nature research

Corresponding author(s): Jasmina Wiemann

Last updated by author(s): Mar 24, 2022

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐	☒	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Fossil hard tissues (total n=51), sediments (n=15) and modern skeletal elements (n=35) were surface-cleaned and subjected to in situ Raman microspectroscopy (532 nm, 500-1800 cm-1, 10s, 10 replicates; acquisition software: LabSpec 5), and a subset (n=4) to in situ ATR FT-IR spectroscopy (632 nm, 500-3000 cm-1, 32 replicates; acquisition software: Agilent Firmware). Spectra were standard-processed and analyzed in SpectraGryph 1.2 (see Supplementary Information Sections 2a, b for baselining and normalization). Relative intensities at 24 band positions were selected (using the Multi-Cursor function in SpectraGryph 1.2) to determine the endogenous nature and completeness of the metabolic signal in fossils: resulting variance-covariance matrices for fossil-sediment, fossil eggshell-tooth-bone, and fossil femora sample sets were subjected to ordination methods using PAST 3.0 software (Supplementary Information Sections 2, 3). Signal intensities from metabolic markers at 670, 1585, and 1685 cm-1 were extracted for fossil and modern femora (using the Multi-Cursor function in SpectraGryph 1.2), and crosslink-to-peptide signal ratios (MCin vivo) were calculated from these relative signal intensities in Microsoft Excel (Supplementary Information Section 2c, functional spreadsheet can be found as Source Data file).
Data analysis	MCin vivo values of modern femora were regressed (PAST 3.0) against published metabolic rates and the fit was used for converting values into metabolic rates. Intraskeletal variation of signals was characterized for Chironectes and Iguana (box-and-whiskers plot generated in PAST 3.0). A linear regression (PAST 3.0) for femora of fossils with extant relatives (n=8) (Supplementary Data 'Calibration') determined a function that converts fossil MCin vivo values into metabolic rates. Based on this conversion function, mass specific metabolic rates were calculated for all fossil and modern long bones (in Microsoft Excel) and plotted (log-log) against published body masses (Microsoft Excel): A 95%-confidence interval calculated in PAST 3.0 was based on known endotherms in this sample. Variation in metabolism was assessed for non-avian ornithodirans, crown mammals, birds, and lepidosaurs. Metabolic rates were superimposed on a time-scaled phylogenetic framework (R) and ancestral states were reconstructed (R, Supplementary Information Section 2d-f; all R code is available in the Source Data). Additional non-time-scaled analyses of alternative trees can be found in the Source Data spreadsheet, and ancestral states were calculate using Mesquite 3.61. Figure elements were combined and labeled in Adobe Photoshop CS5.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The Supplementary Information and Supplementary Data contain the source data required for the calculation of metabolic rates as shown in the Figs. 1b, d, e, 2, Extended Data Figs. 1, 2, 3, 7, 8, and Supplementary Fig. 2. Specimen information, extracted S- and N-crosslink Raman intensities, formulas and conversions used to calculate the metabolic rates, details of regressions, data used for principal component and discriminant analyses, published body masses/body mass estimates, correlation analyses, assessments of the prediction performance of various used regression models, assessments of potential data biases, labeled plots of all the analyses performed, R code and data for time-scaling the phylogenetic tree and maximum likelihood ancestral reconstruction. Raman and ATR FT-IR spectra are plotted in the main text Fig. 1a and Extended Data Figs. 4, 5, and (as a net enrichment plot in) 6.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description

1. Spectral acquisition and standard processing (spike removal, baselining, smoothing, normalization) for fossil hard tissues (total n=51 of biological replicates, each analysed with n=10 technical replicates), sediments (n=15 of geological replicates, each analysed with n=10 technical replicates) and modern skeletal elements (n=35 of biological replicates, each analysed with n=10 technical replicates). Femora of n=4 (biological replicates of) representative taxa were additionally subjected for in situ Fourier-Transform Infrared spectroscopy (every sample was analyzed with n=32 technical replicates) to corroborate band assignments (Extended Data Fig. 4).

2. Selection of n=24 bands from the spectra to capture heterogeneities and characterize the total organic composition (based on Wiemann et al. 2020) for all fossil eggshells, bones, teeth, and sediments.

3. Subjecting the fossil and sediment taxon-character matrix to a Non-linear Multidimensional Scaling Analysis (shown in Extended Data Fig. 1a – fossil tissues, and Supplementary Information Section 3).
Result 1: Organic matter in the analysed samples is endogenous.

4. Assessing the biplot trajectories for bands encoding S- and N-crosslinks in fossil tissues which reveals that bone and dentine are characterized by increased amounts of ALEs (consistent with the results of Wiemann et al. 2020, Fig. 3b).

5. Noting that the analyses in Wiemann et al. 2020 (specifically Figs. 1a, 3b, 4b) show that the increased abundance of ALEs in bone corresponds to in vivo increased concentrations of ALEs which sort taxa in a ChemoSpace according to their presumed thermal strategy (Extended Data Fig. 1b, also in Wiemann et al. 2020), and therefore record metabolic information. Wiemann et al. (2020) explored biosignature fidelity in different tissue types, and showed that bone preserves a more accurate metabolic signal than dentine. Therefore, we chose bone as the target tissue for extracting metabolic information in ALEs in fossil and modern taxa.
Result 2: ALEs in fossil bone appear to preserve metabolic information.

6. Focus on femora (identified as most suitable through assessments of intraskeletal variability of metabolic signals) of endothermic and ectothermic amniotes.

7. Calculating the crosslink-to-protein ratio (MCin vivo) for all extant taxa and compiling metabolic rates for all analyzed taxa from published physiological data (see Source Data spreadsheet and Supplementary Information for details).

8. Running a (linear) regression of MCin vivo values versus available, published metabolic rates for extant taxa, and assessing prediction performance with three distinct measures: Pearson's r, r2, and Spearman ranks. There is a significant correlation between the abundance of ALEs (known markers of metabolic oxidative stress) and the metabolic rate in extant taxa. The resulting regression function is used as calibration function for extant taxa (yielding values equivalent to metabolic rates).
Result 3: ALEs are present in modern bones in a relative abundance that reflects the metabolic rate. ALE crosslink-to-protein ratios can be transformed into metabolic rates using two different calibrations for fossil and extant bone.

9. Selecting fossils from the data set with close extant relatives which have published metabolic rates.

10. Calculating crosslink-to-protein ratios (MCin vivo) for these selected fossils and performing a separate bivariate fit (=linear regression) against the published metabolic rates. Model performance shows a significant correlation between fossil crosslink-to-protein ratios (= MCin vivo values), and residuals are quantified to assess error margins (Supplementary Data). The resulting regression function is used to calibrate fossils.

11. Calculating crosslink-to-protein ratios (MCin vivo values) for all other fossils and applying the fossil calibration function (yielding values equivalent to metabolic rates).

12. Calculating mass-specific metabolic rates for modern and extinct crown mammal and birds and plotting them against corresponding body masses to generate the 95%-confidence interval for endothermy.

13. Calculating mass specific metabolic rates for all other fossil taxa, plotting them against corresponding body masses, and projecting them onto the 95% confidence interval for endothermy.

14. Quantifying the uncertainty associated with Raman-based metabolic rates for extant taxa (shown in Fig. 1e), revealing that the Raman approach tends to slightly underestimate metabolic rates.

15. Assigning endothermy as the thermoregulatory strategy for fossils that plot at the lower boundary of, within, or above the endothermy confidence interval.

16. Downstream analyses of calculated metabolic rates (Ancestral State Reconstruction and correlation analyses).
Result 4: Figs. 1c-e, 2 and Supplementary Data 'Correlation analyses' provide insights into how amniote metabolic rates evolved.

Research sample

Modern, adult specimens (n=25 femora, n=10 ulnae, metatarsals, skull bones, ribs, vertebrae) were selected from the Yale Peabody Museum Division of Vertebrate Zoology (Supplementary Data 'Extant Taxa') for maximum phylogenetic and tissue type coverage. Fossil specimens (n=13 eggshells, n=8 teeth, n=30 long bones including 28 femora, 1 humerus, a cetacean, and 1 vertebra, a snake) were selected for macroscopic evidence of 'carbonaceous' residues and phylogenetic diversity. Specimens with preserved organic matter were identified on the basis of previously published criteria (Wiemann et al. 2018 Nature Communications). In addition, a diversity of different sediment types (n=15; Supplementary Data 'Sediments') was included to assess the endogeneity of preserved carbonaceous fossil films.

Sampling strategy

No sample size calculation was performed, instead, modern and fossil samples were selected for maximum phylogenetic (Amniota) and tissue type (intraskeletal variation) coverage. As a requirement for the analysis of fossil organic matter, we focused primarily on fossils that preserve carbonaceous materials with a diagnostic dark brownish or black discolouration which stands out against a light-coloured sediment matrix. Light-coloured sediments are usually low in sedimentary organic matter which could potentially 'contaminate' endogenous, fossil organic matter, and an extensive endogeneity assessment is shown in the Supplementary Fig. 2. Most fluvial, alluvial, aeolian, and shallow marine sediments are known to have fostered early diagenetic oxidative crosslinking as a prerequisite to biomolecule fossilization. We have augmented specimens known to preserve fossil organic matter based on analyses published in Wiemann et al. (2018a: fossil vertebrate teeth, bones, eggshells), Wiemann et al. (2018b: fossil vertebrate eggshells), and Wiemann et al. (2020), with additional specimens from suitable depositional environments. Wiemann et al. (2020) confirmed the presence and endogeneity of fossil organic matter for most samples used in this study (please compare specimen lists: Wiemann et al. 2020, Supplementary Data 'Specimen data').

This study presents the most comprehensive, integrated assessment of modern and fossil materials in the context of reconstructing the evolution of molecular mechanisms behind metabolic strategies to this date.

Data collection

In situ Raman (Horiba LabRam800 with a holographic notch filter and a 532 nm laser in the Department of Earth and Planetary Sciences at Yale University) and ATR Fourier-Transform Infrared (Agilent Technologies Cary 660 Series FT-IR Spectrometer with a diamond crystal Pike GladiATR module at the Yale West Campus Analytical Core [WCAC]) spectra were collected between early 2019 and July 2021. Raman spectra were collected by J. Wiemann (and independently replicated in collaboration in the Yale Materials Characterisation Core [see Wiemann et al. 2022, Bioessays]), and ATR FT-IR spectra were collected by J. Wiemann under technical guidance of T. Wu and M. Ghosh at the Yale West Campus Analytical Core.

Timing and spatial scale

The taxa included in our analysis of the evolution of amniote metabolic strategies represent the diversity of amniotes, have a global distribution, and range in age from Permian to Recent. For individual details, please see the tabs on 'Fossil taxa' and 'Extant taxa' in the Supplementary Data spreadsheet.

Data exclusions

No data were excluded: we performed extensive outlier and uncertainty assessments (all available in the Supplementary data spreadsheet, with detailed explanations in the Supplementary Information), and included all data in our reconstruction of the evolution of amniote metabolic rates. Our analyses of intraskeletal variability of a recorded metabolic signal in extant amniotes led us to focus our analyses on homologous bone elements, and we focused on femora due to the completeness of the metabolic signal as revealed by our ChemoSpace assessments (shown in the Extended Data Fig. 1a). To ensure maximum comparability of the metabolic signals extracted, samples were analysed consecutively, ideally in one run. Extant samples that were added during the revision of this manuscript are highlighted in the Supplementary Data ('Extant taxa') and were scaled (+ 0.3) to match the original data set. All the details as accessible in our Supplementary Data spreadsheet and in the Supplementary Information under paragraph 2d.

Reproducibility

All Raman spectra were collected with n=10 technical replicates, and signals were validated with complementary ATR Fourier-Transform Infrared spectra collected for n=4 representative samples with n=32 technical replicates each. Metabolic signals are found in both Raman and FT-IR fingerprints. All major groups of amniotes are represented in this study by multiple taxa (multiple biological replicates) and consistent patterns in metabolic signals were obtained. There is a significant positive correlation between MCin vivo values and available, independently published metabolic rates for extant taxa. Independent follow-up projects successfully detected a metabolic signal using the here presented approach.

Randomization

Randomization is not relevant for this project, as the identities of the analyzed fossil or modern samples are known, and the reconstruction of the evolution of metabolic strategies in amniotes requires analysis of metabolic signals in a phylogenetically informed framework.

Blinding

Spectral processing, and the extraction of normalized, relative spectral intensities contributing to the calculated crosslink-to-protein

Blinding

ratios (MCin vivo) were effectively performed 'blindly', and sample identities were attached to the resulting MCin vivo values. Also, ordination methods, i.e., PCA (Principal Component Analysis), analyzed the variance-covariance matrices without any implementation of environmental variables, and thereby extracted features 'blindly'.

Did the study involve field work? ☐ Yes ☒ No

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☐	☒ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Palaeontology and Archaeology

Specimen provenance

The n=30 fossil, and n=25 modern amniote bones included in this study come from the historical Vertebrate Paleontological and Zoological Collections of the Yale Peabody Museum in New Haven, CT, USA.

All specimen catalogue numbers and sample ages are listed below and in the Source Data spreadsheet, and individual details on specimen provenance, collection dates, and fossil localities can be assessed via the online specimen catalogue of the Yale Peabody Museum, or per request to the authors or collections managers.

Specimen deposition

Bones of fossil specimens included in this study are deposited in the Vertebrate Paleontological Collections of the Yale Peabody Museum:

Anseriform bird, Yale Peabody Museum, To be catalogued
Diplodocid, Yale Peabody Museum, To be catalogued
Rhamphorhynchoid, Yale Peabody Museum, YPM VP 9150
Hesperornis, Yale Peabody Museum, To be catalogued
Pachycephalosaur, Yale Peabody Museum, To be catalogued
Allosaurus, Yale Peabody Museum, YPM VP 4944
Deinonychus, Yale Peabody Museum, YPM VP 5220
Brontothere, Yale Peabody Museum, YPM VP 11122
Nodosaurus, Yale Peabody Museum, To be catalogued
Telmatornis, Yale Peabody Museum, YPM VP 902
Saniwa, Yale Peabody Museum, To be catalogued
Marsupial, Yale Peabody Museum, To be catalogued
Insectivore, Yale Peabody Museum, YPM VP PU 14645
Cetacean, Yale Peabody Museum, YPM VP 53059
Tyrannosaurus, Yale Peabody Museum, To be catalogued
Dryosaurus, Yale Peabody Museum, YPM VP 7324
Pteranodon, Yale Peabody Museum, YPM VP 002271
Polyglyphanodon, Yale Peabody Museum, To be catalogued
Odaxosaurus, Yale Peabody Museum, To be catalogued
Plesiosaur, Yale Peabody Museum, YPM VP 065733
Melanosaurus, Yale Peabody Museum, YPM VP PU 21801
Crocodylomorph, Yale Peabody Museum, YPM VP 065732
Hadrosaur, Yale Peabody Museum, To be catalogued
Xanthusiid, Yale Peabody Museum, To be catalogued
Triceratops, Yale Peabody Museum, To be catalogued
Edaphosaurus, Yale Peabody Museum, YPM VP PU 014686
Stegosaurus, Yale Peabody Museum, YPM VP PU 14556
Champsosaur, Yale Peabody Museum, To be catalogued
Cteniogenys, Yale Peabody Museum, YPM VP 7858
Paleophis, Yale Peabody Museum, To be catalogued

Bones of extant specimens included in this study are deposited in the Vertebrate Zoological Collections of the Yale Peabody Museum:

Anthrozous, Yale Peabody Museum, YPM VZ MAM 6545
Aotus, Yale Peabody Museum, YPM VZ MAM 1503
Ara, Yale Peabody Museum, YPM VZ ORN 84773

Bos*, Yale Peabody Museum, Cat. no. to be added
Chironectes*, Yale Peabody Museum, Cat. no. to be added
Columbina, Yale Peabody Museum, YPM VZ ORN 137085
Crypturellus, Yale Peabody Museum, YPM VZ ORN 102518
Dendrocygna, Yale Peabody Museum, YPM VZ ORN 84805
Iguana*, Yale Peabody Museum, Cat. no. to be added
Marmosa, Yale Peabody Museum, YPM VZ MAM 12712
Mustela, Yale Peabody Museum, YPM VZ MAM 15091
Oceanodroma, Yale Peabody Museum, YPM VZ ORN 109108
Ochotona, Yale Peabody Museum, YPM VZ MAM 14835
Passer, Yale Peabody Museum, YPM VZ ORN 109464
Platypus, Yale Peabody Museum, YPM VZ MAM 3932
Puma*, Yale Peabody Museum, Cat. no. to be added
Rattus, Yale Peabody Museum, YPM VZ MAM 6589
Spheniscus, Yale Peabody Museum, YPM VZ ORN 103791
Tachyglossus, Yale Peabody Museum, YPM VZ MAM 6799
Tenrec, Yale Peabody Museum, YPM VZ MAM 4170
Topaza, Yale Peabody Museum, YPM VZ ORN 101486
Agama, Yale Peabody Museum, Cat. no. to be added
Furcifer, Yale Peabody Museum, Cat. no. to be added
Varanus, Yale Peabody Museum, Cat. no. to be added
Gavialis (juvenile), Yale Peabody Museum, Cat. no. to be added

Additional information and catalogue numbers for specimens included in the ChemoSpace shown in Extended Data Fig. 1a, and sediments included in the ChemoSpace shown in Supplementary Fig. 1 can be found in the Source Data Spreadsheet (sediments), and in the Supplementary materials for Wiemann et al. 2020, Science Advances (teeth and eggshells).

Dating methods

No new dates are provided.

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Ethics oversight

No ethical approval or guidance was required, as all metabolic rate analyses were entirely non-destructive, and only historical specimens housed at the Yale Peabody Museum were included.

Note that full information on the approval of the study protocol must also be provided in the manuscript.