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1	Title: Synchrotron-X-ray fluorescence analysis reveals diagenetic alteration of fossil
2	melanosome trace metal chemistry
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13	
11	Abstract: A low feature of the nigment melonin is its high hinding effinity for trace metal
14	Abstract. A key readure of the pigment meranin is its high binding armity for trace metar
15	ions. In modern vertebrates trace metals associated with melanosomes, melanin-rich
16	organelles, can show tissue- and taxon-specific distribution patterns. Such signals preserve in
17	fossil melanosomes, informing on the anatomy and phylogenetic affinities of fossil
18	vertebrates. Fossil and modern melanosomes, however, often differ in trace metal chemistry;
19	in particular, melanosomes from fossil vertebrate eyes are depleted in Zn and enriched in Cu
20	relative to their extant counterparts. Whether these chemical differences are biological, or
21	taphonomic, in origin is unknown, limiting our ability to use melanosome trace metal
22	chemistry to test palaeobiological hypotheses. Here, we use maturation experiments on eye
23	melanosomes from extant vertebrates and synchrotron rapid scan-X-ray fluorescence analysis

to show that thermal maturation can dramatically alter melanosome trace element chemistry.
In particular, maturation of melanosomes in Cu-rich solutions results in significant depletion
of Zn, likely due to low pH and competition effects with Cu. These results confirm fossil
melanosome chemistry is susceptible to alteration due to variations in local chemical
conditions during diagenesis. Maturation experiments can provide essential data on
melanosome chemical taphonomy required for accurate interpretations of preserved chemical
signatures in fossils.

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32 Keywords: fossil, soft tissue, taphonomy, Synchrotron-X-ray fluorescence.

33

MELANIN is an essential biomolecule in animals that supports immunity (Agius and Roberts 34 2003; Nappi and Christensen 2005) and facilitates key physiological processes including 35 directional photoreception in the eye (Oakley et al. 2015) and the protection of tissues from 36 UV damage (Brenner and Hearing 2007). Melanin may also contribute to metal homeostasis 37 via its high binding affinity for metal ions such as Ca^{+2} , Fe^{+3} , Cu^{+2} and Zn^{+2} (Hong and 38 Simon 2007; Wogelius et al. 2011; Rossi et al. 2019) (albeit the two common forms of 39 melanin, eumelanin and phaeomelanin, differ in metal affinity) (Wogelius et al. 2011, 40 41 Manning et al. 2019). Remarkably, evidence of melanin has been reported from many 42 vertebrate fossils, usually in association with preserved melanosomes (micron-sized organelles) (e.g. Lindgren et al. 2012; McNamara et al. 2016). As with melanin in extant 43 44 animals (Hong and Simon, 2007, fossil melanin can associate with metals, especially Cu (Wogelius et al. 2011) and Zn (Manning et al. 2019). Preserved associations between these 45 metals and melanosomes can be tissue- and taxon-specific (Rogers et al. 2019; Rossi et al. 46 2019), thus offering the potential to inform on the internal anatomy (Rossi et al. 2019) and 47

phylogenetic affinities (Rogers *et al.* 2019) of fossils and the functional evolution of melanin
(Rossi *et al.* 2019).

50

51 The metal inventory of fossil melanin, however, differs from that of modern analogues, even 52 in closely related taxa (Rossi et al. 2019; Rogers et al. 2019). In the case of vertebrate eye melanosomes, concentrations of Zn are markedly lower in fossils relative to modern 53 vertebrate eyes, and concentrations of Cu and Fe are higher (Rogers et al. 2019). The origins 54 55 of these differences are unclear, thus impacting our ability to confidently interpret preserved melanosome chemistry. Previous work on fossils acknowledged the possibility of diagenetic 56 57 alteration of the trace metal inventory of melanosomes (Wogelius et al. 2011); this is supported by experimental evidence that the trace element chemistry of melanin is sensitive 58 59 to fluctuations in pH (Hong and Simon 2007) and high metal concentrations (Chen et al. 2009). Characterization of the impact of diagenesis on melanosome trace metal chemistry is 60 61 therefore essential to identify taphonomic biases in the melanosome fossil record.

62

Here, we resolved these issues by conducting maturation experiments on melanosomes from 63 extant vertebrate eyes at elevated pressures and/or temperatures and with different chemical 64 media in order to simulate how different pore fluids (i.e. sources of metal ions) interact with 65 melanosomes during diagenesis. Analysis of the results using synchrotron rapid scan-X-ray 66 fluorescence analysis (SRS-XRF) and X-ray absorption spectroscopy (XAS) reveals which 67 68 melanosome-associated elements are susceptible to changes in concentration during diagenesis. Critically, the experimental data aid interpretations of the chemical differences 69 between fossil and modern eye melanosomes, thus informing on key biases in the 70 melanosome fossil record. 71

73 MATERIALS and METHODS

74 *Modern specimens*

Specimens of European sea bass (*Dicentrarchus labrax* n = 14) were obtained from
commercial suppliers and dissected within 24 h after death. One eye from each animal was
bisected and melanin was enzymatically extracted from the posterior half of each eye
(including the melanosome-rich choroid and retinal pigment epithelium (RPE)) using the
method in Rossi *et al.* (2019). The extraction process breaks down cellular material and can
degrade phaeomelanin, yielding extracts that are biased towards eumelanin (Liu et al. 2005).

81

82 Fossil specimens

Preserved melanosomes from the eyespot of a specimen of *Dapalis macrurus* (CKGM F 6
427; Actinopterygii: Perciformes, from Alpes de Haute-Provence, France (Oligocene)) and
eyespots from the fossil teleosts Tetradontiformes indet. (NHMD 199 838; from the Fur
Formation, Denmark (Eocene)) and *Knightia* (FOBU 17 591; Actinopterygii: Clupeiformes
from the Fossil Butte member of the Green River formation, Colorado/Utah/Wyoming
(Eocene)) were reported in Rogers *et al.* (2019) and analyzed further as below.

The respective fossil localities vary in lithology and diagenetic history. NHMD 199 838 is hosted in diatomite that was deposited in a restricted marine basin (Pedersen and Buchardt, 1996) and experienced at least 40–45°C and ~33–49 bar during diagenesis (McNamara et al. 2013). FOBU 17 591 is hosted in laminated limestone that was deposited in a restricted terrestrial basin with marked salinity fluctuations during deposition (Loewen and Buchheim 1998). Data on diagenetic history are not available for the Fossil Butte Member (part of the Fossil Basin); sediments from the Uinta Basin of the Green River Formation experienced

burial conditions of up to 65–180°C and ~400–2000 bar (McNamara et al. 2013). CKGM F
6427 is preserved in a laminated limestone but lacks stratigraphic data; specimens of this
taxon are common in the freshwater/brackish lacustrine limestones of the Campagne-Calavon
Formation of Alpes de Haute-Provence (Gaudant, 2015), though data on burial history are not
available.

102

Institutional abbreviations: NHMD, Natural History Museum of Denmark; CKGM, Cork
Geological Museum at University College Cork; FOBU, Fossil Butte National Monument.

106 EDTA treatment of melanin

Unmatured melanin (50-100 mg) extracted from a single Dicentrarchus eye was added to an 107 108 aqueous solution of EDTA (one ml) for 24 h at room temperature and then centrifuged. This process was repeated six times followed by washing four times in biomolecular-grade water 109 in order to remove excess EDTA. The EDTA-treated extract was then added to one ml of 16 110 mmol Cu-solution for 24 h at room temperature before being washed twice in 10⁻³ M HCl to 111 precipitate any Cu still in solution (Hong et al. 2004). The extract was washed twice in 112 biomolecular water and once in acetone. Half of the extract was experimentally matured (see 113 below). 114

115

116 *Experimental maturation*

Taphonomic studies investigating the impact of maturation on soft tissues typically use
elevated temperatures and pressures for relatively short periods of time (usually < 24 h) to
artificially simulate aspects of the maturation process (McNamara *et al.* 2013; Colleary *et al.*2015). Such experiments are known to yield morphological and chemical phenomena similar
to those exhibited by fossils (Stankiewicz *et al.* 2000). Melanosome extracts for experimental

maturation (n = 12) were each inserted into separate Au capsules. To each capsule was added 122 one ml of experimental medium, defined as follows. The EDTA-treated extract used distilled 123 deionized (DD) water. All other extracts used either DD water, "Cu-solution" (Trace Cert 124 Copper standard for AAS (1 000 mg l⁻¹)), "Zn-solution" (Trace Cert Zinc standard for AAS 125 (1 000 mg l⁻¹)) or "Cu-Zn-solution" (50:50 mixture of Trace Cert Copper and Zinc standards 126 for AAS (Rogers et al. 2020 (Table S1))). The inclusion of metal ion solution in some 127 128 experiments is designed to test how variation in diagenetic pore fluid chemistry impacts melanin chemistry. All metal ion solutions have a concentration of 16 mmol and are at pH 3. 129 130

Brushes were used to remove any extract or experimental medium from the termini of each capsule prior to them being welded shut with a Lampert PUK 4 microwelding system. Sealed capsules were thermally matured for 24 h at 200°C in either an oven at one bar or, for some samples (including EDTA-treated extracts) a custom-built high-pressure high-temperature rig (StrataTech, UK) at 120 bar. It was not possible to measure the pH of the experimental medium at the end of the experiment due to the small size of the capsules and unpredictable escape of fluid from the capsules upon opening.

138

139 Synchrotron Rapid Scanning-X-ray Fluorescence (SRS-XRF) analysis

X-ray fluorescence spectra were collected from matured and untreated extracts and fossils at
beamline 2-3 at the Stanford Synchrotron Radiation Lightsource (SSRL). Extracts and small
samples (ca. one mm²) of dark brown carbonaceous material from the eyespot of the fossil
fish *Dapalis macrurus* (Oligocene, CKGM F 6 427) were mounted on kapton tape. Samples
were spatially rastered by a microfocused beam of two µm² provided by an Rh-coated
Kirkpatrick-Baez mirror pair with 20–50 ms/pixel dwell time. A Si (111) double crystal
monochromator was used to set incident X-ray energy at 11 keV; the storage ring was in top-

off mode at three GeV and contained 500 mA. Samples were mounted at 45° to the incident
X-ray and the intensity of the beam was measured using a nitrogen-filled ion chamber. At
each data point, the intensity of fluorescence lines for selected elements (P, S, Ca, Ti, Mn, Fe,
Ni, Cu and Zn) was collected and monitored using an Xpsress3 pulse processing system
(Quantum Detectors) coupled to a silicon drift Vortex detector (Hitachi, USA) for energy
discrimination.

153

154 SRS-XRF data processing

155 MicroAnalysis Toolkit software (Webb 2011) was used to normalize fluorescence spectra and calibrate concentrations of each element in $\mu g/cm^2$ against NIST traceable thin-film 156 elemental standards. Mean concentrations and standard deviation values for each element 157 were calculated for selected regions of interest. Inspection of SRS-XRF spectra from the 158 Multi-Channel Analyzer (MCA) data reveals that in extracts the concentrations of certain 159 elements (P, S, Ti and Mn) are too low to be discriminated confidently from the background 160 (Rogers et al. 2020 (Figs S1–S7)). Concentration data from these elements were therefore 161 excluded from further analyses. Ni was identified in the MCA data but concentrations (< nine 162 μ g/cm²) were markedly lower than those of other elements and thus Ni was also excluded 163 from further analysis. 164

165

The SRS-XRF data were analyzed further using Linear Discriminant Analysis (LDA) in
PAST (Hammer *et al.* 2001) to visualize variations in the chemistry of selected elements (Ca,
Fe, Cu and Zn) among the melanin extracts and fossil melanosomes. The significance of
differences in concentration among samples was assessed using ANOVA or non-parametric
alternatives (Kruskal-Wallis and Welch's F-test) and appropriate pairwise post hoc analyses

171 (Tukey, Mann Whitney and Dunn, respectively). Differences in elemental concentrations172 among extracts were visualized using box plots.

173

174 *X-ray absorption spectroscopy (XAS)*

EDTA-treated extracts and samples from the eyespot of *Dapalis* were mapped at beamlines 175 2-3 and 10-2 at the Stanford Synchrotron Radiation Lightsource (SSRL). At beamline 2-3 176 177 extracts were analyzed as above. At beamline 10-2 extracts were analyzed using a 25-200 µm beam using a series of tungsten apertures. Four to six points of interest were selected from 178 179 each SRS-XRF map. XAS spectra were collected from these points by driving the emitted intensity from ~8 787 eV to ~9 827 eV across the Cu K edge (set at 8 987 eV using a Cu foil) 180 in a stepwise fashion (step size of 10 eV from 8 749 to 8 958 eV, except for between 8 959 to 181 9 006 eV (i.e. across the Cu K edge) where step size of 0.35 eV was used. Between two and 182 seven repeat scans (15 min each) were collected at each point. 183

184

A Cu foil was used to calibrate the energy of the monochromator. X-ray absorption near edge structure spectroscopy (XANES) was used to investigate the oxidation state of Cu associated with the melanosomes in our dataset. Each spectrum was monitored for loss of intensity and photo-reduction associated with exposure to the electron beam. No evidence for photoreduction was observed among replicate scans and repeat scans at each point are mutually consistent. Spectra were processed as standard (i.e. via normalization and background removal) in Athena (Ravel and Newville 2005).

192

193 **RESULTS**

194 Trace metal concentrations in matured melanosome extracts

Linear Discriminant Analysis (LDA) plots of the SRS-XRF data show extensive overlap of
data from unmatured and matured melanosomes in chemospace (Fig. 1A–B). Differences in
trace element chemistry between untreated extracts and extracts experimentally matured in
DD water are not statistically significant (*p-values*=0.1034–0.8206, Rogers et al. 2020 (Table
S2A–C)); these two samples cannot therefore be distinguished chemically.

200

Concentrations of Ca (Welch F: df = 16.18, F = 73.39, p = 7.91⁻¹¹) and Cu (df = 17.47, F = 201 118.8, $p = 5.792^{-13}$) are significantly different between unmatured extracts and extracts 202 matured in Cu and/or Zn-solutions (Fig. 2, Rogers et al. 2020 (Table S2A)). More 203 specifically, concentrations of Ca are significantly lower in extracts matured in Zn-solution 204 and mixed Cu-Zn-solution (Dunn's post hoc p = 0.002-0.006). Concentrations of Cu are 205 higher in extracts matured in Cu-solution and mixed Cu-Zn-solution (p = 0.001-0.002) 206 (Rogers et al. 2020 (Table S2B–C)). Concentrations of Zn are significantly lower in all 207 extracts matured with Cu- and/or Zn-solution (Kruskal-Wallis H (chi^2) = 23.33, p = 0.001; 208 209 Dunn's post hoc p = 0.001-0.006). Variations in Fe concentrations among untreated and experimentally matured extracts are not significant (ANOVA df = 2.115, F = 17.5, p = 210 0.1034). 211

212

213 Comparison with fossil trace element chemistry

The principal component analysis plot of the fossil data reveals that the eyespot data for individual specimens plot close to the data for the associated host sediment (Figure S9); the data for individual specimens and their sediment do not overlap with those for other fossils. Variation in concentrations of Ca, Fe and Cu among specimens are significantly different (Ca: Welch F: df = 2.668, F = 37.62, p = 0.01109; Fe: ANOVA: df = 8, F= 162, p = 6.01⁻⁰⁶;
Cu: Welch F: df = 2.983, F = 13.7, p = 0.03136; posthoc tests: Rogers et al. 2020 (Table S4)).
Concentrations of Zn do not differ significantly among specimens (Kruskal-Wallis H (chi²) = 0.3556, p = 0.8371).

222

Differences in concentrations of Cu between the fossil eyespots and the host sediment are
significantly different for each fossil specimen (Rogers et al. 2020 (Table S4)). In addition,
concentrations of Fe (and, in *Dapalis*, Ca) are significantly different between the eyespot of *Knightia* and the respective host sediment; similarly, concentrations of Fe and Ca differ
significantly between the eye of *Dapalis* and the host sediment. Differences in concentrations
of Zn between eyespots and host sediment are not significantly different for any of the three
fossils analyzed (Rogers et al. 2020 (table S4)).

230

LDA plots of the total dataset reveal three major groupings in the LDA chemospace (Fig. 231 3A): (1) fossil melanosomes, (2) melanosome extracts matured in Cu-solution and Cu-Zn-232 solution, and (3) melanosome extracts matured in DD water, in Zn-solution and untreated 233 234 melanosomes. The primary elements driving this chemical variation are Ca, Cu and Zn (Fig. 3B); in particular, fossil melanosomes have significantly lower concentrations of Zn than 235 untreated modern equivalents (Kruskal-Wallis (H (chi^2) = 12.79, 0.0003). Fossil and 236 237 untreated modern melanosomes do not differ significantly in concentrations of Ca (Kruskal-Wallis H (chi²) = 1.421, p = 0.233)), Cu (H (chi²) = 0.002, p = 0.965)) or Fe (F = (1, 16) = 238 3.478, p = 0.081) (Rogers et al. 2020 (Table S5)). In the total dataset, however, differences in 239 240 Cu concentrations between samples are significant (Kruskal-Wallis (H (chi^2) = 7.2, 0.02732); differences in concentrations of Ca (Kruskal-Wallis (H (chi^2) = 5.422, 0.06646), Fe (Kruskal-241

Wallis (H (chi²) = 5.6, 0.06081) and Zn (Kruskal-Wallis (H (chi²) = 0.3556, 0.8371) are not
significant.

244

Cu-XANES spectra for all samples analyzed have a dominant peak centred at 8 997.5 \pm 1 eV showing a major contribution from Cu(II) (Fig. 3C). Only the Cu-XANES profile from the eyespot of *D. macrurus* shows a distinct pre-edge feature at 8 984.5 eV, indicating a contribution from Cu(I).

249

250 **DISCUSSION**

The results of our study reveal that the metal inventory of eye melanosomes is not significantly altered by maturation in DD water (Fig. 1A–B). This suggests that melanosomemetal associations should persist in natural diagenetic scenarios where pore fluids have low concentrations of metal ions and, since pH decreases with increasing temperature, where pH does not vary markedly. In contrast, melanosome extracts matured in Cu and/or Zn solutions differ in chemistry to untreated samples, and to each other. This confirms that the metal inventory of melanosomes is highly sensitive to local ionic concentrations during diagenesis.

258

Metals commonly bind to three functional groups within the eumelanin molecule (OH⁻, NH₄⁺
and COOH⁻) (Hong *et al.* 2004); Cu can also be accommodated within the eumelanin
porphyrin structure, which may survive diagenesis in at least some fossils (Wogelius *et al.*2011). In *Sepia* melanin, Fe(III) can bind to NH₄⁺ or OH⁻ (Hong *et al.* 2004); Ca(II) and
Zn(II) bind to COOH⁻ and Cu(II) binds primarily to OH⁻. In all cases eumelanin-bound
metals are strictly co-ordinated to light elements (i.e., O/N). Unlike eumelanin, phaeomelanin

comprises monomers (benzothiazine and benzothiazole) that contain S and can chelate Zn
(Manning et al. 2019); whether the phaeomelanin S-groups (and other groups present, e.g.
NH⁻) commonly bind other metals is unclear.

268

269 Extracts matured in Cu-solution are enriched in Cu and depleted in Zn relative to unmatured melanosomes. Experiments at room temperature and pressure indicate that where Cu 270 concentrations exceed 10 mmol, additional Cu(II) can bind to COOH⁻ sites normally 271 272 occupied by Ca(II) and Zn(II) (Hong and Simon 2006), displacing the latter two elements. This is consistent with evidence of Cu-binding to melanin COOH⁻ groups in fossil feathers 273 (Wogelius et al. 2011). In our experiments it is possible that the concentration of the Cu-274 solutions used (16 mmol) was sufficiently high to trigger replacement of Zn(II) by Cu(II) at 275 COOH⁻ sites during maturation. Experiments on artificial eumelanin, however, show a 276 decrease in COOH⁻ with thermal maturation (Ito et al. 2013). Elevated Cu concentrations in 277 our experiments may therefore reflect (at least in part) the binding of Cu to unoccupied OH-278 or NH₄⁺ rather than COOH⁻. 279

280

Surprisingly, Zn concentrations decrease even when melanosome extracts are matured in 281 solutions containing Zn. Following saturation of COOH⁻ groups with Zn(II), NH₄⁺ and OH⁻ 282 groups can present secondary binding sites for Zn(II) (Hong and Simon 2006). Melanin, 283 however, has a much higher overall binding affinity for Cu(II) than for Zn(II) (Hong and 284 285 Simon 2007) and thus Zn(II) may be unable to bind to OH⁻ groups in melanin when they are already occupied by Cu(II). Low Zn concentrations in melanosomes matured in Cu-Zn-286 solutions may therefore reflect the following: (1) saturation of OH⁻ groups by Cu(II) from the 287 experimental medium and the inability of Zn(II) to displace melanin-bound Cu(II) at these 288

sites, and (2) a decrease in the abundance of COOH⁻ bonds due to thermal maturation (Ito *et al.* 2013).

291

The decrease in Zn concentrations in melanosomes matured in Zn-solutions (i.e., where saturation of OH^- groups by Cu(II) cannot occur, as in experiments with Cu-solution) is also consistent with a maturation-induced decrease in available COOH⁻ (Ito *et al.* 2013). This indicates that the original COOH⁻-Zn bond present in the untreated extracts was unstable under the conditions used in our experiments.

297

298 Concentrations of Fe do not differ among untreated and treated melanosomes. This is somewhat surprising because melanin has a higher affinity for Cu(II) than Fe(III) (Hong and 299 Simon, 2007) and thus Cu(II) may be expected to replace Fe(III) at the OH⁻ group (to which 300 Cu commonly binds (Hong and Simon 2007)) and the NH₄⁺ group (to which it binds when 301 present in concentrations <10 mmol (Hong and Simon 2006)). Instead, our data suggest that 302 the additional Cu(II) has not displaced Fe(III) at OH⁻ and NH₄⁺ groups but is bound 303 elsewhere, either to unoccupied functional groups or to COOH⁻ groups in which Cu(II) 304 replaces previously bound metals such as Zn(II). 305

306

Patterns of Ca enrichment and depletion in our dataset are complex. Relative to untreated
samples, melanosome extracts are depleted in Ca when matured in Zn and Cu-Zn-solutions
but concentrations of Ca are the same when extracts are matured in Cu-solution (Fig. 2). This
may reflect changes in pH during the experiments. Metal binding sites in *Sepia* melanin are
sensitive to changes in pH; more specifically, increased acidity (e.g. a change from pH 7 to

pH 2) results in a decrease in the concentration of elements bound to COOH⁻ groups (Liu *et al.* 2004). This reflects protonation of COOH⁻ groups by H⁺ and associated replacement of
chelated metals such as Ca(II) (Liu *et al.* 2004). The exact reasons why this occurs in
melanosome extracts matured with Zn and Cu-Zn-solutions but not in extracts matured only
in Cu solutions is unclear but it may reflect differences in temporal changes in pH evolution
in different experimental settings.

318

319 The suspension of melanin in low pH Cu- and/or Zn-solutions in our experiments may have resulted in the replacement of COOH⁻-bound Ca(II) and Zn(II) by H⁺. This process cannot, 320 however, explain the high Ca concentrations in extracts matured in Cu-solution, the origin of 321 which remains unclear. The persistence of Ca(II) in thermally matured samples implies either 322 the survival of some COOH⁻ groups under conditions understood to induce decarboxylation 323 (Ito et al. 2013) or binding of Ca(II) to other functional groups in melanin or associated 324 proteins within the melanosome. It is possible that the Ca signal could reflect contributions 325 326 from other recalcitrant tissue components of the eye, but this is unlikely given that the 327 extracts comprise near-pure agglomerations of melanosomes.

328

Our experiments provide an empirical basis for interpreting the chemistry of fossil melanosomes and, in particular, can help us interpret key chemical differences between these and modern melanosomes. Differences in chemistry between the fossil specimens studied may reflect taxonomic or biota-level differences; the small sample size prevents discrimination of these interpretations. Given other evidence for pervasive biota-level control on fossil melanosome chemistry (Rossi et al., 2020), the chemical differences among the specimens in this study likely represent a sedimentary signal (Rogers et al. 2020 (Fig. S9)). The consistent offset in the chemical data for eyespots (especially enrichment of Cu) relative to the host sediment could indicate retention of a component of original chemistry (i.e. naturally elevated concentrations of Cu similar to those *in vivo*) or concentration of elements, especially Cu, in fossil melanosomes during diagenesis.

340

Direct comparison of the fossil and experimental data reveal that both fossil and thermally matured melanosomes are strongly depleted in Zn relative to unmatured samples. This supports our experimental data on Zn mobility during diagenesis and suggests that the Zn concentrations in the fossils are not original but likely lower than *in vivo*. In natural settings, any Zn(II) displaced as a result of a decrease in pH is likely to be mobilized and lost from the system. Alternatively, displaced Zn may be rendered unavailable for re-chelation by fossil melanin via incorporation into inorganic mineral precipitates in the host sediment.

348

A decrease in pH could occur relatively early *postmortem* via the release of organic acids 349 during decay (Briggs and Kear 1993) or through contact with pore waters rich in H⁺ ions. 350 Higher temperatures at deeper burial conditions would also lower pH, further promoting the 351 loss of Zn from fossil melanosomes. Degradation of phaeomelanin (and loss of key Zn 352 binding sites including S^{2-} and OH^{-} (Manning *et al.* 2019)) during diagenesis could lead to a 353 further depletion of Zn. This process is unlikely to contribute significantly to our 354 experimental data, however, as the eye melanin of D. labrax is dominated by eumelanin in 355 356 vivo (Rogers et al. 2019) and most or all phaeomelanin originally present in the tissue is likely to have been degraded during enzymatic extraction (Liu et al. 2005). Uplift and 357 exposure would presumably result in an increase in local pH, but it is unclear whether 358 thermally altered melanin could bind available Zn in this scenario, especially given that 359

thermal maturation is associated with decarboxylation of melanin (Ito *et al.* 2013) and thus
loss of preferred functional groups for Zn-melanin chelation.

362

Ca, Fe and Cu concentrations are similar in fossil melanosomes and unmatured extracts. This 363 364 suggests that original associations between Ca(II), Fe(III) and Cu(II) and melanin may have been retained. Melanin, however, has a lower binding affinity for Ca(II) than Zn(II) (Hong 365 and Simon 2007); given that Zn was lost from matured melanosomes in our experiments it 366 367 thus seems unlikely that original associations between Ca(II) and melanin would survive diagenesis. Instead, it is plausible that Ca concentrations in the matured melanosome extracts 368 may reflect loss early during diagenesis and subsequent re-binding of Ca(II) once local pH 369 and ionic conditions become more favourable. This suggests that the melanin molecule may 370 retain a dynamic relationship with its environment long into diagenesis. Other metals such as 371 Fe and Cu, originally bound to melanin, could also be lost and rebound in this fashion. 372 Alternatively, some Ca, Fe and Cu associated with fossil eye melanosomes may have been 373 374 bound to non-melanin labile organic components in vivo. Breakdown of those compounds during diagenesis and subsequent incorporation of metal ions into remnants of the melanin 375 molecule (Wogelius et al. 2011) could contribute to concentrations of these metals in fossil 376 melanosomes, but this hypothesis requires testing. Although biomolecules are understood to 377 undergo the loss of functional groups during diagenesis (Eglingtonet al. 1991, Ito et al. 2013), 378 our results suggest at least some of these functional groups must survive this process and 379 remain viable binding sites for various metal ions. 380

381

Application of these SRS-XRF data to fossils strongly suggests that low Zn concentrations
and low Zn: Cu ratios in some fossil vertebrate eye melanosomes are a diagenetic artefact

(Fig. 4). Our previous study revealed that eye melanosomes in fossil vertebrates have low Zn 384 relative to extant analogues (Rogers et al. 2019). Our experimental data reveal that this likely 385 reflects the loss of Zn and/or the replacement of Zn by Cu during diagenesis. Variation in the 386 chemistry of melanosomes preserved in fossils from different biotas likely reflects different 387 ionic concentrations, pH and burial regimes during diagenesis (Rogers et al. 2019). Variation 388 in diagenetic regime, however, is less likely to explain spatial heterogeneity in melanin 389 390 chemistry within a single fossil. In the latter scenario chemical variation is more likely to reflect biological factors, e.g. original variation in eu- and phaeomelanin content due to 391 392 integumentary patterning (Wogelius et al. 2011) and/or enrichment in various internal organs (Rossi et al. 2019, 2020). Higher concentrations in some fossil vertebrates, especially those 393 hosted within concretions (such as those from the Mazon Creek), may reflect rapid 394 cementation of the host sediment, limiting interactions of pore fluids with melanosomes (and 395 thus loss of Cu and Zn) later during diagenesis. 396

397

398 Cu-XANES spectra for matured and untreated melanosomes are dominated by signals for Cu(II). The local binding environment of melanosomal Cu clearly does not alter substantially 399 during maturation (Fig. 3C). The presence of a Cu(I) signal in fossil melanosomes from 400 Dapalis could reflect an artefact of the XANES analysis whereby some Cu(II) was reduced 401 during analysis. Other vertebrate fossils (Wogelius et al. 2011; Rogers et al. 2019) and 402 403 modern melanosomes analyzed under identical conditions, however, do not show a contribution from Cu(I). Instead, the Cu(I) contribution to the *Dapalis* XANES spectrum may 404 405 be a real signal, reflecting local conditions during diagenesis.

In conclusion, our study reveals the effects of diagenesis on trace element chemistry of 407 melanosomes. Specifically, our data confirm that relatively low concentrations of Zn in fossil 408 vertebrate eye melanosomes is likely to be a diagenetic feature and that this depletion in Zn 409 relative to modern eye melanosomes is controlled, in large part, by local ionic and pH 410 conditions during diagenesis and not by elevated temperatures and pressures alone. Our 411 experiments also show that original concentrations of Ca and Cu in melanosomes are 412 413 susceptible to changes during maturation. The fossils we studied show considerable variation in the concentrations of Ca and Cu, likely due to variations in the diagenetic history of the 414 415 deposit. Our data also reveal that the oxidation state of Cu is not altered under conditions of our maturation experiments, but that it may be altered during fossilization. These results 416 confirm that components of the preserved trace element chemistry of fossil melanosomes 417 could be a diagenetic artefact and that maturation experiments can yield essential insights 418 into the chemical taphonomy of melanosomes that should be incorporated into future 419 interpretations of preserved chemical signatures in fossils. 420

421

Collectively, our data emphasise the dynamic nature of links between fossil chemistry and the
host sediment, in particular variations in pore fluid chemistry, through time. Melanin
elemental chemistry is clearly plastic during diagenesis. Other, more labile, components of
fossil chemistry, e.g. proteins (Asara et al. 2007, Schweitzer et al. 2007, Schweitzer et al.
2013, Schroeter et al. 2017) and nucleic acids (Schweitzer et al. 2013, Bailleul et al. 2020),
would presumably be equally, if not more, susceptible to alteration during decay and
diagenesis.

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- 437 *Author contributions.* M.E.M. and C.S.R. conceived the study; C.S.R., M.E.M. and S.M.W.
- 438 performed synchrotron rapid scanning-X-ray fluorescence and X-ray absorption spectroscopy
- 439 (XAS); C.S.R. and M.E.M. wrote the manuscript with input from S.M.W.
- 440 *Supplementary data.* All data is provided in the main text or via the Dryad Digital
- 441 Repository:
- 442 https://datadryad.org/stash/share/H5aMNvcCsDvpSa94b2Wf8T69ZSdwlLc3Cb9zMAI0hn4.

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549

550 **Figure captions:**

551 Figure 1. Effect of temperature and pressure on the trace element chemistry of eye

552 melanosomes of *Dicentrarchus* (Actinopterygii: Perciformes). A, Linear Discriminant

553 Analysis chemospace plot based on measured concentrations of Ca, Fe, Cu and Zn in

untreated and experimentally matured melanosomes. B, Biplot showing the contribution of

555 each element to variation in A.

Figure 2. Differences in the concentrations of key elements Ca, Fe, Cu and Zn in

557 melanosome extracts from eyes of *Dicentrarchus*. X-axis labels refer to untreated extracts

558 (UNMAT) and extracts matured under various conditions as follows: Cu T only, Cu-solution

with elevated temperature; Cu T&P, Cu-solution with elevated temperature and pressure; Cu

560 & Zn, Cu-Zn-solution with elevated temperature and pressure; DD T only, distilled water

with elevated temperature; DD T&P, distilled water with elevated temperature and pressure;

562 Zn, Zn-solution with elevated temperature and pressure; UNMAT, untreated melanosomes.

563 Concentration units are μ g/cm².

Figure 3. Comparison of the trace element chemistry of melanosome extracts and fossil
melanosomes. A, Linear Discriminant Analysis chemospace plot including data for Ca, Fe,

566 Cu and Zn in unmatured and matured melanin extracts from eye melanosomes of

567 *Dicentrarchus* and from fossil Teleostei (*Dapalis, Knightia* and Tetradontiformes). B, Biplot 568 of key elements and their contribution to variation in A. C, XANES spectra at the Cu K edge 569 at 8987 eV (dashed line).

570 **Figure 4.** A, Comparison of data on Zn and B, log Zn and Cu chemistry concentrations and

571 log Zn:Cu ratios among matured and unmatured melanosome extracts and fossil eyespot

572 melanins. X-axis labels refer to fossil specimens, untreated extracts (UNMAT) and extracts

573 matured under various conditions as follows Cu T only, Cu-solution with elevated

temperature; Cu T&P, Cu-solution with elevated temperature and pressure; Cu & Zn, Cu-Zn-

solution with elevated temperature and pressure; DD T only, distilled water with elevated

576 temperature; DD T&P, distilled water with elevated temperature and pressure; Zn, Zn-

solution with elevated temperature and pressure; UNMAT, untreated melanosomes. Data

578 from Mazon Creek (MC) vertebrates, Tetradontiformes and *Knighita* are from Rogers *et al.*

579 2019. Concentration units are $\mu g/cm^2$.



Figure 2.







¹ Supplementary Information for

2

Synchrotron-X-ray fluorescence analysis reveals diagenetic alteration of fossil melanosome trace metal chemistry

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- 10 This PDF file includes:
- 11
- 12 Figures S1 to S9
- 13 Tables S1 to S5
- 14



Fig. S1. MCA spectra from regions of interest in SRS-XRF maps of unmatured melanin extracts A–C.
X-axis, X-ray emission energy (keV). Y-axis, counts. Yellow line denotes measured data; green line
denotes the computed fit.





X-axis, X-ray emission energy (keV). Y-axis, counts. Yellow line denotes measured data; green line

denotes the computed fit.





31 denotes the computed fit.



Figure S4. MCA spectra from regions of interest in SRS-XRF maps of unmatured melanin extracts H,
I, J. X-axis, X-ray emission energy (keV). Y-axis, counts. Yellow line denotes measured data; green
line denotes the computed fit.



Fig. S5. MCA spectra from regions of interest in SRS-XRF maps of unmatured melanin extracts K, L.
X-axis, X-ray emission energy (keV). Y-axis, counts. Yellow line denotes measured data; green line

65 denotes the computed fit.





Fig. S6. MCA spectra from regions of interest in SRS-XRF maps of unmatured melanin extracts M,

69 N. X-axis, X-ray emission energy (keV). Y-axis, counts. Yellow line denotes measured data; green

- 70 line denotes the computed fit.
- 71
- 72





Fig. S7. MCA spectra from regions of interest in SRS-XRF maps of unmatured melanin extracts O, P.
X-axis, X-ray emission energy (keV). Y-axis, counts. Yellow line denotes measured data; green line
denotes the computed fit.





81

82 Fig. S8. MCA spectra from regions of interest in SRS-XRF maps of fossil eyespots of *D. macrurus*,

83 NHMD 199838 (Tetrodontiformes indet.) and UGF 2015-16 X-axis, X-ray emission energy (keV). Y-





Table S1. List of maturation treatments.

Melanosome extracts	Experimental maturation treatment
A-C	Unmatured
D-E	Matured in DD water, with elevated temperature only
F-G	Matured in DD water, with elevated temperature and pressure
H-J	Matured in Zn, with elevated temperature and pressure
K-L	Matured in Cu and Zn, with elevated temperature and pressure
M-N	Matured in Cu, with elevated temperature only
O-P	Matured in Cu, with elevated temperature and pressure

107	Table S2. Statistical analyses of trace element concentrations in melanosome extracts. A, ANOVA /
108	Welch's F-test (*); B, Kruskal-Wallis; C, Post-hoc analyses for Welch F test and Kruskal-Wallis
109	(Dunn's post hoc). Cu T only, extract with added Cu-rich solution treated to elevated temperature; Cu
110	T&P, extract with added Cu-rich solution treated to elevated temperature and pressure; Cu & Zn,
111	extract with added Cu-and Zn-rich solution treated to elevated temperature and pressure; DD T only,
112	extract with added distilled water treated to elevated temperature; DD T&P, extract with added
113	distilled water treated to elevated temperature and pressure; Zn, extract with added Zn-rich solution
114	treated t elevated temperature and pressure; UNMAT, untreated melanosomes. [†] indicates
115	concentration data that were log-transformed prior to statistical testing to make the data conform to
116	normality.

Element concentration	df	F	р
Fe	2.115^{\dagger}	17.5†	0.1034^{\dagger}
Ca	16.18*†	73.39*†	7.91 E-11* [†]
Cu	17.47*†	$118.8^{*\dagger}$	5.792 E-13* [†]

	В
	~

Element concentration	H (chi ²)	р
Zn	23.33	0.000693

118

C

C		UNMAT	DD T only	DD T & P	Zn	Cu & Zn	Cu T & P	Cu T only
	UNMAT		2.43E-01	1.57E-01	4.44E-03	1.03E-03	1.23E-01	1.12E-01
	DD T only	2.43E-01		8.21E-01	2.06E-04	4.86E-05	1.34E-02	1.19E-02
G	DD T & P	1.57E-01	8.21E-01		7.48E-05	1.80E-05	6.91E-03	6.10E-03
Ca	Zn	4.44E-03	2.06E-04	7.48E-05		4.61E-01	3.17E-01	3.39E-01
	Cu & Zn	1.03E-03	4.86E-05	1.80E-05	4.61E-01		1.12E-01	1.22E-01
	Cu T & P	1.23E-01	1.34E-02	6.91E-03	3.17E-01	1.12E-01		9.67E-01
	Cu T only	1.12E-01	1.19E-02	6.10E-03	3.39E-01	1.22E-01	9.67E-01	
		UNMAT	DD T only	DD T & P	Zn	Cu & Zn	Cu T & P	Cu T only
	UNMAT		7.07E-01	5.32E-01	6.49E-01	8.75E-04	2.41E-03	1.78E-03
	DD T only	7.07E-01		8.21E-01	4.34E-01	7.21E-04	1.85E-03	1.39E-03
C	DD T & P	5.32E-01	8.21E-01		3.02E-01	3.08E-04	8.37E-04	6.20E-04
Cu	Zn	6.49E-01	4.34E-01	3.02E-01		3.49E-03	8.60E-03	6.57E-03
	Cu & Zn	8.75E-04	7.21E-04	3.08E-04	3.49E-03		7.89E-01	8.53E-01
	Cu T & P	2.41E-03	1.85E-03	8.37E-04	8.60E-03	7.89E-01		9.34E-01
_	Cu T only	1.78E-03	1.39E-03	6.20E-04	6.57E-03	8.53E-01	9.34E-01	
		UNMAT	DD T only	DD T & P	Zn	Cu & Zn	Cu T & P	Cu T only

		UNMAT		7.68E-01	1.75E-01	6.19E-03	1.79E-03	1.79E-03	5.61E-03
		DD T only	7.68E-01		8.10E-01	1.12E-01	9.27E-02	6.56E-02	6.56E-02
,	7	DD T & P	1.75E-01	8.10E-01		8.75E-02	5.08E-03	8.24E-03	3.06E-02
4	Zn	Zn	6.19E-03	1.12E-01	8.75E-02		6.80E-01	5.96E-01	2.16E-01
		Cu & Zn	1.79E-03	9.27E-02	5.08E-03	6.80E-01		9.36E-01	4.71E-01
		Cu T & P	1.79E-03	6.56E-02	8.24E-03	5.96E-01	9.36E-01		4.71E-01
		Cu T only	5.61E-03	6.56E-02	3.06E-02	2.16E-01	4.71E-01	4.71E-01	
119									
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126	Table S3. Statis	stical analyse	es of trace el	ement conce	entrations in	n fossil eye	espots. A, V	Welch's Al	NOVA;
127	B, Kruskal-Wal	lis test; C, D	unn's post h	oc analyses	for both W	elch's AN	OVA and	Kruskal-W	′allis. †
128	indicates concer	ntration data	that were lo	g-transform	ed prior to	statistical t	esting due	to non-noi	mality.

df	F	p
2660	27.62	0.01100
2.008	37.62	0.01109
8^{\dagger}	162^{\dagger}	0.03136^{\dagger}
2.983^{\dagger}	13.7†	0.8371^{\dagger}
	2.008 8 [†] 2.983 [†]	$\begin{array}{cccc} 2.008 & 57.02 \\ 8^{\dagger} & 162^{\dagger} \\ 2.983^{\dagger} & 13.7^{\dagger} \end{array}$

В			
	Element concentration	H (chi ²)	р
	Zn	0.3556	0.8371

С				
		CKGM F 6327	FOBU 17 591	NHMD 199838
Ca	CKGM F 6327		0.8815	0.05263
	FOBU 17 591	0.8815		0.03689
	NHMD 199838	0.05263	0.03689	
		CKGM F 6327	FOBU 17 591	NHMD 199838
Fe	CKGM F 6327		0.8815	0.03689
	FOBU 17 591	0.8815		0.05263
	NHMD 199838	0.03689	0.05263	
		CKGM F 6327	FOBU 17 591	NHMD 199838
Cu	CKGM F 6327		0.1797	0.00729
	FOBU 17 591	0.1797		0.1797
	NHMD 199838	0.00729	0.1797	

- 134 Table S4. Statistical analyses of trace element concentrations between fossil eyespots and associated
- 135 sedimentary matrix. A, t-test; B, Welch's F-test; C, Mann Whitney-U.[†]indicates concentration data
- that were log-transformed prior to statistical testing due to non-normality.

А				
Element concentration	Specimen	df	t	р
Ca	CKGM F 6327	6	3.9178	0.0078
Ca	FOBU 17 591	4	0.0813	0.93913
Fe	CKGM F 6327 [†]	6†	3.2465 [†]	0.0228 [†]
Cu	NHMD 199838 †	4 [†]	7.0917 [†]	0.0020877^{\dagger}
7	FOBU 17 591	4	0.0308	0.96693
ZII	NHMD 199838	4	1.454	0.21963
В				
Element concentration	Specimen	df	F	р
Ca	NHMD 199838	2.043	3.073	0.219
r.	FOBU 17 591	2.07	106	0 008268
1/-				0.000200
Fe	NHMD 199838	2.062	0.6704	0.4967
Fe	NHMD 199838 CKGM F 6327 [†]	2.062 2.004 [†]	0.6704 2.07 [†]	0.4967 0.01327 [†]
Fe Cu	NHMD 199838 CKGM F 6327 [†] FOBU 17 591	2.062 2.004 [†] 2.07	0.6704 2.07 [†] 0.0813	0.4967 0.01327 [†] 0.93913
Fe Cu	NHMD 199838 CKGM F 6327 [†] FOBU 17 591	2.062 2.004 [†] 2.07	0.6704 2.07 [†] 0.0813	0.4967 0.01327 [†] 0.93913
Fe Cu C Element concentration	NHMD 199838 CKGM F 6327 [†] FOBU 17 591 Specimen	2.062 2.004 [†] 2.07 df	0.6704 2.07 [†] 0.0813 U	0.4967 0.01327 [†] 0.93913 p

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Table S5. Statistical analyses of differences between the trace element concentrations in unmatured
 melanosome extracts and fossils. A, ANOVA; B, Kruskal-Wallis. [†] indicates concentration data that
 were log-transformed prior to statistical testing due to non-normality.

Element concentration	df	F	р
Fe	17†	3.478 [†]	0.08063†
В			
Element concentration	H (chi ²)		р
Са	1.421		0.2332
Cu	0.001949		0.9648
Zn	12 79		0.000349