In situ SEM/EDS compositional characterization of osteocytes and blood vessels in fossil and extant turtles on untreated bone surfaces; different preservational pathways microns away.

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Abstract

Osteocytes and blood vessels are the main cellular and tissue components of the bone tissue of vertebrates. Evidence of these soft-tissue microstructures has been widely documented in the fossil record of Mesozoic and Cenozoic turtles. However, all these studies have characterized morphologically and elementally these microstructures via isolation from the fossilized bone matrix where they were preserved or in ground sections, which could raise skepticism about the results due to potential cross-contamination or reagents effects. Fossil turtle bones from three different localities with distinct preservation environments and geological settings,; including *Mongolemys elegans* from the Late Cretaceous of Mongolia,; *Allaeochelys crassesculpta* from the Eocene of Germany,; and a podocnemidid indet.; from the Miocene of Colombia are studied here. Bone from two extant turtle species, *Lepidochelys olivacea* and *Podocnemis lewyana*, as well as a commercial chicken *Gallus gallus*, were used for modern comparisons. Scanning Electron Microscopy-Energy Dispersive Spectroscopy analyses performed directly on untreated fresh surfaces showed that osteocytes in the fossil turtle bone are mostly composed of iron and manganese. In contrast, the *in situ* blood vessels of the fossil turtles, as well as those from the extant taxa, are rich in elements typically organic in origin (carbon and nitrogen), which are absent to minimum in the surrounding bone or rock matrix; ~~suggesting~~ this suggests a possible endogenous composition ~~in origin~~ for these fossil structures. Also, the results presented here show that although originally both (osteocytes and blood vessels) are organic soft components of bone, as evidenced in the extant turtles and chicken, they can go through completely different preservational pathways only microns away from each other in the same fossil bone.

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Keywords: Fossil cells, exceptional preservation, osteoblasts, Testudines, Deep time, Mongolia, Colombia, Germany

Introduction

Bone is a complex biological tissue that characterizes extant and fossil vertebrates, and consists of a mineralized (calcium, phosphorus) and a non-mineralized (collagen and non-collagenous proteins) extracellular matrix, plus water and some lipids (Boskey & Gehron 2013; Rey et al. 2009). Cells involved in bone tissue are osteoclasts, osteoblasts, and the most abundant of them: ~~are~~ osteocytes (Bonewald 2011). Osteocytes are embedded within the hard-mineralized component of bone ~~for~~ throughout life (exceptions being when released by fracture or during remodeling) (Robling & Bonewald 2020), making them highly susceptible to be preserved in fossil bones, ~~as it~~which has been extensively documented in different groups of vertebrates (e.g., Bailleul et al. 2019; Enlow & Brown 1956; Pawlicki & Nowogrodzka-Zagorska 1998; Schweitzer 2011; Schweitzer et al. 2013; Surmik et al. 2019). Similar preservation of osteocytes and blood vessels has~~ve~~ also been documented in fossil turtles, showing that their preservation is independent of geologic time, paleoenvironment, lithology, lineages, and latitude (Cadena 2016; Cadena et al. 2013; Cadena & Schweitzer 2012, 2014~~; Cadena & Schweitzer 2014~~)

Something in common to all aforementioned studies are the analytical tools used to study and characterize these fossil bone microstructures, which include principally: 1) ground sections and observation under transmitted and polarized microscopy (Cadena & Schweitzer 2012; Surmik et al. 2019); 2) bone demineralization using ethylenediaminetetraacetic acid (EDTA) as a chelating agent (0.5 M, pH 8.0), allowing to release the osteocytes, blood vessels, and any other cells or soft-tissue fibers from the bone matrix for ~~their posterior~~ensuing study by transmitted and/or polarized light, scanning and/or transmission electron microscopy and any coupled elemental analyzer, Raman spectroscopy, Fourier-transform infrared spectroscopy (FTIR), immunological and antibody studies (e.g., Alfonso-Rojas & Cadena 2020; Bailleul et al. 2019, 2020~~; Bailleul et al. 2020~~; Cadena 2016; Saitta et al. 2019; Schweitzer et al. 2013; Surmik et al. 2019; Wiemann et al. 2018)

The preservation of these soft-tissue microstructures (osteocytes and blood vessels) and their potential original constituents (proteins and DNA) has been questioned and considered a consequence of microbial interactions within fossil bone and its microenvironment, or even as a result of cross-contamination in the laboratory (Buckley et al. 2017; Kaye et al. 2008; Saitta et al. 2019). The biofilm hypothesis as a source for soft-tissue preservation in dinosaur bones has been rigorously tested, ~~showing~~ which identified fundamental morphological, chemical and textural differences between the resultant biofilm structures and those derived from dinosaur bone, and offering rejection for this hypothesis (Schweitzer et al. 2016). ~~The~~ Issues concerning cross-contamination

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Boles, Z.M., 2016. Vertebrate taphonomy and paleoecology of a Cretaceous-
Paleogene marine bonebed. PhD Thesis. Drexel University, 263 pp.

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and ~~issues with~~ replications, timing of sample collections, and reagents have also been addressed ~~in~~ by Schweitzer et al. (2019).

Compositionally, ~~the~~ osteocytes and blood vessels from different groups of fossil vertebrates have been shown to commonly be enriched in iron (Cadena 2016; Schweitzer et al. 2014; Surmik et al. 2019; Ullmann et al. 2019), an element that has been suggested to play~~s~~ a key role in preserving and even masking identification of proteins in fossil tissues via Fenton reactions (Schweitzer et al. 2014). Other elements typically found in these fossil bone microstructures are carbon, calcium, and silicon (Cadena 2016; Ullmann et al. 2019). At present, all these studies of elemental characterization have been conducted using SEM/EDS on isolated (post~~after~~-demineralization) osteocytes and blood vessels, or from polished ground sections, which implies some degree of manipulation or contact with reagents or preparation tools, potentially raising skepticism on the~~ir~~ elemental results.

Here, I explore the *in situ* (directly on fresh and untreated surfaces) preservation and elemental composition of bone microstructural elements (cells and blood vessels) of fossil turtle bones from three localities~~,~~ which have completely different geological settings~~,~~ including: 1) Gobi Desert, Mongolia, from the Late Cretaceous (late Campanian–early Maastrichtian); 2) Messel Pit, Germany, from the Eocene; and 3) La Venta fauna, Colombia, from the Miocene. Comparison samples include bone from two extant turtles and a domesticated chicken. I discuss herein the results of these analyses and the advantages of using *in situ* SEM/EDS for understanding preservation of bone microstructures in fossils.

Materials & Methods

Fossil and extant samples. All the fossil and extant samples analyzed here were free of any resin, glue, or stabilizing additives since field collection. Two small shell pieces donated by Dr. M. Norell (American Museum of Natural History, AMNH) from a partially ~~–~~articulated shell (carapace and plastron) of *Mongolemys elegans*~~,~~ (IGM-90/42) ~~specimen~~ were used for this study. Specimen IGM-90/42 ~~specimen~~ has been previously figured, including ground sections that show excellent preservation of osteocytes under transmitted light microscopy (Cadena et al. 2013~~,~~; figs. 7, 9). This fossil material was collected by the AMNH and the Mongolian Academy of Sciences joint field expeditions at the Bugin Tsav locality, Gobi Desert, Mongolia~~,~~; from fine-grained sandstones ~~deposits~~ representing pond deposits ~~belonging to~~within the Nemegt Formation, considered to be late Campanian–early Maastrichtian (~ 80 Ma) in age (Jerzykiewicz 2000, and references therein).

Small isolated fragments ~~of~~ from the carapace of an *Allaeochelys crassesculpta* (SMF ME 2449~~) specimen~~ ~~was~~ were donated ~~from~~ by Dr. K.~~rister~~ Smith (Senckenberg Naturmuseum Frankfurt, SMF); these were collected from the ~~worldwide~~ well-known locality of Messel Pit, which represents volcanic lake deposits from the early-middle Eocene (~ 48 Ma) (Lenz et al. 2015). Osteocytes, blood vessels, and collagen fibers from

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this specimen were previously described and elementally characterized ~~for this specimen in~~ by ~~(~~Cadena (2016~~,~~; figs, 4–7).

Carapace fragments from a podocnemidid indet. specimen, UR-CP-0043, as well as the surrounding rock matrix, were collected in 2018 directly from an excavation site (approximately 1.5 m from the surface) ~~under~~ using strict aseptic techniques (nitrile gloves, face mask, wrapped in sterilized aluminum foil and kept in glass containers with silica gel for moisture control until analyses were performed). This fossil material was collected from the Repartidora locality, La Victoria Formation, middle Miocene (13.6 ± 0.2 Ma), Tatacoa Desert, Colombia, from what are interpreted as fluvial deposits (Cadena et al. 2020). Permits for collecting and study of the samples were granted by the Colombian Geological Survey (Radicado Nº 20193800017321).

For comparisons, two extant turtle carcasses were sampled directly in the field following the same aseptic protocols used for specimen UR-CP-0043 ~~specimen~~. The first corresponds to carapace fragments from an individual of the sea turtle *Lepidochelys olivacea* (uncatalogued specimen) collected in January 2017 at the Pacific coast, Santa Elena Province, Ecuador, permit granted by Yachay Tech University. The second specimen sampled corresponds to a carcass of the side-necked turtle *Podocnemis lewyana* found in a sand ~~-~~bed of the Magdalena River, close to La Victoria village, Huila Department, Colombia, under a permit granted by the ethics committee of Universidad del Rosario (~~-~~Resolución DVO005 672-CV1066) and the Colombian Autoridad Nacional de Licencias Ambientales (Technical concept Nº 02263, 2019). A third sample corresponds to a femur fragment from a commercial chicken *Gallus gallus* obtained directly from a local market. Muscle tissue was removed and small bone fragments were cut using a sterilized scalpel and dried out at room temperature for several days.

Institutional abbreviations. AMNH; American Museum of Natural History, New York, USA; IGM~~:~~, Geological Institute of the Mongolian Academy of Sciences, Ulaan Baatar, Mongolia; SMF ME, Senckenberg Naturmuseum, Frankfurt, Germany; UR-CP~~,~~; paleontological collection, Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia.

Scanning electron microscopy and elemental analysis (SEM/EDS). Each of the fossils, rock matrix and extant bone samples were placed between two disposable sterilized lab-weighing boats and gently hit ~~it~~ with a rock hammer for breaking it in smaller pieces. Using tweezers (sterilized before every mounting process), one of the smaller pieces of broken bone was transferred to an SEM holder with adjustable screws and secured. To prevent any potential particles or dust ~~to enter~~from entering the SEM chamber, ~~the~~ each sample was gently air cleaned before placing it in the SEM carousel. Elemental analysis was performed ~~combined~~ in combination with high resolution imaging of the bone surfaces, ~~and~~ as well as (in some cases) the rock matrix attached to it, using a scanning electron microscope coupled with an energy-dispersive X-ray spectroscopy analyzer (Phenom ProX, at the Paleontological Lab of Yachay Tech University (YTU), San Miguel de Urcuqui, Ecuador). Imaging was performed at 5 kV using different magnification settings, and point–and-map analyses of elemental

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composition of selected regions or features were performed at 15 kV. At least 5 or more elements points were explored for each osteocyte or blood vessel, as well as the surrounding bone matrix or rock. Quality of EDS analyses was evaluated considering only those with one~~l~~ million counts or higher. Full raw data is presented in Data S1.

Bone demineralization. In order to control the occurrence and preservation of endogenous osteocytes and blood vessels in some of the samples, small bone pieces of *Mongolemys elegans*~~,~~ (IGM-90/42) ~~specimen~~ and the podocnemidid indet. specimen (UR-CP-0043) were demineralized using disodium ethylenediaminetetraacetic acid (EDTA) (0.5 M, pH 8.0, filter-sterilized using a 0.22 µm filter) as previously described (Cadena 2016; Cleland et al. 2015) for a period of 5 days to 2 weeks, or until osteocytes and blood vessels were detected. Photographs of the recovered osteocytes were taken using a transmitted light microscope (Olympus BX-63) and a polarized light microscope (Olympus BX-53)~~,~~ at the paleontological lab of YTU. Some of the isolated osteocytes ~~of~~ from IGM-90/42 were mounted in a stub and analyzed following the same protocol and SEM/EDS machine aforementioned.

Results

***Mongolemys elegans*, Late Cretaceous of Mongolia.** The *in situ* osteocytes of *Mongolemys elegans* (specimen IGM-90/42) under SEM~~,~~ exhibit a different contrast with the~~ir~~ surrounding bone matrix, which is exclusive of their three-dimensional volume, because when they left their lacuna, this space exhibited the same contrast as the bone matrix (Figs. 1A–B). Compositionally, they are predominantly constituted by iron, calcium, carbon, manganese, and in minor occurrences, barium and nitrogen (Figs. 1C–K; 2A; Data S2; and Fig. S1). There is no~~t~~ evidence of any of these elements in ~~the~~ empty osteocyte~~s~~ lacunae, which are composed of calcium and phosphorus, like the bone matrix (Figs. 1L–N). The isolated osteocytes show~~s~~ that ~~the~~ iron is concentrated o~~i~~n their external surface and the manganese in ~~the~~ an internal layer~~;,~~ this is clearly ~~showed~~ evident in ~~the~~ mapping and a cross-line elemental profile (Figs. 1O–P; S1). ~~The~~ ~~o~~Observation of some of the isolated (~~after~~ post-demineralization) osteocytes under transmitted and polarized light ~~show their~~revealed excellent morphological preservation, with some of them emitting low~~–~~degree~~d~~ birefringence colors under polarized light (Fig. 3).

***Allaeochelys crassesculpta*, Eocene of Germany.** The most abundant bone microstructures preserved in this sample are blood vessels and the walls that formed the Haversian-Volkmann (H-V) canals~~;,~~ also, in some, there is evidence of very small (2.5 µm diameter) structures with a striated margin ~~resembling~~ which resemble the morphology of osteoblast cells (Figs. 4A–D; S2). The blood vessels exhibit a width ~~between~~ of 1~~ to~~ 3 µm, with an average wall thickness of 0.2 µm (Fig. 4D). Compositionally, the blood vessels are mainly composed of carbon and nitrogen, with minor amounts of calcium, phosphorus, and iron (Figs. 2B; 4E–G; 4J–N~~;~~ S2; Data S2). The bone matrix surrounding them lacks nitrogen and carbon, and it is exclusively characterized by calcium, phosphorus, and iron (Figs. 4E,I). A bone sample with rock matrix attached shows that the bone is composed of calcium, phosphorus, iron, and

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Commented [P23]: Perhaps just 'points' or 'locations'

Commented [P24]: I think you mean 'test for', correct? Or are you considering the normal demin process here as a method to obtain independent confirmation of the SEM findings? Needs to be clarified somehow…

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Commented [P26]: Perhaps 'microstructures' as presumably images were collected of the vessels too, and 'microstructures' would cover both

Commented [P27]: How? Using a cellulose filter? Drying a drop of demineralization solution onto a silica chip? Please add a sentence or two discussing how the samples were mounted after demin. Also, were images collected of the microstructures still in solution in the EDTA in the wells, or were they transferred to separate slides and cover slipped? Finally, were any water (or other solution) rinses performed after demin and before imaging? I know some of these details might be included in your citations to Cleland et al. 2015 and Cadena 2016, but it would be really nice to include a brief description of them in the text here so the reader doesn't have to go digging for these methods in other papers.

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Commented [P32]: By 'minor occurrences' do you mean only some osteocytes show these elements as present, or that all of them have these two elements (Ba & N) present in small amounts? Clarify please

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nitrogen, and, in contrast, the rock matrix is rich in aluminum and silicon (Figs. 2C–D; 4O–Q; Data S2).

Podocnemidid ~~i~~indet., Miocene of Colombia. The sample of the side-necked turtle from La Venta, Colombia, shows at the bone external cortex preservation of walls that formed the H-V canals, blood vessels, and osteocytes tightly embedded in the very homogenous bone matrix (Figs. 5A–B; 5F–G). Elementally, the blood vessels and H-V canal walls are rich in carbon, nitrogen, and calcium, with minor amounts of phosphorous and silicon (Figs. 2B; 5C–D; 5H–I; Data S2). In contrast, the osteocytes are composed of iron, calcium, aluminum, manganese, phosphorus, and minor amounts of silicon (Figs. 2B; 5H–J; Data S2). The bone matrix lacks carbon and nitrogen, and it is constituted by calcium and phosphorus mainly (Figs. 2C; 5C,E,H). An isolated bone fragment (~~after~~ post-demineralization) shows some of the osteocytes still embedded in the matrix, varying in color from orange to black, the darker ones located closer to black, dendritic mats (Figs. 5K–M).

***In situ* extant turtle and chicken bone microstructures.** The carapace bone fragment of the extant side-necked turtle *Podocnemis lewyana* shows osteocytes ~~inside their osteocytes~~ within lacunae (Figs. 6A–B). Their composition is rich in carbon, nitrogen, calcium, and phosphorus (Figs. 2A; 6C–D; Data S2). The bone matrix is relatively richer in calcium (Figs. 2C; 6C,E). The H-V canals exhibit a distinct wall and a high concentration of blood vessels and red blood cells, which are rich in carbon and nitrogen (Figs. 2B; 6F–G; S3; S4; Data S2). ~~A S~~similar spatial patterns and compositions ~~is~~ are shared by the bone of the extant marine turtle *Lepidochelys olivacea* (Figs. 2; 6H–M; Data S2), and the bone of *Gallus gallus* (chicken) (Figs. 2; 6N–P; Data S2).

Discussion

As previously shown (Cadena 2016; Schweitzer et al. 2014; Surmik et al. 2019; Ullmann et al. 2019), I validated via *in situ* analyses, that iron is a very common constituent of fossil osteocytes, such as those found in ~~those from~~ the Late Cretaceous *Mongolemys elegans* and the Miocene podocnemidid indet. bone samples studied herein (Figs. 1, 5). However, this composition is not always homogenous and varies ~~from~~ between the external ~~to the~~and internal layer of ~~the~~ osteocytes, as shown in ~~the~~ a broken and folded osteocyte from *M. elegans*~~,~~ which exhibit~~s~~ing richer content of manganese internally and iron externally (Figs. 1O–P). High levels of manganese were also detected in ~~the~~ osteocytes from the Miocene side-necked turtle from Colombia, indicating that besides iron as initially suggested by Schweitzer et al. (2014), manganese ~~is~~ may also be involved in the preservation of these bone microstructures in deep time. The source for this rich content of manganese seems to be ~~derived~~ from manganese oxides such as ~~the~~ pyrolusite ~~that could penetrate~~penetrating bone microfractures, as I showed here occurred in some fragments of the Miocene podocnemidid indet. from Colombia (Figs. 5K–M), and also has been characterized to occur in dinosaur fossil bones ~~of~~ from the same Formation (Nemegt~~) Formation,~~ from which the *M. elegans* ~~came~~ studied herein was collected from (Owocki et al. 2016). The color variation exhibited by the fossil osteocytes of *M. elegans* and podocnemidid indet. seems to be related to relative enrichment of manganese (darker

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Commented [P38]: But your citations include work by others, so this can't be "I". "As previously shown, I validated…" just doesn't work either grammatically or scientifically here. I think it's a product of the sentence structure here, where you mean to say something like 'the analyses presented here concur with prior reports that iron is a very common constituent'. Please rephrase the sentence to correctly reflect this meaning

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Commented [P39]: 'may vary' would be more appropriate I think because it allows for differences by sample better than just saying 'varies'

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ones). In contrast to the osteocytes of the extant turtle and chicken bone, which are rich in carbon and nitrogen (Figs. 2, 6), these elements only appear in minor amounts in the fossil ~~ones~~osteocytes. However, ~~they~~ these cells exhibited a very distinct composition when compared to the surrounding bone matrix and even the surface of ~~their osteocytes~~ lacunae, indicating that their mineralized preservation occurred at micro-scale inside the bone, a hypothesis that should be tested by future studies using additional tools (e.g., ~~as for example~~ Raman and FTIR spectroscopy).

The blood vessels and H-V canal walls preserved in the Eocene *Allaeochelys crassesculpta* from the Messel Pit and the Miocene podocnemidid indet. specimen from ~~the~~ La Venta not only ~~preserved~~ exhibited a similar morphology, but also exhibited the same elemental composition as the ones ~~from~~ in extant turtle and chicken bone (Figs. 2, 4, 5, S2, S3, S4; Data S2), being rich in carbon and nitrogen, and differing from the surrounding bone matrix which is richer in calcium and phosphorus, ~~or~~ and differing from the rock matrix which is rich in silicon and aluminum (~~,~~without any traces of carbon, calcium, or nitrogen), ~~to~~ which suggests that carbonates or nitrates were in the surrounding microenvironment. The *in situ* measurements performed on some of the preserved blood vessels from *A. crassesculpta*~~,~~ ~~showing~~ exhibiting uniform fabric and thin walls of 0.2 µm thickness (Fig. 4D) suggest that they are not consistent with the characteristics of biofilms, which tend to be amorphous and bigger in size (Schweitzer et al. 2016). Blood vessels constitute one of the most promising microstructures preserved in fossil turtles for molecular paleontology studies, and future studies should focus~~e~~ on their molecular *in situ* characterization using ToF-SIMS mass spectrometry, for example, similarly as has been used in dinosaurs and other fossil vertebrates (Alfonso-Rojas & Cadena 2020; Henss et al. 2013; Lindgren et al. 2018; Schweitzer et al. 2019).

For the first time, I herein report the preservation of osteoblasts ~~are reported~~ in fossil vertebrates, particularly in the Messel Pit turtle *A. crassesculpta.* Their oval with striated margins in shape occurring at the walls of the H-V canals (Figs. 5B–D; S2), and thus resemble the morphology and size of ~~those~~ osteoblasts observed in electron micrographs of human bone (Nakamura 2007; Schmidt et al. 2002). At the same time, ~~the~~ evidence here provided~~,~~ from the Miocene podocnemidid indet. turtle from Colombia (Figs. 5F–J) shows that, in the same bone specimen,~~;~~ osteocytes and blood vessels that are only 20 microns away from each other are compositionally different. This indicates that ~~both~~ each microstructure~~s~~ went through a different preservational pathway~~s~~. Osteocytes seem to be more mineralized than blood vessels in these fossil samples, with high amount of iron and manganese, and less organic components ~~in contrast to~~than blood vessels (Figs. 2, 3). In the extant bone of turtles and chicken, osteocytes and blood vessels exhibit similar elemental compositions under SEM/EDS, both being rich in carbon and nitrogen, which are typical compounds of proteins (Figs. 2, 6) (Torabizadeh 2011). A~~,~~ similar composition was detected herein in fossil blood vessels ~~of~~ from *A. crassesculpta* and the podocnemidid indet.~~,~~ from Colombia.

Traditionally, it has been suggested that ~~the~~ SEM/EDS has to be performed on homogenous or polished surfaces to avoid topographic~~y~~ effects on ~~the~~ EDS analyses (Goldstein et al. 2003). However, as I showed here, ~~this~~ such effects were ~~is~~ minimum ~~in~~

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Commented [P47]: Make this more formal. Maybe something like 'large in diameter'?

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for the analyzed sample, with composition and values being very similar in both the fossil and extant samples (Fig. 2). A more critical condition for EDS analysis on untreated samples is to ~~have~~ acquire the ~~highest~~ maximum count rate ~~as~~ possible: ~~,~~ above 1 million counts is ideal.

Conclusion

This study provided evidence that *in situ* analyses using a conventional technique, ~~as~~ SEM/EDS, on untreated fresh surfaces of fossil and extant bones constitutes a protocol that should be added to the rigorous plethora of proxies and tools (e.g., those recently reviewed and summarized by Schweitzer et al. [2019]) to support and demonstrate the preservation of cells, soft- tissues, and their original constituents in deep time~~; which have been recently reviewed and summarized (Schweitzer et al. 2019)~~. Furthermore, *in situ* analyses of fossil and extant bone samples ~~contribute to~~may also help eliminate ~~minimize the~~ any potential skepticism o~~f~~ ~~the~~ results obtained by molecular paleontology studies, because, as ~~supported~~ demonstrated here, ~~there is~~it requires minimal~~um~~ sample preparation/manipulation, use of reagents ~~use~~, or contact with lab tools that could cause possible contamination.

Acknowledgements

I thank M. Norell, K. Smith, S. Schaal and A. Vanegas for access to samples. I also thank ~~to~~ M. Schweitzer for some preliminary feedback on some of the results presented here. Thanks are also extended to Yachay Tech and the~~,~~ Colombian Geological Survey and Ethics Committee at Universidad del Rosario for the permits to collect and analyze the fossil samples.

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Commented [P55]: Maybe 'signal intensities'?

Commented [P56]: This is in your opinion since there's no citation, right? If yes, then change the start of the sentence to something like 'I therefore suggest that a more critical condition…'

Commented [P57]: I strongly urge the author to consider using these suggested changes to make this closing sentence easier to read and to remove any potentially confrontational connotation that could be inferred by the (admittedly illogical) 'skeptics' still out there

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Figure 1. SEM/EDS analyses of *Mongolemys elegans* IGM-90/42 bone.
(A) Micrograph of one of the osteocytes and an empty ~~osteocyte~~ lacuna nearby. (B) EDS of the bone region shown in (A), in which orange indicates bone matrix (calcium)~~,~~ and blue-yellow denotes the osteocyte (iron and oxygen). (C) Micrograph of one osteocyte, indicating the regions where EDS mapping and point analyses were performed. (D–E) Composite elemental map (D) and individual element maps (E) for the rectangle labeled as 1 in (C), in which osteocytes show~~ing~~ high amounts of iron and nitrogen. (F) Elemental point values ~~of~~ for point 7 (bone matrix) shown in (C). (G) Elemental ~~point values of~~ for point 4 (osteocyte) shown in (C). (H) Micrograph of a broken osteocyte inside its lacuna. (I) Individual elements maps from rectangle 1 shown in (H), with the broken osteocyte showing a high content of manganese. (J) Elemental point values ~~of~~ for point 2 (bone matrix) shown in (H). (K) Elemental point values ~~of~~ for point 6 (osteocyte body) shown in (H). (L) Micrograph of an empty ~~osteocyte~~ lacuna. (M–N) Composite elemental map and individual element maps for the rectangle labeled as 1 in (L), in which the wall surface of the ~~osteocyte~~ lacuna exhibits the same composition as the bone matrix. (O) An isolated, broken, and folded osteocyte showing a high amount of iron at ~~the~~ its external surface and manganese ~~at~~ in its~~the~~ internal region. (P) Cross-line elemental profile ~~of~~ across the broken and folded osteocyte shown in (O), revealing ~~the~~ a switch ~~in~~ between iron and manganese content between the external and internal surfaces of the fossil cell. Full EDS results ~~of~~ for the points shown in (C, H, and L) are presented in Fig. 2 and Data S2.

Commented [P58]: Which is blue and which is yellow?

Commented [P59]: Part (E) needs some sort of note about the darker the color, the more of the element present. Then that can be mentioned as applying to all the later figures too.

Commented [P60]: And oxygen according to panel (E) too

Commented [P61]: Perhaps 'signal intensities'? Also, since some of the columns don't sum to 100% you should probably note that the chart depicts only the four most prevalent elements. Same goes for part (G)

Commented [P62]: See previous comment, and it applies to parts (J) and (K) below and in captions for Figs. 4, 5, and 6 too

Commented [P63]: The caption for part (O) needs a brief note that the red line labeled P denotes the transect where elemental data were collected for part (P)

Commented [P64]: What is the Y axis of the graph, wt%? Label it please

Commented [P65]: Or cell wall?

Commented [P66]: Caption to the figure needs to close with a 'Scale bars as indicated.' Same for all the other figure captions too

Figure 2. %Wt of elements for fossil and extant turtle bone.
(A) EDS point analyses of osteocytes, indicating high amounts of iron and manganese in the fossil ~~ones~~ cells (from *Mongolemys elegans* and the podocnemidid indet. specimens), whereas carbon and nitrogen dominate the ~~ones~~ cells from extant taxa (*Podocnemis lewyana* and *Lepidochelys olivacea*), including those from a ~~also~~ chicken (*Gallus gallus*). (B) EDS point analyses of blood vessels, with fossil and extant vessels showing a similar elemental composition rich in carbon and nitrogen. (C) EDS point analyses of the bone matrix surrounding ~~the~~ osteocytes or blood vessels. Note how f~~F~~ossil and extant samples exhibit ~~exhibiting~~ similar %Wt values for calcium, carbon, and phosphorus. Fossils show relative enrichment in iron and a lower amount of nitrogen in comparison to the modern bone matrix samples. (D) EDS point analyses of the surrounding rock matrix, which show~~s~~~~ing~~ an absence of carbon, calcium, and nitrogen, ~~and~~ but ~~enrichment in~~abundant silicon and aluminum ~~instead~~. Full data ~~of~~ for these EDS point analyses ~~is~~ are presented in Data S1, S2.

Figure 3. Fossil osteocytes ~~of~~ from *Mongolemys elegans* specimen IGM-90/42.
(A–F) Isolated (~~after~~ post-demineralization) osteocytes viewed under transmitted light microscopy (A, C, E) and polarized light microscopy (B, D, F), these last showing low to moderate birefringence. All photographs taken with a 100X-oil immersion objective lens.

Figure 4. SEM/EDS analyses of *Allaeochelys crassesculpta* SMF ME 2449 bone.
(A–D) Micrographs of two Haversian canals, in which (B, D) show~~ing~~ ~~the~~ blood vessels ~~system~~ outlined in red ~~in (B, D)~~ and osteoblasts are outlined in green ~~in (B, D)~~. M~~m~~easurements of the width of a blood vessels ~~width~~, wall thickness, and osteoblast~~s~~ diameters are also shown in (D). (E–G) Micrograph~~s~~ (E) and EDS elemental maps (F–G) of one of the blood vessels. (H) Elemental point values ~~of~~ for point 3 (blood vessel) shown in (E), showing it to be rich in carbon and nitrogen. (I) Elemental point values ~~of~~ for point 5 (bone matrix) shown in (E), showing ~~rich amount~~an abundance of calcium, phosphorus, and iron, and absence of carbon and nitrogen. (J) Bone fragment placed in the SEM holder. (K) Micrograph showing a blood vessel embedded in the bone matrix, from the yellow region indicated in (J). (L) Close-up micrograph of the blood vessel shown in the red rectangle in (K). (M) Elemental point values ~~of~~ for point 3 (blood vessel) shown in (L), showing a high amount of carbon. (N) Elemental maps of the blood vessel shown in (L), indicating a high amount of carbon and nitrogen; these elements~~, which~~ are absent in the surrounding bone matrix, which is characterized by calcium and phosphorus. (O–Q) Micrographs (O–P) and EDS elemental maps of a bone margin in contact with rock matrix, the latter of which exhibits a relatively higher amount of aluminum and absence of calcium, phosphorus, and iron. Full data ~~of~~ for these EDS point analyses ~~is~~ are presented in Data S1, S2.

Figure 5. SEM/EDS analyses of ~~the~~ podocnemidid ~~i~~indet. UR-CP-0043 bone.
(A) Bone sample mounted in the SEM holder. (B–C) Micrograph (B) and EDS ~~individual~~ element maps (C) of one of the blood vessels embedded in the bone matrix, showing high amounts of carbon and nitrogen, a minor amount of silicon; this differs~~, and differing~~ from the bone matrix, which is dominated by calcium and phosphorus. (D) Elemental point values ~~of~~ for point 2 (blood vessel) shown in (B). (E) Elemental point values ~~of~~ for point 5 (bone matrix) shown in (B), showing a high amount of calcium and phosphorus. (F–G) Micrographs showing an osteocyte and blood vessel 20 microns away from each other, both embedded in the bone matrix. (H) EDS ~~individual~~ element maps of the region shown in (F), in which the blood vessel ~~showing~~ exhibits high amounts of carbon and nitrogen~~,~~ and the osteocyte is richer in iron ~~and~~ but lacks significant~~ing of~~ carbon and

Commented [P67]: I'd suggest putting a period after the (B, D, F) and then making the latter though a standalone new sentence: 'Under polarized light, the osteocytes can be seen to show low to moderate birefringence.'

Commented [P68]: What does the broad orange swath in parts (B) and (D) mark?

Commented [P69]: Unless I'm mistaken, the green rectangle in (J) is not mentioned anywhere. Can it be removed?

Commented [P70]: Perhaps 'composed mainly of'

Commented [P71]: Again, can the green rectangle be removed since it isn't mentioned?

Commented [P72]: Seems more like moderate than minor, but ok

Commented [P73]: And oxygen

Commented [P74]: This either needs some note about the blue and brown coloring in (G) being artificial or how those colors correspond to particular elements (do they?)

Commented [P75]: Use µm instead of 'microns'

Commented [P76]: And oxygen

nitrogen. (I) Elemental point values ~~of~~ for point 2 (blood vessel) shown in (F). (J) Elemental point values ~~of~~ for point 7 (osteocyte body) shown in (F). (K) An isolated bone fragment after ~~4~~ four days of demineralization, viewed under transmitted light microscopy (at 20x). (L) Close-up image of the red rectangle region shown in (K), viewed under polarized light microscopy (at 40x), showing darker osteocytes closer to where dendritic pyrolusite mats ~~appear~~. (M) Close-up image of the red rectangle region shown in (L) (at 100x), showing ~~indicating~~ the dendritic pyrolusite and some of the osyteocytes in detail. Full data ~~of~~ for these EDS point analyses ~~is~~ are presented in Data S1, S2.

Figure 6. SEM/EDS analyses of the extant turtle and chicken ~~extant~~ bones. (A) Bone fragment of *Podocnemis lewyana* in the SEM holder. (B–C) Micrograph (B) and EDS ~~individual~~ element maps (C) of ~~some~~ osteocytes embedded in the bone matrix, showing high amounts of carbon and nitrogen; these~~, and~~ differ~~ing~~ from the bone matrix, which is dominated by calcium and phosphorus. (D) Elemental point values ~~of~~ for point 3 (osteocyte) shown in (B). (E) Elemental point values ~~of~~ for point 4 (bone matrix) shown in (B). (F) Micrograph of one of the Volkmann canals and a blood vessel ~~system of~~ in the sample of *P. lewyana* bone. (G) Close-up image ~~of~~ taken mostly within the Volkmann canal ~~wall and~~in which blood vessels are ~~system (~~ outlined in red~~) *P. lewyana*~~. (H) Bone fragment of *Lepidochelys olivacea* in the SEM holder. (I) Micrograph of a region of the cancellous bone shown in the yellow rectangle in (H). (J–K) EDS composite (J) and individual elemental (K) analys~~e~~is of the bone region shown in (I). (L) Elemental point values ~~of~~ for point 2 (blood vessel) shown in (I), showing high amount of carbon and nitrogen. (M) Elemental point values ~~of~~ for point 5 (bone matrix) shown in (I), showing high amounts of calcium and carbon. (N) Bone fragment ~~of~~ from a femur of *Gallus gallus* in the SEM holder. (O) Micrograph of the bone region shown in the yellow rectangle in (N), showing ~~some~~ osteocytes embedded in the bone matrix. (P) EDS elemental maps of one of the osteocytes (in the red rectangle) shown in (O), showing a high amount of carbon within the fossil cell. Full data ~~of~~ or these EDS point analyses ~~is~~ are presented in Data S1, S2.

Commented [P77]: How about 'adjacent to'

Commented [P78]: Can the green box be removed?

Commented [P79]: Again, (J) needs a note about whether the color coding is artificial for easier visualization or if the colors correspond to enrichment in particular elements