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Integrating fish scale and bone isotopic compositions for 'deep time' retrospective studies

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ABSTRACT

Isotopic studies of archived fish scales have tremendous potential to develop long-term retrospectives that provide important insights into how humans have altered aquatic ecosystems. However, fish specimens in museum archives and other repositories typically date to time periods when the impacts of industrial societies may have already caused profound environmental changes. Archaeological fish bones offer an opportunity to bridge this key temporal gap by providing samples spanning from the recent past to as far back as the Pleistocene. Collagen is the primary protein component of both fish scale and bone, but the comparability of isotopic compositions from these tissues has not been established experimentally. To lay the framework for integrating isotopic datasets from these tissues, we compare $\delta^{13}C$ and $\delta^{15}N$ of bone and scale collagen, as well as other tissues, from fish with life-time controlled diets. Results show that while there is no difference in $\delta^{13}C$ between scale and bone collagen, there may be a very slight but meaningful inter-tissue offset in $\delta^{15}N$ (<0.3‰). We discuss potential sources of $\delta^{15}N$ variation in scale and bone collagen measurements. Because there is no difference in scale and bone $\delta^{13}C$, and the observed offset in $\delta^{15}N$ is very small (less than analytical uncertainty in many studies), our findings demonstrate that collagen isotopic compositions from these tissues should be directly comparable when integrating datasets from modern and ancient samples to build more powerful, millennium-scale isotopic times series. In linking isotopic compositions of collagen from modern, historical (scales), and archaeological (bones) fish, our findings open the way for more nuanced contextualization of how ecosystems functioned prior to large-scale exploitation and how they have responded to mounting anthropogenic pressures in the intervening centuries.

1. Introduction

Comparing isotopic compositions between tissues from modern, historical, and ancient animal populations can provide century-, or even millennium-scale retrospectives for exploring Holocene environmental change (e.g., Braje, et al., 2017; Burton et al., 2001; Guiry and Buckley, 2018; Newsome et al., 2007). By providing longer-term isotopic retrospectives, these studies have tremendous potential for making important contributions to our understanding of past animal behaviour (Guiry et al., 2020; Szpak et al., 2013), nutrient dynamics (e.g., Guiry, et al., 2018; Szpak et al., 2018), and productivity (Schell, 2000). Such information can provide context for organism- and ecosystem-level response to changing conditions, which may be valuable for guiding future environmental restoration efforts and improving conservation policy (Rick and Lockwood, 2013; Swetnam et al., 1999). However, because many of the most significant human impacts began as local societies underwent industrialization (dating as early as the eighteenth century), historical specimens archived in museum collections and other repositories (typically dating to the nineteenth and twentieth centuries) may have isotopic compositions reflecting environments that have already been profoundly altered (Szabó and Hédl, 2011). Archaeological specimens can offer an excellent alternative, often providing abundant sample material and sufficient taxonomic and spatiotemporal control (Lyman 1996, 2006, 2012; Wolverton and Lyman, 2012). With respect

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to aquatic ecosystems, by linking isotopic data from fish scales, collected in the last several decades, and bones, deposited over millennia, there is outstanding potential to build retrospective time series for exploring many dimensions of environmental change in greater detail (Guiry et al. In Review; Guiry et al., 2020; Guiry et al., 2016).

Several studies have begun to explore the potential of integrating modern and historical scale materials with archaeological bone archives based on the assumption that bones and scale isotopic compositions record fish diets and environmental conditions in the same way (e.g., Guiry et al., 2016). Although there have been numerous studies that explore differences in $\delta^{13}C$ and $\delta^{15}N$ between tissues (e.g., Kelly, et al., 2006; MacNeil et al., 2006; Matich et al., 2010; Vollaire et al., 2007), no study that we are aware of has systematically compared the isotopic compositions of bone and scale to establish whether they are directly comparable. Given the importance of bone and scale isotopic compositions in archaeological and ecological research, respectively, it may be surprising that the comparability of collagen from these tissues has not been explored. This may reflect a general division between the kinds of materials that are typically available for analyses in ecological and archaeological research. Ecological research on fish has established isotopic offsets between scale collagen and soft tissues, like muscle and fins (e.g., Kiriluk, et al., 1995; Sinnatamby et al., 2008; Vašek et al., 2017; Wainright et al., 1993). Soft tissues are less laborious to analyse than hard tissues and have shorter integration times (weeks to months) which are relevant to many present day ecological questions. However, soft tissues are typically not well preserved in ancient deposits and as a result the archaeological literature has focused more on establishing isotopic offsets between hard tissues like bone, and tooth, and hair (Ambrose and Norr, 1993; O'Connell et al., 2001).

As marine and freshwater ecologists begin to work more with bone (e.g., Matsubayashi, et al., 2017; Skovrind et al., 2019; Turner Tomaszewicz et al., 2017; Vokhshoori et al., 2019), exploiting some of its unique qualities (see below), there may be increasing value in understanding isotopic relationships between bone and other tissues. One of the most distinctive aspects of the isotopic composition from bone collagen is the long temporal perspective that it can provide (Geyh, 2001; Hedges et al., 2007; Matsubayashi and Tayasu, 2019; Stenhouse and Baxter, 1979; Wild et al., 2000). Once cellular bone is formed, it remodels slowly, and this gentler pace of turnover, relative to most other biological tissues, means that the isotope composition of bone will reflect a longer-term average of dietary intake. Acellular bone (characteristic of many teleosts), which lacks the osteocytes responsible for bone turnover in cellular bone, can still undergo remodelling (Cohen et al., 2012; Dean and Shahar, 2012; Horton and Summers, 2009), allowing for some exchange of new and old collagen. Nonetheless, provided that a representative cross section of growth is sampled, the isotopic compositions of acellular bone should still provide a long-term averaged perspective similar to cellular bone. While the longer time integration represented by bone collagen isotopic compositions is not well suited to studies that seek to explore environmental change on a seasonal or other sub-annual scale, it may be ideal for investigating longer-term (multi-annual), regional-scale environmental shifts (e.g., Hobson, 2019).

The comparability of isotopic compositions from different tissues is typically established through experimental comparisons between relevant tissues within the same individual organism (e.g., Ambrose and Norr, 1993; Hobson and Clark, 1992). In order to establish accurate inter-tissue offsets between the isotopic compositions of different tissues, these experimental comparisons must be performed on an animal that has had a consistent diet for the full length of time required for all relevant tissues to fully turn over. The unique temporal qualities of bone collagen makes this a challenging material to study systematically. The turnover rate of collagen in cellular bone will depend on a wide range of variables, and is therefore not as well understood as that of other tissues (Geyh, 2001; Hedges et al., 2007). It is likely that much of the protein (and its constituent carbon and nitrogen) in a given bone will have been laid down when an animal underwent the bulk of its growth (i.e., transition from juvenile to adult forms). Subsequently, the addition and exchange of new amino acids in a mature animal occurs slowly over the balance of the animal's lifetime as required by cellular repair, continued growth, and modifications to musculoskeletal architecture (Hedges et al., 2007). Therefore, experiments seeking to establish the comparability of isotopic compositions of bone and other tissues must use samples from organisms that have consumed the same foods, in the same environment for the bulk of their growth and during their adult lives. This means that bone and scale materials from fish with unknown (wild caught) dietary histories should not be used to establish inter-tissue $\delta^{13}C$ and $\delta^{15}N$ offsets.

In order to observe inter-tissue offsets, we compared the isotopic composition of collagen from bones and scales as well as muscle tissues, and lipids from captive raised adult fish that had consistent diets throughout their lifetimes.

1.1. Methodological context

Bone Collagen. Protein typically makes up ~21% of fish bone by weight (after lipids are removed; Guiry et al., 2016c). In addition to collagen, which makes up 90% of bone protein, bone contains several other sources of carbon and nitrogen, including lipids, carbonates, and non-collagenous proteins (NCPs) (Herring, 1972). These materials will significantly skew $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen if they are not fully removed (Guiry et al., 2016c) and, for this reason, collagen must be extracted and purified prior to analyses of bone collagen isotopic compositions.

It is worth pointing out that a number of recent studies have sought to investigate potential effects of demineralization protocols (using acidification) on bone collagen $\delta^{13}C$ and $\delta^{15}N$ (Bas and Cardona, 2018; Carrasco et al., 2018; Turner Tomaszewicz et al., 2015). These experiments have utilized bone samples from wild animals (rather than animals with known, isotopically consistent lifetime diets). Animals, wild or domesticated, can have isotopically significant dietary shifts and, due to intra-and inter-bone variations in collagen turn over time, this can result in greater heterogeneity in collagen isotopic compositions even between adjacent areas of the same bone (Guiry et al., 2016a; Matsubayashi et al., 2019). Unless animal diets are controlled, or bone samples are thoroughly homogenized (e.g., Guiry, et al., 2016c) prior to being separated into aliquots, any variation or pattern observed in bone collagen isotopic compositions may actually reflect slight differences in the average collagen turnover rate between samples rather than the effects of differing methodological protocols. While acidification may affect the isotopic compositions of some samples by selectively altering or removing proteins from bulk tissues (Schlacher and Connolly, 2014), this should not the case with extracted bone collagen, which is not soluble during acidification at the temperatures that collagen extractions are performed (Brown et al., 1988). Indeed, the demineralization step in bone collagen extraction protocols is specifically designed to remove, among other things, contaminant proteins (NCPs) that are not collagen, producing a purer collagen extract. Although, theoretically, it is possible to induce selective amino acid loss by using extraction protocols that are suboptimal for a given sample (e.g., powdering of bone or suboptimal pH or temperature controls, Collins and Galley, 1998; particularly when followed by ultrafiltration, Guiry, et al., 2016c), the lack of any effect of appropriate demineralization protocols (Longin, 1971; Olsson et al., 1974) on modern and ancient bone collagen isotopic compositions has long been established (Ambrose, 1990), as have quality control (QC) indicators of collagen degradation and contamination (DeNiro, 1985; Guiry and Szpak, 2020; Van Klinken, 1999).

Scale Collaegn: Teleost fish scales are composed primarily of protein and mineral, typically in a two part structure including a mineralized exterior layer and an underlying collagen-rich fibrilar layer (for review see, Trueman and Moore, 2007). The protein component of fish scales is primarily composed of collagen, with smaller amounts of NCPs than

bone (Nishimoto et al., 2003; Redruello et al., 2005). For this reason, isotopic analyses of fish scales might not need to account for NPC contamination during collagen extractions (i.e., by omitting the demineralization step). Although a variety of lipid compounds are present in fish scales (Grahl-Nielsen and Glover, 2010), their abundance relative to other organic components has not been described in detail. However, unlike bone, fish scales do not appear to serve a major lipid storage function and the amount of lipids in scales is thought to be too small to impact scale isotopic compositions (Espinasse et al., 2019). Thus, the only potentially important source of non-collagenous carbon contamination is mineral carbonates.

It has been estimated that the mineralized external layer can make up about 30% of a scales weight (Trueman and Moore, 2007). However, the actual ratio between mineral and protein in fish scales is not well characterized, and the two could vary within and between individuals and species based on differences in scale function and architecture. Previous studies have taken a range of approaches in accounting for the potential effect of carbon contributions from mineral to fish scale $\delta^{13}C$, including demineralization (Estep and Vigg, 1985). While many continue to use a demineralization step, some omit this procedure based on: 1) the assumption that the quantity of mineral-derived carbon contributing to scale $\delta^{13}C$ is inconsequentially small (Kiriluk et al., 1995; Trueman and Moore, 2007); and 2) experimental studies comparing analyses of mineralized and demineralized scales have found that demineralization can have little or no effect on scale isotopic compositions (Rennie et al., 2009; Sinnatamby et al., 2007; Ventura and Jeppesen, 2010).

Some studies have in fact observed isotopic differences between demineralized and non-demineralized scale samples (Perga and Gerdeaux, 2003; Syväranta et al., 2008). This is typically attributed to selective removal during demineralization of carbonate rich mineral as well as nitrogenous materials (NCPs in the case of scales), both of which are contaminants of collagen. However, as with bone collagen (above), the underlying cause of isotopic variability between samples may be difficult to interpret in the case of wild-caught fish with unknown dietary histories. Scales also grow slowly over the lifespan of the fish and have isotopic compositions reflecting diet during the bulk of growth (Dixon et al., 2015; Hutchinson and Trueman, 2006). For a variety of reasons, fish may move between areas with very different isotopic baselines with relative ease (for a review of environmental variables see, Guiry, 2019). Intra-individual isotopic analyses of separate scales (or different samples from the same scale) that grew at slightly different times/rates could therefore exhibit substantial differences in isotopic composition. Moreover, because scale protein is composed primarily of collagen, the elemental composition of which is not impacted by demineralization (see above), there is no *a priori* reason to anticipate that this step should influence resulting isotopic compositions. Given the above, a possible factor contributing to differences observed between demineralized and non-demineralized scales is that the scale material used in each analysis may be reflecting slightly different growth histories, incorporating carbon and nitrogen atoms sourced at slightly different times and/or locations.

Demineralization could have some benefits for analyzing scales that have been archived over longer periods of time. Fish scales obtained from museum collections may be a century or more old and can have a wide range of curatorial and preservational histories, ranging from storing dried scales in envelopes, to taxidermied mountings, to formalin fixation and ethanol storage. Removal of a scale's external layer may help to dislodge unwanted materials that have become adhered to a scale's surface and is therefore a potentially important step for pre-treatment protocols that include historical scale materials. In addition, although occurring in low abundance, scales do contain NCPs that are potential contaminants for collagen extractions, such as osteocalcin (Nishimoto et al., 2003) and osteonectin (Redruello et al., 2005), both of which are at least partly removed during demineralization (e.g., Romberg, et al., 1985; Termine et al., 1981). We therefore included a demineralization step in our scale pre-treatment.

2. Methods

2.1. Sample collection

No live fish were sampled in this study. Samples were taken from portions of seven adult Chinook (*Oncorhynchus tshawytscha*) provided by Yellow Island Aquaculture on Quadra Island, British Columbia, Canada in December of 2017. All fish were four years old and had been raised together from hatchlings at Yellow Island Aquaculture under the same conditions and on a consistent, nutritionally replete feed (Taplow Organic Grower brand; 43% protein, 28% lipid, 8% carbohydrate). Feed samples were also analyzed to characterize the isotopic composition of their diet. Feed type and supplier remained consistent over the course fish growth. Although raised using the same commercial feed source for their entire lives, we were not able to longitudinally analyse feed samples from multiple periods. However we see no *a priori* reason to anticipate that the isotopic composition of feed will have changed meaningfully through time. As fish were kept in ocean pens, there may have been some opportunity to acquire other foods in addition to feed; however, we do not expect this to have made a substantial contribution to overall diet. By controlling for dietary variation, these fish provide suitable specimens for assessing isotopic relationships between tissues regardless of tissue-specific turn over times. All materials were stored frozen until the processing of the respective tissues as outlined below.

2.2. Sample preparation

In this study, bones were steamed lightly to remove adhering flesh and left to air dry. One vertebra from each fish was then cut into small cubes ($\sim 2\ mm^3$) and soaked in 2:1 chloroform methanol in an ultrasonic bath (solution refreshed every 15 min until solution remained clear) to remove lipids (Folch et al., 1957). The resulting lipid-rich chloroform methanol solutions were then dried in a fumehood and the lipid extract was saved for isotopic analyses. Following a modified Longin (1971) collagen extraction protocol, samples were then soaked in 0.5M HCl for several days (solution refreshed every 24 h until visible signs of reaction ceased) to remove the mineral phase of bone as well as most NCPs, and then rinsed to neutrality in Type 1 water (Resistivity = 18 MΩ cm). Samples were then refluxed in a 10^{-3} M HCl (pH3) solution at 70 °C for 36 h to release the acid soluble fraction of bone protein (primarily collagen) into solution. Samples were then centrifuged to separate out remaining solids and the solution pipetted into a new vial and subsequently frozen before freeze-drying.

Scales were collected from the same standard position (posterior to the dorsal fin just above lateral line on the left side) on each fish and soaked three times in Type 1 water for 10 min in an ultrasonic bath. Scales were then demineralized by soaking in 0.5M HCl for 2 min in an ultrasonic bath. Finally, scales were rinsed to neutrality in Type 1 water and left to air dry.

Muscle. Although numerous studies have established isotopic offsets between muscle and scale collagen, comparisons of muscle and bone collagen are rare and we are not aware of any in the published literature for fish with controlled diets. Therefore, while not the focus of this study, we also include isotopic compositions for muscle from the same fish prepared following two protocols – delipidated and non-delipidated.

All samples were cut from dorsal muscle using a scalpel and rinsed. For the delipidated group of samples, lipids were then removed following the Bligh and Dyer (1959) method. All samples were dried in an oven at 50 °C for 48 h.

Feed. In order to establish diet-tissue offsets for these fish we also analyzed 10 feed pellets prepared following two protocols – delipidated ($n = 5$) and untreated ($n = 5$).

Untreated pellets received no pre-treatment. For the delipidated group of samples, lipids were removed through soaking in 2:1

chloroform methanol following Bligh and Dyer (1959).

2.3. Isotopic analyses

Stable carbon and nitrogen elemental and isotopic compositions for collagen (0.5 mg subsamples) from bone and scale samples as well as bulk bone lipid extracts (0.3 mg subsamples) were measured in triplicate on a Vario MICRO Cube elemental analyser coupled via continuous flow to an Isoprime isotope ratio mass spectrometer (Elementar, Hanover, Germany) in the Department of Anthropology at the University of British Columbia. A two point calibration anchored to USGS40 and USGS41a (Electronic Supplementary Materials [ESM] 1, Table S1; Qi et al., 2003; Qi et al., 2016) was used to calibrate $\delta^{13}C$ and $\delta^{15}N$ relative to VPDB and AIR, respectively. Table S2 (ESM) provides standard deviations and numbers for calibration standards. We monitored analytical accuracy using three internal check standards (Table S1, ESM). Table S3 (ESM) reports mean and standard deviations for check standards and Table 1 reports standard deviations for all sample replicate analyses. Following Szpak et al. (2017b), for $\delta^{13}C$ and $\delta^{15}N$, respectively, we calculated: random errors ($uR_{(w)}$) to be ±0.06‰ and ±0.10‰; systematic errors ($u_{(bias)}$) to be ±0.10‰ and ±0.12‰; standard uncertainty to be ±0.11‰ ±0.16‰.

Stable carbon and nitrogen elemental and isotopic compositions for muscle samples and feed pellets were measured commercially at the University of Victoria Isotope Facility using a Costech 4010 elemental analyzer (Costech, Florence, Italy) coupled via continuous flow to a Thermo Finnegan Delta Advantage isotope ratio mass spectrometer (Thermo-Finnigan, Bremen, Germany). A two point calibration anchored with internal reference materials ('Caffeine' and 'Dorm', themselves determined using IAEA N1, N2, and CH-6 as well as NBS-22; ESM Table S1) was used to calibrate $\delta^{13}C$ and $\delta^{15}N$ relative to VPDB and AIR. Replicates ($n = 5$ per treatment type) were run for feed pellets. Standard deviations for calibration standards for $\delta^{13}C$ and $\delta^{15}N$, respectively, were ±0.05‰ and ±0.21‰ for Caffeine ($n = 3$) and ±0.05‰ and ±0.24‰ for Dorm ($n = 5$).

2.4. Collagen integrity

Regardless of taxon, the ratio of percent carbon-to-nitrogen (C:N_{Atomic}) in collagen is highly conservative across all vertebrates. Archaeologists routinely use a C:N_{Atomic} range of 2.9–3.6 as the QC cut-off within which a sample is considered sufficiently well preserved and free of contamination for isotopic analyses (Ambrose, 1990; DeNiro, 1985; Van Klinken, 1999). Recent experimental evidence from modern fish bone collagen (Guiry et al., 2016c) has demonstrated that collagen samples with C:N_{Atomic} values that fall outside 3.00 and 3.30 are

Table 1
Isotopic and elemental compositions for samples presented in this study. Abbreviations: VPDB (Vienna Pee Dee Belemnit), AIR (Ambient Inhalable Reservoir), %Lip (% lipids), and %Col. (% collagen). $\delta^{15}N$ is absent from bulk bone lipids as N is not measurable at the analyzed quantities.

Material	Fish No.	IUBC	n =	$\delta^{13}C$ (‰, VPDB)	$\delta^{15}N$ (‰, AIR)	%C	%N	C:$N_{atm.}$	% Lip. Yld.	% Col. Yld.
Bone collagen	50	4506	3	−14.41 ± 0.02	15.10 ± 0.03	41.47	15.42	3.14	22.9	17.1
Bone collagen	51	4507	3	−14.36 ± 0.01	15.35 ± 0.05	43.29	16.02	3.15	21.0	20.2
Bone collagen	52	4508	3	−14.29 ± 0.06	14.89 ± 0.02	43.12	15.68	3.21	20.4	18.3
Bone collagen	53	4509	3	−14.60 ± 0.05	15.03 ± 0.03	42.52	15.61	3.18	21.6	17.5
Bone collagen	54	4510	3	−14.36 ± 0.04	15.13 ± 0.01	42.69	15.59	3.19	15.9	18.4
Bone collagen	55	4511	3	−14.20 ± 0.07	15.13 ± 0.06	42.60	15.60	3.18	18.9	17.7
Bone collagen	56	4512	3	−14.24 ± 0.04	15.19 ± 0.01	43.10	15.95	3.15	25.7	17.1
Scale collagen	50	4513	3	−14.55 ± 0.09	15.54 ± 0.02	43.68	16.20	3.14		
Scale collagen	51	4514	3	−14.41 ± 0.06	15.40 ± 0.09	42.93	16.17	3.10		
Scale collagen	52	4515	3	−14.16 ± 0.03	15.23 ± 0.02	42.89	16.09	3.11		
Scale collagen	53	4516	3	−14.35 ± 0.05	15.42 ± 0.04	42.98	16.23	3.09		
Scale collagen	54	4517	3	−14.06 ± 0.02	15.20 ± 0.02	42.74	16.21	3.07		
Scale collagen	55	4518	3	−14.06 ± 0.03	15.50 ± 0.01	42.64	16.05	3.10		
Scale collagen	56	4519	3	−14.35 ± 0.03	15.54 ± 0.09	42.75	16.03	3.11		
Bone lipid	50	4506L	3	−25.54 ± 0.02		71.99				
Bone lipid	51	4507L	3	−25.51 ± 0.03		74.63				
Bone lipid	52	4508L	2	−25.45 ± 0.01		72.81				
Bone lipid	53	4509L	3	−25.51 ± 0.04		69.44				
Bone lipid	54	4510L	3	−25.69 ± 0.09		72.72				
Bone lipid	55	4511L	4	−25.39 ± 0.03		73.88				
Bone lipid	56	4512L	3	−25.41 ± 0.03		72.51				
Muscle (w/o lipid)	50	NA	1	−17.30	15.36	47.04	13.90	3.95		
Muscle (w/o lipid)	51	NA	1	−17.03	15.43	48.01	14.36	3.90		
Muscle (w/o lipid)	52	NA	1	−16.99	15.15	47.27	14.13	3.90		
Muscle (w/o lipid)	53	NA	1	−16.99	14.87	43.98	13.23	3.88		
Muscle (w/o lipid)	54	NA	1	−17.03	15.51	46.86	14.12	3.87		
Muscle (w/o lipid)	55	NA	1	−16.81	15.31	45.45	13.57	3.91		
Muscle (w/o lipid)	56	NA	1	−17.04	15.13	46.35	13.82	3.91		
Muscle (w/lipid)	50	NA	1	−21.04	14.43	56.09	9.63	6.79		
Muscle (w/lipid)	51	NA	1	−18.87	14.61	50.70	12.61	4.69		
Muscle (w/lipid)	52	NA	1	−20.85	14.23	56.37	9.59	6.85		
Muscle (w/lipid)	53	NA	1	−20.33	14.41	55.51	10.33	6.27		
Muscle (w/lipid)	54	NA	1	−20.08	14.74	54.42	10.88	5.83		
Muscle (w/lipid)	55	NA	1	−19.86	14.64	53.88	10.67	5.89		
Muscle (w/lipid)	56	NA	1	−19.71	14.62	52.46	11.13	5.50		
Feed (w/o lipid)	1	NA	1	−20.34	11.58	42.33	7.62	6.48		
Feed (w/o lipid)	2	NA	1	−19.71	11.81	39.40	7.80	5.89		
Feed (w/o lipid)	3	NA	1	−20.10	11.55	40.49	7.63	6.19		
Feed (w/o lipid)	4	NA	1	−19.74	11.77	40.73	8.03	5.92		
Feed (w/o lipid)	5	NA	1	−19.80	11.53	40.73	7.97	5.96		
Feed (w/lipid)	1	NA	1	−22.31	10.93	48.21	5.82	9.66		
Feed (w/lipid)	2	NA	1	−22.21	10.92	49.75	5.89	9.85		
Feed (w/lipid)	3	NA	1	−22.37	11.26	49.79	6.73	8.63		
Feed (w/lipid)	4	NA	1	−21.90	11.46	49.18	6.47	8.86		
Feed (w/lipid)	5	NA	1	−22.39	10.85	49.31	5.79	9.93		

contaminated with lipids or other non-collagenous materials and are not acceptable for isotopic analyses (Guiry and Szpak, 2020).

2.5. Statistical analyses

Quantitative analyses were performed using the PAST version 3.22 (Hammer et al., 2001). A Shapiro-Wilk test (Shapiro and Wilk, 1965) was used to assess normality of distribution among all tissues. For comparisons between normally distributed data, a One Way ANOVA was performed followed by either a Tukey's post hoc test (if variances are equal; Tukey, 1949) or individual Welch's *t* tests (if variances are unqual; Welch, 1947) to compare differences in $\delta^{13}C$ and $\delta^{15}N$ between tissues. Homogeneity of variance was assessed using a Levene's test (Levene, 1960). Comparisons of isotopic compositions of feed prepared using different treatments were made using Student's *t* tests (if variances were equal) or Welch's *t* tests (if variances are unqual; Welch, 1947).

3. Results

All bone and scale collagen analyses produced $C:N_{Atomic}$ values ($n = 14$; mean = 3.14 ± 04; range = 3.07–3.21) falling within the range for acceptable collagen integrity. Results for all analyses are presented in Table 1. The difference between $\delta^{13}C$ and $\delta^{15}N$ means between bone collagen and other tissues and feed are presented in Table 2 and Fig. 1. Shapiro-Wilk tests did not reject the null hypotheses, suggesting that $\delta^{13}C$ and $\delta^{15}N$ for all tissue types and feed were normally distributed ($p > 0.05$; see ESM, Table S4 for full results). A One Way ANOVA for $\delta^{15}N$ ($F_{3,24} = 35.08$, $p = <0.0001$) was followed by Tukey's post hoc test (Table 3), as Levene's tests showed that homogeneity of variance were not unequal ($p = 0.404$). Following a Levene's test showing that variances were unequal across all groups for $\delta^{13}C$ ($p < 0.003$), a Welch *F* tests was used to compare $\delta^{13}C$ means between all groups ($F = 33.66$, $df = 13.18$, $p < 0.001$). Across all $\delta^{13}C$ groups, the unequal variances stemmed from greater variation in the non-delipidated muscle group. For this reason, individual Welch *t* tests were used to compare the non-delipidated muscle group $\delta^{13}C$ with that of the bone ($t = 20.45$, $df = 10$, $p < 0.001$), scale ($t = 20.39$, $df = 10$, $p < 0.001$), and delipidated muscle ($t = 10.91$, $df = 10$, $p < 0.001$) groups. For all other groups, a One Way ANOVA ($F_{2,28} = 703.3$, $p < 0.001$) was followed by Tukey's post hoc test (Table 3), as Levene's tests showed that homogeneity of variance were not unequal ($p = 0.294$). For comparisions of delipidated and untreated feed samples, a Student's *t*-test and Welch *t*-test showed significant differences for both $\delta^{13}C$ ($t = 15.17$, $df = 8$, $p < 0.001$; Levene's test, $p = 0.285$) and $\delta^{15}N$ ($t = 4.28$, $df = 8$, $p = <0.005$; Levene's test, $p = 0.0410$), respectively.

Relative to bone collagen, scale collagen was on average 0.07 ± 0.17‰ higher and 0.29 ± 0.16‰ lower for $\delta^{13}C$ and $\delta^{15}N$, respectively. The mean difference between these tissues was less than analytical uncertainty for $\delta^{13}C$ (and not statistically significant) but slightly greater than analytical uncertainty (and statistically significant) for $\delta^{15}N$ suggesting that a very slight but meaningful offset may occur between bone and scale collagen for $\delta^{15}N$. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ was on average 2.68 ± 0.16‰ higher (significantly different) and 0.13 ± 0.19‰ lower (not significantly different), respectively, than dilapidated muscle tissue and 5.75 ± 0.16‰ higher and 0.59 ± 0.19‰ lower (both significantly different), respectively than non-delipidated muscle. Bone lipids exhibited a large systematic offset from bone collagen averaging 11.13 ± 0.16‰ higher than lipids for $\delta^{13}C$. Diet-tissue offsets for delipidated and untreated feed are shown in Table 4.

Table 2
Observed inter-tissue offsets.

Fish No.	Bone-Scale		Bone-Lipid	Bone-Muscle (w/ o Lipids)		Bone-Muscle (w/ Lipids)	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
50	0.14	−0.44	11.14	2.89	−0.26	6.63	0.67
51	0.05	−0.04	11.15	2.67	−0.08	4.51	0.74
52	−0.13	−0.33	11.17	2.70	−0.26	6.56	0.66
53	−0.26	−0.39	10.90	2.39	0.16	5.73	0.62
54	−0.30	−0.07	11.33	2.67	−0.38	5.72	0.39
55	−0.13	−0.37	11.19	2.61	−0.18	5.66	0.49
56	0.10	−0.35	11.16	2.80	0.06	5.47	0.57
Mean	***−0.07***	***−0.29***	***11.15***	***2.68***	***−0.13***	***5.75***	***0.59***
±1σ	*0.17*	*0.16*	*0.13*	*0.16*	*0.19*	*0.71*	*0.12*

4. Discussion

This study provides the first direct inter-tissue comparison of the isotopic composition of collagen from fish with known, consistent diets throughout their lifespans. For this reason, our data offer a valuable opportunity to establish clear offsets between the isotopic compositions of collagen, other proteins, and lipids from different tissues without complications related to dietary variation (as with wild-caught specimens). We first compare findings from muscle and lipids with inter-tissue and diet-tissue isotopic differences observed in previously published research. Second, we offer a more detailed discussion of results from the isotopic compositions of scale and bone collagen as well as their comparability for future retrospective isotopic time-series studies.

Offsets observed between muscle, lipids, and other tissues are generally consistent with and add to the body of previously published inter-tissue isotopic comparisons for fish. Results from non-delipidated muscle $\delta^{13}C$ (mean = −20.1 ± 0.73‰) were more variable and were much lower relative to delipidated muscle (mean = −17.0 ± 0.14‰) and collagen samples (mean scale = −14.3 ± 0.18‰; mean bone = −14.4 ± 0.13‰), as would be expected for samples contaminated to varying degrees with heavily ^{13}C-depleted lipids (mean = −25.7 ± 0.01‰). Significant differences between $\delta^{15}N$ for non-delipidated muscle (mean = +14.5 ± 0.18‰) and all other groups were less clear (mean for non-delipidated muscle ranges between 0.60 and 0.88 lower than other tissues). In contrast, $\delta^{15}N$ for delipidated muscle (mean + 15.3 ± 0.22‰) showed no significant differences with other tissues (mean scale +15.4 ± 0.14‰; bone +15.1 ± 0.14‰). It seems likely, therefore that this difference stems from the lack of processing received by non-delipidated muscle samples, potentially making them more heterogeneous (i.e., including a greater quantity and diversity of extra-cellular proteins) with fewer 'trophic' amino acids (O'Connell, 2017) than collagen or treated muscle samples. Contamination with phosphatidylcholines, which have a single N atom, may also be a contributing factor (Svensson et al., 2016). The observed mean $\delta^{13}C$ offset between treated muscle and scale collagen (mean = +2.7‰, range = +2.7 to +3.0‰) is in the range expected based on other studies of similar salmonids (e.g., Coll, 2015 [mean = +2.7‰]; Espinasse et al., 2019 [mean = +3.5‰]; Johnson and Schindler, 2012 [no mean given, range = +2.2 to +4.0‰]; Satterfield and Finney, 2002 [mean = +3.7‰]).

Our dataset also provides new estimates for diet-tissue offsets (Table 4). Non-delipidated muscle was on average 0.2‰ lower for $\delta^{13}C$ and 2.9‰ higher for $\delta^{15}N$ relative to diet, which fall in line with previous observations for salmonids (Dempson and Power, 2004) and other taxa (Caut et al., 2009; Post, 2002; Vander Zanden and Rasmussen, 2001). As would be expected, diet-tissue offsets were in a similar range for delipidated muscle $\delta^{15}N$ (elevated by 3.6‰ over diet) but much higher for $\delta^{13}C$ (elevated 2.9‰ over diet). We are aware of no published life-time controlled feeding studies (although see Matsubayashi et al., 2019) that compare fish diet and collagen isotopic compositions but the observed elevations in scale and bone $\delta^{13}C$ (5.6 and 5.6 respectively) and $\delta^{15}N$ (3.8 and 3.5, respectively) relative to diet are also in line with those observed in other vertebrate taxa (Ambrose and Norr, 1993; Tieszen and Fagre, 1993). Higher $\delta^{13}C$ in delipidated feed (mean = −19.9 ± 0.27‰) relative to untreated feed (mean = −22.24 ± 0.20‰) suggests that the pre-treatment successfully removed lipids from feed pellets. However, as is evident from statistically significant difference in $\delta^{15}N$ between delipidated (mean = +11.65 ± 0.13‰) and untreated feed (mean = +11.08

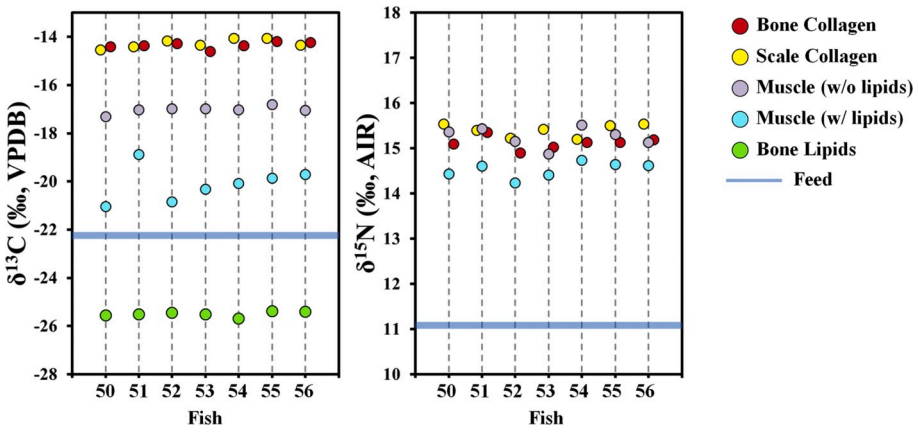

Fig. 1. Isotopic compositions of all tissues and feed (untreated).

Table 3
Results ($p =$) of one-way ANOVA, post hoc Tukey's tests comparing fish tissues. p values < 0.05 are bolded. See text for muscle (with lipids) $\delta^{13}C$ comparisons with other tissues.

Material		Scale Collagen	Muscle w/o Lipids	Muscle w/ Lipids
$\delta^{13}C$	Bone Collagen	0.6538	< **0.0000**	NA
$\delta^{13}C$	Scale Collagen	–	< **0.0000**	NA
$\delta^{13}C$	Muscle w/o Lipids	–	–	NA
$\delta^{15}N$	Bone Collagen	**0.0221**	0.4741	< **0.0000**
$\delta^{15}N$	Scale Collagen	–	0.3628	< **0.0000**
$\delta^{15}N$	Muscle w/o Lipids	–	–	< **0.0000**

Table 4
Tissue-diet offsets (calculated by subtracting mean tissue δ from mean diet δ).

Tissue	Feed (with lipids)		Feed (without lipids)	
	$\delta^{13}C_{TISSUE-DIET}$	$\delta^{15}N_{TISSUE-DIET}$	$\delta^{13}C_{TISSUE-DIET}$	$\delta^{15}N_{TISSUE-DIET}$
Bone collagen	−5.6	−3.5	−7.9	−4.0
Scale collagen	−5.6	−3.8	−8.0	−4.3
Bone lipid	5.6	NA	3.3	NA
Muscle (w/o lipid)	−2.9	−3.6	−5.2	−4.2
Muscle (w/ lipid)	0.2	−2.9	−2.1	−3.4

± 0.26‰), the chloroform-methanol treatment also altered the nitrogenous component of the feed (Post et al., 2007), indicating that the protein compositions had also been affected (as, aside from phosphatidylcholines, lipids do not have nitrogen). Because this shift could have implications for $\delta^{13}C$ as well as $\delta^{15}N$ we give no further consideration to diet-tissue offsets for the delipidated feed.

Results show that bone and scale collagen are directly comparable with respect to $\delta^{13}C$ but may have a very slight offset of ~0.29‰ for $\delta^{15}N$. This offset in $\delta^{15}N$ could reflect a variety of factors. First, theoretically, a small difference in the relative proportion of 'source' (lower $\delta^{15}N$) and 'trophic' (higher $\delta^{15}N$) amino acid compositions (O'Connell, 2017) between collagen in scale and bone could explain the observed pattern. However, several comparative studies (e.g., Ahmed, et al., 2019; Gistelinck et al., 2016; Wang et al., 2014) as well as meta-analyses of the amino acid compositions of collagen from a variety of fish tissues has shown that fish bone and scale collagen amino acid compositions do not differ (Fig. 2; Guiry and Szpak, 2020). Second, it could also be that scale resorption has altered the isotopic composition of scales. Resorption of scale collagen would also, in theory, preferentially remove isotopically light ^{14}N, leaving scales relatively more enriched with ^{15}N. Scales are an important calcium reservoir for fish and may be resorbed when calcium is needed under a variety of conditions (Mugiya and Watabe, 1977), particularly during times of nutritional or physical stress (Kacem et al., 2013; Persson et al., 1995). However, as adults near the peak of their growth, and with a nutritionally replete diet, scale resorption would be an unlikely explanation for the slight elevation of scale collagen $\delta^{15}N$.

A third possibility could be that residual NCPs are present in greater abundance in bone collagen than anticipated. Collagen has a much lower $C:N_{Atomic}$ relative to NCPs typically associated with ossified tissues due to a larger proportion of glycine in collagen (about a third of constituent amino acids), which has a particularly low ratio of carbon to nitrogen atoms (2:1) (Table 5) (Guiry and Szpak, 2020). The slight difference in the $C:N_{Atomic}$ for bone collagen, at 3.17, and scale collagen, at 3.10, observed here, would be consistent with inclusion of relatively more glycine-poor NCPs in bone collagen for two reasons. First, this interpretation is consistent with key aspects of the relationship between fish collagen and environmental adaptations (Eastoe, 1957). It is well documented that collagen in cold water fish taxa is distinctive in having less hydroxyproline (with a C:N of 5:1) and more serine (with a C:N of

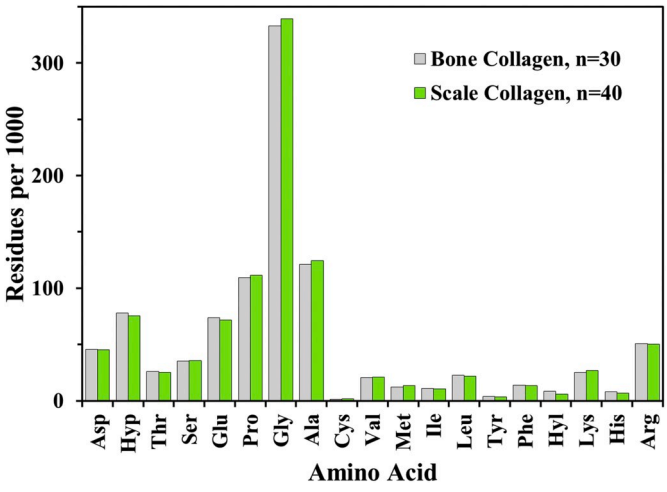

Fig. 2. Previously published amino acid compositions of bone ($n = 30$, including 17 species) and scale ($n = 40$, including 25 species) warm water (tropical and subtropical, defined using FishBase, Froese and Pauly, 2000) fish collagen from published literature (see ESM 1 Table S5) Following Shapiro-Wilk tests (showing data for most groups are not normally distributed), Mann-Whitney U tests comparing respective amino acids from scale and bone collagen show no statistically significant differences ($p > 0.05$; see ESM1, Table S6).

Table 5
$C:N_{Atomic}$ calculated for major non-collagenous proteins based on mean amino acid counts from complete and reviewed amino acid sequences (see ESM Table S7) from a variety of taxa available from UniProt (UniProt Consortium, 2018). Calculations are intended to give a general sense $C:N_{Atomic}$ variation and are considered approximate as they are based on amino acid sequences that may include small signal peptides. *$C:N_{Atomic}$ calculated for core protein only.

Non-Collagenous Bone Proteins	$C:N_{Atomic}$	n =
Asproin	3.73 ± 0.01	3
Bone Sialoprotein	3.53 ± 0.05	6
Biglycan	3.68 ± 0.01*	8
Decorin	3.63 ± 0.02*	12
Dentin matrix acidic phosphoprotein 1	3.24 ± 0.04	4
Fibromodulin	3.67 ± 0.03*	5
Lumican	3.84 ± 0.03*	6
Osteoadhearin	3.79 ± 0.04*	4
Osteocalcin	3.83 ± 0.1	18
Osteonectin	3.74 ± 0.02	9
Osteopontin	3.49 ± 0.08	8
Osteoregulin	3.15 ± 0.05	3
Periostin	3.65 ± 0.01	2
Versican	3.74 ± 0.03*	4

3:1) relative to the amino acid compositions of warmer water taxa (Fig. 3) (Gustavson, 1955; Rigby, 1967; Rigby and Spikes, 1960). This difference in amino acid compositions means that cold water fish like *O. tshawytscha* (a temperate species) typically have a collagen $C:N_{Atomic}$ at the lower end of the range observed for all fish (3.00–3.30, $n = 290$ including 193 species, mean 3.16 ± 0.06) (Guiry and Szpak, 2020). Although, amino acid compositions have not been published for *O. tshawytscha* specifically, those from other *Oncorhynchus* spp. inhabiting the North Pacific Ocean have particularly low $C:N_{Atomic}$: 3.07 (Matsui et al., 1991) and 3.10 (Kimura et al., 1988) for *O. keta* and 3.07 (Avena-Bustillos et al., 2006) for *O. gorbuscha*. While these $C:N_{Atomic}$ are in line with our results from scale collagen, they suggest that our bone collagen extracts include trace amounts of residual NCPs that were not removed during the demineralization and reflux steps of collagen extraction. Second, this interpretation of $\delta^{15}N$ differences between bone and scale is consistent with the isotopic shift that should be caused by NCP contamination. The amino acid compositions of key NCPs in bone, like osteocalcin and osteonectin, suggests that they should have higher $\delta^{15}N$ than collagen. Because NCPs invariably have much less glycine (although variable, typically considered a 'source' amino acid, O'Connell, 2017), and are often also proportionately richer in some 'trophic' amino acids (e.g., leucine/isoleucine, aspartic acid/asparagine, alanine,

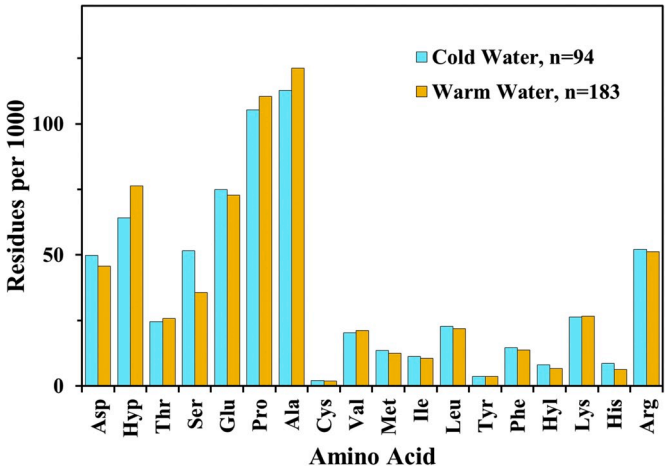

Fig. 3. Previously published amino acid compositions for warm and cold water fish bones, scale, and skin collagen (see ESM Table S7). Warm (tropical, subtropical) and cold (temperate, polar, boreal, and deep-water) water defined using FishBase (Froese and Pauly, 2000). Glycine not shown to improve scale.

Guiry and Szpak, 2020) it is likely that NCP contamination would result in a $\delta^{15}N$ elevation that is in line with the observed difference between bone and scale collagen.

Incomplete removal of NCPs may be an issue peculiar to collagen extractions from some modern bones. In contrast, issues with NCP contamination are typically not encountered with ancient samples (possibly reflecting lower abundances of NCP preservation). It may therefore be advisable to include an additional pre-treatment that specifically targets and removes residual NCPs that remain bound to collagen extracted from fresh bone, such as soaking in a sodium hydroxide solution (Lowry et al., 1941). Sodium hydroxide pre-treatments are routinely used to remove NCPs from collagen in food chemistry studies (Nagai and Suzuki, 2000) and to remove base-soluble contaminants in archaeological studies (Szpak et al., 2017) but are not currently included in collagen extraction protocols for modern bone.

5. Summary and conclusion

Whatever the cause, the differences in $\delta^{15}N$ observed in our comparisons between bone and scale collagen are extremely small and may fall below measurement uncertainty thresholds for some analytical sessions. Moreover, most research questions in historical ecology and archaeology deal with patterns in $\delta^{15}N$ that are >0.29‰. Thus, for the intents and purposes of most research projects, this $\delta^{15}N$ offset is likely be inconsequential such that, as with $\delta^{13}C$, collagen $\delta^{15}N$ values from bone and scale samples will be effectively comparable.

Our results focus on a small set of analyses from a single species and we therefore encourage additional comparisons involving collagen from scales and bone from other taxa to further explore these findings. However, as highlighted in Fig. 2, there is broad similarity between the amino acid compositions of collagen extracts from fish scale and bone. While a wide range of physiological and other factors can lead to isotopic differences between tissues, a primary source of inter-tissue isotopic variation should be the relative proportion of amino acids with different metabolic histories (for review see, O'Connell, 2017). Therefore, to the extent that variation in isotopic compositions between tissues is driven by relative differences in contributions from amino acids with different metabolic histories, the similarity between the amino acid compositions of scale and bone collagen observed by a number of studies (Ahmed et al., 2019; Gistelinck et al., 2016; Guiry and Szpak, 2020; Wang et al., 2014) allow us to anticipate, at least from a theoretical perspective, that our findings should be widely applicable.

Scale and other tissue archives often represent a wealth of spatiotemporally and taxonomically well-contextualized specimens and are increasingly being used to investigate environmental change on a multidecadal scale (e.g., Blanke, et al., 2018; Espinasse et al., 2019; Fera et al., 2017; Jacobs et al., 2017; Kishe-Machumu et al., 2017; Oczkowski et al., 2020; Olden et al., 2019). Bone collagen is the most abundant protein available from archeologically and paleontologically preserved fish remains and therefore holds significant promise for extending isotopic times series further back in time, well beyond the retrospective scope of even the earliest museum and natural history collections. In this context, integrating bone and scale collagen isotopic data can help provide deeper insights into a variety of environmental processes that, most importantly, span the timeframes before and after the Industrial Revolution (e.g., Guiry, et al. In Review; ; Guiry et al., 2016). This kind of longer-term perspective has tremendous potential to provide a more nuanced understanding of how ecosystems functioned prior to large-scale exploitation and how they have responded to mounting anthropogenic pressures in the intervening centuries (e.g., Braje, et al., 2017; Rosania, 2012). Such information may provide valuable new insights into planning effective environmental restoration efforts and developing better conservation policy (Rick and Lockwood, 2013; Swetnam et al., 1999).

Samples

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Declaration of competing interest

The authors declare no competing interests.

CRediT authorship contribution statement

Eric J. Guiry: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Brian P.V. Hunt:** Resources, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2020.104982.

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