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Organic preservation of fossil musculature with ultracellular detail

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The very labile (decay-prone), non-biomineralized, tissues of organisms are rarely fossilized. Occurrences thereof are invaluable supplements to a body fossil record dominated by biomineralized tissues, which alone are extremely unrepresentative of diversity in modern and ancient ecosystems. Fossil examples of extremely labile tissues (e.g. muscle) that exhibit a high degree of morphological fidelity are almost invariably replicated by inorganic compounds such as calcium phosphate. There is no consensus as to whether such tissues can be preserved with similar morphological fidelity as organic remains, except when enclosed inside amber. Here, we report fossilized musculature from an approximately 18 Myr old salamander from lacustrine sediments of Ribesalbes, Spain. The muscle is preserved organically, in three dimensions, and with the highest fidelity of morphological preservation yet documented from the fossil record. Preserved ultrastructural details include myofilaments, endomysium, layering within the sarcolemma, and endomysial circulatory vessels infilled with blood. Slight differences between the fossil tissues and their counterparts in extant amphibians reflect limited degradation during fossilization. Our results provide unequivocal evidence that high-fidelity organic preservation of extremely labile tissues is not only feasible, but likely to be common. This is supported by the discovery of similarly preserved tissues in the Eocene Grube Messel biota.

Keywords: exceptional faunas; taphonomy; muscle; organic preservation; biomolecules

1. INTRODUCTION

The non-biomineralized, decay prone, tissues of organisms are preserved in the fossil record either as organic remains or via replication in authigenic minerals (Allison & Briggs 1991; Briggs 2003). Recalcitrant (decay resistant) non-biomineralized tissues, such as cuticles, are often preserved as organic remains, and, occasionally, retain some of their original biomolecules (Stankiewicz *et al.* 1997; Briggs 1999). However, almost all unequivocal examples of more labile (decay prone) metazoan tissues, e.g. musculature, are preserved in authigenic minerals; these replicate tissue structure with varying degrees of fidelity (depending on the mineral phase and the timing of mineralization relative to decay). Organic preservation of tissues inside amber (Poinar & Hess 1982; Grimaldi *et al.* 1994) can result in retention of cellular ultrastructures (Henwood 1992), but is rare and reflects a suite of extremely unusual circumstances. Charcoalification can preserve labile tissues, but has, to date, been recorded only in fossil plants (Crepet *et al.* 1992; Edwards & Axe 2004). Debate continues over the significance of the organically preserved structures recovered by Schweitzer *et al.* (2005) after acid dissolution of samples of dinosaur bone. These structures were interpreted first as blood vessels and osteocytes largely on the basis of their general morphological similarity to examples of these in the extant ostrich. The fossil structures, however, have been reinterpreted as the remains of recent biofilms that lined, but did not infill completely, voids inside the bone, thus generating hollow structures with a similar geometry to blood vessels and osteocytes (Kaye *et al.* 2008). Further, the robustness of the original biochemical analyses that purported to identify collagen peptide sequence fragments in the fossil structures (Asara *et al.* 2007; Schweitzer *et al.* 2007) has been much debated (Asara & Schweitzer 2008; Asara *et al.* 2008; Buckley *et al.* 2008; Organ *et al.* 2008; Pevzner *et al.* 2008; see also Schweitzer *et al.* 2009). Suggestions that labile biomolecules are likely to survive over geological time scales when organisms are encapsulated within natural resins (Poinar *et al.* 1996) have also been questioned (Stankiewicz *et al.* 1998). Sulphur-rich organic remains recovered from inside the bones of fossil frogs hosted within approximately 10 Myr old (Miocene) lacustrine sediments that crop out near Libros, northeast Spain, were interpreted as the first (and to date only) examples of fossilized bone marrow (McNamara *et al.* 2006). Others, however, have been more equivocal in their interpretation of these structures

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(Stokstad 2006). There is therefore no consensus as to whether the preservation of very labile non-biomineralized tissues as organic remains is possible other than via encapsulation within natural resins (amber, copal). Confirming this is a prerequisite to any investigation of the processes responsible, including any systematic analysis of the fate of the original biomolecules under different diagenetic conditions. Herein, we describe, to our knowledge, the first record of organically preserved musculature including its sedimentological context. The muscle's gross morphology resembles that of an extant analogue, but this, alone, is not the basis for our conclusion. Remarkably, despite some degradation before fossilization, diagnostic macromolecular ultrastructural features have been retained.

2. MATERIAL AND METHODS

The fossilized musculature is from the trunk of a specimen (Museo Nacional de Ciencias Naturales, Madrid (MNCN) 12555) of the salamander *Chelotriton* sp. from the Lower- to Middle Miocene (Agustí *et al.* 1988) Ribesalbes Lagerstätte, near Castellón de la Plana, northeast Spain. This deposit, hosted within thermally immature, sulphur-rich oil shales (kerogen Type I-S: atomic $S_{org}/C > 0.04$; atomic $H/C > 1.5$; Sinninghe Damsté *et al.* 1993), yields abundant exceptionally preserved plants and insects. Rare amphibians are preserved as articulated skeletons enclosed in a thin, black carbonaceous layer that defines the outline of the soft tissues (figure 1*a*). Specimen 12555 was recovered during commercial mining of the oil shales at the beginning of the twentieth century and does not appear to have been treated or prepared prior to our analysis.

(a) *Fossilized muscle tissue*

Samples of muscle tissue identified under a binocular microscope were picked from the specimen using sterile scalpels and needles. For scanning electron microscopy (SEM), samples were not prepared further; they were mounted onto aluminium stubs using double-sided carbon tape, gold or carbon sputter-coated, and examined with a Hitachi S-3500N variable pressure microscope equipped with an EDAX Genesis energy dispersive spectrometer. Sample conductivity was enhanced by applying small quantities of silver dag to carbon-coated samples. Backscattered- and secondary electron images were produced from carbon-coated samples, and secondary electron images from gold-coated samples. Observations were made at an accelerating voltage of 15 kV.

For transmission electron microscopy (TEM), samples were impregnated with TAAB EM resin in an aluminium mould under vacuum in the following resin/ethanol mixtures, each for 2 h: 10, 30, 50, 70, 90, 100, 100, 100 per cent resin. Ultrathin (80–90 nm thick) microtome sections were cut with a Drukker 2 mm 45° diamond knife, picked up on Cu grids and allowed to dry. Selected sections were stained with 2 per cent uranyl acetate in water for 20 min, washed with distilled water and further stained with lead citrate (0.01 g lead citrate in 10 ml water and 0.1 ml 10 M NaOH) for 10 min. Grids were washed again with distilled water, allowed to dry and examined using a JEOL 2000TEMSCAN operating at 80 kV with an objective aperture of 10 µm diameter. Differences in tone (electron contrast) in transmission electron micrographs indicate relative differences in atomic number between features: the darker the feature, the higher the atomic number of its components. Contrast is calibrated automatically by the camera detector and thus subtle differences in electron contrast between features may not be apparent if an extremely electron-dense feature is in the field of view.

(b) *Modern muscle tissue*

Muscle tissue from the extant salamander *Ambystoma mexicanum* supplied in 100 per cent ethanol (after primary fixation in gluataraldehyde) was treated as follows: immersion in osmium tetroxide (60 min), buffer (a mixture of sodium dihydrogen phosphate and disodium hydrogen phosphate) (10 min), 70 per cent ethanol (10 min (twice)), 90 per cent ethanol (10 min (twice)) and 100 per cent ethanol (20 min (twice)). For TEM, fixed and dehydrated samples were prepared further as follows: immersion in propylene oxide (15 min (twice)), 50 : 50 propylene oxide: TAAB EM (60 min) and 100 per cent resin (120 min at 37°C). Resin impregnation was conducted under a vacuum of 86–90 kPa using a Buehler Vacuum Impregnation Unit. Samples were then placed in an aluminium mould with fresh 100 per cent resin and polymerized under vacuum for 18 h at 60°C. Ultrathin (80–90 nm thick) sections were placed on Cu grids, stained with uranyl acetate and lead citrate and examined as for the fossil material. SEM samples were prepared and analysed as for the fossil material.

3. FOSSIL MUSCLE

Hypaxial skeletal muscle fibres are preserved immediately adjacent to the thoracic vertebrae (figure 1*b*,*c*). As in modern salamanders (Brainerd & Azizi 2005), the fibres are closely and regularly spaced, parallel and stacked in sheets separated by endomysium (figure 1*d*–*g*). Hollow tubes with circa 3 µm thick walls orientated orthogonally to the axis of some fibres (figure 1*d*, arrow) represent sections through vascular structures, probably arterioles and venules. Each fibre is straight, unbranched, 15–25 µm wide and exhibits a chevron-shaped pattern of elongate cracks, one set on either side of the medial axis (figure 1*d*,*e*). In transverse section, each fibre is square to rectangular in outline, has concave sides and an irregular, circular to oval-shaped, central void (figure 1*f*). These voids, and the cracks, are attributed to diagenetic shrinkage of the fibres, probably via condensation of the cytosol. In stained transverse TEM sections, myofilaments are apparent as two sets of densely packed, electron-dense, 'spots' (figure 2*a*): spots of one set are larger (10–14 nm diameter as opposed to 5–8 nm diameter) and more electron dense. The spots are sections through thick (myosin-rich) and thin (actin-rich) myofilaments; the presence of both indicates the plane of sectioning has passed through the A-band of a fibre. The structure in the fossil material is slightly less ordered than the regular hexagonal arrangement found in its modern counterpart (compare figure 2*a* and *b*); the loss of fidelity probably originated during initial, limited, decay before fossilization. Myofibrils are not apparent in the fossil material, even in stained sections, but this is not unexpected. In many extant amphibians, the trunk muscles are tonic, and the myofilaments are weakly or not bundled into myofibrils (Gans 1981). The sarcolemma is typically 800–1200 nm thick, slightly greater than in extant salamanders (650–850 nm thick) (compare figure 2*c*,*e* with 2*d*,*f*). This difference may be

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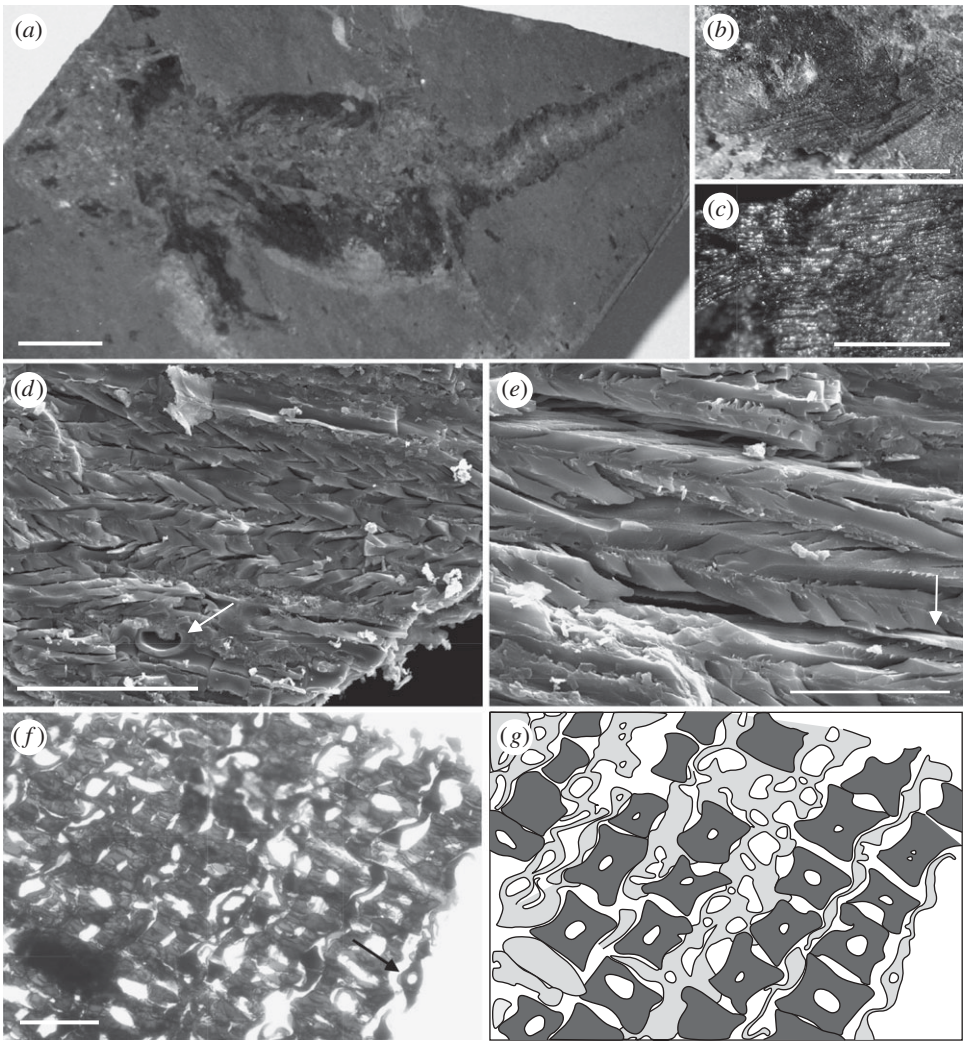

Figure 1. Organically preserved musculature in a fossil salamander. (*a*) *Chelotriton* sp. (MNCN 12555) with an outline of soft tissues defined by a carbonaceous layer. Scale bar, 10 mm. (*b*) Musculature *in situ* adjacent to vertebrae and (*c*) a freshly fractured surface through a sample. Scale bar, 1 mm. (*d*, arrow) SEM images of muscle illustrating chevron pattern of cracks, hollow blood vessel in transverse section and (*e*, arrow) separation of sarcolemma from sarcoplasm. Scale bars: (*d*) 100 μm and (*e*) 30 μm. (*f*) Semithin transverse section through the muscle with (*g*) explanatory drawing, showing the arrangement of fibres (dark grey) in parallel sheets separated by endomysium (light grey); arrow in (*f*), blood vessel in the transverse section. (*f*) Scale bar, 20 μm.

real; alternatively, the thickness of the sarcolemma in the fossil muscle may have increased during minor post-mortem separation of collagen fibres (Taylor *et al.* 1995). Internally, the sarcolemma can be either structureless with a very thin, relatively electron-dense, margin (figure 2*c*), or multi-layered (figure 2*e*); both conditions occur in extant material (figure 2*d*,*f*). The multi-layered structure is more apparent where the sarcolemma has detached from the interior of the fibre and is convoluted; in such examples, the sarcolemma often separates further into a series of layers (figure 2*e*), a characteristic of the initial stages of its decay (Taylor *et al.* 1995).

Adjacent sheets of muscle fibres are separated by a thin (2–6 μm thick) endomysium. The endomysium is often laminated and can separate into its component layers (figure 2*g*). Endomysial circulatory vessels (4–10 μm in diameter and circular in cross section) (figure 2*g*) can be filled with a friable, highly electron-dense, material (figure 2*g*, inset). This infill is extremely unstable under the electron beam and usually disintegrates during examination, producing resin-free voids. The infill occurs only within these circulatory vessels, not other void spaces, and has been observed in unstained sections. Collectively, these observations confirm the infill is of biological origin, not a diagenetic infill of void space or an artefact of sample preparation; circulatory vessels in modern salamander muscle are infilled with blood (figure 2*h*). The fossil blood is structureless, and there is no evidence for spherical features, e.g. red blood cells (most purported fossil examples of which have been demonstrated to be pyrite framboids, Martill & Unwin 1997). The composition of the fossil blood is unknown; its high electron density indicates that it is unlikely to be entirely carbonaceous. EDX spectra of the musculature exhibit peaks for only C and S. The musculature is therefore not replicated in authigenic minerals. It is, at least primarily, the diagenetically altered remains of the original organic tissues.

4. WIDER IMPLICATIONS

The detail revealed by TEM imaging unequivocally identifies the organic remains as fossilized musculature from

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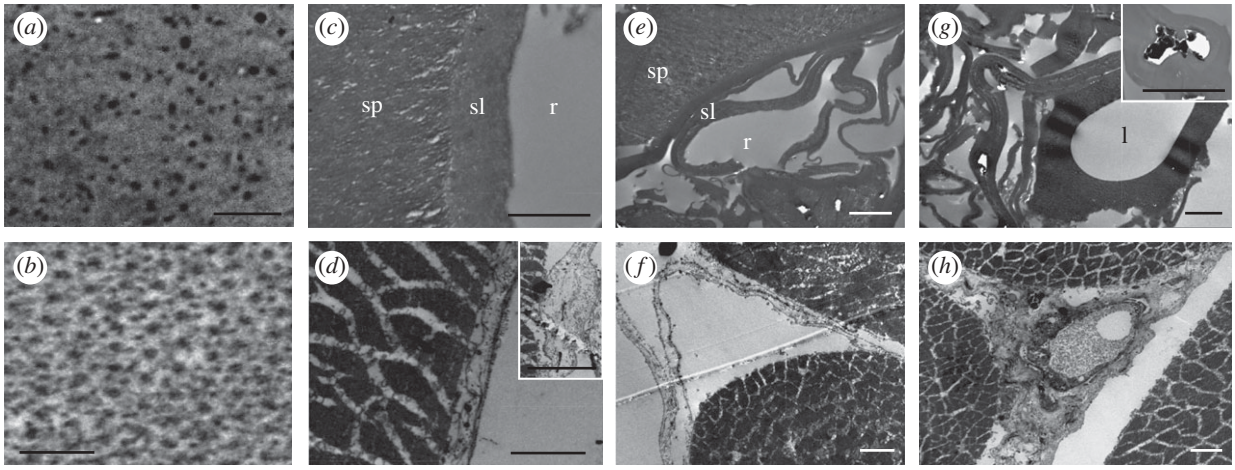

Figure 2. (*a*,*c*,*e*,*g*) TEM images of transverse sections of skeletal muscle tissue from MNCN 12555 and (*b*,*d*,*f*,*h*) corresponding features in the extant salamander *A. mexicanum*. Images except (*c*), (*e*) and (*g*) are from stained sections. (*a*,*b*) Sarcoplasm showing myofilaments in the transverse section at A-band. (*c*,*d*) Margin of fibre showing sarcolemma (sl) attached to sarcoplasm (sp); inset in (*d*) shows local thickening of sarcolemma. (*e*, *f*) Margin of fibres showing sarcolemma (sl) detached from sarcoplasm (sp). (*g*,*h*) Endomysial circulatory vessels surrounded by endomysium. Inset in (*g*) shows electron-dense, friable, solidified blood residue within the circulatory vessel. l, lumen of capillary; r, resin. Scale bars (*a*,*b*) 50 nm and 2 μm for all other images.

the salamander itself. This therefore confirms, for the first time, to our knowledge, that the high-fidelity fossilization of extremely decay-prone tissues as organic remains is not only feasible but can occur in the absence of protective encapsulating agents such as bone (in the case of the bone marrow, McNamara *et al.* 2006) and amber.

Preservation of the musculature as a sulphur-rich organic residue is attributed to sulphurization of organic molecules within the tissue (McNamara *et al.* 2006). Sulphurization of organic matter is a common diagenetic phenomenon and has been documented in various carbonate, evaporite and siliceous ooze-dominated, marine, and non-marine environments (Killops & Killops 2005); this includes environmental contexts known to host exceptional faunas (Sinninghe Damsté *et al.* 1993; de las Heras *et al.* 2003). Further, this mode of exceptional preservation, articulated skeletons encased in a carbonaceous layer that defines the body outline, characterizes other Mesozoic and, especially, Cenozoic lacustrine-hosted biotas from Europe and elsewhere; examples include the Messel (Eocene, Germany) (Franzen 1990), Enspel (Oligocene, Germany) (Toporski *et al.* 2002), Bechlejovice (Oligocene, Czech Republic) (Roček 2003) and Shiobara (Pleistocene, Japan) (Allison *et al.* 2008) biotas. We therefore predict that careful examination of specimens will confirm that high-fidelity organic preservation of labile soft tissues is relatively common in the fossil record, particularly in lacustrine settings. This is supported by the preliminary investigation of organically preserved tissues from several taxa from the Messel biota (see the electronic supplementary material). Fauna from such biotas are prime targets for the recovery of other examples of high-fidelity, organically preserved soft tissues, and, potentially, labile biomolecules.

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