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Intra-Body Variations of Stable Isotope Ratios ($\delta^{13}C$, $\delta^{15}N$) and Influence of Storage Methods in Aquatic and Post-Aquatic Stages of the Common Toad, *Bufo bufo*

Andrey N. Reshetnikov * and Daniil I. Korobushkin

A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninskiy pr. 33, 119071 Moscow, Russia; dkorobushkin@yandex.ru

* Correspondence: anreshetnikov@yandex.ru

Abstract: Isotopic signatures of carbon and nitrogen are widely used for analysis of the structure of food webs in aquatic ecosystems. The study of animals raises a number of methodological questions, including choice of representative tissues and organs for sampling as well as storage of the studied organisms. Furthermore, the impacts of preservation methods can be tissue-specific, age-specific, and even taxon-specific; thus, studies of these impacts on particular taxa are necessary. We focused on the C and N isotope composition of the common toad (*Bufo bufo*), one of the most widespread European anuran amphibians. We hypothesized that its different tissues and organs may vary in isotopic composition, and ethanol and freezing may have different effects on isotopic values. Our results showed that both "tissue" and "storage method" factors significantly affected the $\delta^{13}C$ values of tadpoles and postmetamorphic juveniles, whereas only the "tissue" factor had a significant effect on the $\delta^{15}N$ values. The two stages, tadpoles and postmetamorphs, should be analyzed separately despite the brief postmetamorphic period of the juveniles. The skin, legs, muscles, and tail in tadpoles and legs, muscles and heart in juveniles can be used for $\delta^{13}C$ and $\delta^{15}N$ analysis regardless of the method of storage. The results will serve for the optimization of future study designs in isotopic ecology.

Keywords: ethanol storage; isotopic ecology; lake Glubokoe; trophic discrimination factors; trophic ecology

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1. Introduction

Isotopic signatures of carbon and nitrogen are widely used for analysis of the structure of food webs in both aquatic and terrestrial ecosystems [1–3]. The carbon $^{13}C/^{12}C$ ratio (usually expressed as $\delta^{13}C$) allows tracing basal carbon sources [4,5], whereas $^{15}N/^{14}N$ ratio ($\delta^{15}N$) increases as nitrogen passes from one consumer to another, indicating the trophic level of an animal in the trophic chain [6]. These phenomena make stable isotope analysis (SIA) a suitable tool for solving a wide range of questions in trophic ecology [7,8]. The unique isotope composition ("isotopic signature") of an animal reflects its diet over time with a certain (sometimes significant) delay. In this case, carbon and nitrogen of different tissues and functional systems of the body are updated at different rates [9–11].

In small-sized animals of terrestrial and aquatic taxa (e.g., collembolans, mites, microcrustaceans), the isotopic composition can be measured in the whole organism without selection of a representative tissue or organ [12–14]. The use of a whole organism of large-sized animals is not possible and raises a number of methodological questions. The isotopic composition of different organic compounds that make up the tissues of organisms is not the same. For example, lipids are depleted in ^{13}C and ^{15}N in comparison with proteins and sugars [15,16]. Different tissues and body parts of animals differ significantly in the mass content of proteins, lipids, and may contain bones (vertebrates) or chitin (invertebrates). These differences determine the variation in the isotopic composition of various

tissues and organs [14,17]. Indeed, a significant variation in the isotopic composition of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) within one organism was shown for a number of invertebrates and vertebrates, including diplopods [18], orthopterans [19], aphids [20], fishes [21], reptiles [22,23], birds [24] and mammals [25–27]. However, we know little about such differences between tissues in amphibian [28–30], which play an important role in trophic webs of both aquatic (in larval stage) and terrestrial (as adults) ecosystems.

Muscles are considered the most convenient tissue for stable isotope study of an animal because structural elements are assumed to have lower rates of isotopic incorporation than splanchnic tissues and organs [31]. Muscles have been often used in studies on vertebrates [21], including amphibians [29,32]. However, the extraction of muscle tissue from the legs of tadpoles is possible only in the final developmental stages. The extraction of muscles from the legs of juveniles can also be difficult due to the small sizes of the specimens. However, the use of a homogenized individual for isotopic analysis may result in an incorrect assessment due to the possible chemical differences between tissues and organs.

Another important question relates to the methods of material preservation prior to isotopic analysis. Freeze drying and subsequent dry storage is considered the most convenient conservation method [33], which does not affect the isotopic composition of the material [34–36]. However, the use of a freeze-dryer for the preservation of samples during field studies is very difficult, since this is a piece of stationary laboratory equipment that is difficult to transport to the field. Under these conditions, samples can be frozen in the freezer of a household or mobile refrigerator (usually at −18 °C) or fixed with preservative liquids, among which ethanol is most frequently used [37]. However, ethanol is known to alter stable isotope ratios in samples [27,36,38]. Furthermore, the impacts of storage methods are taxon-specific, age-specific (see e.g., [33,39,40]) and can even be tissue-specific. Thus, studies on the impacts of these storage methods on particular taxa are necessary.

The influence of preservation methodology on stable isotope ratios has also been investigated in various taxa, such as terrestrial invertebrates [33,41], birds [42] and mammals [27], as well as aquatic microorganisms [39,43], invertebrates [36,44], fishes [45], and mammals [46]. Nevertheless, the influence of the storage method on the ratio of stable isotopes of such an important taxon as amphibians is not clear. Amphibian larvae occupy basal trophic niches, can reach significant seasonal biomass in fresh water bodies, and, after metamorphosis, move a great volume of organic matter to land, linking aquatic and terrestrial ecosystems [47,48]. After metamorphosis, anuran amphibians change their feeding habits from omnivorous to predatory [48]. The same is true for toads (Bufonidae) which are periodically the object of ecological studies with stable isotope analysis [49,50]. Moreover, there is practically no data on the effect of the storage method on different tissues of toads, as fixation with ethanol can alter the isotopic values of one tissue, but not affect the other [27]. Thus, in addition to choosing a particular tissue for isotope analysis, it is necessary to compare the methods of its preservation.

In our study, we focused on isotope composition of the common toad (*Bufo bufo*), one of the most widespread European anuran species. We hypothesized that different tissues may differ in isotopic composition, and ethanol may affect the isotopic values of C and N, and formulated the following tasks: (i) to assess the differences in the C and N isotopic composition of tissues and organs within the same developmental stages for tadpoles and separately for juveniles; (ii) to test the impact of the most common preservation method (ethanol) on isotope composition of different tissues. Additionally, we compared isotopic values of the same tissues and organs of larval ("tadpoles") and newly-metamorphosed juvenile (hereafter referred to as "juveniles") toads.

2. Materials and Methods

2.1. Experimental Design and Sampling

We collected *B. bufo* samples at the Lake Glubokoe Hydrobiological Station (N 55°45′; E 36°30′) of the A.N. Severtsov Ecology and Evolution Institute, the Russian Academy of

Sciences, located 70 km west from the center of Moscow [51]. Toads of two ages (tadpoles and juveniles; hereafter the factor is referred to as "stage") were caught simultaneously on 19 June 2019. The Glubokoe lake has a 60 m wide nearshore shallow waters, overgrown by macrophytes with a domination of water horsetail *Equisetum fluviatile* L., 1753, common reed *Phragmites australis* (Cav.) Trin. ex Steud., 1871, and broadleaf cattail *Typha latifolia* L. (1753). The lake and surroundings belong to Glubokoe Lake Nature Reserve. There are no significant anthropogenic local pollutants, such as sewage, which can potentially affect isotopic composition of basal food of tadpoles in the studied lake. The region is characterized by warm summers and moderately cold winters, with the mean monthly temperatures ranging from −10 °C (January) to +17 °C (July) and an average annual precipitation of about 700 mm [52].

Tadpoles were collected close to the shoreline and newly-metamorphosed juveniles were collected on the bank near the shoreline. The collected tadpoles were at Gosner's (1960) stage 37.4 ± 0.9 (mean ± standard error is presented here and below) with sizes: L = 33.6 ± 0.3 mm; m = 441.6 ± 10.1 mg; n = 10. All the collected juveniles (L = 12.3 ± 0.3 mm; m = 181.6 ± 9.0 mg; n = 10) completed metamorphosis and had resorbed their tail. After collection, tadpoles and juveniles were left starving in separate containers without food for two days to evacuate the digestive tract. The studied specimens were sacrificed by immersion in ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich). This anesthetic does not have significant influence on isotope ratios of tissues [30]. Half of the caught specimens were placed in a freezer and stored at −18 °C, other half of the samples were fixed in 96% ethanol (hereafter the factor is referred to as "storage method"). We used the same quality and quantity of ethanol for all the samples. The samples were processed in February 2020, with a duration of storage of 8 months. Furthermore, the tissues and organs were extracted from each specimen (hereafter the factor is referred to as "tissue"). The replicate of each tissue or organ was five (from five different specimens). Thus, we used 10 larval individuals (5 for analysis of frozen and 5 for analysis of ethanol-stored tissues) as well as 10 juveniles (5 for analysis of frozen and 5 for ethanol-stored tissues). Additionally, 10 larval specimens were homogenized as a whole for analysis of freezing and ethanol storage effects (5 and 5, respectively) and 10 juvenile specimens were used as a whole to study freezing and ethanol storage effects (5 and 5, respectively). The following tissues and organs were analyzed in tadpoles: muscles from back, hind legs, skin, heart, tail, digestive tract including intestines, and larval stomach, the *manicotto glandulare*, (hereafter referred to as "muscles", "legs", "skin", "heart", "tail" and "guts", respectively) and the whole homogenized specimens (hereafter referred to as "whole organism"). Unfortunately, we were not able to analyze heart samples from tadpoles after freezing because of destruction of this organ upon thawing. Thus, heart was excluded from some pairwise comparisons. The following tissues and organs were analyzed in juveniles after freezing and separated after ethanol fixation: muscles, legs, skin, heart, digestive tract (including guts and well-developed stomach) and whole homogenized specimens (hereafter referred to as "muscles", "legs", "skin", "heart", "guts" and "whole organism", respectively). The collection and processing of the organisms are in line with all current regulations of the Russian Federation and approved by the Animal Care and Use Committee of the A.N. Severtsov Ecology and Evolution Institute, the Russian Academy of Sciences (permit # 12 from 14 May 2018).

2.2. Stable Isotope Analysis

All tissue samples were separately oven-dried at 50 °C for 3 days. To assess the mean isotopic values of whole specimen, we homogenized samples after drying using a ball mill (Retsch MM200, Retsch GmbH, Haan, Germany). Then, samples were weighed (approx. 300 μg) and wrapped in tin foil. Their isotopic compositions were determined using a Thermo-Finnigan Delta V Plus continuous-flow mass spectrometer (Thermo Electron GmbH, Bremen, Germany) coupled with an elemental analyzer (Thermo Flash 1112, Thermo Electron) at the Joint Usage Center "Instrumental Meth-

ods in Ecology" at the IEE RAS. The isotopic composition of N and C was expressed in the δ-notation relative to the international standards (atmospheric nitrogen and VPDB): $\delta X(‰) = [(R_{sample}/R_{standard}) - 1] \times 1000$, where R is the molar ratio of the heavier isotope to the lighter isotope. Samples were analyzed with reference gases calibrated against IAEA (Vienna, Austria), reference materials USGS 40 and USGS 41. The drift was corrected using an internal laboratory standard (casein). The standard deviation of $\delta^{15}N$ and $\delta^{13}C$ values in the reference material was <0.15‰ ($n = 8$). Along with stable isotope composition, the average mass values of nitrogen and carbon (%) were determined. A total of 125 samples and 20 internal laboratory standards (casein) were analyzed for $\delta^{13}C$, $\delta^{15}N$, C and N.

2.3. Data Analysis

Thus, the experiment had a factorial design with the three following treatments: the factors "stage", "tissue", and "storage method", the influence of which was tested on carbon and nitrogen isotopic values, as well as on C/N ratios. To assess the effect of the factors "tissue", "storage method", and their interaction (shown as "Storage method × Tissue") on the $\delta^{13}C$, $\delta^{15}N$ and C/N values of tadpoles and juveniles separately ($n = 60$ with exception of 5 frozen heart samples and $n = 60$ samples, respectively), we applied general linear modelling (GLM) with the forward stepwise predictor selection method which permutes combinations of variables to find the best model built from variables with significant effects [53].

To estimate differences between $\delta^{13}C$ and $\delta^{15}N$ values of *B. bufo* tadpoles and juveniles, we applied GLM, forward stepwise predictor selection method, with factors "stage", "tissue", and their interaction (shown as "Stage × Tissue"). For this aim we used 100 samples in total, of which 50 were frozen and 50 were ethanol-fixed. The analyses were provided separately for frozen ($n = 50$) and ethanol-fixed ($n = 50$) samples. The comparison was made for samples of muscles, skin, guts, and legs taken from amphibians of both stages and whole specimens were used.

Before analyses, for assessing homogeneity and normality of the selected model, the data were tested using the Statistica residuals tool. All dependent variables were also checked for normal distribution with the Shapiro-Wilk test and showed to be normally distributed and homogenous and required no transformation, which allows us to apply GLM. After GLM, the significance of differences between means was assessed using Tukey's HSD test. All statistical hypotheses were tested at the $p < 0.05$ significance level. Data processing was performed using Statistica 13.3 (TIBCO Software, Palo Alto, CA, USA).

3. Results

3.1. "Tissue" and "Storage Method" Factors in Tadpoles

Both "tissue" and "storage method" factors significantly affected the $\delta^{13}C$ values of tadpoles (Table 1), but not the interaction of these factors. Fixation in ethanol increased $\delta^{13}C$ values as compared to frozen tadpoles by an average of 0.4‰ (Figure 1A). However, pairwise comparison of different tissues and organs showed significant differences only for comparisons of the digestive tract. Fixation with ethanol of other tissues and organs did not affect $\delta^{13}C$ values (Figure 1A). The range of $\delta^{13}C$ mean values between different tadpole organs and tissues averaged about 1.5‰. The digestive tract was significantly depleted in ^{13}C relative to the other organs (Figure 1A).

Only the "tissue" factor had a significant effect on the $\delta^{15}N$ values of tadpoles (Table 1). The highest $\delta^{15}N$ values were observed in tail, muscles, and the whole body (Figure 1B). The lowest $\delta^{15}N$ values were in the digestive tract, which on average differed from the tail, muscles and the whole homogenized organism by 1.2‰, 0.7‰, and 0.7‰, respectively.

Storage of the sample in ethanol significantly reduced the C/N ratio in the tissues of the digestive tract, muscles, legs, tail, and the whole organism, but not in the skin (Figure 1C).

Table 1. Results of GLM (forward stepwise selection of factors) for mean $\delta^{13}C$, $\delta^{15}N$ and C/N values of *Bufo bufo* tadpoles and juveniles. Heart values were removed from the "tadpole" dataset. "SS"—sums of the squares, "p"—probability levels, "Error" refers to the total unexplained variance remaining in the model. "n.s." indicates lack of significant results ($p > 0.05$).

Factors	$\delta^{13}C$		$\delta^{15}N$		C/N	
	SS	p	SS	p	SS	p
Tadpoles						
Storage method	2.42	0.037		n.s.	3.28	<0.0001
Tissue	10.3	0.0044	11.78	<0.0001	9.68	<0.0001
Storage method × Tissue		n.s.		n.s.	0.80	0.0084
Error	28.05		12.8		2.16	
Model R^2	0.31		0.479		0.864	
Model p		0.0022		<0.0001		<0.0001
Juveniles						
Storage method	6.99	0.0004		n.s.	3.2	<0.0001
Tissue	7.48	0.016	6.3	0.0001	2.67	<0.0001
Storage method × Tissue		n.s.		n.s.	1.37	<0.0001
Error	22.5				1.01	
Model R^2	0.386		9.2		0.878	
Model p		0.00057	0.406	0.0001		<0.0001

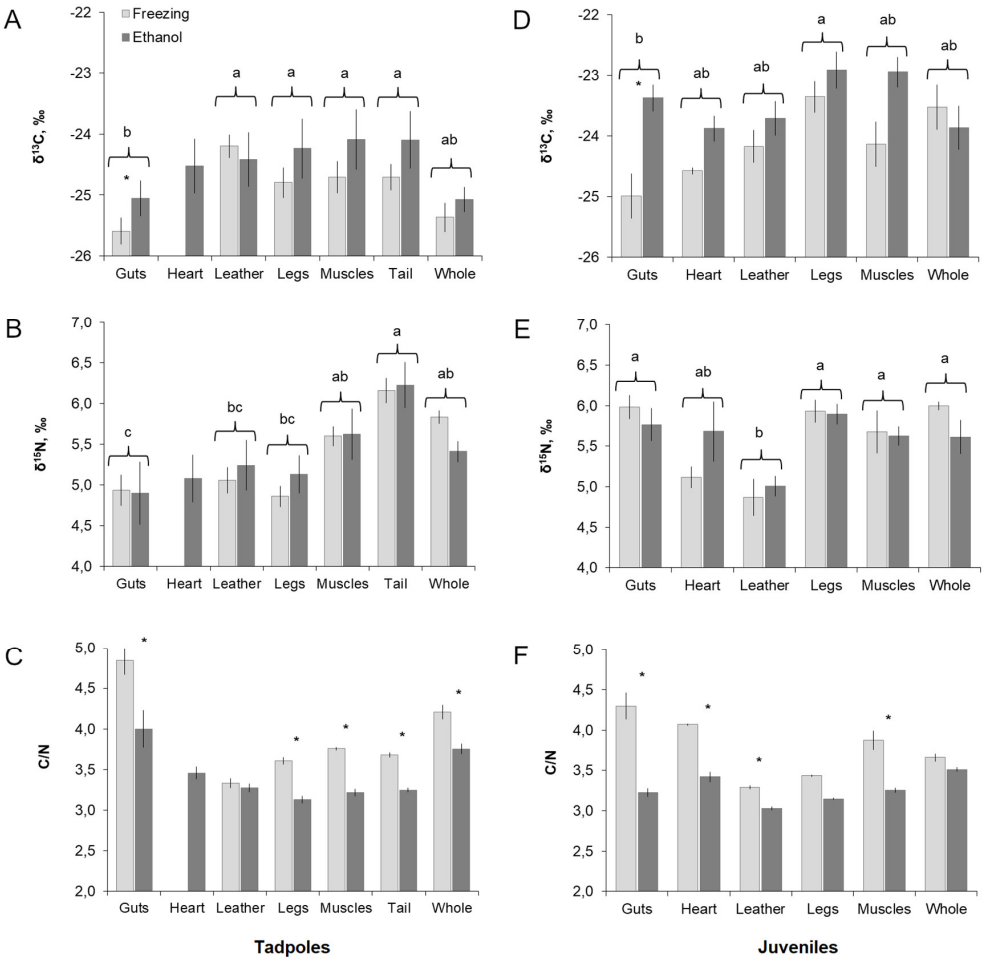

Figure 1. Stable isotope composition $\delta^{13}C$, $\delta^{15}N$ (‰ ± SE) and C/N of *B. bufo* tadpoles (**A**–**C**) and juveniles (**D**–**F**). Asterisks indicate significant differences between frozen and ethanol-fixed tissues according to Tukey HSD test after GLM, $p < 0.05$. Different letters indicate significant differences between mean values of certain type of tissues by Tukey HSD test. Heart of frozen tadpoles was not analyzed.

3.2. "Tissue" and "Storage Method" Factors in Juveniles

Both factors "tissue" and "storage method" significantly influenced the $\delta^{13}C$ values in juveniles (Table 1), but not the interaction of the factors. Storage of samples in ethanol on average increased the $\delta^{13}C$ values by 0.68‰. The highest difference between frozen and ethanol-fixed samples was in the digestive tract (Figure 1D). Mean $\delta^{13}C$ values of different tissues varied in the range of 1‰, but only $\delta^{13}C$ values of digestive tract (about −24.1‰) and legs (about −23.1‰) differed significantly (Figure 1D).

The $\delta^{15}N$ values were influenced only by the "tissue" factor (Table 1). The mean $\delta^{15}N$ values were significantly lower in the skin than in the digestive tract, legs, muscles, and the whole organism (Figure 1E), with the spread of mean values about 0.97‰.

The juvenile C/N ratio significantly depended on the interaction of factors "tissue" and "storage method" (Table 1). As in tadpole tissues fixed with ethanol, the C/N ratios of ethanol-fixing tissues of juveniles were significantly lower compared to frozen ones, including skin but not legs and whole homogenized organism (Figure 1F).

3.3. The Tissue Isotope Composition and Effect of Ethanol: Tadpoles vs. Juveniles

The $\delta^{13}C$ values significantly differed (factor "stage") in both frozen and ethanol-fixed tadpoles and juveniles (Table 2). In general, juveniles had higher $\delta^{13}C$ values as compared to tadpoles (Figure S1A,C). The muscles, legs, and whole organisms showed lower $\delta^{13}C$ values in frozen tadpoles compared to frozen juveniles (Figure S1A). The digestive tract and whole organism of individuals fixed with ethanol were also significantly different (Figure S1C). The skin did not differ significantly between stages for any of the fixation methods.

Table 2. Results of $\delta^{13}C$ and $\delta^{15}N$ values comparison between the same tissues of *B. bufo* tadpoles and juveniles using GLM (forward stepwise selection of factors). "n.s." indicates lack of significant results ($p > 0.05$).

Factor	Freezing				Ethanol			
	$\delta^{13}C$		$\delta^{15}N$		$\delta^{13}C$		$\delta^{15}N$	
	SS	p	SS	p	SS	p	SS	p
Stage	9.26	0.000015	2.165	0.000293	18.49	0.000001	1.292	0.044
Tissue	8.96	0.000807	4.378	0.000087	6.79	0.030980		n.s.
Stage × Tissue	4.68	0.025843	3.258	0.000784		n.s.		n.s.
Error	13.84		5.011		25.44		14.54	
Model R^2	0.61		0.66		0.498		0.082	
Model p		0.00002		0.000002		0.000008		0.044

The $\delta^{15}N$ values significantly differed (factor "stage") in both frozen and ethanol-fixed tadpoles and juveniles (Table 2). The $\delta^{15}N$ difference between the tissues of tadpoles and juveniles showed a similar trend in both frozen and ethanol-fixed animals (Figure S1B,D). Significant $\delta^{15}N$ differences between frozen tadpoles and juveniles were found in the digestive tracts and legs (Figure S1B).

4. Discussion

4.1. Differences in Stable Isotope Signatures of Tissues/Organs and Influence of Storage Methods

4.1.1. Tissues/Organs

The results show differences in stable isotope signatures of some tissues/organs of the common toad at both age stages. The digestive tract of tadpoles was significantly depleted in ^{13}C relative to other organs and depleted in ^{15}N relative to the muscles and tail; the digestive tract of juveniles was significantly depleted in ^{13}C relative to the legs and enriched in ^{15}N relative to the skin (Figure 1A,B,D,E). These differences in isotope compositions can most likely be caused by the remains of food or presence of parasites and symbionts in the digestive tract (as special cleaning procedures were not performed for this isotope analysis). Indeed, we noted that the digestive tract of all tadpole and some juvenile individuals

contained the remains of food despite the 2-day forced starving period. Tadpoles could repeatedly consume faeces due to caprophagy [48] from the bottom of containers and juveniles could most likely digest slowly or hunt small flying invertebrates. In amphibian studies, gut contents are often analyzed separately to assess the isotopic composition of their food [54]. The differences between consumer's tissues and its food are common phenomenon conditioned by diet-tissue discrimination factors [55]. Moreover, there is no comprehensive information about the possible influence of symbiotic intestinal macro- and microorganisms and microbial fermentation processes in tadpole intestines [56–59] on isotope assimilation by an organism and on whole gut content chemical composition. Theoretically, parasites, symbionts and microbial intestinal components may affect the resulting isotope signature of gut content.

Earlier, Cloyed et al. [29] assessed tissue-specific trophic discrimination factors in the adult green frog *Lithobates clamitans* (Latreille, 1801) and found that bone collagen is the most affected tissue, compared to the skin and whole blood. However, we could not compare our results with those of Cloyed et al. [29] because taking of blood and bone collagen samples is rather difficult in small-sized amphibians.

4.1.2. Storage Method

We compared the two common storage methods that may be useful in absence of the freeze-drying device. As a whole, preservation of samples with ethanol can increase $\delta^{13}C$ values but not $\delta^{15}N$ values relative to freezing samples. Despite that freezing is the more preferable method for investigation of $\delta^{13}C$ values [41], the choice of tissue preservation method is less important for analysis of $\delta^{15}N$ signature in tadpoles and juveniles: both methods provide similar results and are well applicable. The greater impact of ethanol on the ^{13}C value may be conditioned by presence, in this preservative, of carbon with its own specific isotopic signature, which can alter the isotope ratios of the analyzed tissues by dissolving and extracting lipids, which have lower ^{13}C content relative to carbohydrates and proteins [33].

Importantly, we studied samples after a comparatively long storage period (8 months). Earlier, investigations of samples after a brief (5 days) storage did not reveal any impact of the ethanol method upon neither $\delta^{13}C$ nor $\delta^{15}N$ of tissues (e.g., [41]). Additionally, the effect of preservation methods on stable isotope composition may depend on storage duration [38,60].

In our study, storage of samples in ethanol significantly reduced the C/N ratio in the tissues of separate organs and the whole organism, but not in the skin of tadpoles and legs of juveniles (Figure 1C,D). Obviously, these organs contain a minimal amount of fat [61] resulting in a non-significant impact of ethanol. Similar to the above-discussed impact to $\delta^{13}C$ value, this is apparently associated with the destruction of lipids and adsorption of C from fat-containing tissues [33].

4.2. Practical Recommendations Concerning Choice of Tissues/Organs and Preservation Methods (Freezing vs. Ethanol) for Stable Isotope Analysis

In tadpoles, any organ other than the digestive tract can be used for $\delta^{13}C$ analysis, regardless of the method of storage, because the digestive tract of tadpoles and whole homogenized specimens have reduced $\delta^{13}C$ relative to muscles, which are most commonly used in isotopic analysis. Excluding the digestive tract from the stable isotope analyses avoids the potential problems associated with cleaning the digestive tract from food residues, parasites, symbionts, and microbes. When analyzing tadpoles for $\delta^{15}N$ values, we can disregard the method of fixation. However, deleting the digestive tract is necessary because it may significantly differ in ^{15}N relative to other organs and, in particular, muscles. Additionally, the heart is not a convenient organ for analysis of samples after freezing because of post-freezing decomposition. The most popular tadpole tissues in stable isotope studies are muscles from the tail [62–64], but small-sized tadpoles are often used as a whole but without the digestive tract [62] or after a gut clearing period [65]. Our results showed that the above-listed approaches are appropriate for stable isotope analysis, however

special attention should be given to gut clearing procedures because caprophagy is typical for many larval amphibians [48] and symbionts, parasites and microbes may have the potential to impact the resulting isotope signature.

In juveniles, any organs other than the digestive tract or whole organism may be used for $\delta^{13}C$ analysis regardless of the method of fixation. Any organs of juveniles may be also used for $\delta^{15}N$ analysis regardless of the method of storage, but it is better to avoid analysis of the skin, which is depleted in ^{15}N relative to other organs. Restriction concerning usage of the digestive tract for isotope analysis in tadpoles is also important for juveniles. Our results suggest that the traditional usage of muscle tissue in stable isotope studies of amphibian juveniles (e.g., [37]) is quite a convenient method.

The results also showed that different developmental stages have to be analyzed separately as these stages differ significantly in both studied isotope values. Additionally, significant $\delta^{15}N$ differences between frozen tadpoles and juveniles were found in the digestive tracts and legs. The guts of tadpoles and juveniles could contain remains of principally different food objects, as tadpoles are omnivorous, whereas juveniles are obligatory predators [48,66]. This also provides some restrictions for using the whole organism samples. Legs present as rudiments in larval amphibians but rapidly develop and grow during metamorphosis and in early juveniles [48]; so these organs undergo a faster incorporation of chemical elements and their isotopic composition does not reflect averaged food assimilation during entire larval period.

In general, we do not recommend the usage of the digestive tract in the analysis of both tadpoles and juveniles, even after a starvation period. The usage of whole homogenized organisms should be avoided, as the digestive tract or internal fat can significantly affect mean $\delta^{13}C$ values. The use of muscle tissues may be most accurate for analysis in tadpoles and juveniles but some non-muscle organs are also well suited for analysis. Our experience suggests that the most convenient or easiest method would be using the muscle tissues from back part (closely to vertebral column) of tadpoles or juveniles because this body part is not transformed drastically during metamorphosis [61].

Importantly, non-lethal sampling of tissues for stable isotope analysis (small pieces of fins for larvae or fingers for adults) is possible and preferable for large-sized specimens of amphibians [30,54] because this group of vertebrates is recognized to be declining since the second half of 20th century [67]. However, when sampling small-sized larvae or juveniles it is difficult to adhere to this guidance. Therefore, we hope our findings will serve for optimization of future study designs in trophic ecology and hence will decrease the number of used amphibian individuals.

5. Conclusions

In tadpoles, skin, legs, muscle and tail can be used for joint ^{13}C and ^{15}N analysis regardless of the method of storage. Whole homogenized individuals may also somewhat differ in ^{13}C and ^{15}N from muscle tissues due to the large proportion of the digestive tract relative to the whole body. It is likely that, for small-sized larvae and newly-metamorphosed juveniles of anuran amphibians, one of the optimal approaches is to remove the digestive tract (or all viscera), homogenize the remaining organism and then use it to estimate $\delta^{13}C$ and $\delta^{15}N$ isotope values.

In the absence of a freeze-dryer, the most convenient method is freezing of amphibian samples for storage before stable isotope analysis. Ethanol is also an appropriate storage method for some of the above-listed tissues and organs, or a whole organism after deletion of the digestive tract, however, long-term storage in ethanol may slightly affect $\delta^{13}C$.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13182544/s1, Figure S1: Stable isotope composition ($\delta^{13}C$ and $\delta^{15}N$ values) of *B. bufo* tadpoles and juveniles after freezing (A and B) and ethanol-fixation (C and D). Asterisks indicate significant differences between tadpoles and juveniles according to the Tukey HSD test after GLM, $p < 0.05$.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the A.N. Severtsov Institute of Ecology and Evolution, the Russian Academy of Sciences (protocol # 12 from 14 May 2018).

Informed Consent Statement: Not applicable.

Data Availability Statement: The primary data are available upon request to the authors.

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Conflicts of Interest: The authors have no conflicts of interest/competing interests.

References

1. Peterson, B.J.; Fry, B. Stable isotopes in ecosystem studies. *Annu. Rev. Ecol. Evol. Syst.* **1987**, *18*, 293–320. [CrossRef]
2. Layman, C.A.; Araujo, M.S.; Boucek, R.; Hammerschlag-Peyer, C.M.; Harrison, E.; Jud, Z.R.; Matich, P.; Rosenblatt, A.E.; Vaudo, J.J.; Yeager, L.A.; et al. Applying stable isotopes to examine food-web structure: An overview of analytical tools. *Biol. Rev.* **2011**, *87*, 545–562. [CrossRef]
3. Boecklen, W.J.; Yarnes, C.T.; Cook, B.A.; James, A.C. On the use of stable isotopes in trophic ecology. *Annu. Rev. Ecol. Evol. Syst.* **2011**, *42*, 411–440. [CrossRef]
4. Araujo-Lima, C.; Forsberg, B.R.; Victoria, R.; Martinelli, L. Energy Sources for Detrivorous Fishes in the Amazon. *Science* **1986**, *234*, 1256–1258. [CrossRef] [PubMed]
5. Anderson, W.B.; Polis, G.A. Marine subsidies of island communities in the Gulf of California: Evidence from stable carbon and nitrogen isotopes. *Oikos* **1998**, *81*, 75–80. [CrossRef]
6. Post, D.M. Using stable isotopes to estimate trophic position: Models, methods, and assumptions. *Ecology* **2002**, *83*, 703–718. [CrossRef]
7. Hobson, K.A. Tracing origins and migration of wildlife using stable isotopes: A review. *Oecologia* **1999**, *120*, 314–326. [CrossRef]
8. Newsome, S.D.; Yeakel, J.D.; Wheatley, P.V.; Tinker, M.T. Tools for quantifying isotopic niche space and dietary variation at the individual and population level. *J. Mammal.* **2012**, *93*, 329–341. [CrossRef]
9. Schmidt, O.; Scrimgeour, C.M.; Curry, J.P. Carbon and nitrogen stable isotope ratios in body tissue and mucus of feeding and fasting earthworms (*Lumbricus festivus*). *Oecologia* **1999**, *118*, 9–15. [CrossRef] [PubMed]
10. Phillips, D.L.; Eldridge, P.M. Estimating the timing of diet shifts using stable isotopes. *Oecologia* **2006**, *147*, 195–203. [CrossRef]
11. Gratton, C.; Forbes, A. Changes in $\delta^{13}C$ stable isotopes in multiple tissues of insect predators fed isotopically distinct prey. *Oecologia* **2006**, *147*, 615–624. [CrossRef]
12. Klarner, B.; Maraun, M.; Scheu, S. Trophic diversity and niche partitioning in a species rich predator guild—Natural variations in stable isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$) of mesostigmatid mites (Acari, Mesostigmata) from Central European beech forests. *Soil Biol. Biochem.* **2013**, *57*, 327–333. [CrossRef]
13. Schilder, J.; Tellenbach, C.; Möst, M.; Spaak, P.; van Hardenbroek, M.; Wooller, M.J.; Heiri, O. The stable isotopic composition of Daphnia ephippia reflects changes in $\delta^{13}C$ and $\delta^{18}O$ values of food and water. *Biogeosciences* **2015**, *12*, 3819–3830. [CrossRef]
14. Potapov, A.M.; Tiunov, A.V.; Scheu, S. Uncovering trophic positions and food resources of soil animals using bulk natural stable isotope composition. *Biol. Rev.* **2019**, *94*, 37–59. [CrossRef]
15. Deniro, M.J.; Epstein, S. Influence of diet on distribution of carbon isotopes in animals. *Geochim. Cosmochim. Acta* **1978**, *42*, 495–506. [CrossRef]
16. Webb, S.C.; Hedges, R.E.M.; Simpson, S.J. Diet quality influences the $\delta^{13}C$ and $\delta^{15}N$ of locusts and their biochemical components. *J. Exp. Biol.* **1998**, *201*, 2903–2911. [CrossRef]
17. Shipley, O.N.; Matich, P. Studying animal niches using bulk stable isotope ratios: An updated synthesis. *Oecologia* **2020**, *193*, 27–51. [CrossRef] [PubMed]
18. Semenyuk, I.I.; Tiunov, A.V. Intraspecific dispersion and age changes of isotopic composition ($^{15}N/^{14}N$, $^{13}C/^{12}C$) of diplopod tissues (Myriapoda, Diplopoda). *Izv. Penz. Gos. Pedagog. Univ. Im. V.G. Belinskogo* **2011**, *25*, 428–436. (In Russian)
19. Wehi, P.; Hicks, B. Isotopic fractionation in a large herbivorous insect, the Auckland tree weta. *J. Insect Physiol.* **2010**, *56*, 1877–1882. [CrossRef] [PubMed]

20. Perkins, M.J.; McDonald, R.A.; van Veen, F.J.F.; Kelly, S.D.; Rees, G.; Bearhop, S. Important impacts of tissue selection and lipid extraction on ecological parameters derived from stable isotope ratios. *Methods Ecol. Evol.* **2013**, *4*, 944–953. [CrossRef]
21. Pinnegar, J.K.; Polunin, N.V.C. Differential fractionation of $\delta^{13}C$ and $\delta^{15}N$ among fish tissues: Implications for the study of trophic interactions. *Funct. Ecol.* **1999**, *13*, 225–231. [CrossRef]
22. Murray, I.W.; Wolf, B.O. Diet and growth influence carbon incorporation rates and discrimination factors ($\Delta^{13}C$) in desert box turtles, *Terrapene ornata luteola*. *Herpetol. Conserv. Biol.* **2013**, *8*, 149–162.
23. Warne, R.W.; Wolf, B.O. Nitrogen stable isotope turnover and discrimination in lizards. *Rapid Commun. Mass Spectrom.* **2021**, *35*, e9030. [CrossRef]
24. Hobson, K.A.; Clark, R.G. Assessing avian diets using stable isotopes 2. Factors influencing diet-tissue fractionation. *Condor* **1992**, *94*, 189–197. [CrossRef]
25. Lesage, V.; Hammill, M.O.; Kovacs, K.M. Diet-tissue fractionation of stable carbon and nitrogen isotopes in phocid seals. *Mar. Mam. Sci.* **2002**, *18*, 182–193. [CrossRef]
26. Ehrich, D.; Tarroux, A.; Stien, J.; Lecomte, N.; Killengreen, S.; Berteaux, D.; Yoccoz, N.G. Stable isotope analysis: Modelling lipid normalization for muscle and eggs from arctic mammals and birds. *Methods Ecol. Evol.* **2011**, *2*, 66–76. [CrossRef]
27. Javornik, J.; Hopkins, J.B.; Zavadlav, S.; Levanič, T.; Lojen, S.; Polak, T.; Jerina, K. Effects of ethanol storage and lipids on stable isotope values in a large mammalian omnivore. *J. Mammal.* **2019**, *100*, 150–157. [CrossRef]
28. Caut, S.; Angulo, E.; Courchamp, F. Variation in discrimination factors ($\delta^{15}N$ and $\delta^{13}C$): The effect of diet isotopic values and applications for diet reconstruction. *J. Appl. Ecol.* **2009**, *46*, 443–453. [CrossRef]
29. Cloyed, C.S.; Newsome, S.D.; Eason, P.K. Trophic discrimination factors and incorporation rates of carbon- and nitrogen-stable isotopes in adult green frogs, Lithobates clamitans. *Physiol. Biochem. Zool.* **2015**, *88*, 576–585. [CrossRef] [PubMed]
30. Bélouard, N.; Petit, E.J.; Huteau, D.; Oger, A.; Paillisson, J.-M. Fins are relevant non-lethal surrogates for muscle to measure stable isotopes in amphibians. *Knowl. Manag. Aquat. Ecosyst.* **2019**, *420*, 2–8. [CrossRef]
31. Martinez del Rio, C.; Wolf, N.; Carleton, S.A.; Gannes, L.Z. Isotopic ecology ten years after a call for more laboratory experiments. *Biol. Rev.* **2009**, *84*, 91–111. [CrossRef]
32. Smith, R.L.; Beard, K.H.; Shiels, A.B. Different prey resources suggest little competition between non-native frogs and insectivorous birds despite isotopic niche overlap. *Biol. Invasions* **2017**, *19*, 1001–1013. [CrossRef]
33. Krab, E.J.; Van Logtestijn, R.S.P.; Cornelissen, J.H.C.; Berg, M.P. Reservations about preservations: Storage methods affect delta C-13 signatures differently even in closely related soil fauna. *Methods Ecol. Evol.* **2012**, *3*, 138–144. [CrossRef]
34. Barrow, L.M.; Bjorndal, K.A.; Reich, K.J. Effects of preservation method on stable carbon and nitrogen isotope values. *Physiol. Biochem. Zool.* **2008**, *81*, 688–693. [CrossRef]
35. Fleming, N.E.C.; Houghton, J.D.R.; Magill, C.L.; Harrod, C. Preservation methods alter stable isotope values in gelatinous zooplankton: Implications for interpreting trophic ecology. *Mar. Biol.* **2011**, *158*, 2141–2146. [CrossRef]
36. Le Bourg, B.; Lepoint, G.; Michel, L.N. Effects of preservation methodology on stable isotope compositions of sea stars. *Rapid Commun. Mass Spectrom.* **2020**, *34*, e8589. [CrossRef] [PubMed]
37. Bissattini, A.M.; Buono, V.; Vignoli, L. Disentangling the trophic interactions between American bullfrogs and native anurans: Complications resulting from post metamorphic ontogenetic niche shifts. *Aquat. Conserv. Mar. Freshw. Ecosyst.* **2019**, *29*, 270–281. [CrossRef]
38. Jesus, F.M.; Pereira, M.R.; Rosa, C.S.; Moreira, M.Z.; Sperber, C.F. Preservation methods alter carbon and nitrogen stable isotope values in crickets (Orthoptera: Grylloidea). *PLoS ONE* **2015**, *10*, e0137650. [CrossRef] [PubMed]
39. Carabel, S.; Verísimo, P.; Freire, J. Effects of preservatives on stable isotope analyses of four marine species. *Estuar. Coast. Shelf. Sci.* **2009**, *82*, 348–350. [CrossRef]
40. Vanderklift, M.A.; Ponsard, S. Sources of variation in consumer-diet $\delta^{15}N$ enrichment: A meta-analysis. *Oecologia* **2003**, *136*, 169–182. [CrossRef]
41. Butler, O.M.; Rashti, M.R.; Chen, C. Influence of storage and drying methods on invertebrate elemental and isotopic measurements. *Comm. Soil Sci. Plant Anal.* **2018**, *49*, 1–7. [CrossRef]
42. Bugoni, L.; Mcgill, R.A.R.; Furness, R.W. Effects of preservation methods on stable isotope signatures in bird tissues. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 2457–2462. [CrossRef] [PubMed]
43. Oczkowski, A.; Thornber, C.S.; Markham, E.E.; Rossi, R.; Ziegler, A.; Rinehart, S. Testing sample stability using four storage methods and the macroalgae Ulva and Gracilaria. *Limnol. Oceanogr. Methods* **2015**, *13*, 9–14. [CrossRef]
44. Fanelli, E.; Cartes, J.E.; Papiol, V.; Rumolo, P.; Sprovieri, M. Effects of preservation on the $\delta^{13}C$ and $\delta^{15}N$ values of deep sea macrofauna. *J. Exp. Mar. Biol. Ecol.* **2010**, *395*, 93–97. [CrossRef]
45. Durante, L.M.; Sabadel, A.J.M.; Frew, R.D.; Ingram, T.; Wing, S.R. Effects of fixatives on stable isotopes of fish muscle tissue: Implications for trophic studies on preserved specimens. *Ecol. Appl.* **2020**, *30*, e02080. [CrossRef] [PubMed]
46. Kiszka, J.; Lesage, V.; Ridoux, V. Effect of ethanol preservation on stable carbon and nitrogen isotope values in cetacean epidermis: Implication for using archived biopsy samples. *Mar. Mamm. Sci.* **2014**, *30*, 788–795. [CrossRef]
47. Gibbons, J.W.; Winne, C.T.; Scott, D.E.; Willson, J.D.; Glaudas, X.; Andrews, K.M.; Todd, B.D.; Fedewa, L.A.; Wilkinson, L.; Tsaliagos, R.N.; et al. Remarkable amphibian biomass and abundance in an isolated wetland: Implications for wetland conservation. *Conserv. Biol.* **2006**, *20*, 1457. [CrossRef]
48. Wells, K.D. *The Ecology and Behavior of Amphibians*; The University of Chicago Press: Chicago, IL, USA; London, UK, 2007.

49. Glos, J.; Ruthsatz, K.; Schroder, D.; Riemann, J.C. Food source determines stable isotope discrimination factor dN and dC in tadpoles. *Amphib. Reptil.* **2020**, *41*, 501–507. [CrossRef]
50. Reshetnikov, A.N.; Korobushkin, D.I.; Gongalskiy, K.B.; Korotkevich, A.Y.; Selskaya, A.N.; Kotov, A.A.; Tiunov, A.V. Trophic positions and niche segregation of two anuran species in the ecosystem of a forest lake. *Hydrobiologia* **2021**. [CrossRef]
51. Smirnov, N.N. Lake Glubokoe (Moscow region, Eastern Europe), general characteristics. *Hydrobiologia* **1986**, *141*, 1–6. [CrossRef]
52. Annenskaya, G.N.; Zhichkova, V.K.; Kalinina, V.P.; Mamay, I.I.; Nizovtsev, V.A.; Khrustaleva, M.A.; Tseselchuk, Y.N. *Landscapes of Moscow Region and Their Current Condition*; SGU: Smolensk, Russia, 1997; 296p. (In Russian)
53. Miller, J.; Haden, P. *Statistical Analysis with the General Linear Model*; Department of Psychology, University of Otago: Dunedin, New Zealand, 2006; Available online: https://www.otago.ac.nz/psychology/otago039309.pdf (accessed on 1 July 2021).
54. Trakimas, G.; Jardine, T.D.; Barisevičiūtė, R.; Garbaras, A.; Skipitytė, R.; Remeikis, V. Ontogenetic dietary shifts in European common frog (*Rana temporaria*) revealed by stable isotopes. *Hydrobiologia* **2011**, *675*, 87–95. [CrossRef]
55. Kadye, W.T.; Redelinghuys, S.; Parnell, P.S.; Booth, A.J. Exploring source differences on diet-tissue discrimination factors in the analysis of stable isotope mixing models. *Sci. Rep.* **2020**, *10*, 15816. [CrossRef] [PubMed]
56. Beebee, T.J.C.; Wong, L.-C. Leucine uptake by enterobacterial and algal members of larval anuran gut flora. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **1992**, *101B*, 527–530. [CrossRef]
57. Pryor, G.S.; Bjorndal, K.A. Effects of the nematode Gyrinicola batrachiensis on development, gut morphology, and fermentation in bullfrog tadpoles (*Rana catesbeiana*): A novel mutualism. *J. Exp. Zool.* **2005**, *303A*, 704–712. [CrossRef] [PubMed]
58. Pryor, G.S.; Bjorndal, K.A. Symbiotic fermentation, digestal passage, and gastrointestinal morphology in bullfrog tadpoles (Rana catesbeiana). *Physiol. Biochem. Zool.* **2005**, *78*, 201–215. [CrossRef]
59. Vences, M.; Lyra, M.L.; Kueneman, J.G.; Bletz, M.C.; Archer, H.M.; Canitz, J.; Handreck, S.; Randrianiaina, R.-D.; Struck, U.; Bhuju, S.; et al. Gut bacterial communities across tadpole ecomorphs in two diverse tropical anuran faunas. *Sci. Nat.* **2016**, *103*, 25. [CrossRef]
60. Korotkevich, A.Y.; Kuznetsova, N.A.; Tiunov, A.V. Changes in Carbon and Nitrogen Isotope Ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in Springtails during Long-term Storage of Soil Samples. *Russ. J. Ecol.* **2016**, *47*, 572–574. [CrossRef]
61. Terentiev, P.V. *Frog*; Sovetskaya Nauka: Moscow, Russia, 1950; 345p.
62. Caut, S.; Angulo, E.; Díaz-Paniagua, C.; Gomez-Mestre, I. Plastic changes in tadpole trophic ecology revealed by stable isotope analysis. *Oecologia* **2013**, *173*, 95–105. [CrossRef]
63. Arribas, R.; Díaz-Paniagua, C.; Caut, S.; Gomez-Mestre, I. Stable isotopes reveal trophic partitioning and trophic plasticity of a larval amphibian guild. *PLoS ONE* **2015**, *10*, e0130897. [CrossRef]
64. Vassilieva, A.B.; Sinev, A.Y.; Tiunov, A.V. Trophic segregation of anuran larvae in two temporary tropical ponds in southern Vietnam. *Herpetol. J.* **2017**, *27*, 217–229.
65. San Sebastián, O.; Navarro, J.; Llorente, G.A.; Richter-Boix, Á. Trophic strategies of a non-native and a native amphibian species in shared ponds. *PLoS ONE* **2015**, *10*, e0130549. [CrossRef] [PubMed]
66. Kuzmin, S.L. *The Amphibians of the Former Soviet Union*; Pensoft: Moscow, Russia; Sofia, Bulgaria, 2013; 383p.
67. Houlahan, J.E.; Findlay, C.S.; Schmidt, B.R.; Meyer, A.H.; Sergius, L.; Kuzmin, S.L. Quantitative evidence for global amphibian population declines. *Nature* **2000**, *404*, 752–755. [CrossRef] [PubMed]