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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 ^{13}C values of fin and mucus but not dorsal muscle. The ^{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.

KEYWORDS
Anguillid, chemical preservation effects, critically endangered, stable isotope analysis, trophic ecology

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 & 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 (SI) 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, 2019b).

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*., 2006a, 2006b). 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 SI ratios (Sweeting *et al*., 2004), this is not always logistically possible in field conditions. Consequently, chemical-based preservation of fish or tissue samples is often the most feasible option to prevent samples from spoiling (Kelly *et al*., 2006a, 2006b). 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., 2006a, 2006b). Nonetheless, 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*., 2006a, 2006b; Carabel *et al*., 2009).

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*., 2006a, 2006b; 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% C.I.: 61.6 ± 5.9 g, range: 0.6–228.8 g) were collected from a sidestream 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 (Smith-Root LR-24, Vancouver, WA, USA). After their capture, the eels were euthanised and weighed (to 0.01 g), and dorsal muscle, fin and epidermal mucous samples were taken (*cf*. Winter *et al*., 2019a, 2019b; Winter & Britton, 2021). These initial tissue samples were considered as 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"–"I"). On days 15, 30, 45, 90 and 140 (T_{15}–T_{140}), each eel was removed from ethanol and re-weighed (after drying to remove excess ethanol), and new tissue samples were 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°C for 48 h. Although a mucous sample was taken on T_0 and T_{15}, no mucus was available on any eel to sample thereafter. The tissue and mucous samples were then bulk analysed for $\delta^{13}C$ and $\delta^{15}N$ in a Thermo Delta V isotope ratio mass spectrometer (Thermo Scientific, Waltham, MA, USA) interfaced to an NC2500 elemental analyser (CE Elantach Inc., Lake- wood, NJ, 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 S.D. 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 Information 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; Supporting Information Table S1). Significant shifts in fin and mucus $\delta^{13}C$ were found between T_0 and each subsequent time step (T_{15}–T_{140}) but not between the time steps of T_{15} and T_{140} (Figure 1; Supporting Information 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 Information Figure S3).

With the preservation-induced shifts in $\delta^{13}C$ occurring between T_0 and T_{15} only 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 was significantly correlated (Pearson's correlation coefficient: $r = 0.87$, $P < 0.01$), they were not included together in models (Supporting Information Table S3). Model fitting procedures (AIC) identified the best-fitting model as ~tissue + individual + mass change (Table 1; Supporting Information Table S3). This final model also provided the mean difference (±95% C.I.) 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 ^{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% C.I.: 0.58 ± 0.23‰; Supporting Information 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 ^{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*., 2006a, 2006b; 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.

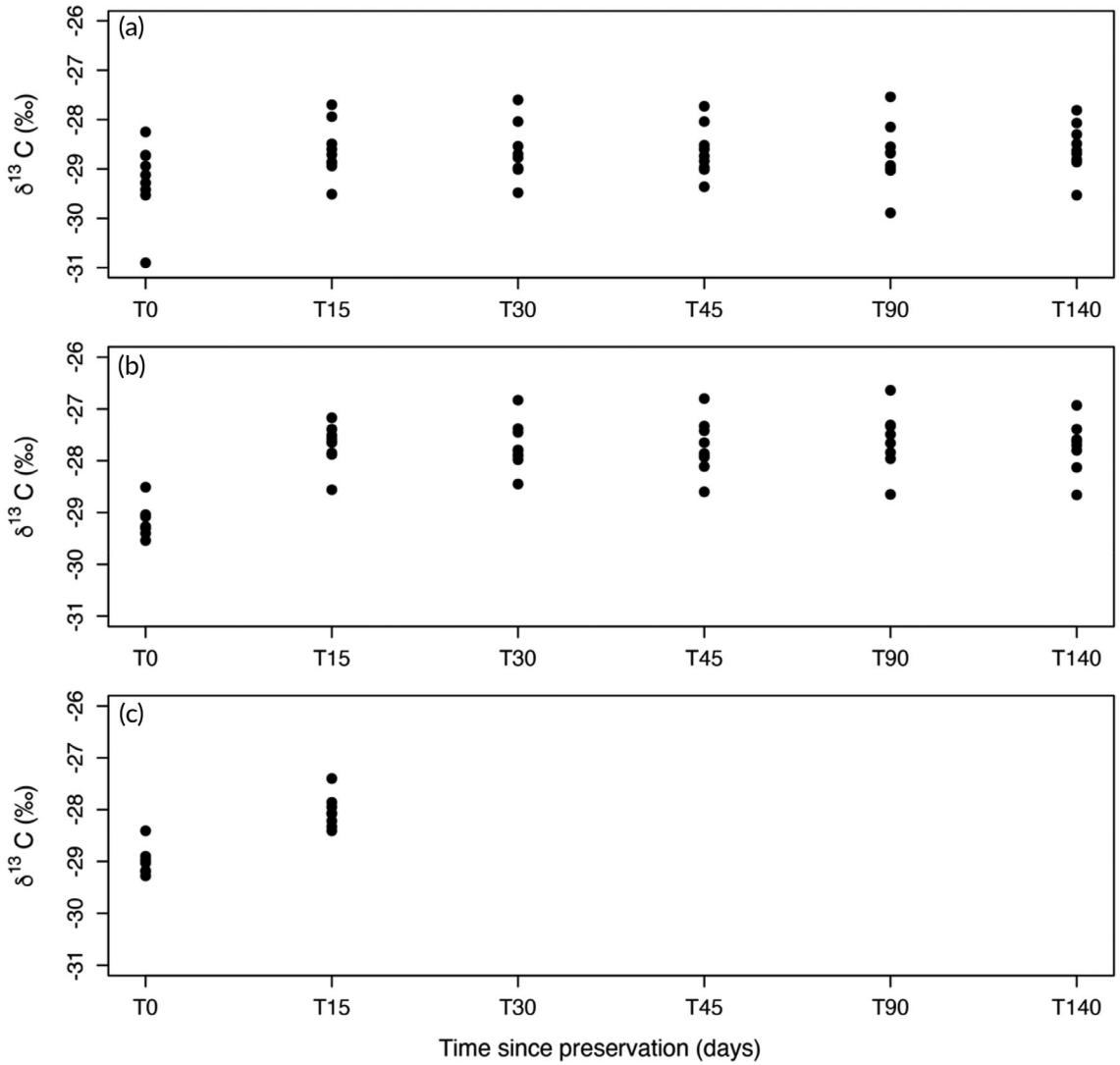

FIGURE 1 Time since ethanol preservation *vs.* $\delta^{13}C$ of each tissue on days T_0–T_{140} (a) dorsal muscle, (b) fin and (c) mucus. Note that mucus could not be extracted on days 30–140. See Supporting Information Figure S1 for plots of individual eel

TABLE 1 Results of the best-fitting linear mixed effects model (~tissue + individual + mass change; *cf*. Supporting Information Table S3) testing the effect of ethanol preservation on $\delta^{13}C$ values between T_0 and T_{15}

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

Note: "Estimate" represents the mean difference in $\delta^{13}C$ between the two time steps for use as a correction factor.

The effect of preservation time is an important consideration for SI studies that incorporate the use of preserved animal samples. There is consensus that the effects of ethanol preservation on $\delta^{13}C$ usually occur rapidly, usually within the first 2–3 weeks of preservation (Kaehler & Pakhomov, 2001), but are independent of storage time thereafter. Indeed, SI tissue samples from a range of taxa preserved over a periods of years have revealed no isotopic changes as their preservation time increased (Edwards *et al*., 2002; Rennie *et al*., 2012; Syväranta *et al*., 2008), 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. The authors recommend that where ethanol-preserved samples comprise 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.

AUTHOR CONTRIBUTIONS

All authors were involved in the conceptualisation of the study and in writing and editing the manuscript. R.M.B., J.R.B. and A.C.P. completed

all sampling. J.R.B. and R.M.B. completed sample preparation, and R.M.B. completed all data analyses and evaluation. All authors agreed to submission of the manuscript.

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CONFLICT OF INTEREST

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

ETHICS STATEMENT

This study was performed after obtaining all relevant ethical and legislative approvals (UK Home Office Project Licence P47216841; Environment Agency permit reference EP/EW027-C-042/19919/01).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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