004). The model presented here goes a step further by providing a way to generate, from information on isotopic variances, estimates of standardized indices of individual specialization (Bolnick et al. 2002) that can be compared among different populations or used to evaluate gut-content variation in different species and/or systems. This method requires thorough sampling of the isotope ratios of the prey community, but is otherwise straightforward. Individual specialization is a phenomenon with important ecological and evolutionary implications for populations. In a review of the incidence of individual specialization, Bolnick et al. (2003) make the case that most studies on individual specialization up to now were only able to test the null hypothesis that individuals in a population are all generalists and that we should be able to actually measure and compare the degrees of individual specialization across different populations. For instance, it is still unclear how widespread this phenomenon is among natural populations, as well as what ecological conditions will favor its evolution and maintenance. Quantifying individual specialization in a comparable manner is a necessary step in any attempt to answer these questions.

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