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*Preservation of feather fibers from the Late
Cretaceous dinosaur Shuvuuia deserti raises concern
about immunohistochemical analyses on fossils*

Massachusetts Institute of Technology

1   Preservation of feather fibers from the Late Cretaceous dinosaur *Shuvuuia*

2   *deserti* raises concern about immunohistochemical analyses on fossils

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4   Evan T. Saitta[a,1,*], Ian Fletcher[b], Peter Martin[c], Michael Pittman[d], Thomas

5   G. Kaye[e], Lawrence D. True[f], Mark A. Norell[g], Geoffrey D. Abbott[h], Roger E.

6   Summons[i], Kirsty Penkman[j], and Jakob Vinther[a,k]

7

8   [a] School of Earth Sciences, University of Bristol, Bristol BS8 1RJ, UK

9   [b] School of Natural and Environmental Sciences, Drummond Building,

10   Newcastle University, Newcastle upon Tyne NE1 7RU, UK

11   [c] School of Physics, University of Bristol, Bristol BS8 1TL, UK

12   [d] Vertebrate Palaeontology Laboratory, Department of Earth Sciences, The

13   University of Hong Kong, Pokfulam, Hong Kong, China

14   [e] Foundation for Scientific Advancement, Sierra Vista, AZ 85650, USA

15   [f] Department of Pathology, University of Washington, Seattle, WA 98195,

16   USA

17   [g] Division of Paleontology, American Museum of Natural History, New York,

18   NY 10024, USA

19   [h] School of Natural and Environmental Sciences, Drummond Building,

20   Newcastle University, Newcastle upon Tyne NE1 7RU, UK

21   [i] Department of Earth, Atmospheric and Planetary Sciences, Massachusetts

22   Institute of Technology, Cambridge, MA 02139, USA

23   [j] Department of Chemistry, University of York, York YO10 5DD, UK

24 k School of Biological Sciences, University of Bristol, Bristol BS8 1TQ, UK

25 l Present address: Integrative Research Center, Section of Earth Sciences,

26 Field Museum of Natural History, Chicago, Illinois 60605, USA

27

28 * Corresponding author at: Integrative Research Center, Section of Earth

29 Sciences, Field Museum of Natural History, Chicago, Illinois 60605, USA.

30   *E-mail address:* evansaitta@gmail.com (E. T. Saitta).

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33   ABSTRACT

34   White fibers from a Late Cretaceous dinosaur *Shuvuuia deserti* stained

35   positive for ß-keratin antibodies in a 1999 paper, followed by many similar

36   immunological Mesozoic protein claims in bones and integument. Antibodies

37   recognize protein epitopes derived from its tertiary and quaternary

38   structure, so such results would suggest long polypeptide preservation

39   allowing for sequencing with palaeobiological implications. However,

40   proteins are relatively unstable biomacromolecules that readily hydrolyze

41   while amino acids exhibit predictable instability under diagenetic heat and

42   pressure. Furthermore, antibodies can yield false positives. We reanalyzed a

43   *Shuvuuia* fiber using focused ion beam scanning electron microscopy,

44   energy-dispersive X-ray spectroscopy, time-of-flight secondary ion mass

45   spectrometry, and laser-stimulated fluorescence imaging, finding it to be

46   inorganic and composed mainly of calcium phosphate. Our findings are

47   inconsistent with any protein or other original organic substance

48   preservation in the *Shuvuuia* fiber, suggesting that immunohistochemistry

49   may be inappropriate for analyzing fossils due to issues with false positives

50   and a lack of controls.

51

52   Keywords: fossils; feathers; antibodies; keratin; calcium phosphate; protein

53

1. Introduction

Organic 'soft tissue' preservation in Mesozoic fossils has been reported for over three decades (De Jong et al., 1974; Gurley et al., 1991; Schweitzer et al., 2005, 2007, 2009; Asara et al., 2007; Bertazzo et al., 2015; Moyer et al., 2016a, 2016b; Pan et al., 2016). Organically-preserved, tens-of-millions-of-years-old proteins, cells, and tissues would be groundbreaking, with potential for studying molecular evolution through geologic time and illuminating extinct animal physiologies. Early work reported β-keratin in bird and non-avian dinosaur integumentary structures (Schweitzer et al., 1999a, 1999b), such as IGM 100/977 (Mongolian Institute of Geology, Ulaan Bataar, Mongolia), a specimen of the small, bipedal Late Cretaceous alvarezsaurid theropod dinosaur *Shuvuuia deserti* from the Mongolian Djadochta Formation with multiple thin fibers associated with the skeleton interpreted as primitive feathers (Schweitzer et al., 1999b).

However, proteins are unstable over deep geologic time (Kriausakul and Mitterer, 1978; Armstrong et al., 1983; Lowenstein ,1985; Mitterer, 1993; Bada, 1998; Briggs and Summons, 2014; Saitta et al., 2017). Partially intact proteins persist for 3.4 Ma in exceptionally cold environments (Rybczynski et al., 2013). Biomineralized calcite crystals can act as closed systems with respect to intracrystalline biomolecules although degradation of these biomolecules still occurs (Curry et al., 1991) (e.g., in a survey of New Zealand brachiopod shells, immunological signals correlated to peptide hydrolysis but were lost by 2 Ma (Walton, 1998; Collins et al., 2003)). The

oldest, uncontested, low-latitude peptide sequence is a short, acidic peptide

found within ~3.8 Ma ratite eggshells (Demarchi et al., 2016). The sequence

was a disordered aspartic acid-rich region of the eggshell protein

struthiocalcin. Its survival is exceptional since carbonate-bound proteins

degrade rapidly and predictably (Curry et al., 1991; Walton, 1998; Collins et

al., 2003), explaining their utility in amino acid racemization dating, and

the surviving region was independently predicted by molecular simulation

from four candidate binding regions. The disordered and otherwise unstable

sequence adopted a stable configuration when bound to the calcite which in

effect 'froze' the peptide to the surface, dropping the local system

temperature by ~30 K, as described by (Demarchi et al., 2016). While short

peptide sequences are relatively common from younger Pleistocene sub-

fossils (Orlando et al., 2013), these are over two orders of magnitude

younger than purported antibody-based Mesozoic peptides.

Antibodies are immune system proteins with unique molecular

structures that can bind to distinct epitopes on antigens, which are

exogenous substances triggering an immune response, such as pathogens.

Antibody-antigen binding specificity has been co-opted for research and

medical purposes, but false positives are common and of concern for any

experiment (True, 2008). Binding specificity is the basis of

immunohistochemical experiments performed on *Shuvuuia* fibers and other

fossils reporting the presence of ß-keratin (Schweitzer et al., 1999a, 1999b;

Moyer et al., 2016a, 2016b). A true positive would suggest long, likely intact,

peptide sequence preservation with higher-order protein folding, given the

conformation-based nature of antibody-antigen binding specificity (Laver et

al., 1990). Beyond the unlikelihood of Mesozoic protein folding preservation,

another issue with these immunochemistry studies is that molecular dating

of the evolution of keratin proteins indicates that modern feather ß-keratins

diverged ~143 Ma, meaning that the feathers of non-avian dinosaurs such

as *Shuvuuia* might not have been composed of the same family of avian ß-

keratins as that of modern feathers (Greenwold and Sawyer, 2011). This

would mean that modern feather keratin is the wrong antigen to raise

antibodies against for experiments on *Shuvuuia* keratin.

The possibility that these results represent false positives is

investigated here. Accordingly, we analyzed *Shuvuuia* fiber composition by

examining another fiber from IGM 100/977 (see Schweitzer et al., 1999b;

Moyer et al., 2016a for immunochemistry result on fibers from this same

specimen). A small piece of matrix containing the fiber studied here was

originally removed from the specimen in 2009 by Amy Davidson who

originally prepared the specimen, stored at room temperature within paper

towel wrapping, and analyzed from 2016 onwards (potentially increasing

the likelihood of detecting proteinaceous signatures in the form of

contamination). The original report of ß-keratin in IGM 100/977 was

published in 1999 (Schweitzer et al., 1999b), and the fiber used in this study

received no further preparation prior to our study. If the fibers indeed

contain endogenous keratin, then the ~17 years between studies is unlikely

123   to result in much degradation, considering their putative persistence since

124   the Late Cretaceous and withstanding diagenesis, environmental moisture

125   in the surface or subsurface, climatic and geothermal high temperatures,

126   and weathering. Therefore, although atmospheric exposure might be argued

127   to impose different degradation stresses on the fossil (e.g., increased

128   oxidation or decay from newly introduced aerobic microbes) than do burial

129   and diagenesis that could result in a loss of original protein over ~17 years,

130   we consider this to be unlikely. If the fiber is indeed composed of keratin,

131   then it should contain signatures consistent with proteins and organic

132   material more generally. Therefore, this study examines the basic structure

133   and chemistry of the fiber. We analyzed the fiber using light microscopy,

134   laser-stimulated fluorescence (LSF) imaging (Kaye et al., 2015; Wang et al.,

135   2017), time-of-flight secondary ion mass spectrometry (TOF-SIMS),

136   scanning electron microscopy (SEM), and energy-dispersive X-ray

137   spectroscopy (EDS). The untreated fiber and surrounding sediment matrix

138   underwent light microscopy and LSF imaging and a fragment of fiber and

139   matrix underwent TOF-SIMS. After discovering that the fiber and matrix

140   were covered by cyanoacrylate consolidant, a focused ion beam (FIB) trench

141   of the fiber fragment was analyzed under SEM and EDS. Finally, the

142   fragment of the fiber and surrounding sediment matrix were resin-

143   embedded, polished, and the cross-section was analyzed with SEM, EDS,

144   and TOF-SIMS.

145

2. Material and methods

2.1. Light microscopy and LSF

LSF images were collected using a modified version of the protocol of Kaye et al. (2015). The untreated fiber and surrounding sediment matrix was illuminated with a 405 nm, 500 mw violet laser diode and imaged with a Leica DFC425 C digital camera under magnification from a Leica M205 C stereomicroscope. An appropriate long pass blocking filter was fitted to the objective of the stereoscope to prevent image saturation by the laser. The laser diode was defocused to project a beam cone that evenly lit the specimen during the photo's time exposure in a dark room. The images were post-processed in Photoshop CC 2016 for sharpness, color balance, and saturation.

2.2. SEM and EDS

Following light microscopy and LSF, a fragmented portion of the fiber and surrounding sediment matrix was sputter-coated with a 50 nm thick layer of gold using an Edwards ScanCoat system to prevent specimen-induced charging and difficulties with imaging/cutting. FIB preparation was performed with an FEI Helios NanoLab 600 dual FIB-SEM system. Before the cross-section was produced (approximately 40 µm × 20 µm × 20 µm), a protective strip of platinum was deposited over the region bordering the cut to prevent against any potential ion-beam damage to the sample surface.

168 Milling was conducted at 30 kV with progressively lower beam currents (6.0

169 nA to 0.9 nA) to produce an artefact-free surface.

170   The same fragment of fiber and surrounding sediment matrix was

171 later embedded in EpoThin 2 epoxy resin and polished using silicon carbide

172 paper to reveal a cross-section of the fiber. SEM and EDS analysis of the

173 FIB-trenched, and subsequently, the resin-embedded polished sample was

174 conducted within a Zeiss SIGMA-HD VP scanning electron microscope with

175 associated EDS instrumentation from EDAX Ltd. The EDS system consisted

176 of an Octane Plus Si-drift detector alongside TEAM control and analysis

177 software.

178   Spot compositional analysis was performed on the samples at 15 kV

179 and 1.7 nA electron beam current for a duration of 100 s per point before

180 progressing onto the next user-defined point. Elemental mapping of the ion-

181 beam cut surface was conducted using the same instrument to visually

182 identify regions of compositional similarity and variation. All EDS analyses

183 were taken in cross-sectional or top-down view in order to mitigate artefacts

184 deriving from the orientation angle of the detector relative to one axis of the

185 specimen.

186

187 *2.3. TOF-SIMS*

188   The fiber and surrounding sediment matrix underwent TOF-SIMS

189 untreated and, later, resin-embedded polished. The samples were mounted

190   directly onto a sample holder using double-sided carbon tape or clean

191   stainless steel screws and clips as appropriate.

192   Static SIMS analyses were carried out using an ION-TOF 'TOF-SIMS

193   IV – 200' instrument (ION-TOF GmbH, Münster, Germany) of single-stage

194   reflectron design (Schwieters et al., 1991). Positive and negative ion spectra

195   and images were obtained using a Bi_3^+ focused liquid metal ion gun at 25

196   keV energy, incident at 45° to the surface normal and operated in 'bunched'

197   mode for high mass resolution. This mode used 20 ns wide ion pulses at 10

198   kHz repetition rate. Charge compensation was effected by low-energy (~20

199   eV) electrons provided by a flood gun. The total ion dose density was less

200   than 1×10^{16} ions m^{-2}. The topography of the sample surface and the ion

201   gun mode of operation limited the mass resolution in this work to

202   approximately m/Dm = 2000. The spatial resolution was limited by the

203   primary ion beam diameter to ~4 µm.

204   Initial analysis of untreated sample: Positive and negative ion static

205   SIMS spectra and images were recorded from the outermost ~1 nm of the

206   sample surface at room temperature. Raw data containing the secondary

207   ions recorded at each pixel was acquired with a 256 pixel × 256 pixel raster

208   and a field of view of 256 µm × 256 µm.

209   Subsequent analysis of resin-embedded polished sample: The area to

210   be analyzed was sputtered using the Bi_3^+ ion beam in 'continuous' or 'DC'

211   mode for 240 s in an attempt to remove any contamination from the

212   polishing process. The ion beam current was 0.5 nA and the ion dose density

213 was less than 1×10^{19} ions m^{-2}. Positive and negative ion static SIMS

214 spectra and images were recorded from the outermost ~ 1 nm of the sample

215 surface at room temperature. Raw data containing the secondary ions

216 recorded at each pixel was acquired with a 256 pixel × 256 pixel raster and

217 a field of view of 500 µm × 500 µm.

218   Images were regenerated from selected peaks in the raw datasets

219 following a full recalibration of the mass scale using the 'Ionspec' and

220 'Ionimage' software from ION-TOF GmbH. The images are presented un-

221 normalized with a linear intensity scale and after Poisson correction for the

222 detector dead-time effects. The images are shown in thermal scale which

223 runs from black through red, orange, and yellow to white with increasing

224 signal intensity.

225   Using the software to select pixels in regions of interest from the total

226 ion image allowed spectra of the fiber and matrix regions in the sample to

227 be generated from the raw data collected by the ION-TOF 'TOF-SIMS IV –

228 200'.

229

230 **3. Results**

231 *3.1. Light microscopy and LSF*

232   The fiber resides within loosely-consolidated, predominantly sand-

233 sized quartz grains (Fig. 1A–C). The fiber is a pale, white cylinder with

234 unidirectional tapering and occasional breakages. White residues similar in

235 color and texture to the fiber also appear in adjacent sediment near the

236   fiber. Some fiber segments are missing through breakage (as evidenced by

237   the fact that the specimen has multiple fibers preserved that vary in

238   continuity and consistent with the loosely consolidated nature of the

239   sediment) and others deflect around sediment grains. The fiber is 7.7 mm in

240   linear length and ranges in width from 0.25 mm basally to 0.03 mm

241   apically. LSF reveals that the fiber fluoresces a different color to the

242   surrounding sediment grains (Fig. 1D).

243

244   *3.2. TOF-SIMS*

245   In the untreated sample, both the fiber and sediment grains have

246   strong CN^- ($m/z = -26$) peaks as well as strong peaks at $m/z = -112$

247   consistent with $C_5H_6NO_2^-$ (Supplementary Figs. S135–S136). The sediment

248   grains and fiber have very similar spectra over the entire m/z range

249   examined, consisting largely of organic secondary ions (Supplementary Figs.

250   S137–S140).

251   In the resin-embedded polished sample (Fig. 2A), secondary ions such

252   as Ca^+, CaO_X^+, $CaPO_X^+$, and PO_X^- were strongly localized to the fiber (Fig.

253   2B–F). Secondary ions such as Mg^+, Fe^+, K^+, Al^+, Na^+, Si^+, Si^-, SiO_X^-, and Cl^-

254   were present in the sediment grains at elevated levels compared to the fiber,

255   although low levels of Na^+, relative to the grains, show some non-specific

256   localization to the fiber as well (Fig. 2G–O). Secondary ions related to epoxy

257   (both positive and negative) and cyanoacrylate (negative) residues appear

258   on the grains, fiber, and embedding resin (Fig. 2P–R). Of the secondary ions

259 examined that can potentially relate to amino acids such as those identified

260 by Hedberg et al. (2012) (Fig. 2S-JJ), total N^+ (Fig. 2KK), and S^- (Fig. 2LL),

261 none showed preferential localization to the fiber, and most were detected at

262 low levels broadly across the grains, fibers, and embedding resin, while CN^-

263 strongly localized to the embedding resin (Fig. 2S–MM). Furthermore, peaks

264 at $m/z = 76.02$ that could possibly come from $C_2H_6SN^+$ are not strongly

265 present in the fiber and are more expressed in the sediment grains and

266 embedding resin.

267

268 *3.3. SEM and EDS*

269　　　To reveal the internal composition in a minimally intrusive fashion, a

270 trench was milled using FIB. SEM reveals the fiber's corrugated surface and

271 platy core textures (Fig. 1E), although the potential for 'curtaining' artifacts

272 from FIB should be kept in mind. EDS up the FIB section from the more

273 interior region onto the uncut surface shows consistent elemental makeup

274 with strong Ca, C, Si, Al, O, and P peaks (Supplementary Figs. S1–S22,

275 Supplementary Tables S1–S7). Other small peaks detected include Fe, Mg,

276 and Na. S peaks are weak in all spectra. Ga peaks are a result of

277 implantation from FIB. Top-down elemental maps (i.e., trench viewed

278 perpendicular to the fiber surface as opposed to viewing the wall of the FIB

279 trench which is perpendicular to the fiber surface) show potential

280 preferential localization of C to the uncut surface (Supplementary Figs.

281 S23–S41, Supplementary Tables S8–S12).

282   The resin-embedded polished sample shows a similar, yet stronger

283   signal, without topographic artifacts inevitably present in the EDS of the

284   FIB trench. The platy texture of the fiber core still seems apparent (Fig. 3A).

285   Ca and P are strongly localized in the fiber, and although Si, Al, and O are

286   also present, they are more prevalent in adjacent sediment grains. S is

287   weakly expressed in the fiber and sediment. Mg is present in small amounts

288   in the fiber. Na is more prevalent in the sediment than the fiber, while Fe

289   mostly appears in the space between the fiber and sediment grains (Fig.

290   3B–I). No substantial C was detected (Fig. 3J).

291

292   **4. Discussion**

293   The analyses performed here reveal four key aspects of the fiber's

294   chemistry: it is covered in cyanoacrylate, it is largely calcium phosphate, it

295   lacks chemical signatures consistent with protein, and it likely has clay

296   mineral infiltration within the possibly endogenous calcium phosphate.

297   TOF-SIMS suggested that the fiber and surrounding sediment are

298   encapsulated in ethyl-cyanoacrylate polymer by the presence of $C_5H_6NO_2^-$

299   secondary ions (Supplementary Figs. S135–S140), deriving from the

300   consolidant used in the original preparation of the fossil to stabilize the

301   specimen prior to the Schweitzer et al. (1999b) study (Krazy Glue™ 201

302   ethyl-cyanoacrylate, white cap, low viscosity, Borden Inc., purchased in

303   1994, and used in addition to Butvar B-76 [polyvinyl butyral]; Amy

304   Davidson personal communication). The presence of cyanoacrylate first

became apparent when analyzing the untreated sample with TOF-SIMS,

prior to embedding in resin and polishing. Cyanoacrylate was detected

strongly in the untreated sample, but was detected as a diffuse pattern

(along with epoxy embedding resin; Fig. 2P,Q) across the entire surface of

the polished sample likely due to grinding (Fig. 2R). EDS detected surface

consolidant as C (Supplementary Figs. S1–S41, Supplementary Tables S1–

S12).

EDS of the fiber core revealed Ca, P, and O, consistent with calcium

phosphate (Fig. 3.B–D,J). TOF-SIMS corroborated this composition with

preferential localization of Ca^+, CaO_X^+, $CaPO_X^+$, and PO_X^- to the fiber, while

the lack of Ca^- was consistent with Ca derived from the cation of $CaPO_4$

(Fig. 2.B–F). TOF-SIMS carried out by Schweitzer et al. (1999b) also

detected Ca^+ and CaO_2^+ in *Shuvuuia* (IGM 100/977) fibers.

Very little S was observed in the fiber through EDS, which would

have been present in stable disulfide bonds of keratin protein *in vivo*

(Goddard and Michaelis, 1934); no significant N was observed, strongly

suggesting the absence of protein since N is incorporated into peptide bonds

(Fig. 3I,J). TOF-SIMS showed a lack of preferential localization of any of the

examined potential amino acid fragment ions from Hedberg et al. (2012)

(work which relates secondary ions to the particular amino acid they can

derive from), total N^+, or S^- to the fiber (Fig. 2S–MM). Relatively uniform,

low-intensity distributions across the polished section of some of these

fragment ions suggests that they largely came from spreading of applied

328 consolidant residues during grinding. Given the lack of S^-, peaks at $m/z =$

329 76.02 are likely due to $C_5H_2N^+$ (m/z = 76.018) and/or $C_2H_4O_3^+$ (m/z =

330 76.016) rather than $C_2H_6SN^+$ deriving from the cysteine found abundantly

331 in keratin and responsible for forming disulfide bridges. Regardless, $m/z =$

332 76.02 peaks do not strongly appear in the fiber, but rather in the sediment

333 grains and embedding resin (Fig 2CC). What little S is present in the fiber

334 could potentially be in an inorganic form such as pyrite, which is commonly

335 found in fossils. Some of the S atoms themselves, however, could have

336 originally been released from keratin breakdown products. Previously-

337 published, positive-ion TOF-SIMS data of *Shuvuuia* (IGM 100/977) fibers

338 revealed the presence of various organic secondary ions, interpreted as

339 deriving from endogenous keratin amino acids (Schweitzer et al., 1999b),

340 which are likely derived from either non-amino acid molecules or from

341 amino acid contamination, possibly bound with cyanoacrylate. A further

342 issue with the TOF-SIMS analysis of Schweitzer et al. (1999b) is that it did

343 not present the results from the sediment matrix control in the main text

344 that might elucidate localization patterns, or lack thereof, of these organic

345 secondary ions.

346   Potential clay presence in the fiber suggested by EDS as Si, Al, and O

347 (Fig. 3D–F,J) is consistent with SEM observations of a platy texture within

348 the FIB trench and resin-embedded polished sample, keeping in mind the

349 potential for FIB artifacts (Fig. 1E). Furthermore, the observed

350 ultrastructural texture and internal structure do not match descriptions of

351   those from modern feathers (Davies 1970; Lucas & Stettenheim, 1972).

352   TOF-SIMS did not reveal clay markers in the fiber as EDS did, perhaps due

353   to lower resolution mapping (e.g., Mg^+, Fe^+, Al^+, Na^+, Si^+, Si^-, SiO_X^-), but did

354   show non-specific Na^+ presence in the fiber at lower levels than the

355   sediment grains (Fig. 3G–O). Ultimately, calcium phosphate dominance in

356   the fiber is consistent with the differing fiber autofluorescence to the

357   surrounding silicate-dominated sediment matrix during LSF (Fig. 1D).

358   Similar to the data here, Schweitzer et al. (1999b) detected Na^+, Al^+, and Si^+

359   in *Shuvuuia* (IGM 100/977) fibers using TOF-SIMS.

360

361   *4.1. Calcium phosphate preservation of fossil keratinous structures: updating*

362   *the taphonomic model*

363   Endogenous organic carbon in *Shuvuuia* fibers cannot be confirmed,

364   especially in light of the detection of applied cyanoacrylate. Their white

365   coloration is consistent with a lack of organic carbon. Similarly, fossil bone

366   from the Djadochta Formation is also white, again consistent with organic

367   loss.

368   Keratinous structures (i.e., the anatomical structure/tissue originally

369   containing keratin protein *in vivo*) commonly fossilize as white, fluorescing

370   material, such as claw sheaths preserved in the Djadochta Formation

371   (Moyer et al., 2016b) and other Mesozoic localities (Martin et al., 1998;

372   Schweitzer et al., 1999a). For example, *Psittacosaurus* integument appears

17

373   to preserve as fluorescent calcium phosphate with associated fossil melanin

374   (Mayr et al., 2016; Vinther et al., 2016).

375   Moyer et al. (2016b) ran EDS on white claw sheaths from the

376   oviraptorid theropod dinosaur *Citipati*, similarly preserved from the same

377   formation as *Shuvuuia* and also claimed to contain keratin protein based on

378   immunohistochemistry. Their study similarly revealed the *Citipati* claw

379   sheaths to be predominantly composed of Ca and O, with P also present,

380   indicating preservation as calcium phosphate. EDS on *Citipati* claw sheaths

381   did not reveal S, suggesting that phosphatic preservation of keratinous

382   tissues need not contain S. Platy textures found in *Citipati* claw sheaths

383   similar to those observed here in the *Shuvuuia* fiber were interpreted as

384   keratin by Moyer et al. (2016b), but our study suggests that such textures

385   are likely not indicative of protein preservation. EDS on *Citipati* claw

386   sheaths similarly revealed the presence of Si, Al, and Mg (Moyer et al.,

387   2016b), consistent with clay mineral infiltration.

388   Most research into fossil feathers focuses on organically-preserved

389   specimens, as in the field of paleo-color reconstruction (Vinther, 2015),

390   probably partly related to the richness of exceptional carbonaceous fossils

391   from China (Norell and Xu, 2005) and similar lagerstätten. However,

392   calcium and phosphorous are known to be concentrated in *Archaeopteryx*

393   feather rachi (Bergmann et al., 2010) and an isolated Liaoning feather

394   (Benton et al., 2008). North American Mesozoic fossil discoveries in

395   particular, unlike those from Konservat-Lagerstätten with extensive

396   carbonaceous preservation as in some Chinese deposits, provide an example

397   of alternative preservational modes for keratinous structures. Contrary to

398   finer-grained, anoxic depositional environments that retain and preserve

399   organic molecules like fossil melanin (Fürsich et al., 2007), North American

400   Mesozoic keratinous fossils are often from coarser-grained, oxidized

401   depositional environments averse to organic retention and preservation

402   (Parry et al., 2018). Instead, Mesozoic keratinous fossils in North America

403   are often preserved as simple skin impressions in the sediment (Bell, 2012)

404   – possibly explaining why large Chinese tyrannosaurs are preserved with

405   carbonaceous fossil feathers (Xu et al., 2012), while large North American

406   tyrannosaurs only preserve scale impressions (Bell et al., 2017). In some

407   cases, however, North American fossil keratinous structures appear to

408   preserve in a manner consistent with phosphatized sheaths. Ungual and

409   beak sheaths on hadrosaurs (Murphy et al., 2006) and osteoderm sheaths in

410   stegosaurs (Christiansen and Tschopp 2010) are potentially some examples

411   of phosphatized keratinous fossils, deserving of further chemical analyses.

412   The North American ankylosaur *Borealopelta* has osteoderm sheaths with

413   preserved melanin and calcium phosphate, although this rare specimen was

414   found in an exceptional carbonate concretion formed under euxinic

415   conditions (Brown et al., 2017). As for filamentous keratin, Late Miocene

416   phosphatized baleen has been reported from South America (Gioncada et

417   al., 2016). Regarding this *Shuvuuia* specimen, the loosely consolidated,

418   sand-sized quartz grains of the matrix would not be expected to retain

419   organics well due to the potential leaching of breakdown products through

420   the relatively large pore size or through relatively greater exposure to

421   adverse conditions such as oxidation, so phosphate-dominated preservation

422   of fibers is not unexpected.

423   A phosphatic preservational mode of fossil keratinous structures is

424   unsurprising since many different keratinous tissues are hardened via

425   calcium phosphate deposition *in vivo* to varying degrees. For example,

426   calcium phosphate has been measured or detected in fresh/modern claw,

427   horn, beak, and hoof sheaths, nails, baleen, hair, quills, whiskers, and

428   feathers, especially feather calami (Blakey et al., 1963; Pautard, 1963, 1964,

429   1970; Blakey and Lockwood 1968; Lucas and Stettenheim 1972; Szewciw et

430   al., 2010). Calcification of keratinous structures appears to change their

431   material properties of the integumentary structure, positively correlating

432   with hardness and presumably related to the biological function of the

433   structure (Baggott et al., 1988; Bonser, 1996; Szewciw et al., 2010).

434   Fossil keratinous tissues likely undergo significant volume loss due to

435   protein degradation, with resistant calcium phosphate and pigments

436   remaining (Saitta et al., 2017). Volume loss would allow for clay minerals to

437   precipitate into the fossil as evidenced by clay signatures in *Shuvuuia* fibers

438   and *Citipati* claw sheaths (Moyer et al., 2016b). Future work should be

439   conducted to determine to what degree phosphatic preservation of

440   keratinous structures represents endogenous calcium phosphate with

441   subsequent infilling of other materials (e.g., clay minerals), or if endogenous

442 calcium phosphate can act as a nucleus for additional, secondary calcium or

443 phosphate precipitation during fossilization. If the former, then

444 phosphatized keratinous structure fossils could provide important

445 paleobiological data regarding the shape, size, and degree and distribution

446 of calcification of integumentary structures *in vivo*. It might be argued that

447 the keratin protein could be phosphatized through authigenic

448 mineralization as a result of decay, as is the case with fossil muscle tissue

449 (Briggs et al., 1993; Briggs and Wilby, 1996; Briggs, 2003; Parry et al.,

450 2015). However, invoking authigenic mineralization as an explanation for

451 keratinous structure fossilization is unnecessary since they can contain

452 calcium phosphate endogenously. Our taphonomic model is consistent with

453 the fact that phosphatic keratinous tissue fossils are often structures

454 expected to be hardened *in vivo* (e.g., claw, osteoderm, and beak sheaths).

455 Furthermore, authigenically phosphatized tissue tends to preserve small

456 structural details with high fidelity, which does not appear to be the case for

457 some fossil keratinous structures such as the *Shuvuuia* fibers.

458   IGM 100/977 fibers likely derive from feathers rather than the

459 taphonomic environment based on their distinct, tapering morphology as

460 well as their distribution and orientation around the bones. Furthermore,

461 the structures likely do not represent tissue types other than integumentary

462 structures, such as dermal collagen or muscle. The claim that dermal

463 collagen has been found fossilized as fibers has been heavily refuted

464 (Smithwick et al., 2017), and although muscle tissue can phosphatize via

465   authigenic mineralization (Briggs et al., 1993; Briggs and Wilby, 1996;

466   Briggs, 2003; Parry et al., 2015), the sparse distribution of the *Shuvuuia*

467   fibers and their orientation around the bones is inconsistent with

468   phosphatized masses of muscle. Given that the fibers are calcium

469   phosphate, *Shuvuuia* feathers were likely hardened *in vivo*. As rachi might

470   be expected to be preferentially calcified (Pautard, 1964; Benton et al., 2008;

471   Bergmann et al., 2010; also see Supplementary Fig. S141 for an LSF image

472   of a modern feather rachis with prominent fluorescence consistent with

473   calcification), *Shuvuuia* feathers could have consisted of a well-developed

474   rachis with less calcified barbs being unpreserved in this specimen, except

475   possibly for some calcium phosphate residues in the matrix. Such an

476   interpretation is consistent with alvarezsaurid phylogenetic position, since

477   closely related maniraptoriformes are known to have pinnate feathers (Cau

478   et al., 2015) more closely resembling the morphology of many modern

479   feathers (Lucas and Stettenheim, 1972) than to simple monofilaments. In

480   this case, former interpretations of morphologically simple monofilaments

481   typically assumed for alvarezsaurids (Xu et al., 2014) may be

482   taphonomically biased. Therefore, a more complex feather morphology is

483   potentially a better supported hypothesis than simple, calcified

484   monofilaments such as those in *Psittacosaurus*. Unlike the short fibers of

485   *Shuvuuia*, *Psittacosaurus* bristles are hyper-elongated and erect, suggesting

486   a unique display or signaling function (Mayr et al., 2016). However, another

487   alternate hypothesis might involve calcified, simple filaments that represent

488   vestigial structures as a result of secondary

489   terrestrialization/glidelessness/flightlessness, in a manner resembling the

490   calamus-like wing feathers on cassowaries (Prum, 2005). Thus, in a rather

491   unusual reversal of the typical direction of evidence and interpretations for

492   this topic of study, better resolved phylogenies and investigations

493   pinpointing the appearance and loss of arboreality/gliding/flight using

494   osteological data may be integral in working out feather morphology for this

495   taxon.

496

497   *4.2. False positives in paleo-immunohistochemistry*

498   We argue that previous paleontological studies using polyclonal

499   antibodies raised against keratin (Schweitzer et al., 1999a, 1999b; Moyer et

500   al., 2016b, 2016b; Pan et al., 2016) have mistaken false positive results for

501   evidence of original keratin protein preservation in fossils. Two obvious

502   sources might yield false positive stains in *Shuvuuia* fibers and other

503   fossilized keratinous structures: calcium phosphate or cyanoacrylate

504   polymer.

505   Calcified material has been stated to yield both false negatives and

506   positives with immunohistochemistry (Sedivy and Battistutti, 2003), while

507   biomedical research has used cyanoacrylate nanoparticles to adsorb

508   antibodies (Illum et al., 1983). The original study of *Shuvuuia* fibers

509   showing positive antibody stains performed no demineralization and instead

510   rinsed them in 100% ethanol prior to analysis (Schweitzer et al., 1999b),

which might not be expected to act as a strong enough solvent to remove

cyanoacrylates through rinsing, although other consolidants applied to the

fossil in the field or lab might exhibit different solubility, such as Butvar B-

76. A more recent analysis resulting in positive antibody stains of *Citipati*

(formerly IGM 100/979) claw sheaths (Moyer et al., 2016b), likely

consolidated during collection or preparation in a similar manner to IGM

100/977, did use demineralization and revealed fibrous structures,

consistent with applied organic polymers, as well as mineral grains.

Antibody binding to the *Shuvuuia* fiber rim and throughout the fiber (Moyer

et al., 2016a) suggests that both cyanoacrylates, or other consolidants like

Butvar B-76, and calcium phosphate could be responsible for false positives.

Another issue for paleo-immunohistochemistry is that antibodies

show non-specific binding to melanoidins, condensation products formed

during degradation of protein and polysaccharide mixtures that can be

present in fossils and sediments (Collins et al., 1992). Although we do not

suspect melanoidin presence in *Shuvuuia* fibers given the limited organic

content of the fibers, this could explain antibody staining of other fossils

that do have organic preservation.

Since antibodies typically bind to a specific 3-dimensional epitope of

the target protein, a positive result on Mesozoic fossils would suggest not

only that peptides are present, but also highly preserved protein with

surviving 3° or 4° structures. Higher order protein folding is unlikely to

persist through deep time (Bada, 1998), therefore false positives are of

concern. In an immunological study of subfossil human skeletal material for three target proteins, antibody cross-reactivity was observed with non-human mammal bone, certain collagens, and bacterial cells (Brandt et al., 2002), calling into question the utility of immunochemistry on ancient material. False positives from immunohistochemistry are even possible in recent, biological samples (True, 2008). The observation that "...in [a] feather treated for 10 years at 350°C, [Moyer et al.] were able to demonstrate weak positive binding of anti-feather antibodies localized to the tissues" (Moyer et al., 2016a, p. 7/18) can either be interpreted as epitope survival or the propensity for nonspecific cross-reactivity of antibodies as proteins degrade. Attempts to replicate these 350 °C conditions in a laboratory oven revealed that feathers rapidly start to smoke at this temperature and turn into a black ash (i.e., within minutes) (Supplementary Figs. S142–S143), consistent with melanoidin and other browning condensation reactions and the inferable loss of protein structure.

Ultimately, immunohistochemistry was used to support the claim that *Shuvuuia* fibers consisted of keratin protein (Schweitzer et al., 1999b; Moyer et al., 2016a). If this claim were true, then other techniques should be capable of providing multiple, independent lines of evidence that support or are consistent with a keratin protein composition. We show here that this is not the case, thereby questioning the validity the evidence evoked from immunohistochemistry.

4.3. Suggestions for future practice

557

558   Immunohistochemistry of fossils is insufficient to confirm protein

559   preservation because of its high susceptibility to false positives. These

560   complications are more readily diagnosed and addressed when analyzing

561   recent, biological samples with well-established protocols and controls

562   (True, 2008). (However, see Collins et al. (1992) for an alternative

563   experimental procedure with antibodies, whereby antibody cross-reactivity

564   with experimentally-produced melanoidins was observed and suggestive of

565   Maillard-type pathways for protein-polysaccharide degradation occurring

566   naturally in diagenesis). Other chemical tests (e.g., chromatography and

567   mass spectrometry) can detect unique protein markers. For example,

568   pyrolysis-gas chromatography-mass spectrometry is a sensitive, cheap

569   method used for geologic samples, yielding a distinct suite of pyrolysates for

570   intact or degraded proteins, particularly amides, succinimides, and

571   diketopiperazines (Saitta et al., 2017). Additionally, liquid chromatography

572   can be used to search for amino acids, and racemization can indicate their

573   relative age (Bada and Schroeder 1975). Methods such as these might not be

574   necessary, however, if the sample in question does not first show clear

575   indication of an organic composition. Note that these methods were not used

576   to study the *Shuvuuia* fiber since the sample failed to give fundamental

577   chemical and structural signatures consistent with an organic, let alone

578   proteinaceous, composition. It is also important to thoroughly extract

579   conserving agents from fossils prior to any analysis (Pinder et al., 2017).

580   Our study highlights that, ideally, multiple tests should be run to produce

581   robust claims of fossil proteins that search for organic elemental and

582   molecular signatures, protein degradation products, and predictable amino

583   acid racemization profiles before sequencing peptides or attempting

584   immunohistochemical experiments.

585   Questionable usage of histochemical staining on fossil samples has

586   implications beyond that of dinosaur or feather evolution. For example,

587   Kemp (2002) used a positive result from picrosirius red (a dye used to stain

588   collagen and amyloid in tissues) of conodont hyaline tissue as one line of

589   evidence to conclude that collagen was originally present in the tissue,

590   refuting homology with vertebrate enamel and leading others to claim that

591   conodonts were not vertebrates (Turner et al., 2010). Like the antibody

592   staining of fossil tissues, might picrosirius red staining also be susceptible

593   to false positives in such samples, weakening this line of evidence? Further

594   highlighting the difficulties in attempts to determine the original protein

595   composition, or lack thereof, in a fossil tissue is the more recent proposition

596   that conodont hyaline tissue actually contained keratin, not collagen, in

597   early growth stages while the majority of the tissue lacked organics (Terrill

598   et al., 2018). However, the S and organic signatures detected in the tissue

599   through EDS and X-ray photoelectron spectroscopy by Terrill et al. (2018)

600   are inconclusive evidence, insufficient to diagnose a keratin protein source.

601

602   **5. Conclusions**

603   *Shuvuuia* fibers from IGM 100/977, previously identified through

604   immunohistochemistry as preserving keratin, are composed of calcium

605   phosphate by virtue of their color, texture, fluorescence, elemental

606   composition, and secondary ion mass spectra. Hardened keratin contains

607   calcium phosphate *in vivo*. Thus, one hypothesis is that the fibers

608   potentially derive from the remains of calcified feather rachi, raising the

609   possibility of a more complex, pennaceous feather morphology in

610   alvarezsaurids than previously thought, although simple monofilaments

611   cannot be ruled out. Endogenous organic material or amino acid signatures

612   could not be confirmed in the fiber. Detected organic material likely derived

613   from applied consolidants rather than endogenous proteins based on the

614   lack of localization of any distinct organic secondary ions to the fiber. The

615   fiber and associated sediment matrix are covered in cyanoacrylate, likely

616   derived from the fossil's preparation, in which cyanoacrylate and Butvar B-

617   76 were applied. Cyanoacrylates and other substances, potentially including

618   calcium phosphate, can accumulate antibodies, likely explaining previous

619   reports of keratin preservation in these fibers as false positives. As

620   suggested by our study, immunohistochemistry on fossils is likely

621   inappropriate for studying ancient, putative proteins without independent,

622   corroborative evidence, and even then, the possibility of contamination in

623   corroborating methods should be controlled for.

624

625   **Acknowledgements**

642

643   **Appendix A. Supplementary material**

644   Supplementary data associated with this article can be found, in the

645   online version at . These include a supplementary PDF file along with four

646   .XLSX/.CSV and four .TXT raw data files.

647

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914 **Figure legends**

915 **Fig. 1.** *Shuvuuia* (IGM 100/977) fiber analyzed. **(A–D)** Untreated fiber. (A–

916 C) Light micrographs. (A) Whole fiber. (B) Basal end. (C) Apical region. (D)

917 LSF image of basal portion showing unique fluorescence in color and

918 intensity relative to the matrix. (E) Scanning electron image of FIB trench

919 in fiber.

920

Fig. 2. Resin-embedded polished sample TOF-SIMS. Fiber in center.

Fragment ions associated with (B–F) fiber, (G–O) sediment matrix, (P–R)

applied residues, and (S–MM) potential amino acids or their derivatives,

including peaks identified by Hedberg et al. (2012). (B) Sum of Ca^+, total

CaO_X^+, and total $CaPO_X^+$. (C) Sum of $CaOH^+$, Ca_2O^+, and $Ca_2O_2H^+$. (D) Sum

of $CaPO_2^+$, $CaPO_3^+$, $Ca_2PO_3^+$, $Ca_2PO_4^+$, $Ca_3PO_4^+$, and $Ca_3PO_5^+$. (F) Sum of

PO_2^- and PO_3^-. (N) Sum of SiO_2^-, SiO_3^-, $HSiO_3^-$, $Si_2O_4^-$, $Si_2O_4H^-$, and

$Si_2O_5H^-$. (P) Sum of CH_3O^+, $m/z = 135$, and $m/z = 191$. (Q) Sum of $m/z = -$

211 and $m/z = -283$. (R) Sum of $C_5H_6NO_2^-$ and $C_{10}H_{12}N_2O_4^-$. (KK) Sum of

images (S) to (BB) and (DD) to (JJ) inclusive. (LL) Sum of S^-, HS^-, SO_2^-, and

SO_3^-.

932

Fig. 3. EDS of resin-embedded polished sample. Fiber in center. Significant

peaks of region analysis of fiber (A) labelled in spectrum (J).