**RESEARCH ARTICLE**

**Comparison of structural, architectural and mechanical aspects of cellular and acellular bone in two teleost fish**

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**SUMMARY**

The histological diversity of the skeletal tissues of fishes is impressive compared with that of other vertebrate groups, yet our understanding of the functional consequences of this diversity is limited. In particular, although it has been known since the mid-1800s that a large number of fish species possess acellular bones, the mechanical advantages and consequences of this structural characteristic – and therefore the nature of the evolution of this feature – remain unclear. Although several studies have examined the material properties of fish bone, these have used a variety of techniques and there have been no direct contrasts of acellular and cellular bone. We report on a comparison of the structural and mechanical properties of the ribs and opercula between two freshwater fish – the common carp *Cyprinus carpio* (a fish with cellular bone) and the tilapia *Oreochromis aureus* (a fish with acellular bone). We used light microscopy to show that the bones in both fish species exhibit poor blood supply and possess discrete tissue zones, with visible layering suggesting differences in the underlying collagen architecture. We performed identical micromechanical testing protocols on samples of the two bone types to determine the mechanical properties of the bone material of opercula and ribs. Our data support the consensus of literature values, indicating that Young’s moduli of cellular and acellular bones are in the same range, and lower than Young’s moduli of the bones of mammals and birds. Despite these similarities in mechanical properties between the bone tissues of the fish species tested here, cellular bone had significantly lower mineral content than acellular bone; furthermore, the percentage ash content and bone mineral density values (derived from micro-CT scans) show that the bone of these fishes is less mineralized than amniote bone. Although we cannot generalize from our data to the numerous remaining teleost species, the results presented here suggest that while cellular and acellular fish bone may perform similarly from a mechanical standpoint, there are previously unappreciated differences in the structure and composition of these bone types.

Key words: acellular bone, teleost fish, mechanical properties.

**INTRODUCTION**

Bone forms a tough and protective load-bearing framework for the bodies of vertebrates. The magnitude and direction of the principal loads to which bones are exposed can vary. Furthermore, bone is loaded repeatedly and cyclically during the animal’s life; therefore, fatigue damage in the form of microcracks is bound to accumulate within the bone material. Most vertebrate bones are able to cope with a changing mechanical environment and damage accumulation through adaptation of their morphology and self-repair (termed modeling and remodeling, respectively). Both processes depend on the ability of bone to sense local changes in its mechanical environment, such as increasing strain or strain energy density levels, which result from the accumulation of microcracks or changing loads. This mechanosensory ability is widely thought to be provided by bone cells – the osteocytes (Adachi et al., 2009; Bonewald, 2011).

Osteocytes arise from the bone-forming cells (osteoblasts) (Franz-Odendaal et al., 2006). In the bones of mammals, some osteoblasts become embedded within the extracellular matrix they deposit (osteoid), and this matrix then becomes mineralized. Once embedded and trapped within the matrix, the osteoblasts become known as osteocytes, residing within small spaces named osteocytic lacunae. The bone matrix is permeated by an interconnected network of microscopic channels (canaliculi), which contain cellular processes of osteocytes, and thus embedded osteocytes are able to communicate with their neighbors.

In the middle of the 19th century it was observed that in the great majority of teleost fish species (and thus a large proportion of vertebrates), bone is acellular and does not contain osteocytes. The ubiquity of osteocytes throughout vertebrate animals, including mammals, birds, amphibians and reptiles, and their proposed mechanosensory function make this observation surprising. Interestingly, the bone of basal teleosts is cellular; therefore, the loss of osteocytes is a derived property (Parenti, 1986; Kranenbarg et al., 2005a); as the main function of bone is mechanical, this suggests that the evolution of acellularity confers a mechanical advantage to the skeleton. However, based on current knowledge of mammalian bone biology, lack of cells would hinder the functionality of fish bone, restricting its ability to respond to changing loading conditions and to repair microdamage. And yet, various reports suggest that acellular fish bone functions mechanically in a manner similar to its cellular counterpart. For example, several authors have reported that the acellular bone of
neoteleosts – the eight most-derived superorders of bony fishes, which include the Percomorpha, the largest and most diverse group of fishes – responds to mechanical loads by the process of modeling, as a cellular bone would (Huysseune et al., 1994; Hegrenes, 2001; Kilhara et al., 2002; Kranenborg et al., 2005a; Kranenborg et al., 2005b; Nemoto et al., 2007; Kitamura et al., 2010; Totland et al., 2011). Furthermore, although osteocytic lacunae are believed to act as stress concentrators (e.g. Nicoella et al., 2006), and therefore their absence could conceivably decrease the rate of microdamage accumulation, it is reasonable to assume that acellular bone still accumulates some fatigue damage from repeated cyclic mechanical loads, and therefore would benefit from the ability to replace damaged areas. However, the manner in which acellular fish bone is able to undergo modeling and remodeling without the strain-sensing osteocytes is currently unknown, although osteoblasts and bone lining cells are reasonable candidates for mediators of these processes (Witten and Huysseune, 2009).

The structure and mechanical properties of bones from a large variety of vertebrates from different taxonomic levels (mammals, amphibians, reptiles and birds) have been extensively studied. It is thus somewhat surprising that so little is known about the structure and mechanical properties of the bones of modern teleost fish, as the number of teleost fish species likely represents at least 50% of the rest of the vertebrate species combined (Currey, 2010). Very few studies have examined the material properties of fish bone, with cellular fish bone being the most studied (Roy et al., 2000; Rho et al., 2001; Erickson et al., 2002; Wang et al., 2002; Zhang et al., 2002; Paxton et al., 2006) and, to the best of our knowledge, with only one study providing values for acellular fish bone (Horton and Summers, 2009). However, none of these previous studies tested and compared the two bone types, and valid comparisons among the studies are difficult because they employed a wide diversity of methods and focused on disparate species and different skeletal elements. The dearth of data regarding the mechanics of fish bone tissue most likely stems from the fact that fish bones are typically small and their shape is extremely irregular, making sample preparation and testing very difficult (Currey, 2010). In order to properly compare the mechanical properties of cellular and acellular bone, it is imperative that samples of the two bone types be tested by exactly the same method using multiple replicates.

In the study reported here, we examined in detail the structure and mechanics of two bones (operculum and rib) obtained from fish with acellular bones [the tilapia Oreochromis aureus (Steindachner, 1864); Acanthopterygii] and from fish with cellular bones (the common carp, Cyprinus carpio L.; Ostariophysi). We used various techniques to describe their morphological features, mean mineral density and microstructural features. These analyses are paired with standardized mechanical testing methods to determine and compare the mechanical properties of cellular and acellular fish bone tissue.

**MATERIALS AND METHODS**

**Specimens and sample preparation**

Sixteen freshly killed, healthy 18-month old carp weighing 672–1281 g, and 15 freshly killed, healthy 14-month old tilapia, weighing 356–576 g, were obtained from a commercial fishery (Madan, Maagan Michael, Israel).

The operculum and fifth rib were then harvested from all fish by manual dissection within 1 h. Samples of all bones were collected for histological study and immediately fixed in 4% paraformaldehyde solution (Sigma, St Louis, MO, USA). Samples were also collected for light microscopy, micro-computed tomography (micro-CT) scanning and mechanical testing. These samples were wrapped in saline-soaked gauze and stored at –20°C until further testing.

**Histology and light microscopy**

Rib and operculum bone samples, taken from four carp and four tilapia, were fixed overnight in 4% paraformaldehyde in PBS at 4°C. They were then decalcified in 0.5 mol l⁻¹ EDTA, pH 7.4. Once decalcified, they were dehydrated in graded ethanol solutions and Histo-clear® (National Diagnostics, Atlanta, GA, USA). They were then embedded in Paraplast (SPI supplies, West Chester, PA, USA), cut by microtome into 5 μm sections and mounted on glass slides (Superfrost, Thermo Scientific, Barrington, IL, USA). The embedded sections were deparaffinized in xylene, washed twice with ethanol, rehydrated through a graded series of ethanol solutions and stained with hematoxylin and eosin (H&E).

Five histological sections from each of the four carp opercula were used to obtain estimates of osteocyte density within the bone. To determine osteocyte density, a large rectangle was outlined digitally over a high-resolution image of each sample such that it covered as much of the section as possible, the area of the rectangle was determined and all osteocytes within this region were counted manually. Osteocyte density was determined as the number of osteocytes within the rectangle divided by its area.

In addition, several unstained, fresh samples of ribs and opercula from four carp and four tilapia were prepared for light microscopy. Transverse and axial rib slices were sectioned from the proximal region of the fifth rib, and both axial and dorso-ventral strips were prepared from the operculum. The regions from which the samples in the different bones were obtained are schematically shown in Fig. 1. Bone samples were prepared using a slow-speed water-cooled diamond-blade saw (Isomet® low speed saw, Buehler, Lake Bluff, IL, USA), and their surfaces ground and polished using a grinding–polishing device (Minimet®1000, Buehler). The prepared surfaces were examined by a reflected-light microscope (Olympus® BX 51 microscope, Olympus, Tokyo, Japan) at different magnifications, and images were captured using a high-resolution camera (Olympus® DP 71 digital camera).

**Water, organic and ash content**

Opercula from eight mature carp and eight mature tilapia were used for determination of ash content. Small pieces (800–1200 mg) of each operculum were carefully dissected and cleaned of all soft tissue, then placed in acetone solution for 12 h to remove all lipids. Samples were weighed with a semi-analytical scale (±0.05 mg) to determine lipid-free wet mass, then placed in a ceramic cup and heated in an oven (Tuttner, Jerusalem, Israel) to 100°C for 3 h to remove all unbound water. After heating, the samples were immediately weighed to determine their dry mass (organic and mineral content) and placed (within their ceramic cups) in an oven at 500°C for 10 h, so that all organic material was burned. The remaining ash was weighed immediately, so that the dry bone ash content (%) of each sample could be determined. Water (wet mass – dry mass), organic (dry mass – ash mass) and ash content were also calculated by dividing by wet mass to determine a percentage.

**Mechanical testing**

Mechanical testing was performed with the samples immersed in saline, using a custom-built micromechanical testing device (see Fig. 2). All samples were thawed immediately before testing for 24 h at 4°C, then for 1 h at room temperature. The operculum beams were tested in three-point bending; rib samples were tested in axial compression because three-point bending tests require long, straight
samples with a consistent cross-section, and these conditions could not be adequately met for ribs.

**Operculum samples**

Two beams were prepared from opercula from each of eight carp and seven tilapia: beams from the left operculum were oriented along the axial orientation of the operculum, while beams prepared from the right operculum were oriented in a perpendicular (dorso-ventral) orientation (see Fig. 1). Thirty beams of roughly similar size were prepared in all (14 from tilapia opercula and 16 from carp opercula). The length of the axial beams, for both types of fish, ranged between 11.4 and 21.93 mm, and that of the dorso-ventral beams ranged between 12.31 and 23.73 mm. The width of the dorso-ventral beams was 4.94±0.42 mm (mean ± s.d., here and for all subsequent values unless otherwise stated) for the carp and 5.03±0.20 mm for the tilapia. The width of the axial beams was 5.25±0.30 mm for the carp and 5.11±0.07 mm for the tilapia. The thickness of the beams was the native thickness of the operculum, and was measured for each beam at several locations. Thickness ranged between 0.60 and 1.04 mm in the carp samples and between 0.48 and 0.84 mm in the tilapia samples; however, there was never more than 5% variation in depth across the length of any given beam.

**Rib samples**

Eight samples of the left fifth rib of carp and seven samples of the left fifth rib of tilapia were prepared. A tubular segment (carp segment length 2.66±0.04 mm; tilapia segment length 2.65±0.19 mm) was obtained from the proximal, reasonably straight part of each rib, using the Isomet® slow-speed water-cooled diamond-blade saw (see Fig. 1). In compression testing (see below), stress calculation requires knowledge of the cross-sectional area of the sample; as biological samples do not have a perfectly uniform cross-sectional area (CSA), we chose samples with relatively small CSA variation (<10%) and set the effective transverse bone CSA for each sample (mm²) as an average of the CSA of the proximal and distal ends, as determined from digital images (ImageJ, NIH, Bethesda, MD, USA, version 1.44I).

**Three-point bending of operculum beams**

The operculum beams were tested by three-point bending. The beams were placed in the custom-built micromechanical testing device within a saline-containing test chamber (see Fig. 2A). A stationary anvil was attached to the wall of the test chamber. This anvil consisted of two supports, which were 8 mm apart. A moving anvil with a single prong was attached to a load cell (model 31, Honeywell, Sensotec, Columbus, OH, USA), which was attached in turn to a high-precision linear motor (Physik Instrumente (PI) GmbH, Karlsruhe, Germany). The tip of the moving prong was centered between the bottom supports (see Fig. 2A). All parts of the testing system (including the chamber itself) were made of stainless steel. The parts contacting the sample (bottom supports and upper prong) had rounded profiles (1 mm diameter) to reduce stress concentration. The upper prong was brought into contact with the tested beams at a predetermined preload (2N), the chamber was filled with physiological saline solution at room temperature until the sample was fully immersed, and bending tests were conducted under displacement control at a rate of 500μm·m⁻¹·s⁻¹ up to a final displacement of 1500μm (at this displacement the beams were in the plastic, post-yield zone, but had not fractured yet). Force–displacement data were collected by custom-written software (LabView, National Instruments, Austin, TX, USA) at 50 Hz. The resulting load–displacement curves were used to estimate the Young’s modulus of the beam material. It should be noted that as shear deformation was not taken into consideration, estimated values of the Young’s modulus are slightly underestimated. However, as the mean span-to-depth ratio of all tested beams was 12.06±3.52, this effect is relatively minor (Spatz et al., 1996; Draper and Goodship, 2003). Young’s modulus...
was calculated based on the following relationship obtained from beam theory:

\[ E = \frac{S \times L^3}{48I} \],

(1)

where \( E \) is the Young’s modulus of the bone material in the direction coinciding with the long axis of the beam (N mm\(^{-2}\)), \( S \) is the slope of the linear part of the force–displacement curve (N mm\(^{-1}\)), \( L \) is the distance between the stationary supports (mm) and \( I \) is the moment of inertia (mm\(^4\)) of each operculum beam. The moment of inertia \( I \) of a rectangular beam is calculated as follows:

\[ I = \frac{b \times d^3}{12} \],

(2)

where \( b \) is the beam width (mm) and \( d \) is the beam depth (mm).

Compression testing of rib samples

All rib samples were tested by axial compression under displacement control in the same micromechanical testing device (Fig. 2B). Each sample was placed between two anvils, one of which was stationary and attached to the wall of the test chamber, while the other was movable and attached to a load cell, which was attached in turn to a high-precision linear motor. The moving anvil had a well-lubricated ball-like edge of 10 mm diameter upon which a stainless steel platen with a corresponding lubricated half-sphere cavity was fitted, such that the platen could rotate to adjust for slight misalignment of the sample’s edges (see Fig. 2B). One end of the sample was placed against the platen and the movable anvil was moved carefully until it contacted the opposite end of the sample, and a desired preload was obtained (2 N). The sample was then fully immersed in saline and loading proceeded at a constant rate of 60 \( \mu \)m min\(^{-1}\), up to a total displacement of 60 \( \mu \)m. Force–displacement data were collected by custom-written software (LabView). The resulting load–displacement curves were analyzed to calculate the Young’s modulus of the rib sections tested.

The modulus was calculated as the ratio of stress to strain, where the stress was determined by:

\[ \sigma = \frac{F}{A} \],

(3)

where \( \sigma \) is stress (N mm\(^{-2}\)), \( F \) is the applied force (N) and \( A \) is the average bony cross-sectional area of the rib section (mm\(^2\)) determined as described above. The strain was determined by:

\[ \varepsilon = \frac{\Delta L}{L} \],

(4)

where \( \varepsilon \) is the strain (dimensionless), \( \Delta L \) is the shortening of the compressed rib segment under load (mm) and \( L \) is its original length (mm). Thus the modulus could be extracted from the results of the experiment as:

\[ E = \frac{FL}{A \Delta L} \].

(5)

As the rib segments were quite stiff, part of the movement of the anvil was due to compression of the load cell. Load cell compliance was determined by compressing the two stainless anvils against each other, and evaluating the resulting load–deformation curve. Thus for a given load, sample compliance could be determined by subtracting the load cell compression displacement from the total sample-load cell compression displacement. Because for the same load the deflection of the load cell was found to be about 50% of that of the sample-load cell unit, sample stiffness was similar to that of the load cell, making the correction valid (if the load cell was much more compliant than the sample, such a correction would be inaccurate).

In order to validate the results obtained by this experimental setup, first a hollow circular tube was prepared from Delrin\textsuperscript{®} (polyoxymethylene). This material has known mechanical properties (the Young’s modulus of Delrin\textsuperscript{®} is 3.2 GPa, quite similar to that of the bones tested here). The tube was 2.5 mm long, with 1.3 mm o.d. and 0.95 mm i.d., and was thus similar in dimensions to the tested rib sections. The tube was compressed 4 times and the Young’s modulus for Delrin\textsuperscript{®} determined by these experiments was 2.92±0.096 GPa, within an acceptable margin of error of the material’s actual stiffness.

Micro-CT scanning

The tubular rib sections and opercular beams were scanned using a micro-CT scanner (1174 scanner, SkyScan\textsuperscript{®}, Kontich, Belgium). The X-ray source was set at 50 kVp (peak kilovoltage) and 800 \( \mu \)A. In total, 450 projections were acquired over an angular range of 180 deg. The operculum samples of both carp and tilapia were scanned with an isotropic voxel size of 27.6 \( \mu \)m and an integration time of 3900 ms, while the carp and tilapia rib samples were scanned at an isotropic voxel size of 11.1 and 8.6 \( \mu \)m, with integration times of 3600 and 3500 ms, respectively. All scans were performed with a 0.25 mm aluminum filter to decrease beam-hardening effects. Scans were reconstructed and analysis was performed using commercial software (NRecon\textsuperscript{®} SkyScan\textsuperscript{®} software, version 1.6.1.2 and CT analyser\textsuperscript{®} SkyScan\textsuperscript{®} software, version 1.9.3.2, respectively). Bone mineral density (BMD) was determined based on calibration with two phantoms of known mineral density (0.25 and 0.75 mg cm\(^{-3}\)) supplied by SkyScan\textsuperscript{®}, which were scanned under exactly the same conditions as the operculum and rib specimens.

Statistics

Statistical analyses of quantitative data (BMD, ash content and mechanical testing results) were made to find differences between carp and tilapia groups, as well as between ribs and opercula within each species of fish. The quantitative data were compared between and within groups by the non-parametric Mann–Whitney test. The level of significance was set at \( P \leq 0.05 \).

RESULTS

Histology and light microscopy

The current study focused primarily on the comparison of the material properties of the bones of these two teleost species. However, as the mechanical behavior of bone is strongly linked to its structure, we also provide a brief description of some features discerned from a light microscopy study of stained and unstained bone samples.

Fish opercula are bony plates, thin and slightly curved in the medio-lateral direction. Both tilapia and carp opercula exhibited three distinct tissue zones, observable under reflected light microscopy in either coronal sections (i.e. opercula divided into rostral and caudal sections) or transverse sections (i.e. divided into dorsal and ventral sections). The lateral (outer) and medial (inner) thirds of the thickness of the bone showed clear lamellar organization (see area bracketed in Fig. 3B), sandwiching a middle, less-ordered (isotropic) tissue (see area bracketed in Fig. 3A), similar in appearance to ‘woven’ bone (Currey, 2008). The blood supply to all bones in both types of fish is quite sparse, but blood vessels tended to be localized in the disordered woven zone. The lateral and medial lamellar zones appeared thicker and more compact in...
tilapia than in carp. A similar layering of tissue zones was also observed in frontal (i.e. perpendicular to the long axis) and longitudinal cross-sections of ribs of both species, where a less-organized inner zone (sometimes perforated by the central lumina of the ribs) was surrounded by an outer zone of concentrically ordered layers (Fig. 3F).

When samples of opercula of both species were polished on their medial or lateral faces (i.e. perpendicular to the sections mentioned above), no distinct tissue layering or zonal arrangement was visible (Fig. 4). However, on these polished surfaces, we consistently saw dense collections of what appeared to be small voids in the tissue, similar in size and density in the two bone types and typically smaller and with a much higher density than the osteocytic lacunae in the cellular carp bone. The voids appeared as small ‘pores’ (2–4µm wide) and/or wavy and occasionally branched ‘lines’ (~15–40µm long) (Fig. 4); we took these to be the same structural features, but viewed in different orientations, and therefore interpret the structures as tapered or blind-ended tubules. As tubules were only visible in median (and not coronal or transverse) sections of opercula, we verified them to be consistent structural features and not preparation artifacts by examining multiple additional tissue sections from individuals of both species, prepared using a variety of methods (embedded vs non-embedded samples, polished vs unpolished, etc.), but the appearance described above remained consistent. Although
of the operculum was significantly (P<0.001) lower than the BMD of the tilapia opercula (median 1066 mg cm\(^{-3}\), range 737–824 mg cm\(^{-3}\) and median 912 mg cm\(^{-3}\), range 883–963 mg cm\(^{-3}\), respectively, see Fig. 7A). Water, ash and organic content relative to lipid-free wet mass were 14.2%, 49.4% and 36.4%, respectively, for carp, and 14.0%, 55.5% and 30.5%, respectively, for tilapia (Fig. 7B).

**DISCUSSION**

In this study we compared the material composition (BMD; ash, water and organic content), structure and mechanical properties between the acellular opercula and ribs of a neoteleost fish (*O. aureus*) and those of a more basal teleost (the carp *C. carpio*) whose...
bones are cellular. Whereas material composition was shown to vary significantly between cellular and acellular bone in the species tested here, and gross structure to vary to some degree, the mechanical properties were shown to be largely similar (with the exception of the orientation-dependent moduli of tilapia opercula).

Structurally, the tissues are easily visibly distinguished from one another by the presence and absence of osteocytes, but are more similar to each other in gross, histological morphology than they are to the more-studied cellular bone types of amniotes. In particular, differences in osteocyte presence and associated canalicular density are striking, even for the cellular boned carp where osteocytes are much less densely packed than those of birds and mammals (424 vs 700–1800 cells mm\(^{-2}\)) (Li et al., 1991; Skedros, 2005). The low osteocyte density, coupled with the apparent relative scarcity of blood vessels in both tilapia and carp bone, make both fish bone types observed here appear, at lower magnifications, as more solid (less porous) blocks of tissue compared with amniote bone, also suggesting they may have a lower metabolic rate. Our data and published work on medaka (Ekanayake and Hall, 1988) point to a relative lack of blood vessels in teleost bone; however, Moss reported extremely vascular regions in both acellular and cellular bone (Moss, 1963) and so it remains unclear whether fish bone as a rule is less vascular than amniote bone.

Although lacking the rich cellular patterning of most amniote bones, carp and tilapia bones still exhibited pronounced structural features. The obvious tissue zones, viewed in coronal/transverse cross-sections of opercula and ribs, suggest differences in underlying collagen organization. In particular, the disorganized central (woven) region may be the remains of the earliest primary bone formed during development, whereas the laminated lateral and medial layers point to a later process of layered tissue deposition in both types of bone. This finding agrees with those of Moss (Moss, 1960; Moss, 1961), who described fish bone (both cellular and acellular) as a mixture of lamellar and woven bone, and is further supported by the common use of opercular annuli to age post-hatch individuals of many species of bony fishes (e.g. Frost and Kipling, 1959; Vilizzi and Walker, 1999; Khan and Khan, 2009). The observed histological differences therefore likely reflect underlying structural patterning, as collagen organization has been shown to play a role in mineral platelet packing, size and distribution in vertebrate bones (Weiner et al., 1999; Currey, 2008; Bigi et al., 2000). It is unclear to what degree fish endoskeletal bones exhibit the hierarchical structural organization of amniote bony tissues, but one study of endoskeletal bone in carp (Bigi et al., 2000) and studies of intramuscular fish bone (mineralized myoseptal tendons) (e.g. Lee and Glimcher, 1991; Zhou et al., 2007) and scales (made of acellular bony tissue in both cellular and acellular boned species, yet perhaps dental in evolutionary origin) (e.g. Meunier, 1987; Bigi et al., 2000; Sire and Huysseune, 2003) point to structural systems in fish bone types that are similar to but simpler than amniote bone, where tissue layers differ in their collagen orientation (Lee and Glimcher, 1991; Currey, 2008). The visible evidence of tissue organization in the ribs and opercula of the two species in our study provides a roadmap for future biological and material science examinations into fundamental questions of growth, mineralization and organization in fish bones. The observed interspecific differences in tissue layers (e.g. the thicker woven region in carp opercula relative to that of tilapia) also offer a valuable opportunity for testing and modeling basic tissue-specific structure–function relationships.

The dense network of narrow tubules (2–4 μm wide, ~15–40 μm long), a major structural feature observed under reflected light microscopy in ground and polished sections, remains more difficult to interpret (Fig.4). These structures do not appear to be artifacts, as we observed them in rib and opercular bones of both species (albeit with a higher density in opercular bone) and under multiple forms of sample preparation. However, their structural interpretation and the extent to which they perforate the thickness of the skeletal element are not clear. The tubules are apparently not associated with osteocytes or vascular tissue, and bear strong resemblance to the canaluli of Williamson (~2–4 μm wide canals with an apparent nutritive function in the cellular dermal skeletons and endoskeletons of basal bony fishes (Sire and Meunier, 1994)) and endoskeletal tubules (~2–8 μm wide) observed in the bones of several teleost...
species and containing thin Sharpey’s-like fibers, probably periosteal in origin (Kölliker, 1857) [figs 11,12 in Moss (Moss, 1961); figs 4–6 in Hughes et al. (Hughes et al., 1994)]. It is possible also that the features we observed are the bundles of ‘uncalcified collagen fibers’ described by Moss (Moss, 1960; Moss, 1963) [which may also be the ‘T-fibers’ of Hughes et al. (Hughes et al., 1994)], anchored directly in the matrix but not housed in tubes; however, the pored end-on perspective of these structures argues they are tubular in nature (e.g. Fig. 4B,C). We could not verify the contents or detail the structure and distribution of these tubules; however, their presence in both an acanthopterygian fish with acellular bones (tilapia) and an osteriophyly fish with cellular bones (carp), and the reported presence of similar structures in bones/scales of basal cellular bony fishes and several other species of acellular acanthopterygian fishes, suggests these are extremely widespread structural features. A three-dimensional analysis of tissue patterning would provide fundamental insight into the material organization of fish bone, and the simple shape of the opercula and ribs make them ideal skeletal elements for examination. Studies are currently underway to examine these features in three dimensions using high-resolution X-ray imaging and scanning electron microscopy.

Despite gross structural similarities between the bone types here (aside from cell presence or absence), the acellular operculum and ribs of tilapia have a much higher mineral density (by as much as 25%) and ash content (by about 10%) than the cellular bones of the carp. The lower mineral density of cellular bone could be attributed to its higher porosity due to the presence of lacunae (Currey, 2008); however, the low percentage volume occupied by the osteocytic lacunae in the carp [estimated in our study to be around 1.6%, compared with ~5–9% in anniotes (Currey, 1988)] makes such an explanation unlikely. A literature review of percentage ash values for acellular and cellular fish bone (N=20 species for each bone type; M.D., J. W. C. Dunlop and R.S., in preparation) suggests that a lower mineral content is a consistent feature of cellular bone (~50% ash) relative to acellular bone (~58% ash); these differences are also evident in Toppe et al.’s (Toppe et al., 2007) comparison of several acellular and cellular species. Furthermore, compared with amniote bones, which have ~61–74% dry ash content (Currey, 2008; Szpak, 2011), fish bones are in general less well mineralized (Biltz and Pellegrino, 1969; Lees, 1987a; Szpak, 2011; Toppe et al., 2007) (Fig. 7B). This supports theoretical conjectures that mineral is less tightly packed among collagen fibrils in fish bone relative to mammalian bone (Lees et al., 1984; Lees, 1987a; Lees, 1987b), but the reasons for the differences among fish species are unclear; a much wider array of species must be studied to understand this feature in a phylogenetic context and studies correlating bulk tissue mineral density and nanoscale mineral packing would be very instructive.

As the BMD of cellular fish bone is significantly lower than that of acellular fish bone, we expected Young’s modulus to also be much lower. The Young’s modulus of bone is determined by its composition (mostly mineral density and porosity) and by its microarchitecture. However, counter to our predictions, values of Young’s moduli of bone material obtained from samples of ribs and opercula from the two fish types tested here were quite similar: for both cellular and acellular fish bone, median Young’s modulus values were found to be in the range 4.1–8.5 GPa. The reason for the similarity of material stiffness in bones with such a large difference in mineral density is not clear, but surely relates to some degree to the organization and interactions of mineral and organic components in the tissues (Fig. 7B).

There are very few studies reporting the material properties of fish bones; however, the values for Young’s moduli observed in this study are within the range reported (3.7–8.4 GPa) recently for the acellular rib bone of another fish species, the great sculpin, Myoxocephalus polyacanthocephalus (Horton and Summers, 2009) (Fig. 8A). Such values are lower than those reported for the cortical bones of birds and mammals. For instance, Young’s modulus of equine cortical bone was reported to be between 17.4 and 23.5 GPa in the axial direction, and between 9.3 and 13.9 GPa in the transverse direction (Shahar et al., 2007). Currey reported on the Young’s modulus of a wide range of amniote bones (but no fish), which were all tested by a similar protocol, and with the exception of antlers (which are particularly compliant because of their exceptionally low mineral content), the range of values reported fell between 14.2 and 28.2 GPa (Currey, 1999). Even in 4 week old mice, which could be expected to have low moduli as a result of their young age and consequent low mineral content, the axial Young’s modulus of
cortical bone was reported to be 8.9±1.1 GPa (Lev-Tov Chattah et al., 2009).

The relatively low stiffness of fish bone is likely related to its comparatively low mineral content, but whether the lower stiffness in fact confers a functional advantage on fishes (e.g. whether more compliant bone is better suited to fish ecologies) is unknown. Fish bone stiffness is probably also related to some unique features of its microarchitecture. In the carp (cellular bone), the moduli in orthogonal directions within the median plane of the operculum were similar, and substantially lower than the axial modulus measured in the rib. In tilapia (acellular bone), the axial modulus in the rib and the modulus in the antero-posterior orientation in the transverse plane of the operculum were similar, and much higher (by about 50%) than the modulus in the dorso-ventral orientation in the transverse plane of the operculum. These findings suggest that there may be an inherent difference between the hierarchical architecture of acellular and cellular bone, at least in the bones of these two fishes, beyond the presence/absence of osteocytes and their lacunae. We believe this will be clarified through analyses of the ontogeny of tissue mineralization and of
crystal orientation in species with cellular and acellular bone types. It would be of particular interest to examine how tissue material properties and mineral microstructure change in fish bones that show large-scale ontogenetic changes or spatial grades in the degree of cellularity (Moss, 1961; Moss, 1963; Witten et al., 2001; Witten and Huysseune, 2009).

It is important to note that material property data for bone are highly dependent on sample preparation and testing techniques, and therefore comparisons among studies that used different methodologies may not be valid. To the best of our knowledge, only eight studies (including ours) have performed mechanical tests aimed at determining material properties of fish bone, and only two of these (ours and that of Horton and Summers (Horton and Summers, 2009)) have focused on acellular bone (see Fig. 8). Ignoring for a moment the differences in sample orientation (i.e. axial vs transverse tests), the skeletal element examined and phylogeny (Fig. 8A), the combined data from these studies suggest that cellular bone, on average, is stiffer than acellular bone (11.4±2.6 vs 7.0±1.3 GPa) (Fig. 8B). However, the majority of studies examining cellular bone tested dried samples using nanoindentation, a technique for measuring nanoscale material moduli. Not only is the indentation modulus often higher for dry than for hydrated biological samples (Guidoni et al., 2006; Enders et al., 2004) but also it is unclear how it can be compared with Young’s moduli obtained by common techniques used to test materials at the micrometer scale, such as bending, tension and compression tests (Hengsberger et al., 2003). When all available fish material property data are plotted according to testing method, the values generated by nanoindentation (i.e. the majority of data for fish cellular bone) are higher and show a wider range of variation (Fig. 8B). This effect of testing method is underlined by nanoindentation data of carp (Roy et al., 2000), which provided an indentation modulus more than twice our reported Young’s modulus in compression from axial tests, and by nanoindentation data of the same tilapia opercula used in this study but tested dry (A.S., unpublished data), which provided an indentation modulus of 14.5±1.5 GPa (Fig. 8B), approximately twice as high as the Young’s modulus we report from three-point bending of the same tissue. Based on the likelihood that existing data for fish bone material properties are test dependent and on our comparison of tilapia and carp bone, we posit that cellular and acellular bone are, in general, equivalent from the standpoint of material stiffness.

Our study presents the first comparison of acellular and cellular fish bone materials, performed by uniform methodology on samples of standardized size. Yet, although these species provide valuable reference points for acellular and cellular bone, they are quite phylogenetically disparate (Fig. 8A), with one a derived perciform neoteleost (tilapia: Acanthopterygii) and the other a more basal teleost (carp: Ostariophysi). In fact, the scant data on fish bone material properties have been drawn from a very wide phylogenetic sampling, made up of one polypeterform fish [Polypeterus senegalensis (Erickson et al., 2002)] and one to two representatives each from four superorders of Teleostei: Clupeomorpha [Clupea harengus (Rho et al., 2001)], Ostariophysi [Danio rerio (Wang et al., 2002; Zhang et al., 2002); Cyprinus carpio (this study) (Roy et al., 2000)], Protacantheropygi [Oncorhynchus mykiss, Salmo trutta (Paxton et al., 2006)] and Acanthopterygii [Myxocyprinopelus polycyanthrocephalus (Horton and Summers, 2009); Oreochromis aureus (this study)]. All of the studied species are freshwater fishes except M. polycyanthrocephalus and C. harengus, and the two acanthopterygian species are the only acellular boned fish that have been examined (Fig. 8).

Our data suggest that fish bone has fundamental structural, compositional and material performance differences relative to other bony tissues; yet it remains unclear what selective pressures might have led to the loss of osteocytes in some fish groups. We are currently exploring the microstructure of cellular and acellular fish bone in multiple species. These analyses will allow us to test the hypothesis that differences in fibril structure and fibril-array arrangement are responsible for the differences found between fish bone types (i.e. mineral density and isotropy), and between fish bone and that of other vertebrates (e.g. low stiffness and cellularity). To provide a useful phylogenetic perspective and allow accurate comparisons of mechanical property data for acellular and cellular fish bone, it would be valuable for future work on fish bone to standardize material testing methodology and, if possible, to choose test species according to those phylogenetic groups for which there are existing data. It would be particularly useful to see comparisons within the Perciformes, which have many species of convenient size for testing and, although largely acellular boned, also contain several reportedly cellular-boned species within the Scombrinae (Köhlker, 1857; Moss, 1961). The Protacantheropygi also represent a particularly interesting group for study as they possess nearly equal numbers of cellular- and acellular-boned species (Moss, 1961; Kranenburg et al., 2005a; Kranenburg et al., 2005b).

In the comparison of carp and tilapia bone, the lack of cells does not seem to confer an obvious material performance advantage relative to cellular fish bones; as such, the cause of the evolutionary development towards acellularity in neoteleost fish bone remains unclear. An explanation previously offered was that acellularity could increase the stiffness of fish bone, which is low relative to that of mammalian bone (Horton and Summers, 2009). Our data support Horton and Summers’ rejection of this hypothesis because the bones of the more basal teleost, which are cellular, were shown here to have a similar stiffness to that of acellular bones from a neoteleost, despite the presence of osteocytic lacunae. Alternatively, the primary function of osteocytes may be osteocytic osteolysis (Clark et al., 2005; Bonucci, 2009; Witten and Huysseune, 2009; Bownwald, 2011). As fish are unlikely to face calcium deficiency given the high availability of minerals in their natural habitat (both marine and fresh water), selective pressure would have favored the loss of osteocytes, as the high metabolic cost of their maintenance would outweigh the benefit. The loss of osteocytes in fishes then implies that the other functions typically performed by these cells are either of lesser importance to fish bone or are addressed through as yet unidentified mechanisms.

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