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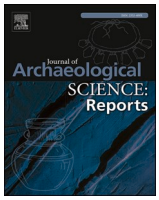

Does exposure to heat alter stable isotopes in ostrich eggshell? A controlled experiment and analysis☆

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ABSTRACT

Little research explores the effect of heat on the isotopic composition of ostrich eggshell (OES), though several studies use stable isotopes in OES to assist in paleoclimatic reconstruction. Archaeological OES often shows signs of heat exposure, though distinguishing lower-temperature exposure remains challenging. This controlled study uses modern OES heated in an electric kiln to examine if exposure to low through high degrees of heat shifts $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values in the organic and mineral components of ostrich eggshell. Results indicate that the total organic portion of the shell conserves original isotopic signatures with exposure below ~220 °C, while the mineral portion conserves signatures up to 500 °C. Additionally, the study provides C:N ratios as a reliability criterion for the organic portion of archaeological OES. Data produced in this study allows for a more discriminating selection of archaeological samples for paleoclimatic reconstructions.

1. Introduction

Ostrich eggshell (OES) is found in many archaeological sites in Africa and Asia, most often in the form of fragments discarded after the nutritious part of the egg has been consumed. Researchers use the isotopic composition of OES to reconstruct regional paleoclimatic conditions (Ecker, 2015; Janz et al., 2009; Johnson, 1997; Lee-Thorp and Ecker, 2015; Niespolo, 2020; Wriston and Haynes, 2013; Routledge, 2020; Johnson, 1995). Paleoclimatic reconstructions derived from OES rely heavily on carbon (expressed as $\delta^{13}C$) and nitrogen (expressed as $\delta^{15}N$) isotopes in the total organic fraction (TOF) fraction, and carbon and oxygen (expressed as $\delta^{18}O$) isotopes in the mineral (carbonate) fraction (Ecker, 2015; Johnson, 1997; Lee-Thorp and Ecker, 2015; Niespolo, 2020; Wriston and Haynes, 2013; Routledge, 2020; Johnson, 1995; Johnson et al., 1998). Archaeological OES often exhibits variation in colors suggesting some degree of heat exposure, so assessing the extent to which thermal alteration biases these isotopic signals is critical for ensuring the integrity of paleoenvironmental interpretations.

Most previous studies rely on stable isotopes of carbon and oxygen within the mineral portion of the shell, but more recent studies analyze carbon and nitrogen isotopic composition in the organic portion of the shell to reconstruct localized microclimates at archaeological sites in southern and eastern Africa (Ecker, 2015; Johnson, 1997; Lee-Thorp and Ecker, 2015; Niespolo, 2020; Johnson, 1995). While some studies include images of the fragments selected for analysis, many do not, and the condition of the fragments remains ambiguous. Oftentimes, OES is discolored and darkened, indicating pieces may have been exposed to high degrees of heat, either from cooking or from secondary hearth fires (Collins and Steele, 2017; Diehl et al., 2022; Janssen et al., 2011). Other times the discoloration is lighter, making it difficult to discern whether the shell had been exposed to more moderate amounts of heat. This type of heat exposure produces only slight visual evidence, which may appear similar to sediment staining because the change is only a slight darkening of the creamy ivory color of the natural eggshell.

To date, there is little work investigating whether $\delta^{13}C$ and $\delta^{15}N$ values in heated fragments of OES are affected by heat exposure (Johnson, 1995; Hodgkins, 2018; Miller, 2016). Johnson (Johnson, 1995) and colleagues used heat to simulate diagenesis in the mineral portion of OES [page 121–160]. To achieve this, they exposed modern shell to low degrees of heat (<150 °C) over long periods of time (up to 8 weeks) (Johnson, 1995). They found that the $\delta^{13}C$ value of the total organic fraction increased 0.5 ‰, with the change occurring early in the heating process; once changed the values remained consistent through the remaining 500 h of heat exposure (Johnson, 1995). While some researchers have documented the relationship between OES color changes and heating temperatures (Collins and Steele, 2017; Texier,

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2013; Texier, 2010), a systematic study on the effects of high degrees of heat on the C, N, and O isotopes in ostrich eggshell, similar to what would be encountered in an open hearth, has not been explored. Consistent with these previous studies, our heating determined that heat-affected OES will change in color, darkening around 260 °C, attaining a deep brown color around 340 °C. Near 420 °C OES begins to lighten, turn greenish/khaki, and finally producing a blue/grey color near 500 °C. These last two colors are sometimes overlaid with an opalescent rainbow effect (Fig. 1). At temperatures greater than 500 °C, the eggshell calcines and turns white (Collins and Steele, 2017). Over long periods of time, buried in sediment, the surface color of the OES can change further, especially if the original deposition was in an ash bed in a hearth area. Knowing how varied and degraded OES can be when recovered, we aim here to investigate how the stable isotopes in the organic and inorganic components of OES react to heat.

2. Materials and methods

2.1. Heating ostrich eggshell

Whole ostrich eggs were purchased from Ostrichland USA, an ostrich farm located in Solvang, California, about 65 km northwest of Santa Barbara. Two separate purchases were made at different times of the year; the first purchase was in March 2019 for three eggs, designated eggs A, B, and C, and the second was in July 2021 for eggs D and E. When the eggs were purchased, we (PJM) confirmed with the staff at the farm that the ostriches' diets were regular and consistent, and therefore we could assume that the OES' isotopic values should be consistent because they were living on the same farm with the same resources. All the eggs were stored in a standard kitchen refrigerator until they were prepared for heat exposure.

To prepare the OES for this experiment, the whole eggs were washed with cold tap water and perforated with a sharp stone to remove the edible portion of the egg. Once emptied, the inside of the egg was washed with cold tap water and the membrane was removed by carefully pulling the rubbery film away from the shell. The shell was allowed to air dry before being fragmented and labeled. When eggs D and E were removed from the refrigerator to be washed and prepared for the kiln, we noted that mold had infiltrated both the surface and the inside of the eggshell. The inside of the egg was discarded, and the shell was washed inside and out with cold tap water to remove as much of the mold as possible. The mold was growing directly through the shell (Fig. S1) and could not be eliminated, so the shells were photographed to document the mold, and the eggs were processed in the same way as eggs A-C. This development was concerning because we were unsure if different amounts of mold present in an individual fragment would affect the ability to compare fragments, but we decided that the data from the heated fragments could still be compared to data from unheated fragments of the same egg to discern if the mold would cause changes in the stable isotope ratios within an egg, so we did not remove the eggs from the project.

We fragmented the eggshell by hand and produced fragments approximately similar in size to be sure there would be adequate sample mass for the planned analyses (Fig. 1C and D). A total of 115 fragments of each egg were labeled and weighed in preparation for exposure to heat inside of the kiln. Fragments were assembled into sets of 5 and each set was assigned an alphanumeric identifier that each of the five fragments would share, followed by a unique numeric identifier (e.g., A1.3 is egg A, set 1, fragment 3). The set identifier represents a specific temperature to which the fragments in the set were exposed (Fig. 1I).

A Skutt Kilnmaster Automatic Kiln model KM818 with onboard controller was used for this experiment. The shelf was positioned in the vertical center of the kiln and several sets of fragments were laid out in an arrangement that would allow us to remove the sets of 5 one at a time as the kiln was ramped up in temperature at a rate of 300 °C per hour to the designated temperature for that set (Fig. 1B and C). When heat reached the target temperature, the kiln was opened, the five fragments of that designated set were removed, and the kiln closed so that the temperature could continue to ramp up. We removed the first set at 100 °C and the next every 20 °C, up to 500 °C. The fragments were not held at the target temperature for any length of time but were removed from the heat source immediately upon reaching the target temperature. Approximately 50 fragments each from eggs A and B were heated in 2 batches including ten temperature sets (100 fragments total), and the three remaining eggs (C, D, E) were done together. As each set of 5 fragments was removed from the kiln, the fragments were allowed to cool before being photographed (Fig. 1G and H).

Fig. 1. A: Ostrich eggshell after removing the edible portion and washing in preparation to fragment (Egg A).**B:** McNeill arranging OES fragments into the kiln.**C:** Fragments from eggs C, D, and E arranged in the kiln in preparation for heating. Fragments were photographed inside surface facing up so the sample numbers could be included, but they were flipped over before heating.**D:** Egg C with all 21 sets displayed, plus the unheated set 0, located in the upper left of the arrangement. Some sets have fewer fragments because they were already prepared for isotope processing.**E:** Egg A, sets 16–20, showing the opalescence of the surface that is sometimes present on the higher numbered sets that were heated to higher temperatures.**F**: Egg C, sets 17–20, illustrating the variability in color displayed when heated to the higher temperatures.**G and H:** Egg B, set 10 front and back, after being heated in the kiln.**I:** Set numbers and their corresponding temperatures in degrees Celsius.

2.2. Eggshell demineralization for determination of $\delta^{13}C$ and $\delta^{15}N$ in proteins

One fragment from sets 0–10 from each egg was selected for total organic fraction (TOF) C and N isotope analyses and the protein was isolated by acid demineralization (Niespolo, 2020; Johnson, 1995). A pilot study showed that proteins from the organic portion of the shell could not be isolated in samples that were heated to temperatures above 220 °C (set 7). These fragments did not contain enough organic material for analysis after exposure to the high temperatures, so fragments from sets 1–10 were included, but fragments from sets 11–21 were excluded. Samples were individually demineralized in 0.5 M HCl at 4 °C for up to two weeks, with the acid changed every other day. No base step was used because Niespolo, et al. (2015) found that the use of sodium hydroxide (NaOH), destroys and degrades the organic component of OES, resulting in low C and N and altered $\delta^{13}C$ and $\delta^{15}N$ values (Niespolo, 2020; Niespolo, 2015). After the eggshell demineralized, samples were rinsed with deionized H_2O three times and approximately 10 ml of H_2O with a pH of 3 was added to the vial. Samples were placed in a 70 °C oven for up to two weeks to allow the proteins in the TOF to denature. We pipetted liquid with the denatured proteins from the sample vials every other day, and H_2O with a pH of 3 was replaced as needed. The liquid containing the proteins was stored under refrigeration at 4 °C.

Once sample dissolution was complete, the samples were frozen, then lyophilized to isolate the organic portion.

The freeze-dried samples were submitted to the University of California Davis Stable Isotope Facility. Each was analyzed for total C, total N, $^{13}C/^{12}C$, and $^{15}N/^{14}N$ using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Long-term instrument standard deviation is 0.2 ‰ for $\delta^{13}C$ and 0.3 ‰ for $\delta^{15}N$.

2.3. Eggshell preparation for determination of $\delta^{13}C$ and $\delta^{18}O$ in carbonate

We chose a 0.15 mg section from each temperature set from eggs A-D for carbonate $^{13}C/^{12}C$ and $^{18}O/^{16}O$ analysis at the UC Davis Stable Isotope Facility. To prepare the sample, a small portion of shell was powdered in a mortar and pestle, then placed into a small glass scintillation vial and dried at 60 °C in a drying oven overnight. Isotopic measurements were carried out using a GasBench II system interfaced to a Delta V Plus IRMS (Thermo Scientific, Berlin, Germany). The long-term instrument standard deviation is 0.1 ‰ for $\delta^{13}C$ and 0.2 ‰ for $\delta^{18}O$.

2.4. Data analysis

In preparation for the paleoclimatic reconstruction evaluation, the $\delta^{18}O$ data from the heated and unheated fragments were converted from Vienna Pee Dee Belemnite (VPDB), the standard typically used for solid carbonates like eggshell and provided by the UC Davis Stable Isotope Facility, to Vienna Standard Mean Ocean Water (VSMOW), which is traditionally used in paleoclimatic reconstructions. The conversion was made in R using the following equation: $\delta^{18}O_{VSMOW} = (\delta^{18}O_{VPDB} + 29.98)/0.97002$). This allows for direct comparison of $\delta^{18}O$ values with those of local water sources (Sharp, 2017). Miller and Fogel (2016) determined that $\delta^{18}O$ values in OES primarily reflect the bird's drinking water, making VSMOW the preferred standard for ensuring consistency in paleoclimate studies (Miller and Fogel, 2016). The values from this conversion will be plotted along with $\delta^{13}C$ from the carbonate and $\delta^{15}N$ from the total organic fraction following the methods used in Niespolo (2020).

3. Results

The aim of this project is to understand how heat affects the outcome of an isotopic analysis of ostrich eggshell used in paleoclimatic reconstructions. A summary of these results can be found in Table 1, and all isotopic results by fragment and temperature are in Table S2. Stable isotope values are reported in delta (δ) notation relative to internationally recognized standards: $\delta^{13}C$ and $\delta^{18}O$ values are referenced to Vienna Pee Dee Belemnite (VPDB), while $\delta^{15}N$ values are reported relative to atmospheric nitrogen (AIR).

3.1. Egg A

The unheated sample returned a $\delta^{13}C$ value of −7.2 ‰, with the heated samples (n = 21) showing a mean decrease of 0.6 ‰ relative to the unheated sample. The standard deviation of 0.2 ‰ suggests some variability in the isotopic shift, but the overall trend remains consistent with the unheated sample (Fig. 2A). Oxygen isotope values ($\delta^{18}O$) of the unheated sample returned a value of −4.1 ‰ with a 0.2 ‰ average difference relative to the heated sample (n = 21) with a standard deviation of 0.1 ‰ (Fig. 2B). In the organic fraction, the $\delta^{13}C$ value of the unheated sample was –23.5 ‰, with the heated samples (n = 7) showing a mean decrease from the unheated sample of only 0.1 ‰. The $\delta^{15}N$ value of the unheated sample is 6.8 ‰, with the mean of the heated samples (n = 7) decreased slightly by 0.6 ‰, suggesting a loss of the heavier nitrogenous compounds, which is expected as the protein-bound N transforms into gaseous N (Fig. 3). Standard deviations are 0.1 ‰ for $\delta^{13}C$ (Fig. 3A) and 0.3 ‰ for $\delta^{15}N$ (Fig. 3B).

The unheated sample of Egg A produced a C:N ratio of 3.90, and the mean of this value from the heated samples is 3.93, producing a

Table 1
Summary of Stable Isotope Metrics for Ostrich Eggshell Samples (Eggs A-E). This table presents the mean, standard deviation (SD), variance, minimum, maximum, and coefficient of variation (CV) for stable isotope values measured from both the organic and carbonate fractions of ostrich eggshells. Metrics are provided for $\delta^{13}C$ in the total organic fraction ($\delta^{13}C_{TOF}$) and carbonate ($\delta^{13}C_{carbonate}$), $\delta^{15}N$ in the organic fraction, and $\delta^{18}O$ in the carbonate fraction. Each egg is analyzed separately, with color coded sections corresponding to individual eggs: Egg A (red circle), Egg B (yellow square), Egg C (green diamond), Egg D (blue triangle), and Egg E (purple inverted triangle), and this color coding is carried out through all of the figures. Egg E is only included in organic fraction analyses due to the absence of carbonate data. The complete dataset is provided in Table S2.

Egg-A Metric	$\delta^{13}C_{tof}$	$\delta^{15}N$	$\delta^{13}C_{carbonate}$	$\delta^{18}O$	Egg-B Metric	$\delta^{13}C_{tof}$	$\delta^{15}N$	**$\delta^{13}C_{carbonate}$**	**$\delta^{18}O$**
Unheated (Control)	−23.5	6.8	−7.2	−4.1	Unheated (Control)	−25.3	7.3	−11.6	−2.4
Mean (Heated Samples)	−23.6	6.2	−7.8	−3.9	Mean (Heated Samples)	−25.4	7.6	−11.7	−2.3
Standard Deviation (SD)	0.1	0.3	0.2	0.1	Standard Deviation (SD)	0.1	0.1	0.2	0.1
Variance	0.0	0.1	0.1	0.0	Variance	0.0	0.0	0.0	0.0
Difference from Control	−0.1	−0.6	−0.6	0.2	Difference from Control	−0.1	0.3	−0.1	0.1
Min Value	−23.7	5.9	−8.5	−4.1	Min Value	−25.5	7.5	−12.0	−2.6
Max Value	−23.5	6.7	−7.4	−3.7	Max Value	−25.3	7.9	−11.3	−2.1
Coefficient of Variation (CV)	0.00	0.04	−0.03	−0.03	Coefficient of Variation (CV)	0.00	0.02	−0.01	−0.04
Egg-C Metric	$\delta^{13}C_{tof}$	$\delta^{15}N$	**$\delta^{13}C_{carbonate}$**	**$\delta^{18}O$**	**Egg-D Metric**	$\delta^{13}C_{tof}$	$\delta^{15}N$	**$\delta^{13}C_{carbonate}$**	**$\delta^{18}O$**
Unheated (Control)	−23.5	4.8	−9.5	−4.1	Unheated (Control)	−22.5	6.6	−9.2	−4.5
Mean (Heated Samples)	−24.1	4.5	−10.4	−4.8	Mean (Heated Samples)	−23.0	6.7	−9.2	−4.7
Standard Deviation (SD)	0.1	0.3	0.6	0.4	Standard Deviation (SD)	0.1	0.1	0.2	0.2
Variance	0.0	0.1	0.3	0.2	Variance	0.0	0.0	0.0	0.0
Difference from Control	−0.6	−0.3	−0.9	−0.7	Difference from Control	−0.5	0.1	−0.1	−0.2
Min Value	−24.2	4.2	−11.0	−5.5	Min Value	−23.1	6.5	−9.5	−5.0
Max Value	−24.0	5.1	−9.5	−4.0	Max Value	−22.9	6.8	−8.9	−4.5
Coefficient of Variation (CV)	0.00	0.08	−0.05	−0.09	Coefficient of Variation (CV)	0.00	0.01	−0.02	−0.03
Egg-E Metric	$\delta^{13}C_{tof}$	$\delta^{15}N$							
Unheated (Control)	−21.9	5.9							
Mean (Heated Samples)	−22.3	5.8							
Standard Deviation (SD)	0.1	0.1							
Variance	0.0	0.0							
Difference from Control	−0.4	−0.1							
Min Value	−22.6	5.5							
Max Value	−22.1	5.9							
Coefficient of Variation (CV)	−0.01	0.02							

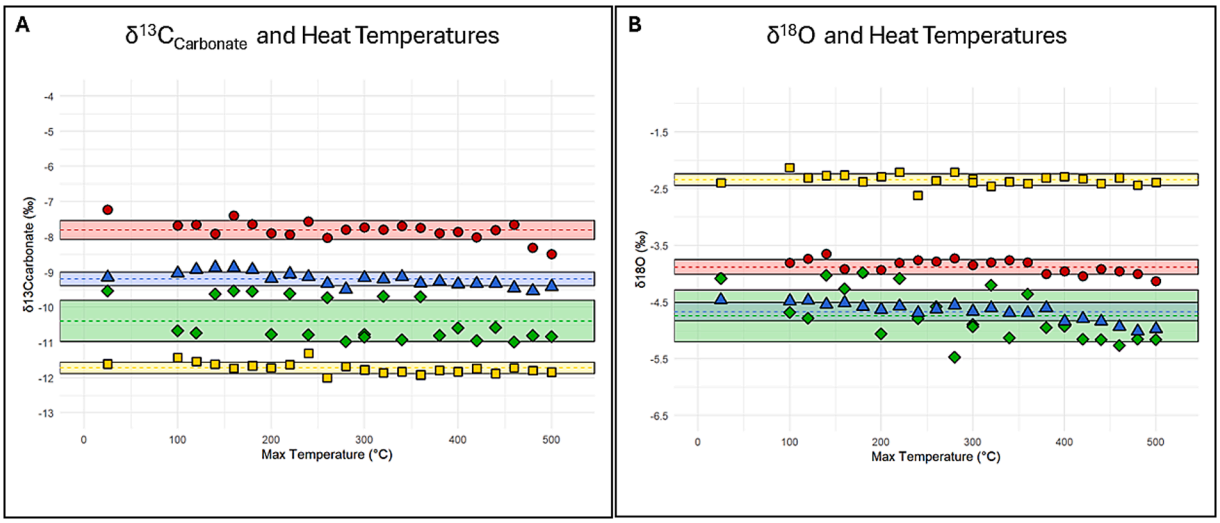

Fig. 2. Stable isotope values of $\delta^{13}C$ and $\delta^{18}O$ in the carbonate fraction of OES across different maximum heating temperatures: Legend: Egg A (red circle), Egg B (yellow square), Egg C (green diamond), Egg D (blue triangle), and Egg E (purple inverted triangle) **A:** $\delta^{13}C_{Carbonate}$ values plotted against maximum temperature (°C), with shaded regions indicating the mean ±1 standard deviation from the unheated sample (control sample) for each egg. **B:** $\delta^{18}O$ values in the carbonate fraction plotted against maximum temperature (°C), with shaded regions indicating the mean ±1 standard deviation from the unheated sample (control sample) for each egg.Results show that in most of the eggs both of these sets of values in the carbonate fraction remain relatively stable up to 500 °C, suggesting that heating does not meaningfully alter these isotopic signatures in OES. These findings reinforce the reliability of the carbonate fraction for paleoclimatic reconstructions, even in contexts where heating may have occurred. Section 4.3 discuss Egg C in more detail.

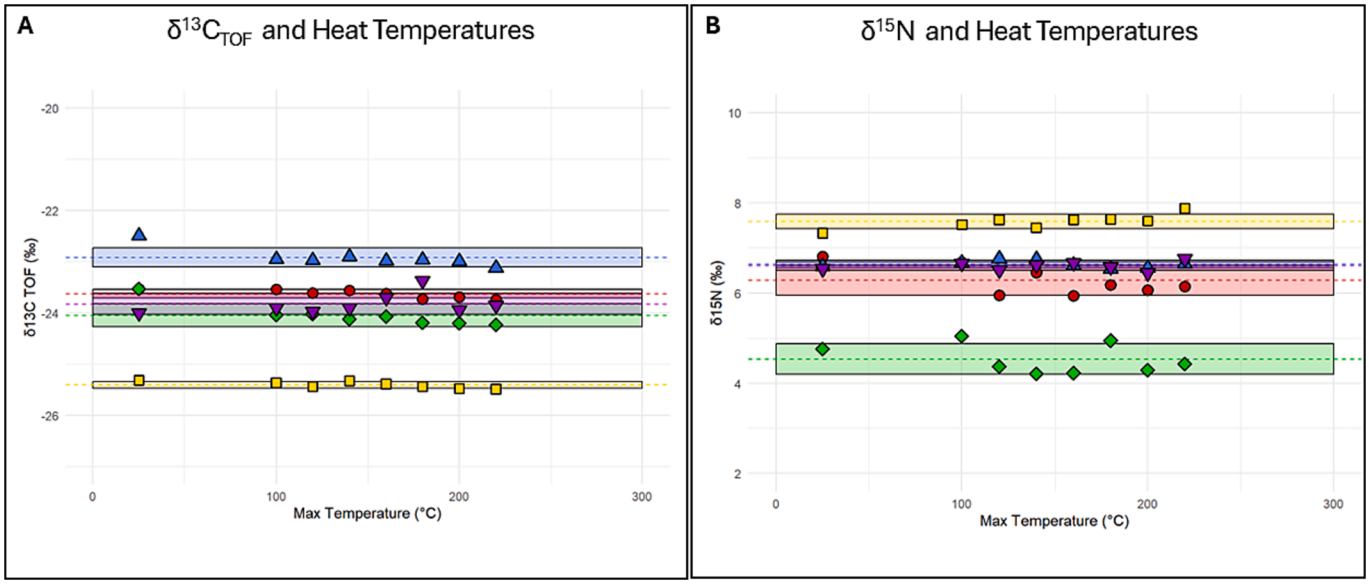

Fig. 3. Stable isotope values of $\delta^{18}C$ and $\delta^{15}N$ in the Organic Fraction of OES across different maximum heating temperatures: Legend: Egg A (red circle), Egg B (yellow square), Egg C (green diamond), Egg D (blue triangle), and Egg E (purple inverted triangle) **A:** $\delta^{13}C_{TOF}$ values plotted against maximum temperature (°C), with shaded regions indicating the mean ±1 standard deviation from the unheated sample (control sample) for each egg. **B:** $\delta^{15}N$ values plotted against maximum temperature (°C), with shaded regions indicating the mean ±1 standard deviation from the unheated sample (control sample) for each egg. Despite exposure to increasing temperatures, the isotopic values remain relatively stable, demonstrating that heat exposure up to 240 °C does not meaningfully alter $\delta^{13}C_{TOF}$ or $\delta^{15}N$ in the organic fraction. However, above this temperature threshold, organic material becomes too degraded for reliable isotopic analysis. The variations observed between individual eggs highlight natural inter-egg differences in isotopic composition.

difference of + 0.03 (Table S1).

3.2. Egg B

The carbonate $\delta^{13}C$ values in Egg B show a smaller change then Egg A, with the unheated sample returning a value of −11.6 ‰. The mean of the heated samples (n = 21) decreased by only 0.1 ‰ from the unheated sample, suggesting these samples may have been slightly more resistant to heating effects (Fig. 2A). $\delta^{18}O$ values remained relatively stable with the unheated sample returning a value of −2.4 ‰, and the mean difference of the heated samples (n = 21) was only 0.1 ‰, which is negligible and within machine error (0.2 ‰) (Fig. 2B). The standard deviation of the heated samples from the unheated sample is 0.2 ‰ from the $\delta^{13}C$ and 0.1 ‰ from the $\delta^{18}O$ values. The $\delta^{13}C$ value in the organic fraction of the unheated sample is −25.3 ‰, showing only minimal fluctuation of only 0.1 ‰ mean difference in the heated samples (n = 7) from the unheated eggshell, and a standard deviation of 0.1 ‰. The $\delta^{15}N$ value of the unheated sample is 7.3 ‰, with the mean values of the

heated samples (n = 7) shows an increase by 0.3 ‰ from the unheated sample with a standard deviation of 0.1 ‰ (Fig. 3B).

The unheated sample from Egg B produced a C:N ratio of 3.95, and the mean for the heated samples is 3.93. (Table S1).

3.3. Egg C

The unheated sample from Egg C produced carbonate $\delta^{13}C$ values of −9.5 ‰ with the mean difference in the heated samples (n = 21) of only −0.9 ‰, with a standard deviation of 0.6 ‰ (Fig. 2A). Oxygen isotopes ($\delta^{18}O$) in the unheated sample returned a value of −4.1 ‰, showing minimal change of −0.7 ‰ in the mean of the heated samples (n = 21) from the unheated sample with a standard deviation of 0.44 ‰ (Fig. 2B). In the organic fraction, $\delta^{13}C$ value from the unheated sample is −23.5 ‰ with a mean difference in the heated samples (n = 7) of −0.6 ‰, with a standard deviation of only 0.1 ‰ (Fig. 3A). $\delta^{15}N$ value of the unheated sample is 4.8 ‰, showing a mean decrease in the heated samples (n = 7) of 0.3 ‰ from the unheated sample, and a standard deviation of 0.4 ‰, implying some loss of nitrogen (Fig. 3B).

The unheated sample from Egg C produced a C:N ratio of 4.23, and the mean for the heated samples is 3.96, producing a difference of −0.27. This egg has a higher C:N than the rest of the eggs, so it is possible this decrease could be a result of small amounts of lipids from the external membrane which can remain in the pores of the egg and possibly not removed by the pretreatment (Table S1).

3.4. Egg D

The $\delta^{13}C$ value of the unheated sample is −9.2 ‰, with the mean difference of the heated samples (n = 21) only −0.1 ‰ from the unheated sample, and a standard deviation of 0.2 ‰. (Fig. 2A). Oxygen isotopes ($\delta^{18}O$) also showed minimal change, with the unheated sample returning a value of −4.5 ‰ and a mean difference in the heated samples (n = 21) from the unheated sample of −0.2 ‰, with a standard deviation of 0.2 ‰ (Fig. 2B). In the organic fraction, $\delta^{13}C$ values remained relatively stable, with the unheated sample returning a value of −22.5 ‰ and a mean difference from the unheated eggshell of −0.5 ‰ with a standard deviation of 0.1 ‰ (Fig. 3A). The mean of the $\delta^{15}N$ values from the heated samples increased slightly by 0.1 ‰ from the unheated sample 6.6 ‰, with a standard deviation of 0.1 ‰, suggesting minimal thermal impact (Fig. 3B).

The unheated sample from Egg D produced a C:N ratio of 3.91, and the mean for the heated samples is 3.91, reflecting minimal change and indicating a stable sample (Table S1).

3.5. Egg E

For Egg E, we only have data from the organic portion of the shell, and the $\delta^{13}C$ values showed minimal variation with heating (mean difference from the unheated sample (−21.9 ‰) is −0.4 ‰), suggesting relative stability in the carbon isotope composition despite thermal exposure (standard deviation of 0.1 ‰) (Fig. 3A). $\delta^{15}N$ values exhibited less change, with the unheated sample returning a value of 5.9 ‰, showing a mean difference from the heated samples (n = 7) of only −0.1 ‰ with a standard deviation of 0.1 ‰, which is below instrument error of 0.2 ‰ (Fig. 3B).

The unheated sample from Egg E produced a C:N ratio of 3.87, and the mean for the heated samples is 3.93, producing a difference of +0.06 (Table S1).

3.6. Summary of individual eggs

The findings collectively indicate that while heating induces some changes in both organic and mineral stable isotopes, the extent of alteration varies between eggs (Figs. 4 and 5). The C:N ratio in the organic fraction does not change at higher temperatures, a particularly significant result. This suggests that amino acids and/or entire proteins are being lost as complete units rather than breaking down into intermediate organic fragments. All nitrogen and carbon within the proteins are volatilizing as gasses as chemical bonds break, rather than leaving behind partial organic residues composed only of carbon. Despite these transformations, the data remain relatively stable across temperature changes.

3.7. Paleoclimatic reconstruction

To further evaluate the impact of heat exposure on stable isotope values in OES and the implications for paleoclimatic reconstructions, the experimental data are examined using figure designs adapted from Niespolo (2020). The plots illustrate $\delta^{13}C$ values in carbonate, $\delta^{15}N$

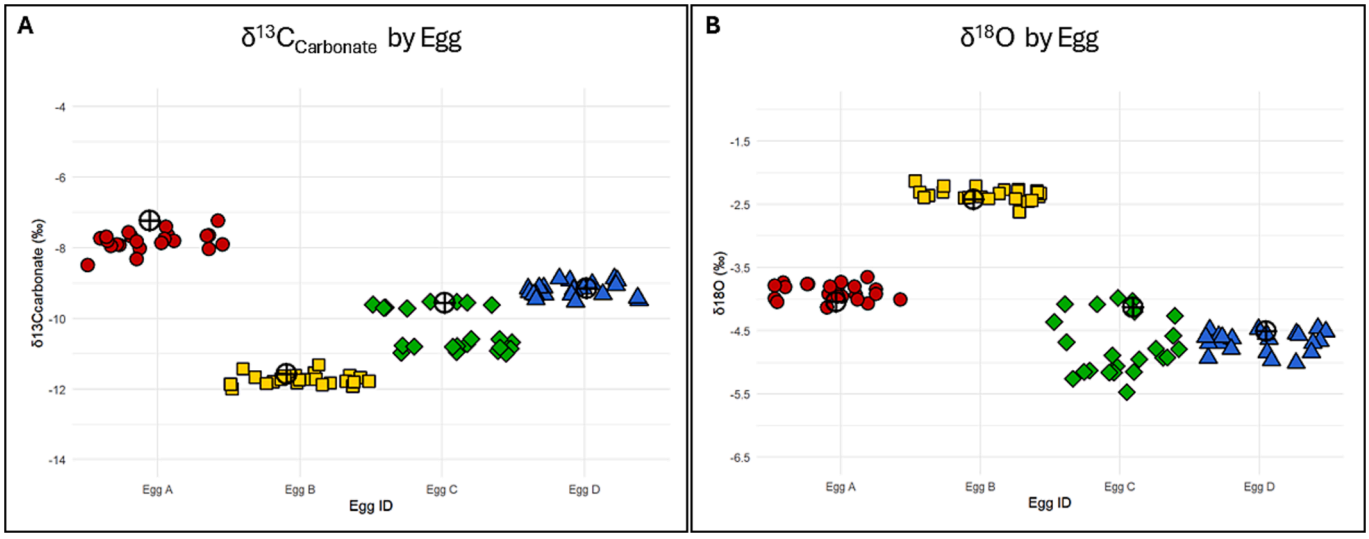

Fig. 4. Stable isotope values of the carbonate fraction in OES: Legend: Egg A (red circle), Egg B (yellow square), Egg C (green diamond), Egg D (blue triangle), and Egg E (purple inverted triangle) The black marker within each Egg ID represents the unheated control sample value, serving as a baseline for comparison. **A:** $\delta^{13}C$ values of the carbonate fraction show differences in response to heating, with some eggs exhibiting more variation than others. These shifts may reflect thermal alteration of carbonate-bound carbon and potential fractionation effects. **B:** $\delta^{18}O$ values illustrate the extent of oxygen isotope variation across eggs. Some samples display greater dispersion, indicating potential isotopic exchange or structural modifications due to heating.

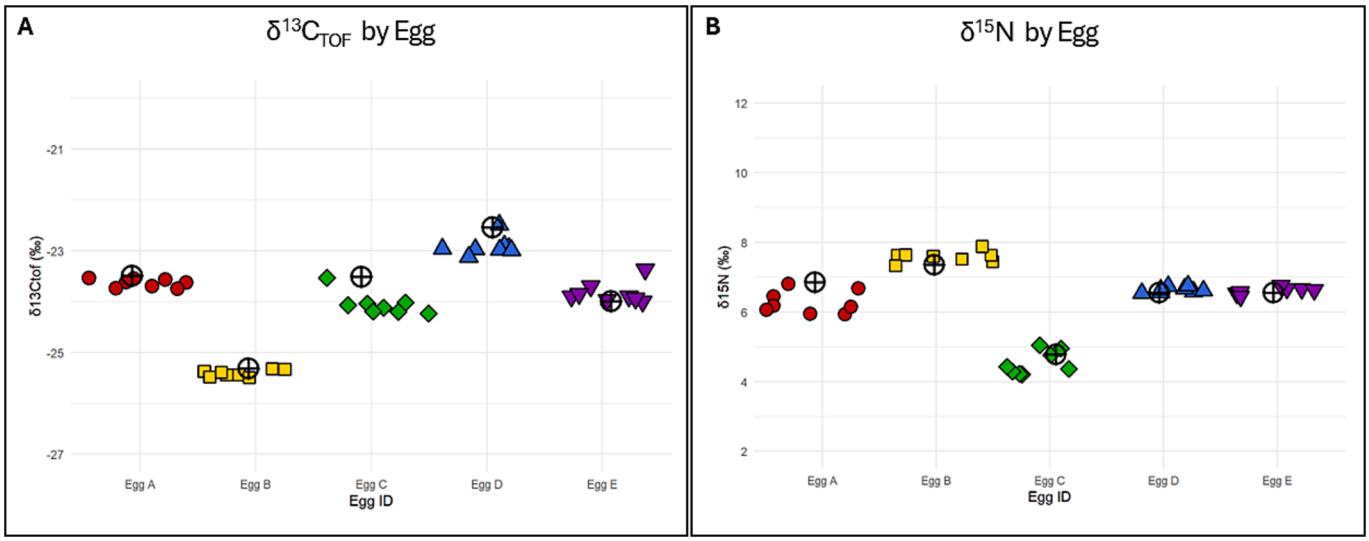

Fig. 5. Stable isotope values of the Total Organic Fraction in OES: Legend: Egg A (red circle), Egg B (yellow square), Egg C (green diamond), Egg D (blue triangle), and Egg E (purple inverted triangle) The black marker within each Egg ID represents the unheated control sample value, serving as a baseline for comparison. **A:** $\delta^{13}C$ values exhibit varying degrees of dispersion across eggs, indicating differences in how heating affects the organic carbon fraction. Some eggs show minimal variation, while others display greater isotopic shifts, suggesting some potential thermal alteration of carbon composition. **B:** $\delta^{15}N$ values show the extent of nitrogen isotopic varation due to heating. The degree of dispersion varies between eggs, with some maintaining relatively stable $\delta^{15}N$ values and others exhibiting noticeable shifts, potentially due to nitrogen loss or fractionation.

values in the total organic fraction, and $\delta^{18}O$ values in carbonate, allowing us to directly compare with established paleoclimate methodologies (Fig. 6). Unlike Niespolo et al., where the Y-axis represents time, these plots instead use maximum temperature exposure to directly track the effects of heat exposure on isotopic values. Panel A visualizes $\delta^{13}C$ values in carbonate alongside estimated dietary $\delta^{13}C$ values, contextualizing shifts within the C_3 and C_4 vegetation framework. Panel B presents $\delta^{15}N$ values in relation to precipitation amount (PMAP), a key variable in aridity reconstructions. Panel C examines $\delta^{18}O$ variations, highlighting the relationship between oxygen isotopes and evaporative processes.

3.8. Reliability of the data

The reliability of paleoclimatic reconstructions made using archaeological OES is dependent on the preservation of endogenous compounds. To further test if our heated eggshell had been meaningfully isotopically modified, we used the reliability criteria previously established for OES by Johnson (Johnson, 1995; Johnson et al., 1998) and used by Niespolo (2020). The carbon fractionation between the mineral and total organic fraction (TOF) of ostrich eggshell is constant in modern OES ($\Delta^{13}C_{Calcite\text{-}TOF} = 15 \pm 2$ ‰) (Johnson, 1995; Johnson et al., 1998), so we used this value to test for the preservation of the isotopic ratios across increasing temperature gradients (Johnson, 1995; Johnson et al., 1998). We performed this test on Eggs A-D and the results are illustrated

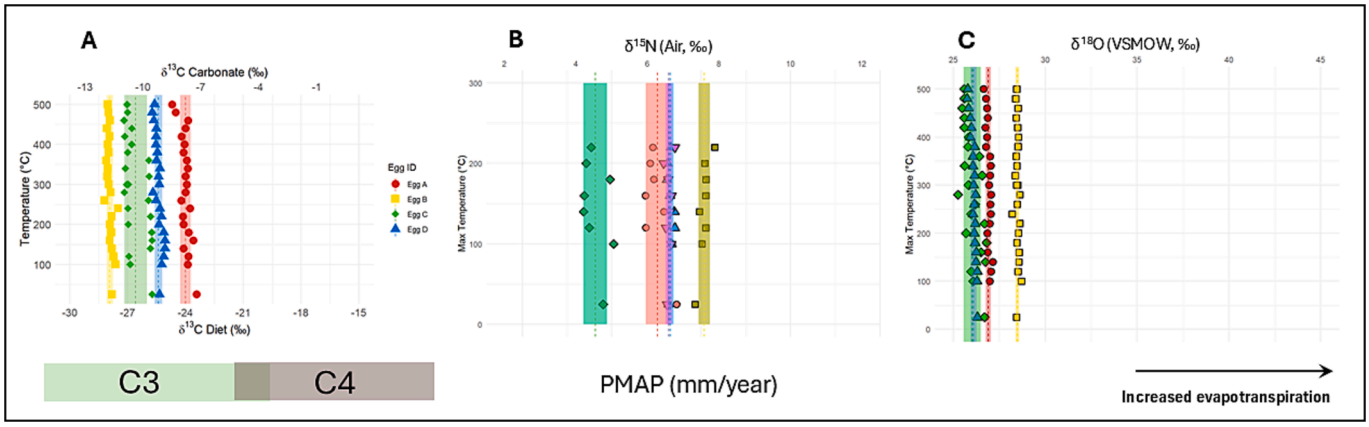

Fig. 6. $\delta^{13}C$ and $\delta^{18}O$ values of ostrich eggshell carbonate and $\delta^{15}N$ values from the organic fraction across different heating conditions, following the analytical framework of Niespolo et al. (2020); here temperature replaces time. Legend: Egg A (red circle), Egg B (yellow square), Egg C (green diamond), Egg D (blue triangle), and Egg E (purple inverted triangle). The shaded vertical bands indicate the isotopic ranges for unheated samples. A. $\delta^{13}C$ values of the carbonate fraction plotted against dietary $\delta^{13}C$ values ($\delta^{13}C_{Carbonate} - 16.2$ (Johnson, 1995), demonstrating minimal isotopic shifts with increasing temperature. The separation between C3 and C4 plant ranges highlights that despite heating, the overall dietary signal remains distinguishable. B. $\delta^{15}N$ values as a function of maximum temperature, showing limited variation across heating conditions, suggesting that heating effects on oxygen isotopes remain within a narrow range. C. A comparison of $\delta^{18}O$ values across heating temperatures, reinforcing the observation that thermal alteration dies not significantly impact the isotopic fidelity necessary for paleoclimatic reconstructions. Together, these results confirm that the minor isotopic variations observed in heated samples do not meaninigfully alter the paleoenvironmental interpretations derived from ostrich eggshell carbonate, supporting the robustness of this proxy in paleoclimatic studies.

in Fig. 7. As can be seen in Fig. 7, all temperature category-$\Delta^{13}C$ pairs fall within the acceptable published range. This validates the carbon isotope values in both the mineral portion as well as the TOF in our samples and illustrates that the carbon isotope values are not meaningfully impacted by increases in temperature (see Fig. 8).

4. Discussion

4.1. Evaluating the impact of heat exposure on the outcome of a paleoenvironmental reconstruction

To assess the impact of heat exposure on the stable isotopes in OES, we evaluated the results within the framework of standard paleoclimatic reconstruction methods using OES as seen in Niespolo (2020). By comparing heated and unheated samples, this study evaluates the extent to which heating affects $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values and determines whether these alterations mimic or obscure environmental trends. Graphing experimental results using established methods for isotopic interpretation allows for direct comparisons with archaeological and paleontological datasets, providing insights into the reliability of heat altered OES in reconstructing past climates.

Stable isotope ratios provide insights into past climatic and ecological conditions. $\delta^{13}C$ values in the carbonate and organic fractions reflect the proportion of C3 versus C4 vegetation but originate from different biochemical sources: the organic portion reflects mainly dietary protein sources, while the carbonate portion records the isotopic composition of bicarbonate in body fluids (Tieszen, 1991; Farquhar et al., 1982; Heaton, 1999). While $\delta^{13}C$ is commonly used to distinguish between C_3 and C_4 plant consumption, it also provides information on environmental conditions such as water availability and vegetation structure (Tieszen, 1991; Farquhar et al., 1982; Heaton, 1999; Klein, 2014; Linares and Camarero, 2012). Plants experiencing water stress or growing in arid environments often exhibit higher $\delta^{13}C$ values due to changes in photosynthetic efficiency and stomatal conductance (Tieszen, 1991; Farquhar et al., 1982; Heaton, 1999; Klein, 2014; Galmés, 2007). These physiological responses can be reflected in both the organic and carbonate fractions of ostrich eggshell, offering insight into past aridity and habitat conditions beyond just plant type. Higher $\delta^{13}C$ values in either fraction can indicate a greater presence of C_4 plants, often associated with drier environments, but can also result from increased water-use efficiency in C_3 plants under stress (Tieszen, 1991; Farquhar et al., 1982; Heaton, 1999; Klein, 2014; Galmés, 2007). $\delta^{18}O$ values in OES carbonate are linked to water balance and aridity, influenced by evaporation rates and precipitation sources (Tieszen, 1991; Farquhar et al., 1982; Heaton, 1999). In the organic portion, $\delta^{13}C$ values capture dietary inputs beyond plant type, including contributions from animal protein or aquatic resources, while $\delta^{15}N$ values provide insights into trophic level and nitrogen cycling, with elevated values often associated with increased aridity, water stress, or shifts in available protein sources within the ecosystem (Farquhar et al., 1982; Galmés, 2007).

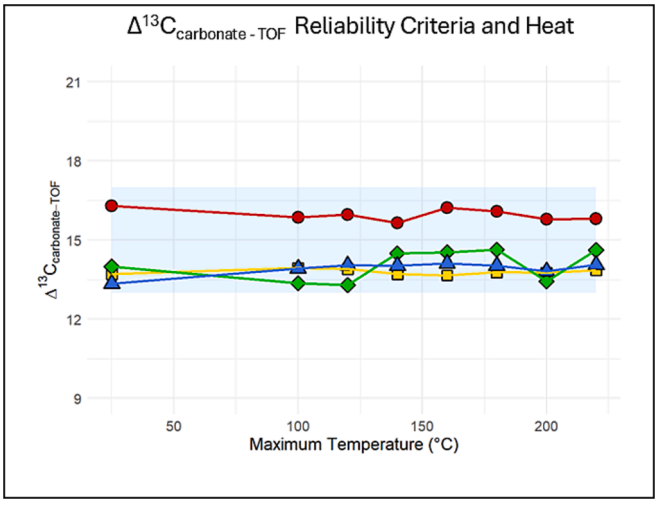

Fig. 7. Reliability criteria using the difference in $\delta^{13}C$ values between the carbonate and total organic fractions ($\Delta^{13}C_{Calcite-TOF}$) of ostrich eggshell samples as a function of maximum heating temperature: Legend: Egg A (red circle), Egg B (yellow square), Egg C (green diamond), Egg D (blue triangle), and Egg E (purple inverted triangle). The shaded blue region represents the range of acceptable $\Delta^{13}C_{Calcite\text{-}TOF}$ values, established by Johnson (1995) and utilized by Niespolo (2020).

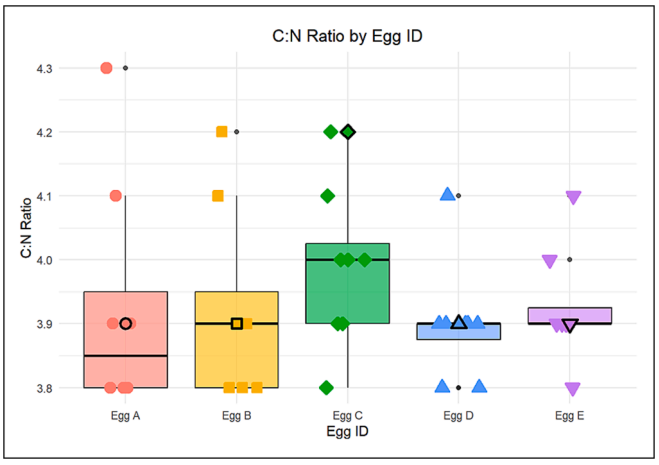

Fig. 8. Box plots of the C:N ratio in the TOF of OES: Legend: Egg A (red), Egg B (yellow), Egg C (green), Egg D (blue triangle), and Egg E (purple). The boxes represent the interquartile range (middle 50% of data), with whiskers indicating data spread. Individual points correspond to measured C:N ratios with shape and color distinguishing different eggs. Markers with black outlines represent unheated samples. The plot indicates that Egg C exhibits the greatest variation in C:N ratios, while Eggs D and E display relatively low variability, suggesting that heating may have influenced C:N ratios differently across eggs.

Stable isotope analysis of OES is widely used in paleoclimatic reconstructions due to its ability to preserve environmental and ecological signals over time. The $\delta^{13}C$ values in both the carbonate and organic fractions provide complementary insights into past vegetation dynamics, dietary intake, and ecosystem shifts, while $\delta^{18}O$ values in carbonate serve as proxies for hydrological conditions and climatic aridity (Johnson, 1997; Niespolo, 2020; Johnson, 1995; Johnson et al., 1998; Miller and Fogel, 2016). The application of these isotopic systems to archaeological OES relies on the assumption that diagenetic alterations, including those caused by heating, do not meaningfully distort the original environmental signals.

Despite variations in isotope values across different maximum heating temperatures, the results plotted in Fig. 6 demonstrate that these shifts do not meaningfully alter the overall outcome of the reconstruction. This suggests that even when subjected to high temperatures, OES retains its potential as a reliable paleoenvironmental archive. By employing established graphing methods, these figures contextualize the experimental data within broader paleoclimatic studies, reinforcing the applicability of heated OES in reconstructing past environments.

4.2. Variation between eggs

In analyzing the data from this study, we observed a second possible source that could introduce variation into paleoclimate reconstructions: one driven by natural biological differences among individual ostriches. Thermal alteration does not meaningfully impact the reliability of paleoclimatic reconstructions, as isotopic values remain relatively stable during heating events. However, natural variation among individual ostriches still presents a factor to consider; while the data from each

individual egg remained tightly clustered, there was considerable variation between eggs. All eggs in this study were sourced from the same ostrich farm, where birds graze across a system of pastures but receive the same supplemental feed (per conversation PJM had with employee at Ostrichland USA), ensuring some dietary consistency. In contrast, wild ostriches experience even greater variability in diet and physiology due to unrestricted foraging behaviors. This highlights an important consideration for archaeological sampling. Researchers need to ensure a sufficiently large sample size for each context of interest (i.e. stratigraphic layer) to capture the full range of natural isotopic variation and improve the robustness of the paleoenvironmental interpretations.

4.3. Egg C

Egg C exhibits inconsistencies across both isotopic analyses in the carbonate fraction, raising concerns about the reliability of its data. Throughout this study, some of its values deviated from expected trends observed in the other eggs, suggesting that unknown factors may influence its isotopic composition. The bimodal distribution observed in the isotopic data from Egg C is unique from the other eggs included in this study. Fig. S2 illustrates this pattern in the data, but the trend is likely not related to the heat exposure from this experiment.

The isotopic composition of eggshell reflects a snapshot of the ostrich diet of the previous 3–5 days, with any dietary changes becoming apparent in the stable isotope values within approximately 4 days (Johnson, 1995; Johnson et al., 1998; Hobson, 1995). The distinct bimodal clustering in Egg C suggests the ostrich may have experienced a shift in dietary resources shortly before laying this egg. The data form two tightly clustered groups, each exhibiting minimal internal variation. This pattern indicates a clear separation in isotopic signatures, likely corresponding to two distinct dietary phases. Under nonexperimental conditions, such a distribution could be misinterpreted as representing eggs from different individuals rather than a single egg subjected to varying dietary influences; however wild birds are unlikely to experience such a dramatic shift during egg formation. Despite the bi-modal distribution of the data from this egg, all of the data remained within acceptable ranges and passed all of the reliability criteria.

4.4. C:N ratio in ostrich eggshell

The carbon-to-nitrogen (C:N) atomic ratio is a key indicator of protein composition in biological materials. Ostrich eggshells, however, contain a distinct protein matrix, incorporating Type X collagen along with non-collagenous proteins such as osteopontin and osteocalcin (Pérez-Huerta, 2023). These proteins differ in amino acid compositions and structural function compared to bone collagen, which likely results in a higher and more variable C:N ratio for OES. Based on its biochemical composition, our data indicate that a well-preserved ostrich eggshell C:N ratio is expected to range between approximately 3.70 and 4.35 (Table 2). These experimental data are within the acceptable range of the reliability criteria established by Johnson (1995) and Johnson et al. (1998) and discussed in section 3, validating the carbon isotope values in both the mineral portion as well as the TOF in our samples. None of the OES C:N baseline values fall within the acceptable range for bone collagen, reinforcing that the standards applied to Type I collagen-based materials in bone and teeth cannot be directly transferred to eggshell. Given the complexity of the ostrich's diet and physiology, additional factors contribute to variability in the C:N ratio. Unlike obligatory drinkers, ostriches obtain a significant portion of their water from metabolic and dietary sources, which influences nitrogen retention and processing in ways distinct from mammals and other avian species (Johnson, 1995; Milton et al., 1994; Williams, 1993). Furthermore, the incorporation of dietary nitrogen from plants, each with its own isotopic variability, alongside the fractionation effects occurring during the short span of eggshell formation suggest that the acceptability criteria for bone and tooth total organic fraction carbon and nitrogen should not be applied to ostrich eggshell.

Table 2
C:N Ratio Data This table contains data to calculate the C:N Ratio of the TOF.

SampleID	TotalC	TotalN	C:N
A0 1	530.68	158.76	3.9
A1 5	277.73	84.72	3.8
A2 2	415.03	128.01	3.8
A3 5	491.68	151.05	3.8
A4 3	473.87	145.71	3.8
A5 2	306.82	92.53	3.9
A6 3	168.8	48.53	4.1
A7 4	129.28	35	4.3
B01	142.32	42.02	3.95
B1 2	295.32	89.74	3.8
B2 3	348.07	105.59	3.8
B3 2	124.91	35.23	4.1
B4 1	344.24	105.02	3.8
B5 5	364.3	109.47	3.9
B6 4	393.39	117.7	3.9
B7 4	291.04	81.5	4.2
C0 1	95.01	26.22	4.2
C1 2	958.8	288.11	3.9
C2 1	232.53	68.59	4
C3 1	623.11	188.9	3.8
C4 5	505.66	152.99	3.9
C5 4	241.89	70.77	4
C6 3	246.85	71.52	4
C7 1	265.33	74.6	4.1
D0 1	139.76	41.68	3.9
D1 2	535.19	163.35	3.8
D2 1	511.07	155.03	3.8
D3 3	696.83	211.14	3.9
D4 1	415.93	126.02	3.9
D5 5	723.44	215.68	3.9
D6 1	357.09	105.82	3.9
D7 3	209.29	58.84	4.1
E0 1	542.41	163.35	3.9
E1 5	669.33	201.53	3.9
E2 2	552.55	167.33	3.9
E3 1	361.82	109.09	3.9
E4 1	274.13	83.54	3.8
E5 1	428.33	120.63	4.1
E6 2	456.96	135.53	3.9
E7 1	215.02	62.2	4

C:N data from Niespolo et al. (2020) are consistent with our findings (SI Table S1). This estimate is grounded in the protein-dominant nature of the organic fraction, which differs from Type I collagen but remains within a range consistent with proteinaceous materials. Ratios above 4.35 may indicate nitrogen loss due to diagenetic alteration or thermal exposure, while values below 3.70 could suggest contamination, protein degradation, or an altered organic-mineral balance. Given the absence of published C:N benchmarks for the Type X collagen and other proteins found in OES, further research is needed to refine this expected range and assess how other environmental factors influence the preservation of ostrich eggshell's organic fraction.

5. Conclusions

This controlled study demonstrates that even significant heat exposure does not destroy or meaningfully alter the stable isotopes in ostrich eggshell, a finding that had not been previously established. One highlight is that if proteins can be successfully isolated from the organic fraction of the eggshell, they will yield reliable isotopic data despite heat exposure. The carbonate fraction remains stable under temperatures to at least 500 °C, reinforcing its viability for isotopic analysis in paleoclimatic studies. Beyond this point, eggshell begins to calcine, rendering it unsuitable for archaeological analysis due to its friability. Recognizing this issue, the present study was designed to control for inter-egg variation by analyzing individual eggs independently. The results confirm that isotopic data from the organic fraction of OES can be trusted, provided sufficient organic material is extracted and C:N ratios fall within

the expected range for well-preserved OES samples, established here as 3.70 to 4.35. However, above 240 °C, too little organic material remains to yield enough proteinaceous material for analysis.

Overall, findings contribute to our understanding of how heat affects stable isotope preservation in both the organic and mineral portions of eggshell. By establishing the temperature and C:N thresholds for reliable isotopic analysis in heated OES, this study provides critical guidance for researchers selecting OES samples for isotopic investigations, particularly those employed in paleoenvironmental reconstructions.

CRediT authorship contribution statement

Patricia J. McNeill: Project administration, Funding acquisition, Conceptualization, Writing – original draft, Investigation, Formal analysis, Methodology, Data curation. **Bryna E. Hull-McNeill:** Writing – review & editing, Formal analysis, Visualization, Methodology. **Sophia Gilbertson:** Methodology, Investigation. **Teresa E. Steele:** Writing – review & editing, Supervision, Validation, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jasrep.2025.105323.

Data availability

All the data has been included with submission.

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