APPENDIX A.

Materials and methods

For each specimen analyzed, small fragments of bone were collected, using aseptic techniques under a laminar flow, HEPA filtered hood (Envirco, model #TT3624). Because bone is a composite material composed of both an organic and mineral phase, removal of mineral from extant bone reveals micromorphological features the mineral phase obscures. To study organic microstructural features, all specimens were subjected to demineralization using ethylenediaminetetra-acetic acid (EDTA, 0.5 M, pH ~8.0, 0.22µm PES filtered) in sterile polystyrene culture dishes (Costar) with daily buffer changes, as described in Schweitzer *et al*. 2005a. After a period of 2 weeks to 3 months, material remaining after demineralization was rinsed in filtered phosphate buffer (PBS, pH 6.8), imaged using a Zeiss Stemi 2000-C dissecting microscope or a Zeiss Axioskop 2 plus compound microscope with an AxioCam MRc5 digital camera and Axio Vision Software (v.4.3), then collected for further analyses.

With material younger than 20,000 years, enzymatic digestion was employed to remove remnant collagen fibers and liberate vessels and cells. To accomplish this, demineralized bone was digested in sterile culture dishes with collagenase enzyme (5 or 10 mg/ml, Roche Diagnostics, type A) in 25 mM Tris, 5 mM $CaCl_2$ (pH 7.5), for 2-10 days at 37^{o}C, rinsed in PBS or water as above, imaged and collected. A portion of collected ostrich vessels were fixed with 4% paraformaldehyde in PBS to enhance stability, which resulted in improved visualization of endothelial cellular features.

After demineralization, soft tissues and isolated vessels were collected for electron microscopy. For Scanning Electron Microscopy (SEM), samples were rinsed in PBS to remove EDTA and fixed by incubating for 2 hours (24^{o}C) in 2.5% gluteraldehyde (Ted Pella, EM grade) diluted in PBS (pH 6.8). After fixation, tissues were rinsed repeatedly in deionized organic-free water (Barnstead e-pure system), placed on new glass disks, and allowed to air-dry under a laminar flow hood. Disks were coated with ~10-20 nm Au-Pd or Au, and imaged at a working distance of 10-15 mm using a JEOL 6100 electron microscope operating at 20 kV and 1 nA beam current.

For transmission electron microscopy (TEM), demineralized fixed tissues and/or vessel fragments were thoroughly rinsed with e-pure water, dried and embedded in hard grade acrylic LR White (London Resin Company Ltd) or DER 736/ERL 4221 Epoxy (Electron Microscopy Sciences, Spurr, 1969) resin. Tissues were sectioned with an ultramicrotome (Reichert, Ultracut E) using glass or diamond knives to ~70-90 nm, collected on gold or nickel formvar coated grids (Electron Microscopy Sciences), and imaged using a LEO 912 AB Omega LB-6 M3 at 100 kV or a Phillips/FEI EM208S at 80 kV.