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**Brief Communication**

# Effects of preservation by ethanol on $\delta^{13}C$ and $\delta^{15}N$ of three tissues of the critically endangered European eel *Anguilla anguilla*

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## ABSTRACT

The temporal effects of ethanol preservation on the $\delta^{13}C$ and $\delta^{15}N$ values of tissues excised from European eel *Anguilla anguilla* were assessed. Preservation significantly enriched $\delta^{13}C$ values of fin and mucus, but not dorsal muscle. The $\delta^{13}C$ enrichment occurred in the initial 15 days of preservation and was independent of initial eel mass. Tissue preservation effects on $\delta^{15}N$ values were negligible. These tissue-specific isotopic shifts should be considered when ethanol preserved eel samples are used.


**Key words:** Anguillid, chemical preservation effects, critically endangered species, non-destructive sampling, trophic ecology, stable isotope analysis

The ecological application of stable isotope analysis (SIA) has enabled assessments of the trophic relationships of a wide range of species within fish communities (Jennings and Van der Molen, 2015; McCue *et al.,* 2020). Insights into food-web structure and associated energy flux tend to rely on the relationships between the stable isotope ratios of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) (Fry, 2006), and these can be used to calculate a range of ecologically relevant metrics, such as the isotopic (trophic) niche size (Jackson *et al.,* 2012). A range of fish tissues can be used for deriving $\delta^{13}C$ and $\delta^{15}N$ data, such as dorsal muscle, fin and mucus, with each having a different rate of isotopic turnover (Winter *et al.,* 2019a,b).

An important ecological application of SIA is the assessment of long-term changes in the trophic ecology of fish populations and communities, where preserved archival collections can provide information on historical baselines (Kelly *et al.,* 2006). Also, when contemporary samples are collected in the field, there is a frequent need to immediately preserve samples for subsequent analyses. Although freezing is commonly used, which has minimal effects on stable isotope ratios (Sweeting *et al.,* 2004), this is not always logistically possible in field conditions. Consequently, the chemical based preservation of fish or tissue samples is often the most feasible option to prevent samples from spoiling (Kelly *et al.,* 2006). The application of these fish tissues for SIA then requires determination of the extent to which chemical preservation has altered their SI values, especially if these values are to be used in analyses that also involve non-preserved tissues (Edwards *et al.,* 2002). The chemical preservation effects on SI values have been assessed for a range of aquatic and terrestrial organisms, including birds (Bugoni *et al.,* 2008), mammals (Javornik *et al.,* 2019) and fishes (Kelly *et al.,* 2006). However, the extent of preservation-induced shifts in $\delta^{13}C$ and $\delta^{15}N$ is often species- and tissue-specific, and of varying magnitude (Kelly *et al.,* 2006; Carabel *et al.,* 2008).

The European eel *Anguilla anguilla* L. 1758 ('eel' hereafter) has suffered substantial declines in recruitment and abundance in recent decades and has been assessed as 'critically endangered' on the IUCN Red List of Threatened Species since 2008 (Pike *et al.,* 2020). Investigations into their trophic ecology to better understand the drivers and consequences of population change can incorporate both archived and contemporary samples. In contemporary sampling programmes where trophic analyses of eel are required, muscle tissue can be compared directly with fin and mucus, as there are no significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values of these tissues within individuals (Boardman et al., 2022). Non-lethal tissue sampling is thus recommended due to their critically endangered status (e.g. Boardman *et al.,* 2022), especially as this can now be coupled with non-lethal screening for the invasive nematode parasite *Anguillicoloides crassus* (De Noia *et al.,* 2022). Nevertheless, eel SIA is still often based on dorsal muscle, where tissue samples are usually excised from euthanised fish (Capoccioni *et al.,* 2021; Parzanini *et al.,* 2021). Also, when fin and mucus are used as an alternative tissue to muscle, their SI values are still generally compared with, or converted to, muscle values (e.g., Kelly *et al.,* 2006; Busst *et al.,* 2015).

To understand how ethanol preservation affects the SI values of eel muscle, fin, and epidermal mucus, and how this varies with preservation time, eels (n = 9; mean starting mass ± 95% CI: 61.6 ± 5.9 g, range 0.6 - 228.8 g) were collected from a side-stream in the lower reaches of the River Frome (51° 20' 21″N; 2° 17' 44″W), Southern England, in September 2021 using back-mounted electric fishing (SmithRoot LR24). Following their capture, the eels were euthanised, weighed (to 0.01 g), and dorsal muscle, fin and epidermal mucus samples were taken (*cf.* Winter et al., 2019a,b; Winter and Britton, 2021). These initial tissue samples were considered as the control samples (i.e. without preservation; time $T_0$). Each eel was then transferred into an individual sample bottle filled with 98 % ethanol, ensuring all of the eel was fully immersed

and provided with a reference letter ('A' to 'I'). On days 15, 30, 45, 90 and 140 ($T_{15}$ to $T_{140}$), each eel was removed from the ethanol, re-weighed (after drying to remove excess ethanol), and new tissue samples taken from an area close to where the $T_0$ sample was excised, rinsed of any remaining ethanol, and dried to constant mass at 60 ºC for 48 h. Although a mucus sample was taken on $T_0$ and $T_{15,}$ no mucus was available on any eel to sample thereafter. The tissue and mucus samples were then bulk analysed for $\delta^{13}C$ and $\delta^{15}N$ in a Thermo Delta V isotope ratio mass spectrometer (Thermo Scientific, USA) interfaced to a NC2500 elemental analyser (CE Elantach Inc., USA). Analytical precision of the $\delta^{13}C$ and $\delta^{15}N$ sample runs was estimated against an internal standard sample of animal (deer) material every 10 samples, with the overall standard deviation estimated at 0.08 and 0.04 ‰ respectively.

Preservation in ethanol resulted in reduced eel mass between $T_0$ and $T_{15}$ (mean proportion of lost mass: -0.24 ± 6%), with smaller eels losing proportionally more mass than larger individuals. Changes in mass between $T_{15}$ and $T_{140}$ were minimal (Supporting materials: Figure S1). Ethanol preservation resulted in significant shifts in $\delta^{13}C$ of fin and mucus (ANOVA: Fin: $F_{1,50} = 9.34$, $P = 0.01$; Mucus: $F_{1,14} = 41.45$, $P < 0.01$), but not muscle ($F_{1,52} = 2.07$, $P = 0.15$) (Figure 1, Table S1). Significant shifts in fin and mucus $\delta^{13}C$ were found between $T_0$ and each subsequent time step ($T_{15}$ to $T_{140}$), but not between the time-steps of $T_{15}$ to $T_{140}$ (Figure 1, Supporting materials: Figure S2, Table S2). In contrast, time since preservation did not significantly alter the $\delta^{15}N$ values of any tissue across the entire period (ANOVA: Muscle: $F_{1,52} = 0.54$, $P = 0.46$; Fin: $F_{1,50} = 0.28$, $P = 0.60$; Mucus: $F_{1,14} = 0.07$, $P = 0.93$), so no further analyses were completed (Supporting materials: Figure S3).

With the preservation-induced shifts in $\delta^{13}C$ only occurring between $T_0$ and $T_{15}$ in fin and mucus, a linear mixed effects model (LME4 R-package) was used to test how this shift related

to changes in eel mass and their starting mass. The initial model structure used the difference in $\delta^{13}C$ between $T_0$ and $T_{15}$ as the response variable, with fixed factors of tissue (as fin or mucus), eel starting mass and eel change in mass, and with eel identity included as a random variable to account for individual variability (*cf*. Figure 1). As change in mass and starting mass were significantly correlated (Pearson's correlation coefficient: $r = 0.87$; $p < 0.01$), they were not included together in models (Supporting materials: Table S3). Model fitting procedures (Akaike's Information Criteria (AIC) identified the best-fitting model as ~Tissue + Individual + Mass change (Table 1, Supporting materials: Table S3). This final model also provided the mean difference (± 95% CI) in $\delta^{13}C$ between $T_0$ and $T_{15}$ per tissue that can be applied as correction factors to convert preserved values $\delta^{13}C$ to a predicted non-preserved value (fin: 1.50 ± 0.28 ‰; mucus: 0.95 ± 0.11 ‰) (Table 1). Although differences in $\delta^{13}C$ between $T_0$ and $T_{140}$ were non-significant for muscle, there was some enrichment between $T_0$ and $T_{15}$ (mean ± 95 % CI: 0.58 ± 0.23 ‰; Supporting materials: Table S1), and thus this mean value could also be applied as a correction factor where this was considered to be a concern.

Our results demonstrated that preservation in ethanol does not significantly alter $\delta^{15}N$ values of European eel and thus values from preserved samples can be compared directly with those from non-preserved samples. In contrast, ethanol preservation caused a shift in eel $\delta^{13}C$ values, most notably in fin tissues, which would need accounting for before preserved samples can be compared with fresh samples. Although the reasons for these $\delta^{13}C$ shifts were not explored, this enrichment could have been due to the carbon contained within ethanol and/ or ethanol acting as a solvent dissolving lipid during storage or lipid hydrolysis, as ethanol acts as a lipid-extracting agent (e.g. Kelly *et al.,* 2006; Syväranta *et al.,* 2008). While we acknowledge we used a relatively small sample size (n = 9), this was due to the critically endangered status of eel that meant there was an ethical requirement to limit the numbers removed from the

population. Moreover, the preservation effects on mass and the SI values were relatively consistent across all nine eels, irrespective of their starting mass.

The effect of preservation time is an important consideration for stable isotope studies that incorporate the use of preserved animal samples. There is consensus that the effects of ethanol preservation on $\delta^{13}C$ usually occurs rapidly, usually within the first 2 to 3 weeks of preservation (Sweeting, 2004; Kaehler and Pakhomov, 2001), but is independent of storage time thereafter. Indeed, SI tissue samples from a range of taxa preserved over periods of years have revealed no isotopic changes as their preservation time increased (Edwards *et al.,* 2002; Syväranta *et al.,* 2008; Rennie *et al.,* 2012), implying that the results of our 140 day preservation study are applicable to situations where eel samples have been preserved for considerably longer.

In conclusion, ethanol can be used to preserve eel tissues without compromising their SI data, although consideration to applying correction factors to $\delta^{13}C$ values should be given if these data are to be used in conjunction with SI data derived from non-preserved tissues. We recommend that where ethanol preserved samples comprise of whole eels, fin samples should be taken for SIA in preference to muscle, as this will enable easier comparison with fin tissues collected non-lethally from live eels.

## FUNDING INFORMATION

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## CONFLICTS OF INTEREST

The authors declare that they are not aware of any competing interests.

## SUPPORTING INFORMATION

**Figure S1:** Time since ethanol preservation versus eel mass on days $T_0$ to $T_{140}$ for each individual European eel. Note differences in values on the Y axis that reflect differences in eel starting mass.

**Figure S2:** Time since ethanol preservation versus $\delta^{13}C$ of each tissue on days $T_0$ to $T_{140}$ for each individual European eel, where • mucus, ○ dorsal muscle, • fin.

**Figure S3:** Time since ethanol preservation versus $\delta^{15}N$ of each tissue on days $T_0$ to $T_{140}$ for each individual European eel, where • mucus, ○ dorsal muscle, • fin.

**Table S1:** Mean and range (as minimum ('min') and maximum ('max')) of $\delta^{13}C$ and $\delta^{15}N$ of fin, dorsal muscle ('muscle'), and mucus according to the time since preservation (as $T_0$ to $T_{140}$).

**Table S2:** Tukey post-hoc test P-values from ANOVA testing time since preservation (as $T_0$ to $T_{140}$) versus $\delta^{13}C$. P-values are presented as fin/muscle/mucus. Note mucus could only be extracted on day 0 and day 15.

**Table S3.** Ranking of candidate models testing differences in $\delta^{13}C$. over time since preservation according to Akaike Information Criterion (AIC) values, where the bold font denotes best fitting models according to lowest AIC values. All models include individual random intercepts (1|Individual).

## AUTHOR CONTRIBUTIONS

All authors were involved in the conceptualisation of the study, and in writing and editing the manuscript. RMB, JRB and ACP completed all sampling. JRB and RMB completed sample preparation, and RMB completed all data analyses and evaluation. All authors agree to submission of the manuscript.

## ETHICAL STATEMENT

The study was completed following the gaining of all relevant ethical and legislative approvals (UK Home Office Project Licence P47216841; Environment Agency permit reference EP/EW027-C-042/19919/01).

## REFERENCES

Boardman, R., Pinder, A., Piper, A., Gutmann Roberts, C., Wright, R., & Britton, J. (2022). Non-lethal sampling for the stable isotope analysis of the c ritically endangered European eel *Anguilla anguilla*: how fin and mucus compare to dorsal muscle. *Journal of Fish Biology*, **100**, 847-851.

Bugoni, L., McGill, R., & Furness, R. (2008). Effects of preservation methods on stable isotope signatures in bird tissues. *Rapid Communications in Mass Spectrometry*, **22**, 2457-2462.

Busst, G., Bašić, T., & Britton, J. (2015). Stable isotope signatures and trophic-step fractionation factors of fish tissues collected as non-lethal surrogates of dorsal muscle. *Rapid Communications in Mass Spectrometry*, **29**,1535-1544.

Capoccioni, F., Leone, C., Giustini, F., Brilli, M., Buttazzoni, L., Hanel, R., & Ciccotti, E. (2021). $\delta^{13}C$ and $\delta^{15}N$ in yellow and silver eels (*Anguilla anguilla*, 1758) from different

Mediterranean local stocks and their variation with body size and growth. *Marine and Freshwater Research*, **72**, 1208-1219.

Carabel, S., Verísimo, P., & Freire J. (2009). Effects of preservatives on stable isotope analyses of four marine species. Estuarine*,Coastal and Shelf Science*, **82**, 348–350.

De Noia, M., Poole, R., Kaufmann, J., Waters, C., Adams, C., McGinnity, P., & Llewellyn, M. (2022). Towards an in situ non-lethal rapid test to accurately detect the presence of the nematode parasite, Anguillicoloides crassus, in European eel, Anguilla anguilla. *Parasitology*, **149**, 605-611.

Edwards, M., Turner, T., & Sharp, Z. (2002). Short- and Long-Term Effects of Fixation and Preservation on Stable Isotope Values (δ13C, δ15N, δ34S) of Fluid-Preserved Museum Specimens. *Copeia*, **4**, 1106-1112.

Fry, B. (2006). *Stable isotope ecology*. New York: Springer.

Jackson, M.C., Donohue, I., Jackson, A.L., Britton, J.R., Harper, D.M., & Grey, J. (2012). Population-level metrics of trophic structure based on stable isotopes and their application to invasion ecology. *PloS one*, **7**.

Javornik, J., Hopkins, J., Zavadlav, S., Levanič, T., Lojen, S., Polak, T., & Jerina, K. (2019). Effects of ethanol storage and lipids on stable isotope values in a large mammalian omnivore. *Journal of Mammalogy*, **100**, 150-157.

Jennings, S., & van der Molen, J. (2015). Trophic levels of marine consumers from nitrogen stable isotope analysis: estimation and uncertainty. *ICES Journal of Marine Science*, **72**, 2289-2300.

Kaehler, S., & Pakhomov EA. (2001). Effects of storage and preservation on the δ13C and δ15N signatures of selected marine organisms.*Marine Ecology Progress Series*, **219**, 299–304.

Kelly, B., Dempson, J., &Power, M. (2006). The effects of preservation on fish tissue stable isotope signatures. *Journal of Fish Biology*, **69**,1595-1611.

Kelly, M., Hagar, W., Jardine, T., & Cunjak, R. (2006). Nonlethal Sampling of Sunfish and Slimy Sculpin for Stable Isotope Analysis: How Scale and Fin Tissue Compare with Muscle Tissue. *North American Journal of Fisheries Management*, **26**,921-925.

McCue, M.D., Javal, M., Clusella-Trullas, S., Le Roux, J.J., Jackson, M.C., Ellis, A.G., Richardson, D.M., Valentine, A.J., & Terblanche, J.S. (2020). Using stable isotope analysis to answer fundamental questions in invasion ecology: progress and prospects. *Methods in Ecology and Evolution*, **11**,196-214.

Parzanini, C., Arts, M. T., Power, M., Rohtla, M., Skiftesvik, A. B., Koprivnikar, J., Brownman, H.I., Milotic, D., & Durif, C.M. (2021). Trophic ecology of the European eel (*Anguilla anguilla*) across different salinity habitats inferred from fatty acid and stable isotope analysis. *Canadian Journal of Fisheries and Aquatic Sciences*, **78**, 1721– 1731.

Pike, C., Crook, V., & Gollock, M. (2020). *Anguilla anguilla*. The IUCN red list of threatened species.

Rennie, M. D., T, Ozersky., & D.O,Evans. (2012). Effects of formalin preservation on invertebrate stable isotope values over decadal time scales.*Canadian Journal of Zoology*. **90**, 1320–1327.

Sweeting, C. J., N. V, Polunin., & S, Jennings. (2004). Tissue and fixative dependent shifts of $\delta^{13}C$ and $\delta^{15}N$ in preserved ecological material. *Rapid Communications in Mass Spectrometry*,**18**,2587–2592.

Syväranta, J., Vesala, M., Rask, J., Ruuhijärvi, J., & Jones RI. (2008). Evaluating the utility of stable isotope analyses of archived freshwater sample materials. *Hydrobiologia*. **600**,121–130.

Winter, E. R., & Britton, J. R. (2021). Individual variability in stable isotope turnover rates of epidermal mucus according to body size in an omnivorous fish. Hydrobiologia, **848**, 363–370

Winter, E.R., Nolan, E.T., Busst, G., & Britton, J.R. (2019). Estimating stable isotope turnover rates of epidermal mucus and dorsal muscle for an omnivorous fish using a diet-switch experiment. *Hydrobiologia*, **828**,245-258.

Winter, E.R., Nyqvist, M., & Britton, J.R. (2019). Non-lethal sampling for stable isotope analysis of pike Esox lucius: how mucus, scale and fin tissue compare to muscle. *Journal of fish biology*, **95**,956-958.

**Table 1:** Results of the best-fitting linear mixed effects model (~Tissue + Individual + Mass change; *cf.* Table S3) testing the effect of ethanol preservation on $\delta^{13}C$ values between $T_0$ and $T_{15}$. 'Estimate' represents the mean difference in $\delta^{13}C$ between the two time-steps for use as a correction factor.

| Tissue | Estimate | t value | P-value |
|---|---|---|---|
| **Fin** | 1.50 ± 0.28 | 7.77 | <0.001 |
| **Mucus** | 0.95 ± 0.11 | 5.32 | <0.001 |

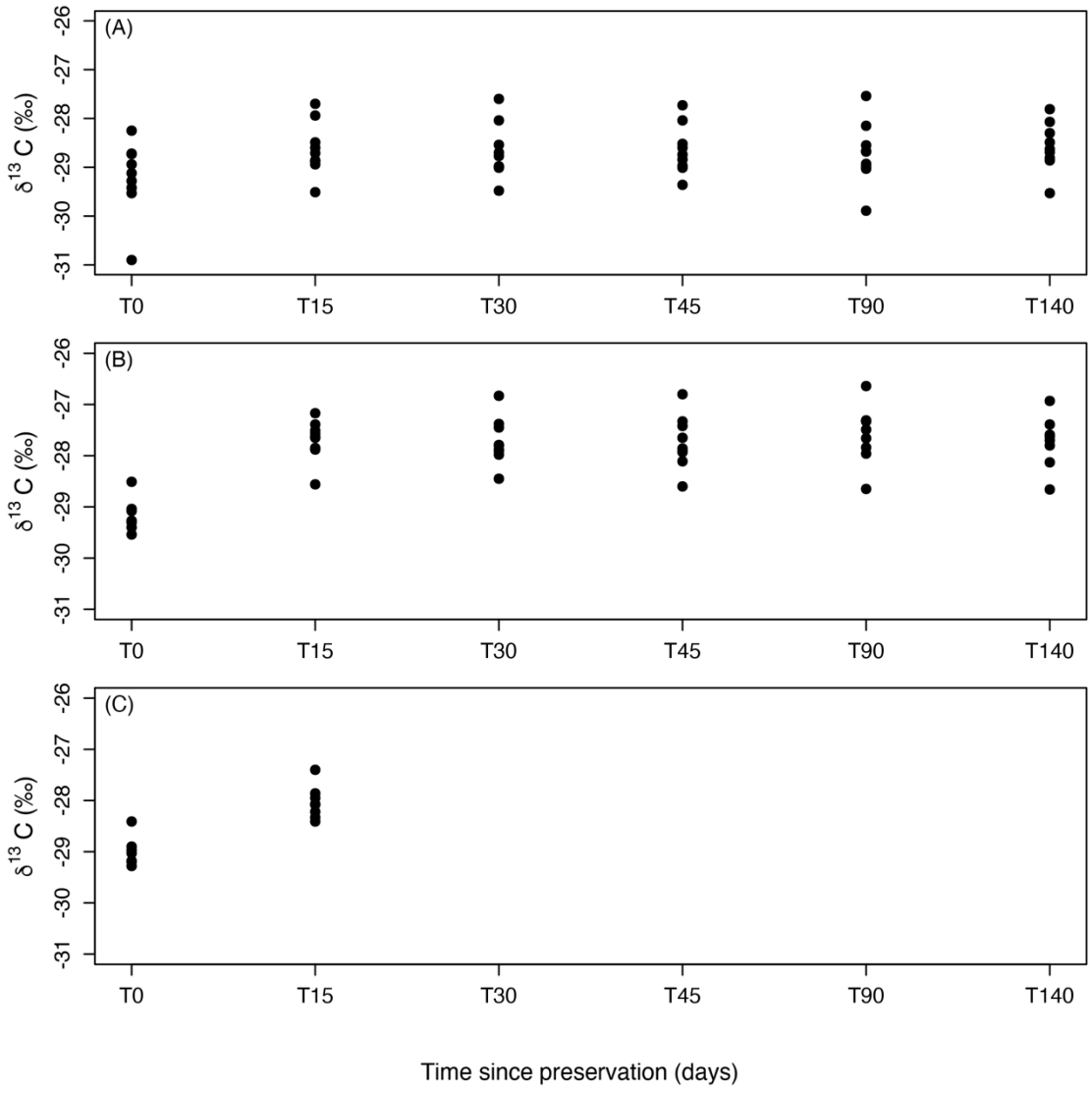


**Figure 1.** Time since ethanol preservation versus $\delta^{13}C$ of each tissue on days T0 to T140 (A) dorsal muscle, (B) fin, (C) mucus). Note that mucus could not be extracted on days 30-140. Please see Figure S1 for plots of individual eel.