Stable Oxygen Isotopes from Skeletal Carbonates as a Proxy for Habitat in Extant and Extinct Testudines

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Tasman Rosenfeld, April 27, 2022
Introduction

Fossil turtles – an enigmatic group

Turtles, with their distinctive bony external shells, anapsid skulls, edentulous beaks, and notorious long lifespans, are one of the most enigmatic groups of vertebrates. Indeed, due to their derived morphology, the exact placement of turtles within the amniote tree of life has proven to be one of the greatest challenges in vertebrate systematics. Different phylogenetic analyses throughout the past century, including morphological, molecular, and combined datasets, have recovered them in a variety of positions, including as the sister of other amniotes alongside other anapsids (e.g., Romer 1956), as the sister of other reptiles alongside other anapsids (e.g., Gauthier et al. 1988; Laurin and Reisz 1995; Lee 1995, 1997), as the sister of lepidosaurs (e.g., Hedges 1994), as the sister of Sauropterygia (deBraga and Rieppel 1997), and more recently as the sister of archosaurs (e.g., Kumizawa and Nishida 1999) or as the sister of crocodilians (e.g., Hedges and Poling 1999).

Though Testudines as the sister of archosaurs has in recent years become the most widely supported hypothesis in molecular datasets, at the beginning of the 21st century, the primary competing hypotheses for the position of turtles were that they lay a) within an anapsid, terrestrial clade at the base of Reptilia (Gauthier et al. 1988; Laurin and Reisz 1995; Lee 1995, 1997) or b) as the sister of aquatic Sauropterygia (deBraga and Rieppel 1997). Ancestral habitat was proposed as a means of testing the plausibility of these conflicting hypotheses (Lee 1996; Rieppel and Reisz 1999), i.e., if the closest extinct relatives of turtles were terrestrial, their affinity with the Parareptilia might be more likely, while if an aquatic origin became apparent, they might indeed be sister of Sauropterygia. Thus, reconstructing the habitat of turtles lying just
outside the crown became a major theme of investigation.

Autochthony cannot be assumed of fossils found in fluvial or marine deposits since pre-burial transport into basins is common. Indeed, fossil turtles have been discovered in marine deposits, such as the Platy limestones of France and Germany, whose assemblages also happen to include terrestrial vascular plants, dinosaurs, and lepidosaurs (Joyce et al. 2021). Clearly, sedimentology alone does not provide a complete picture of an organism’s habits in life. To supplement the sedimentary record, authors commonly cite anatomical correlates as indices of habitat preferences in life. Some of such anatomical features are less robust than others; as first discussed by Ernst and Barbour 1989, though highly domed shells are most commonly in terrestrial turtles and dorsoventrally depressed ones are generally found in aquatic species, there are exceptions to each, like the highly domed shell of aquatic Cuora amboinensis and the greatly depressed shells of the tortoises Malacochersus tornieri and Manouria impressa. Dziomber et al. (2020) performed a quantitative analysis of shell ecomorphology that recovered overlapping morphologies in distinctly terrestrial and aquatic species, supporting the notion that shell morphology alone is not a reliable index of habitat. Given that the evolution of the compartmentalized middle ear of turtles has been regularly attributed to underwater hearing (Hetherington 2008; Christensen-Dalsgaard et al. 2012; Willis et al. 2013), Foth et al. (2019) compared the morphology of extant terrestrial and aquatic species’ middle ear cavities, though ultimately found no relationship between shape or size of the middle ear cavity and habitat. The most successful anatomical proxies for habitat have proven to be forelimb proportions (Joyce and Gauthier 2004; Dudgeon et al. 2021) and microstructure of shell bone (Scheyer and Sander
Both lines of evidence are in agreement that the closest extinct relatives of crown turtles lived in terrestrial settings.

One drawback of all the aforementioned morphological proxies for habitat, however, is that they are incapable of discriminating between marine, brackish, and freshwater habitats. The forelimb studies, for example, grouped together turtles from large bodies of water (oceans and lakes) and moving bodies of water (oceans and rivers) together, as the way a turtle swims—the function in which the forelimb is involved—is apparently independent of whether it occurs in marine or meteoric waters. That is to say that, from a morphological perspective, the large sideneck and softshell turtles are just as competent swimmers as sea turtles. Thus, if we are interested in reconstructing the paleoecology of fossil turtles at a finer scale than terrestrial vs. aquatic, we must identify non-anatomical proxies.

We are currently in the midst of something of a revolution in vertebrate paleontology: that is, the recognition that fossils preserve more information than just morphology, and specifically that they can serve as powerful geochemical records that can be at least as informative about the habits and habitats of extinct organisms. Stable isotopes are one such component of this. The bioapatite in vertebrate bone includes both phosphate and carbonate portions, and each of which include all three of the stable oxygen isotopes, whose fractionations are well understood (see below).

$^{18}O$ Signatures in Natural Waters
Lighter isotopes in water, $^{16}\text{O}$ and H, evaporate more readily than their heavier stable counterparts, $^{17}\text{O}$, $^{18}\text{O}$, and D, respectively. Conversely, the heavier isotopes condense before the lighter isotopes. This leads to the ocean being enriched in $^{18}\text{O}$ and meteoric waters being enriched in $^{16}\text{O}$ over the course of the hydrologic cycle. $^{18}\text{O}$ values are typically reported as $\delta^{18}\text{O}$, which is defined by the following equation:

$$\delta^{18}\text{O} = \left( \frac{\left(\frac{^{18}\text{O}}{^{16}\text{O}}\right)_{\text{sample}}}{\left(\frac{^{18}\text{O}}{^{16}\text{O}}\right)_{\text{reference}}} - 1 \right) \times 1000 \text{‰}$$

Barrick et al. (1999) showed that $^{18}\text{O}$ ratios measured from turtle shell phosphate did indeed recover a slope of increasing $\delta^{18}\text{O}$ values from freshwater to marine turtles, as would be expected from the isotope ratios of the waters in their environments.

**Utility of Triple Oxygen**

The two stable isotopes of oxygen, $^{18}\text{O}$ and $^{17}\text{O}$, are assumed by mass dependent fractionation to follow a fixed proportion to each other in natural systems, such that:

$$\frac{^{17}\text{R}_{s}}{^{17}\text{R}_{r}} = \left(\frac{^{18}\text{R}_{s}}{^{18}\text{R}_{r}}\right)^{\lambda}$$

where $^{17}\text{R}$ represents the ratio $^{17}\text{O}/^{16}\text{O}$, $^{18}\text{R}$ represents $^{18}\text{O}/^{16}\text{O}$, $s$ and $r$ represent the sample and reference, respectively, and the exponent $\lambda$ represents the terrestrial fractionation line (TFL), that in natural waters has been found to be $\approx 0.528$ (Meijer and Li 1998; Barkan and Boaz 2005).

Deviations from the TFL due to particular causes of fractionation (varies by process) are reported as $\Delta^{17}\text{O}$, that is calculated as the following:

$$\Delta^{17}\text{O} = \delta^{17}\text{O} - 0.528 \times \delta^{18}\text{O}$$
where $\delta^{17}\text{O}$ is the ratio of $^{17}\text{O}/^{16}\text{O}$ in a sample relative to the ratio in Vienna standard mean ocean water (VSMOW) and $\delta^{18}\text{O}$ is the ratio of $^{18}\text{O}/^{16}\text{O}$ in a sample relative to the ratio in VSMOW (Passey and Levin 2021). Such deviations, measured from the carbonates of animals, can also provide a signal of the origin of the water in which the carbonates were formed (Passey et al. 2014).

**Hypotheses and predictions**

By measuring both the $\delta^{18}\text{O}$ and $\Delta^{17}\text{O}$ incorporated in the carbonate portion of the bioapatite in extant turtle bones, we hypothesized that we could recover and calibrate a signal of a turtle’s habitat in life based on the values of these metrics that could then be used to reconstruct the paleoecology of a fossil turtle. Given that meteoric waters are depleted in $^{18}\text{O}$ relative to the ocean and have a positive deviation from the TFL of 0.033‰ (Luz and Barkan 2010), we predicted that freshwater turtles would have lower $^{18}\text{O}$ values and higher $\Delta^{17}\text{O}$ than marine turtles, with brackish turtles displaying intermediate values. We expected terrestrial turtles to carry an O isotope signal predominantly representative of atmospheric $\text{O}_2$.

**Methods**

*Selecting extant specimens*

Bone material was selected from three specimens of marine (*Caretta caretta* and *Dermochelys coriacea*), brackish (*Malaclemys terrapin*), freshwater (*Chrysemys picta*), and terrestrial (*Terrepene carolina*) turtles from the Division of Vertebrate Zoology of Yale Peabody Museum (YPM). All aquatic specimens were collected from Connecticut waters, while the terrestrial turtles were collected from Rhode Island. Where possible, we chose to destructively sample the
most physically damaged and disarticulated specimens (i.e., those that would be relatively less informative for future anatomical studies).

**Selecting fossil specimens**

We selected fossil shell fragments—composed of metaplastic bone—from two Cretaceous *Bothremydae* side-neck turtles from Division of Vertebrate Paleontology at YPM: *Taphrosphys sulcatus* (YPM VPPU.018706) collected from the lowermost portion of the Hornerstown Formation in Sewell, New Jersey, and *Chedgihaii barberi* (YPM VP.003608), formerly *Bothremys barberi*, from the Niobrara Formation in Wallace County, Kansas. Both fossils were inferred as being marine/estuarine in habits on a sedimentological basis despite the challenges of assumptions of autochthony described above (Gaffney 1975; Gaffney et al. 2006).

**Collecting carbonate samples**

Using a Dremel tool with a brush bit, we cleared away organics and/or loose sediment from the surface of the bone. Then, with a diamond-tip bit, we shaved off cortical bone dust and collected it in sample vials, avoiding less dense inner trabecular bone that is likely to include more organics. Bones that were too small to shave bone dust from were crushed in mortar and pestle. We used the same procedure for fossil shell fragments, except we also cleared away an outer layer of material that visibly appeared to be more diagenetically altered than the underlying cortical bone using the diamond-tip bit prior to collecting any the material in sample vials. This step also cleared away any adhesives or other coatings that may have been present on the surface of the fossil.
Purifying carbonate samples

Each sample, fossil and Recent, was then reacted with 8% NaClO solution to remove organic material. After 20 minutes, the solution was diluted with deionized water and decanted five times. Fossil samples were also reacted with 2ml of 0.1M acetic acid solution for 45 minutes to react away secondary carbonates and other mineral contaminants (Koch et al. 1997), and then were diluted with deionized water and decanted five times. Wet samples were placed in an oven overnight at 60ºC to dry. We used shorter reaction intervals than other authors in order to avoid isotopic exchange reported to occur with the same reactants at much longer time intervals (e.g. Koch et al. 1997).

Purifying CO₂

Each 20-25mg sample was then degassed in a split flask with 2ml of 105% H₃PO₄ and placed in a 25ºC water bath to equilibrate. Flasks were then tipped so that the samples could be digested by the acid within the bath overnight. The released CO₂ was then purified using cryogenic (liquid nitrogen and dry ice) traps along a pump line to remove H₂O and non-condensable gases. This process is similar to that used for bulk carbonates (McCrea, 1950) except that we used a larger sample due to the low concentration of CO₃ in bioapatite.

Purifying O₂

Following Wostbrock et al. (2020), CO₂ was cryogenically transferred into Ni tubes for fluorination. 30-times more gas than stoichiometrically necessary for a complete reaction of BrF₅ was then added as an oxidizing reagent and the tubes were then heated to 700ºC for 4 days to ensure complete fluorination. The tubes were then frozen with liquid nitrogen and the O₂ gas
then cryogenically purified along a vacuum line. The tubes were warmed and refrozen to release any remaining O2 that may have been trapped in the initial BrF5 ice. Purified O2 was adsorbed onto a 5 Å mol sieve cooled to liquid nitrogen temperature. Note that pure O2 is necessary for measuring $^{17}$O values because it is indistinguishable from $^{13}$C in CO2.

**Mass spectrometry**

Purified O2 was carried from the mol sieve to a second mol sieve at the mass spectrometer in a He stream set at a constant flow of 6.0 ml/min at room temperature through a 6ft, 1/8in diameter, 5 x mol sieve gas chromatograph. The He/O2 mixture was cooled to liquid nitrogen temperature at the mol sieve, allowing all He to be pumped away. The O2 was then expanded into a Thermo MAT 253mass spectrometer at the Yale Analytical Stable Isotope Center in New Haven, Connecticut specifically configured for O2 gas. The oxygen was measured in dual inlet mode using 26 second integration with 20-30 iterations per analysis. Sample gas was measured against an in-house O2 reference gas calibrated for triple oxygen isotope values based on the standard published in Sharp and Wostbrock (2021).

**Results**

Some results were not replicable due to contamination of the sample at some point during the purification process or analysis in the mass spectrometers. These data were accordingly discarded. As a result, we only ended up with two marine turtles, three brackish turtles, three freshwater turtles (though one of the $\Delta^{17}$O values was unrepeatable), and two terrestrial turtles in the sample. Mean values for all measurements are reported in Table 1.
<table>
<thead>
<tr>
<th>Mean</th>
<th>$\delta^{18}O$ (CO$_2$) (%)</th>
<th>$\delta^{17}O$ (CO$_3$) (%)</th>
<th>$\Delta^{17}O$ (CO$_3$) (per meg)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine</td>
<td>37.561</td>
<td>14.043</td>
<td>$-0.139$</td>
<td>2</td>
</tr>
<tr>
<td>Brackish</td>
<td>35.148</td>
<td>12.790</td>
<td>$-0.146$</td>
<td>3</td>
</tr>
<tr>
<td>Freshwater</td>
<td>34.032</td>
<td>12.191</td>
<td>$-0.161$</td>
<td>3 (2 for $\Delta^{17}O$)</td>
</tr>
<tr>
<td>Terrestrial</td>
<td>35.145</td>
<td>13.290</td>
<td>$-0.161$</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1. Mean values and sample sizes for all measurements from each habitat type.

$\delta^{18}O$ from CO$_2$

Marine turtles showed the highest $\delta^{18}O$ values, with a mean of 37.561‰. Brackish and freshwater turtles had mean $\delta^{18}O$ of 35.148‰ and 34.032‰, respectively. The mean $\delta^{18}O$ of terrestrial turtles was 36.145‰.

$\Delta^{17}O$ from CO$_3$ (via O$_2$)

$\delta^{17}O$ values are plotted against $\delta^{18}O$ for each sample in Figure 1a to show the consistency of our data with TFL. $\Delta^{17}O$ values of marine turtle carbonates were the least negative with a mean of $-0.139$ per meg. Brackish turtles were slightly more negative with a mean of $-0.146$ per meg. Freshwater and terrestrial turtles both had a mean of $-0.161$ per meg. $\Delta^{17}O$ values of each sample are plotted against $\delta^{18}O$ values in Figure 1b.
**Figure 1.** a) $\delta^{17}\text{O}$ (from carbonate) plotted against $\delta^{18}\text{O}$ (from CO$_2$); b) $\Delta^{17}\text{O}$ (from carbonate) plotted against $\delta^{18}\text{O}$ (from CO$_2$), $y = 0.033$ represents the deviation from TFL in meteoric waters and $y = 0$ represents oceanic water given the reference standard (VSMOW). Circles represent extant turtle samples and triangles represent fossil turtle samples. Red circles are marine; purple circles are brackish; blue circles are freshwater; green circles are terrestrial; the black triangle is YPM VP.003608; and the orange triangles are measurements taken from two scutes from YPM VPPU.018706. Sample labels corresponding to each data point are shown at $y = 10\%$ and $y = -0.075$ per meg, respectively.

*Fossil data*
Pictures of the sampled bony shell material are shown in Figure 2. YPM VP.003608 had $\delta^{18}O$ of 34.628‰ and $\Delta^{17}O$ of $-0.152$ per meg. The two shell-bone fragments from YPM VPPU.018706 did not have identical values for $\delta^{18}O$ or $\Delta^{17}O$ ($\delta^{18}O$: 40.025‰ and 39.394‰; $\Delta^{17}O$: $-0.164$ and $-0.185$ per meg, respectively).

**Figure 2.** a) Scute from YPM VP.003608; b) two scutes from YPM VPPU.018706. Both specimens shown post-sampling. Surrounding material is representative of what was sampled. Scale bars are equivalent to 1cm.

**Discussion**

$\delta^{18}O$ values from extant turtles were consistent with our hypothesis that skeletal carbonates in aquatic turtles would preserve a roughly consistent signal of O isotope ratios in environmental waters, resulting in the highest $\delta^{18}O$ values being found in marine turtles and the lowest being found in freshwater turtles. The $\Delta^{17}O$ results were in stark contrast to these, however: all values we recovered were heavily negative, suggesting that incorporation of atmospheric oxygen strongly influences the O makeup of skeletal carbonates, as atmospheric oxygen is depleted in $^{17}O$ relative to the TFL (average $\Delta^{17}O$ of air = $-0.447$; Sharp and Wostbrock 2021). Essentially, we found that $\delta^{18}O$ provides a record of the environmental water, while $\Delta^{17}O$ records the degree
to which turtles are incorporating atmospheric O (presumably via breathing) over the O of the water in which they live (which is presumably incorporated through drinking and diet). Brackish turtles displayed intermediate values between freshwater and marine turtles for both metrics, but their $\delta^{18}O$ values were slightly closer to our freshwater values while their $\Delta^{17}O$ were slightly closer to our marine values. This may be explained by the nature of brackish habitats, or at least the saltmarshes that Malaclemys terrapin inhabits in the northeastern USA. These habitats have a regular, if not constant, influx of freshwater that may lead to animals preferentially spending time in the proximate (relative to the continent) margins of the habitat. This is observed in non-obligate brackish-dwelling turtles, such as Chelydra serpentina, though these animals lack the cranial salt-excretory glands present in M. terrapin. These habitats may, however, not have as many exposed surfaces upon which turtles can bask, meaning that brackish turtles spend more of their time in the water, consuming a greater proportion of oxygen from the water than from the atmosphere, more like a marine turtle rather than one living in freshwater or on land. Additionally, it is known that oxygen consumption and salinity are positively correlated in M. terrapin (Bentley et al. 1967), and the periodic intrusion of saltwater into brackish habitats via tidal flow provides a regular source of elevated salinity. It thus makes sense that a greater proportion of oxygen that has been incorporated into skeletal carbonates originates in water rather than air if a) there are fewer sites at which the turtle can be exposed to air for extended periods of time and b) high salinity makes brackish turtles consume more oxygen cutaneously from the water.

Given that the ocean is a larger reservoir than rivers, lakes, and other terrestrial accumulations of meteoric waters, we expected the proportion of environmental O$_2$ in skeletal carbonates from
marine animals to be more consistent because perturbations to isotope ratios in the ocean are generally better buffered. The fact that the two marine turtle specimens sampled (both Caretta caretta), one an adult and one a sub-adult as judged by the relative size of bone elements, had δ¹⁸O and Δ¹⁷O values that were more disparate than extant samples from any other habitat type (even after re-running material from the samples to confirm repeatability) prompted us to consider possible ontogenetic sources of variability. The sub-adult animal, whose δ¹⁸O value was ~1.5‰ lower than the adult and Δ¹⁷O value, lay within the range of the three brackish samples, is, as a result, suspected to have belonged to a population of North Atlantic C. caretta that spends their pre-breeding foraging life-stage in the brackish waters of the Indian River Lagoon (Reece et al. 2006). This suggests that the environmental signals in O isotope ratios from earlier life stages are not rapidly overwritten in skeletal carbonates and, as such, we suggest that only putative adult specimens of extant or extinct species be sampled for isotopic ratios in skeletal carbonates.

The δ¹⁸O and Δ¹⁷O values of YPM VP.003608 cluster within the range of the extant freshwater and brackish turtles we sampled. Thus, our data suggest that Bothremys barberi was distinctly not marine and may have been freshwater or estuarine in habit. The values recovered from YPM VPPU.018706, however, are not only inconsistent across shell fragments sampled, but inconsistent in the environmental signal they provide. The δ¹⁸O values were higher than in any of the extant turtles we sampled and thus were most similar to the marine turtles. The Δ¹⁷O values, however, were among the most negative of the values we recovered from extant turtles. In other words, the δ¹⁸O suggests marine habits for Taphrophys sulcatus, while the Δ¹⁷O values suggest freshwater or terrestrial habits. Thus, our data are inconclusive regarding the
paleoecology of *T. sulcatus*. We lacked the time and funding this spring to analyze any of the fossil samples multiple times to test for repeatability.

Clearly, more data are required to confirm or reject the habitat signals we identified in the ratios of stable O isotopes in *Testudines* skeletal carbonates. Future plans include sampling more specimens from these habitat types in Connecticut, as well as sampling along other transects within *Malaclemys terrapin* range, as it is the only living obligate brackish-water turtle. In these future analyses, we also hope to test the triple O isotopes in the phosphate portion of bioapatite. We also plan to resample the fossils used in this study. Comparing values recovered from carbonates and phosphates may illuminate differences in the degree of corruption of environmental signals due to diagenesis in the fossil samples.

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