Received: 19 March 2020 | Accepted: 28 May 2020

DOI: 10.1111/2041-210X.13433

RESEARCH ARTICLE

Methods in Ecology and Evolution BRITISH ECOLOGICAL SOCIETY

Quality control for modern bone collagen stable carbon and nitrogen isotope measurements

Eric J. Guiry[1,2] | Paul Szpak[1]

[1]Department of Anthropology, Trent University, Peterborough, ON, Canada

[2]School of Archaeology and Ancient History, University of Leicester, Leicester, UK

Correspondence
Eric J. Guiry
Email: eguiry@lakeheadu.ca

Funding information
Social Sciences and Humanities Research Council of Canada (SSHRC) Banting Post-Doctoral Fellowship; SSHRC Insight Devlopment, Grant/Award Number: 430-2017-01120; Natural Sciences and Engineering Research Council of Canada Discovery, Grant/Award Number: RGPIN-2020-04740

Handling Editor: David Soto

Abstract

1. Isotopic analyses of collagen, the main protein preserved in subfossil bone and tooth, has long provided a powerful tool for the reconstruction of ancient diets and environments. Although isotopic studies of contemporary ecosystems have typically focused on more accessible tissues (e.g. muscle, hair), there is growing interest in the potential for analyses of collagen because it is often available in hard tissue archives (e.g. scales, skin, bone, tooth), allowing for enhanced long-term retrospective studies. The quality of measurements of the stable carbon and nitrogen isotopic compositions of ancient samples is subject to robust and well-established criteria for detection of contaminants and diagenesis. Among these quality control (QC) criteria, the most widely utilized is the atomic C:N ratio ($C:N_{Atomic}$), which for ancient samples has an acceptable range between 2.9 and 3.6. While this QC criterion was developed for ancient materials, it has increasingly being applied to collagen from modern tissues.
2. Here, we use a large survey of published collagen amino acid compositions (n = 436) from 193 vertebrate species as well as recent experimental isotopic evidence from 413 modern collagen extracts to demonstrate that the $C:N_{Atomic}$ range used for ancient samples is not suitable for assessing collagen quality of modern and archived historical samples.
3. For modern tissues, collagen $C:N_{Atomic}$ falling outside 3.00–3.30 for fish and 3.00–3.28 for mammals and birds can produce systematically skewed isotopic compositions and may lead to significant interpretative errors. These findings are followed by a review of protocols for improving $C:N_{Atomic}$ criteria for modern collagen extracts.
4. Given the tremendous conservation and environmental policy-informing potential that retrospective isotopic analyses of collagen from contemporary and archived vertebrate tissues have for addressing pressing questions about long-term environmental conditions and species behaviours, it is critical that QC criteria tailored to modern tissues are established.

KEYWORDS

amino acids, bone, carbon isotope, collagen, historical ecology, lipid, nitrogen isotope, non-collagenous protein

The peer review history for this article is available at https://publons.com/publon/10.10.1111/2041-210X.13433

1 | INTRODUCTION

Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope analysis of plant and animal tissues is a powerful tool for quantifying ecological relationships and animal behaviour at a wide range of spatial and temporal scales. Although the isotopic compositions of any carbon- and nitrogen-bearing tissue can be analysed, ecologists studying contemporary and historical ecosystems generally focus analyses on readily available muscle and organ tissues that require relatively little processing (Newsome, Clementz, & Koch, 2010). In contrast, research investigating earlier ecosystems has been extensively focused on analysing the isotopic composition of proteins extracted from hard tissues, such as bones and teeth, which are more readily available because they are generally the only vertebrate structures that preserve in archaeological and paleontological deposits (Ambrose, 1990). In this context, Type I collagen (hereafter, 'collagen'), which makes up 90% of bone and tooth protein (Herring, 1972), has been the primary focus of a vast majority of protein-based isotopic research on ancient environments (Guiry, 2019; Szpak, Metcalfe, & Macdonald, 2017).

Although collagen has been analysed for decades in archaeology, collagen from a wider range of species and tissues has been increasingly studied in contemporary contexts (e.g. Dixon, Dempson, & Power, 2015; Guiry, Royle, et al., 2020; Skovrind et al., 2019; Turner Tomaszewicz, Seminoff, Price, & Kurle, 2017; Vokhshoori et al., 2019). In part, this is because collagen (Table 1) is the most abundant protein in many tissues that are routinely archived in biological and conservation programmes (e.g. fish scales, skin, bone, teeth; and is also typically abundant in natural history collections) and, therefore, can provide unparalleled opportunities for retrospective research reaching back years, decades or centuries into the past (e.g. Guiry & Hunt, 2020; Nelson, Quakenbush, Mahoney, Taras, & Wooller, 2018; Newsome et al., 2007; Wainright, Fogarty, Greenfield, & Fry, 1993). Moreover, the isotopic composition of collagen from modern and archived tissues is directly comparable (i.e. without adjustments for inter-tissue trophic enrichment variables) with that of collagen preserved in archaeological and paleontological contexts, thereby opening the possibility for substantially longer, 'deep time' retrospectives studies (e.g. Burton et al., 2001; Guiry, Needs-Howarth, et al., 2016; Guiry, Orchard, Royle, Cheung, & Yang, 2020; Misarti, Finney, Maschner, & Wooller, 2009; Szpak, Orchard, McKechnie, & Gröcke, 2012). These studies are particularly important because they can allow for more accurate reconstructions of preindustrial environmental conditions as well as provide detailed insights into how humans have altered ecosystem dynamics throughout the Anthropocene (Braje et al., 2017; Guiry, Beglane, et al., 2018; Guiry, Buckley, et al., 2020; Szpak, Buckley, Darwent, & Richards, 2018; Szpak et al., 2019)—both of which, in turn, can provide context for guiding future conservation policy and environmental restoration efforts (Rick & Lockwood, 2013; Swetnam, Allen, & Betancourt, 1999).

Collagen has a number of well-established quality control (QC) indicators for isotopic measurements of ancient materials (Table 2), which enable detection of contaminating non-collagenous materials and post-burial degradation/alteration (Ambrose, 1990; DeNiro, 1985). These QC indicators were established based on observations of anomalies in the relationship between the isotopic and elemental compositions of modern and ancient collagen (for reviews see, Szpak, 2011; Van Klinken, 1999), which show that the concentrations and ratios of carbon and nitrogen vary little between collagen from different tissues and taxa (Neuman, 1949). Two of the QC criteria for ancient collagen are yield (the wt% collagen extracted from bone, with 1% being the most frequently cited minimum value) and minima for wt% C (13%) and wt% N (4.5%) in the analysed collagen. Both of these criteria identify degraded, rather than contaminated, collagen

TABLE 1 Terminology for collagen sourced from different types of specimens along with some of the major contamination sources common to each

		Contamination potential			
Time frame	Description	Lipid	Non-collagenous proteins	Soil borne	Preservative
Modern	Samples extracted from frozen or fresh tissues that will usually have been taken explicitly for the purpose of stable isotope (or related) analysis and have spent minimal time under any kind of curation	High	High	NA	NA
Archival	Samples extracted from tissues (preserved by any means) held in natural history archives or other repositories that, at the time of their collection, were taken from living animals or recently deceased animals. These will be preserved specimens or skeletons. Flesh and soft tissues are still on the specimens or these were removed from the specimens partly or wholly by curators. Generally, these specimens have been collected from CE 1700 onwards	Moderate to high	High	NA	Possible (if fixatives were used)
Ancient	Samples collected from archaeological or paleontological sites, typically dating to pre CE 1900. No or minimal adhering soft tissues. Samples were in contact with the burial environment for a prolonged period of time such that contamination from soil-borne compounds is a major concern as is selective loss of amino acids	Low	Unknown	High	NA

TABLE 2 Established collagen quality control indicators for ancient collagen extracts

Collagen quality control indicator	Observed in modern collagen[a]	Acceptable for ancient collagen
%C	41.91 ± 0.39%	>13%
%N	15.40 ± 0.20%	>4%
$C:N_{Atomic}$	3.17 ± 0.17	2.9–3.6

[a]Mean and one standard deviation derived from survey of 436 skin, bone and scale collagen amino acid compositions from 193 vertebrate taxa (Supporting Information 1, Table S1).

and are therefore only applicable to ancient samples. For ecologists, the most relevant collagen QC criteria will be the ratio of percent carbon-to-nitrogen expressed as $C:N_{Atomic}$. For studies analysing several specimens from a single taxon, an observation of a correlation between $C:N_{Atomic}$ and $\delta^{13}C$ can provide an additional indicator for collagen contamination (Ambrose, 1990). $C:N_{Atomic}$ represents that ratio of carbon to nitrogen atoms in a sample and can be calculated by multiplying the molecular C:N (i.e. wt% C/wt% N as measured during elemental analyses) by the ratio between the average atomic mass of C and N (14.007/12.011). Because $C:N_{Atomic}$ can be calculated using elemental data that are determined in tandem with $\delta^{13}C$ and $\delta^{15}N$ at no extra cost, this collagen QC measure is also easily obtainable. Moreover, the elemental data used to calculate $C:N_{Atomic}$ are resilient to inter-lab differences in calibration (even if the absolute wt% C and N differ among labs, the $C:N_{Atomic}$ will be the same provided the calibration standard used contained both C and N).

While the $C:N_{Atomic}$ collagen QC indicator was initially established to aid with identification of bone diagenesis in archaeological and paleontological research (DeNiro, 1985), it has also become the primary means used by ecologists to evaluate the integrity of isotopic compositions measured on modern collagen samples (e.g. Bas & Cardona, 2018; Turner Tomaszewicz, Seminoff, Avens, & Kurle, 2016). However, analyses of collagen from ancient and contemporary samples require different analytical considerations that render QC indicators developed from the former unsuitable for latter. For archaeological collagen, shifts in $C:N_{Atomic}$ are typically caused by the presence of additional unwanted endogenous (e.g. non-collagenous materials such as lipids) or exogenous (e.g. humic acids from the burial environment) carbon sources or collagen deterioration (selective amino acid loss) resulting in a disproportionate loss of either carbon or nitrogen (for reviews, see Collins & Galley, 1998; Collins et al., 2002; Collins, Riley, Child, & Turner-Walker, 1995; Van Klinken, 1999). A broader $C:N_{Atomic}$ range of 2.9–3.6 was therefore deemed acceptable for archaeological collagen specifically to account for the possibility of the selective loss of certain amino acids through centuries or millennia of leaching, hydrolysis and microbial activity. The rationale behind selecting this range of cut-offs was that the isotopic compositions of ancient samples with $C:N_{Atomic}$ within the 2.9–3.6 range appeared to be unaltered by contamination or degradation and therefore are less likely to present interpretive issues (Ambrose, 1990; DeNiro, 1985; note that Van Klinken, 1999 recommended a narrower range from 3.1 to 3.5 but this has not been widley adpoted). Although there has been relatively little research on the effect of selective amino acid loss on the isotopic composition of ancient collagen (although see, Dobberstein et al., 2009), low levels of collagen deterioration that do not result in large shifts in $C:N_{Atomic}$ are thought to have minimal impact (Van Klinken, 1999). Unlike archaeological bones, collagen from recently collected tissues should not have undergone deterioration and, therefore, any deviation from the natural $C:N_{Atomic}$ range observed in modern collagen should result from inclusion of non-collagenous materials. This kind of contamination can occur in a variety of osseous vertebrate tissues and fish scales where other materials (lipid and non-collagenous proteins [NCPs]) that may have very different elemental and isotopic compositions have not been fully removed and can result in substantially skewed collagen isotopic compositions (Guiry, Szpak, & Richards, 2016).

As interest in the isotopic analyses of collagen from contemporary and archived tissues continues to grow, it is imperative that collagen QC criteria tailored to modern tissues are established. The purpose of this paper is to characterize the $C:N_{Atomic}$ ranges observed in different vertebrate collagens and use these to define acceptable $C:N_{Atomic}$ ranges for evaluating collagen quality for major vertebrate groups. First, we survey published amino acid compositions from 193 vertebrate taxa to characterize the observed $C:N_{Atomic}$ range for major vertebrate classes (mammals, birds, fish). Second, we draw on experimental data to demonstrate how the acceptable $C:N_{Atomic}$ range for evaluating the integrity of isotopic measurements on modern collagen can be narrowed to provide a more sensitive indicator for contamination. Finally, we discuss the analytical and interpretive implications of analysing isotopic compositions in collagen prepared using different extraction protocols.

2 | MATERIALS AND METHODS

2.1 | Establishing an observed range for vertebrate collagen $C:N_{Atomic}$

To establish the natural range of $C:N_{Atomic}$ for vertebrate collagen, we surveyed a wide body of data from the food chemistry literature. Amino acid compositions are from both acid and pepsin solubilized collagens. Comparing $C:N_{Atomic}$ ratios from studies that performed both methods on collagen from 13 species, Szpak (2011) found that amino acid compositions measured using acid and pepsin solubilized collagen were nearly identical with no statistically significant differences. Data from both types of collagen extraction techniques should therefore produce amino acid compositions that are comparable for our purposes. Because fish collagens are adapted to environmental conditions (Eastoe, 1957; Gustavson, 1955), we further grouped fish based on habitat preferences using classifications provided on FishBase (Froese & Pauly, 2000) as follows: warm water (tropical, subtropical) and cold water (temperate, polar, boreal and deep-water). Building on Szpak (2011), the survey located 436 amino acid compositions (see Supporting Information 1, Table S1), which

TABLE 3 Number of species and analyses included in survey of skin, bone and scale collagen amino acid compositions shown by taxonomic class (see Supporting Information 1, Table S1)

	Total		Bone		Skin		Scales	
Class	Species	Analyses	Species	Analyses	Species	Analyses	Species	Analyses
Actinopterygii	136	287	31	51	116	186	29	50
Amphibia	6	8	0	0	6	8	0	0
Aves	3	12	2	6	2	6	0	0
Cephalaspidomorphi	1	3	0	0	1	3	0	0
Elasmobranchii	19	31	6	6	16	25	0	0
Holocephali	1	1	0	0	1	1	0	0
Mammalia	20	86	11	24	14	62	0	0
Myxini	1	2	0	0	1	2	0	0
Reptilia	3	3	1	1	2	2	0	0
Sacropterygii	3	3	0	0	3	3	0	0
Total	193	436	51	88	162	298	29	50

we categorized by tissue type (skin, scale, bone) and by taxonomic class (Table 3). All amino acid compositions were compared as residues per 1,000.

2.2 | Establishing an acceptable range for $C:N_{Atomic}$

To establish a $C:N_{Atomic}$ range within which modern collagen stable isotope compositions have not been meaningfully skewed by contamination with non-collagenous materials, we used $\delta^{13}C$ and $C:N_{Atomic}$ data from recent studies (Guiry, Szpak, et al., 2016; Szpak & Guiry, in prep.) comparing collagen extracted from modern bones prepared following different protocols designed to produce collagen contaminated to varying degrees with residual bone lipids. For fish, data are derived from analyses of four separate collagen extractions (total n = 288) from each of 72 bones taken from 35 individual fish representing 17 marine and freshwater taxa. For mammals (n = 25 samples) and birds (n = 25 samples), data are derived from five separate collagen extractions (total n = 250) from high lipid-content bones of 50 individuals representing 19 taxa. Because lipids are carbon rich and are significantly depleted in ^{13}C relative to collagen, lipid contamination is easily detectable by comparing $\delta^{13}C$ and $C:N_{Atomic}$, which will be skewed lower and higher, respectively, when collagen is contaminated with residual lipids. For each set of analyses (i.e. four to five different collagen extractions per bone), we quantified the effect of different amounts of lipid contamination on stable carbon isotope composition by determining the difference in $\delta^{13}C$ between the sample with the lowest $C:N_{Atomic}$ (this will be nearest to the theoretical $C:N_{Atomic}$ observed in collagen; Szpak, 2011) and the other three to four samples. This produced 413 (216 for fish and 197 for birds and mammals) individual comparisons of the relationship between $C:N_{Atomic}$ and $\delta^{13}C$ resulting from different levels of contamination with non-collagenous materials (in this case, primarily lipids). We also explored the extent to which $\delta^{13}C$ may be affected even when samples produce $C:N_{Atomic}$ values within the acceptable range by quantifying the relationship between positive shifts in $C:N_{Atomic}$ and skewing of $\delta^{13}C$ at a smaller scale. To accomplish this, we compared $\Delta^{13}C_{clean\text{-}contaminated}$ (i.e. for each sample, $\delta^{13}C$ of the extract with the lowest $C:N_{Atomic}$ subtracted from the $\delta^{13}C$ of extracts with higher $C:N_{Atomic}$) and $\Delta C:N_{Atomic\ (clean\text{-}contaminated)}$ (i.e. for each sample, $C:N_{Atomic}$ of the extract with the lowest $C:N_{Atomic}$ subtracted from the $C:N_{Atomic}$ of extracts with higher $C:N_{Atomic}$) within each set of collagen extractions. This produced 413 (216 for fish and 197 for birds and mammals) individual comparisons of the relationship between $\Delta^{13}C_{clean\text{-}contaminated}$ and $\Delta C:N_{Atomic\ (clean\text{-}contaminated)}$ for establishing the point at which small deviations in $C:N_{Atomic}$ begin to impact collagen $\delta^{13}C$.

2.3 | Statistics

Statistical analyses were performed with PAST Version 3.22 (Hammer, Harper, & Ryan, 2001). For amino acid residue and $C:N_{Atomic}$ data, we used a Shapiro–Wilk test to assess normality of distribution (Supporting Information 1, Table S2). When comparing groups where one or more samples were not normally distributed, we used a Mann–Whitney U test. For comparisons between group with normal distributions, we used either a Student's t test (where variances were equal) or a one-way ANOVA followed by either a Dunnett's (when variances were not equal) or a Tukey's (when variances were equal) post hoc test. A Levene's test was used to assess homogeneity of variance. For determining the significance of correlations between bone collagen $C:N_{Atomic}$ and $\delta^{13}C$ data we used Spearman's ρ.

3 | RESULTS

Excluding two Antarctic icefish (*Chionodraco hamatus* and *Racovitzia glacialis*, with unique physiological adaptations; see, Szpak, 2011), mean collagen $C:N_{Atomic}$ for all species and tissues based on amino acid compositions was 3.17 ± 0.08 and ranged from 3.00 to 3.33 (Table 4; Figure 1, for full list see Supporting Information 1, Table S1). Before

TABLE 4 Mean collagen amino acid compositions and corresponding $C:N_{Atomic}$ for ray-finned fish (Actinopterygii), mammal (Mammalia) and bird (Aves) tissues (n = 382; see Supporting Information 1, Table S1). For other vertebrate classes, see Supporting Information 1, Table S5

Class	Actinopterygii				Mammalia			Aves		
Tissue	Bone	Scale	Skin	All tissues	Bone	Skin	All tissues	Bone	Skin	All tissues
n	51	50	186	287	24	62	86	6	3	9
Asp	48.3 ± 7.0	45.3 ± 5.0	47.2 ± 6.7	47.0 ± 6.6	47.6 ± 2.5	45.9 ± 3.3	46.4 ± 3.2	54.4 ± 21.5	46 ± 1.7	51.6 ± 17.5
Hyp	73.2 ± 13.0	75.0 ± 16.4	71.3 ± 13.0	71.9 ± 14.1	95.5 ± 8.3	93.4 ± 12.4	94.0 ± 11.4	90.5 ± 22.2	103 ± 5.3	94.6 ± 18.8
Thr	26.2 ± 4.0	24.9 ± 2.3	25.0 ± 6.6	25.3 ± 5.8	18.9 ± 2.3	19.0 ± 3.5	18.9 ± 3.2	20.7 ± 1.8	19 ± 0	20.1 ± 1.6
Ser	42.9 ± 12.4	35.7 ± 7.0	41.8 ± 11.8	41.2 ± 11.7	34.7 ± 3.7	34.4 ± 4.2	34.5 ± 4.1	29.9 ± 2.9	29.7 ± 0.6	29.8 ± 2.3
Glu	74.7 ± 6.4	71.8 ± 6.3	73.6 ± 7.0	73.9 ± 8.3	75.1 ± 4.3	74.4 ± 3.5	74.6 ± 3.7	76.9 ± 8.3	74 ± 1	75.9 ± 6.8
Pro	106.8 ± 10.4	110.7 ± 12.9	109.1 ± 12.1	108.8 ± 12.1	119.1 ± 7.1	124.0 ± 7.4	122.6 ± 7.6	115.0 ± 3.2	119 ± 1.7	116.3 ± 3.4
Gly	330.4 ± 19.3	338.7 ± 19.3	334.5 ± 23.2	333.9 ± 23.4	328.9 ± 11.5	329.7 ± 15.5	329.5 ± 14.4	331.5 ± 6.6	331.3 ± 0.6	331.4 ± 5.2
Ala	116.5 ± 11.4	125.1 ± 9.7	117.8 ± 16.4	118.5 ± 15.2	114.4 ± 9.0	112.6 ± 9.3	113.1 ± 9.2	113.3 ± 12.1	116 ± 6	114.2 ± 10.1
Cys	1.4 ± 1.2	1.7 ± 1.6	2.2 ± 3.8	2.0 ± 3.0		0.5 ± 1.4	0.5 ± 1.4			
Val	21.0 ± 4.4	20.9 ± 4.0	20.7 ± 4.5	21.0 ± 4.9	22.8 ± 2.0	22.3 ± 3.5	22.4 ± 3.2	20.1 ± 5.5	17 ± 1	19 ± 4.7
Met	12.9 ± 3.2	14.4 ± 7.0	12.7 ± 3.9	13.1 ± 4.5	4.3 ± 1.6	6.3 ± 1.6	5.7 ± 1.8	7.1 ± 1.8	7.3 ± 0.2	7.2 ± 1.4
Ile	11.5 ± 3.3	10.4 ± 2.5	10.6 ± 3.0	10.9 ± 3.5	10.9 ± 1.5	11.2 ± 2.0	11.1 ± 1.8	10.9 ± 3.7	10.4 ± 2.5	10.7 ± 3.2
Leu	23.5 ± 4.1	21.9 ± 3.0	21.8 ± 4.1	22.4 ± 5.1	25.7 ± 1.7	23.8 ± 3.1	24.4 ± 2.9	24.2 ± 4.0	24.3 ± 0.6	24.3 ± 3.2
Tyr	4.1 ± 2.2	3.4 ± 1.8	3.5 ± 2.5	3.6 ± 2.3	2.8 ± 1.9	3.5 ± 1.8	3.3 ± 1.8	1.9 ± 0.5	2.3 ± 0.7	2.0 ± 0.5
Phe	14.6 ± 3.3	13.9 ± 2.2	13.8 ± 3.1	14.0 ± 3.2	13.5 ± 1.4	10.7 ± 4.4	11.5 ± 4.0	13.8 ± 1.1	13.3 ± 1.2	13.7 ± 1.1
Hyl	8.6 ± 4.2	5.9 ± 2.2	6.9 ± 6.8	7.0 ± 5.8	5.8 ± 2.6	6.2 ± 1.6	6.1 ± 2.0	8.5 ± 6.2	14.4 ± 13.5	10.5 ± 8.8
Lys	24.5 ± 6.0	26.5 ± 4.7	27.1 ± 6.5	26.6 ± 6.2	25.9 ± 3.0	27.7 ± 4.4	27.2 ± 4.1	25.1 ± 4.3	19 ± 13	23.1 ± 7.9
His	9.1 ± 6.8	6.5 ± 2.3	6.6 ± 3.3	7.1 ± 4.1	4.6 ± 1.0	5.8 ± 3.4	5.5 ± 3.0	3.6 ± 1.8	4.4 ± 0.5	3.9 ± 1.5
Arg	50.7 ± 3.4	50.0 ± 3.3	52.1 ± 4.9	51.4 ± 4.6	49.0 ± 3.7	49.3 ± 2.7	49.3 ± 3.0	51.1 ± 2.7	50.3 ± 1.2	50.8 ± 2.3
%C	41.9 ± 0.3	41.8 ± 0.4	41.8 ± 0.4	41.8 ± 0.4	42.2 ± 0.2	42.1 ± 0.3	42.2 ± 0.3	42.0 ± 0.3	42.1 ± 0.04	42.1 ± 0.3
%N	15.4 ± 0.2	15.4 ± 0.2	15.5 ± 0.2	15.4 ± 0.2	15.3 ± 0.1	15.3 ± 0.1	15.3 ± 0.1	15.3 ± 0.1	15.27 ± 0.05	15.3 ± 0.1
%H	7.2 ± 0.1	7.3 ± 0.0	7.2 ± 0.1	7.3 ± 0.1	7.2 ± 0.0	7.3 ± 0.1	7.2 ± 0.1	7.2 ± 0.0	7.2 ± 0.1	7.2 ± 0.0
%O	35.1 ± 0.3	35.0 ± 0.3	35.1 ± 0.4	35.1 ± 0.4	35.2 ± 0.3	35.1 ± 0.3	35.2 ± 0.3	35.3 ± 0.4	35.2 ± 0.0	35.3 ± 0.3
%S	0.38 ± 0.09	0.43 ± 0.21	0.38 ± 0.12	0.39 ± 0.14	0.12 ± 0.05	0.18 ± 0.05	0.17 ± 0.05	0.21 ± 0.05	0.21 ± 0.01	0.21 ± 0.04
$C:N_{atm}$	3.17 ± 0.05	3.17 ± 0.06	3.15 ± 0.05	3.16 ± 0.06	3.23 ± 0.04	3.21 ± 0.05	3.22 ± 0.04	3.21 ± 0.02	3.22 ± 0.01	3.21 ± 0.02

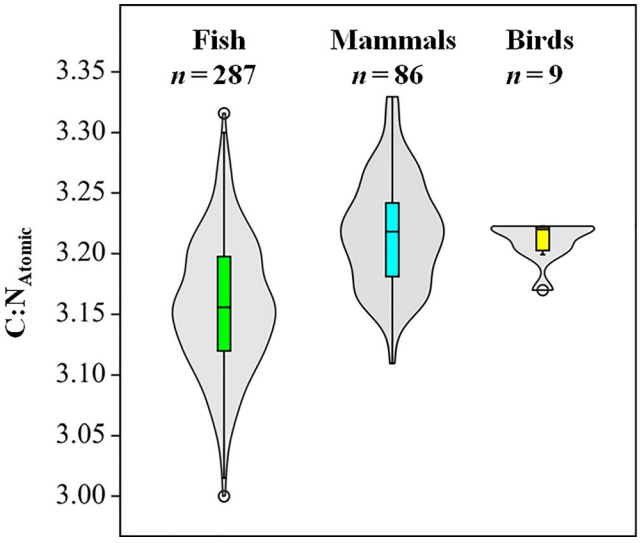

FIGURE 1 Collagen $C:N_{Atomic}$ for ray-finned fish (Actinopterygii), mammals (Mammalia) and birds (Aves) observed in survey of amino acid compositions (n = 382; for data see Supporting Information 1, Table S1)

comparing mean $C:N_{Atomic}$ between taxonomic groups, we compared mean $C:N_{Atomic}$ for tissues with five or more amino acid compositions within each class to establish whether differences occur in amino acid compositions between tissues. No statistically significant differences were observed between mean $C:N_{Atomic}$ of skin versus bone collagen in Elasmobranchii (t = 1.43, df = 85, p = 0.16) and Mammalia (t = 0.04, df = 28, p = 0.97). Following a one-way ANOVA ($F_{2,282}$ = 4.07, p = 0.02), post hoc Tukey's tests also showed no differences between means for Actinopterygii scale and bone (p = 0.72) or skin (p = 0.15) collagen $C:N_{Atomic}$. The same test showed a significant (p = 0.03) but extremely small (c. 0.019) difference between the mean $C:N_{Atomic}$ of skin and bone collagen for Actinopterygii. This difference appears to be driven by the large number of skin collagen amino acid compositions from cold water fish, which typically have less hydroxyproline (carbon rich) and more serine (carbon poor; see Supporting Information 1, Figure S1) relative to warmer water fish. Lower $C:N_{Atomic}$ and hydroxylproline abundances and higher serine abundances in the collagen of cold water fish taxa is a well-established observation and is likely related to thermal properties of collagens adapted to colder environments (Rigby, 1967; Rigby & Spikes, 1960). Given the small size of this difference, we proceeded with grouping amino acid composition from all tissues within each taxonomic group for comparisons of mean $C:N_{Atomic}$ between classes (Actinopterygii, Elasmobranchii, Mammalia and Aves). Shapiro–Wilk tests showed that $C:N_{Atomic}$ data for all groups are normally distributed, except for Aves (Supporting Information 1, Table S2). Comparing Aves collagen $C:N_{Atomic}$ with other classes, a Mann–Whitney U test showed no difference with Mammalia (U = 351, p = 0.65) and Elasmobranchii (U = 351, p = 0.14) groups but that mean Actinopterygii $C:N_{Atomic}$ is significantly lower (U = 93, p < 0.001). For this reason, for further comparisons, data from Mammalia and Aves are grouped and compared separately from Actinopterygii. A one-way ANOVA ($F_{2,402}$ = 45.69, p < 0.001), followed by post hoc Tukeys tests showed that Actinopterygii $C:N_{Atomic}$ are also significantly lower than all other taxa (for Elasmobranchii p = 0.02, for Mammalia p < 0.001). Elasmobranchii also have lower mean $C:N_{Atomic}$ than other taxa but this difference is only significant when compared to Mammalia (p = 0.005).

A strong correlation was found between $C:N_{Atomic}$ and $\delta^{13}C$ in our comparison of experimental data from fish (Spearman's ρ = −0.875, p < 0.001) and mammals and bird (Spearman's ρ = −0.718, p < 0.001) bone collagen with varying degrees of lipid contamination (Figure 2). To establish the cut-off point at which $C:N_{Atomic}$ can be used to indicate contamination with non-collagenous materials, we compared $C:N_{Atomic}$ and $\delta^{13}C$ for data grouped by $C:N_{Atomic}$ into cumulative iterations starting at 3.10 and increasing by intervals of +0.01 (i.e. 3.10–3.11, 3.10–3.12, 3.10–3.13 and so on) until significant correlations were identified. Significant correlations were not found in $C:N_{Atomic}$ and $\delta^{13}C$ for sample groups with a $C:N_{Atomic}$ of 3.30 and lower for fish and 3.28 and lower for mammals and birds. All groups including $C:N_{Atomic}$ values >3.30, for fish, and 3.28, for mammals and birds, show significant correlations

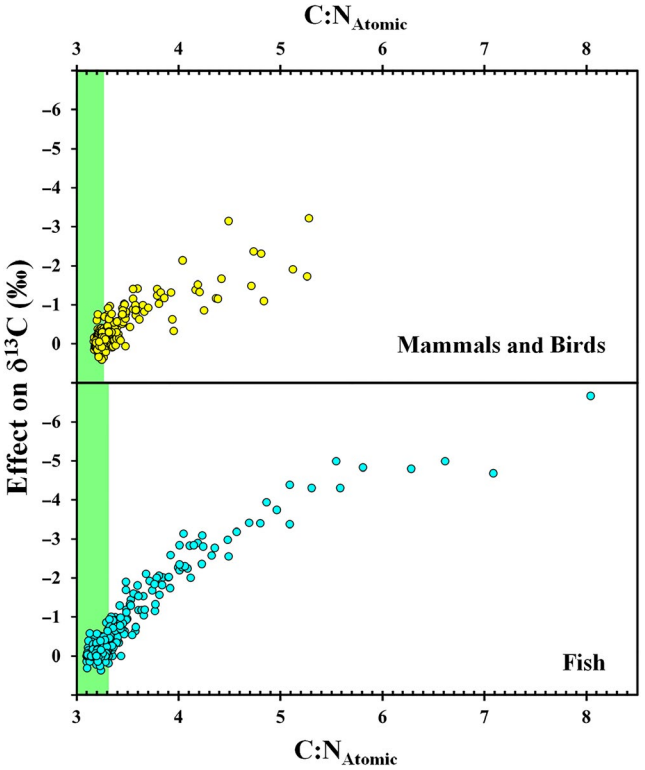

FIGURE 2 Relationship between lipid contamination, as indicated by $C:N_{Atomic}$, and negative skewing of collagen $\delta^{13}C$. Plots compare data generated by recent studies (Guiry, Szpak, et al., 2016; Szpak & Guiry, in prep.) on the effects of collagen extraction methods on the elemental and isotopic compositions of 122 bones from 85 fish, mammal and bird specimens. Four to five extraction procedures were applied to subsamples from each bone. Within each group of four to five samples per bone, the $\delta^{13}C$ of the sample with the lowest $C:N_{Atomic}$ was subtracted from the $\delta^{13}C$ of the other samples and are plotted against their respective $C:N_{Atomic}$. The shaded box shows the acceptable range based on Spearman's ρ (see Supporting Information 1, Table S3)

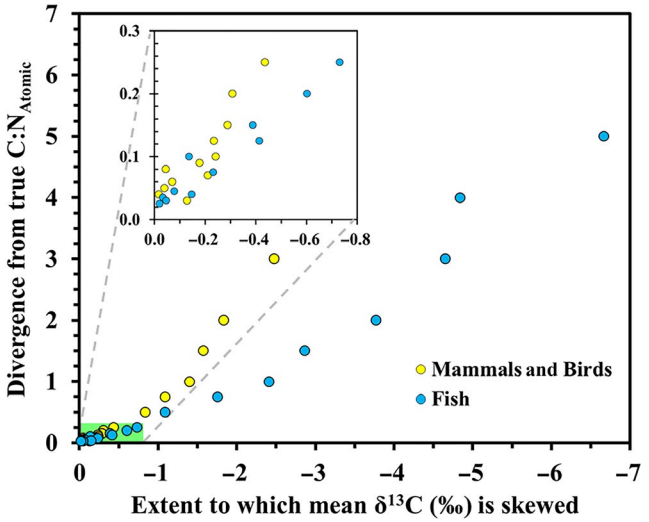

FIGURE 3 Plot of mean difference in $\delta^{13}C_{clean\text{-}contaminated}$ (horizontal axis) and $\Delta C{:}N_{Atomic\ (clean\text{-}contaminated)}$ (vertical axis) for groups binned by ascending $\Delta C{:}N_{Atomic\ (clean\text{-}contaminated)}$. Data from Figure 2. Comparisons provided in Supporting Information 1, Table S4. Inset shows enlargement of area highlighted in green

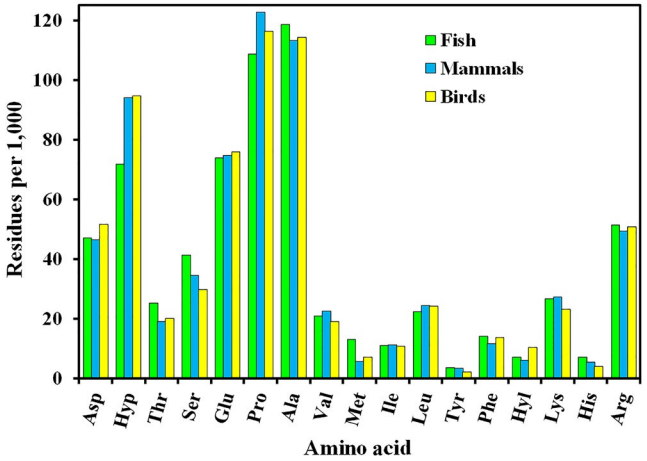

FIGURE 4 Mean amino acid compositions of fish (n = 287), mammal (n = 86) and bird (n = 9) collagen (see Supporting Information 1, Table S1 for data). Gly not shown in order to improve visualization of scaling for comparison of other amino acids

($p < 0.05$) with correlation strength (as defined by Spearman's ρ) growing as higher $C{:}N_{Atomic}$ comparisons are included (see Supporting Information 1, Table S3).

We also found a strong negative correlation between $\Delta^{13}C_{clean\text{-}contaminated}$ and $\Delta C{:}N_{Atomic\ (clean\text{-}contaminated)}$ among different intra sample fish (Spearman's $\rho = -0.937$, $p < 0.001$) and mammal and bird (Spearman's $\rho = -2.472$, $p < 0.001$) collagen extracts falling along a continuum of lipid contamination. To quantify the impact that smaller shifts in $C{:}N_{Atomic}$ (even those occurring within the acceptable $C{:}N_{Atomic}$ range) may have on $\delta^{13}C$ (Figure 3), we compared $\Delta^{13}C_{clean\text{-}contaminated}$ and $\Delta C{:}N_{Atomic\ (clean\text{-}contaminated)}$ at cumulative $\Delta C{:}N_{Atomic\ (clean\text{-}contaminated)}$ iterations starting at 0.010 and increasing by intervals of +0.005 (i.e. 0.010–0.015, 0.010–0.020, 0.010–0.025 and so on) until significant correlations were identified. Significant correlations were not found in bins with a $\Delta C{:}N_{Atomic\ (clean\text{-}contaminated)}$ difference of 0.030 and lower for fish and 0.060 and lower for mammals and birds. All bins with greater $\Delta C{:}N_{Atomic\ (clean\text{-}contaminated)}$ show significant correlations ($p < 0.05$) with correlation strength (as defined by Spearman's ρ) growing as $C{:}N_{Atomic}$ difference increases (see Supporting Information 1, Table S4).

4 | DISCUSSION

4.1 | Establishing $C{:}N_{Atomic}$ QC criteria for fish, mammal and bird bone collagen

Relative to fish, mammal and bird collagens are characterized by much less variation in amino acid compositions and lower proportions of relatively carbon-poor serine, threonine, alanine, and glycine and higher proportions of relatively carbon-rich proline and hydroxyproline, on average (Figure 4). For this reason, mean $C{:}N_{Atomic}$ for bird and mammal bone collagen have a narrower range of $C{:}N_{Atomic}$ and are on average slightly elevated by 0.05 over that of fish (Figure 1). The positive correlation between $C{:}N_{Atomic}$ and $\delta^{13}C$ observed for comparisons that include analyses with $C{:}N_{Atomic} > 3.30$ in fish and 3.28 in mammals and birds provides useful benchmarks above which collagen stable isotope compositions are likely compromised due to contamination with non-collagenous materials. The utility of these cut-off values is supported by their close agreement with the observed $C{:}N_{Atomic}$ means for fish skin, bone and scale collagen (Table 4, n = 290, 3.16 ± 0.06, range = 3.00–3.30) as well as mammal and bird skin and bone collagens (Table 4, n = 95, 3.22 ± 0.04; range = 3.11–3.33). With respect to using $C{:}N_{Atomic}$ to evaluate the quality of isotopic measurements made on bone collagen, we can therefore use 3.30 for fish and 3.28 for mammals and birds as the upper limit (a cut-off value) for acceptable stable carbon and nitrogen isotope compositions.

Establishing a lower cut-off $C{:}N_{Atomic}$ value is comparatively straightforward because the main sources of contamination for collagen for contemporary and archived historical materials is likely to be carbon rich and will therefore cause an increase, rather than a decrease, in $C{:}N_{Atomic}$. As outlined above, these contamination sources include lipids as well as mineral (for bone), neither of which contain a substantive nitrogen component (although some lipids, such as phosphatidylcholines have a single N atom), as well as NCPs. Owing to its higher glycine content (with its low $C{:}N_{Atomic}$ of 2), relative to most other proteins, collagen also has a lower $C{:}N_{Atomic}$ than potential sources of NCP contamination (Table 5; Figure 5). While sources of nitrogen-rich, carbon-poor contamination are unlikely in collagen extracts, they could be introduced through instrumental issues and therefore, low $C{:}N_{Atomic}$ still provides a QC indicator for flagging compromised isotopic measurements. For fish, the lowest observed $C{:}N_{Atomic}$ observed in bone collagen is 3.06 based on analyses of modern collagen extracts by Guiry, Szpak, et al. (2016)

TABLE 5 Details for major non-collagenous proteins found in ossified tissues. $C:N_{Atomic}$ were calculated based on mean amino acid counts from complete and reviewed amino acid sequences available from The Uniprot Consortium (2018; see Supporting Information 1, Table S5) and were processed using Bioedit v 7.2 (Hall, 1999). Because complete amino acid sequences may include small signal peptides (usually 16–30 amino acids long) not found in the mature protein, calculated $C:N_{Atomic}$ will deviate slightly from the true $C:N_{Atomic}$ and are intended as estimates illustrating broad variability in elemental composition between collagen and different non-collagenous proteins. Unless otherwise noted, molecular weight data are from Robey and Boskey (2013)

Non-collagenous bone proteins	$C:N_{Atomic}$	*n*=	Size (kDa)
Asproin	3.73 ± 0.01	3	67
Bone sialoprotein	3.53 ± 0.05	6	46–75
Biglycan	3.68 ± 0.01[a]	8	270 (38–45 core)
Decorin	3.63 ± 0.02[a]	12	130 (38–45 core)
Dentin matrix acidic phosphoprotein 1	3.24 ± 0.04	4	54[b]
Fibromodulin	3.67 ± 0.03[a]	5	59 (42 core)
Lumican	3.84 ± 0.03[a]	6	70–80 (37 core)
Osteoadhearin	3.79 ± 0.04[a]	4	85 (47 core)
Osteocalcin	3.83 ± 0.1	18	5.8
Osteonectin	3.74 ± 0.02	9	35–45
Osteopontin	3.49 ± 0.08	8	44–75
Osteoregulin	3.15 ± 0.05	3	50[b]
Periostin	3.65 ± 0.01	2	90
Versican	3.74 ± 0.03[a]	4	NA (360 core)

[a]$C:N_{Atomic}$ calculated for core protein only.
[b]Size calculated based on complete amino acid sequences in Bioedit v 7.2 (Hall, 1999).

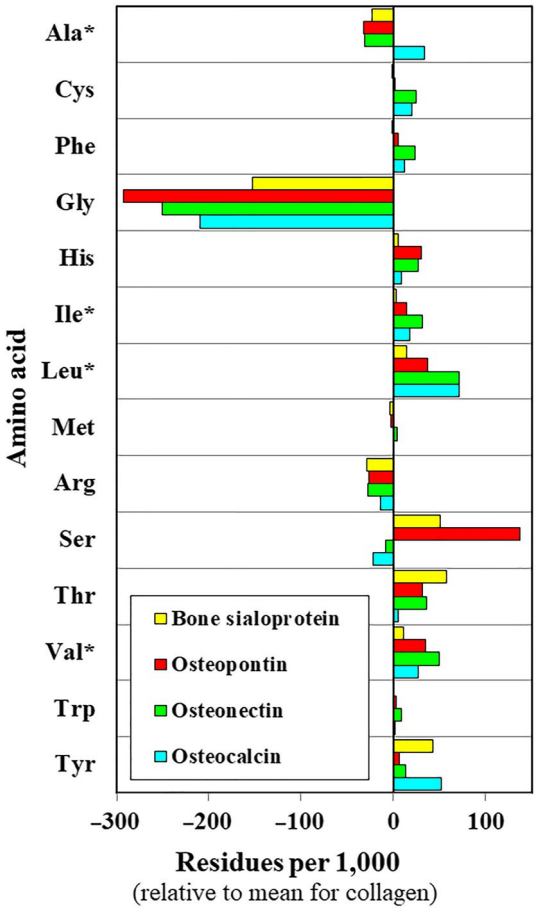

FIGURE 5 Select amino acid compositions (see Table 4; Supporting Information 1, Table S6) of non-collagenous proteins prevalent in bone compared to the mean for collagen (all species, *n* = 436, Supporting Information 1). *Amino acids commonly considered to undergo trophic ^{15}N enrichment (O'Connell, 2017)

and 3.00 based on the survey of published amino acid composition assays (*n* = 290, Supporting Information 1, Table S1). The lowest observed $C:N_{Atomic}$ observed in mammal and bird bone collagen is 3.15 based on analyses of modern collagen extracts by Szpak and Guiry (in prep.) and 3.11 based on the survey of published amino acid composition assays (*n* = 95, Supporting Information 1, Table S1). However, the sample size of published amino acid compositions of mammals and birds is smaller in comparison to fish and therefore may not capture the full range of variation in $C:N_{Atomic}$. For this reason, a lower $C:N_{Atomic}$ limit of 3.00 for fish, mammals and birds can be established as a conservative acceptable collagen stable carbon and nitrogen isotope compositions.

It is important to bear in mind that the ranges of acceptable $C:N_{Atomic}$ identified here are only a general guideline and that even collagen extracts with $C:N_{Atomic}$ that fall within this acceptable range may still have skewed isotopic compositions. Significant correlations in $C:N_{Atomic}$ and $\delta^{13}C$ shifts among different extracts from the same samples show that elevations in $C:N_{Atomic}$ of as little as 0.03 can be accompanied by significant negative shifts in $\delta^{13}C$. For instance, on average a $C:N_{Atomic}$ increase of 0.25, which would fall within the envelope of acceptable $C:N_{Atomic}$ identified here, was associated with $\delta^{13}C$ values skewed by approximately 0.5‰. For this reason, it is critical that close attention is paid to optimizing collagen purification protocols.

4.2 | Best practices for modem bone collagen extractions

With respect to characterizing the isotopic composition of collagen from any tissue, it is critical that sample pre-treatment protocols are tailored to remove non-collagenous materials as effectively as possible. This is particularly important for ossified tissues like bones and teeth, which are composite materials that include other carbon-rich components (mineral, lipids, NCPs). A variety of techniques have been developed for extracting and purifying collagen from modern and ancient bone and typically include steps for removing bone mineral (demineralization in dilute HCl, Longin, 1971; or calcium chelation with EDTA, Olsson, El-Daoushy, Abdel-Mageed, & Klasson, 1974), lipids (solvent wash, usually with a chloroform and methanol mixture, Bligh & Dyer, 1959; Folch, Lees, & Sloane-Stanley, 1957) and

acid insoluble NCPs (soaking in NaOH, Haynes, 1967; and refluxing in weak acids, Longin, 1971).

Recently, there has been discussion in the literature suggesting that analysing δ^{15}N of whole bone powder produces more reliable results relative to analyses of extracted and purified bone collagen (e.g. Bas, García, Crespo, & Cardona, 2019). While there are some cases where δ^{15}N analyses of whole bone of tooth powder may be a desirable alternative to analyses of extracted bone collagen, these are limited to circumstances wherein small sample size negates the ability to extract a sufficient amount of purified collagen from the sample (e.g. Fahy et al., 2014; Guiry, Hepburn, & Richards, 2016; Guiry, Jones, et al., 2018; Rossman et al., 2015). In most cases, however, analyses of δ^{15}N from untreated bone powder samples will *de facto* produce less predictable and more heterogeneous results. This is because a substantial fraction (10% of extracellular proteinaceous material) of whole bone or dentine protein is composed of a variable mixture of NCPs (for reviews, see Gorski, 2011; Orsini et al., 2009; Robey & Boskey, 2013), the most abundant of which include osteocalcin (Gundberg, Hauschka, Lian, & Gallop, 1984; Hauschka, Lian, & Gallop, 1975; Price, Poser, & Raman, 1976), osteonectin (Termine et al., 1981), osteopontin (Senger, Wirth, & Hynes, 1979; Sodek, Ganss, & McKee, 2000) and bone sialoprotein (Ganss, Kim, & Sodek, 1999; Herring, 1972).

Incomplete removal of NCPs creates two major problems for interpreting the isotopic composition of whole bone powder. First, the relative proportions of major NCPs are known to vary significantly within and among bones, individuals and species (e.g. Gorski, 1998; Roach, 1994). This variation in the relative proportions of different NCPs means that the contribution of nitrogen from different amino acids cannot be anticipated. Second, the amino acid compositions of these NCPs each differ from that of collagen (Table 5; Fisher, Hawkins, Tuross, & Termine, 1987; Gundberg et al., 1984), with differing proportions of 'source' and 'trophic' amino acids (Figure 5) that, in turn, will skew the δ^{15}N of whole bone protein relative to extracted, purified collagen. These two levels of variation (in relative proportions of different NCPs and their variable contributions of amino acids with different trophic discrimination factors) will necessarily result in more heterogeneous and less predictable δ^{15}N values from analyses of whole bone protein.

In addition to removing the mineral and lipid fractions of bones and teeth, collagen extraction protocols help to remove NCPs, thereby producing a more consistently homogeneous material that is better suited for comparing isotopic compositions within and among anatomical elements, individuals and species. In particular, the demineralization and refluxing steps should remove a substantial fraction NCPs, like osteocalcin, which are tightly bound in the mineral phase (bioapatite) of bone and dentine (Gundberg et al., 1984). Other important NCPs, such as osteonectin, that have a strong affinity for binding to both mineral and collagen, are also at least partly removed through standard HCl- or EDTA-based collagen extraction protocols (e.g. Romberg, Werness, Lollar, Riggs, & Mann, 1985; Termine et al., 1981). Although there has been little work quantifying the NCP composition of bone collagen extracts prepared using different protocols (although see 2018), it is likely that NCPs, while present, remain in very low quantities (Linde, Bhown, & Butler, 1981).

Additional steps have also been recommended to further purify bone collagen and may help to improve collagen QC indicators. Brown, Nelson, Vogel, and Southon (1988) recommended 'ultrafiltration' with 10 or 30 kDa molecular weight cut-off (MWCO) filters for the extraction of collagen for AMS radiocarbon dating, a suggestion that has widely been taken up for collagen extraction protocols for IRMS analyses (Dobberstein et al., 2009; Sealy, Johnson, Richards, & Nehlich, 2014). Ultrafiltration removes low molecular weight contaminants, concentrating higher molecular weight collagen molecules (*c.* 120 kDa per peptide once the helix is unwound) in the resulting extract. While recent experimental research has confirmed that ultrafiltration does not have a meaningful impact on removal of a number of key contaminants (e.g. lipids, Guiry, Szpak, et al., 2016; humic acids, Szpak, Krippner, & Richards, 2017), there has been little research investigating the effects of ultrafiltration on the removal of NCPs from collagen extracts (although see Wadsworth & Buckley, 2018). However, as shown in Table 4, nearly all major NCPs present in bone, including osteonectin (32 kDa; Termine et al., 1981) osteopotinin (44 kDa; Rangaswami, Bulbule, & Kundu, 2006) and bone sialoprotein (33–34 kDa; Ganss et al., 1999) are too large for removal through ultrafiltration (even at the 30 kDa MWCO). One exception is ostecalcin (5.8 kDa; Price et al., 1976), a much smaller mineral-bound NCP, but this molecule will likely already have been effectively removed through demineralization (Gundberg et al., 1984). For this reason, ultrafiltration is unlikely to improve NCP removal for collagen purification.

A more effective method for removal of NCPs may be the addition of a NaOH pre-treatment step (Lowry, Gilligan, & Katersky, 1941), which is routinely applied for collagen purification in food chemistry in the process of characterizing collagen amino acid compositions (e.g. Nagai & Suzuki, 2000). This step is also commonly applied during collagen extractions from archaeological materials (between the demineralization and refluxing steps) because it removes base-soluble contaminants like humic acids derived from the burial environment. While use of the NaOH pre-treatment step can reduce collagen yields in ancient samples (Chisholm, Nelson, Hobson, Schwarcz, & Knyf, 1983), this should not be an issue for well-preserved, modern bones. The NaOH step does not induce selective loss of amino acids (Katzenberg, 1989; Kennedy, 1988) and, for this reason, should not impact collagen isotopic compositions (Ambrose, 1990).

In summary, isotopic analyses of whole bone should not be supplemented for analyses of extracted bone collagen because the 10% NCP fraction of bone protein will skew isotopic compositions. While standard collagen extraction protocols should remove all major contaminants, including most NCPs, a small fraction of NCPs may remain (bonded directly to collagen). Decades of hindsight in archaeological and paleontological isotopic analyses of collagen extracts from a wide range of taxa show that NCPs are rarely considered to be an important source of contamination for ancient collagen isotopic compositions. However, it is not clear what effect time and burial conditions might have on the survival of NCPs and it is therefore possible that, while NCPs are not a serious issue for ancient tissues,

they may still present an issue for effective purification of collagen extracts from modern tissues (for further discussion, see Guiry & Hunt, 2020). A NaOH treatment step may serve to further reduce contamination of collagen with NCPs and could be used to help ensure that extracts do not fail the $C:N_{Atomic}$ QC criterion for isotopic analyse of modern collagen samples.

5 | CONCLUSIONS

Stable carbon and nitrogen isotope analyses of collagen from bones, scales and other tissues are becoming increasingly important in ecological research for a variety of reasons such as access to archived tissues for retrospective research (Dietl et al., 2015) and providing longer-term intra-individual perspectives for migratory studies (Hobson, 2019). While isotopic analysis of collagen has been an integral and well-established component of archaeological and paleobiological research for decades, techniques for assessing data quality in ancient materials are not directly transferable to analyses of collagen from modern tissues. In this study, we have used observations of amino acid compositions from a wide range of species as well as experiment results from fish, mammal and bird bone collagen isotopic compositions to better characterize $C:N_{Atomic}$ QC criteria for isotopic analyses of modern collagen. Whereas widely accepted QC criteria for ancient collagen based on $C:N_{Atomic}$ are between 2.9 and 3.6, for modern tissues we establish new cut-offs between 3.00 and 3.30 for fish and 3.00 and 3.28 for mammals and birds. However, it is important to recognize that even for collagen extracts with $C:N_{Atomic}$ falling within this range isotopic compositions could still potentially be skewed and it is therefore critical to optimize collagen purification protocols. With respect to collagen extraction protocols, we have also reviewed key processes for ensuring that collagen extractions from modern bone are better able to meet these new collagen quality criteria. In particular, it is important that efforts are made not only to remove lipid and mineral contaminants (known to effect $\delta^{13}C$) but also to ensure that NCPs are removed as these can have important consequences for $\delta^{13}C$ and $\delta^{15}N$ measurements.

ACKNOWLEDGEMENTS

E.J.G. has been supported by a Social Sciences and Humanities Research Council of Canada Banting Post-Doctoral Fellowship.

AUTHORS' CONTRIBUTIONS

E.J.G.: conceptualization, methodology, investigation, formal analysis, data curation, funding acquisition, visualization, writing—original draft, writing—review and editing. P.S.: methodology, writing—review and editing.

DATA AVAILABILITY STATEMENT

All data presented in this paper are publicly archived on Dryad (https://doi.org/10.5061/dryad.ffbg79crm; Guiry & Szpak, 2020) and are also available in Supporting Information 1 and in referenced sources.

ORCID

Eric J. Guiry https://orcid.org/0000-0002-1467-1521
Paul Szpak https://orcid.org/0000-0002-1364-6834

REFERENCES

Ambrose, S. H. (1990). Preparation and characterization of bone and tooth collagen for isotopic analysis. *Journal of Archaeological Science*, *17*(4), 431–451. https://doi.org/10.1016/0305-4403(90)90007-R

Bas, M., & Cardona, L. (2018). Effects of skeletal element identity, delipidation and demineralization on the analysis of stable isotope ratios of C and N in fish bone. *Journal of Fish Biology*, *92*(2), 420–437. https://doi.org/10.1111/jfb.13521

Bas, M., García, N. A., Crespo, E. A., & Cardona, L. (2019). Intraskeletal variability in stable isotope ratios of C and N among pinnipeds and cetaceans. *Marine Mammal Science*, *36*(1), 375–385. https://doi.org/10.1111/mms.12644

Bligh, E., & Dyer, W. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*, 911–917. https://doi.org/10.1139/o59-099

Braje, T. J., Rick, T. C., Szpak, P., Newsome, S. D., McCain, J. M., Smith, E. A. E., … Hamilton, S. L. (2017). Historical ecology and the conservation of large, hermaphroditic fishes in Pacific Coast kelp forest ecosystems. *Science Advances*, *3*(2), e1601759.

Brown, T. A., Nelson, D. E., Vogel, J. S., & Southon, J. R. (1988). Improved collagen extraction by modified Longin method. *Radiocarbon*, *30*(2), 171–177. https://doi.org/10.1017/S0033822200044118

Burton, R. K., Snodgrass, J. J., Gifford-Gonzalez, D., Guilderson, T., Brown, T., & Koch, P. L. (2001). Holocene changes in the ecology of northern fur seals: Insights from stable isotopes and archaeofauna. *Oecologia*, *128*(1), 107–115. https://doi.org/10.1007/s004420100631

Chisholm, B. S., Nelson, D. E., Hobson, K. A., Schwarcz, H. P., & Knyf, M. (1983). Carbon isotope measurement techniques for bone collagen: Notes for the archaeologist. *Journal of Archaeological Science*, *10*(4), 355–360. https://doi.org/10.1016/0305-4403(83)90073-0

Collins, M. J., & Galley, P. (1998). Towards an optimal method of archaeological collagen extraction: The influence of pH and grinding. *Ancient Biomolecules*, *2*, 209–222.

Collins, M. J., Nielsen-Marsh, C. M., Hiller, J., Smith, C. I., Roberts, J. P., Prigodich, R. V., … Turner-Walker, G. (2002). The survival of organic matter in bone: A review. *Archaeometry*, *44*(3), 383–394. https://doi.org/10.1111/1475-4754.t01-1-00071

Collins, M. J., Riley, M. S., Child, A. M., & Turner-Walker, G. (1995). A basic mathematical simulation of the chemical degradation of ancient collagen. *Journal of Archaeological Science*, *22*(2), 175–183. https://doi.org/10.1006/jasc.1995.0019

DeNiro, M. J. (1985). Postmortem preservation and alteration of in vivo bone collagen isotope ratios in relation to palaeodietary reconstruction. *Nature*, *317*, 806–809. https://doi.org/10.1038/317806a0

Dietl, G. P., Kidwell, S. M., Brenner, M., Burney, D. A., Flessa, K. W., Jackson, S. T., & Koch, P. L. (2015). Conservation paleobiology: Leveraging knowledge of the past to inform conservation and restoration. *Annual Review of Earth and Planetary Sciences*, *43*, 79–103. https://doi.org/10.1146/annurev-earth-040610-133349

Dixon, H. J., Dempson, J. B., & Power, M. (2015). Assessing the use of different marine growth zones of adult Atlantic salmon scales for studying marine trophic ecology with stable isotope analysis. *Fisheries Research*, *164*, 112–119. https://doi.org/10.1016/j.fishres.2014.10.009

Dobberstein, R. C., Collins, M. J., Craig, O. E., Taylor, G., Penkman, K. E., & Ritz-Timme, S. (2009). Archaeological collagen: Why worry about collagen diagenesis? *Archaeological and Anthropological Sciences*, *1*(1), 31–42. https://doi.org/10.1007/s12520-009-0002-7

Eastoe, J. (1957). The amino acid composition of fish collagen and gelatin. *Biochemical Journal*, *65*(2), 363. https://doi.org/10.1042/bj0650363

Fahy, G. E., Richards, M. P., Fuller, B. T., Deschner, T., Hublin, J.-J., & Boesch, C. (2014). Stable nitrogen isotope analysis of dentine serial sections elucidate sex differences in weaning patterns of wild chimpanzees (*Pan troglodytes*). *American Journal of Physical Anthropology*, *153*(4), 635–642.

Fisher, L. W., Hawkins, G. R., Tuross, N., & Termine, J. D. (1987). Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. *Journal of Biological Chemistry*, *262*(20), 9702–9708.

Folch, J., Lees, M., & Sloane-Stanley, G. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, *226*(1), 497–509.

Froese, R., & Pauly, D. (2000). *FishBase 2000: Concepts, design and data sources*. Los Banos, CA: ICLARM.

Ganss, B., Kim, R. H., & Sodek, J. (1999). Bone sialoprotein. *Critical Reviews in Oral Biology & Medicine*, *10*(1), 79–98. https://doi.org/10.1177/10454411990100010401

Gorski, J. P. (1998). Is all bone the same? Distinctive distributions and properties of non-collagenous matrix proteins in lamellar vs. woven bone imply the existence of different underlying osteogenic mechanisms. *Critical Reviews in Oral Biology & Medicine*, *9*(2), 201–223. https://doi.org/10.1177/10454411980090020401

Gorski, J. P. (2011). Biomineralization of bone: A fresh view of the roles of non-collagenous proteins. *Frontiers in Bioscience*, *16*, 2598. https://doi.org/10.2741/3875

Guiry, E. (2019). Complexities of stable carbon and nitrogen isotope biogeochemistry in ancient freshwater ecosystems: Implications for the study of past subsistence and environmental change. *Frontiers in Ecology and Evolution*, *7*(313). https://doi.org/10.3389/fevo.2019.00313

Guiry, E. J., Beglane, F., Szpak, P., Schulting, R., McCormick, F., & Richards, M. P. (2018). Anthropogenic changes to the Holocene nitrogen cycle in Ireland. *Science Advances*, *4*(6), eaas9383. https://doi.org/10.1126/sciadv.aas9383

Guiry, E. J., Buckley, M., Orchard, T. J., Hawkins, A. L., Needs-Howarth, S., Holm, E., & Szpak, P. (2020). Deforestation caused abrupt shift in Great Lakes nitrogen cycle. *Limnology and Oceanography*. Online First. https://doi.org/10.1002/lno.11428

Guiry, E. J., Hepburn, J. C., & Richards, M. P. (2016). High-resolution serial sampling for nitrogen stable isotope analysis of archaeological mammal teeth. *Journal of Archaeological Science*, *69*, 21–28. https://doi.org/10.1016/j.jas.2016.03.005

Guiry, E. J., & Hunt, B. (2020). Integrating fish scale and bone isotopic compositions for 'deep time' retrospective studies. *Marine Environmental Research*, *160*. https://doi.org/10.1016/j.marenvres.2020.104982

Guiry, E. J., Jones, B. M., deFrance, S., Bruseth, J. E., Durst, J., & Richards, M. P. (2018). Animal husbandry and colonial adaptive behavior: Isotopic insights from the la belle shipwreck fauna. *Historical Archaeology*, *52*(4), 684–699. https://doi.org/10.1007/s41636-018-0142-7

Guiry, E. J., Needs-Howarth, S., Friedland, K. D., Hawkins, A. L., Szpak, P., Macdonald, R., … Richards, M. P. (2016). Lake Ontario salmon (*Salmo salar*) were not migratory: A long-standing historical debate solved through stable isotope analysis. *Scientific Reports*, *6*. https://doi.org/10.1038/srep36249

Guiry, E. J., Orchard, T. J., Royle, T. C. A., Cheung, C., & Yang, D. Y. (2020). Dietary plasticity and the extinction of the passenger pigeon (*Ectopistes migratorius*). *Quaternary Science Reviews*, *233*, 106225. https://doi.org/10.1016/j.quascirev.2020.106225

Guiry, E., Royle, T. C. A., Matson, R. G., Ward, H., Weir, T., Waber, N., … Szpak, P. (2020). Differentiating salmonid migratory ecotypes through stable isotope analysis of collagen: Archaeological and ecological applications. *PLoS ONE*, *15*(4), e0232180. https://doi.org/10.1371/journal.pone.0232180

Guiry, E. J., & Szpak, P. (2020). Data from: Quality control for modern bone collagen stable carbon and nitrogen isotope measurements. *Dryad Digital Repository*, https://doi.org/10.5061/dryad.ffbg79crm

Guiry, E. J., Szpak, P., & Richards, M. P. (2016). Effects of lipid extraction and ultrafiltration on stable carbon and nitrogen isotopic compositions of fish bone collagen. *Rapid Communications in Mass Spectrometry*, *30*(13), 1591–1600. https://doi.org/10.1002/rcm.7590

Gundberg, C., Hauschka, P., Lian, J., & Gallop, P. (1984). Osteocalcin: Isolation, characterization, and detection. *Methods in Enzymology*, 516–544. https://doi.org/10.1016/0076-6879(84)07036-1

Gustavson, K. (1955). The function of hydroxyproline in collagens. *Nature*, *175*(4445), 70–74. https://doi.org/10.1038/175070a0

Hall, T. A. (1999). *BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT* (pp. 95–98). London, UK: Information Retrieval Ltd.

Hammer, Ø., Harper, D. A., & Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, *4*(1), *9*.

Hauschka, P. V., Lian, J. B., & Gallop, P. M. (1975). Direct identification of the calcium-binding amino acid, gamma-carboxyglutamate, in mineralized tissue. *Proceedings of the National Academy of Sciences of the United States of America*, *72*(10), 3925–3929. https://doi.org/10.1073/pnas.72.10.3925

Haynes, V. (1967). *Bone organic matter and radiocarbon dating* (pp. 163–168). Vienna, Austria: International Atomic Energy Agency.

Herring, G. (1972). The organic matrix of bone. In G. Bourne (Ed.), *The biochemistry and physiology of bone* (pp. 127–189). New York, NY: Academic Press.

Hobson, K. A. (2019). Application of isotopic methods to tracking animal movements. In K. A. Hobson & L. I. Wassenaar (Eds.). *Tracking animal migration with stable isotopes* (2nd ed., pp. 85–115). New York, NY: Academic Press.

Katzenberg, M. A. (1989). Stable isotope analysis of archaeological faunal remains from southern Ontario. *Journal of Archaeological Science*, *16*(3), 319–329. https://doi.org/10.1016/0305-4403(89)90008-3

Kennedy, B. V. E. (1988). *Variation in 13 C values of post-medieval Europeans: Archaeology*. Calgary, Canada: University of Calgary.

Linde, A., Bhown, M., & Butler, W. T. (1981). Non-collagenous proteins of rat dentin. Evidence that phosphoprotein is not covalently bound to collagen. *Biochimica Et Biophysica Acta (BBA) - Protein Structure*, *667*(2), 341–350. https://doi.org/10.1016/0005-2795(81)90200-2

Longin, R. (1971). New method of collagen extraction for radiocarbon dating. *Nature*, *230*, 241–242. https://doi.org/10.1038/230241a0

Lowry, O. H., Gilligan, D. R., & Katersky, E. M. (1941). The determination of collagen and elastin in tissues, with results obtained in various normal tissues from different species. *Journal of Biological Chemistry*, *139*(2), 795–804.

Misarti, N., Finney, B., Maschner, H., & Wooller, M. J. (2009). Changes in northeast Pacific marine ecosystems over the last 4500 years: Evidence from stable isotope analysis of bone collagen from archeological middens. *The Holocene*, *19*(8), 1139–1151. https://doi.org/10.1177/0959683609345075

Nagai, T., & Suzuki, N. (2000). Preparation and characterization of several fish bone collagens. *Journal of Food Biochemistry*, *24*(5), 427–436. https://doi.org/10.1111/j.1745-4514.2000.tb00711.x

Nelson, M. A., Quakenbush, L. T., Mahoney, B. A., Taras, B. D., & Wooller, M. J. (2018). Fifty years of Cook Inlet beluga whale feeding ecology from isotopes in bone and teeth. *Endangered Species Research*, *36*, 77–87. https://doi.org/10.3354/esr00890

Neuman, R. (1949). The amino acid composition of gelatine, collagens and elastins from different sources. *Archive of Biochemistry*, *24*, 289–298.

Newsome, S. D., Clementz, M. T., & Koch, P. L. (2010). Using stable isotope biogeochemistry to study marine mammal ecology. *Marine Mammal Science*, *26*(3), 509–572. https://doi.org/10.1111/j.1748-7692.2009.00354.x

Newsome, S. D., Etnier, M. A., Kurle, C. M., Waldbauer, J. R., Chamberlain, C. P., & Koch, P. L. (2007). Historic decline in primary productivity in western Gulf of Alaska and eastern Bering Sea: Isotopic analysis of northern fur seal teeth. *Marine Ecology Progress Series*, *332*, 211–224. https://doi.org/10.3354/meps332211

O'Connell, T. C. (2017). 'Trophic' and 'source' amino acids in trophic estimation: A likely metabolic explanation. *Oecologia*, *184*(2), 317–326. https://doi.org/10.1007/s00442-017-3881-9

Olsson, I. U., El-Daoushy, M. F. A., Abdel-Mageed, A. I., & Klasson, M. (1974). A comparison of different methods for pretreatment of bones. I. *Geologiska Föreningen i Stockholm Förhandlingar*, *96*(2), 171–181. https://doi.org/10.1080/11035897409454271

Orsini, G., Ruggeri Jr., A., Mazzoni, A., Nato, F., Manzoli, L., Putignano, A., … Breschi, L. (2009). A review of the nature, role, and function of dentin non-collagenous proteins. Part 1: Proteoglycans and glycoproteins. *Endodontic Topics*, *21*(1), 1–18. https://doi.org/10.1111/j.1601-1546.2012.00270.x

Price, P. A., Poser, J. W., & Raman, N. (1976). Primary structure of the gamma-carboxyglutamic acid-containing protein from bovine bone. *Proceedings of the National Academy of Sciences of the United States of America*, *73*(10), 3374–3375. https://doi.org/10.1073/pnas.73.10.3374

Rangaswami, H., Bulbule, A., & Kundu, G. C. (2006). Osteopontin: Role in cell signaling and cancer progression. *Trends in Cell Biology*, *16*(2), 79–87. https://doi.org/10.1016/j.tcb.2005.12.005

Rick, T. C., & Lockwood, R. (2013). Integrating paleobiology, archeology, and history to inform biological conservation. *Conservation Biology*, *27*(1), 45–54. https://doi.org/10.1111/j.1523-1739.2012.01920.x

Rigby, B. (1967). Correlation between serine and thermal stability of collagen. *Nature*, *214*(5083), 87–88. https://doi.org/10.1038/214087a0

Rigby, B., & Spikes, J. (1960). Hydroxyproline and the shrinkage temperature of collagen. *Nature*, *187*(4732), 150–151. https://doi.org/10.1038/187150a0

Roach, H. (1994). Why does bone matrix contain non-collagenous proteins? The possible roles of osteocalcin, osteonectin, osteopontin and bone sialoprotein in bone mineralisation and resorption. *Cell Biology International*, *18*(6), 617–628. https://doi.org/10.1006/cbir.1994.1088

Robey, P. G., & Boskey, A. L. (2013). The composition of bone. In J. C. Rosen (Ed.), *Primer on the bone metabolic diseases and disorders of mineral metabolism* (8th ed., pp. 49–58). New York, NY: Wiley-Blackwell.

Romberg, R., Werness, P., Lollar, P., Riggs, B. L., & Mann, K. (1985). Isolation and characterization of native adult osteonectin. *Journal of Biological Chemistry*, *260*(5), 2728–2736.

Rossman, S., Ostrom, P., Stolen, M., Barros, N., Gandhi, H., Stricker, C., & Wells, R. (2015). Individual specialization in the foraging habits of female bottlenose dolphins living in a trophically diverse and habitat rich estuary. *Oecologia*, *178*(2), 415–425. https://doi.org/10.1007/s00442-015-3241-6

Sealy, J., Johnson, M., Richards, M., & Nehlich, O. (2014). Comparison of two methods of extracting bone collagen for stable carbon and nitrogen isotope analysis: Comparing whole bone demineralization with gelatinization and ultrafiltration. *Journal of Archaeological Science*, *47*, 64–69. https://doi.org/10.1016/j.jas.2014.04.011

Senger, D. R., Wirth, D. F., & Hynes, R. O. (1979). Transformed mammalian cells secrete specific proteins and phosphoproteins. *Cell*, *16*(4), 885–893. https://doi.org/10.1016/0092-8674(79)90103-X

Skovrind, M., Castruita, J. A. S., Haile, J., Treadaway, E. C., Gopalakrishnan, S., Westbury, M. V., … Lorenzen, E. D. (2019). Hybridization between two high Arctic cetaceans confirmed by genomic analysis. *Scientific Reports*, *9*(1), 7729. https://doi.org/10.1038/s41598-019-44038-0

Sodek, J., Ganss, B., & McKee, M. (2000). Osteopontin. *Critical Reviews in Oral Biology & Medicine*, *11*(3), 279–303. https://doi.org/10.1177/10454411000110030101

Swetnam, T. W., Allen, C. D., & Betancourt, J. L. (1999). Applied historical ecology: Using the past to manage for the future. *Ecological Applications*, *9*(4), 1189–1206. https://doi.org/10.1890/1051-0761(1999)009[1189:AHEUTP]2.0.CO;2

Szpak, P. (2011). Fish bone chemistry and ultrastructure: Implications for taphonomy and stable isotope analysis. *Journal of Archaeological Science*, *38*(12), 3358–3372. https://doi.org/10.1016/j.jas.2011.07.022

Szpak, P., Buckley, M., Darwent, C. M., & Richards, M. P. (2018). Long-term ecological changes in marine mammals driven by recent warming in northwestern Alaska. *Global Change Biology*, *24*(1), 490–503. https://doi.org/10.1111/gcb.13880

Szpak, P., & Guiry, E. (in prep.). Improved collagen extraction protocols for modern bone. *Oecologia*.

Szpak, P., Julien, M. H., Royle, T. C., Savelle, J. M., Yang, D. Y., & Richards, M. P. (2019). Sexual differences in the foraging ecology of 19th century beluga whales (*Delphinapterus leucas*) from the Canadian High Arctic. *Marine Mammal Science*, *36*(2), 451-471. https://doi.org/10.1111/mms.12655

Szpak, P., Krippner, K., & Richards, M. (2017). Effects of sodium hydroxide treatment and ultrafiltration on the removal of humic contaminants from archaeological bone. *International Journal of Osteoarchaeology*, *27*(6), 1070–1077. https://doi.org/10.1002/oa.2630

Szpak, P., Metcalfe, J. Z., & Macdonald, R. A. (2017). Best practices for calibrating and reporting stable isotope measurements in archaeology. *Journal of Archaeological Science: Reports*, *13*, 609–616. https://doi.org/10.1016/j.jasrep.2017.05.007

Szpak, P., Orchard, T. J., McKechnie, I., & Gröcke, D. R. (2012). Historical ecology of late Holocene sea otters (*Enhydra lutris*) from northern British Columbia: Isotopic and zooarchaeological perspectives. *Journal of Archaeological Science*, *39*(5), 1553–1571. https://doi.org/10.1016/j.jas.2011.12.006

Termine, J. D., Kleinman, H. K., Whitson, S. W., Conn, K. M., McGarvey, M. L., & Martin, G. R. (1981). Osteonectin, a bone-specific protein linking mineral to collagen. *Cell*, *26*(1), 99–105. https://doi.org/10.1016/0092-8674(81)90037-4

Turner Tomaszewicz, C. N., Seminoff, J. A., Avens, L., & Kurle, C. M. (2016). Methods for sampling sequential annual bone growth layers for stable isotope analysis. *Methods in Ecology and Evolution*, *7*(5), 556–564. https://doi.org/10.1111/2041-210X.12522

Turner Tomaszewicz, C. N., Seminoff, J. A., Price, M., & Kurle, C. M. (2017). Stable isotope discrimination factors and between-tissue isotope comparisons for bone and skin from captive and wild green sea turtles (*Chelonia mydas*). *Rapid Communications in Mass Spectrometry*, *31*(22), 1903–1914.

The UniProt Consortium. (2018). UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Research*, *47*(D1), D506–D515. https://doi.org/10.1093/nar/gky1049

Van Klinken, G. J. (1999). Bone collagen quality indicators for palaeodietary and radiocarbon measurements. *Journal of Archaeological Science*, *26*(6), 687–695. https://doi.org/10.1006/jasc.1998.0385

Vokhshoori, N. L., McCarthy, M. D., Collins, P. W., Etnier, M. A., Rick, T., Eda, M., … Newsome, S. D. (2019). Broader foraging range of ancient short-tailed albatross populations into California coastal waters based on bulk tissue and amino acid isotope analysis. *Marine Ecology Progress Series*, *610*, 1–13. https://doi.org/10.3354/meps12839

Wadsworth, C., Buckley, M. (2018). Characterization of proteomes extracted through collagen-based stable isotope and radiocarbon dating methods. *Journal of Proteome Research*, *17*(1), 429–439. https://doi.org/10.1021/acs.jproteome.7b00624

Wainright, S. C., Fogarty, M., Greenfield, R., & Fry, B. (1993). Long-term changes in the Georges Bank food web: Trends in stable isotopic compositions of fish scales. *Marine Biology*, *115*(3), 481–493. https://doi.org/10.1007/BF00349847

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Guiry EJ, Szpak P. Quality control for modern bone collagen stable carbon and nitrogen isotope measurements. *Methods Ecol Evol*. 2020;11:1049–1060. https://doi.org/10.1111/2041-210X.13433