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$\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ isotope values from preserved elasmobranch jaws: Implications for ecological studies from existing collections

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ABSTRACT

Stable isotope analysis has become a widely used biogeochemical tool owing to its capacity to reveal predator foraging habitats, trophic level, and prey preferences. The breadth of applicable tissue types is quickly growing across taxa, including for elasmobranchs, with tooth isotopes gaining traction to trace within-individual variation in trophic ecology. Jaws in museums and private collections present a unique opportunity to access samples from rare or protected species and size classes. However, most of these jaws are chemically treated to prevent degradation and to whiten teeth and cartilage for aesthetic and long-term display. Prior to using stable isotopes from these jaws, we need to understand the impacts of chemical treatments on carbon, nitrogen, and sulphur isotopes. We compared the tooth preparation process (acid digestion) and $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$ values of teeth from dried jaws to jaws preserved in ethanol, bleach, or hydrogen peroxide. We investigated the effects of preservation methods across three elasmobranch species with distinct tooth morphologies: cownose rays (*Rhinoptera bonasus*) with tooth plates, gummy sharks (*Mustelus antarcticus*) with small plate-like teeth, and broadnose sevengill sharks (*Notorynchus cepedianus*) with larger serrated teeth. Preservation had no impact on tooth digestibility or $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$ values across all dentition types. These findings support the use of display jaws from private collections and museums in ecological studies using isotopes.

1. Introduction

Dietary biomarkers have become a widely used tool for quantitative analysis of dietary composition and foraging patterns of predators, particularly in environments that preclude direct observations of feeding events (Drew et al., 2024; Jackel et al., 2023; Raoult et al., 2020). The feeding ecology of marine predators is increasingly assessed using carbon, nitrogen, and more recently sulphur stable isotopes (Raoult et al., 2024; Calver and Loneragan, 2024; Matich et al., 2014; Munroe et al., 2018; Griffiths, 1991), owing to limitations in stomach content analysis which only provides information about recently ingested meals and is skewed by differences in digestion rate across ingested preys (Carbia et al., 2020; Tieszen et al., 1983). The use of stable isotopes as dietary tracers relies on the occurrence of natural isotopes, which exist as both heavy (^{13}C, ^{15}N, ^{34}S) and light isotopes (^{12}C, ^{14}N, ^{32}S), the ratios of which differ predictably across trophic levels and habitats enabling their use as biomarkers in trophic ecology (Croisetiere et al., 2009; Hussey et al., 2010; Raoult et al., 2024; Munroe et al., 2018; Roberts et al., 2024).

As isotopes are incorporated into all tissues, muscle, skin, liver, blood, blubber, vertebrae, teeth, (Olin et al., 2014; Burke et al., 2023; Hussey et al., 2012), a variety of tissues have been used for stable isotope analysis (Koch et al., 2007; Kim and Koch, 2011; Matich et al., 2014; Guiry and Hunt, 2020; Shipley et al. 2021; Smith et al., 2023). Certain tissues assimilate isotope ratios from prey sources at different rates, thus reflecting diet over unique time-scales and encouraging the careful consideration of which tissue or tissues are most applicable (Kim et al., 2012; Logan and Lutcavage, 2010). Isotopic signatures can be influenced by a range of biotic, e.g., development stage and sex, and abiotic factors, e.g., sample storage temperatures and preservation solutions (McCutchan et al., 2003; Planas et al., 2020; Wolf et al., 2009). Preservation solutions like ethanol, formalin, and lugol's iodine are commonly used by scientists and museums (Komoroske et al., 2017) to store tissues for later biochemical and genetic analysis (Olin et al.,

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2014). However, such preservation methods have variable tissue- and taxa-specific effects on both $\delta^{13}C$ and $\delta^{15}N$ values (Davenport and Bax, 2002; Willert et al., 2020; Nagy, 2010; Peiman et al., 2021; Sarakinos et al., 2002). The extent to which preservation impacts carbon and nitrogen stable isotope ratios remains unclear, and limited to a few preservation methods, taxa, and tissues. Furthermore, the effects on sulphur isotope ratios has not been investigated to date, despite its growing use (Raoult et al., 2024). The impact of preservation must be assessed to expand the range of preserved tissues accessible for stable isotope studies, especially for taxa and tissues that are difficult to sample.

Elasmobranchs (sharks, rays, and chimaeras) are one of the most geographically widespread and diverse groups of meso- and top-predators (Heupel et al., 2014; White and Last, 2012). However, our understanding of the trophic role of elasmobranchs is often limited by sample accessibility, highlighting the need for new techniques or alternative tissues that provide additional information about an individual's trophic ecology (Harahush et al., 2012). This is especially true for rare and threatened species from which biopsies might be logistically difficult to obtain due to ecological scarcity (Meyer et al., 2020; Smart et al., 2013). Stable isotopes from elasmobranch teeth (shark) and plates (rays) are a novel source of stable isotope tissue which are growing in popularity (Grainger et al., 2022; Shipley et al. 2021; Valdez et al., 2022). Dentin from these structures encapsulates diet from amino acids in the blood when the tooth was formed (Smith et al., 2013; Guiry and Szpak, 2020; Trayler et al., 2023; Valdez et al., 2022; Zeichner et al., 2017). Elasmobranch teeth and plates can also provide a unique opportunity to understand an individual's trophic ecology across time as new teeth are continuously formed and capture trophic signature at different time points (Valdez et al., 2022; Hulsey et al., 2020). While teeth can be easily obtained from species commercially or recreationally targeted, teeth from rare or protected species are logistically more difficult to obtain. Preserved jaws from private collections and museums provide an alternative source of teeth that can be used for feeding ecology studies. This is particularly relevant for charismatic megafauna that have been targeted by game fishers seeking trophy jaws (e.g., white shark, *Carcharodon carcharias*; shortfin mako, *Isurus oxyrinchus*; tiger shark, *Galeocerdo cuvier*). Teeth can also act as potential source for historic samples as shark's cartilaginous skeletons and soft tissues easily degrade (Luer et al., 1990; Ahonen and Stow, 2008).

Scientists, museum curators, and private collectors use various methods and chemicals to preserve elasmobranch jaws and whiten teeth for aesthetic and long-term display, e.g., natural drying, hydrogen peroxide (H_2O_2), or bleach (NaClO) (Komoroske et al., 2017; Correa, 2012). Researchers have also used chemicals like hydrogen peroxide to remove soft tissue from shark teeth prior to digestion and isotope analysis with the assumption that they do not impact tooth isotopes (Shipley et al. 2021). However, the natural crystallinity and physical hardness of the enameloid of shark teeth and ray plates may be impacted by these treatments (Shipley et al. 2021). Carbon and nitrogen rich compounds (e.g., lipids, proteins including amino acids) are present in some of the preservation solutions, e.g., ethanol and formalin (Lau et al., 2012; Xu et al., 2011), and thus could alter the isotopic signatures of samples stored in these solutions. Storage duration may also further affect stable isotopes. For example, the difference in $\delta^{15}N$ values between frozen and ethanol-stored fin tissue of smalltooth sawfish (*Pristis pectinata*) changed over time (Olin et al., 2014), highlighting the complicated relationships between preservation methods and sample storage. How common preservation methods and storage duration affect tooth stable isotopes is unknown, preventing the use of these archived samples to infer the trophic ecology of elasmobranchs, yet trophy jaws may be the most abundant source of samples for some species.

Measuring organic carbon isotopes in teeth requires the removal of inorganic carbon when using teeth for stable isotope analysis (Shipley et al. 2021; Trayler et al., 2023). There are several acids in use for inorganic carbon removal within elasmobranch tooth structures, including acetic, hydrochloric, sulphurous sulfuric, or phosphoric acid (Carrier et al., 2018; Schlacher and Connolly, 2014; Grainger et al., 2022; Shipley et al. 2021). However, digestion methods vary substantially between studies (Carrier et al., 2018; Schlacher and Connolly, 2014; Grainger et al., 2022; Shipley et al. 2021), and often lack key details including soak times, acid concentration, initial sample weight, or resulting percentage of sample remaining. Specific digestion protocols are necessary to avoid the risk of over-digestion samples, leaving insufficient quantities for analysis, or and under-digesting a sample which leaves an excess of inorganic carbon thus impacting the resulting isotope data. The lack of specific information and inconsistent acid digestion protocols makes it difficult to replicate studies, or decide protocols should be used to remove inorganic carbon from specific tissues.

Our study, therefore, aimed to advance acid digestion protocols and assess the impacts of common jaw preservation methods (ethanol, bleach, and hydrogen peroxide) and storage time (no storage *versus* stored for six months) on the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values of elasmobranch teeth and plates of different tooth morphologies. We tested changes in isotopic values across three phylogenetically distinct species with varying tooth morphology (Fig. 1): cownose ray (*Rhinoptera bonasus*) with tough plates, gummy shark (*Mustelus antarcticus*) with denticle plate-like shaped teeth, and broadnose sevengill shark (*Notorynchus cepedianus*) with cockscomb shaped teeth. Incorporating species with different feeding mechanics and detention morphologies aided in assessing if the impact of jaw preservation on stable isotopes is consistent across dentition type. Our aims were to (1) advance acid digestion protocols for elasmobranch tooth demineralisation, (2) test if preservation method affects acid digestion and tooth isotope ratios, (3) determine if and how time after preservation impacts $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values, and (4) assess the consistency of these effects across different dentition types (shark teeth, shark plates, or ray plates).

2. Methodology

2.1. Animal sampling

Elasmobranchs were donated by commercial fishers in Australia. Eight gummy sharks (*Mustelus antarcticus*; size unknown but specimens were subadults and adults) and nine broadnose sevengill sharks (*Notorynchus cepedianus*; 164.3–178.5 cm total length, but also included unmeasured small juveniles of ~80 cm total length) were collected in Robe, South Australia, and nine cownose rays (*Rhinoptera bonasus*; 72–110 cm disc width) from coastal New South Wales that were bycatch caught through the NSW Shark Meshing (Bather Protection) Program. These phylogenetically distinct species were selected due to their varying tooth morphologies, type of prey consumed, and feeding mechanics (cownose rays use tooth plates to crush squid, bivalves, and crustaceans, gummy sharks have small plate-like teeth and feed on small fish, crustaceans, and squid, and broadnose sevengill sharks use broad serrated teeth to feed on sharks, rays, and seals (Barnett et al., 2010; Abrantes and Barnett, 2011; Chan et al., 2022; Collins et al., 2007) (Fig. 1).

2.2. Determining optimal acid digestion treatment

A pilot study was conducted to determine the most appropriate acid digestion protocol to remove inorganic carbon from elasmobranch teeth. Two acids (ethylenediaminetetraacetic acid [EDTA; Sigma-Aldrich®] and hydrochloric acid [HCl]), and four soak times were tested to determine which best demineralised the teeth from each species. This was achieved by removing three to six tooth files from the preserved jaws and grinding them into a fine powder using a ball-mill (as described in Shipley et al., 2021). Using the ground tooth files, two acid digestion solutions (5 mL of 0.5 M pH 8.0, EDTA) (Grainger et al., 2022) and 1 mL, 5 mL, and 10 mL of 1 M HCl (Bosley and Wainright, 1999; Shipley et al., 2021b) were used to determine which acid and concentration resulted in

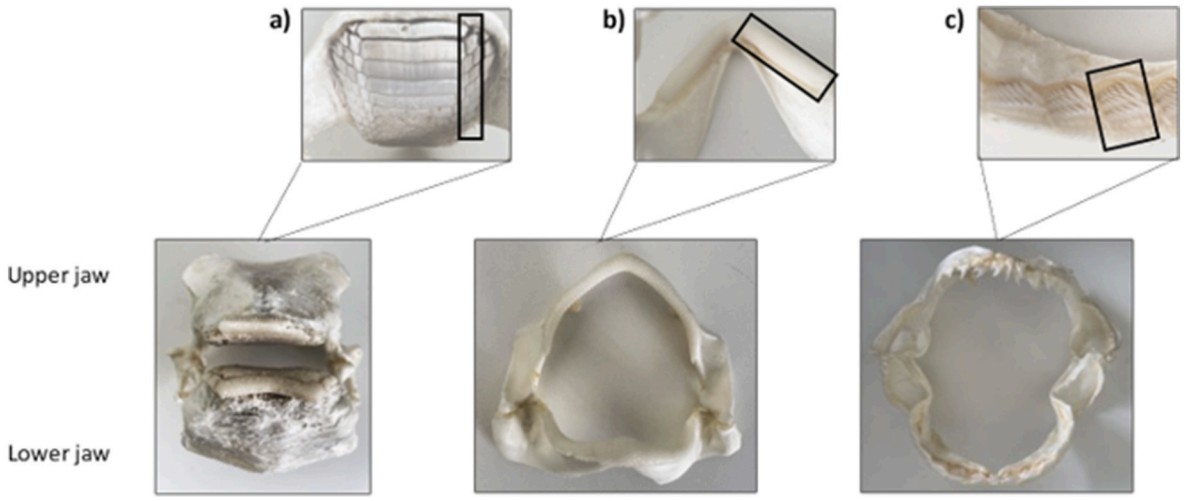

Fig. 1. (a) Jaws of a cownose ray (*Rhinoptera bonasus*); (b) gummy shark (*Mustelus antarcticus*); and (c) broadnose sevengill shark (*Notorynchus cepedianus*). Black box shows the tooth file(s) collected for each jaw.

sufficient demineralisation while retaining enough organic material for carbon, nitrogen, and sulphur (CNS) isotope analysis. Refrigerated HCl was the preferred demineralisation acid as it digested the samples to 20% of the original sample within the shortest amount of time (1–2 days) compared to refrigerated EDTA (1–3 weeks). Out of the four HCl concentrations, 5 mL provided the most consistent digestion compared to the 10 mL concentration that over-digested and the 1 mL that under-digested the samples. Refrigerated soak time in 5 mL HCl solution was then trialled over four durations (6 h, 12 h, 18 h, and 24 h) using three 50 mg ($\pm$ 1.5 mg) replicates per species. The replicates were taken from a homogenised mixture containing multiple teeth from multiple individuals of the same species to eliminate the influence of within-individual and within-tooth variation, while ensuring sufficient tooth material was available for all four treatments. Twelve hours of refrigerated immersion produced the most consistent sample digestion that approached the 20% post-digestion weight target (Fig. S4). Following results from these trials, samples were digested in 5 mL HCl for 6 h at 4 °C, and those that remained under-digested (>60% of the original weight) were subsequently redigested for an additional 6 h at 4 °C. We categorised digested samples into groups of over-digested (<20% of original weight), fully-digested (20–35% of original weight), and under-digested (>35% of original weight). Samples that were under-digested were excluded from analysis to eliminate influence of residual inorganic carbon on the isotope values (Grainger et al., 2022). This level of sample digestion was conservative as it adequately digested or over-digested the tooth, ensuring excess inorganic carbon was removed and could not impact the isotope results.

2.3. Tooth extraction and preservation

To assess the impacts of preservation we excised six jaws of each species, which were manually cleaned by removing any soft tissue and cartilage to expose the teeth and plates (Fig. 2). After cleaning, we sectioned jaws into quarters for subsequent treatment. We selected the preservation methods, concentrations, and soak time based on the most common methods and chemical solutions used by museums, private collectors, and commercial jaw cleaners (Wolfehunt, 2023; De Marchi, 2022). Prior to extracting the tooth files, we preserved sections of shark

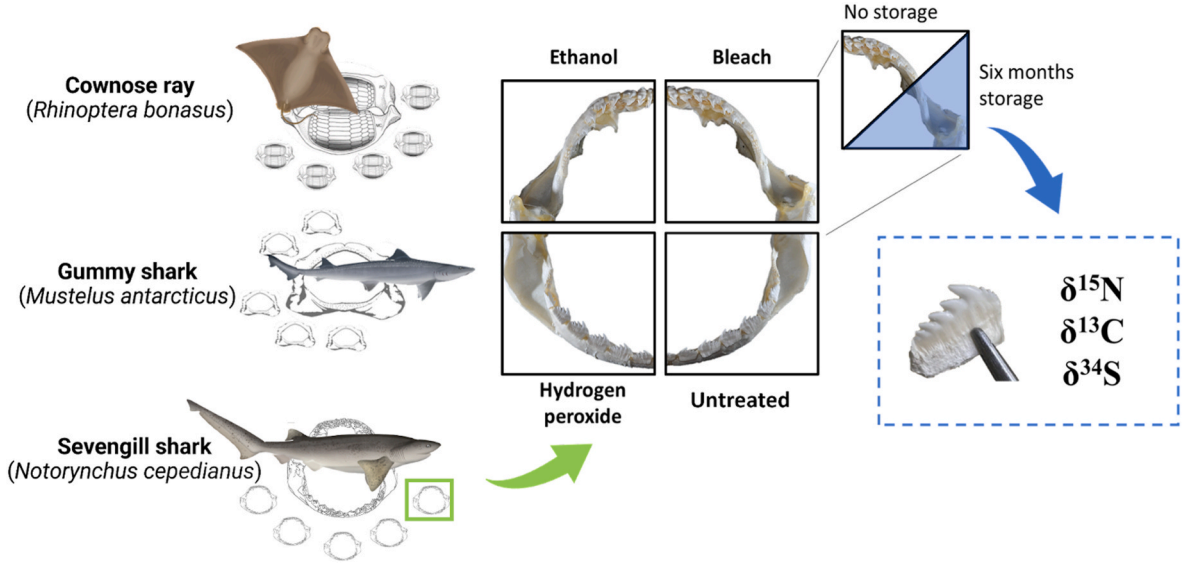

Fig. 2. Experimental design showing the *species*, location of tooth file, the assigned preservation method per jaw, the stable isotopes measured in each treatment, and storage time (indicated in the blue rectangle). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

jaws in hydrogen peroxide (6% H_2O_2), household bleach (10% NaOCl), and ethanol (10% C_2H_5OH), and compared those to frozen and naturally air-dried jaws as a control untreated jaw (Fig. 2). We soaked the jaws for 4 h, air-dried them overnight, then soaked them again for 4 h.

We divided each two segments in two to assess impact of storage time on tooth isotopes. Both halves went through the same preservation process, but one half was analyzed immediately, while the other half was stored for six months in ambient conditions prior to analysis (Fig. 2). For smaller jaws, we used two tooth files to ensure sufficient organic matrix (5 mg) was available after demineralisation for all $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ analysis. If insufficient organic matrix was obtained (<5 mg), a smaller 2 mg sample was used for $\delta^{13}C$ and $\delta^{15}N$ analysis only.

2.4. Tooth demineralisation

We extracted collagen from tooth files using the demineralisation procedure established in the pilot study. We freeze-dried teeth at −50°C for 48 h and homogenised into a fine powder using a 35 ml zirconium oxide capsule in a planetary ball mill (Retsch, MM400). We immersed 80 mg of powdered tooth samples (or the whole sample if < 80 mg) in 5 mL of 1 M hydrochloric acid (HCl), vortexed mixed for 30 s, and then left at 4 °C for 12 h. We re-digested samples that required further digestion (>35% pre-digestion weight) in HCl for 6 h. We then centrifuged the solution at 3500 rpm for 5 min and decanted the supernatant. We rinsed the remaining organic residue using deionized water, removed the supernatant, and centrifuged again for 5 min, and repeated this over three rinse cycles. We then placed the samples in the oven at 50 °C until dry, which took 6–8 h depending on sample weight.

2.5. Stable isotope analysis

Depending on targeted isotopes, between 2 and 12 mg of tooth collagen was weighed into tin capsules. Nitrogen, carbon and sulphur stable isotopes were analyzed using flash combustion isotope ratio mass spectrometry (varioPYRO cube coupled to Isoprime100 mass spectrometer). After combustion at 1120 °C, the bulk sample gas passes through the system and is stripped of H_2O in the water traps, while SO_2 and CO_2 are retained in 'purge and trap' columns. The different components are subsequently fed into the mass spectrometer and measured against a reference gas. As the relative amounts of the gases evolved from a sample at a given weight need to match the linear range of the detectors in the mass spectrometer, the simultaneous determination of all three isotopes is only possible if the ratio of their elemental percentages is not too different. For organic matrices, carbon tends to be the most abundant element, hence a dilutor was used to lower the CO_2 load entering the IRMS source, in the present method with a dilution factor ranging from 2.7 to 8.3, depending on sample size.

Stable isotope abundances are reported in delta (δ) values as the deviations from conventional standards in parts per mil (‰) from the following equation:

$$\delta X\ (‰) = [(R_{sample}/R_{standard} - 1) \times 1000]$$

where X = ^{13}C, ^{15}N or ^{34}S and R = the ratio $^{13}C/^{12}C$, $^{15}N/^{14}N$ or $^{34}S/^{32}S$.

$\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ values are reported respective air, PDB (Pee Dee Belemnite) and CDT (Canyon Diablo Trollite), respectively. Primary Reference Materials (for nitrogen: IAEA-N1, IAEA-N2, USGS40, USGS41 and USGS-25, for sulphur: IAEA-S-1, IAEA-S-2, and IAEA-S-3, IAEA-SO5, NBS 123 and NBS 127; for carbon: NBS 21, USGS 24, USGS 40 and USGS 41) were used to correct for instrumental drift and Quality Control purposes. EA standards (sulfanilamide) and isotope standards were run every 6th sample during analysis.

The analytical precision of the δ values, determined by repetitive measurements of at least three international standards, was around 0.15‰ for carbon, 0.20‰ for nitrogen, and 0.45‰ for sulphur. The precision for blank corrected and factored element content was 0.15% for carbon and nitrogen and 0.25% for sulphur.

2.6. Data analysis

The following samples were excluded from the analysis: (1) samples that were under-digested (>35% sample remaining after HCl digestion; Shipley et al. 2021; Grainger et al., 2022) to eliminate influence of residual inorganic carbon on the isotope values; (2) samples less than 1.8 mg due to lack of sufficient elemental nitrogen to determine $\delta^{15}N$ isotopes; and (3) samples less than 5 mg were excluded from the $\delta^{34}S$ analyses for not containing sufficient elemental sulphur. Gummy shark samples immediately after preservation (i.e., no storage) were over-digested and were therefore not included in the analysis.

We tested whether *preservation* method or *species* influenced the digestion process by a generalised linear model (GLM), using the glm function in the lme4 package (version 1.1–34; Bates et al., 2015). We then assessed whether preservation method or tooth morphology affected tooth stable isotopes using a GLM with *preservation* method (fixed, 4 levels) and *species* (fixed, 3 levels) as independent variables, including the interaction between these two factors, for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values. The effect of storage time was not included in the model because the analytical method that produced the isotope data differed between storage times. Instead, we ran the same models for samples processed immediately and those processed following six months of storage and compared model output to assess whether the impact of *preservation* method and *species* varied between the two storage periods. We selected the most appropriate statistical family and transformation by examining the distribution of the response variable and upon visual inspection of the model residuals. Data were inspected visually, and a square-root transformation was applied for nitrogen and sulphur isotope values, no transformation was required for the carbon values. We ran all model combinations of fixed effect terms using the dredge function (package MuMIn, version 1.43.17; Barton and Barton 2020) and ranked using Akaike's information criterion corrected for small sample size (AIC_c) (Burnham et al., 2011). To explain the variance of the response variable when added to the model, we used the contributions of the fixed variable (marginal R^2) using the r.squaredGLMM function in the lme4 package (Bates et al., 2015). All modelling analyses were done in the R statistical environment (version 4.2.1).

3. Results

3.1. Acid digestion approach

The removal of inorganic carbon, measured as the percentage of samples remaining after digestion, was influenced by *species* (top-ranked model $wAIC_c$ = 0.4), explaining 5% of the variance (Table 1). More dense tooth structures, i.e., cownose ray plates, had the highest mean weight (~19.8 g remaining after digestion) and were the least digested, followed by sevengill sharks (~17.2 g), while the softer teeth from gummy sharks (~14.3 g) were the most digested (Fig. 3). *Preservation*

Table 1
Top five generalised linear models (GLM) assessing the effects of preservation and species on teeth digestion. df, degrees of freedom; LL, log-likelihood; AIC_c, Akaike's information criterion corrected for small sample size; $wAIC_c$, model probability; and variance explained by R_m, marginal (fixed effects). Top-ranked model based on $wAIC_c$ values is shown in bold.

Model	df	LL	AIC_c	$wAIC_c$	R_m
(a) six months storage time					
~ *Species*	**4**	**−411.55**	**831.49**	**0.36**	**0.05**
~ *Preservation* + *Species*	7	−408.37	831.87	0.30	0.10
~1 (intercept - only)	2	−414.44	833.00	0.17	0.00
~ *Preservation*	5	−411.66	833.92	0.11	0.05
~ *Digestion (%)* + *Preservation* + *Species*	13	−402.39	834.65	0.07	0.18

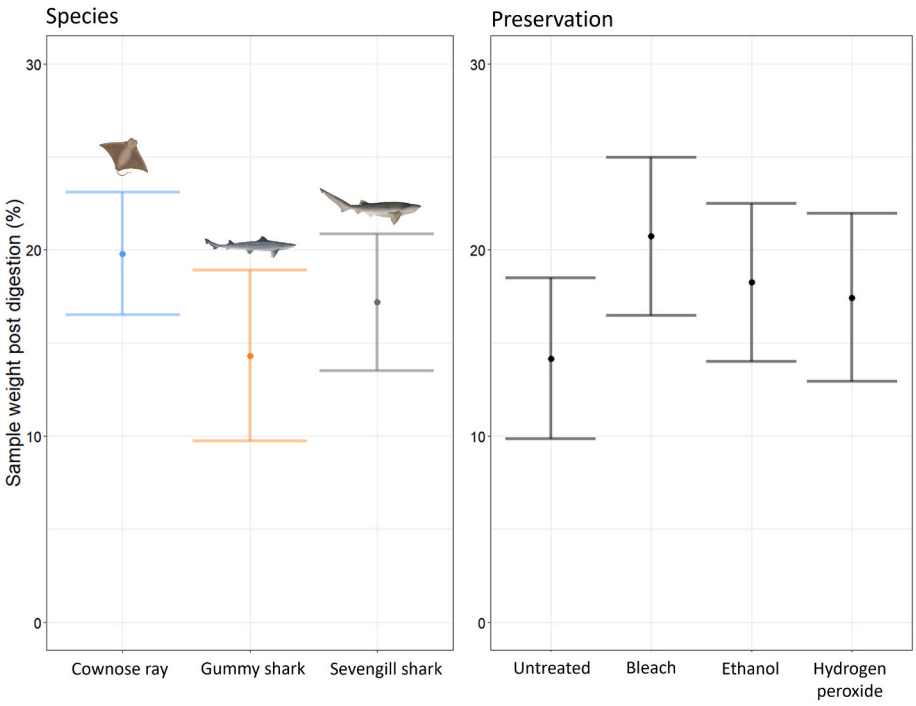

Fig. 3. Predicted weight (estimated marginal means from **first-** [*species*] and **second-ranked** [*preservation*] generalised linear model) of sample remaining after digestion in hydrochloric acid (%) for preserved elasmobranch teeth. Error bars indicate 95% confidence intervals.

Table 2
Top five generalised linear models (GLMs) to effects of preserved elasmobranch teeth on $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$ values. *df*, degrees of freedom; LL, log-likelihood; AIC_c, Akaike's information criterion corrected for small sample size; $wAIC_c$, model probability; and variance explained by R_m, marginal (fixed effects). Top-ranked model based on $wAIUC_c$ values is shown in bold.

Model	*df*	LL	AIC_c	$wAIC_c$	R_m
$\delta^{13}C$					
(a) no storage time					
~ *Species*	**3**	**−21.57**	**50.41**	**0.60**	**0.48**
~ *Digestion* (%) + *Species*	4	−20.64	51.50	0.35	0.50
~ *Preservation* + *Species*	6	−19.66	56.56	0.03	0.52
~ *Preservation* * *Species*	8	−15.62	57.52	0.02	0.62
(b) six months storage time					
~ *Species*	**4**	**−42.65**	**94.56**	**0.64**	**0.20**
~ *Digestion* (%) + *Species*	5	−42.64	97.22	0.17	0.20
~1 (intercept - only)	2	−47.00	98.35	0.10	<0.01
~ *Preservation* + *Species*	7	−41.10	100.06	0.04	0.24
$\delta^{15}N$					
(a) no storage time					
~ *Species*	**3**	**24.06**	**−40.86**	**0.77**	**0.92**
~ *Digestion* (%) + *Species*	4	24.29	−38.36	0.22	0.92
~ *Preservation* + *Species*	6	24.28	−31.30	0.01	0.91
~ *Digestion* (%) + *Preservation* + *Species*	7	24.75	−28.03	<0.01	0.91
(b) six months storage time					
~ *Species*	**4**	**15.66**	**−22.07**	**0.46**	**0.75**
~ *Digestion* (%) + *Species*	5	16.52	−21.10	0.29	0.75
~ *Preservation* + *Species*	7	19.06	−20.25	0.19	0.77
~ *Digestion* (%) + *Preservation* + *Species*	8	19.61	−18.07	0.06	0.77
$\delta^{34}S$					
(a) no storage time					
~ *Species*	**3**	**31.82**	**−56.39**	**0.65**	**0.36**
~ *Digestion* (%) + *Species*	4	32.33	−54.44	0.24	0.37
~ *Preservation* + *Species*	6	34.38	−51.52	0.06	0.44
~ *Digestion* (%) + *Preservation* + *Species*	7	35.28	−49.09	0.02	0.47
(b) six months storage time					
~1 (intercept - only)	**2**	**14.90**	**−25.45**	**0.66**	**< 0.01**
~ *Digestion* (%)	3	15.05	−23.37	0.23	0.01
~ *Species*	4	14.99	−20.73	0.06	<0.01
~ *Preservation*	5	15.26	−18.59	0.02	0.02

method and *species* influenced the removal of inorganic carbon (second-ranked model $wAIC_c$ = 0.3), with 10% of model variance explained (Table 1). Untreated teeth had the least amount of sample remaining after digestion (<15%), compared to other methods (>15–30%).

3.2. Impact of preservation on tooth isotopes

Preservation method was not included in the top-ranked models for $\delta^{13}C$, $\delta^{15}N$, or $\delta^{34}S$ values, suggesting that it did not drive variation of stable isotope values (Table 2). There was no difference in tooth isotopes between the untreated teeth and those preserved in bleach, ethanol, and hydrogen peroxide (Fig. 4). *Preservation* method did not have an impact on tooth isotopes at either storage time and was consistent across dentition type (Table 2). $\delta^{13}C$ ranged from −16.0 to −13.2‰, −18.5 to −12.6‰, and −18.4 to −13.2‰ for cownose rays, sevengill sharks, and gummy sharks, respectively. $\delta^{15}N$ ranged from 9.3 to 12.9‰, 12.0–17.8‰, and 10.9–14.4‰ for cownose rays, sevengill sharks, and gummy sharks, respectively. $\delta^{34}S$ ranged from 15.1 to 19.3‰, 13.7–19.1‰, and 15.0–19.1‰ for cownose rays, sevengill sharks, and gummy sharks, respectively.

3.3. Isotopic analysis

3.3.1. Carbon

Species was the only factor included in the top-ranked model of $\delta^{13}C$ value for teeth processed immediately after being treated for preservation and after six months of storage ($wAIC_c$ = 0.60 and 0.64, respectively; Table 2), with the model explaining 48% and 20% of the variance, respectively. For both storage times, sevengill sharks had the lowest $\delta^{13}C$ values (−16.3‰ and −15.8‰, respectively). Cownose rays had the highest $\delta^{13}C$ (−14.9‰) immediately after being treated for preservation and was similar to gummy sharks when this species was included in the model with samples stored for six months (Fig. 5).

3.3.2. Nitrogen

Species was also the only factor included in the top-ranked model of $\delta^{15}N$ values for teeth processed immediately after being treated for

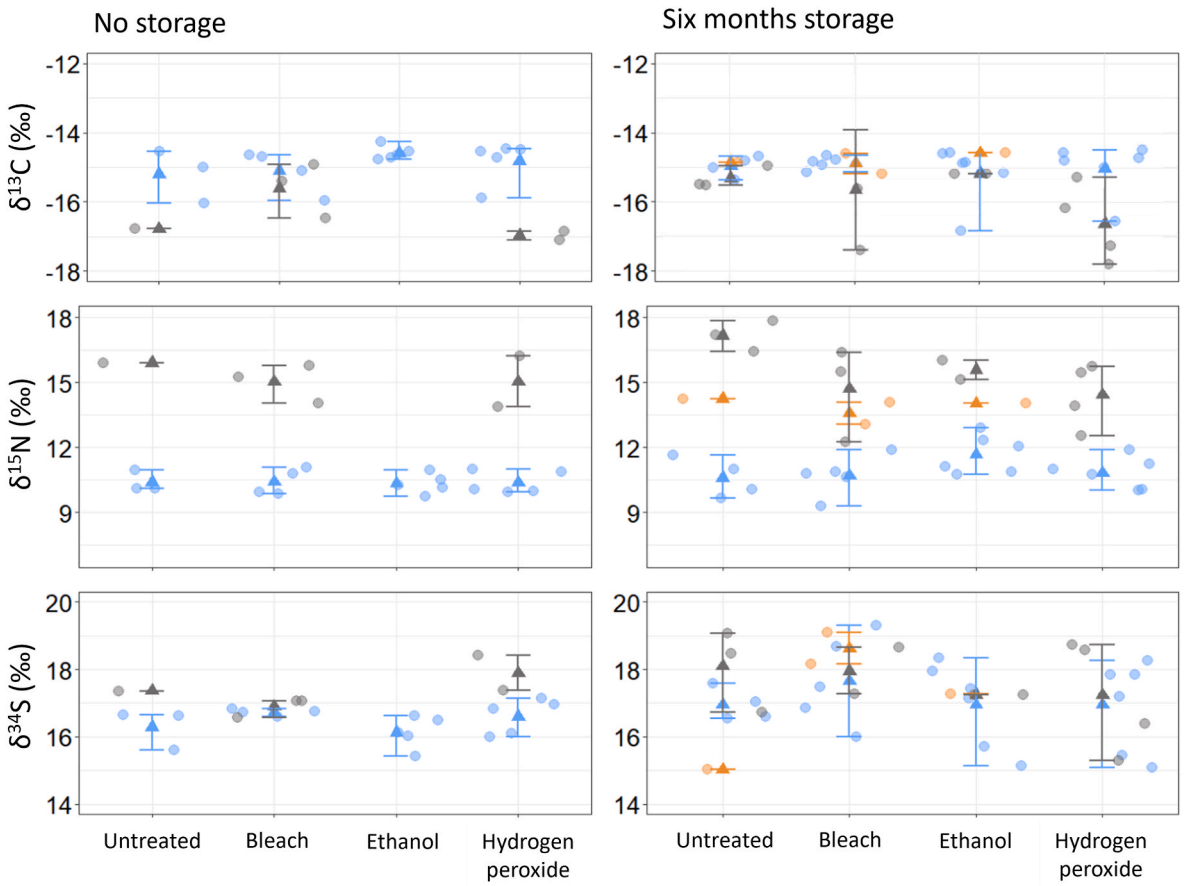

Fig. 4. $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ (‰) values for elasmobranch teeth immediately after preservation and after six months of storage across different dentition structures. Error bars indicate 95% confidence intervals. Blue represents cownose rays, orange represents gummy sharks, and grey represents sevengill sharks. Circles represent isotope values, and triangles represent mean isotope values for each species. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

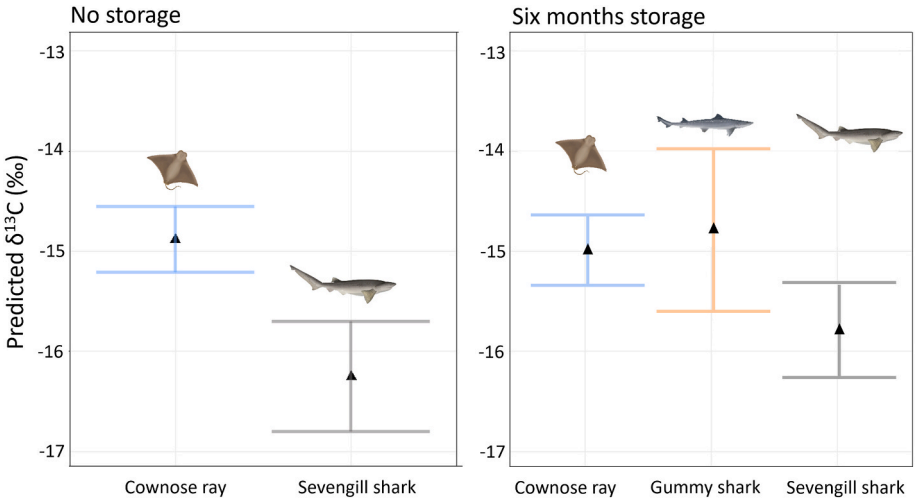

Fig. 5. Predicted $\delta^{13}C$ value (‰) (estimated marginal means from **top-ranked** generalised linear model) for elasmobranch teeth immediately after being treated for preservation (left) and after six months of storage (right) across different dentition structures. Error bars indicate 95% confidence intervals.

preservation and after six months of storage ($wAIC_c$ = 0.77 and 0.46, respectively; Table 2), with the model explaining a large amount of the variance (92% and 75%, respectively). In both time periods, cownose ray plates had the lowest $\delta^{15}N$ value (~10.4‰ and ~11.0‰), while sevengill shark teeth had the highest $\delta^{15}N$ value which was similar across time periods (~15.2‰) (Fig. 6). Gummy sharks had $\delta^{15}N$ values between that of cownose rays and sevengill sharks (~13.9‰).

3.3.3. *Sulphur*

The only factor that influenced $\delta^{34}S$ values for teeth processed immediately after being treated for preservation was *species* ($wAIC_c$ = 0.65; Table 2), which explained 36% of the variance. The $\delta^{34}S$ value of

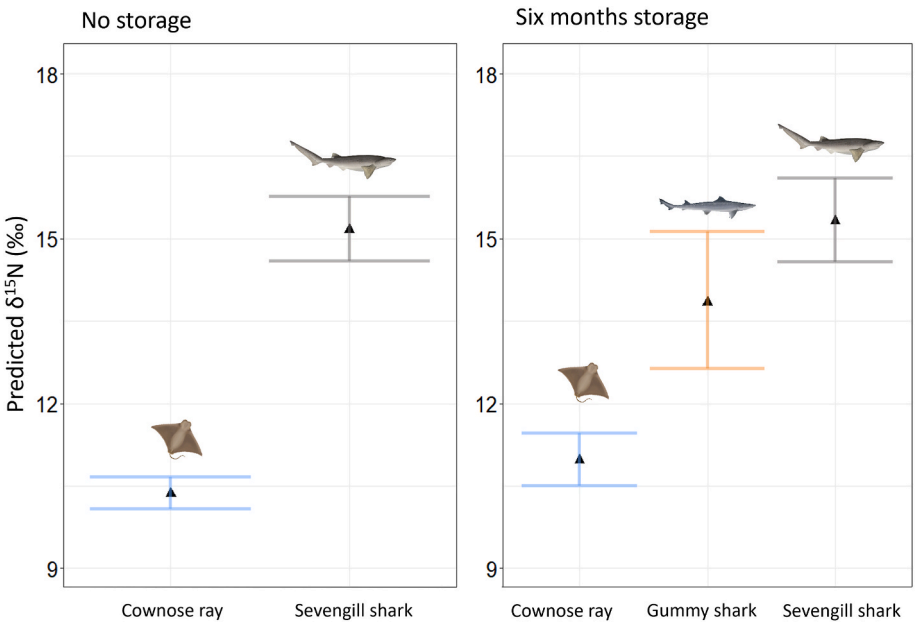

Fig. 6. Predicted $\delta^{15}N$ value (‰) (estimated marginal means from **top-ranked** generalised linear model) for preserved elasmobranch teeth immediately after being treated for preservation (left) by storage time; no storage; and after six months of storage (right) across different dentition structures. Error bars indicate 95% confidence intervals.

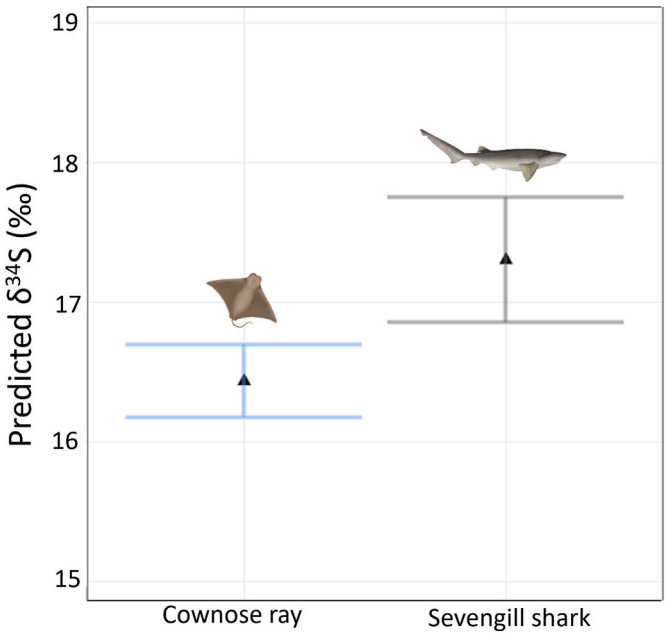

Fig. 7. Predicted $\delta^{34}S$ value (‰) (estimated marginal means from **top-ranked** generalised linear model) for preserved elasmobranch teeth immediately after being treated for preservation across different dentition structures. Error bars indicate 95% confidence intervals.

sevengill shark teeth (17.3‰) was higher than that of cownose ray plates (16.4‰) (Fig. 7). However, the top-ranked model after six months of storage was the null model ($wAIC_c = 0.66$; Table 2), suggesting that $\delta^{34}S$ values were similar across species.

4. Discussion

Our study demonstrates that the primary chemical treatments used by museum and private collectors to preserve shark jaws (i.e., ethanol, bleach, and hydrogen peroxide) do not affect elasmobranch tooth $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ regardless of storage duration. The lack of impact from these chemicals was ubiquitous across all three species, which encompassed a range of dentition structures including small, soft, flattened teeth (gummy shark), large dense serrated teeth (sevengill shark), and hard, very dense plates (cownose ray). However, dentition type affected the removal of inorganic carbon, as the dense cownose ray plates were less digested compared to the soft gummy shark teeth. While 12-hr digestion in refrigerated 1 M HCl yielded the most consistent digestion, the persistent differences in digestibility between species and preservation methods advocates for ongoing exploration of tooth demineralisation protocols and target amounts of samples remaining post-digestion. Each species had unique $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values, in line with their trophic position and basal food source, highlighting the utility of elasmobranch teeth in trophic ecology studies. However, $\delta^{34}S$ values were no longer distinct between species after six months of storage, suggesting a biologically, kinetically, or thermodynamically driven fractionation process of sulphur isotopes in collagen.

4.1. Removal of inorganic carbon

Removal of inorganic carbon from tooth material is necessary to enable accurate assessments of animal feeding ecology using tooth isotopes, as the structural carbonate of teeth contains inorganic carbon, which can bias estimates of the organic $\delta^{13}C$ values used in ecological studies (Trayler et al., 2023; Guiry and Szpak, 2020). Despite the importance of inorganic carbon removal, different acids, concentrations, and soak times have been used to reach various target digestion amounts. Those using EDTA to digest teeth reported slower and more consistent digestion rates than with HCl, enabling greater control over the most appropriate soak times to minimise the risk of over-digestion that can result in insufficient material for C, N, and S analysis (Grainger et al., 2022). Our trial found that the EDTA protocol resulted in variable digestion amounts. Many samples crystallised onto the glass vials such that they could not be removed, and of the samples that could be removed and weighted, most were over-digested to the extent that too little tissue remained for subsequent isotope analysis. Additionally, the EDTA protocol was substantially more time intensive, requiring two-week soak times and several hours of preparation per sample. Such logistical and time burdens preclude the use of EDTA digestion in

projects with large sample sizes. While soaking samples in HCl for 12 h improved digestion reliability compared to other soak times and to EDTA digestion, the variation in the amount of tissue remaining post-digestion remained higher than anticipated (8.8–18.7% at 12 h) and differed among species. We recommend using a weak acid solution of 5 mL of 1 M hydrochloric acid (HCl), with shorter soak times (i.e. 10 h) for soft teeth (i.e., gummy shark) and longer soak times (24 h) for dense teeth (i.e., ray plates) to reach a post-digestion sample weight 20% of the initial sample size.

Tooth structure affected the rate of acid digestion, with soft teeth digesting quicker than the tough ray plates. Gummy sharks have very soft tooth denticles which may be more permeable compared to the dense tooth plates in cownose rays (Enax et al., 2012; Kolmann et al., 2015), explaining the rapid but inconsistent digestion observed here. The likely influence of tooth structure, hardness, and permeability on digestion rate may explain why our EDTA trial resulted in severe over-digestion. The EDTA protocol was optimized by Grainger et al. (2022) for juvenile white shark teeth which may be tougher and less permeable than the shark teeth and ray plates used in our study. The species-specific results highlight that advances in acid digestion must be treated with caution, as they may have limited applicability across species with different detention, and particularly between taxa with different tooth compositions (e.g. enameloid vs. enamel) (Carrasco et al., 2018; Mateo et al., 2008). These limitations have been detailed for other taxa including otters, whose unique tooth composition prevented the use of established acid digestion protocols (Carrasco et al., 2018), and human bones and teeth whereby the isotope values differed between acid-digestion methods (Demény et al., 2019). Given that acid digestion effectiveness is species- and taxa-specific, we recommend isotope ecologists undertake, and report the results of, digestion trials, even when protocols optimized for similar species are available. This is particularly important as the use of hard structures, like teeth, grows in popularity amongst ecologists.

4.2. Does preservation method impact tooth isotopes?

The impacts of preservation type, i.e., ethanol, formalin, salt, lugol's iodine, and freezing, on $\delta^{13}C$ and $\delta^{15}N$ values have been assessed across different tissues (i.e., muscle, skin, fin clips, blood, and whole organisms) and taxa (i.e., invertebrates, Osteichthyes, Mollusca, Mammalia, and Chondrichthyes) (Bosley and Wainright, 1999; Davenport and Bax, 2002; Hidalgo-Reza et al., 2019; Willert et al., 2020). These studies demonstrate that the effects of chemical treatments or other preservation methods vary among species and treatments/methods. However, most of these studies have focused on the isotope ratios of soft tissues (e.g., muscle and liver) with more permeable surfaces with the potential to absorb external chemicals at a faster rate than harder structures, like teeth and vertebrae (Enax et al., 2012). These hard structures provide a unique opportunity to use less permeable and isotopically exchangeable tissues for trophic ecology studies. In contrast to previous studies using soft tissues (e.g., Hidalgo-Reza et al., 2019; Willert et al., 2020), we found that preservation did not impact the isotope ratios of teeth.

Ethanol typically has the greatest impact on chondrichthyan soft tissue isotopes, with lower $\delta^{13}C$ values and higher $\delta^{15}N$ values in preserved muscles compared to unpreserved muscles (Kim and Koch, 2011; Olin et al., 2014; Macleod, 2015), likely from removal of urea and TMAO. From this, we expected ethanol to impact tooth isotopes, but found consistent $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values across all preservation methods. Hydrogen peroxide and bleach react poorly or not at all to other molecules, thus reducing the potential for introduced carbon (Crowley and Wheatley, 2014; Winterbourn, 2013). This likely explains the lack of changes in $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values after teeth were preserved in bleach or hydrogen peroxide. As these methods are commonly used by private collectors and commercial jaw cleaners, findings from our study are directly applicable to jaws in private collections and confirm that isotopic results from preserved teeth are not compromised by the most common chemical treatments and preservation methods.

Published literature on the impacts of chemical preservation by museums and researchers on isotope values focuses on prolonged soak times and storage in chemicals like ethanol for weeks to years (Bosley and Wainright, 1999; Davenport and Bax, 2002; Hidalgo-Reza et al., 2019; Willert et al., 2020; Olin et al., 2014). Our study, however, focussed on the short soak time of ~8 h, replicating the soak times used by museums and private collectors when preparing elasmobranch jaws for display (De Marchi, 2022)(S. De Marchi, personal communication). Although our study showed that an 8-h ethanol soak does not change tooth isotope values, extended soak times of weeks to years, typical in museum and research specimens, could still affect the isotopes used in trophic studies. Similarly, bleach can modify the organic compounds in teeth with prolonged treatment exceeding 4 h (Sibert et al., 2017). Nevertheless, collectors immerse jaws in bleach for 1–4 h, avoiding the impacts observed with prolonged bleach use (Sibert et al., 2017). Chemically, hydrogen peroxide loses effectiveness over time and rapidly decomposes Watts et al., 1999). Therefore, prolonged soak time in hydrogen peroxide may not increase the impact on tooth isotope values. Overall, this study confirms that the chemical soak times commonly used by private collectors to produce display jaws do not jeopardise the isotopic composition of the teeth, and thus expands the range of jaws available for isotopic research.

4.3. Does dentition type influence the effects of preservation on isotopes?

We tested changes in isotopic values across three phylogenetically distinct species with dentition structures of varying densities and structures to assess whether preservation impact on tooth isotopes was consistent across detention types. In line with previous studies, we expected dentition to influence the response of $\delta^{13}C$ and $\delta^{15}N$ values to preservation methods, attributed to the natural properties of the tissues, i.e., collagen structure, permeability, and metabolic activity specific to species (Kelly et al., 2006; Nagy, 2010; Peiman et al., 2021; Pinnegar and Polunin, 1999; Sarakinos et al., 2002). However, the effect of preservation on isotope values did not vary across tooth structure and density. Even in the case of the small and comparatively soft gummy shark teeth for which samples immediately after treatments were not available due to over-digestion, the stable isotope values were not affected by preservation treatments.

4.4. Does storage time impact tooth isotopes of preserved jaws?

We expected hydrogen peroxide to have an impact on tooth isotopes over time as it is a stable chemical solution, unlike ethanol which rapidly evaporates after application (Robinson et al., 2021) and was therefore less likely to impact isotopes over time. However, neither $\delta^{13}C$ and $\delta^{15}N$ values were affected by storage time, regardless of chemical treatment. For sulphur isotopes, preservation did not impact tooth values at either storage time, but $\delta^{34}S$ values were no longer different between species after six months of storage. This raises the question of whether sulphur isotopes are an informative tracer following six months or longer of natural degradation. Sulphur stable isotope fractionation is closely tied to bacterial processes (Fry et al., 1986), which may naturally occur during ambient exposure over time. Differences in sample sizes due to loss of samples to over-digestion might have also contributed to the lack of detectable differences between species after six months storage. It is also possible that the slightly larger measurement error for sulphur isotope ratios compared to nitrogen isotope ratios (0.25‰ vs 0.15‰, respectively) and small sample size contributed to these patterns. Overall, irrespective of their chemical preservation or natural drying, tooth isotopes did not undergo significant biochemical changes after six months of storage due to preservation, which agrees with most of the literature about long-term storage (Carabel et al., 2009; Edwards et al., 2002; Syväranta et al., 2008).

4.5. Isotopic ratios and ecological applications

4.5.1. Carbon

Species was included in the top-ranked model, indicating that preserved teeth are useable to distinguish differences in species with distinct foraging habitats. Cownose rays had the largest $\delta^{13}C$ range (~−14.0‰ to −12.6‰), followed by gummy sharks (−14.3‰ to −12.6‰), and sevengill sharks the lowest (~−14.5‰ to −14.0‰). Although the gummy sharks and cownose rays were each sampled from the same geographical region and presumed to have similar $\delta^{13}C$ within individuals of the group, we observed considerable individual variation. This suggests a range of carbon sources available to benthic feeders like rays and small benthic sharks (Chan et al., 2022). However, this stems from only six individuals and may not be representative of the species' full trophic niche. Sevengill sharks and gummy sharks have distinct foraging habitats, behaviours, and prey items (Barnett et al., 2010; Shaw et al., 2016), explaining why $\delta^{13}C$ values in sevengill sharks (−14.5‰) were lower than gummy sharks (−13.5‰), reflecting that sevengill sharks forage within comparatively offshore food webs (Barnett et al., 2010; Funes et al., 2023) or have a greater reliance on seagrass-driven food webs (Davenport and Bax, 2002; Heithaus et al., 2013). This may also be true for cownose rays who had varying degrees of overlap of $\delta^{13}C$ values to the other elasmobranch species, linking to use of benthic and pelagic habitats for primary producer consumption (Collins et al., 2007; Kolmann et al., 2015).

4.5.2. Nitrogen

Lower $\delta^{15}N$ values were observed in cownose rays (~11.0‰) compared to gummy sharks and sevengill sharks (~13.9‰ and ~15.3‰, respectively). This was expected as cownose rays consume a wide variety of low trophic level benthic organisms (Collins et al., 2007) compared to sevengill sharks that typically feed on smaller elasmobranch species (including gummy sharks), small pinniped, teleosts, and cephalopods. The difference in $\delta^{15}N$ values between cownose rays (~10.7‰) and sevengill sharks (~15.2‰) was the equivalent of a whole trophic level (3.4‰ enrichment of $\delta^{15}N$; Hussey et al., 2014), which is supported by previous studies (Barnett et al., 2010; Raoult et al., 2019). Gummy sharks had the widest $\delta^{15}N$ range (~12.3–15.1‰) out of the three species, which overlaps with the $\delta^{15}N$ values of sevengill sharks (~14.6–16.1‰). While it was expected for these two species to have non-overlapping nitrogen values based on the known diet of these species, the sevengill sharks included in this species were relatively small, potentially explaining the slight overlap observed.

4.5.3. Sulphur

Sulphur stable isotopes have been previously used for food-web modelling in conjunction with $\delta^{13}C$ and $\delta^{15}N$ values to better discriminate between benthic (~1‰) and pelagic (~20‰) productivity (Raoult et al., 2019; Connolly et al., 2004), providing insights into the basal food sources of elasmobranchs. The tooth plates from cownose rays had slightly lower $\delta^{34}S$ values (~16.4‰) than the sevengill shark teeth (~17.3‰), reflecting the benthic foraging quintessential of cownose rays (Raoult et al., 2019). Yet, when comparing our results to previous studies, lower $\delta^{34}S$ values (~14.9‰; Chan et al., 2022) have been reported from fin clips of similar sized cownose rays caught off New South Wales. As preservation did not have an impact on the tooth biochemistry, our higher $\delta^{34}S$ values in cownose plates suggest a greater reliance on pelagic productivity for the individuals sampled in our study.

Application of $\delta^{34}S$ values in elasmobranch research remains relatively novel, and with that comes a range of challenges. This study highlighted challenges of simultaneously analysing the three isotopes (^{13}C, ^{15}N, and ^{34}S) as each isotope has different elemental compositions within the tooth material. We found that for stable isotope analysis of elasmobranch tooth material, $\delta^{13}C$ and $\delta^{15}N$ analysis should be run separately to $\delta^{34}S$ to allow for differences in elemental composition and readability. This is the result of considerably lower concentrations of sulphur in the tooth protein matrix, e.g., 1% for sulphur *versus* 10% and 40% for nitrogen and carbon, respectively. Using this knowledge, we were still able to produce reliable $\delta^{34}S$ values that can be applied to further ecological insights.

4.6. Implications and future research

As chemical preservation did not have an impact on the tooth isotopes, we can infer that elasmobranch teeth or plates with differing dentition can be used for isotope studies even after being treated with bleach, ethanol, and hydrogen peroxide and stored for up to six months. This is particularly useful for rare or threatened species for which standard tissue samples may not be readily available but for which trophy jaws have been collected e.g., white sharks and shortfin mako. As most preserved jaws in private collections and especially museums are typically stored for longer than six months, future studies should determine whether the lack of changes we observed after six months of storage still applies after longer periods. Our study was also limited to elasmobranch teeth. Preserved teeth from other taxa groups, e.g., cetaceans like killer whales (*Orcinus ocra*) or sperm whales (*Physeter macrocephalus*), and pinnipeds including fur seals (*Arctocephalus forsteri*) and sea lions (*Neophoca cinerea*), are also available and may be used in trophic studies. However, these species might have different collagen matrices and acid digestion protocols and preservation methods could impact these teeth differently to elasmobranch teeth, and should be investigated using a similar framework to the methods we used.

5. Conclusion

This study explored stable isotope analysis in elasmobranch tooth material, highlighting the usability of jaws from private collections and museums for trophic ecology studies using $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$ values. Notably, our study reveals that preservation does not affect tooth isotope values, implying that preserved teeth can be used for stable isotope analysis. Minimal differences in tooth isotopes were observed between the two storage times, with the exception of sulphur, suggesting that storage time may not inherently degrade tooth isotopes. We also found that dentition type did not impact how tooth isotopes changed in response to preservation, while still producing ecologically distinguishable isotopic values representative of dietary sources. Furthermore, depending on detector capabilities, tooth isotope analysis may need to be run separately to account for difference in elemental composition within the tooth material (Enax et al., 2012), with $\delta^{15}N$ and $\delta^{13}C$ together and $\delta^{34}S$ values separately. We recommend having a minimum of 80 mg of homogenised tooth sample prior to digestion in HCl for 12 h with the expectation that 20–30% of that sample will remain after digestion. This will allow for sufficient material for analysis of $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$ values. Moreover, the findings encourage the use of preserved teeth from jaws in private collections for future research, given the lack of impact chemical preservation had on tooth isotopes. This study, therefore, contributes valuable insights to the field of stable isotope analysis in elasmobranch teeth, offering practical recommendations and broadening the range of samples available for investigation.

CRediT authorship contribution statement

Laura Holmes: Writing – review & editing, Writing – original draft, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Charlie Huveneers:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Vincent Raoult:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Thomas M. Clarke:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Christian Dietz:** Writing – review & editing,

Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lauren Meyer:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2025.107063.

Data availability

Data will be made available on request.

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