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Supplementary Materials for

Eggshell geochemistry reveals ancestral metabolic thermoregulation in Dinosauria

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

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Data S1 (Microsoft Excel format). Raw data for both carbonate samples and standards and CO_2 standards.

SUPPLEMENTARY MATERIALS

Supplementary Materials and Methods

Geological Setting of Fossil Eggshell and Mollusks

We analyzed seven fossil eggshell fragments, representing the three major clades of dinosaurs (Fig. 1; Theropoda, Sauropodomorpha, and Ornithischia). These samples come from upper Campanian deposits of Alberta, Canada (Fig. S1; Theropoda, Ornithischia) and upper Maastrichtian deposits of Transylvania, Romania (Sauropodomorpha or Ornithischia) (Fig. S2). Based on screening for diagenetic alterations described below, two out of the seven eggshell samples are considered poorly-preserved. For the others, Δ_{47} values were used to estimate dinosaur body temperatures (Δ_{47} is defined in detail below).

Eggshell fragments representing Theropoda (*Troodon formosus*) and Ornithischia (Hadrosaurs: unidentified lambeosaurine, *Hypacrosaurus stebingeri,* and *Maiasaura peeblesorum*) were recovered from the upper Campanian Oldman Formation in southeastern Alberta (Fig. S1) from three distinct localities: Wann's Hill (WH) in the Milk River Natural Area, Little Diablo's Hill at Devil's Coulee (DC), and Lost River Ranch (LRR). All three sites are thought to be roughly contemporaneous (*61*). The Oldman Formation is part of the Belly River Group, an eastward-thinning clastic wedge deposited in the Western Canada Basin in response to tectonic uplift of the Canadian Cordillera. In southernmost Alberta, the Oldman Formation is over 160 meters thick and consists of interbedded mudstones, siltstones, and sandstones deposited in an ephemeral, low-sinuosity fluvial system (*62*). Structural dip of the formation is very close to 0^{o} but dips slightly to the west. The prevalence of paleosols containing carbonate nodules and slickensides indicates that the climate at the time was semi-arid to arid and characterized by seasonal precipitation (*62*), although the presence of hydromorphic paleosols suggests that wetlands were present, at least seasonally (*63*). Radiometric dating of volcanic ash deposits has revealed that the upper Oldman Formation of southern Alberta was deposited between 76.2 Ma and 74.9 Ma (*62*).

All eggshell fragments and fossil mollusk shells come from the same interval of overbank deposits known to produce hadrosaur eggs and embryonic remains (63) and most are catalogued as part of the Royal Tyrrell Museum of Palaeontology (TMP) collection. Eggshells identified as *Maiasaura peeblesorum* (TMP 2009.153.1), *Hypacrosaurus stebingeri* (TMP 1989.69.10), and *Troodon formosus* (TMP 2008.75.127) were recovered from Devil's Coulee (*30, 64*). Eggshell fragments identified as a 'lambeosaurine hadrosaur' (TMP 1988.121.41) and *Troodon formosus* (TMP 1995.21.4) were recovered from Wann's Hill. An additional *Troodon formosus* eggshell fragment (TMP 2003.81.1) was recovered from Lost River Ranch (Table S1). The three different *Troodon formosus* eggshell fragments allow us to compare Δ_{47} results from the same taxon across contemporaneous localities that likely experienced similar paleoenvironmental conditions, but possibly different preservation histories. Vitrinite reflectance analyses of the Campanian-Maastrichtian deposits of the southeastern Alberta plains suggest that these sediments were never heated above 80°C (namely, never reached the oil window; ref. (*44*)). As such, Δ_{47} alteration by solid state diffusion is unlikely in these fossil eggshells and associated mollusks fossils (*65*).

Mollusks shells from the stratigraphically contemporaneous (upper Oldman Formation) sites were also analyzed to characterize paleoenvironmental temperatures. A freshwater gastropod shell (W20) was collected from Wann's Hill (TMP collection, not yet catalogued). A freshwater bivalve was collected from the southern side of the Milk River Natural Area (TMP 2009.149.5).

Eggshell fragments thought to belong to the oospecies *Megaloolithus* cf. *siruguei* (TMP 1991.175.2) were collected from the Tuştea locality, which preserves exposures of the Maastrichtian Densuş-Ciula Formation, in the Haţeg Basin of Transylvania, Romania. The eggshell was preserved in red, pedogenically-modified mudstones preserving caliche nodules. The paleosols at this locality exhibit slickensides and carbonate nodules, indicative of a semi-arid to arid climate characterized by seasonal precipitation (*66*). The sedimentary sequence at Tuştea is indicative of a braided stream with well-drained over-bank deposits (*66*). It is unlikely that solid state diffusion affected the preservation of the *Magyarosaurus* eggshell as the Maastrichtian Densuş-Ciula Formation was buried only up to 500 m for the last 65 Ma (*45*) and therefore experienced temperatures well below 100°C (assuming an average geothermal gradient (*46*) of ~25°C/km) over its burial history.

Bulk Trace Metal Concentrations

Carbonate samples were prepared and analyzed for trace elements in a Picotrace Clean Lab at the Yale Metal Geochemistry Center. In preparation for analysis, powdered samples (~6mg each) were weighed into 1.5 ml acid-cleaned centrifuge tubes and then leached by three 0.5 ml steps of 0.35N HCl in sequence, with each leaching step lasting 1 hour, 30 minutes and 10 minutes, respectively. After each leaching step, the sample was centrifuged and its supernatant combined into sample-specific acid-cleaned Teflon beakers. After completing the leaching, centrifuge tubes with the leached solid were heated on a hotplate at 60ºC for three days until dry and then weighed to calculate the carbonate leaching yield. The combined supernatants were heated on a hotplate at 100ºC for 12 hours, until dry and then re-dissolved in 5% HNO_3 with a 2ppb Indium spike for trace element analysis. The volume of dilute nitric acid was adjusted for each sample using the carbonate yield data to reach a dilution factor of 1000. These solutions were analyzed by comparison to in-house trace element analytical standards, on a Thermo Element XR ICP-MS. Analytical standard solutions were made in-house using single element standards to match the typical carbonate trace element range. In addition, sample solutions were measured alongside two geostandards, USGS COQ-1 carbonatite and USGS BHVO-2 basalt standard with a Ca spike to match the matrix of our sample solutions. All standard values were within 5% of their expected values. Procedural blanks were below detection limit with the exception of Zn.

CL Images and Color Thresholding Using ImageJ

We estimate the portion of the sample that is affected by recrystallization or over growth using the ImageJ software (*67*) with a Fiji platform (*68*) to analyze the CL images. We set a color threshold using hue, saturation and brightness levers, to select and measure only the number of pixels that glow with some yellow in the images (Fig. S5). Then using a touch screen computer and stylus, we traced the outlines of the whole eggshell in cross-section to measure the total number of pixels that represent the whole shell. This approach assumes that the image of the shell in cross-section is representative of the composition of the whole shell.

Clumped isotope (Δ_{47}) analysis

Laboratory Protocol and Standardization

Carbonate clumped isotope thermometry is expressed via the parameter Δ_{47} that is related to the temperature of $CaCO_3$ formation and is independent of the isotopic composition of the water in which it forms. Δ_{47} is a measure of the thermodynamic preference of two heavy isotopes to bind to each other, reflecting the over-abundance of (mostly) $^{13}C^{18}O^{16}O$ in CO_2 extracted from $CaCO_3$ relative to the abundance expected from the random distribution of ^{13}C and ^{18}O among all CO_2 isotopologues. This relative abundance of ^{13}C-^{18}O bonds in the carbonate lattice is temperature-dependent, providing a robust paleothermometer (8, *27*).

Eggshell and mollusk shell samples were ground using a mortar and pestle. Powdered carbonate samples (3.5-5mg) were reacted in McCrea type reaction vessels with 1-2ml of 105% phosphoric acid overnight at 25 °C. The extracted CO_2 was cryogenically separated on a vacuum line and passed through a gas chromatograph column (Supelco Q-plot, 30m X 0.53mm) at -20 °C to remove volatile organic compounds (*69*, *70*).

Isotopic analyses were performed at the Yale Analytical and Stable Isotopic Center. Clean CO_2 samples were analyzed for $\delta^{13}C$, $\delta^{18}O$, and Δ_{47} using a Thermo MAT-253 dual inlet gas source isotope ratio mass spectrometer, configured to simultaneously measure masses 44-49. Each sample was analyzed for 90 cycles of sample-reference gas comparison, with 8-second signal integration time. The shot-noise Δ_{47} precision limit for samples measured at 16V (for mass 44) is 0.006‰. Samples were analyzed as 3-5 replicates. Analyses were performed in 5 batches: February to April, 2014, May to July, 2014, September, 2014 to April, 2015, May to October, 2015, and May to July 2018 during which five different heated gas lines were used to standardize results. This is aimed to minimize systematic errors associated with short-term variations due to sample preparation and mass spectrometric conditions. See Table S1 for averages of sample replicates and Data S1 for raw values of samples and standards and heated gas lines used for different analytical time periods.

Δ_{47} values were calculated from the measured ratios of masses 45, 46, and 47 to mass 44 (R^{45}, R^{46}, R^{47} respectively), relative to the stochastic distribution of all isotopologues (R^{45*}, R^{46*}, R^{47*}) following ref. (*27*)

$$\Delta_{47} = \left[\left(\frac{R^{47}}{R^{47*}} - 1\right) - \left(\frac{R^{46}}{R^{46*}} - 1\right) - \left(\frac{R^{45}}{R^{45*}} - 1\right) \times 1000\right] \quad \text{(Eq. 1)}$$

Standardization of sample CO_2 gas was performed as described in ref. (*8*). Δ_{47} values are converted to the absolute reference frame following the methods outlined in the supplementary material of ref. (*58*). In addition, internal laboratory standards including Carrera marble (YCM) were analyzed to test for instrument stability and quantify external precision. See Data S1 for raw values of the standards. The long-term average (±1SD) Δ_{47} value in the absolute reference frame ($\Delta_{47(abs)}$) for Carrera marble is 0.400 ±0.028‰ (n=49, ref. (*71*)). The average (±1SE) Carrera marble $\Delta_{47(abs)}$ values during the 5 periods of analysis were 0.407 ±0.010‰ (n=6), 0.402 ±0.009‰ (n=12), 0.407 ±0.008‰ (n=20), 0.401 ±0.007‰ (n=16) and 0.418 ±0.011‰ (n=7) (see Data S1 for raw values). The pooled error on all YCM analyses during these 5 periods (n=60) is ± 0.016‰ (± 1SD of residuals). Temperatures were calculated from $\Delta_{47(abs)}$ values based on the calibration line of laboratory precipitated $CaCO_3$ produced at Yale (*8*; Fig. 2). Statistical tests for comparison of Δ_{47} values were conducted in PAST (*72*). Mann-Whitney tests were conducted to compare dinosaurian body temperatures to average mollusk-derived environmental temperatures based on Δ_{47} results.

$\delta^{18}O_{VPDB}$ and $\delta^{13}C_{VPDB}$ were measured simultaneously as part of the Δ_{47} analysis, using an Oztech reference gas CO_2 with a composition of $\delta^{18}O_{VPDB}$ = -15.80‰ and $\delta^{13}C_{VPDB}$ = -3.64‰. Measurements of NBS-19 result in $\delta^{18}O_{VPDB}$ = -2.17±0.04‰ and $\delta^{13}C_{VPDB}$ = +2.11±0.13 ‰ (1 SD, n=12), comparable with the recommended IAEA values of -2.20 ‰ and 1.95 ‰, respectively (*73*). The $\delta^{18}O_{VPDB}$ values of the measured CO_2 were converted to those of carbonate using an acid fractionation factor at 25°C, $\propto_{CO_2-calcite} = 1.01030$ (*74*).

^{17}O Correction and Temperature Calibration

Since there is agreement within the clumped isotope community that reporting Δ_{47} values calculated using the Brand parameters for ^{17}O is preferred, we convert our data (using the equation of ref. (*26*)) to be based on the Brand parameters, and provide the raw data that will allow future studies to be compared with our results regardless of the parameter set used (see Data S1). The Brand values of Yale internal laboratory standards (YCM, 9-25-G, Cylinder CO_2) and heated gases were determined based on averages of these standards when measured together with CO_2 equilibrated with water, that in turn serve to define the absolute reference frame. These values were used to convert sample data to the Brand parameters absolute reference frame ($\Delta_{47(abs)}$). The Δ_{47} values using Brand parameters for the 3 standards plus the theoretical value of heated gases in the absolute reference frame are 0.396‰, 0.708‰, 0.923‰, and 0.027‰, respectively.

Generally, for samples and standards with $\delta^{13}C$ and $\delta^{18}O$ close to that of the reference (working) gas the choice of parameters exerts only a small control on Δ_{47} (note that the heated gases used at Yale vary in both $\delta^{13}C$ and $\delta^{18}O$ so that this effect is not a simple function of sample $\delta^{13}C$ and $\delta^{18}O$). For example, the absolute Δ_{47} values for samples reported here would shift on average by -0.007‰ when switching from the Gonfiantini to Brand parameter sets (raw Δ_{47} data using both parameter sets are given in Data S1). This offset in Δ_{47} is close to the typical analytical uncertainty (±1SE; see above). The laboratory precipitation data of ref. (*8*), on the other hand, shift significantly on average, by +0.021‰, resulting in a mismatch between this calibration and the marine biogenic data after conversion to Brand parameters.

However, due to this mismatch we cannot reasonably use a calibration based on the Brand recalculated laboratory precipitated carbonates to estimate paleotemperatures from biogenic carbonate. We therefore consider three possible Δ_{47} - T calibrations for our Brand parameter dataset. The first is a recently proposed universal Δ_{47} - T calibration, based on laboratory-precipitated carbonate (*75*), the second is the linear fit to biogenic carbonate analyzed over the last 7 years at Yale University ($\Delta_{47} = (0.0519)\frac{10^6}{T^2} + 0.1125$; Fig. S3B), and the third is from a recent clumped isotope thermometer calibration using extant large benthic foraminifera (LBF) measured at Yale University (*59*) and given in the Brand parameter reference frame ($\Delta_{47} = (0.0537)\frac{10^6}{T^2} + 0.09$). When using either one of these three equations the change in temperature, relative to the original Gonfiantini parameters, is on average less than 1°C (Data S1). We therefore report our data using the Gonfiantini parameters and the Gonfiantini based Δ_{47} – temperature calibration (Fig. 2) from ref. (8) in order to enable direct comparison with previously published dinosaur body temperatures *(5, 6)*.

Supplementary Figures

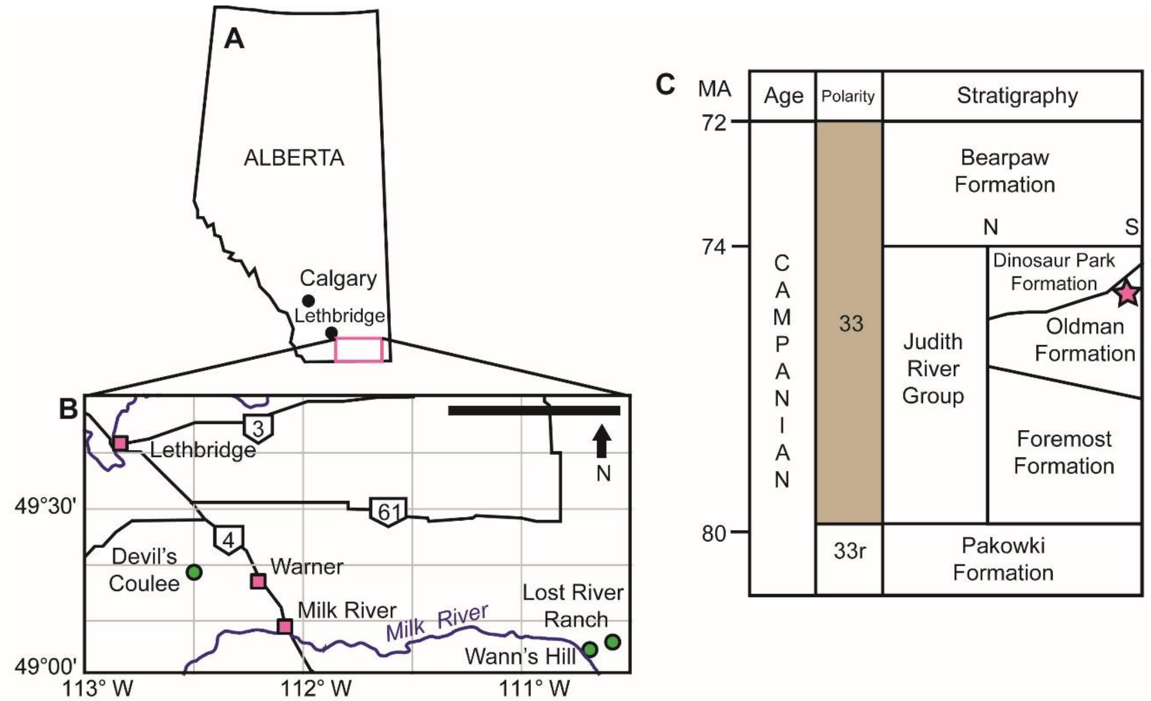

Fig. S1. A) Fossil localities in southern Alberta, Canada (pink rectangle in A). **B)** Detailed map of pink rectangle inset from **(A)**, showing location of the three fossil sites (modified from ref. (*30*)). Scale bar is 50 kilometers. **C)** Simplified stratigraphic column indicating age of Oldman Formation (pink star; ref. (*61*)).

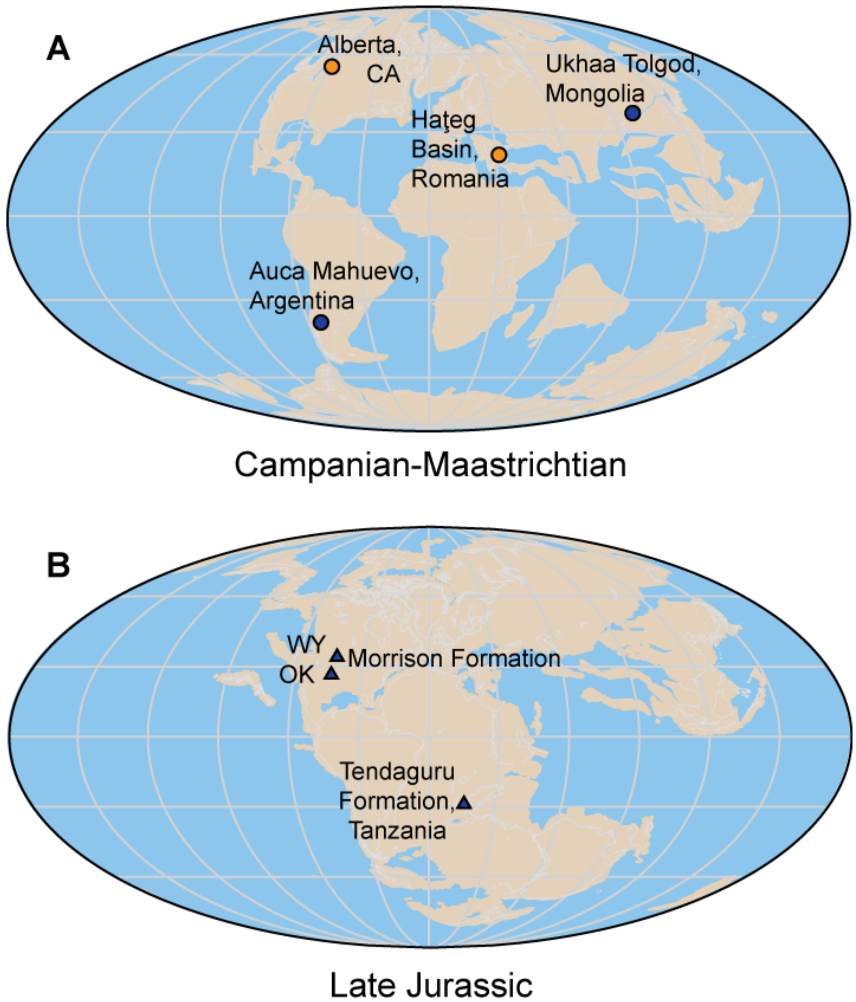

Fig. S2. Paleogeographic map reconstruction of the **A**) Campanian-Maastrichtian and **B**) Jurassic (modified from the Paleobiology Database). Localities of samples analyzed in this study are orange and those from previous studies (*5*, *6*) are blue. Localities of sampled fossil eggshell are shown as circles and fossil teeth as triangles.

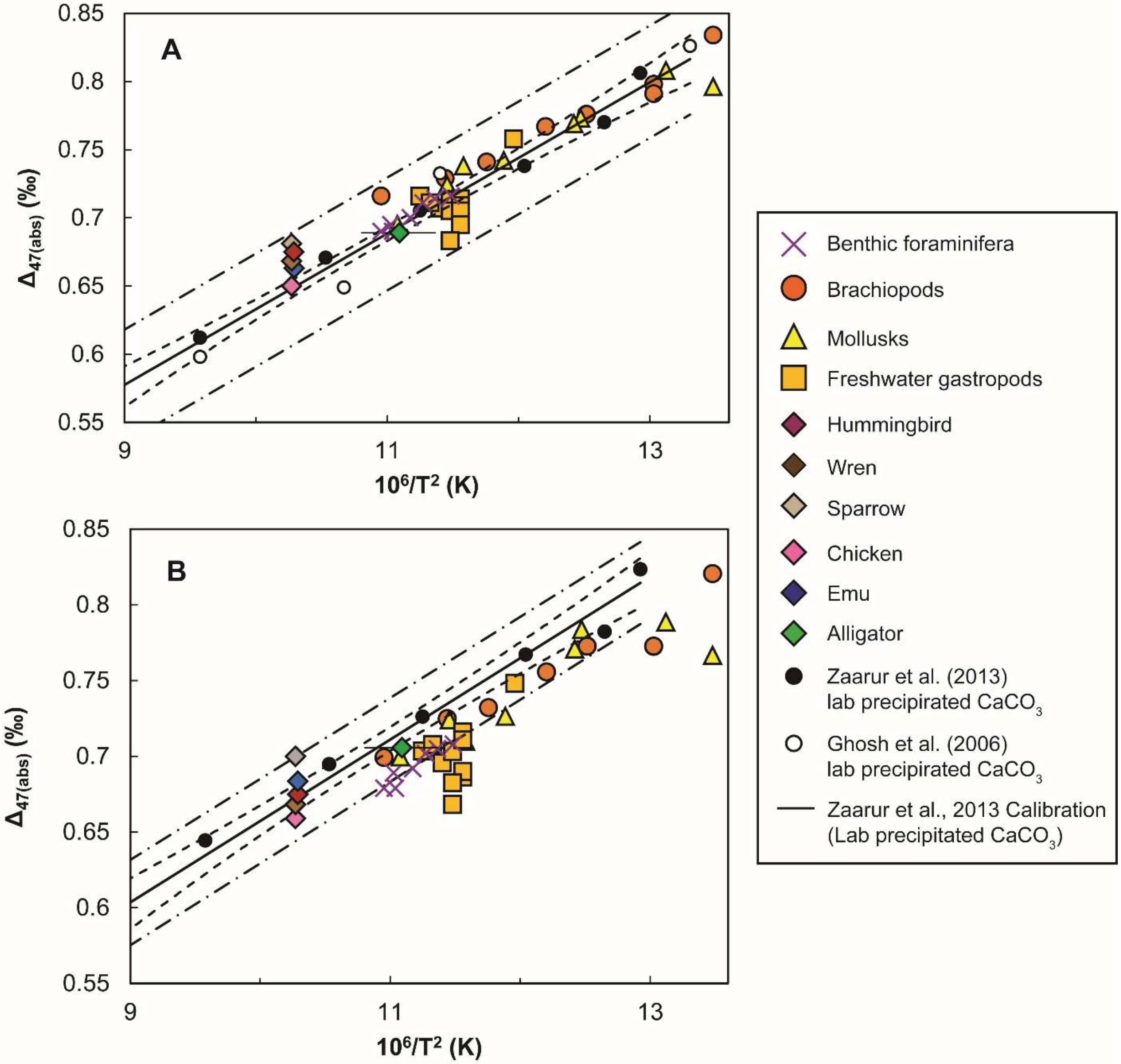

Fig. S3. Clumped isotope [$\Delta_{47(abs)}$] value versus temperature relationship of laboratory-precipitated carbonate calibration data (*8*). A) using Gonfiantini parameters and **B**) same dataset but using Brand parameters. Calibration line (solid), 95% confidence interval (dotted line). Δ_{47} data of biogenic carbonates analyzed between 2010-2018 at Yale University (8, *56-59*), including our extant eggshells (diamonds), are calculated using either Gonfiantini or Brand parameters, respectively, and shown for comparison.

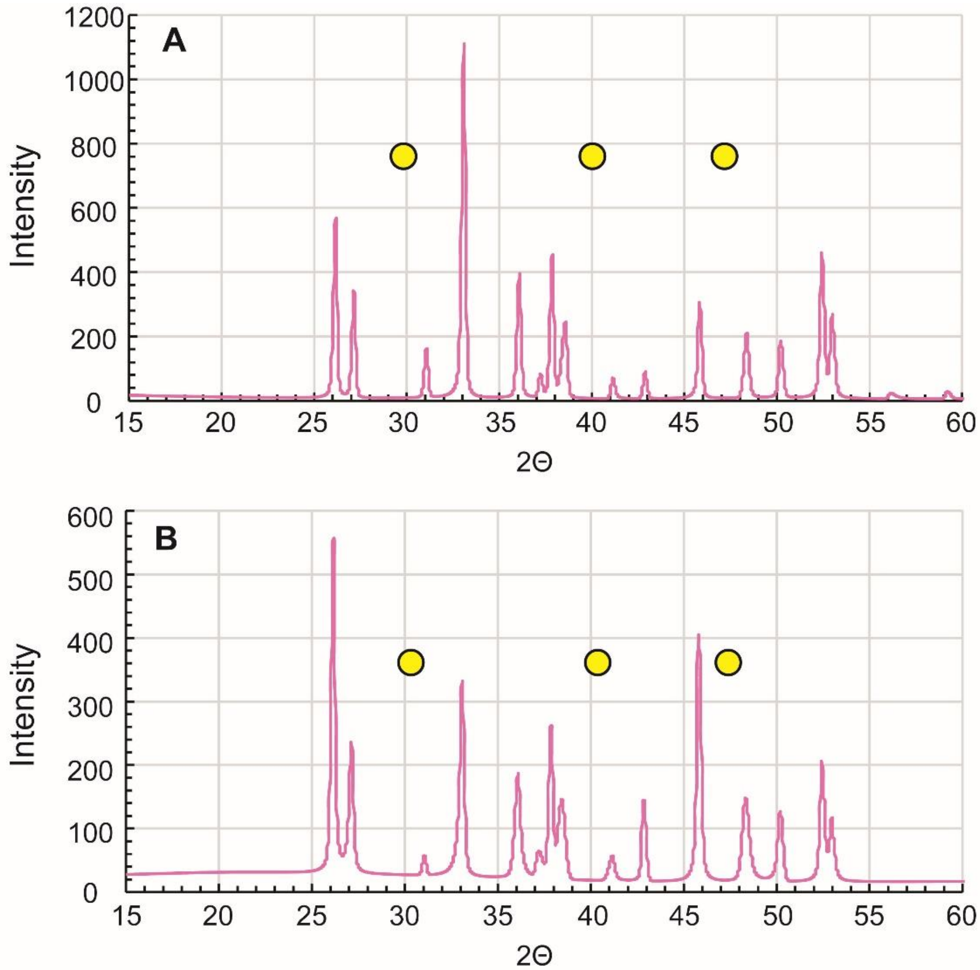

Fig. S4. X-ray diffraction spectra of fossil mollusks. (**A**) Milk River Bivalve (TMP 2009.149.5). (**B**) Wann's Hill Gastropod (TMP uncatalogued). Yellow circles indicate where calcite peaks would have appeared if the aragonitic shells had recrystallized to calcite.

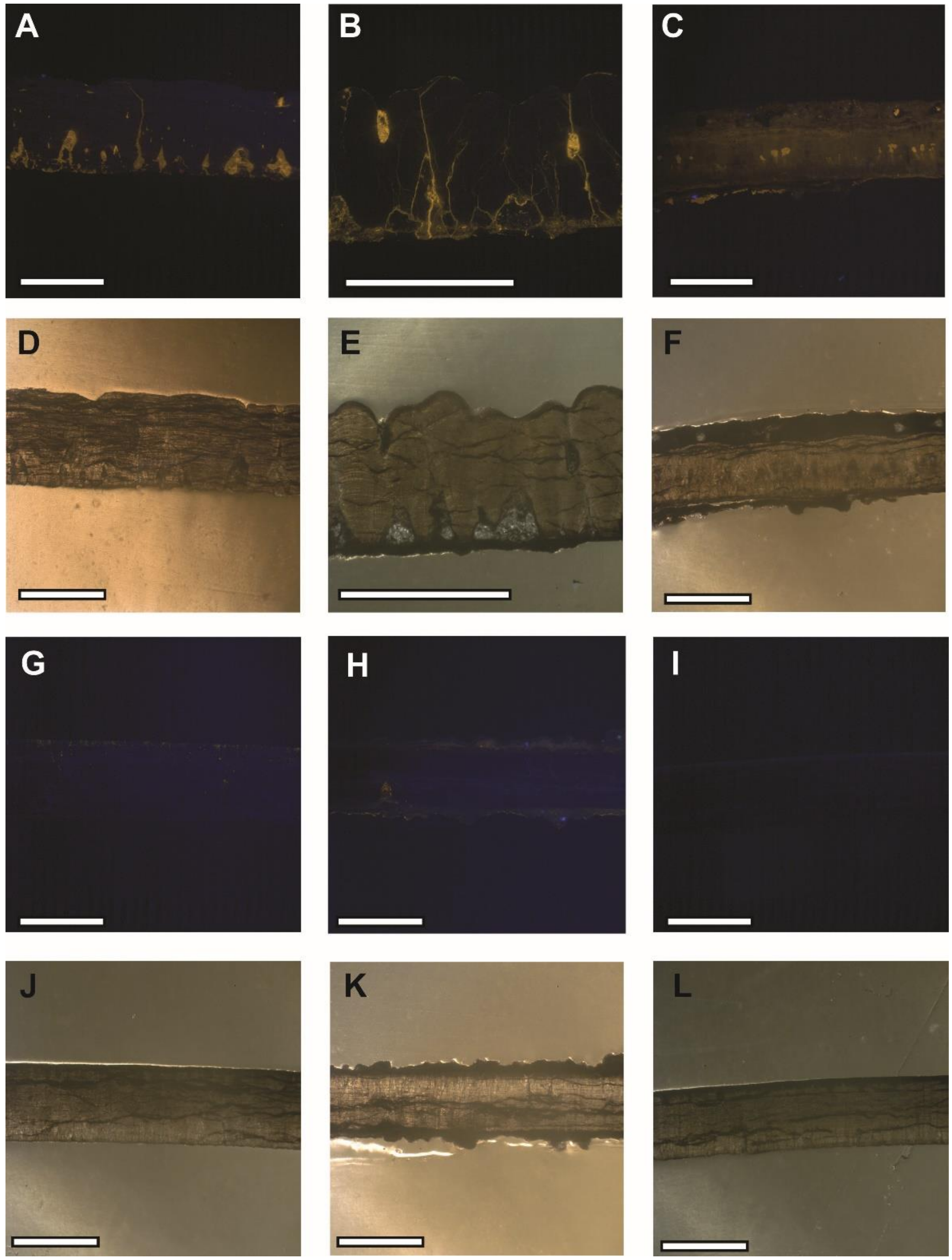

Fig. S5. Photomicrographs of dinosaur eggshell samples. Scale bars are all 1 mm. (**A-C**) Cathodoluminescence (CL) microscopy images of (A) Devil's Coulee *Maiasaura* eggshell, (B) Tuştea, Romania eggshell, (C) Wann's Hill lambeosaurine eggshell. (**D-F**) Transmitting Plane-Light (PL) microscopy images of (D) Devil's Coulee *Maiasaura* eggshell, (E) Tuştea, Romania eggshell, (**F**) Wann's Hill lambeosaurine eggshell. (**G-I**) CL microscopy images of (G) Devil's Coulee *Troodon* eggshell, (H) Wann's Hill *Troodon* eggshell, (I) Lost River Ranch *Troodon* eggshell. (**J-L**) PL microscopy images of (J) Devil's Coulee *Troodon* eggshell, (K) Wann's Hill *Troodon* eggshell, (L) Lost River Ranch *Troodon* eggshell.

Table S1. Specimen descriptions, localities, and isotopic values.

Eggshell	Locality	Age	Paleolatitude	Analytical Replicates	$\delta^{13}C_{vpdb}$ (‰)	$\delta^{18}O_{vpdb}$ (‰)	$\Delta_{47(abs)}$*	T (°C)†
Alligator mississippiensis YPM HERR 015203	Rockefeller Wildlife Refugee, LA	Modern	NA	5	-12.38 ± 0.02	-3.06 ± 0.04	0.689 ± 0.003	28.2 ± 0.8
Dromaius novaehollandiae (Emu)	Gill, MA	Modern	NA	6	-8.2 ± 0.06	-8.42 ± 0.06	0.663 ± 0.008	35 ± 2.2
Gallus gallus (Chicken)	New Haven, CT	Modern	NA	6	0.15 ± 0.01	-6.61 ± 0.05	0.650 ± 0.006	38.4 ± 1.7
Passer domesticus (House Sparrow) YPM ORN uncatalogued	New Haven, CT	Modern	Na	5	-10.82 ± 0.06	-2.68 ± 0.10	0.682 ± 0.004	30 ± 1.1
Troglodytes aedon (House Wren) YPM ORN uncatalogued	Iowa	Modern	NA	3	-12.31 ± 1.47	-4.66 ± 0.23	0.668 ± 0.013	33.6 ± 3.3
Archilochus colubris (Ruby-throated Hummingbird) YPM ORN 132487	New Haven, CT	Modern	NA	3	-12.84 ± 0.89	-4.82 ± 0.23	0.675 ± 0.013	31.9 ± 3.2
Troodon formosus (Theropod) TMP 2008.75.127	Devil's Coulee, AB Canada	Campanian	55 °N	6	-11.5 ± 0.01	-11.15 ± 0.03	0.653 ± 0.014	37.8 ± 3.8
Maiasaura peeblesorum (Hadrosaur) TMP 2009.153.1	Devil's Coulee, AB Canada	Campanian	55 °N	5	-12.01 ± 0.04	-14.39 ± 0.03	0.632 ± 0.009	43.6 ± 2.4
Hypacrosaurus stebingeri (Hadrosaur) TMP 1989.69.10	Devil's Coulee, AB Canada	Campanian	55 °N	5	-9.33 ± 0.04	-10.67 ± 0.07	0.681 ± 0.012	30.4 ± 3.0
Troodon formosus (Theropod) TMP 1995.21.4	Wann's Hill, AB Canada	Campanian	55 °N	6	-11.02 ± 0.03	-9.46 ± 0.11	0.691 ± 0.012	28.1 ± 2.8
Indeterminate lambeosaurine (Hadrosaur) TMP 1988.121.41	Wann's Hill, AB Canada	Campanian	55 °N	4	-10.68 ± 0.01	-9.11 ± 0.14	0.691 ± 0.009	27.7 ± 2.2
Troodon formosus (Theropod) TMP 2003.81.1	Lost River Ranch, AB Canada	Campanian	55 °N	4	-9.61 ± 0.04	-9.37 ± 0.24	0.695 ± 0.017	27.0 ± 4.0

Table S1 cont. Locality information and isotopic values of modern and fossil samples.

Eggshell	Locality	Age	Paleolatitude	Analytical Replicates	$\delta^{13}C_{vpdb}$ (‰)	$\delta^{18}O_{vpdb}$ (‰)	$\Delta_{47(abs)}$*	T (°C)†
Magyarosaurus dacus (Sauropod) TMP 1991.175.2	Tuştea, Haţeg Basin, Romania	Maastrichtian	23 °N	3	-13.08 ± 0.30	-1.89 ± 0.08	0.660 ± 0.004	35.6 ± 1.0
Gastropod W20	Wann's Hill, AB Canada	Campanian	55 °N	3	-1.93 ± 0.01	-9.52 ± 0.03	0.704 ± 0.006	24.6 ± 1.4
Bivalve TMP 2009.149.5	Milk River, AB Canada	Campanian	55 °N	3	-1.13 ± 0.01	-17.49 ± 0.10	0.692 ± 0.006	27.5 ± 1.6

*All Δ_{47abs} values calculated using Gonfiantini parameters and reported with ± 1 standard error, whereas δ value averages are reported with ± 1 s.d.

† Temperatures calculated from laboratory precipitated $CaCO_3$ calibration of ref. (8).

Caption for Data S1. (Separate file).

Raw data for both carbonate samples and standards and CO_2 standards used to put sample unknowns into the absolute reference frame, such as heated gases, equilibrated gases and cylinder CO_2. Raw data is calculated using both Gonfiantini and Brand ^{17}O parameters, with separate tabs for each as well as separate tabs corresponding to samples, heated gases and other standards.