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Fish bone chemistry and ultrastructure: implications for taphonomy and stable isotope analysis

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ABSTRACT

This paper reviews the ultrastructure and chemistry of fish bone, with an emphasis on zooarchaeology and stable isotope analysis. On the basis of the chemical composition of the collagen and the relationships between the collagen and mineral phases, fish bone is more susceptible to biotic and abiotic degradation than mammalian bone and is therefore less likely to be recovered in archaeological deposits. The amino acid composition of fish bone differs from that of mammals, most notably with respect to hydroxyproline content. The C:N ratio of fish collagen is, however, very similar and slightly lower than mammalian collagen, and thus the traditional range of acceptable C:N ratios for archaeological bone collagen (2.9–3.6) should not be shifted or extended for fish on the basis of the amino acid composition of collagen. An extensive survey of published archaeological bone collagen C:N ratios demonstrates that fish collagen from archaeological contexts tends to have significantly higher C:N ratios than mammalian collagen. The elevated C:N ratios in fish bone collagen may be the result of abiotic degradation processes that occur within the bone after death, the presence of exogenous humic contaminants, or endogenous lipid contaminants.

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1. Introduction

A thorough understanding of the processes relevant to the preservation of bone and its constituent materials is critical in archeology, paleontology, forensics and related disciplines. While still relatively understudied, these processes have received some attention within the context of mammalian bone (see Child, 1995; Collins et al., 2002). Conversely, the chemistry of fish bone as it relates to archeology has not been examined in detail. More generally, the physical and chemical properties of fish bone have received less attention than their mammalian counterparts (Currey, 2010). This is particularly problematic because it limits our understanding of post-depositional processes affecting zooarchaeological or paleontological assemblages (cf. Noto, 2011).

The importance of fish remains in the reconstruction of local environmental conditions and subsistence practices has long been recognized (e.g. Colley, 1990; Moss and Cannon, 2011; Rick and Erlandson, 2000). Moreover, isotopic analyses are being applied to archaeological fish remains with increasing regularity, both to address the need for baseline isotopic data in the interpretation of human diet (e.g. Richards and Hedges, 1999; van der Merwe et al., 2003), and in their own right to address a wide variety of archaeological and paleoecological questions (e.g. Barrett et al., 2008, 2011; Ishimaru et al., 2011; Misarti et al., 2009; Szpak et al., 2009). The impact of cultural activities (e.g. fish processing methods) on the distribution of fish remains in archaeological sites has been examined in some detail (e.g. Barrett et al., 1999; Butler, 1996; Zohar et al., 2008, 2001). The differences between fish and mammalian bone from archaeological contexts have been discussed at length in the literature, although usually as they relate to the physical properties of the bones (size, shape and density) (e.g. Butler and Chatters, 1994; Nicholson, 1992; Rojo, 1987; Wigen and Stucki, 1988) and recovery methods employed during excavation (e.g. Nagaoka, 2005; Partlow, 2006; Reitz, 1988; Stewart and Wigen, 2003; Vale and Gargett, 2002; Zohar and Belmaker, 2005). Conversely, the influences of the chemical and ultrastructural properties of fish bone on determining their survival in the archaeological record have not been examined in detail. In some cases, there is an apparent difference in the recovery of fish bones from archaeological sites in comparison with mammals, with the former being under-represented, or presumed to be under-represented *a priori* (e.g. Henshaw, 1995; Howse, 2008; Wheeler, 1977; Wheeler and Jones, 1989). In a comparative degradation experiment, Nicholson (1996a) found that fish tended to be more poorly preserved than mammals or birds of comparable size, but

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was unable to offer an explanation for this pattern. Conversely, Jarman et al. (1982: 5) point out that the assumption that fish bones are comparatively vulnerable to degradation is not empirically supported. The purposes of this paper are to: (1) review the chemistry of fish bone in general, and particularly as it relates to (2) the preservation of fish remains at archaeological sites, and (3) the criteria utilized for verifying the integrity of isotopic data derived from bone collagen, which is discussed in more detail below.

Adequate criteria for the inclusion or exclusion of isotopic data from archaeological bone collagen are essential. To date, a number of studies have attempted to define and refine such criteria (Ambrose, 1990; DeNiro, 1985; DeNiro and Weiner, 1988; Dobberstein et al., 2009; Grupe and Turban-Just, 1998; Grupe et al., 2000; Harbeck and Grupe, 2009; Schoeninger et al., 1989; Tuross et al., 1988, 1989; van Klinken, 1999), but all have focused exclusively on mammalian bone. One notable exception is the work of Nehlich and Richards (2009), who suggested that because of differences in the amino acid composition of collagen, the quality criteria for sulfur isotopic analysis (C:S ratio) should be different for fish and mammalian bone. The most commonly utilized preservation criteria for bone collagen in stable isotope analysis are: atomic C:N ratio, collagen yield and minimum percentages of carbon and nitrogen. All of these measurements can be obtained in the process of conventional isotope ratio mass spectrometry, without additional effort or cost. Some authors have discussed the analysis of the amino acid composition of collagen (e.g. Bocherens et al., 1994; Stafford et al., 1991), but these analyses come with significant additional costs. The C:N ratio was initially discussed as a useful marker of collagen preservation by DeNiro (1985), who found that archaeological specimens with bone collagen C:N ratios outside the range of 2.9–3.6 were characterized by isotopic compositions that were inconsistent with their ecology. The C:N ratio range of 2.9–3.6 remains the most commonly used measure to assess collagen preservation, and in many cases is the *only* criteria for preservation that is reported. In a more comprehensive study of a large number of African mammals, Ambrose (1990) found that in addition to C:N ratio, minimum concentrations of carbon (13%) and nitrogen (4.8%) in the extracted collagen were also robust indicators of collagen preservation. In addition, Ambrose (1990) found that the amount of collagen extracted from the bone (collagen yield) was also informative with respect to collagen preservation, with those samples yielding less than 3.5% collagen being more poorly preserved. As discussed by van Klinken (1999), however, the relationship between collagen yield and collagen ‘intactness’ is likely context specific, and suggested a minimum acceptable yield for temperate climates to be set at 1%.

Generally, fish tend to exhibit more variability in the chemical composition of their bone than mammals (Karim and Bhat, 2009; Moss, 1963). Furthermore, on the basis of a wide body data produced by the food chemistry industry, it is well established that fish collagen possesses different characteristics than mammalian collagen (Avena-Bustillos et al., 2006; Gudmundsson, 2002; Haug et al., 2004). As a result of this, there are questions with respect to the application of traditional collagen preservation criteria (e.g. C:N ratio) to fish, since these are based entirely on the chemical composition of mammalian bone. For example, Grupe et al. (2009) suggested that fish with bone collagen C:N ratios outside of the typically accepted range (2.9–3.6), particularly those >3.6, should not necessarily be excluded on this basis. It is therefore imperative to establish whether or not such criteria can be applied to fish.

2. The chemistry and ultrastructure of bone

This section briefly outlines some general characteristics of bone chemistry and ultrastructure. For a more comprehensive overview, the reader is referred to a number of excellent reviews on the subject (Bourne, 1972; Cowin and Doty, 2007; Currey, 2003, 2008; Glimcher, 1984, 2006; Lees, 1989a; Pasteris et al., 2008; Veis, 1984; Weiner and Wagner, 1998).

Bone is a composite material composed of organic (primarily collagen) and inorganic (bioapatite, $Ca_{10}(PO_4)_3(OH)_2$) components, as well as lipids and water. Type I collagen constitutes approximately 20% of bone by mass and 35% by volume (Pasteris et al., 2008), and >90% of the organic matrix of bone (Veis, 1984). There are many other types of collagen, although none are present in significant quantities in calcified tissues, with the exception of Type II collagen, which is the primary structural protein in the skeletons of elasmobranchs (sharks, skates and rays). The main difference between Type I collagen in different tissues (e.g. skin and bone) relates to the arrangement and cross-linking of the collagen fibers, rather than the chemical composition of the fibers themselves (Bailey et al., 1998; Hanson and Eyre, 1996).

Collagen is a triple helical molecule composed of three α chains, which in mammals, consist of two identical $\alpha1$ helices and one $\alpha2$ helix ($(\alpha1)_2\alpha2$ heterotrimer). The existence of $\alpha1\alpha2\alpha3$ heterotrimers, $(\alpha1)_2\alpha2$ heterotrimers as well as an $(\alpha1)_3$ homotrimer has been recorded in several different species of fish (Kimura et al., 1991; Nagai and Suzuki, 2000). The functional difference between these configurations has not been studied extensively and remains poorly understood. There are some differences in the amino acid composition of $\alpha1$, $\alpha2$ and $\alpha3$ chains, but they are minor (see Eastoe, 1967). Glycine comprises about 1/3 of the amino acid residues in all collagens, with the two dominant motifs being Gly-Pro-X and Gly-X-Hyp, where X is any amino acid other than glycine, proline or hydroxyproline. The high hydroxyproline content of collagen is unusual among proteins and is directly related to the stability and insolubility of molecule, which is discussed in more detail below (Section 4.3). The presence of glycine, which is a particularly small amino acid with only a single hydrogen atom side chain, at every third residue facilitates the triple helical structure of collagen and allows hydrogen bonding between chains within the helix (Regenstein and Zhou, 2007). Collagen peptides are also held together by intermolecular cross-links, which can form through an enzymatically-mediated reaction involving aldehydes and lysine or hydroxylysine. Additionally, over time, cross-links are formed non-enzymatically from reactions involving reducing sugars and lipid oxidation products (Danielsen et al., 1986; Eyre and Wu, 2005; Oxlund et al., 1995, 1996).

Compared to collagen, the chemical and mechanical properties of the mineral phase of bone (bioapatite) are not well understood. Bioapatite serves primarily two functions in mineralized tissues, one is largely mechanical (adding stiffness and strength to the structure) and the other is physiological (serving as an easily accessible ion reservoir) (Glimcher, 1998). Bioapatite (also referred to in the literature as hydroxyapatite, hydroxyl apatite, carbonated apatite or dahlite) is a unique mineral phase, characterized by very small ($\sim 50 \times 25 \times 5$ nm) crystals, low OH^- content and poor crystallinity, all of which have functional significance in bone remodeling (see Glimcher, 2006; Noto, 2011; Rubin et al., 2004; Weiner and Wagner, 1998). The size and the ordering of bioapatite crystals in fish and mammalian bone are generally very similar (Jackson et al., 1978; Kim et al., 1995), but it should be pointed out that there is variability in bioapatite crystal size between tissues and taxonomic groups, and much additional work is needed to better understand the full range of variation (Fratzl et al., 2004; Olszta et al., 2007). There has been some debate in the literature regarding the shape of the bioapatite crystals, with some suggesting a needle-like shape (Fratzl et al., 1992, 2004), but the majority of others suggesting a plate-like shape (Burger et al., 2008; Kim et al., 1995; Rubin et al., 2004; Weiner and Price,

1986). Many minor and trace elements occur in bioapatite (Wopenka and Pasteris, 2005), the most notable of which is the substitution of carbonate, commonly for phosphate (B-type substitution) and less commonly hydroxyl (A-type substitution); this substitution is fairly common and as a result carbonate constitutes 5–6% of the molecule by weight (Elliott, 2002; Pasteris et al., 2004).

Mineralized vertebrate bone tends to contain approximately 66% bioapatite by weight and 50% by volume with relatively little variability. Fish differ from other vertebrates in this respect, however, and their bones may contain appreciably less mineral (Biltz and Pellegrino, 1969); this is reflected in lower bone densities in some species of fish (e.g. Butler and Chatters, 1994; Smith, 2008). The relative proportions of organic and inorganic components in the bone, as well as the interactions between these components are largely responsible for the material properties of bone (strength, toughness, stiffness) (Pasteris et al., 2008). The maintenance of collagen concentrations in aging human bone contributes to the resistance of bones to fractures and porosity (Norman et al., 1996; Zioupos et al., 1999), while the degree of mineralization of bone collagen imparts stiffness and strength to the bone (Wang et al., 2000). The relationship between the mineral and organic phase of bone is not well understood, both at the chemical and mechanical levels (Katti et al., 2010). It has been suggested that the majority of the bioapatite crystals in bone occur within collagen fibrils (Jäger and Fratzl, 2000; Weiner and Traub, 1986; Weiner et al., 1999), while others (Fritsch and Hellmich, 2007; Hellmich and Ulm, 2002; Pidaparti et al., 1996; Sasaki et al., 2002) suggest that the mineral occurs primarily outside of the fibril. A number of adsorption forces (hydrogen bonding, van der Waals' interaction, hydrophobic interactions) confer strength upon the collagen–mineral interaction (Walsh et al., 1994). Additionally, it has been suggested that collagen and bioapatite are strongly bound to each other through the sharing of OH-groups, which are plentiful in collagen (especially hydroxyproline) and lacking in bioapatite (Pasteris et al., 2004).

3. Materials and methods

Amino acid compositions of primarily modern collagens were surveyed from existing literature. The number of taxa and specimens for which data were obtained are presented in Table 1 according to class. Habit preferences of bony fish (Actinopterygii) and cartilaginous fish (Elasmobranchii) were compiled using the online database FishBase (Froese and Pauly, 2011), and were classified into one of the following categories: tropical, sub-tropical, temperate or polar. Sub-tropical and tropical fish were in turn grouped together as warm-water fish, and temperate and polar fish were grouped as cold-water fish. Even though bone is the primary focus of this paper, skin collagen amino acid compositions were also surveyed because this allowed for a much more in depth examination of the chemical variability of collagen. As discussed previously, the amino acid composition of bone and skin collagen are very similar, and it is therefore reasonable to compare the two. All amino acid compositions are presented as number of residues per 1000 residues. One-way analysis of variance (ANOVA) was used to assess differences in the calculated C:N ratios, elemental concentrations and relative proportions of amino acids between taxonomic groups.

Table 1
Number of species included in the analysis of amino acid composition by class.

Class	Bone		Skin	
	Species	Analyses	Species	Analyses
Mammalia	10	23	11	33
Actinopterygii	9	18	65	94
Elasmobranchii	2	2	11	17
Aves	2	5	1	2
Reptilia	1	1	2	2
Amphibia	0	0	5	7
Agnatha	0	0	2	5
Sarcopterygii	0	0	2	3

To assess the chemical composition of archaeological fish bone collagen, a survey was conducted of literature containing isotopic data with at least associated C:N ratios and preferably relative percentages of carbon and nitrogen and collagen yields. Where these studies included data from mammalian bone collagen, these were included in the analysis. Studies with only mammalian bone collagen data were not included as this would even more heavily skew the sample size towards mammals. Only bony fish were included in the analysis. In all, data were obtained for 1836 mammals and 362 fish from 23 studies. Fish were generally underrepresented because these taxa are analyzed with much less frequency than mammals in archaeological and paleontological contexts. Where sample size permitted, comparisons were made of bone collagen C:N ratios between taxa, within the same archaeological context. Studies with at least five samples analyzed for fish and mammals were included, those with less than five were excluded from the within-study comparison, but included in the overall comparisons between fish and mammals. One-way ANOVA was used to determine differences in the collagen preservation criteria between studies and taxonomic groups. For the sake of simplicity, the term 'fish' is reserved only for members of Actinopterygii throughout the remainder of the paper.

4. Results and discussion

4.1. Amino acid and elemental composition of collagen

It is first necessary to address the comparability of methods utilized to extract collagen from archaeological bone and methods used in the food chemistry industry. The majority of bone collagen that is extracted in archeology and related disciplines typically utilizes weak hydrochloric acid (0.25–1.0 M) to demineralize the collagen (following Longin, 1971); EDTA is also used for this purpose (e.g. Tuross et al., 1988), but with much less frequency. Demineralization is sometimes preceded by a solvent treatment (typically a chloroform/methanol mixture) to remove lipids, and followed by a treatment with NaOH to remove soil (e.g. humic) contaminants. The demineralized collagen is then heated at 57–105 °C in acidic solution, leaving a water-soluble residue, which is then dried and analyzed by mass spectrometry. In some protocols, the water-soluble residue is passed through a 30 kDa filter to retain only large collagen fragments (Brown et al., 1988), a process that is believed to remove lipids and other low molecular weight contaminants.

In food chemistry, collagen is typically extracted with either an acid (usually acetic acid) or an enzyme (pepsin) at low temperature (4 °C). The enzymatic digestion typically utilizes material that was not extracted during a previous acid extraction (e.g. Kittiphattanabawon et al., 2010a; Singh et al., 2011). NaOH is often used in pretreatments to remove non-collagenous proteins and other base-soluble materials. Lipids are removed with a solvent, which in most cases is butyl alcohol (Nagai and Suzuki, 2000; Nagai et al., 2002). Although the extraction processes differ in archeology and food chemistry, in both cases the end product is the same and is referred to as collagen or gelatin.

Although the majority of amino acid compositions included in this analysis are from acid-soluble collagen, pepsin-soluble

collagen amino acid compositions were also included. To ensure that the amino acid composition or C:N ratio did not differ significantly between the collagen extraction methods, paired acid and pepsin-soluble amino acid compositions were compared for 13 species (data from Heu et al., 2010; Kittiphattanabawon et al., 2010a,b; Liu et al., 2007; Nalinanon et al., 2010; Noitup et al., 2005; Ogawa et al., 2003, 2004; Singh et al., 2011; Sivakumar et al., 2000; Wood et al., 2008). The C:N ratios of the two extraction methods were nearly identical, with the mean acid-soluble collagen C:N ratio (3.17 ± 0.05, $n = 13$) being slightly higher than the mean pepsin-soluble collagen C:N ratio (3.16 ± 0.05, $n = 13$); this difference was not statistically significant ($F_{[1,24]} = 0.3$, $p = 0.57$).

The mean amino acid composition, relative percentages of carbon and nitrogen, and C:N ratios of bone and skin collagen for Mammalia, Actinopterygii and Elasmobranchii are presented in Table 2. A complete list of amino acid compositions, elemental concentrations (%C, %N, %H, %O, %S) and C:N ratios are presented in the Supplementary Material (Table S1). Data were also obtained for other taxa (Aves, Reptilia, Amphibia, Sarcopterygii, Cephalaspidomorphi, Myxini), but due to small sample sizes and/or the fact that these groups tend to be poorly represented in archaeological contexts, these data are presented only in the Supplementary Material (Table S1). The vast majority of the data are for modern animals, but data that are from archaeological contexts are denoted as such in Table S1.

On the basis of amino acid compositions, the C:N ratios of all bone and skin collagens fell well within the typically cited range of 2.9–3.6 for unaltered bone collagen (Fig. 1; Table 2). Two Antarctic icefish (*Chionodraco hamatus* and *Racovitzia glacialis*) were characterized by unusually high skin collagen C:N ratios of 3.58 and 3.56, respectively (Rigby, 1968); these data are, however, considered to be anomalous (see below for discussion). Within taxonomic groups, the C:N ratios of bone vs. skin collagen were not significantly different ($F_{[1,104]} = 0.03$; $p = 0.86$ for fish, $F_{[1,55]} = 0.4$; $p = 0.51$ for mammals). This supports the previous assertion that the chemical composition of bone and skin collagen is very similar and generally comparable. The C:N ratios of fish collagen were significantly lower than those of mammalian collagen ($F_{[1,33]} = 30.5$, $p < 0.001$ for bone; $F_{[1,126]} = 36.0$, $p < 0.001$ for skin); fish collagen had significantly lower %C ($F_{[1,33]} = 27.0$, $p < 0.001$ for bone; $F_{[1,126]} = 36.9$, $p < 0.001$ for skin) and higher %N ($F_{[1,33]} = 20.1$, $p < 0.001$ for bone; $F_{[1,126]} = 23.9$, $p < 0.001$ for skin) relative to mammalian collagen (Table 2).

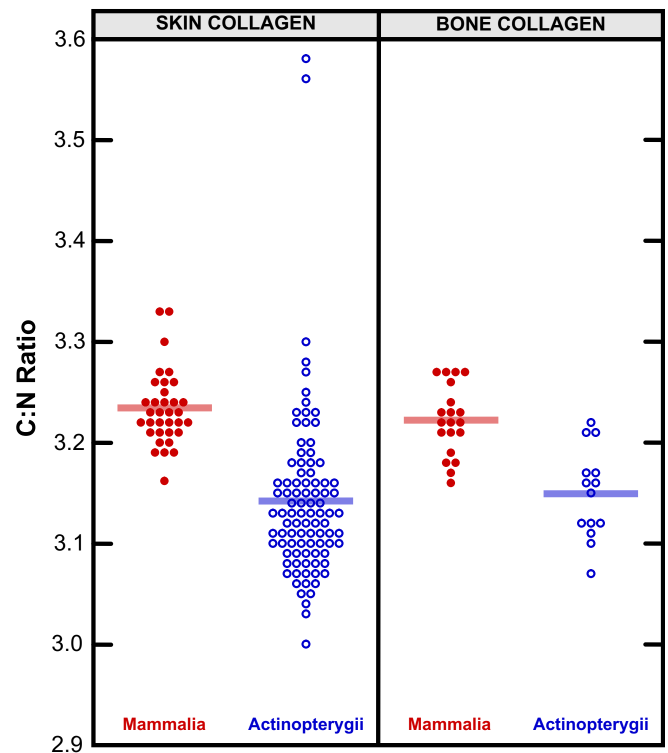

Fig. 1. Calculated C:N ratios based on amino acid compositions for Actinopterygii and Mammalia. Horizontal bars represent the mean value for each class. A complete list of the data used is presented in Table S1.

While the variability in fish collagen C:N ratio is greater than in mammals (Fig. 1), none fell outside, or approached the outer limits of DeNiro's (1985) range of 2.9–3.6. The exception to this are the two icefish species mentioned previously, both of which are highly

Table 2
Mean amino acid compositions and elemental data for mammals, bony fish (Actinopterygii) and cartilaginous fish (Elasmobranchii).

	Bone			Skin		
	Mammalia ($n = 23$)	Actinopterygii ($n = 12$)	Elasmobranchii ($n = 2$)	Mammalia ($n = 34$)	Actinopterygii ($n = 92$)	Elasmobranchii ($n = 17$)
Asp	48 ± 3	46 ± 6	42 ± 9	47 ± 3	47 ± 8	45 ± 10
Hyp	95 ± 8	72 ± 13	77 ± 4	95 ± 7	67 ± 14	74 ± 13
Thr	19 ± 2	26 ± 3	30 ± 11	19 ± 4	26 ± 5	24 ± 3
Ser	35 ± 4	42 ± 14	44 ± 2	36 ± 5	46 ± 12	46 ± 11
Glu	75 ± 4	73 ± 5	79 ± 1	74 ± 3	76 ± 11	77 ± 13
Pro	119 ± 7	105 ± 7	97 ± 20	126 ± 6	108 ± 11	109 ± 6
Gly	330 ± 11	343 ± 11	339 ± 21	329 ± 18	339 ± 26	332 ± 13
Ala	115 ± 9	119 ± 11	117 ± 2	109 ± 8	114 ± 17	114 ± 9
Val	23 ± 2	18 ± 3	23 ± 4	22 ± 3	21 ± 6	24 ± 3
Met	4 ± 2	13 ± 2	11 ± 1	6 ± 2	13 ± 4	13 ± 3
Ile	11 ± 2	10 ± 3	15 ± 5	11 ± 2	11 ± 5	17 ± 4
Leu	26 ± 2	22 ± 3	21 ± 1	24 ± 3	23 ± 7	23 ± 6
Tyr	3 ± 2	3 ± 1	3 ± 1	3 ± 2	3 ± 2	3 ± 2
Phe	14 ± 1	14 ± 4	14 ± 3	13 ± 2	14 ± 4	12 ± 2
Hyl	6 ± 3	9 ± 4	7 ± 1	6 ± 1	8 ± 9	6 ± 1
Lys	26 ± 3	27 ± 3	27 ± 1	29 ± 4	26 ± 5	25 ± 3
His	5 ± 1	7 ± 2	7 ± 2	5 ± 3	7 ± 4	8 ± 2
Arg	49 ± 4	50 ± 3	50 ± 0	49 ± 3	52 ± 6	50 ± 5
C:N ratio	3.23 ± 0.04 (3.16–3.32)	3.15 ± 0.04 (3.07–3.22)	3.17 ± 0.06 (3.12–3.21)	3.23 ± 0.04 (3.16–3.33)	3.14 ± 0.09 (3.00–3.58)	3.18 ± 0.06 (3.08–3.28)
%C	42.2 ± 0.2 (41.2–42.6)	41.7 ± 0.3 (41.2–42.2)	41.8 ± 0.4 (41.5–42.0)	42.3 ± 0.3 (41.8–43.2)	41.7 ± 0.5 (40.8–42.9)	41.9 ± 0.3 (40.8–42.3)
%N	15.3 ± 0.1 (15.0–15.5)	15.5 ± 0.1 (15.3–15.7)	15.4 ± 0.2 (15.3–15.5)	15.3 ± 0.1 (14.9–15.4)	15.5 ± 0.3 (14.8–16.1)	15.4 ± 0.3 (14.7–15.8)

adapted to polar waters, and are characterized by some unique physiological adaptations (see Bilyk and DeVries, 2010; DeVries, 1988). When assessing bone collagen C:N ratios, these anomalous values should be taken into account in instances where Antarctic icefish may be present (e.g. the extreme south of Australia, New Zealand and possibly South America). The geographic distribution of these fish is, however, rather limited (Eastman, 2005) and they are not likely to be encountered in archaeological deposits (but see Bassett, 2004; Colley and Jones, 1987). These unusual amino acid compositions are not typical of polar fish in general, as many other species that live in these conditions were surveyed (see Table S1) and none produced similarly high C:N ratios. Therefore, it should not be expected that archaeological fish bone collagen from cold climates (e.g. the Arctic) would necessarily be expected to possess unusual C:N ratios.

Compared to mammalian collagen, fish collagen is, in general, characterized by relatively high percentages of serine and glycine, and low percentages of hydroxyproline and proline (Table 2). These differences, however, result in lower, rather than higher, C:N ratios in fish compared to mammalian collagen because the C:N ratios of proline (5:1) and hydroxyproline (5:1) are higher than glycine (2:1) and serine (3:1). This is reflected in a very small, but statistically significant difference between fish and mammalian collagen C:N ratios. Overall, the ranges for both collagen carbon and nitrogen content were very small ($\pm$<2% for all tissues and all taxa). There is, therefore, no evidence that the variability in fish collagen amino acid composition results in atypical C:N ratios that deviate from what would be expected for mammalian bone collagen.

Within fish, there is an important distinction in amino acid composition between species living in cold (polar and temperate) and warm (tropical and sub-tropical) waters (Table 3); this applies both to bony and cartilaginous fish (Regenstein and Zhou, 2007). Most importantly, hydroxyproline content is much higher in the collagen of warm-water compared to cold-water fish. Warm- and cold-water fish collagen, however, are not characterized by markedly different C:N ratios, or relative percentages of carbon and nitrogen. Comparatively, mammals possess much higher quantities of hydroxyproline in their collagen (Table 2). These differences are

Table 3
Mean amino acid compositions and elemental data for warm- and cold-water fish.

	Cold-water fish ($n = 54$)	Warm-water fish ($n = 66$)	p[a]
Asp	50 ± 9	44 ± 7	**<0.001**
Hyp	59 ± 12	76 ± 10	**<0.001**
Thr	25 ± 4	26 ± 5	**0.03**
Ser	55 ± 12	38 ± 6	**<0.001**
Glu	77 ± 13	75 ± 8	0.35
Pro	105 ± 10	110 ± 10	**0.005**
Gly	340 ± 25	337 ± 22	0.50
Ala	109 ± 11	119 ± 18	**<0.001**
Val	21 ± 6	21 ± 4	0.38
Met	14 ± 3	13 ± 4	0.12
Ile	12 ± 5	11 ± 5	0.18
Leu	24 ± 9	22 ± 5	0.16
Tyr	3 ± 1	3 ± 2	0.67
Phe	14 ± 4	13 ± 3	0.10
Hyl	6 ± 3	7 ± 3	0.10
Lys	27 ± 4	26 ± 5	0.54
His	9 ± 3	6 ± 3	**<0.001**
Arg	52 ± 5	51 ± 5	0.49
C:N ratio	3.13 ± 0.1 (3.00–3.58)[b]	3.16 ± 0.05 (3.07–3.30)[b]	0.69
%C	41.6 ± 0.5 (40.8–43.8)[b]	41.86 ± 0.36 (41.2–42.9)[b]	0.81
%N	15.5 ± 0.3 (14.2–16.1)[b]	15.44 ± 0.22 (14.8–16.0)[b]	0.43

Statistically significant values ($p < 0.05$) are shown in boldface.
[a] p values for ANOVA comparisons between warm- and cold-water fish collagen amino acid compositions.
[b] Ranges are given in parentheses for C:N ratio, %C and %N.

important with respect to collagen stability, which is discussed in detail below (Section 4.2).

The endoskeleton of elasmobranchs is structurally distinct in comparison to bony fishes. Generally, elasmobranch skeletons have not been extensively studied, but exhibit considerable variability with respect to morphology, both within and between species (Dean and Summers, 2006). The skeleton consists mostly of cartilaginous tissue, which preserves poorly in most archaeological contexts. Several skeletal elements (vertebral centra, teeth, spines and dermal denticles) are, however, characterized by significant calcification and are more similar to mammalian trabecular bone with respect to mineral and collagen contents (Porter et al., 2006); these elements are recovered in archaeofaunal assemblages with some regularity (Rick et al., 2002).

The collagen in elasmobranch skeletons appears to differ from other vertebrates in that it is composed mostly of type II collagen ($\sim$66%), with type I collagen constituting most of the remaining organic fraction (Rama and Chandrakasan, 1984). The most salient difference between types I and II collagen is that the former is typically composed of two $\alpha 1$ and one $\alpha 2$ chains, while the latter is composed of three $\alpha 1$ chains (Seyer et al., 1989). In addition, the $\alpha 1$ chains of type II collagen contain a higher number of hydroxylysine residues than type I collagen ($\sim$20 more residues/1000), which has important functional implications with respect to cross-linking (Miller, 1971), but does not significantly impact the overall C:N ratio of the collagen. The C:N ratio of elasmobranch skin collagen was slightly higher than bony fish skin collagen (Table 2), although this difference was not quite statistically significant ($F_{[1,109]} = 3.8$, $p = 0.05$). Conversely, skin collagen C:N ratio for elasmobranches was significantly lower than for mammals ($F_{[1,49]} = 13.7$, $p < 0.001$).

On the basis of the amino acid composition of bone and skin collagen, the typical range of acceptable C:N ratios is applicable to both bony and cartilaginous fish bone and should not be extended. This is particularly true for the upper limit of this range (>3.6) since collagen C:N ratios are actually slightly, though significantly, lower for fish than for mammals. This measure alone is, however, likely insufficient to adequately assess the quality of archaeological collagen and should be combined with other measures: minimum percentages of carbon and nitrogen and collagen yield (Ambrose, 1990; van Klinken, 1999). Very small ranges of both %C and %N were present in the collagen of fish and mammals, with a range of <2% for both groups. While both %C and %N were significantly different between fish and mammals, the difference between the means was <1% for both elements. On this basis, the minimum concentrations of carbon (13%) and nitrogen (4.5%) as discussed by Ambrose (1990) apply equally well to both fish and mammals. It is not possible to assess any potential differences in collagen yield between fish and mammalian bone on the basis of amino acid compositions. It is important to point out, however, that while there is no reason to alter collagen quality criteria on the basis of amino acid compositions, this does not imply that taphonomic processes affect mammalian and fish bone collagen (and bones in general) equally. As such, additional controlled studies on the influence of various diagenetic processes (e.g. Dobberstein et al., 2008, 2009; Harbeck and Grupe, 2009) on the bones of mammals, fish and birds would be extremely beneficial.

4.2. Archaeological bone collagen C:N ratios

The mean ($\pm 1\sigma$) archaeological bone collagen C:N ratios, collagen yields and relative percentages of carbon and nitrogen for mammals and fish are presented according to study in Table 4. A complete list of all samples surveyed (including other taxonomic groups: Amphibia, Reptilia, Aves) is included in the Supplementary Material (Table S2). Overall, archaeological fish bone collagen C:N

Table 4
Archaeological bone collagen yields, C:N ratios and elemental data by study.

References	Lipid extraction[a]	Mammalia							Actinopterygii						
		C:N ratio		Collagen yield		%C		%N	C:N ratio		Collagen yield		%C		%N
		n	Mean ± 1*σ*	*n*	Mean ± 1*σ*	*n*	Mean ± 1*σ*	Mean ± 1*σ*	*n*	Mean ± 1*σ*	*n*	Mean ± 1*σ*	*n*	Mean ± 1*σ*	Mean ± 1*σ*
Borić et al. (2004)	n.s.	124	3.28 ± 0.34	127	1.9 ± 1.1	124	40.1 ± 8.1	14.3 ± 2.7	14	3.49 ± 0.34	14	1.2 ± 1.1	14	34.7 ± 9.6	11.7 ± 3.3
Bösl et al. (2006)	n.s.	58	3.27 ± 0.30	n.d.		58	42.3 ± 5.6	15.1 ± 1.5	8	3.10 ± 0.38	n.d.		8	37.8 ± 7.4	14.2 ± 2.2
Choy and Richards (2009)	UF	88	3.26 ± 0.10	n.d.		88	40.0 ± 4.0	14.3 ± 1.6	2	3.25 ± 0.13	n.d.		2	42.4 ± 1.7	15.3 ± 1.2
Craig et al. (2009)	UF	140	3.20 ± 0.08	142	6.1 ± 3.4	n.d.		n.d	4	3.50 ± 0.22	4	1.3 ± 0.8	n.d.		n.d.
Drucker and Bocherens (2004)	n.s.	3	3.21 ± 0.05	3	10.1 ± 6.1	3	41.9 ± 0.7	15.2 ± 0.2	7	3.05 ± 0.10	6	0.6 ± 0.4	7	27.6 ± 12.7	10.5 ± 4.7
Eriksson (2004)	UF	55	3.29 ± 0.09	55	3.4 ± 1.8	55	40.5 ± 3.6	14.4 ± 1.3	7	3.37 ± 0.09	8	1.4 ± 0.7	7	36.0 ± 3.7	12.5 ± 1.2
Eriksson et al. (2008)	UF	94	3.15 ± 0.13	100	3.6 ± 2.0	94	38.8 ± 3.6	14.4 ± 1.4	15	3.20 ± 0.10	15	2.4 ± 1.6	15	37.6 ± 3.2	13.7 ± 1.2
Fischer et al. (2007)	UF	114	3.32 ± 0.26	93	10.9 ± 6.0	107	39.3 ± 5.4	13.9 ± 2.1	60	3.63 ± 0.86	58	4.1 ± 3.1	59	35.4 ± 8.6	11.8 ± 3.1
Fornander et al. (2008)	UF	57	3.35 ± 0.19	57	3.3 ± 2.5	57	42.0 ± 3.1	14.7 ± 1.1	6	3.32 ± 0.20	6	2.3 ± 0.8	6	40.0 ± 5.3	14.1 ± 2.2
Grupe et al. (2003)	n.s.	66	3.25 ± 0.33	66	2.1 ± 1.1	66	40.8 ± 6.5	14.7 ± 2.1	9	3.35 ± 0.32	9	1.4 ± 1.2	9	37.3 ± 10.6	13.0 ± 3.2
Grupe et al. (2009)	n.s.	29	3.57 ± 0.35	29	6.6 ± 2.5	29	44.8 ± 5.1	14.9 ± 1.2	53	3.74 ± 0.41	56	1.9 ± 1.9	53	42.2 ± 8.6	13.3 ± 2.8
Jay (2008)	UF	37	3.30 ± 0.10	37	7.2 ± 3.1	37	43.7 ± 1.5	15.5 ± 0.9	12	3.38 ± 0.06	12	2.7 ± 1.4	12	42.2 ± 1.2	14.6 ± 0.5
Jones and Quinn (2009)	n.s.	19	3.14 ± 0.20	n.d.		n.d.		n.d.	4	3.18 ± 0.32	n.d.		n.d.		n.d.
Jørkov et al. (2010)	UF	94	3.23 ± 0.17	94	6.2 ± 3.5	94	42.8 ± 1.7	15.5 ± 1.0	10	3.23 ± 0.07	10	5.3 ± 1.2	10	41.4 ± 1.8	15.0 ± 0.7
King (2006)	None	259	3.34 ± 0.75	268	5.0 ± 5.5	259	19.5 ± 15.4	7.0 ± 5.5	20	4.63 ± 1.25	24	1.6 ± 0.8	20	3.5 ± 6.5	1.2 ± 2.4
Kusaka et al. (2010)	n.s.	107	3.32 ± 0.10	107	2.6 ± 1.5	n.d.		n.d.	8	3.35 ± 0.08	8	3.9 ± 1.2	n.d.		n.d.
Lillie et al. (2011)	n.s.	48	3.26 ± 0.11	n.d.		n.d.		n.d.	4	3.35 ± 0.06	n.d.		n.d.		n.d.
Müldner and Richards (2005)	UF	100	3.33 ± 0.12	100	6.1 ± 3.3	n.d.		n.d.	28	3.31 ± 0.10	28	6.3 ± 2.3	n.d.		n.d.
Müldner and Richards (2007)	UF	239	3.28 ± 0.09	239	5.2 ± 3.1	239	43.5 ± 2.3	15.4 ± 1.0	31	3.42 ± 0.14	31	1.9 ± 1.0	31	42.9 ± 4.3	14.7 ± 1.7
Naito et al. (2010)	n.s.	17	3.21 ± 0.08	17	10.8 ± 5.1	17	43.5 ± 3.0	15.8 ± 1.1	2	3.34 ± 0.01	2	1.6 ± 0.2	2	43.0 ± 1.8	15.0 ± 0.6
Nehlich et al. (2010)	UF	29	3.31 ± 0.07	n.d.		29	43.6 ± 3.1	15.4 ± 1.0	3	3.25 ± 0.07	n.d.		3	40.0 ± 3.2	14.4 ± 1.4
Reitsema et al. (2010)	n.s.	28	3.20 ± 0.01	n.d.		28	39.6 ± 1.5	14.4 ± 0.5	4	3.29 ± 0.04	n.d.		4	38.9 ± 0.6	13.8 ± 0.4
Szpak et al. (2009)	n.s.	31	3.20 ± 0.06	31	10.3 ± 3.3	31	33.8 ± 1.8	12.3 ± 0.7	51	3.31 ± 0.15	51	8.4 ± 3.3	51	36.4 ± 3.3	12.8 ± 1.2
All studies		1836	3.28 ± 0.33	1564	5.13 ± 4.28	1415	37.30 ± 11.58	13.31 ± 11.58	362	3.51 ± 0.59	339	3.70 ± 3.27	313	36.22 ± 11.34	12.37 ± 3.88

[a] UF = ultrafiltration following Brown et al. (1988), n.s. = not specified.

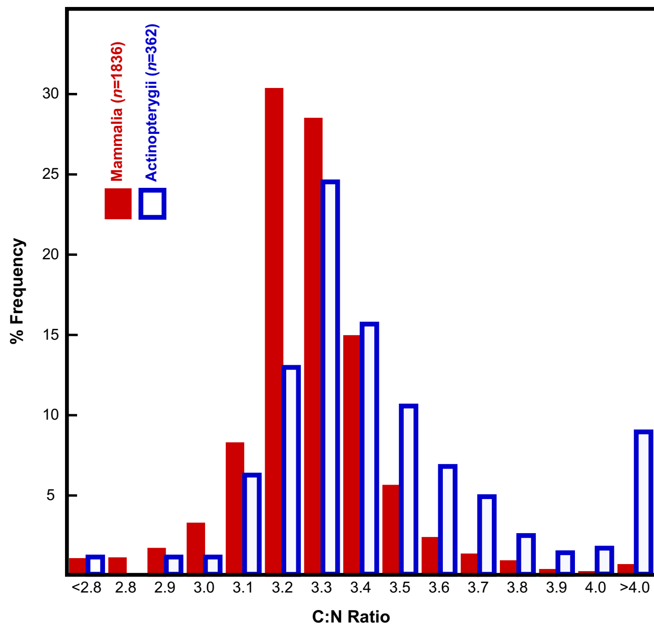

Fig. 2. Archaeological bone collagen C:N ratios for Mammalia and Actinopterygii. A complete list of the data used is presented in Table S2.

ratios (3.51 ± 0.59, $n = 362$) were higher than mammalian bone collagen C:N ratios (3.28 ± 0.33, $n = 1836$) (Fig. 2); this difference was statistically significant ($F_{[1,2196]} = 100.8$, $p < 0.001$).

The use of a 30 kDa ultrafiltration step in collagen extraction reduces the amount of collagen recovered and retains only fragments with large molecular weights (Brown et al., 1988), therefore, these data were treated separately to better control for any potential methodological effects on the relationship between collagen yield and C:N ratio (Table 5). Contrary to what might be expected, however, collagen yield was not significantly different in the filtered and unfiltered samples when collagen C:N ratios were within the range of 2.9–3.6 ($F_{[1,1772]} = 2.9$, $p = 0.09$). Compared to unfiltered collagen, the use of an ultrafiltration step produced collagen with significantly higher %C ($F_{[1,1930]} = 261.9$, $p < 0.001$) and %N ($F_{[1,1930]} = 324.7$, $p < 0.001$); these values were closer to the expected values based on the amino acid composition of modern collagen (Table 2).

For the ultrafiltered collagen, %C was significantly higher for fish compared to mammals ($F_{[1,900]} = 40.0$, $p < 0.001$), while %N was significantly lower ($F_{[1,900]} = 57.0$, $p < 0.001$), as was collagen yield ($F_{[1,1028]} = 29.6$, $p < 0.001$). The pattern observed in the ultrafiltered data for %C and %N is the opposite of what would be expected based on the amino acid composition of modern fish and mammalian collagen (Table 2). Conversely, there were no significant differences in the %C ($F_{[1,597]} = 3.2$, $p = 0.08$), %N ($F_{[1,597]} = 1.9$, $p = 0.17$) or collagen yield ($F_{[1,623]} = 0.9$, $p = 0.36$) between unfiltered fish and mammalian collagen. This suggests that comparisons between elemental data, but not necessarily isotopic data, from collagen extracted with and without an ultrafiltration step may be problematic. Nevertheless, for both ultrafiltered and unfiltered collagen, C:N ratio was significantly greater in fish than in mammals ($p < 0.001$ for both). Moreover, in 16 of the 23 archaeological studies surveyed, fish bone collagen C:N ratios were higher than their mammalian counterparts (Table 3), although many of these studies presented data for very small numbers of fish. Fifteen studies presented sufficiently large numbers of fish and mammals for intra-study comparison, and in seven of these, fish bone collagen C:N ratios were significantly higher than their mammalian counterparts (summarized in Table 6). No studies presented mammalian bone collagen C:N ratios that were significantly higher than fish, which does not accord with what would be expected based on the amino acid compositions of modern collagen (Fig. 2). Thirteen studies presented enough %C and %N data for intra-study comparison. In twelve of these, fish bone collagen was characterized by lower %C and %N, of which seven were significantly different for %C and eight were significantly different for %N ($p < 0.05$). For the 14 studies with collagen yield data, mammalian bone yielded more collagen 12 times (nine of which were statistically significant, $p < 0.05$).

The comparatively high C:N ratios of fish bones from archaeological contexts may be the result of differences in the macromolecular structure of bones, the differential effects of post-burial alteration and/or the suitability of particular laboratory methodologies. Given that fish collagen from modern specimens tends to have *lower* C:N ratios than mammals (Fig. 1), the difference in

Table 5
Archaeological bone collagen quality criteria broken down according to filtration method and taxonomic group.

	All data				Actinopterygii				Mammalia			
	30 kDa Ultrafilter		No filtration		30 kDa Ultrafilter		No filtration		30 kDa Ultrafilter		No filtration	
	n	Mean ± 1σ	*n*	Mean ± 1σ	*n*	Mean ± 1σ	*n*	Mean ± 1σ	*n*	Mean ± 1σ	*n*	Mean ± 1σ
Collagen yield												
C:N < 2.9	8	4.7 ± 3.5	52	1.8 ± 1.0	0		3	0.6 ± 0.4	8	4.7 ± 3.6	49	1.9 ± 1.0
2.9 < C:N < 3.6	1077	5.6 ± 3.8	697	5.2 ± 5.1	144	4.0 ± 2.7	103	5.0 ± 4.3	886	5.9 ± 4.0	522	4.6 ± 4.8
C:N > 3.6	39	2.3 ± 2.1	181	3.7 ± 3.2	25	1.4 ± 1.7	57	1.9 ± 1.3	15	3.6 ± 2.2	63	3.6 ± 3.5
All data	1133	5.4 ± 3.8	948	4.7 ± 4.7	169	3.63 ± 2.69	170	3.77 ± 3.77	917	5.79 ± 3.99	648	4.2 ± 4.5
% Carbon												
C:N < 2.9	9	37.5 ± 11.9	58	21.1 ± 16.7	1	6.4	0		8	41.34 ± 2.71	53	20.85 ± 17.33
2.9 < C:N < 3.6	952	41.4 ± 4	687	34.4 ± 12.1	121	39.18 ± 5.78	107	35.67 ± 9.26	781	41.65 ± 3.65	492	33.27 ± 13.22
C:N > 3.6	34	37.8 ± 9.2	188	33.9 ± 18.8	23	37.18 ± 8.78	57	31.96 ± 19.55	11	38.99 ± 10.49	70	28.71 ± 22.27
All data	995	41.2 ± 4.5	937	33.5 ± 14.4	145	38.64 ± 6.89	168	34.14 ± 13.77	800	41.61 ± 3.81	615	31.68 ± 15.3
% Nitrogen												
C:N < 2.9	9	17 ± 1.4	58	9.3 ± 7.2	1	15.4	0		8	17.19 ± 1.32	53	9.09 ± 7.46
2.9 < C:N < 3.6	952	14.7 ± 1.5	687	12.2 ± 4.2	121	13.8 ± 2.03	107	12.52 ± 3.17	781	14.88 ± 1.36	492	11.87 ± 4.69
C:N > 3.6	34	11 ± 3.4	188	10.3 ± 5.8	23	10.59 ± 3.44	57	9.77 ± 6.1	11	11.89 ± 3.45	70	8.89 ± 6.95
All data	995	14.6 ± 1.7	937	11.6 ± 4.9	145	13.3 ± 2.58	168	11.56 ± 4.58	800	14.87 ± 1.47	615	11.29 ± 5.4
C:N ratio												
All data	1275	3.29 ± 0.25	1127	3.38 ± 0.52	178	3.43 ± 0.53	184	3.58 ± 0.64	1047	3.27 ± 0.15	789	3.3 ± 0.48

Table 6
Summary of archaeological fish–mammal bone collagen comparisons within studies.

References	Lipid extraction[a]	C/N relationship, *p*	%C relationship, *p*	%N relationship, *p*	Collagen yield relationship, *p*
Borić et al. (2004)	n.s.	**F > M, 0.03**	**M > F, 0.02**	**M > F, <0.001**	**M > F, 0.03**
Bösl et al. (2006)	n.s.	M > F, 0.15	**M > F, 0.04**	M > F, 0.15	
Eriksson (2004)	UF	**F > M, 0.03**	**M > F, 0.003**	**M > F, <0.001**	**M > F, 0.002**
Eriksson et al. (2008)	UF	F > M, 0.14	M > F, 0.22	M > F, 0.07	**M > F, 0.03**
Fischer et al. (2007)	UF	**F > M, <0.001**	**M > F, <0.001**	**M > F, <0.001**	**M > F, <0.001**
Fornander et al. (2008)	UF	M > F, 0.74	M > F, 0.16	M > F, 0.31	M > F, 0.34
Grupe et al. (2003)	n.s.	F > M, 0.44	M > F, 0.11	**M > F, 0.01**	M > F, 0.06
Grupe et al. (2009)	n.s.	F > M, 0.06	M > F, 0.15	**M > F, 0.02**	**M > F, <0.001**
Jay (2008)	UF	**F > M, 0.02**	**M > F, 0.003**	**M > F, 0.002**	**M > F, <0.001**
Jørkov et al. (2010)	UF	F ≈ M, 0.96	**M > F, 0.01**	M > F, 0.09	M > F, 0.41
King (2006)	None	**F > M, <0.001**	**M > F, <0.001**	**M > F, <0.001**	**M > F, 0.003**
Kusaka et al. (2010)	n.s.	F > M, 0.40			**F > M, 0.02**
Müldner and Richards (2005)	UF	M > F, 0.33			F > M, 0.78
Müldner and Richards (2007)	UF	**F > M, <0.001**	M > F, 0.27	**M > F, <0.001**	**M > F, <0.001**
Szpak et al. (2009)	n.s.	**F > M, <0.001**	**F > M, <0.001**	**F > M, 0.05**	**M > F, 0.02**

M = mammal, F = fish (Actinopterygii).
Statistically significant differences ($p < 0.05$) are shown in boldface.
[a] UF = ultrafiltration following Brown et al. (1988), n.s. = not specified.

archaeological C:N ratios is *not* the result of variable amino acid compositions of endogenous collagen. More likely, this pattern is the result of differential preservation of fish vs. mammalian bone. Evidence for this notion comes from a consideration of the collagen yields associated with these C:N data. Samples that were characterized by collagen C:N ratios within the range of 2.9–3.6 also had significantly higher collagen yields than those samples with either C:N ratios <2.9 ($F_{[1,1832]} = 32.4$, $p < 0.001$) or >3.6 ($F_{[1,1992]} = 41.7$, $p < 0.001$). Because collagen yield is a fairly robust indicator of collagen intactness (Ambrose, 1990; van Klinken, 1999), it is likely that those samples with bone collagen C:N ratios >3.6 have been subject to some form of diagenetic alteration, and the general trend toward higher C:N ratios in archaeological fish bone collagen merits additional discussion.

It has been established that certain amino acids are preferentially lost from bone in the burial environment. Soil microorganisms with collagenase activity preferentially metabolize larger amino acids with high numbers of carbon atoms from the bone matrix (Grupe, 1995; Grupe et al., 2000). The collagen remaining after bacterial degradation is concentrated in small amino acids (glycine and alanine) (Turban-Just and Schramm, 1998), which are also the amino acids with the lowest C:N ratios. Thus, bone collagen that has been subject to intensive bacterial degradation should possess a lower, rather than higher, C:N ratio. Pfretzschner (2006) has suggested that biotic degradation of bone has a much less significant effect on the loss of collagen than abiotic processes (e.g. chemical hydrolysis). For instance, contamination with humics is a problem of particular importance because it will result in a shift in collagen $\delta^{13}C$ values and be accompanied by a high bone collagen C:N ratio (van Klinken and Hedges, 1995).

Fish bone collagen is likely highly susceptible to contamination with soil humic substances. The extent to which humic contaminants are able to interact with and eventually bind to bone collagen depends primarily upon whether or not these substances are able to penetrate the bone matrix (van Klinken, 1999). Because the collagen in fish bone is loosely packed and not well mineralized, it is more likely that these substances will be able to penetrate the bone. This could explain the high C:N ratios in archaeological fish bone, but protocols for archaeological bone collagen extraction include a step to remove humic contaminants (usually treatment with NaOH, filtration or both). Moreover, according to van Klinken and Hedges (1995), most 'humification' in archaeological bone occurs as a result of *in situ* processes, and not from soil-derived humics. These processes occur naturally in the bone during life when extracellular reducing sugars react non-enzymatically with amino acids to form cross-links, which are referred to as non-enzymatic glycation (NEG) products (Vashishth et al., 2001). Tuross (2002) has found evidence consistent with the presence of these compounds in archaeological bone and suggests that they may be the cause of elevated C:N ratios. It is unclear at this point, however, whether or not fish bone would be more or less likely than mammalian bone to accumulate these NEG products.

An alternative explanation for the generally elevated C:N ratios of fish bone collagen in comparison to mammalian bone relates to the presence of lipid contaminants. Lipids in skeletal tissues include triglycerides, cholesterol and phospholipids (Herring, 1972), none of which contain nitrogen; therefore, their presence results in an increase in only carbon and thus a higher C:N ratio. Moreover, because lipids are relatively depleted in ^{13}C compared to proteins (Ambrose, 1990; Howland et al., 2003; Jim et al., 2004), the presence of lipids would also alter the $\delta^{13}C$ value of the analyzed material. This topic has received considerable attention in the ecological literature (e.g. Hoffman and Sutton, 2010; Logan et al., 2008; Post et al., 2007; Sotiropoulos et al., 2004; Sweeting et al., 2006), although these studies have focused on tissues typically utilized by ecologists: muscle, liver and other soft tissues, which do not preserve in most archaeological contexts. Relatively low quantities of lipids (1–2%) have been reported for human cortical and cancellous bone (Dirksen and Marinetti, 1970; Fages et al., 1994; Pietrzak and Woodell-May, 2005). Although relatively high bone lipid contents have been reported in the literature (e.g. Plumb and Aspden, 2004), these studies report on bone as an organ, rather than bone as a tissue and thus include the marrow, which is rich in lipids (Woodard and White, 1986). In general, with the exception of some marine mammals, mammalian bone contains only small amounts of lipids (Higgs et al., 2011; Tont et al., 1977). Conversely, the bones of fish may act as a significant lipid reserve (Phleger et al., 1976, 1989, 1995; Phleger, 1988) and usually contain a comparatively high percentage of lipids (Table 7).

The isotopic effects of lipid extraction on fish bone has been investigated recently by Miller et al. (2010), who found bone collagen C:N ratios were significantly lower when a lipid extraction step (following Bligh and Dyer, 1959) was utilized. For these fish, most lipid-extracted samples were characterized by C:N ratios within the range of 2.9–3.6, while a larger portion of the untreated samples were not. There are, however, several different methods for extracting lipids (e.g. Ambrose and Norr, 1993; Coltrain et al.,

2004; Erickson, 1993; Hara and Radin, 1978; Jensen et al., 2003; Szpak et al., 2010), each of which is more or less well-suited to particular tissues (Carrapiso and García, 2000).

The two methods predominantly used for the extraction of lipids from biological materials are the Bligh and Dyer (10:5:4 methanol/chloroform/water; Bligh and Dyer, 1959) and Folch (2:1 chloroform/methanol; Folch et al., 1957) methods. Iverson et al. (2001) have demonstrated that for fish tissues with >2% lipids, the Bligh and Dyer method was relatively ineffective, and in samples with high lipid contents, up to 50% of lipids were not successfully extracted. Conversely they found the Folch method to be much more effective at extracting lipids in tissues with high lipid contents. This is particularly relevant since the Bligh and Dyer method (or a modified version of it) has been regularly employed for lipid extraction of bone samples in association with collagen extraction (e.g. Ambrose, 1990; Burton et al., 2002; Harding and Stevens, 2001; Hirons et al., 2001; Newsome et al., 2004; O'Connell et al., 2001).

The Bligh and Dyer method was designed to extract lipids from cod muscle, which has a relatively low (≤2%) lipid content. Given the low lipid content of human cortical bone (1–2%), this method is likely sufficient for this and similar tissues. Conversely, fish bone tends to contain a substantially greater amount of lipids (Table 7). Additionally, the tissue for which the Bligh and Dyer method was designed (cod muscle) consists mostly of phospholipids (Iverson et al., 2001). Fish bone, however, tends to consist primarily of triacylglycerol (~80%) (see Phleger, 1987, 1988; Phleger and Laub, 1989), a nonpolar lipid, which has limited solubility in 1:2 chloroform/methanol (Iverson et al., 2001). Thus the application of the Bligh and Dyer method of lipid extraction to fish bones and other lipid-rich tissues may be inappropriate or require significant modification. Given the generally high C:N ratios observed in archaeological fish bone collagen and the significant lipid component in many fish skeletons, it would be prudent to include a lipid extraction step that has the capacity to extract lipids from tissues with a high lipid content (e.g. the Folch method).

While the employment of a solvent-based lipid extraction step is often included in collagen extraction (following the frequently cited paper by Ambrose, 1990), none of the studies surveyed in this paper utilized such a step. Several studies did, however, utilize a 30 kDa filtration step (see Brown et al., 1988; Richards and Hedges, 1999), which should theoretically remove all lipids from the extracted collagen, although to the best of this author's knowledge, this has not been verified experimentally for fish or mammalian bone. The C:N ratios of filtered collagen were significantly lower than those of unfiltered collagen ($F_{[1,2400]} = 30.6$, $p < 0.001$); this was true for both fish ($F_{[1,360]} = 5.2$, $p = 0.02$) and mammals ($F_{[1,1834]} = 4.5$, $p = 0.03$). This implies that the use of

Table 7
Fish bone lipid content.

Common name	Taxonomic name	% Lipid[a]		References
		Cranium	Vertebrae	
Ocean surgeonfish	*Acanthurus bahianus*	29.7	6.9	Phleger (1988)
Doctorfish tang	*Acanthurus chirurgus*	17.9	4.4	Phleger (1988)
Blue tang surgeonfish	*Acanthurus coeruleus*	10.2	4.3	Phleger (1988)
African pompano	*Alectis ciliaris*	5.5	5.0	Phleger (1987)
Sablefish	*Anoplopoma fimbria*	60.0	38.0	Lee et al. (1975)
Blue antimora	*Antimora rostrata*	0.2	0.2	Lee et al. (1975)
Mexican hogfish (♀)	*Bodianus diplotaenia*	8.7	6.1	Phleger (1987)
Mexican hogfish (♂)	*Bodianus diplotaenia*	5.2	2.3	Phleger (1987)
Spotted-faced rock cod	*Cephalopholis pachycentron*	0.8	2.0	Phleger (1987)
Trilobed Maori wrasse	*Cheilinus trilobatus*	1.2	1.6	Phleger (1987)
Stocky hawkfish	*Cirrhitus pinnulatus*	90.0	64.0	Phleger (1975)
Giant hawkfish	*Cirrhitus rivulatus*	23.9	4.5	Phleger (1987)
Pacific grenadier	*Coryphaenoides acrolepis*	0.2	0.2	Lee et al. (1975)
Peruvian anchoveta	*Engraulis ringens*	12.7	9.2	Phleger and Wambeke (1994)
Honeycomb rock cod	*Epinephelus merra*	3.3	2.1	Phleger (1987)
French grunt	*Haemulon flavolineatum*	7.8	6.9	Phleger (1988)
Chameleon wrasse	*Halichoeres dispilus*	13.5	7.8	Phleger and Wambeke (1994)
Blackbelly rosefish	*Helicolenus dactylopterus*	28.2	13.7	Mendez et al. (1993)
Sand tilefish	*Malacanthus plumieri*	10.1	6.3	Phleger (1988)
Arrowtail	*Melanonus zugmayeri*	1.8	1.3	Phleger and Laub (1989)
Black triggerfish	*Melichthys niger*	0.2	0.6	Phleger (1988)
Yellow-striped squirrelfish	*Neoniphon aurolineatus*	5.0	4.0	Phleger (1975)
Ringtail Maori wrasse	*Oxycheilinus unifasciatus*	52.0	82.0	Phleger (1975)
High-hat	*Pareques acuminatus*	13.6	5.5	Phleger (1988)
Manybar goatfish	*Parupeneus multifasciatus*	6.0	14.0	Phleger (1975)
Glassy sweeper	*Pempheris schomburgkii*	7.4	5.4	Phleger (1988)
Pacific pompano	*Peprilus simillimus*	68.0	47.0	Lee et al. (1975)
Atlantic bigeye	*Priacanthus arenatus*	14.7	10.1	Phleger (1988)
Cardinal snapper	*Pristipomoides macrophthalmus*	4.4	4.0	Phleger and Laub (1989)
South American pilchard	*Sardinops sagax*	16.4	9.0	Phleger and Wambeke (1994)
Parrot fish	*Scarus sordidus*	0.2	0.4	Phleger (1987)
Cornish blackfish	*Schedophilus medusophagus*	21.0	23.0	Lee et al. (1975)
Chub mackerel	*Scomber japonicus*	3.3	6.1	Phleger and Wambeke (1994)
Galápagos sheephead wrasse	*Semicossyphus darwini*	29.5	6.9	Phleger and Wambeke (1994)
Palm ruff	*Seriolella violacea*	26.0	1.6	Phleger and Wambeke (1994)
Redband parrotfish	*Sparisoma aurofrenatum*	2.1	3.7	Phleger (1988)
Threespot damselfish	*Stegastes planifrons*	12.9	5.6	Phleger (1988)
Sand diver	*Synodus intermedius*	2.5	0.4	Phleger (1988)
Hound needlefish	*Tylosurus crocodilus*	1.7	6.5	Phleger (1988)
Yellow stingray	*Urobatis jamaicensis*	0.1	0.6	Phleger (1988)

[a] All data are for modern specimens.

a 30 kDa filter during collagen extraction may reduce the presence of lipid contaminants, although this must be verified experimentally. Conversely, it may indicate that the filtration step is effective at removing other carbon-rich contaminants. Generally speaking, both are likely to be true and the addition of a filtration step should aid in purifying any extracted collagen. The expected patterns in collagen %C (mammal > fish), %N (fish > mammal) and C:N ratio (mammal > fish) were, however, not observed more frequently in extraction protocols that utilized a 30 kDa filtration step (Table 6). Extensive comparative studies examining the effects of various collagen extraction protocols (including ultrafiltration) on C:N ratios, elemental concentrations and isotopic composition in collagen from a wide range of taxonomic groups would be extremely beneficial.

4.3. Fish bone chemistry and zooarchaeology

This section assesses to what extent the differences between mammalian and fish bone may influence their preservation in archaeological deposits. In order to interpret zooarchaeological data, it is essential to understand all of the relevant physical, chemical and cultural factors that act on animal remains after death. A number of excellent reviews exist that deal with the entire suite of taphonomic factors and their role in structuring archaeofaunal assemblages (Gifford, 1981; Lyman, 1994; O'Connor, 2005). While a number of factors such as soil chemistry, local environmental conditions and cultural activities are tremendously important in structuring faunal assemblages, the purpose of this chapter is to deal specifically with intrinsic chemical and structural characteristics of fish, compared to mammalian, bone and to what extent these characteristics may influence to relative abundance of fish in archaeological deposits.

One of the features of fish bone that has been discussed fairly extensively is the fact that most fish skeletons are characterized by acellular bone, lacking the osteocytes that comprise the majority of bone cells in tetrapods. Although there is variability within fish orders, cellular bone tends to be restricted to a portion of the Clupeiformes (herring and anchovies), Elopomorpha (eels, bonefish and tarpons) and Osteoglossomorpha (bony tongues and mooneyes) (Fleming, 1967; Parenti, 1986). The functional and adaptive significance of acellular as opposed to cellular bone in fish is unclear, and comparative studies of material properties have been inconclusive (Horton and Summers, 2009). It is thus difficult to speculate to what extent this difference influences the preservation of particular species of fish (herring for example) in archaeological deposits. The microscopic canals (canaliculi) associated with cell lacunae characteristic of cellular bone may provide additional opportunities for microbial and fungal degradation of bone. Although this is highly speculative, there is evidence that canaliculi play an important role in the expansion of bacteria through the bone after death (Bell, 1990; Jans, 2008), and this may make herring and other species of fish with cellular bone more prone to microbial degradation.

Within the context of the preservation of archaeological bone, the density of bone samples has been studied in a number of taxa and utilized as an explanation for patterns of skeletal element representation (reviewed by Lam et al., 2003; Lam and Pearson, 2005; Lyman, 1984). The bulk of bone density studies have focused on mammalian taxa, with a much smaller body of data available for fish (Butler and Chatters, 1994; Butler, 1996; Nicholson, 1992; Smith, 2008) and birds (Broughton et al., 2007; Cruz and Elkin, 2003; Cruz, 2007; Dirrigl, 2001). Nicholson (1992) found that bone density did not explain degradation of fish skeletal elements in archaeological deposits, or in physical and chemical degradation experiments. Moreover, she found the absolute size of the fish (and by extension, bones) to be insignificant in predicting bone preservation (Nicholson, 1996b). As outlined below, the amount of mineral per unit length as determined in bone density studies is only partly responsible for its physical properties and resistance to both mechanical and chemical degradation.

There is a complex interaction that occurs between the inorganic and organic phases of bone, with the preservation of one necessarily influencing the preservation of the other. Some evidence suggests that the organic component of the bone must first be dissolved before ionic exchange can take place with the bioapatite (Person et al., 1996; Walsh et al., 1994). Most authors, however, have suggested that the bioapatite crystals protect the collagen fibrils within the bone matrix, restricting the activity of enzymatic attack and conferring stability on the collagen molecules (Nielsen-Marsh et al., 2000; Pfretzschner, 2004; Yoshino et al., 1991). While it may not be possible to determine which is more important than the other, it is clear that *both* the mineral and organic phases of bone play a crucial role in its survival.

Although a very limited number of taxa have been studied to date, the arrangement of collagen and bioapatite appears to be similar in mammals and fish (Bigp et al., 2000). Fish are, however, characterized by a larger proportion of uncalcified collagen bundles in their skeletons (Moss, 1961; Moss, 1963; Neuman and Mulryan, 1968). The lack of mineralization in fish bone may be an adaptation related to buoyancy, or it may facilitate easy metabolism of stored lipids within the bones (Mendez et al., 1993). Furthermore, the collagen fibrils in fish bone tend to be less densely packed than in mammals (Lee and Glimcher, 1991). Generally speaking, in mineralized tissues, tightly packed collagen fibrils are more stable and less prone to chemical dissolution (Bonar and Glimcher, 1970; Lees, 1989b). The lack of mineralization and loose packing of collagen fibrils in fish bone are significant because these factors can influence the degree to which microbes can degrade bone. In most cases, the large size of enzymes prohibits them from penetrating the collagen mass (Collins et al., 1995; Rudakova and Zaikov, 1987). Thus, the loosely packed and poorly mineralized collagen fibrils in fish bone may provide bacterial enzymes easier access to the collagen in comparison to mammalian bone.

While microbial degradation of collagen may be an important part of bone deterioration, particularly soon after death (Bell et al., 1996), chemical leaching may account for the majority of collagen lost from bone in the long-term (see Pfretzschner, 2006). The extent of chemical leaching is dependent on the abiotic conditions of the burial environment (e.g. soil pH), as well as the macromolecular nature of the collagen itself (Pfretzschner, 2006). Because of the latter, it is probable that differences in the chemical composition of fish and mammalian bone will have important effects on collagen (and bone) preservation in archaeological contexts. The stability of the collagen helix is largely dependent on the content of imino acids − proline and especially hydroxyproline (Bella et al., 1995; Burge and Hynes, 1959; Burjanadze, 1979; Sakakibara et al., 1973; Vitagliano et al., 2001a,b). This is reflected in collagens with higher imino acid contents being characterized by higher thermal stabilities (melting points) (Fig. 3). In general, the integrity of collagen's molecular structure is facilitated largely by (1) the stereochemical properties of imino acid pyrrolidine rings that add rigidity to the helical structure (Burge and Hynes, 1959; Josse and Harrington, 1964; Ramachandran et al., 1973; Shah et al., 1996; Shoulders et al., 2006), and (2) an extensive hydrogen bonding network that surrounds the collagen chains, in which hydroxyproline is essential (Bella et al., 1994; Burjanadze and Kisiriya, 1982; Collins et al., 1995; Khan et al., 2009). It is thus very significant that fish collagen is substantially depleted in proline and hydroxyproline in comparison to mammalian collagen (Table 2);

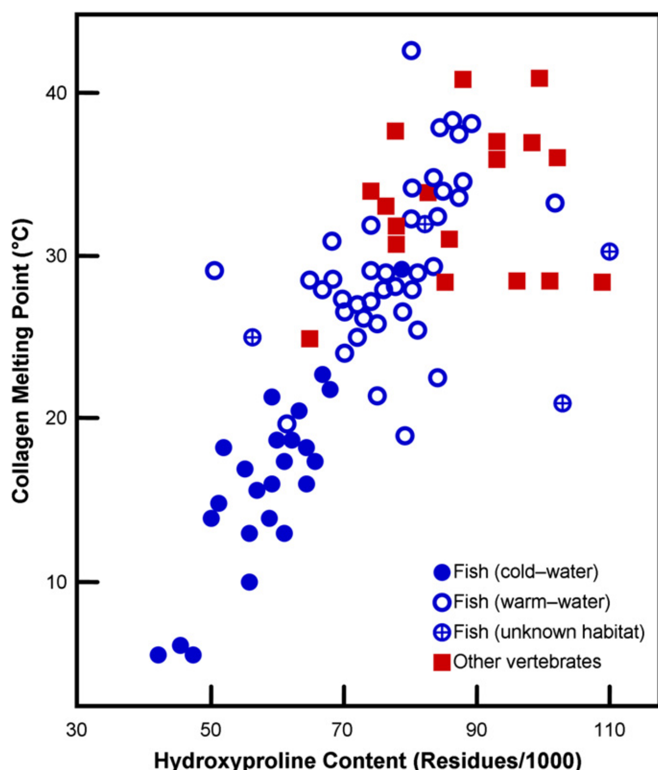

Fig. 3. Hydroxyproline content of vertebrate collagen plotted against collagen melting point. This figure demonstrates the relationship between collagen thermal stability and hydroxyproline content. A complete list of the data used in this figure can be found in Table S3; references are included in the Supplementary Material.

these differences are statistically significant for proline ($F_{[1,126]} = 83.3$, $p < 0.001$ for skin; $F_{[1,33]} = 30.5$, $p < 0.001$ for bone) and hydroxyproline ($F_{[1,126]} = 127.8$, $p < 0.001$ for skin; $F_{[1,33]} = 41.8$, $p < 0.001$ for bone). In general, this results in fish collagen having a lower melting point and less stable arrangement than mammalian collagen (Fig. 3), a fact that has caused considerable difficulty for those in the food chemistry industry wishing to replace mammalian collagens with fish collagens (Regenstein and Zhou, 2007). This is particularly true for cold-water fish, which tend to have the lowest collagen thermal stability among vertebrates. This pattern is also observed for other aquatic poikilotherms (e.g. lampreys, hagfish), but not for mammals (Pikkarainen, 1968). The data presented in Fig. 3 are taken from unmineralized or demineralized collagen, and thus do not take into account the influence of bioapatite on collagen stability. Pasteris et al. (2004) suggested that the bonding at the mineral–collagen interface in bone occurs via the sharing of OH-groups between bioapatite (depleted in OH-groups) and OH-rich amino acids, specifically hydroxyproline. Again, the comparative lack of hydroxyproline in fish compared to mammalian bone collagen implies that the former is more unstable and prone to dissolution than the latter. For this reason, fish bone collagen would be more prone to chemical leaching than mammalian bone collagen, and this effect may be even more pronounced at sites in colder climates.

5. Summary and recommendations for future research

On the basis of the chemical composition of collagen, the typical preservation parameters used in stable isotope analysis (i.e. C:N ratio) should not be expanded or modified for fish. Experiments examining the relationship between changes in isotopic composition, collagen yield and elemental composition for a diverse array of taxa (including fish) are necessary. Because fish bone is particularly rich in lipids, a protocol that is capable of extracting lipids from materials with high lipid contents should be used in association with collagen extraction. Comparative studies examining the effects of different lipid extraction techniques on archaeological bone collagen are also essential.

It is absolutely imperative that studies reporting isotopic data provide sufficient detail with respect to the protocols that were used and include *all* relevant data that may be used to assess collagen preservation: collagen yield, C:N ratio, %C and %N. Although this point was made by Ambrose (1990) over 20 years ago, these data are frequently omitted. With the coupling of mass spectrometers to elemental analyzers, most laboratories generate these data with no additional cost or effort, and as such there is no reason to omit them.

Fish bone appears to be more susceptible to dissolution and degradation than mammalian bone for the following reasons.

1. The extent of mineralization and packing of the collagen. Generally, collagen within osseous tissues that is well mineralized should preserve better than poorly mineralized collagen. Because fish bone collagen is less well mineralized and less densely packed than in mammals, it should be more prone to biotic attack, contamination with exogenous substances and chemical dissolution.
2. The relative instability of the collagen molecule. Because of the differences in amino acid composition between fish and mammalian collagen, the former is much less stable and more prone to chemical hydrolysis than the latter.
3. Weaker collagen–mineral interactions. The strength of the interactions between bone collagen and apatite are governed by the chemical composition of these molecules. The comparative lack of OH-rich amino acids (i.e. hydroxyproline) in fish bone collagen should result in weaker covalent and non-covalent collagen–mineral interactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jas.2011.07.022.

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