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Effects of exposure to nixtamalization liquid on bone collagen $\delta^{13}C$ and $\delta^{15}N$ and archaeological implications

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ABSTRACT

The use of stable isotopes in zooarchaeology is common; however, the effects of many cooking and post-depositional processes on the chemical composition of faunal remains are understood poorly. People of the Americas processed maize through nixtamalization, a method of preparing grains by soaking and cooking them in an alkaline solution. Once discarded, nixtamalization wastewater may have contacted other food waste, such as bone. We examine the effects of alkaline exposure on stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) in fish bone collagen. Bony structures of four modern shortnose gar (*Lepisosteus platostomus*), a commonly identified taxon in eastern North American zooarchaeological assemblages, were exposed to four treatments that varied in alkalinity and duration of exposure. No significant differences were observed between treated and untreated specimens in $\delta^{13}C$ values. Prolonged exposure to a highly alkaline solution caused a shift in bone collagen $\delta^{15}N$ values of approximately -0.44‰. The extreme conditions required to cause this shift suggests that the byproduct of nixtamalization would have negligible effects on archaeological bone collagen $\delta^{13}C$ and $\delta^{15}N$ values.

1. Introduction

Analysis of stable isotopes is an established archaeological method often applied to inorganic materials and the remains of biological organisms to infer diets, migration patterns, subsistence practices, and paleoenvironments (e.g., Ambrose and Krigbaum, 2003; Bentley, 2006; Colaninno, 2012; France et al., 2018; Katzenberg and Waters-Rist, 2018; Reitsema et al., 2015; Robinson and Wadley, 2018; Schoeninger and Moore, 1992). Stable isotopic analyses of osteological materials, typically bone collagen and bone bioapatite, are used to reconstruct the diets of people and animals in the past, as well as aspects of paleoenvironments (DeNiro, 1985; DeNiro and Epstein, 1978, 1981; Guiry et al., 2016; Schoeninger et al., 1983). Stable carbon and nitrogen isotope values in bone collagen reflect the isotopic signatures of the diet of the individual examined (Katzenberg and Waters-Rist, 2018; Schoeninger, 2010; Schoeninger and Moore, 1992). Stable carbon isotopes ($\delta^{13}C$) of organic tissues of animals reflect the isotopic composition of the organic carbon at the base of their food web, with

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predictable levels of isotopic fractionation during metabolic processes (Peterson and Fry, 1987). This makes it possible to examine the types of plants people and animals consumed such as, C_3 plants, plants that undergo C_3 photosynthesis by converting CO_2 into three-carbon molecules (C_3), C_4 plants, those that undergo C_4 photosynthesis converting CO_2 into four-carbon molecules (C), and CAM (crassulacean acid metabolism) plants, plants that use either C_3 or C_4 photosynthesis depending on environmental condition (van der Merwe, 1982). Stable nitrogen isotopes ($\delta^{15}N$) reflect the animal's trophic position (DeNiro and Epstein, 1978, 1981; Minagawa and Wada, 1984). Both $\delta^{13}C$ and $\delta^{15}N$ also are sensitive to environmental variables, including aridity, elevation, temperature, sea-spray/salinity, and vegetation cover, among others (Amundson et al., 2003; Britton et al., 2008; Buchmann et al., 1996; Stewart et al., 1995; Troughton and Card, 1975; van de Water et al., 2002). Together, isotopic analyses of animal tissues can be used to investigate the sources of carbon and nitrogen supporting an ecosystem, e.g., terrestrial versus marine, or C_3 versus C_4 terrestrial plants (DeNiro and Epstein, 1978; Farquhar et al., 1989; Rounick and Winterbourn, 1986).

Isotopic research on zooarchaeological remains rests on the assumption that isotopic ratios remain unaltered from the organism's death to recovery. This assumption does not always hold true (Ambrose, 1990; Andrus and Crowe, 2002; DeNiro et al., 1985; Fernandes et al., 2014; Szpak et al., 2017; van Klinken and Hedges, 1995). Controlled experiments indicate that certain pre- and post-depositional processes cause isotopic fractionation in osteological animal remains, with the potential to skew interpretations derived from these values. For example, DeNiro et al. (1985) noted 5‰ and 4‰ enrichment in ^{13}C and ^{15}N respectively in bone collagen after samples were subjected to extreme heat (200 °C) for an extended period of time (12 h). Less intense heat exposure and indirect heating, characteristics of many common cooking practices like boiling, roasting and grilling carcasses over open flame, and steaming flesh, does not alter the stable isotopic compositions of animal bone collagen beyond the limits of measurement precision (DeNiro et al., 1985; Fernandes et al., 2014).

Although researchers have tested effects of some cooking methods and post-depositional processes on stable isotopic ratios of food waste (Andrus and Crowe, 2002; DeNiro et al., 1985; Fernandes et al., 2014; Szpak et al., 2017), the effects of many traditional food preparation and discard practices remain untested. While there are many unknowns in the taphonomic history of the bone specimens that are sampled for stable isotope assays, the effects of food preparation and discard practices demand further attention.

Here we examine the effects of nixtamalization wastewater on zooarchaeological remains. The practice of nixtamalization, processing maize kernels with an alkaline solution, was widespread among indigenous populations in the Americas who practiced intensive maize agriculture (Katz et al., 1974). Nixtamalization was a common food preparation method prior to European contact and continues today (Hart and Lovis, 2013; Katz et al., 1974; Lovis et al., 2011; Upton et al., 2015). In this process, people soak and boil dried maize kernels in an alkaline solution to improve its nutritional content (Beck, 2001; Katz et al., 1974). The alkaline reagent used varied depending on region and available resources, with people using lime ($Ca(OH)_2$), wood ash (KOH), or lye (NaOH) (Katz et al., 1974). Nixtamalization produces large amounts of wastewater, called nejayote. Today, industrial-scale nixtamalization causes serious pollution problems due to the high pH and biological oxygen demand imparted by organic solids (Castro-Muñoz and Yáñez-Fernández, 2015; Gutiérrez-Uribe et al., 2010; Meraz et al., 2016; Rojas-García et al., 2012). Nejayote retains an estimated 80% of the alkaline reagent originally used during the cooking process and has a pH $>$ 10, making it a difficult wastewater to treat (Gutiérrez-Uribe et al., 2010).

There is little record of how nejayote from small-scale production was discarded in the past. To date there has been no archaeological investigation of nejayote discard practices. An ethnographic account from a modern-day Maya village is informative: one landowner's habit of throwing out the putrid wastewater in his backyard was considered a source of bad odors, insects, and diseases that threatened the welfare of the entire community (Re Cruz, 1996: 65). Given its unpleasant smell and other harmful properties, it is reasonable to assume that people typically disposed of nixtamalization wastewater in a manner similar to other malodorous and noxious waste: in middens, located a safe distance from their homes. If this was the case then many other types of food waste, including bone, would likewise have been exposed to the strongly alkaline solution.

Bone collagen may be susceptible to degradation if exposed to nejayote wastewater. Type I collagen is a trimeric molecule with three left-handed polyproline II helices that twist around each other to form a right-handed triple helix. Amino acids are the structural units that form short polymer chains called peptides. The triple-helical conformation requires a polypeptide sequence with the amino acid glycine (Gly) at every third position in a repeating pattern of the form $(X–Y–Gly)_n$, with the X and Y positions frequently occupied by proline (Pro) and hydroxyproline (Hyp), respectively (Bornstein and Traub, 1979; Kielty and Grant, 2002). Chemical hydrolysis of the peptide bonds is a key process underlying collagen diagenesis. Hydrolysis occurs at random positions along the polypeptide chains, and is catalysed by both hydroxyl (OH-) and hydrogen (H+) ions, with the greatest rates observed at high temperature and pH (Berry et al., 1989; Collins et al., 1995; Rudakova and Zaikov, 1987).

Berry et al. (1989) demonstrated that collagen molecules break down to a mixture of peptides with molecular weights (MW) $<$ 20,000 Da at NaOH concentrations over 0.25 M, with rapid degradation observed at 37 °C. Thus, archaeological bones with prolonged exposure to the strongly alkaline nejayote are expected to have lower collagen yields compared to bones without exposure. The loss of low-MW peptides may alter the relative distribution of amino acids, thereby altering the isotopic makeup of the degraded collagen (e.g., Pinnegar and Polunin, 1999).

We conducted an experiment to test for effects of exposure to alkaline solutions on $\delta^{13}C$ and $\delta^{15}N$ of collagen from fish remains, which are common in archaeological middens. We chose to work with osteological remains of shortnose gar (*Lepisosteus platostomus*), because this species is distributed widely throughout the eastern two-thirds of the United States. They are a top predator among riverine systems, where they opportunistically feed on crayfish, insects, invertebrates, and other fishes (Sutton et al., 2009). A recent study indicates that shortnose gar can live as long as 15 years (King et al., 2018) and individuals have been documented at maximum total lengths of approximately 800 mm (Lee et al., 1980). Gar bones and scales are common in archaeological contexts throughout their range (Colaninno et al., 2017; Martin and Masulis, 1993; Neverett, 2001; Styles, 1981), recovered from middens and ritualized contexts, suggesting that people of the past used this species for purposes beyond that of food (Pauketat et al., 2002; Peres and Deter-Wolf, 2016). Gar have a large number of bones and thousands of bony scales per individual, making this an ideal species to work with for isotopic studies requiring multiple bone collagen samples from a single individual.

2. Experimental design

Our experimental design involved four treatments and a set of control samples. Each treatment exposed shortnose gar vertebrae, scales, and a fleshy segment of the gar carcass that included embedded vertebrae and attached scales to an alkaline solution with varying concentrations and time durations. The four experimental treatments were: Treatment 1) 50% concentration alkaline solution (pH of 8) for 24 h; Treatment 2) 100% alkaline solution concentration (pH of 11) for 24 h; Treatment 3) 50% concentration alkaline solution (pH of 8) for 174 h; and Treatment 4) 100% alkaline solution concentration (pH of 11) for 174 h (Table 1).

Table 1
Parameters of experimental design.

Each treatment contained isolated vertebrae, isolated scales, and articulated vertebrae and scales in fleshy quarters of the individual gar. Low concentration refers to alkaline solution at 50% concentration measuring pH 8 and high concentration was not diluted, measuring pH 11. Short duration is 24 h and long duration is 174 h.	
Treatment 1: Low concentration and short duration	Treatment 2: High concentration and short duration
Treatment 3: Low concentration and long duration	Treatment 4: High concentration and long duration

We selected these concentrations and exposure times to simulate a range of possible scenarios where bone may be subjected to discarded nixtamalization liquid. For example, nixtamalization wastewater may have been diluted prior to or during discard, reducing its pH; hence, our experimental parameters for treatments 1 and 3. We also suspect that bones could have contacted wastewater for a brief period, held in a vessel for a short time (24 h) with other trash, including diluted or undiluted nixtamalization wastewater, prior to tossing all the day's waste (treatment 1 and 2). We also envisioned scenarios where nixtamalization wastewater directly contacted bone for long periods of time, for example, if people discarded bone and wastewater in a lined pit (treatments 3 and 4). Because detailed ethnographic accounts of nejayote disposal practices are limited, we aimed to design treatments that simulated possible disposal scenarios, while confining variability in the experimental design and replicating each treatment with multiple samples.

We exposed the vertebrae, scales, and fleshy segments of four gar individuals, resulting in four samples (isolated vertebrae, isolated scales, vertebrae from flesh, and scales from flesh), per treatment (four treatments), per individual gar (4 individuals) for a projected total of 64 experimental samples, with one sample lost during handling, resulting in 63 experimental samples. We also extracted four samples of vertebrae and scales from each gar prior to experimental treatment resulting in 32 control samples or 8 control samples per fish. All shortnose gar were collected from the Mississippi River near La Crosse, Wisconsin, USA, in June 2017 under Institutional Animal Care and Use Committee (IACUC) reference #14151.

3. Methods

We prepared our nixtamalization liquid following ethnographic accounts of how people of the eastern United States made their alkaline reagent for maize processing (Katz et al., 1974). We used the ashes of hardwood (0.5 kg) that had been combusted and allowed to cool completely. We then passed 4.7 L of water through the ash two times, collecting the water as it drained through the ash after both passes. The resulting solution was alkaline, with a pH of 11.

Prior to treatment, the bodies of the four gar individuals were quartered: one quarter per treatment. For our control samples, we removed vertebra samples and scale samples weighing at least 1 g from each quarter. Gar quarters undergoing the alkaline treatment were baked based on ethnographic accounts that Native Americans consumed gar cooked, rather than raw (Bartram, 2001: 176). The fish quarters were cooked in a dry oven at 95 °C until internal temperatures reached between 65 and 75 °C. Prior research indicates isotopic composition of bone collagen is unaffected by temperatures under 200 °C (DeNiro et al., 1985). After quarters were baked, we extracted 1 g of vertebrae and scales and retained each fleshy quarter with articulated vertebrae and scales for the experimental treatment. For those isolated vertebrae and scales, both control and experimental specimens, we removed all adhering flesh through water maceration and then mechanically with scalpel and forceps. The experimental, cleaned vertebrae and scales from each quarter were placed into test tubes with enough nixtamalization liquid to cover the sample. Flesh quarters were placed in glass jars with solution covering the sample. All experimental treatment samples were covered to prevent outside contamination.

At the termination of all treatments (24 h for treatments 1 and 2 and 174 h for treatments 3 and 4), we decanted the samples and rinsed them with distilled water until added water reached neutral pH. For fleshed samples, we removed all remaining vertebrae and 1 g of scales from the carcass of the gar and rinsed to neutral. These vertebrae and scales were then completely defleshed following the same methods used for the control samples and isolated, experimental vertebrae and scales. All treatment samples then were dried in an oven (80 °C) for 4 h.

The dried bone subsamples were mechanically crushed to reduce whole bones into smaller fragments of approximately 3–5 mm in size and transferred to clean borosilicate test tubes and treated with organic solvents to remove lipids, not normally present in zooarchaeological remains. Each sample was covered with a 2:1 mixture of chloroform/methanol and placed in a warm water bath (50 °C) for 30 min to extract lipids (Folch et al., 1957). The lipid-containing organic solvent then was discarded, and the process repeated, until the organic solvent remained colorless. The samples were then treated with acetone for 30 min in a warm water bath (50 °C) to remove any residual acetone-soluble lipids (Lovern and Olley, 1953). The acetone was decanted, and the samples were allowed to dry in a warm (80 °C) oven for 4 h.

Bone collagen extraction followed a modified Longin (1971) technique. The samples were demineralized in 5–10 mL of cold (2 °C) 1 N HCl for at least 48 h until the bone samples were soft when pressed against the side of the test tube. The demineralizing HCl was decanted from the test tubes and the bone samples were rinsed with distilled water until the pH of the added water reached neutral. We then covered the bone samples with 0.1 M NaOH for approximately 1–2 min. This solution was then decanted and discarded, and the samples were rinsed with distilled water until the added water reached neutral. This was followed by a treatment of 1 N HCl to eliminate atmospheric CO_2. Again, the samples were rinsed with distilled water until they reached a slightly acidic pH (pH = 4). We then covered the samples and heated them to approximately 80 °C for 96 h. Every 24 h, we evaluated the pH of the samples and added a drop of 1 N HCl if the solution measured pH 5 or greater. The resulting acid solution contained suspended bone collagen, which we then filtered through Ezee filters to isolate the total acid insoluble fraction ("collagen"). The remaining water in the samples was evaporated by warming the filtered solution to 80 °C for at least 5 h, until completely dry.

Prepared collagen samples were analyzed on an elemental analyzer-isotope ratio mass spectrometer (EA-IRMS) at the University of Georgia (UGA) Center for Applied Isotope Studies (CAIS). Values are expressed as $\delta^{13}C$ with respect to PDB and $\delta^{15}N$ with respect to AIR. Replicate analyses of internal standards included NIST SRM 1577 bovine liver (consensus values: $\delta^{15}N = +7.95‰$ and $\delta^{13}C = -17.58$; measured values ± 1σ: $\delta^{15}N = +7.95 \pm 0.07‰$ and $\delta^{13}C = -17.59 \pm 0.04‰$) and NIST SRM 1570 spinach (consensus values: $\delta^{15}N = -0.34‰$ and $\delta^{13}C = -27.30$; measured values ± 1σ: $\delta^{15}N = -0.39 \pm 0.04‰$ and $\delta^{13}C = -27.20 \pm 0.07‰$).

To statistically test if the $\delta^{13}C$ and $\delta^{15}N$ values of treatment samples were affected by exposure to the alkaline solution, we conducted a one sample *t*-test on the difference in the means between control and treatment values, with zero as the test value, indicating no significant difference between the difference in the mean of the control subtracted from the mean of the treatment and zero. In this analysis, a difference in the means between the control and treatment that results in a positive value indicates that the treatment decreased the isotopic values, where a negative value would indicate that the treatment increased the isotopic value. We visually compared the differences in the means between control and the four treatments using box-and-whisker plots indicating

Table 2
Descriptive statistics for control and treatment samples.

	n	Average % collagen yield	Average total % carbon	Average total % nitrogen	Average C:N ratio
Control	32	12.39	26.68	10.08	3.1
Treatment 1	16	4.66	32.20	11.93	3.1
Treatment 2	16	14.73	19.12	7.01	3.2
Treatment 3	16	15.03	22.36	8.24	3.2
Treatment 4	15	4.62	26.54	10.22	3.0

the minima, median, maxima, and quartiles of observed values.

4. Results

4.1. Observations from nixtamalization solution treatment

We noted few changes in the appearance or texture and hardness of vertebrae and scales exposed to the alkaline solution, regardless of treatment. Macroscopically, none of the samples showed physical evidence of bone diagenesis such as deterioration, distortion or textural changes as such a brittle or chalky texture (High et al., 2015). The pH of the alkaline solution did change from the start of the experiment to the end for treatments 2, 3, and 4, but not treatment 1, which began the experiment measuring a pH of 8 and after 24 h, remained at pH 8. Treatment 2 dropped from a pH of 11 to start, ending with a pH of 9 after 24 h. Treatment 3 measured between 7 and 8 pH after 174 h, while treatment 4 measured between 7.5 and 9 after 174 h and the solution of treatment 4 turned bright yellowish green in color.

The average percent collagen yield for our controls and treatments varied (Table 2), as did the percent carbon, nitrogen, and atomic ratio of C:N (Table 2). Notably, treatments 1 and 4 had low collagen yields. We believe that for treatment 1, we did not allow enough time for the collagen to fully become suspended, resulting in lower yields. This was not the case for treatment 4, where we allowed collagen enough time to fully become suspended. All averaged C:N ratios for the control and treatment samples are within established norms (Ambrose, 1990; DeNiro et al., 1985; Szpak et al., 2017).

4.2. Results: $\delta^{13}C$ and $\delta^{15}N$ values for vertebrae versus scales and isolated versus articulated

An underlying question that developed during our analysis was whether there would be differences in $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen extracted from (1) vertebrae compared to scales, and (2) isolated elements versus articulated elements. To test this, we ran an independent sample *t*-test comparing the mean of the vertebrae and the mean of the scale values for our control among all sampled fishes for both $\delta^{13}C$ and $\delta^{15}N$. Results indicated no significant differences between scales and vertebrae, or between articulated and isolated elements, in terms of either $\delta^{13}C$ or $\delta^{15}N$ of bone collagen (Table 3). As such, isotopic data from these skeletal elements are combined in subsequent statistical analyses.

4.3. Results: $\delta^{13}C$ and $\delta^{15}N$ values for gar specimens

$\delta^{13}C$ and $\delta^{15}N$ control values (i.e., no alkaline exposure) for the four modern gar individuals are presented in Table 4. $\delta^{13}C$ values of bone collagen from vertebrae and scales ranged from −23.61‰ to −20.81‰. $\delta^{15}N$ values ranged between 11.87‰ to 16.78‰.

4.4. Results: difference in the control and treatment means

For all samples, we found no significant differences between the mean of the control samples and those samples exposed to the alkaline treatment in terms of $\delta^{13}C$ ($p \geq 0.228$, Fig. 1; Table 5). For all four

Table 3
Descriptive statistics and results of independent sample t-test for control vertebra and scale samples and isolated and articulated samples.

Vertebrae and scale samples						
Carbon	*n*	x̄	s	*t*	*df*	*p*
Vertebrae	16	−22.09	1.03	0.187	30	0.853
Scales	16	−22.17	1.51			
Nitrogen	*n*	x̄	s	*t*	*df*	*p*
Vertebrae	16	15.16	1.96	−0.394	30	0.697
Scales	16	15.45	2.23			

Isolated and articulated samples						
Carbon	*n*	x̄	s	*t*	*df*	*p*
Isolated	32	−22.20	1.22	−0.026	61	0.980
Articulated	31	−22.19	1.20			
Nitrogen	*n*	x̄	s	*t*	*df*	*p*
Isolated	32	15.08	1.97	0.074	61	0.941
Articulated	31	15.04	1.99			

Table 4
Descriptive statistics for control $\delta^{13}C$ and $\delta^{15}N$ values for modern gar.

	x̄ $\delta^{13}C$	s $\delta^{13}C$	x̄ $\delta^{15}N$	s $\delta^{15}N$
Gar 0001	−21.33	0.10	16.78	0.62
Gar 0002	−22.76	0.23	15.91	0.15
Gar 0005	−23.61	0.16	11.87	0.10
Gar 0006	−20.81	0.23	16.67	0.34

experimental treatments, the average difference between the $\delta^{13}C$ of the control sample and the $\delta^{13}C$ of the experimental samples were not statistically indistinguishable from zero (Fig. 1).

In terms of $\delta^{15}N$, we observed no difference between the control and experimental treatments for treatments 1, 2, and 3 (Fig. 2). However, the difference between the control and experimental treatment 4 is significantly different from zero ($p = 0.025$), with an average depletion of ^{15}N of 0.44‰. With the exception of treatment 2, the alkaline treatment value is slightly more negative than the mean of the control, indicating that the treatment caused a minor depletion in ^{15}N of bone collagen.

5. Discussion

Our results indicate that $\delta^{13}C$ values of fish bone collagen are not affected by the experimental treatments simulating exposure to the discarded alkaline byproduct from the nixtamalization process. Regardless of alkaline concentration or duration of exposure (up to 174 h), the differences in the means of our $\delta^{13}C$ control from the treatment samples were not statistically different from zero. $\delta^{15}N$ values did show greater effects. Specifically, the differences of all $\delta^{15}N$ means compared to the control trended towards more positive values, indicating that the treatment caused a slight depletion in ^{15}N. Further, the range of the differences are greater than zero for all treatments with the exception of treatment 2. This indicates that $\delta^{15}N$ values for our treatment samples tended to cause a slight depletion in ^{15}N, but this shift is minor. The most extreme example is treatment 4, where the difference in the means between the control and treatment samples is 0.44‰, which would be a treatment effect of −0.44‰ for $\delta^{15}N$.

The low collagen yield associated with treatment 4 suggests degradation had occurred. Low-MW peptides likely were lost via alkaline hydrolysis (Berry et al., 1989). The amino acid composition of the remaining degraded collagen would not mirror the composition of undegraded collagen because the solubility of individual amino acids in water is governed by pH and temperature (Brown and Rousseau, 1994; Carta and Tola, 1996; Gupta and Heidemann, 1990; Pradhan and Vera, 1998; Tseng et al., 2009). Alkaline hydrolysis could alter the isotopic

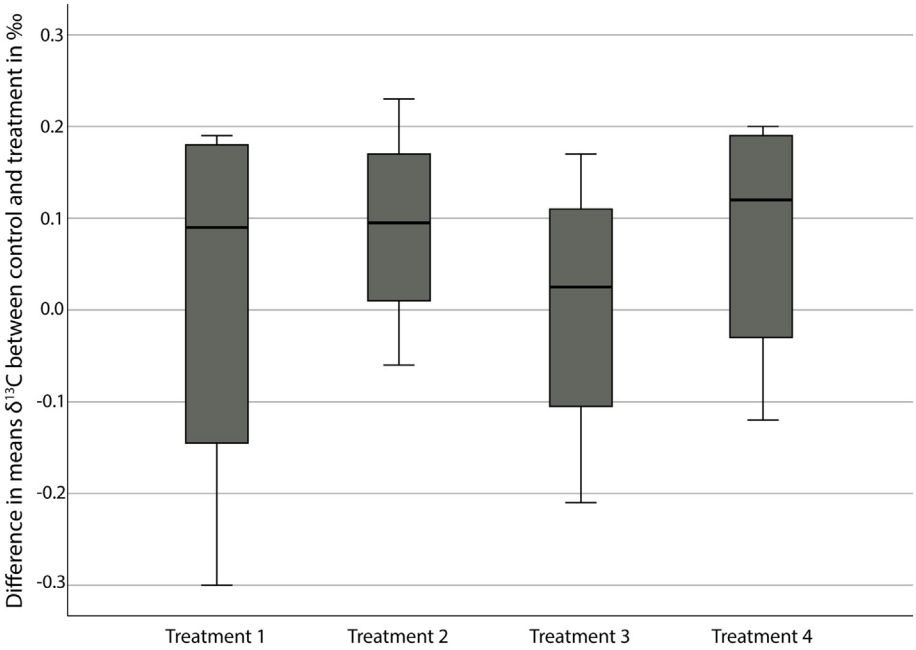

Fig. 1. Box plot of difference in the means between $\delta^{13}C$ for the control and treatment samples.

Table 5
Inferential statistics of one sample test-t comparing the differences in the control mean and treatment mean to zero.

Carbon	*n*	*t*	*df*	*p*	Difference in the mean (control-treatment)	s
Treatment 1	4	0.155	3	0.887	0.02	0.23
Treatment 2	4	1.511	3	0.228	0.09	0.12
Treatment 3	4	0.032	3	0.977	0.00	0.16
Treatment 4	4	1.089	3	0.356	0.08	0.15

Nitrogen	*n*	*t*	*df*	*p*	Difference in the mean (control-treatment)	s
Treatment 1	4	2.720	3	0.073	0.15	0.05
Treatment 2	4	1.404	3	0.255	0.15	0.22
Treatment 3	4	1.552	3	0.218	0.13	0.17
Treatment 4	4	4.178	3	0.025	0.44	0.21

makeup of the degraded collagen by altering its amino acid composition. The differential loss of any amino acids that exhibit high $\delta^{15}N$ values would result in collagen that is depleted in ^{15}N compared to bulk collagen. For example, the non-essential amino acids, including Gly, Pro, and Hyp, among others, are synthesized or modified within the body and are enriched in ^{15}N compared to the dietary source, whereas essential amino acids exhibit little change compared to the dietary source (Gaebler et al., 1966). Glutamic acid (Glu) and alanine (Ala) in particular are the most enriched in ^{15}N in fishes compared to diet (McMahon et al., 2015). The differential loss of any of these relatively ^{15}N-enriched amino acids would result in lowered $\delta^{15}N$ values in the degraded bulk collagen.

We caution that although the shift of −0.44‰ in $\delta^{15}N$ values is statistically significant, it may not hold much interpretive value. There is a well-known trophic level effect among biological organisms, which manifests as a 3‰ enrichment in ^{15}N per trophic level (DeNiro and Epstein, 1981; Schoeninger and Moore, 1992). Additionally, many modern-day aquatic organisms may be enriched in ^{15}N due to wastewater and fertilizer run-off which is known to increase $\delta^{15}N$ values of bone collagen in fishes by at least 2‰. This enrichment effect would obscure any ^{15}N depletion from alkaline exposure; however, this 2‰ enrichment should only affect modern fishes and extracted bone collagen (Brugam et al., 2017). Further, we observed a 4‰ range of variability among control samples from modern individuals. Even within a single individual (gar specimen 0001), $\delta^{15}N$ values of the eight control samples display > 0.45‰ variation. Alkaline treatment, even under the most extreme conditions, did not alter $\delta^{13}C$ and $\delta^{15}N$ outside the range of normal variation observed among individuals within this population.

Under normal patterns of discard, we foresee very few situations where bones would be subjected to more intensive alkaline exposure for greater lengths of time unless people directly placed meat with bones, carcasses, or bony food waste in an alkaline solution. We have yet to test the effects of extremely prolonged exposure (> a month) to a highly basic solution on the isotopic composition of bone collagen. Given that we cannot find evidence that meat and fish-based foods were processed for consumption in an alkaline solution, we suspect that this was not a common practice. Hence, the parameters of our experiment should reflect what bones might be exposed to under normal and even extreme cases. That is, if people discarded nixtamalization wastewater in the same areas where they discarded food waste and bones, these bones likely were not exposed to parameters more extreme compared to treatment 4; that is, a higher concentration of an alkaline solution (pH > 11) for an extended period of time (> 174 h). Our experimental design did not test if bone collagen would be affected by exposure to high alkalinity while in a soil or shell matrix. The different soil types may interact with an introduced alkaline solution differently creating unique chemical conditions for osteological remains. We did not test for these conditions and see this as an area of future research.

Common methods for bone collagen extraction and pretreatment includes demineralizing bone using an HCl dilution and then removing humics with diluted NaOH: an alkaline solution with a pH of 14 (Longin, 1971; Pestle, 2010; Szpak et al., 2017). Researchers have questioned whether collagen extraction and pretreatment protocols effect $\delta^{13}C$ and $\delta^{15}N$ (Pestle, 2010; Szpak et al., 2017). Though we did subject all bones to identical collagen extraction and pretreatment protocols, bone and scale samples in treatments 2 and 4 were exposed to a high concentration of an alkaline agent (pH = 11) for a duration far exceeding that of pretreatment alkaline exposure. As others have suggested (Szpak et al., 2017), short duration of exposure (i.e., < 24 h) to an alkaline solution such as NaOH, and in our experimental case KOH, did not alter $\delta^{13}C$ and $\delta^{15}N$ compared to the control samples. This

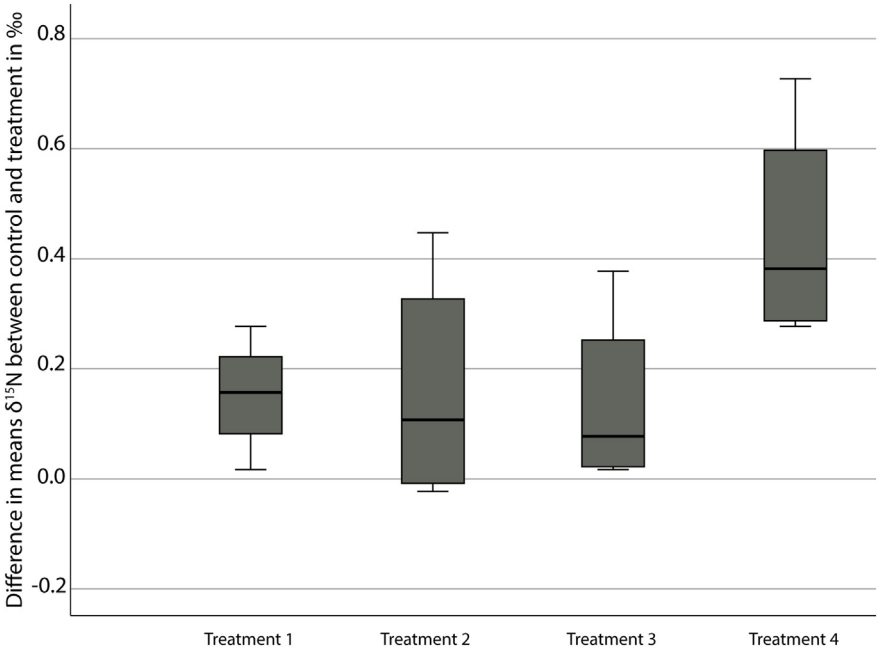

Fig. 2. Box plot of difference in the means between $\delta^{15}N$ for the control and treatment samples.

indicates that short exposure to an alkaline solution (< 24 h) as a pretreatment does not alter isotopic composition beyond that of established pretreatment and extraction protocols (Longin, 1971) and may lend support to studies concluding that NaOH pretreatment does not affect the isotopic composition of endogenous collagen (Szpak et al., 2017).

What is observed in our treatment samples is even under extreme conditions, neither $\delta^{13}C$ and $\delta^{15}N$ values were altered outside the range of normal variation for this species from our sample population. This pattern, particularly the effect of the treatment that is within the range of variation in the population, may not hold true for organisms with a very limited and restricted diet. If a taxon intakes a very restricted range of carbon and nitrogen through their feeding behaviors, we would expect that the $\delta^{13}C$ and $\delta^{15}N$ of bone collagen from such taxon would not vary greatly and exposure to an alkaline solution could impact interpretations of isotopic values. Like all studies that use isotopic values of organisms to make interpretations of past human and animal behavior, it is important to understand how isotopic values in modern populations vary and are impacted by processes that could alter the original isotopic ratios.

6. Conclusion

Our results indicate that $\delta^{13}C$ and $\delta^{15}N$ values of fish bone collagen are not significantly affected when exposed to an alkaline solution for a short duration (≤ 24 h). Extended exposure to an alkaline solution at full concentration causes a slight depletion of ^{15}N of bone collagen, but this depletion is within the range of $\delta^{15}N$ variability of the sample population and likely would not lead to interpretative changes.

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Appendix A. Supplementary data

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