Title

Effects of Lipid Extraction and Ultrafiltration on Stable Carbon and Nitrogen Isotopic Compositions of Fish Bone Collagen

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Abstract

RATIONALE: Fish bone collagen isotopic measurements are increasingly important in palaeodietary and paleoenvironmental studies yet differences in the chemical and physical properties of fish relative to other vertebrate bones are rarely considered. Lipid content in fish bone, which can exceed 50%, may underlie poor collagen integrity criteria typically observed in archaeological studies.

METHODS: We compare stable carbon and nitrogen isotopic and elemental compositions of bone collagen prepared using four different methods from a wide range of modern fish species to: 1) assess the extent to which lipid content influences bone collagen $\delta^{13}C$ and $\delta^{15}N$ values, and 2) evaluate the relative efficacy of chemical (2:1 chloroform-methanol) and physical (30 kDa ultrafilters) methods for removing lipids from bones.

RESULTS: Lower $\delta^{13}C$ values were observed when lipid content exceeded 5% of initial bone mass. Lipid content did not influence $\delta^{15}N$ values. 30 kDa ultrafiltration, a common pretreatment for purifying archaeological collagen, removed fewer lipids and was associated with reduced collagen yields (37% loss) as well as altered amino acid compositions. In contrast, collagen prepared using a 2:1 chloroform-methanol lipid extraction step resulted in significantly improved collagen yields, elemental compositions, and isotopic measurements relative to a control treatment.

CONCLUSIONS: The chemical lipid extraction method (2:1 chloroform-methanol) performed significantly better than physical lipid extraction method (30 kDa ultrafilters). Given the high quantities of lipids in fish bones we recommend the inclusion of a chemical lipid extraction step when isolating collagen from modern and archaeological fish bones.

Introduction

The role of fish (Actinopterygii and Chondrichthyes) in prehistoric human subsistence economies is a topic of great importance in archaeology. The question of when fish became an important part of human or hominin diets is still unclear, as are questions related to the role of fish and other aquatic resources in the development of social complexity [1, 2]. These questions are, to a large extent, addressed primarily using zooarchaeological data, but various taphonomic factors driven by the chemical [3] and physical [4] properties of fish bone, make the quantification of fish remains inherently difficult [5]. Stable isotope analysis (δ^{13}C, δ^{15}N, δ^{34}S) of the organic fraction of human bone (hereafter 'collagen'[1]) has played an important role in better quantifying the importance of marine and freshwater fish in the diets of prehistoric human populations [6-8]. Accurate interpretation of isotopic data derived from human bone is contingent upon a thorough understanding of the isotopic compositions of the foods that may have been consumed, which is frequently accomplished by the isotopic analysis of contemporaneous faunal materials [9-11]. The quality of these data are therefore of considerable importance as they will directly and quantitatively influence the interpretation of human stable isotope values.

Aside from the supporting role fish bone isotopic compositions play in the interpretation of prehistoric human diet, they also have the potential to serve as valuable paleoecological indicators in their own right, which has led to a recent increase in the number of studies utilizing fish bone collagen. Fish bone isotopic compositions may provide insight into dietary shifts driven by natural or anthropogenic disturbances and can serve as indicators of shifts in dominant primary producers or nutrient cycling [12-14]. Studies such as these rely on subtle variations in isotopic compositions on the order of a few ‰ and consequently, accurate determinations of isotopic compositions are paramount. The primary mechanism by which collagen quality is addressed is the ratio of elemental carbon and nitrogen in the analyzed sample (C:N), with a range of 2.9 to 3.6 being characteristic of unaltered collagen [15]. It is common for archaeological fish bone collagen to have C:N ratios near the upper limit of this range and for proportionately more samples (relative to mammalian bone) to have C:N ratios above 3.6 [3].

Reviewing the ultrastructure and chemistry of fish bone, Szpak (2011) hypothesized that two main factors are behind the unusually high C:N ratios observed in archaeological fish bone, namely contamination with carbon-rich sources from (1) humic acids (from endogenous or exogenous sources) and (2) lipids. Structural differences in the mineralization of bone and packing of collagen fibrils likely increase the susceptibility of fish bone to humic acid contamination by providing more binding sites relative to other vertebrate bone. Functionally, fish bones also differ markedly from other vertebrates by acting as a significant reserve for lipids and therefore have potential to preserve greater quantities of lipids. Despite these structural and functional differences, the collagen in fish and other vertebrate (e.g., mammal) bone is nearly identical in terms of chemical composition. Within a single organism, lipids are consistently characterized by significantly lower δ^{13}C values relative to proteins [16]. Additionally, humic acids primarily derived from the decay of C_3 plant organic matter (as would be expected in most environments) would produce δ^{13}C values that are significantly more negative than those recorded in fish bone collagen [17]. Contamination of bone collagen with humics or lipids should therefore manifest in elemental composition and isotope ratios, with lower δ^{13}C values and

[1] We use the term 'collagen' throughout the paper to refer to the acid insoluble proteinaceous component of bone. We recognize that other proteins are present in bone and the term 'gelatine' is more accurate *sensu stricto*, but for the sake of consistency with the larger archaeological literature, we use 'collagen'.

elevated C:N ratios, the latter of which is observed in most published archaeological fish bone collagen datasets.

The problem of isotopically light (in carbon) lipids influencing the $\delta^{13}C$ values of soft animal tissues has been widely acknowledged and studied in ecology [18], but with the exception of a few studies of limited scope [19-22], this problem has not been addressed in any substantive way with bone collagen. This paper provides the first systematic analysis of the effect of relative lipid content on $\delta^{13}C$ values of bone collagen in a wide variety of fish species and compares the effects of common collagen purification methods for dealing with potential lipid contamination. We present the results of a controlled study of four parallel collagen extraction methods (using both chemical and physical lipid removal steps) on 72 bones from modern fish to: (1) assess whether the unusually high levels of lipids in fish bones can have a significant impact on $\delta^{13}C$ values of collagen extracts, (2) explore the extent to which lipid contamination is manifested in traditionally employed collagen quality control measures (C:N, %C, %N, and collagen yield), and (3) determine if either physical (ultrafiltration) or chemical (2:1 chloroform-methanol, v/v) methods for collagen purification provide superior results. We hypothesized that the best results, judged by the similarity of the elemental compositions to those known for fish bone collagen, would be produced when the ultrafiltration and chemical lipid extraction steps were combined. The results of this study demonstrate that (1) lipids can significantly alter fish bone collagen $\delta^{13}C$ values, (2) fish bone collagen samples with altered $\delta^{13}C$ values are also characterized by significantly different %C, C:N, and collagen yields, (3) the chloroform-methanol pretreatment was more successful than the ultrafiltration treatment with respect to removing lipids. On the basis of this study we recommend that a chemical lipid extraction step be included in collagen extraction protocols specifically involving fish.

Materials and Methods

Materials

Contextual information for fish samples used in this study is presented in Table S1.1 (see Supporting Information 1). Samples were collected from 35 individual fish representing 17 marine and freshwater species from both tropical and temperate environments (Table 1). Fish skeletons were carefully prepared through a process of light steaming to loosen flesh, followed by gentle scrubbing under tepid water [23]. Samples were then dried under continuous air flow at room temperature (c. 20°C) for one week. No enzyme or chemical-based treatments were used to extract the bones from the fish carcasses.

Where size permitted, samples were taken from two separate bones per individual fish in order to assess intra-individual variation in lipid content between elements. In most cases articulars and cleithra were sampled (Table 1). Individual bones typically provided enough material (c. 2 g) to perform all four sample pretreatments (see Stable Isotope Analysis section) but in some cases it was necessary to combine the same elements from both sides of an individual to provide sufficient sample material (i.e., both left and right cleithra). Each bone was cut into fragments of roughly equal size (c. 2×2 mm) using a dental drill equipped with a diamond-tipped cutting wheel. These fragments were then mixed and divided into four aliquots of roughly equal mass (450±100 μg) to be used in the four different pre-treatments described below.

Experimental Design

We tested the effect of four analytical procedures on fish bone collagen $\delta^{13}C$ and $\delta^{15}N$ values with a view to understanding how specific steps in collagen extraction procedures influenced contamination from residual lipids in archaeological fish bone. In particular, the pretreatments were designed to test the utility of 2:1 chloroform-methanol [24] and 30,000 Da (Dalton) ultrafiltration [25]. We chose the Folch method of lipid extraction over 10:5:4 chloroform-methanol-water method of Bligh and Dyer [26] because the Folch method has been shown to more effectively extract lipids, particularly for materials with lipid contents >2% [27]. On the basis of previous studies of fish bone lipid contents [3] we expected *a priori* that most fish bones would contain >2% lipids by mass. We measured the quantity of lipids in fish bones (by comparing sample weights before and after the chemical lipid extraction procedures) in order to explore the relationship between initial lipid mass in the bone and elemental and isotopic compositions recorded for the extracted collagen.

The four collagen extraction protocols applied to the treatments were identical except for the variable use of a solvent lipid extraction step (2:1 chloroform-methanol) and an ultrafiltration step using a 30 kDa filtered centrifuge tube (Table 2).

Treatment 1 (hereafter 0/0) used no chloroform-methanol or ultrafiltration so as to provide a comparative baseline for a sample that was maximally contaminated with lipids. Samples were demineralized in a 0.5 M HCl, rinsed to neutrality, and then solubilized in 10^{-3} M HCl (pH ~3) at 75°C for 48 h. The solution was then filtered with a 5−8 μm filter (Elkay, Basingstoke, UK) in order to remove larger particulates. The filtered solution was then frozen and lyophilized.

Treatment 2 (hereafter 0/UF) followed procedures from Treatment 1 but with the addition of an ultrafiltration step [25]. The solution containing the refluxed collagen was filtered using a 15 mL centrifuge tube fitted with a Microsep™ Omega™ modified polyethersulfone membrane (Pall Corporation, Port Washington, NY, USA) to retain the >30 kDa molecular weight fraction. The >30 kDa fraction was frozen and lyophilized.

Treatment 3 (hereafter CM/UF) followed the same procedures as the second treatment but began with a CM pretreatment in order chemically extract lipids prior to demineralization. Samples were soaked in 2:1 chloroform-methanol, which was refreshed every c. 15 min, in an ultrasonic bath until visible evidence of lipids precipitating into solution ceased (i.e., solution remained clear; 1 to 10 sessions). When lipid extraction was complete, chloroform-methanol was removed using a pipette and samples were then air dried for 1-2 days under continuous air-flow to evaporate any residual solution.

Treatment 4 (hereafter CM/0) was the same as the third treatment but with no UF step.

Stable Isotope Analysis

Carbon and nitrogen isotopic and elemental compositions were determined with a Vario MICRO cube elemental analyzer (Elementar, Hanau, Germany) coupled to an Isoprime isotope ratio mass spectrometer (Elementar, Hanau, Germany) in continuous flow mode at the Archaeology Isotope Laboratory, University of British Columbia, Vancouver, Canada. Carbon and nitrogen isotopic compositions were calibrated to VPDB and AIR standards, respectively, using USGS40 and USGS41. Analytical accuracy and precision were monitored using bovine liver (NIST 1577c), internal methionine (Sigma Aldrich, St Louis MO, USA), modern seal collagen, archaeological caribou collagen, and archaeological walrus collagen standards. All samples were analyzed in triplicate. Complete details on the assessment of analytical accuracy and precision are presented in Supporting Information 2.

Analytical Uncertainty

Analytical uncertainties for isotopic and elemental compositions are presented in Table S3.1 (see Supporting Information 1). Measurement uncertainty (u_s) for isotopic and elemental compositions was determined with Equation 1:

$$u_s = \sqrt{(u_a)^2 + \left(u_p\right)^2} \qquad \text{Equation 1}$$

Where u_a is accuracy or reproducibility and u_p is precision or repeatability [28]. Accuracy (u_a) is defined as the mean difference between observed and expected isotopic and elemental compositions for all check (quality assurance) standards analyzed in the same analytical sessions as the samples for which data are presented in this study. Precision was determined with Equation 2:

$$u_p = \sqrt{(p_{srm})^2 + \left(p_{rep}\right)^2} \qquad \text{Equation 2}$$

Where p_{srm} is the average standard deviation of all check and calibration standards analyzed in the same analytical sessions as the samples for which data are presented in this study and p_{rep} is the average standard deviation for all triplicate sample sets analyzed in this study. Analytical uncertainty was assessed separately for each of the four treatments to account for any potential differences in sample heterogeneity produced by the different sample preparation methods.

Amino Acid Analysis

A subset of collagen extracts was selected for amino acid characterization. These samples were selected on the basis of differences in elemental compositions observed between treatments CM/UF and CM/0. Amino acid analysis was conducted at the SPARC BioCentre, The Hospital for Sick Children, University of Toronto, Toronto, Canada.

Collagen extracts were dried in pyrolyzed borosilicate tubes in a vacuum centrifugal concentrator and hydrolyzed by 6 M HCl with 1% C_6H_6O (phenol) for 24 h at 110°C under a continuous flow of pre-purified N_2. Amino acids were then derivatized with a solution consisting of 7:1:1:1 methanol:water:triethylamine:phenyl isothiocyanate (PTIC) at room temperature (c. 20°C) to produce PTC (phenylthiocarbamide) amino acids. The derivatized amino acids were dissolved in a phosphate buffer and analyzed with a Waters ACQUITY ultra high performance liquid chromatography system.

Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistical version 23 for Mac OS X. Differences among isotopic or elemental compositions were assessed using a one-way ANOVA with a post hoc Tukey's HSD (for samples with equal variances) or Dunnett's T3 for samples with unequal variances) test. Homogeneity of variance was assessed using Levene's test. Correlations between initial sample lipid content and difference in isotopic compositions among treatments was assessed using Spearman's ρ.

Results

Molecular Components of Fish Bone

Collagen Content in Fish Bone

Collagen yield, as calculated from whole bone weight (i.e., weight before or without lipid extraction), varied among treatments (Table S3.2, see Supporting Information 3). The mean collagen yield for fish bones recorded for all species and elements was 16.8±2.8 % (CM/0 treatment) with a range between 7.4 and 21.5 % (*n*=72) (Table S3.3, see Supporting Information 3). This more variable and generally lower (relative to mammalian bone) abundance of collagen in fish bone was driven by the wide variation in lipid content observed in fish bones (see Lipid Content in Fish Bone Section). The average collagen yield for the CM/0 treatment calculated with the initial de-fatted bone mass was 21.3±2.8 % (*n*=68), which is comparable to values observed for mammalian bone collagen [29, 30].

Collagen yield declined significantly with the use of ultrafilters (Table S3.4, see Supporting Information 3). Ultrafiltered collagen yields were on average 6.6 % (0/0 vs. 0/UF) and 6.2 % (CM/0 vs. CM/UF) lower (*n*=72) in terms of difference in percent yield. For both comparisons this equated to a loss of 37 % of the initial mass of the acid insoluble protein ('collagen') present in the sample. When the initial sample mass was calculated using the post-chloroform-methanol treated dry sample mass (CM/UF^1 and $CM/0^1$ in Table 4) the difference in percent collagen yield was 7.9 % lower when ultrafilters were used ($CM/0^1$ vs. CM/UF^1), again representing a loss of 37 % of the initial mass of the acid insoluble protein.

The chloroform-methanol step (CM) resulted in comparatively small decreases in collagen yield, with reductions of 1.2 % (0/0 vs. CM/0) and 0.8 % (0/UF vs. CM/UF) in terms of difference in percent yield. These much smaller decreases in collagen yield were likely the product of small amounts of sample loss during the additional pipetting steps employed when refreshing the solvent during the lipid extraction.

Lipid Content in Fish Bone

The amount of lipid extracted from fish bones varied tremendously from 1.1 to 46.1 % by mass. The mean lipid contents of each element according to species are presented in Table S3.5 (see Supporting Information 3). Lipid contents for each individual element are presented in Supporting Information 3. Substantial variation was present among species, with some having negligible lipid contents in the range of 1-3 % by mass (grass carp, northern pike, Atlantic cod) comparable to the relative proportion of lipids in mammalian cortical bone tissue [31, 32]. Conversely, most species had much higher quantities of bone lipids, commonly in the range of 10-30 %, which is comparable to values obtained in previous studies of fish bone lipids [3], although % lipid values much higher than those reported here have been recorded. The comparatively low % collagen yields (i.e., those <20 %) that were recorded for many of the fish (Table S3.3, see Supporting Information 3) were driven primarily by relatively high lipid contents as demonstrated by the linear correlation on the ternary plot presented in Figure 1.

Elemental Compositions

The C:N ratios, %C, and %N for all of the fish in each treatment are summarized in Table S3.6 (see Supporting Information 3) and Figure 2; individual results are presented in Table S4.1 (see Supporting Information 4). The theoretical or expected values are also presented in Table S3.6 (see Supporting Information 3) and Figure 2 for comparative purposes. These values were

calculated on the basis of the carbon and nitrogen present in a variety of Actinopterygii bone collagen [3] and it is therefore significant to note that they do not take into account any other acid insoluble proteins that may be present in bone in addition to collagen. Because collagen has a particularly low C:N ratio relative to other proteins (driven by its high glycine content), these estimates slightly underestimate the C:N ratio and slightly overestimate the %C and %N relative to what would be obtained using collagen extraction procedures typically used for archaeological samples.

There were significant differences among treatments for C:N ratio ($F_{3,284}$=29.50, p<0.001), %C ($F_{3,284}$=33.74, p<0.001), and %N ($F_{3,284}$=138.17, p<0.001). Of all the comparisons among treatments, only two were not significantly different (p>0.05): 0/0 vs. 0/UF for %C and CM/0 vs. CM/UF for %N (Table S3.7, see Supporting Information 3). The 0/0 and 0/UF treatments were more similar to one another than they were to either the CM/UF and CM/0 treatments (Figure 2). Similarly, the CM/UF and CM/0 treatments were more similar to one another than they were to either the 0/0 and 0/UF treatments (Figure 2). Elemental compositions were the least variable and most similar to their expected or ideal values when the CM step was employed (Figure 2). The UF step also created less variable and closer to expected elemental compositions relative to the 0/0 treatment, but was not as effective as the CM step. Not surprisingly, the 0/0 treatment (with no chloroform-methanol and no ultrafiltration) produced the highest C:N ratios (3.94±0.95), consistent with a significant portion of residual lipids being retained in the final collagen extract.

The variation in the elemental compositions, particularly the C:N ratios and %C were driven by the initial lipid content of the sample (Figure 3). The 0/UF treatment, which relied only on physical means of lipid extraction, had an average C:N value (3.55±0.51) that was significantly higher than expected for fish bone collagen (3.15±0.04). Thus, while the 0/UF treatment produced an average C:N value that was closer to the expected value (Table S3.6, see Supporting Information 3) than the 0/0 treatment, it was apparent that the ultrafilters did not completely remove lipids from the samples, particularly those samples with high initial lipid contents.

The CM/0 treatment produced collagen samples with an average C:N value of 3.19±0.06, which was closest to the theoretical value of 3.15. Contrary to expectations, the CM/UF treatment did not produce superior C:N values (i.e., closer to the theoretical value) and produced a higher average C:N value of 3.22±0.10; the difference in C:N ratios between these two treatments was significant (Table S3.7, see Supporting Information 3). Because both treatments used the same chemical lipid extraction step, this difference was likely caused by the selective loss of proteinaceous or other organic materials (presumably with a C:N ratio <3.19) through the ultrafilters rather than the selective retention of carbon-rich lipids.

Isotopic Compositions

Impact of Lipid Extraction and Ultrafiltration on δ^{13}C Values

Isotopic compositions for all samples are presented in Table S4.1 (see Supporting Information 4). Both chemical and physical lipid extraction methods had an influence on collagen δ^{13}C values. Specifically, when samples were either ultrafiltered (UF) or subjected to a chemical lipid extraction step (CM), δ^{13}C values were higher relative to the control (0/0) (Table S3.8, see Supporting Information 3). These differences were, however, only significant for the treatments involving the CM step (Table S3.9, see Supporting Information 3), which suggested

that ^{13}C depleted lipids were most effectively removed by the chemical lipid extraction step. This was supported by the fact that the CM/UF and CM/0 treatments produced statistically indistinguishable $\delta^{13}C$ values (p>0.999). For all treatments the change in $\delta^{13}C$ relative to the control (0/0) was significantly and positively correlated with the initial lipid content of the sample (Table S3.10, see Supporting Information 3). Progressively higher initial lipid contents resulted in correspondingly higher differences in collagen $\delta^{13}C$ values between treatments and controls, although this pattern was much clearer for the treatments involving the CM step (Figure 4). The CM/0 and CM/UF treatments produced nearly identical patterns of difference in $\delta^{13}C$ relative to the control (Figure 4), further confirming the greater effectiveness of the CM step in removing lipids. When the initial lipid content of the sample was <5%, there was little difference in collagen $\delta^{13}C$ between the control (0/0) and any of the treatments (Figure 3).

Impact of Lipid Extraction and Ultrafiltration on $\delta^{15}N$ values

The $\delta^{15}N$ values did not vary systematically according to treatment (Table S3.11, see Supporting Information 3) and the initial lipid content of the sample was not correlated with the difference in $\delta^{15}N$ between any of the treatments and the control (Table S3.12, see Supporting Information 3).

Measurement Uncertainty

Sample repeatability was assessed by comparing the standard deviation of the elemental and isotopic compositions of three aliquots of each sample. To determine a baseline level of variation in a bone collagen sample we produced 10^4 sets of three randomly selected analyses of three internal collagen standards (1/3 from each of the three standards) that were analyzed in the same analytical sessions as the samples presented in this paper (Table S3.13, see Supporting Information 3).

Relative to the baseline level of intra-sample variation, there was greater sample heterogeneity in $\delta^{13}C$ and C:N for 0/0 and 0/UF (Table S3.13, see Supporting Information 3) − those that excluded the lipid extraction step. Comparisons among the four treatments demonstrated significant differences for $\delta^{13}C$ ($F_{3,284}$=14.89, p<0.001), %C ($F_{3,284}$=3.59, p=0.003), %N ($F_{3,284}$=7.24, p<0.001), and C:N ($F_{3,284}$=13.91, p<0.001), but not for $\delta^{15}N$ ($F_{3,284}$=0.53, p=0.66). The presence or absence of the lipid extraction step significantly reduced within sample $\delta^{13}C$ and C:N variation as demonstrated by the lower within sample variation in the CM/UF treatment relative to the 0/UF treatment and CM/0 treatment relative to the 0/0 treatment (Table S3.14, see Supporting Information 3). The only instance in which the ultrafiltration step had a significant effect on within sample variation was for C:N ratio, but the inclusion of an ultrafiltration step with a lipid extraction step (CM/UF relative to 0/UF) resulted in *higher* within sample variation (Table S3.13, see Supporting Information 3).

Amino Acid Compositions

The amino acid compositions for the subsample of bones selected for analysis are presented in Table S3.15 (see Supporting Information 3) and summarized in Figure 5. Although type I collagen is the most abundant protein in bone, the amino acid compositions reflect a mixture of all of the proteinaceous material in the bone. The amino acid compositions were generally similar between the CM/0 and CM/UF treatments, with the only significant difference being for aspartic acid (Figure 5). While these differences were suggestive of some biochemical difference due to the ultrafiltration, they did not account for the observed differences in C:N ratio between the CM/0 and CM/UF treatments (Table S3.15, see Supporting Information 3).

Discussion

The amount of protein in fish bone has not been extensively characterized. The results of this study demonstrate that the % collagen in fish bone varies widely (range of 12.9 to 20.2%), driven largely by the amount of lipid in the bone. This suggests that collagen yields in archaeological fish bone should be systematically lower than those observed in mammalian bones and an expectation of a ~20% collagen yield for 'perfect' fish bone collagen is unrealistic. When lipids are excluded, however, fish and mammal bone compositions are similar with respect to relative collagen and mineral content. Note that when collagen yield is calculated in the manner typical of archaeological studies (i.e., % collagen = collagen mass/initial bone mass), the mineral component (initial bone mass−collagen mass) also includes the bound water that forms the inner hydration shell surrounding the collagen [33]. In human bone, this bound water is a relatively small (c. 1−3 % by mass) but significant component of bone [34].

The ultrafiltration step resulted in a remarkably consistent percentage collagen loss (37% of the initial collagen mass). As is the case with collagen in mammals, fish collagen consists of three α chains, each with a molecular weight of 110−130 kDa [35-38] that are held together by interchain hydrogen bonds [39]; these bonds are cleaved during the reflux step (heating) in the collagen extraction. Since there is no reason to expect that the collagen of modern specimens has been degraded in such a way that the individual chains would be cleaved to produce multiple peptide fragments with a molecular weight of <30 kDa, the loss of collagen must be initiated by selective hydrolysis of the peptides during the demineralization and/or refluxing steps. While no collagen was *lost* during these steps, the collagen was cleaved into fragments, some of which were <30 kDa and these were removed during the ultrafiltration step. Mammalian bone collagen yield data presented by Brown et al. [25] for collagen refluxed at a variety of temperatures do not suggest that refluxing at 75°C (the temperature used in this study) is in and of itself likely to be responsible for significantly reduced collagen recovery, but the results presented in this study indicate otherwise. At the very least, for fish bone collagen, typical conditions used during collagen extraction produce cleaved peptide fragments that are likely to be selectively removed by 30 kDa ultrafilters.

The potential for lipids to influence stable isotope measurements of fish bone collagen has been given little consideration for materials from archaeological contexts. The results of this study clearly demonstrate that fish bone contains substantial quantities of lipids and that if they are not properly removed with a chemical lipid extraction step, they can significantly alter bone collagen isotopic and elemental compositions. Specifically, when a chemical lipid extraction is not performed, collagen extracts produce (1) higher C:N ratios, (2) higher %C and lower %N, (3) less homogenous collagen extracts, and (4) lower $\delta^{13}C$ values. It is therefore imperative to ensure that a set of best practices for fish bone collagen extractions are well-defined and, where feasible, followed.

The results of this study clearly demonstrate that the addition of a 2:1 chloroform-methanol treatment prior to demineralization produces collagen extracts with the best isotopic and elemental measurements. The use of 30 kDa ultrafilters improved the quality of the collagen relative to the control treatment (0/0) but did not effectively or consistently remove a sufficient quantity of lipids from the sample, regardless of the initial lipid content (Figure 3). Therefore, while ultrafiltration may be useful for the removal of other low molecular weight contaminants in archaeological bone collagen, our findings indicate that ultrafiltration (30 kDa MWCO) alone

does not effectively remove lipids (0/UF vs. 0/0) and does not improve the results obtained from samples that have already been subjected to a chemical lipid extraction (CM/UF vs. CM/0).

Based on the results of this study, we recommend the inclusion of a chemical lipid extraction step during collagen extraction for all fish bones. The consistently and significantly high C:N ratios that have been observed in archaeological studies of fish bone collagen suggests lipid contamination may be an issue [3]. Our results demonstrate that the Folch method [24] involving 2:1 chloroform-methanol is sufficient to remove lipids from modern bone. There is no reason to believe that this method would not be effective at removing lipids from archaeological fish bone. The use of an ultrasonic bath aided greatly in the speed with which the lipids were precipitated into solution. We recommend treating the samples with 5−10 ml of 2:1 chloroform-methanol for 15−30 minutes, with the solution being refreshed until no material can be observed precipitating into the solution. The treatment of the samples with chloroform-methanol did not negatively impact the collagen in terms of the yield (aside from small amounts of sample that were lost when the solution was refreshed) or in terms of nitrogen isotopic compositions. Therefore, aside from the small increase in time there is no reason to exclude this step in collagen extraction procedures.

Although we did not perform any lipid extractions on mammalian bone, our results suggest that this step is not likely to be necessary for mammalian bone. Mammalian cortical bone contains a small portion of lipids (<5% by mass) and should therefore not be prone to the type of lipid contamination that might occur in fish. Our results suggest that lipid-driven skewing of fish bone collagen $\delta^{13}C$ values does not occur until the initial lipid content is above 5%, a concentration which is unlikely to be found in modern mammalian cortical bone tissue. It is therefore unlikely that the addition of a chloroform-methanol step would make any difference with respect to isotopic and elemental compositions of mammalian bone collagen.

Conclusion

Fish bones contain substantial quantities of lipids, much higher than those observed in mammalian bone. The collagen content of fish bone is lower than that of mammalian bone. The use of 30 kDa ultrafilters removes some lipids during collagen extraction but substantially reduces the collagen yield (37% loss of initial collagen mass). The addition of a chemical lipid extraction step (2:1 chloroform-methanol) prior to bone demineralization did not reduce collagen yield and resulted in improved elemental and isotopic measurements relative to a control treatment. The combination of the chloroform-methanol and ultrafiltration steps produced results that were superior relative to the collagen extraction protocol that used only ultrafiltration, but were indistinguishable to the protocol that used only chloroform-methanol for these modern fish bones. We recommend the inclusion of a chemical lipid extraction step when isolating collagen from modern and archaeological fish bones.

Acknowledgements

We thank Reba Macdonald and Joe Hepburn for technical assistance. We also thank Alison Harris, Christopher Britt, Edward Cumby, Jacob Jones, Tony Layton, and Stephen Guiry for generously providing some of the fish samples. Author contributions: PS and EG designed research. EG, PS performed the research. PS, EG and MR interpreted the data. PS and EG wrote the paper.

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Table 1. List of species and elements sampled for the present study.

Species		***n*[1]**	**Cleithrum**	**Angular**	**Dentary**	**Preopercle**	**Vertebra**
Barramundi	*Lates calcarifer*	1	✓				✓
Arctic char	*Salvelinus alpinus*	3	✓	✓			
Sockeye salmon	*Oncorhynchus nerka*	3	✓	✓			
Chinook salmon	*Oncorhynchus tshawytscha*	3		✓	✓		
Black rockfish	*Sebastes melanops*	1	✓	✓			
China rockfish	*Sebastes striata*	1	✓				✓
Pacific ocean perch	*Sebastes alutus*	1	✓	✓			
Redbanded rockfish	*Sebastes babcocki*	1	✓	✓			
Tiger rockfish	*Sebastes nigrocinctus*	1	✓	✓			
Lingcod	*Ophiodon elongates*	5	✓	✓			
Pacific halibut	*Hippoglossus stenolepis*	5		✓		✓	
Albacore	*Thunnus alalunga*	1	✓	✓			
Yelloweye rockfish	*Sebastes ruberrimus*	2	✓	✓			
Atlantic cod	*Gadus morhua*	3		✓	✓		
Golden pompano	*Trachinotus auratus*	1	✓				✓
Grass carp	*Ctenopharyngodon idella*	1	✓	✓			
Northern pike	*Esox Lucius*	3		✓	✓		

[1] Number of individual fish sampled for a given species.

Table 2. Summary of variable steps used in the four collagen extraction procedures used in this study.

Treatment		2:1 Chloroform:Methanol	30 kDa Ultrafilter
#	Name		
1	0/0	No	No
2	0/UF	No	Yes
3	CM/UF	Yes	Yes
4	CM/0	Yes	No

Figure 1. Ternary diagram of the three major components of bone by mass. Note that the mineral component also includes the mass of the bound and unbound water in the bone ultrastructure.

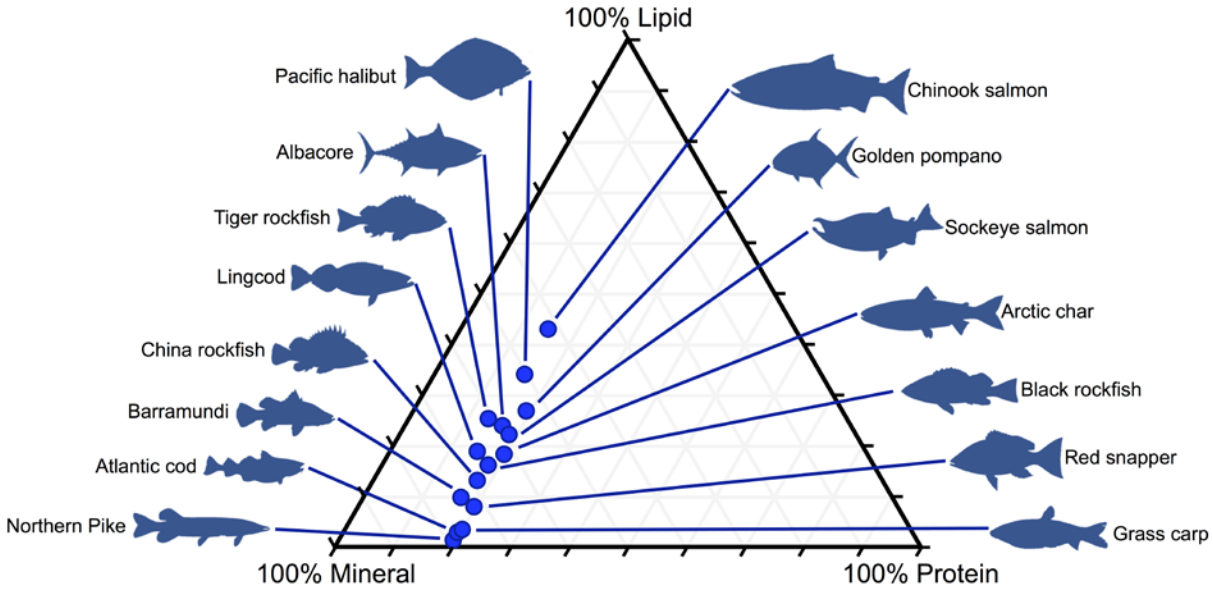

Figure 2. Boxplots for (A) C:N ratio, (B) %C, and (C) %N for the four treatments. Note that for the C:N ratios for Treatment 0/0, several outliers >5.5 have been excluded from the plot for the sake of clarity (5.55, 5.81, 6.61, 7.08, 8.04). The shaded horizontal bar in each plot represents the range of values calculated on the basis of the amino acid composition of Actinopterygii bone collagen (Szpak, 2011).

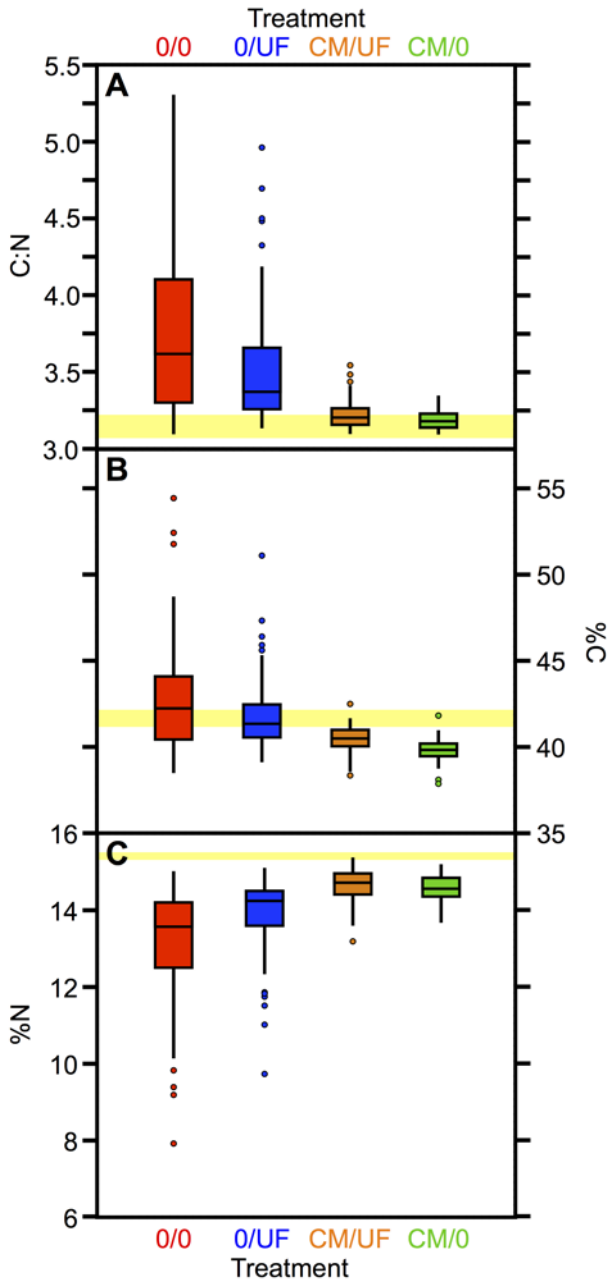

Figure 3. Dot matrix plot of C:N ratio for the four treatments. Each point is shaded on the basis of the lipid content of the initial sample. Open symbols indicate the initial amount of lipid could not be quantified, not that lipids were completely absent. Note that the scale on the y axis expands above 3.6 for the sake of readability.

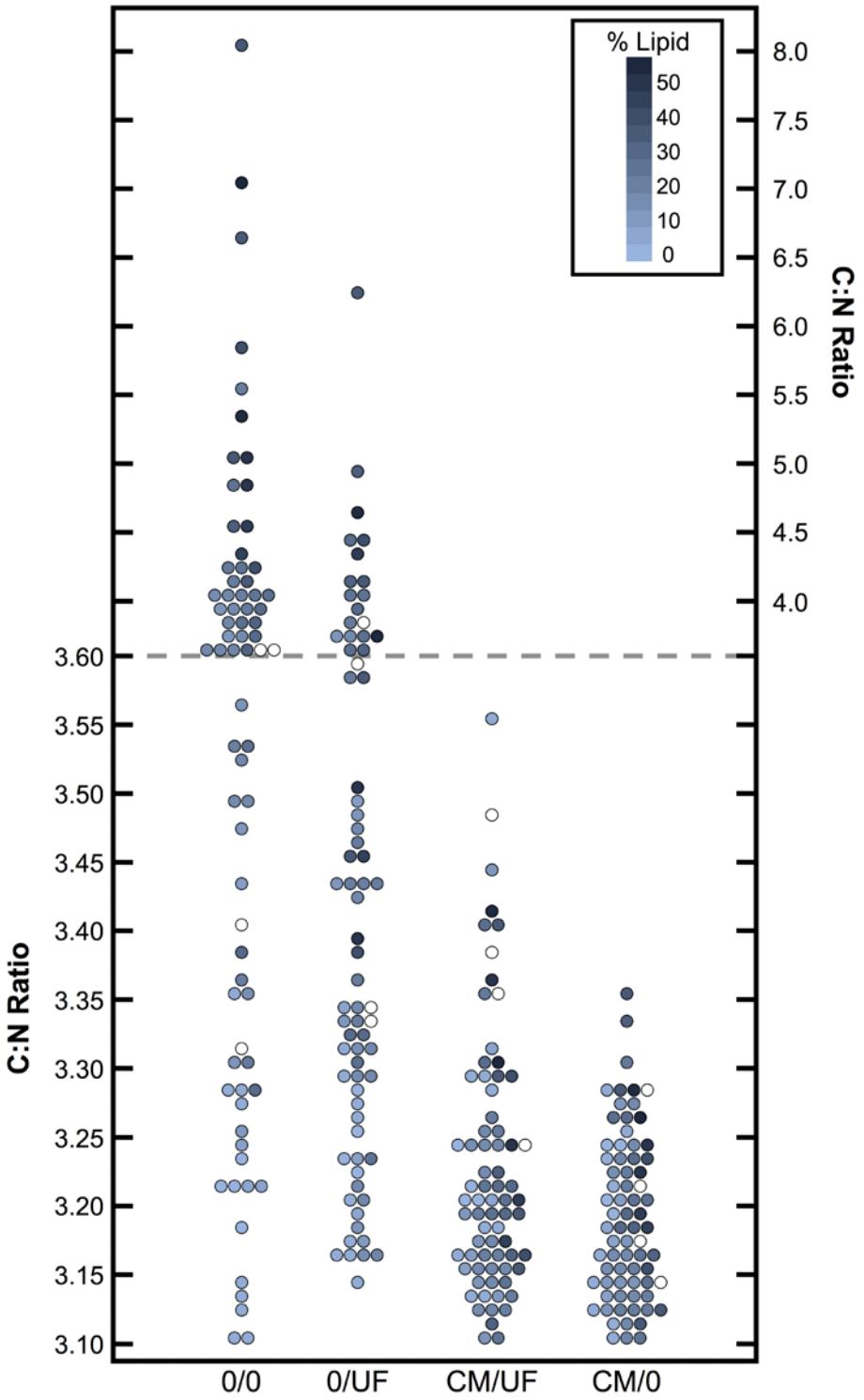

Figure 4. Relationship between initial lipid content and the difference in collagen $\delta^{13}C$ between the three treatments and the control: (A) difference in $\delta^{13}C$ between 0/UF and 0/0, (B) difference in $\delta^{13}C$ between CM/UF and 0/0, (C) difference in $\delta^{13}C$ between CM/0 and 0/0. Each point represents the mean and standard deviation for all samples binned at intervals of 5% for initial lipid content.

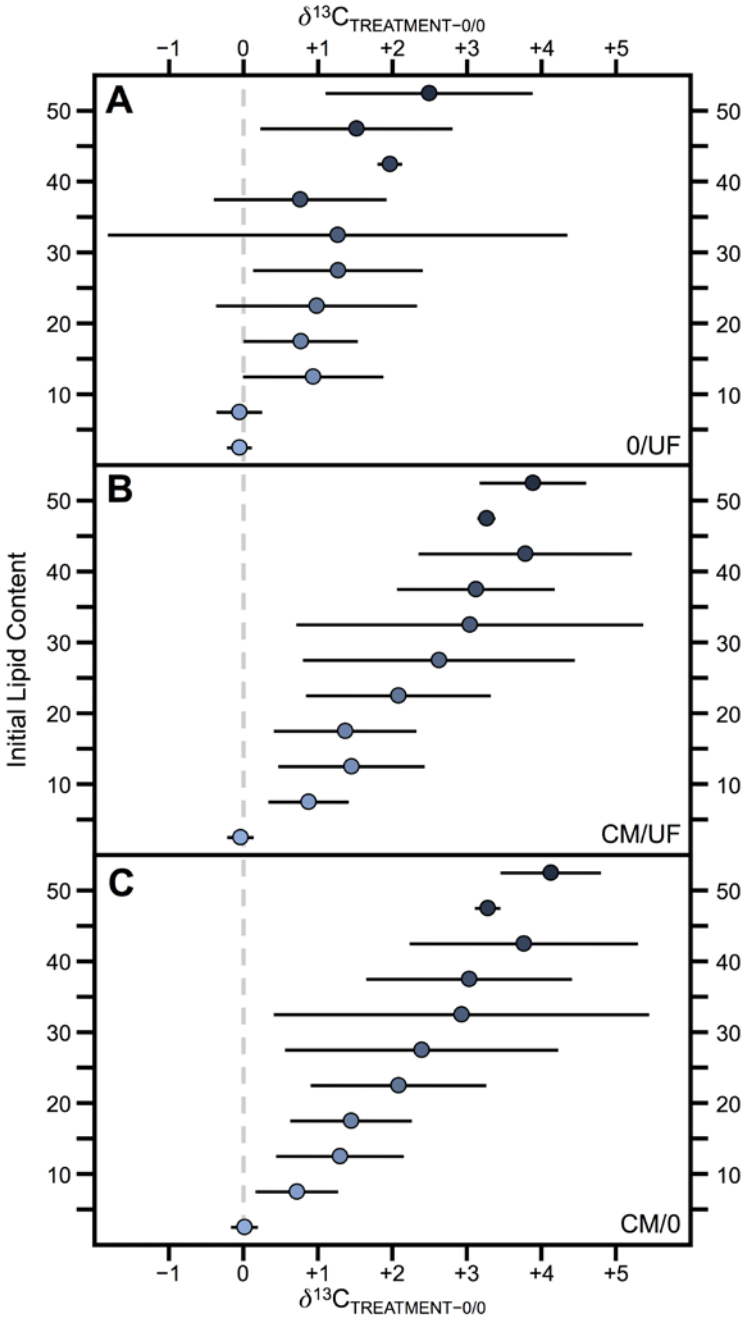

Figure 5. Amino acid compositions for fish bone collagen (residues/1,000 residues). Amino acid compositions for CM/0 (solid bars) and CM/UF (open bars) treatments for: (A) Pacific halibut preopercle, (B) albacore cleithrum, (C) grass carp angular, (D) China rockfish angular, (E) Atlantic cod dentary. The arrow in panels A-E indicates Asx. (F) Average differences in amino acid compositions (*n* residues) in collagen extracted using the CM/0 and CM/UF treatments.