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Soft-tissue evidence for homeothermy and crypsis in a Jurassic ichthyosaur

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The Posidonia Shale Formation of Germany comprises a 40 m thick succession of bituminous mudstones (black shale facies), marls and intercalated limestones of early Toarcian (183–181 Ma, Early Jurassic) age[1,2]. The sediments were deposited in a shallow shelf environment along the northwestern border of the Tethys Ocean[1,2]. A warm, sub-tropical to tropical climate with strong monsoonal influence, seasonal density-stratification of the water column, and siliciclastic input from nearby landmasses ensured a continuous precipitation of detrital minerals and calcareous nanoplankton in the epicontinental basin[1–3]. The fine-grained material accumulated under poorly oxygenated bottom conditions at depths ranging from 50 to 150 m[1,4], resulting in a muddy, water-saturated substrate[5] intermittently disturbed by current activity[6].

Part D. Specimen Details and Taxonomic Assignment

MH 432 was excavated in 1991 from a marine mudstone deposit of the Posidonia Shale Formation in the Kromer Slate Quarry near the village of Ohmden, ~2.5 km northeast of Holzmaden. Because the body surface originally in contact with the seabed is normally better preserved than the side facing the water column[5], the fossil was prepared from beneath without preservatives using a combination of mechanical tools and sandblasting. These procedures uncovered the ventral oblique aspect of a moderate-sized (estimated original snout–tail length ~1.6 m) ichthyosaur that is essentially complete from the orbital region to the base of the tail (Fig. 1a, b).

In addition to extensive soft-tissue structures, MH 432 incorporates an incomplete, yet partially articulated skeleton, comprising the rear part of the skull and mandible, a number of precaudal vertebrae, dorsal ribs, gastralia, pectoral girdle elements, and the pectoral and pelvic fins (Fig. 1a, b).

The posterior portion of the articulated cranium and left mandibular ramus are exposed in oblique left lateral view. The skull includes a posterior maxilla fragment, the lacrimal, jugal, postorbital, and quadratojugal, as well as a virtually complete but somewhat distorted scleral ring. The preserved section of the mandible includes part of the posterior extremity of the dentary, together with the surangular and angular. A prominent suborbital groove is present on the lateral surface of the surangular; the angular is well exposed posteriorly. Other associated cranial elements include a pterygoid and various components of the palate and occipital unit.

The axial skeleton is disarticulated and comprises pre-caudal vertebrae and ribs. Only the gastralia basket, which is accessible in ventral view, remains largely undistorted. A mass of small indeterminate bones within the abdominal cavity probably represents stomach contents.

The pectoral girdle includes parts of the clavicles, interclavicles, scapulae, and coracoids. The complete left scapula is exposed in lateral view, and has a prominent acromial angle. The coracoids lack a distinct posterior notch. The pectoral fins are represented by the humeri and a radius. In contrast, the pelvic fins are mostly intact and articulated. The femora are

approximately 30% smaller than the humeri, and the distal tarsal II is distinctly smaller than both the astragalus and calcaneum.

Following the generic definition of Maxwell[7], MH 432 can be confidently assigned to the parvipelvian ichthyosaur *Stenopterygius* based on: (1) the presence of a long and deep suborbital groove on the surangular; (2) extensive exposure of the angular along the posterior edge of the lower jaw; (3) the prominent acromial angle on the scapula; (4) the lack of a posterior notch on the coracoid; (5) two distal facets for the radius and ulna on the humerus; and (6) the humerus being proportionately about 30% longer than the femur.

Maxwell[7] recognised three nominal species of *Stenopterygius* from the Posidonia Shale Formation of Holzmaden: *S. quadriscissus* (Quenstedt, 1856), *S. triscissus* (Quenstedt, 1856) and *S. uniter* von Huene, 1931. Unfortunately, most of the character states diagnosing these taxa are not preserved in MH 432. The specimen does exhibit noticeable reduction of distal tarsal II relative to the astragalus and calcaneum, which is indicative of *S. triscissus*[7]. However, given that MH 432 likely represents a skeletally immature animal, and that individuals of *S. quadriscissus* are far more numerically abundant than *S. triscissus* in the εII_6 stratigraphic level that yielded the fossil (ref. 7: figure 2), we here elect to retain open species nomenclature pending further refinement of intra-generic differential character states.

Part E. Melanophore Cells and Eumelanin Pigment

One of the most remarkable features of the flank skin is the presence of three-dimensional microbodies with long dendritic processes and a granular internal filling (Figs 2g, l, n, 3a–k, 4g, i, Extended Data Figs 2b, c, 3, 10d and Supplementary Video 1). These are virtually identical in size (~10 μm in diameter, excluding the external projections), geometry and inner fabric to the branched melanophores (melanin-storing cells) of extant reptiles, when imaged under comparable parameters (Fig. 3l–q). The high degree of morphological fidelity (extending to nanometre-scale), combined with our chemical detection of eumelanin (Fig. 3r, s and Extended Data Fig. 3f), permit a confident identification of these ancient microfeatures and their grainy contents as pigment cells and melanosome organelles, respectively. The relict melanophores occur within the deeper layers of the epidermis and superficial dermis (Fig. 2l), and thus coincide topographically with epidermal and dermal melanophores found in modern reptile skin[8] (Fig. 2t).

In addition to discrete cellular structures, scattered melanosome clusters are present throughout the horny layers of the stratum corneum in MH 432, implying a possible relocation of melanic pigments from the epidermal melanophores into keratinised cells[9]. Post mortem leakage of eumelanin into the surrounding tissues and sediments is also apparent (Fig. 3r and Extended Data Fig. 3f). Nonetheless, quantification of chemical markers produced by alkaline hydrogen peroxide degradation (an assay designed to detect eu- and pheomelanins[10]) revealed an organ-specific partitioning that likely reflects their original state[11–13] (Fig. 3r and Extended Data Fig. 3f). Despite some decay-generated dispersal, the bulk of the melanin-derived constituents preserved in MH 432 thus seem to have remained more-or-less in life position; an observation independently verified by ToF-SIMS analysis (Fig. 3s).

Part F. Description of MH 432 Liver

We identify a large patch of red-brown material within the ribcage of MH 432 as remnants of the liver (Extended Data Fig. 8). Demineralisation of the phosphatised (Fig. 1c and Extended Data Fig. 1n) structure liberated pliable organic remains with a predominantly polymeric composition (Fig. 5k and Extended Data Fig. 8k). However, aromatic structures were copious, as opposed to alkyl moieties, which were otherwise much more abundant in the preserved

integument (Extended Data Fig. 4a). Appreciable amounts of eumelanin were also detected (Fig. 3r and Extended Data Fig. 3f). These, together with the anatomical position of the mass, its haematitic colour, and intense fluorescence generated by the anti-haemoglobin antibodies (to the exclusion of all other applied antisera; Extended Data Fig. 8e–h), are compatible with our interpretation of this material as a liver tissue residue[12–14].

Part G. Notes on Analyses and Experiments

Rationale for the identification of proteinaceous matter. The unambiguous identification and characterisation of proteinaceous traces in multimillion-year-old fossils is both controversial and methodologically challenging[15–18]. The foremost issue for our study was contamination, which can occur naturally, or during recovery and handling of the original fossil material[17]. The two most common sources of contamination are environmental microbes and human keratin. To control for these, we tested MH 432 in conjunction with its associated matrix, and other fossils[19], using identical experimental conditions. Blank controls were likewise used to eliminate the possibility that our signals arose from laboratory and/or instrumental artefacts, as well as any contamination introduced from the buffers used to treat our samples during processing.

Different analytical approaches have been employed to assess proteinaceous matter in fossils, e.g. pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), which uses high temperatures to create pyrolysates that might indicate the presence of amino acid-carrying materials[20]. Nonetheless, this method has a comparably low spatial resolution (requiring ~50 mg samples for replicate analyses), and inherently decomposes any residual peptides/proteins, along with other constituents, into a mixture of thermally altered products. An additional concern, especially with kerogen-rich fossils, is that pyrolysis products formed from minute amounts of localised target compounds cannot be separated from the vast co-eluting aliphatic and aromatic moieties that typically comprise the bulk of organic matter. These hydrocarbons dominate even originally protein-rich remains, such as arthropod cuticle[21], and overwhelmingly contribute to pyrolysates, thus creating noise that effectively masks potential diagnostic peptide/protein signals.

Infrared microspectroscopy examines the structure of molecules, and has been found to reveal distinct spectral signatures from organic matter preserved in fossils[22,23]. These include peaks that could be associated with amide bands; however, traces of proteinaceous matter in samples dominated by other organic constituents can be hard to detect. Because amide bands overlap with intensities from many other functional groups, unequivocal identification of proteinaceous compounds is operose, and thus requires corroborating evidence from other methods.

Amino acid analysis (AAA) offers an alternative means of discriminating the presence or absence of particular amino acids. Nevertheless, this technique requires bulk sampling (which is not always possible with rare fossil specimens), uses hydrolysis to reduce peptides into their constituent amino acids, and does not localise these residues to discrete structures[24]. This results in significant information loss; therefore, AAA has limited potential to distinguish between endogenous and exogenous protein sources.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a surface analysis technique that localises molecular signals directly to discrete source structures[25]. ToF-SIMS applies harsh ionisation methods to induce strong fragmentation of proteinaceous compounds during analysis. As a consequence, protein spectra are largely dominated by low-mass fragment ions, some of which are characteristic of the various constituent amino acids. Fragment ions representing dipeptides, or longer amino acid chains, are typically absent (or present only at low signal intensities), and ions corresponding to intact proteins are not

observed. ToF-SIMS is thus unable to identify specific proteins in natural (complex) samples. However, the detection of protein fragment ions does demonstrate the presence of peptides/proteins on the examined surface, and the relative signal intensity distribution between these ions provides information about the amino acid composition.

Immunohistochemistry (IHC) has been applied in previous studies of dinosaur biomolecules, both from extracts[26,27] and in situ bone material[28,29]. IHC capitalises on the principle of antibody binding to specific antigens in tissues, and some antibodies can recognise target proteins from epitopes (antigenic determinants) that are composed of <10 amino acids (in their original conformation)[30,31]. The shortcomings of IHC include potential cross reactivity of antibodies with compounds other than the target protein, as well as induced contamination. It is therefore of utmost importance that assays are accompanied by extensive controls.

Of all mass spectrometric methods, liquid chromatography tandem-mass spectrometry (LC-MS/MS) is often considered the highest standard for protein characterisation from archaeological and palaeontological substrates. This is because it produces sequence data, thereby allowing identification of both the source protein(s) and any co-eluting contaminant biomolecule. Yet, LC-MS/MS is fraught with challenges when applied to multimillion-year-old fossils, as opposed to extant, or geologically very young tissues (which are still relatively protein-rich)[18,24,32]. Paramount is the need for a comparative protein database with which to characterise any experimentally detected peptides/proteins. These are matched against an array of known amino acid sequences, but gaps can create spurious results[33]. This is particularly pertinent for our study because ichthyosaurs are basally branching non-saurian diapsids[34,35], and thus phylogenetically distinct from all living reptiles against which to compare sequence data. This primary obstacle is further compounded by an array of other issues, including: (1) variability in extraction and sample preparation[18]; (2) the presence of co-eluting substances in fossil and soil samples that suppress ionisation (which is critical for detection by the mass spectrometer)[24,32]; (3) diagenetic alteration (e.g. the formation of advanced glycation end products), which is difficult to anticipate during bioinformatic processing of sequence data[36]; and (4) the extremely low abundance and concentration of endogenous proteinaceous matter in fossil tissues relative to exogenous contaminants, which can mask ancient peptides in concentration-dependent analyses[24].

Given these considerations, we specifically designed our analytical protocol for MH 432 to incorporate a novel multi-technique approach in order to optimise the detection and verification of any preserved biomolecular traces, and localise these directly to their source structures. Our results set a new analytical standard that we anticipate will underpin future palaeoproteomic investigations.

Immunohistochemistry (including immunogold labelling). Our manipulation of samples from MH 432 was conducted within a specifically dedicated laboratory facility, within which no extant organismal tissues have ever been handled or stored. All instruments, buffers and solutions were kept entirely separate from those used on our modern controls. An aseptic protocol was applied, and surgical gloves, gowns and face masks were worn at all times. Samples 7 and 8 were demineralised with EDTA (0.5 M, pH 8.0) for about 10 days; samples 12a and 13a were alternatively demineralised for about 4 weeks. Sample 10 was initially treated with 50% hydrogen fluoride, and then EDTA for 10 days. Samples 3, 12, 13, 13c, and 21 were first embedded in 3% agar (Becton Dickinson, Cat# 214530) to stabilise the tissue layers, and then demineralised with EDTA for about 4 weeks. Following these treatments, all tissue residues were washed with 1× phosphate buffered saline (PBS) and prepared for further embedding and sectioning.

Our segregated laboratory set-up designed for processing comparative extant cetacean and leatherback sea turtle tissues was used to sample: (1) short-beaked common dolphin (SAM

M26880) integument; (2) leatherback sea turtle (ZMUC-R2106) integument; (3) harbour porpoise (A2016/05526) integument that had been subjected to artificial maturation (150 °C, 200 bar for 96 h); and (4) leatherback sea turtle (ZMUC-R2106) integument that had been subjected to artificial maturation (150 °C, 200 bar for 96 h). All tissues were fixed for 1 h at room temperature in neutral buffered 10% formalin.

Demineralised (fossil) and fixed (extant) samples were dehydrated in 70% ethanol for 2 × 30 min, then infiltrated with a 2:1 mixture of LR White (EMS, Cat# 14383, hard grade) and 70% ethanol for 1 h. They were then incubated in two changes of undiluted LR White for 1 h each to achieve full penetration of the embedding medium. Infiltrated specimens were placed in gelatine capsules and covered completely with additional embedding medium before being allowed to polymerise at 60 °C for 24 h.

The embedded tissues were sectioned to 200 nm using a Leica EM UC6 Ultramicrotome. The sections were transferred to 6-well Teflon-coated slides and dried on a warming plate until fully adhered to the glass surface. Drying was then completed at 45 °C overnight. The sections were subsequently etched with proteinase K (PCR grade, Roche, 25 µg/ml) in 1× PBS at 37 °C, followed by two sequential incubations in 0.5 M EDTA for epitope retrieval. They were then incubated for 2 × 10 min in 1 mg/ml sodium borohydride ($NaBH_4$) to reduce autofluorescence. These incubations were separated by sequential washes (2 × 5 min) in PBS. Further incubation occurred for 2 h with 4% normal goat serum for rabbit primary antibodies, or 4% normal rabbit serum for goat anti-α-keratin in PBS. This was intended to occupy non-specific binding sites and prevent spurious binding of the primary or secondary antibodies. Specific primary antibodies were diluted to final concentrations (Table S1) in a primary dilution buffer (1% bovine serum albumin (BSA) (Fisher, BP1660-100), 0.1% cold fish skin gelatine (Sigma G7765), 0.05% sodium azide (Sigma S-8032), 0.01 M PBS, pH 7.2), which was applied to the sectioned tissues and allowed to incubate overnight at 4 °C. To further control for spurious binding of the secondary antibody, our sections were also incubated in antibody dilution buffer at identical concentrations, but without primary antibodies. After incubation, all sections were thoroughly washed to remove unbound antibodies, and then incubated with a biotinylated secondary antibody, which was diluted to different specifications: Biotinylated Goat Anti-Rabbit IgG (H+L) (Vector BA-1000) diluted 1:500 for primary antibodies raised in rabbit; Biotinylated Rabbit Anti-Sheep IgG (H+L) (Vector BA-6000) diluted 1:500 for polyclonal goat anti-α-keratin; Biotinylated Goat Anti-Mouse IgG (H+L) (Vector BA-9200) diluted 1:500 for monoclonal anti-peptidoglycan antibodies raised in mouse for 2 h at room temperature. After thorough washing, fluorescein-avidin D (FITC, Vector Laboratories, Cat# A-2001) diluted 1:1000 was applied and allowed to bind for 1 h at room temperature. All incubations were separated by sequential washes (2 × 10 min) in PBS w/Tween 20 (ACROS Organics) followed by 2 × 10 min rinses in PBS. Finally, sectioned tissues were mounted with Vectashield™ anti-fade mounting medium (Vector H-1000) and protected by coverslips. Microscopy examinations were undertaken on a Zeiss Axioskop 2 Plus biological microscope, with images captured using an AxioCam MRc 5 (Zeiss) with 10× ocular magnification, and the Axiovision v. 4.7.0.0 data processing software package.

Both the fossil remains and our comparative extant cetacean and leatherback sea turtle tissues (embedded as described above) were subjected to immunogold staining to validate the immunofluorescence results, and to demonstrate antibody-antigen complexes on tissues at higher resolution. The embedded samples were sectioned to 90 nm using a Leica EM UC6 Ultramicrotome, collected on carbon-coated nickel grids (EMS, Cat# CFT200-NI), and then rinsed for 10 min by placing the grids on large droplets of PBS-Tween20. Incubation was undertaken with 4% normal donkey serum in PBS for 1 h at room temperature to occupy non-specific binding sites and prevent spurious binding. Following this blocking step, the grids were incubated in primary antibody (Polyclonal Goat X-chicken α-keratin diluted 1:10 in primary

dilution buffer) for 3 h at room temperature. They were then washed by being placed on large droplets of PBS-Tween20 for 10 × 2 min to remove unbound antibodies. Further incubation with secondary antibody (18 nm Colloidal Gold AffiniPure Donkey Anti-Goat IgG (H+L) 1:10 (Jackson Immuno Research Inc., Cat# 705-215-147)) was undertaken for 1 h, prior to rinsing with PBS-Tween20 for 10 × 2 min, and E-pure water for 3 × 30 sec, before drying with filter paper. Final staining used 5% methanolic uranyl acetate followed by Reynold's lead citrate. Microscopy examinations utilised an Aberration Corrected STEM–FEI Titan 80-300 electron microscope in the Analytical Instrumentation Facility at North Carolina State University.

Table S1. Primary antibodies with dilutions.

Antigen	Antigen species	Antibody type	Antibody host	Final dilution	Source
α-keratin	chicken (*Gallus domesticus*)	polyclonal	goat	1:75	G-30, courtesy of R. Sawyer
Elastin	bovine (*Bos taurus*)	polyclonal	rabbit	1:75	courtesy of R. Mecham
Haemoglobin	alligator (*Alligator mississippiensis*)	polyclonal	rabbit	1:75	Biosynthesis BSYN6941
Haemoglobin	ostrich (*Struthio camelus*)	polyclonal	rabbit	1:75	GenScript 70594-1
β-keratin	chicken (*Gallus domesticus*)	polyclonal	rabbit	1:100	Biosynthesis BSYN6734
Skin collagen	alligator (*Alligator mississippiensis*)	polyclonal	rabbit	1:75	Biosynthesis BSYN6959
Tropomyosin	chicken (*Gallus domesticus*)	polyclonal	rabbit	1:50	Abcam ab11190
Actin	chicken (*Gallus domesticus*)	polyclonal	rabbit	1:75	Capralogics Inc. P00851
Collagen	chicken (*Gallus domesticus*)	polyclonal	rabbit	1:75	USBiological C7510-13B
Peptidoglycan	*Streptococcus mutans* BHT	monoclonal	mouse	1:75	ABD Serotec 7263-1006

Infrared microspectroscopy. The intensity map of Sample 13a (Fig. 5j) was generated from a Focal Plane Array (FPA) measurement using a 64 × 64 pixel FPA detector. 128 individual scans at 4 cm^{-1} resolution were co-added to achieve a good signal-to-noise ratio. The intensity was integrated over the 2,805–3,016 cm^{-1} region, representing ν(CH) stretch absorptions.

Vibrational spectra acquired from the blubber (Sample 13a), epidermis/dermis (Sample 13a) and liver (Sample 1) showed contributions from both aliphatic and aromatic functional groups (Fig. 5k), including strong signal from $\nu_s(CH_2)$ at 2,853 cm^{-1} and $\nu_{as}(CH_2)$ at 2,924 cm^{-1} (originating from aliphatics) and a weak double-featured peak at ~3,062 and ~3,070 cm^{-1} (assigned to ν(CH) stretch modes of aromatics)[22,37,38]. The fraction of aromatics relative to aliphatics was estimated from the $I{:}\nu(CH_{arom})/I{:}\nu(CH_{aliph})$ ratio[37]. For $I{:}\nu(CH_{arom})$, the region between 3,040 and 3,082 cm^{-1} was integrated, whereas for $I{:}\nu(CH_{aliph})$, the region between 2,750 and 3,040 cm^{-1} was used. A ratio of 0.0010–0.0015 was obtained for the three samples, indicating an aromatic carbon fraction of less than 50%[37]. In addition, deconvolution of the 2,750–3,040 cm^{-1} region was employed to estimate the average length of the aliphatic chains[39]. This was achieved by calculating the ratio of the integrated intensities from the ethyl and methyl groups, $I{:}\nu_{as}(CH_2)/I{:}\nu(CH_3)$, thereby providing a qualitative comparison between the samples[39]. Our data showed that the hydrocarbon chains are distinctly shorter in the blubber ($I{:}\nu_{as}(CH_2)/I{:}\nu(CH_3)$ ~2) relative to those from the epidermis/dermis and liver (the latter producing ratios >10). Finally, based on the narrow line widths in the aliphatic ν(CH) stretch region, we concluded that the blubber has a more homogeneous composition than the two other samples. The intensities of the spectra were normalised with regard to the combined $\delta_{as}(CH_3) + \delta_s(CH_2)$ modes at 1,456 cm^{-1}.

The spectrum of the melanophore in Sample 13 (Extended Data Fig. 3g) was extracted from a FPA map recorded at 4 cm^{-1} resolution using an average of 32 individual scans in order to obtain an adequate signal-to-noise ratio.

Time-of-flight secondary ion mass spectrometry. ToF-SIMS was primarily used to characterise the organic remains of MH 432, with particular emphasis on proteinaceous materials, hydrocarbons, eumelanin, porphyrins, and hopanes/steranes. Nevertheless, inorganic

structures, including calcium phosphate, calcium carbonate and aluminium silicate, were also identified. Proteinaceous remnants were recognised by a number of nitrogen-containing positive fragment ions that are well known to produce strong signal in modern proteins[40,41]. Aliphatic and polyaromatic compounds were likewise detected by various hydrocarbon ions ($C_xH_y^+$, where x<y and x>y denote aliphatic and aromatic ions, respectively), which are typical of these polymers[42]. Eumelanin was distinguished in negative ion mode using the characteristic intensity distribution of fragment ions in the *m/z* 45–175 range[43]. Porphyrins (especially heme) can be similarly identified via their distinctive undulating pattern at *m/z* 450–500 in positive ion mode, and the occurrence of specific iron-containing low mass fragment ions in negative ion mode[19,44]. Hopanes and steranes might also be indicated by diagnostic peaks at *m/z* 191 and 217 in positive ion mode[45], but none of these compounds were discernible in our samples from MH 432.

In order to investigate contributions from the sedimentary matrix, the spatial distribution of aluminium silicates was determined in all samples. In addition, calcium phosphate was identified from $Ca_xPO_y^+$ ions[46]. The close spatial overlap between these ions (Fig. 5l) and negative phosphate ions (Fig. 5m) demonstrates that the latter derive primarily from calcium phosphate (Extended Data Fig. 5c).

Analysis of the thin section (Sample 13a) prepared for TEM (Fig. 5l–n and Extended Data Fig. 5c) required special attention because of the epoxy resin embedding medium, which produced abundant organic signal and thus could potentially be mistaken for ancient matter. Fortunately, it was possible to identify epoxy-specific peaks, including Cl^-, $C_2H_3O_2^-$, $C_3H_3O_2^-$, and $C_3H_8N^+$. Images of these ions showed that the resin was confined to areas outside of the fossil material, demonstrating that no significant infiltration, smearing or spreading of the epoxy had occurred during the preparation process. A low, but noticeable signal of polydimethylsiloxane (PDMS) – a commonly transferred laboratory contaminant – was also observed. However, because PDMS is spectroscopically well characterised[47], the presence of this compound could be carefully considered when evaluating the data collected from Sample 13a. Ion images of PDMS-characteristic peaks further showed a homogeneous distribution across the sample surface, indicating that the contrast between ion images from the molecular components of the fossil, sediment and epoxy was genuine.

Finally, the '*Stenopterygius* melanosomes' spectrum presented in Fig. 3s was obtained from a region with abundant pigment organelles located within the 'Mixed layer' of Sample 13a (Extended Data Fig. 10d—arrows). These data were collected with the instrument optimised for high image resolution, and with peak intensities acquired after nominal mass binning. The synthetic eumelanin reference spectrum was taken from ref. 48.

Alkaline hydrogen peroxide oxidation (AHPO). Quantification of eumelanin markers produced by AHPO yielded low but detectable amounts of pyrrole-2,3,5-tricarboxylic acid (PTCA), pyrrole-2,3-dicarboxylic acid (PDCA) and pyrrole-2,3,4,5-tetracarboxylic acid (PTeCA) in the skin, liver and sediment samples obtained from MH 432 (Fig. 3r and Extended Data Fig. 3f). The highest concentration of PTeCA was found in the liver, with successively lower levels in the skin (~1/2 of that in the liver) and sediment (~1/6 of that in the liver). The PTeCA/PTCA ratio was ~3 for all samples investigated – an increase relative to that of extant *Sepia officinalis* eumelanin – which is consistent with both previously analysed fossil eumelanins[49] and artificially maturated synthetic eumelanins[50]. The presence of trace amounts of eumelanin in the surrounding sediment likely pertains to post mortem leakage. However, the relatively high concentration of melanic pigments in the liver residue most probably reflects its original condition. Appreciable quantities of melanins occur in various tissues and internal organs of extant vertebrates[11,13,51]. In particular, the liver is a rich melanin source[11,13,51].

Amino acid analysis. Hydrolysis and subsequent liquid chromatography-mass spectrometric analysis of samples from the integument (Sample 13d), liver (Sample 7a) and surrounding sedimentary matrix (Sample 14a) revealed the presence of multiple amino acids (Extended Data Fig. 6 and Table S2). The highest concentration was found in the liver (3.813 nmol/mg), with successively lower levels in the integument (2.775 nmol/mg) and sediment (0.397 nmol/mg). Note that asparagine and glutamine readily hydrolyse into aspartic acid and glutamic acid, respectively. On the other hand, cysteine is initially oxidised into cystine, and then cysteic acid[52]. Tryptophan is also degraded when heated in strong acids; these data were therefore omitted from Table S2. In addition, aspartic acid and methionine are susceptible to heat and oxidation[53,54], whereas tyrosine is reactive and can be modified into several other compounds[52]. This might account for the apparent absence of these amino acids in our samples.

Table S2. Amino acid composition of MH 432 (nmol/mg). *not detected

Amino acid	Liver	Integument	Sediment
Alanine	0.448	0.307	0.035
Arginine	0.106	0.055	0.032
Aspartic acid	*	*	*
Glutamic acid	1.191	0.470	0.000
Glycine	0.433	0.500	0.110
Histidine	0.000	0.069	0.000
Isoleucine	0.116	0.044	0.038
Leucine	0.128	0.028	0.058
Lysine	0.056	0.021	0.000
Methionine	*	*	*
Phenylalanine	0.020	0.009	0.021
Proline	0.361	0.147	0.039
Serine	0.393	0.672	0.000
Threonine	0.329	0.309	0.000
Tyrosine	*	*	*
Valine	0.232	0.144	0.064
Total	**3.813**	**2.775**	**0.397**

Part H. Taphonomic Scenario

Judging from the fidelity of the preserved soft parts, the ichthyosaur carcass that would eventually become MH 432 must have arrived intact on the seafloor with its ventral side facing downwards. However, the positioning of the articulated limbs (erect on the left side and parallel to the bedding plane on the right) indicates that the impact occurred at an oblique angle, causing the left-hand extremities to penetrate into the bottom substrate. The viscous consistency of the sediment allowed the cadaver to sink some distance into the mud, and consequently the partly buried animal came to rest belly down, albeit while tilting somewhat towards its left-hand side. As the flesh on the exposed portion of the body decomposed, recalcitrant compounds, such as eumelanin, together with purge fluids and other decay products generated by autolysis, permeated the underlying tissues and surrounding sediments to form a nutrient-rich benthic island, similar to modern whale falls (refs 55, 56; but see also ref. 4). Adipocere (mortuary wax) appears to have developed at an early stage from saponification of released lipids[57,58], and is now evident as irregular patches of amorphous phosphatic matter that cover the external surface of the skin in the abdominal region (Extended Data Fig. 1b—arrows). Rupturing of the exposed body wall enabled decompositional fluids to escape, but also saturated mud to enter the abdominal cavity (Extended Data Fig. 10a–c), thereby shielding the liver and ventral integument from potential scavengers and oxygen-dependant degradation processes. Further weakening or breakdown of tissues resulted in the gravitational collapse of the defleshed backbone, which in turn caused the dorsal ribs to fall forward, and vertebral centra to depart

from their original life positions and drop onto the floor of the body cavity (Fig. 1). The carcass was then completely covered by clay-rich deposits (Extended Data Fig. 10a), and diagenetic compaction subsequently reduced the initially three-dimensional cadaver into a flattened skeleton coated on the underside by vestiges of the integument.

Because the soft-tissues of MH 432 derive exclusively from the lower half of the animal, it is conceivable that early entombment in oxygen-deficient muddy sediments promoted preservation by fixation and/or impediment of organic deterioration[59–62]. This physical barrier also provided environmental isolation, allowing the build-up of chemical gradients necessary for authigenic mineralisation[63–66]. As demonstrated by previous studies[63–66], precipitation of calcium phosphate is controlled by a multitude of concurrent factors, including ocean and sediment chemistry, the degree of degradation, and the nature of the organic substrate, as well as its associated microbiome. Low decay rates and an elevated ambient phosphate concentration influenced by microbial activity are the most feasible drivers for the rapid post mortem mineralisation evidenced by MH 432[64]. Some phosphorous may have been released from the decomposing carcass. However, given the extent of mineral formation, it is tenable that the bulk of the material originated from an externally derived supersaturated solution[64]. Nevertheless, phosphate and/or calcium ions occurring naturally within some tissues and organs (or biomolecules, such as collagen and phospholipids, which possess an inherent ability to bind these inorganics)[67–69] could have served as nucleation sites and/or templates for dissolved environmental phosphorous, inducing precipitation in the cadaver. Local differences in decay rate and/or phosphate availability produced variability in the integumental mineralisation. In some areas, only the epidermis was replicated in calcium phosphate, whereas in others, extensive in situ growth of phosphate nano-crystallites took place. Likewise, certain resistant components of the liver apparently survived degradation long enough to become stabilised and partially replaced by calcium phosphate (presumably because of the relatively high levels of eumelanin and haemoglobin[13,19,70]; Fig. 3r and Extended Data Figs 3f, 8e–h). Notably though, our Py-GC/MS investigation indicated differences in the organic inventory of the liver residue relative to the integument (including a more aromatic content; Extended Data Fig. 4a). These features could plausibly be explained by relatively more advanced biodegradation; however, they might also, at least partially, reflect original compositional differences between the ichthyosaur tissues.

Invasion of the soft-tissues of MH 432 by phosphate-bearing fluids engulfed the remaining organics, thereby protecting them from further microbial degradation[71]. The entrapped carbonaceous material then gradually transformed from its original chemical composition to one dominated by aliphatic and aromatic biopolymers (a process likely facilitated by diagenetic incorporation of sulfur into the geomacromolecules[72,73]; Fig. 5m and Extended Data Fig. 5c). This in situ polymerisation is thought to involve mainly long-chained lipids, such as fatty acids[65,74–79]. Nevertheless, more labile constituents, including proteinaceous moieties, have also been found to survive across geological time via encapsulation within hydrophobic organic matrices[73,80–83]. Complexation with hydrocarbon networks might therefore account for the broad range of epitope/proteinaceous signals retrieved from MH 432 (Figs 4a, e–n, 5g, h and Extended Data Figs 5a, b, 6, 7a–f, 8e–h).

Replacement by nano-crystalline calcium phosphate is evident on inner surface of the integument (which originally faced the water column; Extended Data Fig. 10a, c), where it preferentially preserved connective tissues, such as the reticular dermis and superficial fascia (Fig. 5a, c, d, l, m). This direct style of phosphatisation[64] was followed by calcium carbonate and silicon oxide precipitation (Fig. 2h—arrowheads and Extended Data Fig. 2g), indicating that a sequential shift in the local geochemical conditions took place during the eogenesis of MH 432[63]. While collagenous structures acted as mineral nucleation sites, the antibacterial properties[84] of fatty acids emancipated from the blubber likely retarded the degradation of this

fibro-adipose tissue long enough to permit the onset of polymerisation and polycondensation reactions (Fig. 5i–n). Owing to compaction effects of even slight sediment overburden, the blubber was then compressed (much like our artificially matured porpoise integument; Extended Data Fig. 9e–j), leading to an outward and downward migration of more volatile breakdown products[85] (compare Extended Data Fig. 9n–q). However, the remaining, less mobile organic fraction was polymerised to the point that virtually all microscopic features were obliterated, resulting in the formation of a dense, glossy black layer beneath the cutis (Fig. 5a).

Given that the skin of MH 432 was progressively mineralised from the exterior surface and inwards[64], a zone of weakness was established between the phosphatisation front and underlying polymerised material (corresponding topographically to the loose 'superficial blubber' recovered with our autoclave-treated porpoise integument; Extended Data Fig. 9j). This would have facilitated the entry of various exogenous substances, such as clay and microbes (Extended Data Fig. 10d–h). Microbial activity then reduced the non-mineralised portion of the integument, leaving behind constituents that could not easily be metabolised, including the potentially toxic[86] melanosomes (Extended Data Fig. 10d–f). As a consequence, these were concentrated into discrete layers (Extended Data Fig. 10d–f), along with various protein decay products (Fig. 4e, f), before additional phosphate deposition enclosed the system. The integument of MH 432 thus provides insights into the process leading to a more common type of soft-tissue preservation; that is, as a dense mat of aggregated remnant melanosomes[48,87].

Part I. Endo- and Exogenous Microfeatures in Ichthyosaur Soft-Tissue Remains

Our integrated ultrastructural and biogeochemical investigation, together with other recent published work[48], rigorously identify microbodies previously interpreted as either 'fossilised rod-shaped bacteria'[88] or 'lithified bacteria'[89] as remnant melanosomes (melanin-harbouring cellular organelles). Other microstructures described as 'filamentous prokaryote organisms'[88,90] might alternatively represent vestiges of connective tissue fibres. However, because explaining these structures from earlier published accounts is inherently equivocal, we cannot rule out the possibility that fungi (or some other capillaceous microorganism) could have infested the carbonised ichthyosaur remains illustrated in refs 88, 90. Comprehensive reassessment using multidisciplinary experimental approaches, such as those applied herein, will therefore be necessary to determine any putative microbial contribution to these soft-tissue fossils.

With regard to MH 432, virtually every histological, structural and molecular feature identified by our study is endogenous to this ichthyosaur, including the cholesterol derivatives, protein residues and eumelanic pigment remains. In extant reptile skin, cholesterol participates in the formation and maintenance of the transcutaneous permeability barrier[91], and it is therefore primarily localised with the stratum corneum (notably, this sterol forms a significant component of the hydrophobic lipid matrix that constitutes the intracellular vacuoles and extracellular lamellar membranes[67,91] – features also present in MH 432; Fig. 2k, n and Extended Data Fig. 2b). On the other hand, integumental proteins display a layered vertical distribution, with keratins localised to epidermal structures, while cytoskeletal components (e.g. tropomyosin and actin), haemoglobin and structural proteins (e.g. elastin and collagen) are dispersed throughout the deeper strata[92,93]. Nevertheless, degradation and compaction can cause mixing and spatial overlap of protein-derived breakdown products, which we demonstrate experimentally via our autoclave-treated leatherback sea turtle and porpoise integuments (Extended Data Figs 7i–r, 9n–q).

In addition to genuine animal tissues, small amounts of hopanoids (biomarkers typical of bacteria) were identified in pyrolysis products acquired from the skin of MH 432. Notably, however, their abundance was relatively uniform between the ichthyosaur fossil and surrounding sediment, including the 'remote host rock'. Presumed microbial structures were

observed in regions of the integument characterised by intruding clay minerals and dense melanosome accumulations (Extended Data Fig. 10d–h, j). Significantly, these were proportionately 7–15 times larger than the associated pigment organelles (Extended Data Fig. 10e, g), and rather than possessing a dark, virtually homogenous interior when visualised under TEM (a defining feature of mature melanosomes[94]), were represented exclusively by isolated cellular walls that formed sub-circular to oval microbodies with electron-translucent cores (Extended Data Fig. 10e, g, h).

Part J. References Cited in the Supplementary Information

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