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# Effects of euthanasia method on stable-carbon and stable-nitrogen isotope analysis for an ectothermic vertebrate

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**RATIONALE:** Stable isotope analysis is a critical tool for understanding ecological food webs; however, results can be sensitive to sample preparation methods. To limit the possibility of sample contamination, freezing is commonly used to euthanize invertebrates and preserve non-lethal samples from vertebrates. For destructive sampling of vertebrates, more humane euthanasia methods are preferred to freezing and it is essential to evaluate how these euthanasia methods affect stable isotope results.

**METHODS:** Stable isotope ratios and elemental composition of carbon and nitrogen were used to evaluate whether the euthanasia method compromised the integrity of the sample for analysis. Specifically, the stable isotope and C:N ratios were compared for larval wood frogs (*Rana sylvatica* = *Lithobates sylvaticus*), an ectothermic vertebrate, that had been euthanized by freezing with four different humane euthanasia methods: $CO_2$, benzocaine, MS-222 (tricaine methanesulfonate), and 70% ethanol.

**RESULTS:** The euthanasia method was not related to the $\delta^{13}C$ or $\delta^{15}N$ values and the comparisons revealed no differences between freezing and any of the other treatments. However, there were slight (non-significant) differences in the isotope ratios of benzocaine and $CO_2$ when each was compared with freezing. The elemental composition was altered by the euthanasia method employed. The percentage nitrogen was higher in $CO_2$ treatments than in freezing, and similar (non-significant) trends were seen for ethanol treatments relative to freezing. The resulting C:N ratios were higher for benzocaine treatments than for both $CO_2$ and ethanol. Similar (non-significant) trends suggested that the C:N ratios were also higher for animals euthanized by freezing than for both $CO_2$ and ethanol euthanasia methods.

**CONCLUSIONS:** The euthanasia method had a larger effect on elemental composition than stable isotope ratios. The percentage nitrogen and the subsequent C:N ratios were most affected by the $CO_2$ and ethanol euthanasia methods, whereas non-significant trends suggested that benzocaine and $CO_2$ altered the stable isotope ratios. It appears that the use of MS-222 and freezing with dry ice are the most appropriate euthanasia methods for ectothermic vertebrates.


The development and advancement of stable isotope analysis over recent decades has changed the way in which we view and understand food web dynamics. We can now assess how organisms shift their diet based on changes in the environment,[1] observe how invasive species alter food web structures,[2] and measure the interconnectedness of terrestrial and aquatic habitats.[3,4] As methods advance, more applications of stable isotope analysis continue to be developed. For instance, the concept of isotopic baselines has allowed for comparisons of food webs among sites and ecosystems[5] and recent advances in mixing models have improved our interpretation of food webs despite sources of uncertainty.[6] As stable isotope methods continue to evolve, our understanding of food web ecology will grow.

With the development of stable isotope analysis, concerns have arisen regarding how sample preparation methods may affect both isotope ratios and elemental composition. Previous studies have found that isotope or elemental ratios may be altered by sample preparation, including acidifying samples to remove carbonates,[7] removing gut contents,[7,8] removing lipids,[9–11] and preservation method.[10] The preservation method is interesting because it can introduce new chemicals that may react with the sample. For example, preservation media can hydrolyze proteins in the sample, exchange light isotopes for heavier ones, add light carbon to the sample, or extract lipids from the sample.[10–14]

If the sample preparation method alters isotope or elemental ratios, the subsequent findings and interpretations may be flawed. For example, preservation methods altered the stable isotope ratios for three fish species in China, leading to incorrect estimations of pelagic energy pathways.[15] Formalin preservation overestimated the importance of the pelagic pathway in all three species (by up to 85% in one species), whereas preservation in ethanol or sodium chloride underestimated its importance for all species (by up to 20%).[15] Therefore, accurate interpretation of food webs requires preparation methods that do not contaminate samples.

An additional sample preparation method that has yet to be evaluated, but may pose a concern for analysis, is how the euthanasia method influences the stable isotope and

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elemental composition. Unlike animals where blood, tissue, feathers, or hair may be sampled non-lethally, invertebrates and small ectothermic vertebrates must be sacrificed for stable isotope analysis. Invertebrates are most commonly euthanized by freezing, and this is the most widely accepted method for stable isotope preparation as it limits the possibility of contaminating a sample. However, many regulatory bodies (including organizations such as the American Veterinary Medical Association) do not sanction freezing as a euthanasia method for ectothermic vertebrates. It is thus necessary to compare sanctioned euthanasia methods with freezing to determine if humane euthanasia methods compromise the integrity of the sample for stable isotope and elemental analysis.

This study has assessed how euthanasia method influences stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$ values) and elemental composition (C:N) by comparing the use of dry ice (control) with four commonly used euthanasia methods: $CO_2$, benzocaine, MS-222 (tricaine methanesulfonate), and 70% ethanol. Larval wood frogs (*Rana sylvatica* = *Lithobates sylvaticus*) were used as study organisms as they are small ectotherms common throughout northern North America. Larval frogs have also been used in recent stable isotope studies, yet euthanasia methods have not been considered.[16–18]

## EXPERIMENTAL

Wood frog larvae were raised in mesocosms to ensure similar conditions and food resources. The mesocosms were constructed from plastic cattle-watering tanks (176 cm inside diameter) located in an outside compound at Yale University in New Haven, Connecticut, USA. Three mesocosms were set up following the protocol from Skelly.[19] Briefly, tanks were filled 48 cm deep with well water and 50 g of rabbit food pellets (Hartz Mountain Corporation, Secaucus, New Jersey, USA) was added on 4 March 2012. On 11 March 2012, each mesocosm received 600 g of dried deciduous leaf litter consisting of approximately 550 g oak leaves (*Quercus* spp.) and 50 g American beech leaves (*Fagus grandifolia*), matching local field conditions. On 18 March 2012, the mesocosms were inoculated with phytoplankton and zooplankton concentrates collected from three ponds in the Yale-Myers Forest in Eastford, Connecticut, USA.

On 24 March 2012, approximately 50 embryos were collected from six wood frog egg masses across three temporary ponds in the Yale-Myers Forest. Multiple egg masses and ponds were selected to maximize genetic diversity. Upon collection, the embryos were transferred to mesocosms, where they were placed in floating containers ($38 \times 26 \times 14.5$ cm). The containers had mesh openings on all sides to allow water exchange with the surrounding mesocosm. The embryos were kept separated in their collection ponds until 11 April 2012 when they reached Gosner Stage 25 and began free feeding.[20] At this time, 120 larvae were added to each mesocosm (40 from each of the three collection ponds). The wood frogs were removed from the mesocosms on 1 June 2012, when they reached larval stages 39–40.[20]

Seven wood frog larvae were haphazardly selected from the mesocosms with at least two wood frogs from each mesocosm included for each of the five euthanasia treatments. The five treatments were: dry ice (control), immersion in $CO_2$-saturated water, liquid benzocaine, MS-222, and 70% ethanol. The buffered MS-222 solution was prepared by dissolving 3 g MS-222 with 3 g sodium bicarbonate in 1 L of water. The larvae were immersed in the selected treatment until a few minutes after they stopped moving (<10 minutes for all treatments). Benzocaine left a film residue on larvae; thus, each individual was gently rinsed with deionized water. The larvae were then frozen for five weeks prior to analysis, at which point the guts were removed, the individuals were dried at 60 °C for 48 hours, and then ground to a fine powder with a mortar and pestle. Approximately 1.2 mg of powdered sample was loaded into pressed tin capsules (Costech Analytical Technologies, Valencia, CA, USA) and analyzed for percentage carbon, percentage nitrogen, and $\delta^{13}C$ and $\delta^{15}N$ values in a combustion elemental analyzer (ESC 4010; Costech Analytical Technologies) coupled to an isotope ratio mass spectrometer (Delta Plus Advantage; ThermoFinnigan, Bremen, Germany). The stable isotope ratio results are presented in typical delta ($\delta$) notation in units of per mil (‰) reported relative to the international standards Vienna Pee Dee Belemnite ($\delta^{13}C$ values) and atmospheric air ($\delta^{15}N$ values). The $\delta$-values were computed as:

$$\delta^{13}C \text{ or } \delta^{15}N = \left[\left(R_{\text{sample}}/R_{\text{standard}}\right) - 1\right] \times 1000 \qquad (1)$$

where $R$ is equal to $^{13}C/^{12}C$ or $^{15}N/^{14}N$ [21]. Two reference standards were used: United States Geological Survey (USGS) glutamic acid 41 and an internal laboratory standard of cocoa, which was normalized based on USGS glutamic acid 40 and 41. The precision of analysis for $\delta^{13}C$ values, $\delta^{15}N$ values, % C, and % N was ±0.16‰, ±0.04‰, ±0.23%, and ±0.03%, respectively, based on the standard deviation of replicates of the internal cocoa reference standard.

Multivariate analysis of variance (MANOVA) was used to compare the $\delta^{13}C$ and $\delta^{15}N$ value for the five different treatments. The data met the assumption of multivariate normal distribution with homogeneous covariance matrices as visually assessed with a multivariate chi-square quantile plot. To test for differences between the dry ice control and each of the other treatments, multivariate pairwise contrasts were used. Analysis of variance (ANOVA) and Tukey's *post hoc* contrasts were used to test for differences in C:N ratios, percentage carbon, and percentage nitrogen among the five euthanasia treatments. All analyses were run in R 2.13.1,[22] with the significance set as $\alpha = 0.05$.

## RESULTS

There were no differences in $\delta^{13}C$ or $\delta^{15}N$ values based on euthanasia method (MANOVA, $F_{8,58} = 1.035$, Wilks $p = 0.421$, Fig. 1). There were also no univariate differences for $\delta^{13}C$ values (ANOVA, $F_{4,30} = 0.453$, $p = 0.770$) or $\delta^{15}N$ values (ANOVA, $F_{4,30} = 1.644$, $p = 0.189$). Pairwise multivariate contrasts between the dry ice control and the other treatments also showed no differences (MANOVA, $CO_2$: $p = 0.571$; benzocaine: $p = 0.190$; MS-222: $p = 0.979$; ethanol: $p = 0.920$). However, non-significant trends showed that larvae euthanized with benzocaine had $\delta^{13}C$ and $\delta^{15}N$ values that were 0.22‰ and 0.28‰ lower, respectively, than those for freezing,

  

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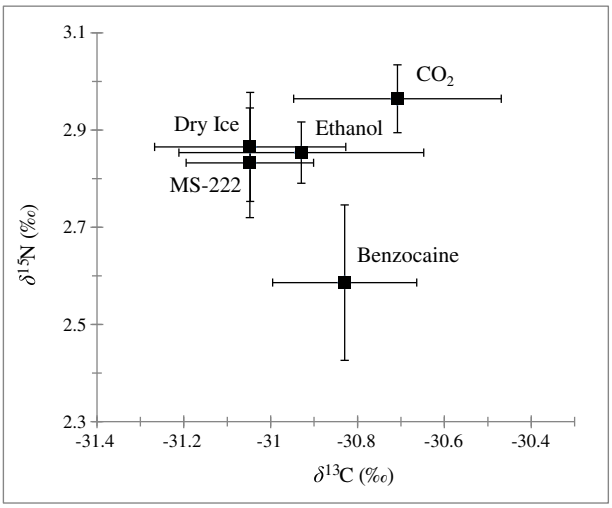


**Figure 1.** Mean $\delta^{13}C$ and $\delta^{15}N$ signatures of larval wood frogs for the five different euthanasia treatments. Bars represent standard error.

and that larvae euthanized by $CO_2$ had $\delta^{13}C$ and $\delta^{15}N$ values that were 0.34‰ and 0.10‰ higher, respectively, than those for freezing.

There were differences among the C:N ratios by euthanasia method (ANOVA, $F_{4,30} = 4.154$, $p = 0.009$, Fig. 2), which were attributed to differences in percentage nitrogen (ANOVA, $F_{4,30} = 4.242$, $p = 0.008$), but not in percentage carbon (ANOVA, $F_{4,30} = 1.107$, $p = 0.371$). The percentage nitrogen was higher in the $CO_2$ treatments than in freezing (Tukey honestly significantly different (HSD) test, $p = 0.029$) and benzocaine (Tukey HSD, $p = 0.030$). There were similar (non-significant) increases in percentage nitrogen for ethanol treatments relative to both freezing (Tukey HSD, $p = 0.112$) and benzocaine (Tukey HSD, $p = 0.115$). The resulting C:N ratios were higher for benzocaine treatments than for both $CO_2$ (Tukey HSD, $p = 0.014$) and ethanol (Tukey HSD, $p = 0.020$) (Fig. 2). Similar (non-significant) trends suggested that the C:N ratios were also higher for freezing treatments than for $CO_2$ (Tukey HSD, $p = 0.219$) and ethanol (Tukey HSD, $p = 0.279$).

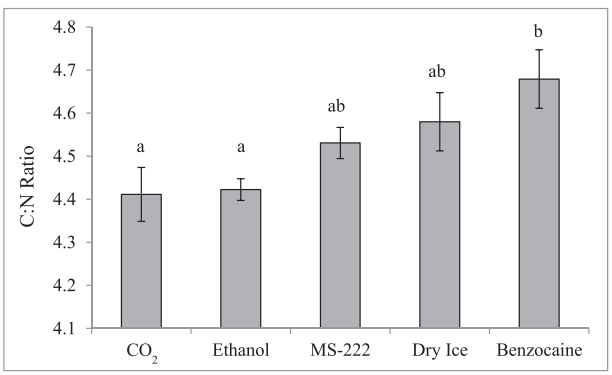


**Figure 2.** Mean (± standard error) C:N ratios of larval wood frogs for the five different euthanasia treatments. Different letters above the bars signify significant differences.

## DISCUSSION

Stable isotope analysis is a useful tool for understanding food web dynamics. However, results and interpretations can be marred if stable isotope ratios or elemental compositions were altered due to the sampling preparation method. This is the first study to evaluate if euthanasia methods compromise samples that will be used for stable isotope studies. The findings of this study suggest that euthanasia method can modify stable isotope ratios and elemental composition to varying extents.

The five well-known euthanasia methods used in this study did not significantly alter the stable isotope response of larval wood frogs. However, non-significant trends suggested that euthanasia with benzocaine or $CO_2$ might be more likely to alter stable isotope ratios than other methods. Benzocaine treatments produced samples that were slightly depleted in both $^{15}N$ and $^{13}C$, whereas $CO_2$ treatments produced samples that were slightly enriched in $^{13}C$ relative to freezing. It is possible that the carbon or nitrogen in benzocaine ($C_9H_{11}NO_2$) allowed lighter isotopes to replace heavier isotopes, thus causing isotope depletion. Another possibility is that some benzocaine residue remained after the larvae had been rinsed, thus introducing extra material to the sample. For $CO_2$, there was a slight enrichment in $^{13}C$, perhaps indicating that the $CO_2$ exchanged lighter isotopes in the sample with heavier ones from the media.

Previous studies have assessed how long-term preservation in ethanol alters stable isotope ratios. The results have varied widely with it being found that ethanol may enrich both $^{13}C$ and $^{15}N$,[10,15,23] just $^{13}C$ and not $^{15}N$,[24] or exhibit no changes compared with controls.[9,14] It is believed that ethanol can remove $^{13}C$-depleted lipids from a sample, and this would explain $^{13}C$ enrichment.[10] The lack of significant effects in this study might be due to the short amount of time for which the larvae were immersed in the euthanasia media (<10 minutes) compared with preservation methods (weeks, months, or years). It thus seems pertinent to remove animals from the euthanasia media in a timely manner, before preserving them either frozen or dried.

In addition to causing possible changes in stable isotope ratios, the euthanasia method can alter the elemental composition of samples. In this study, the percentage nitrogen increased relative to freezing for $CO_2$ and slightly (non-significant) for ethanol treatments. The C:N ratios were lower for $CO_2$ and ethanol treatments than for benzocaine, and a similar (non-significant) trend was seen relative to the freezing controls. The mechanism that causes alteration of the percentage carbon and nitrogen is unknown, but it might entail leaching of amino acids and proteins or the loss of volatile lipid compounds.[25] The elemental composition, specifically the C:N ratios, is critical for modeling lipid extraction for stable isotope analysis[26,27] and for understanding diet and trophic levels through ecological stoichiometry.[28] Therefore, euthanasia methods that alter the elemental composition of a sample could lead to misinterpretation of the food web.

## CONCLUDING REMARKS

This study provides evidence that the euthanasia method has a larger effect on elemental composition than on stable isotope ratios. The percentage nitrogen and the subsequent



  

C:N ratios are most affected by the $CO_2$ and ethanol euthanasia methods. Although not significant, benzocaine and $CO_2$ appear to alter stable isotope ratios compared with those of the freezing controls. The use of MS-222 and freezing with dry ice appear to be the most appropriate euthanasia methods because they protect both elemental composition and stable isotope ratios in larval wood frogs.

Although these results apply only to one species and future work could test the effects of euthanasia method for other species, this study likely applies to most small ectothermic vertebrates. The short immersion time that the animals were exposed to the euthanasia media (<10 minutes) probably prevented samples from being altered further, as can happen when samples are preserved in the media. It is therefore pertinent to remove animals soon after movement ceases as longer immersion times may produce different results. These findings may alleviate concerns of researchers in remote field sites who can now substitute MS-222 when dry ice or freezers are unavailable. MS-222 is also a more humane euthanasia method, suggesting it to be the preferred choice.

Thus, elemental composition appears to be more sensitive to euthanasia methods than stable isotope ratios. For euthanizing vertebrates for isotope studies, MS-222 or freezing are recommended because these two methods did not alter the stable isotope ratios or elemental composition of the sample.

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