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The holocephalan ratfish endoskeleton shares trabecular and areolar mineralization patterns, but not tesserae, with elasmobranchs little skate and catshark

Oghenevwogaga J Atake, Fidji Berio, Melanie Debiais-Thibaud, B Frank Eames

Department of Anatomy, Physiology, and Pharmacology, University of Saskatchewan, Saskatoon, Canada • Institut des Sciences de l'Evolution, University of Montpellier, CNRS, IRD, EPHE, Montpellier, France

eLife Assessment

This **important** study significantly advances our understanding of the skeleton of cartilaginous fishes by using a range of state of the art and complementary approaches to compare the skeleton amongst three cartilagenous fishes (catshark, little skate and ratfish). The evidence presented is **compelling** and likely to impact several fields of study.

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Abstract

Specific character traits of mineralized endoskeletal tissues need to be clearly defined and comprehensively examined among extant chondrichthyans (elasmobranchs, such as sharks and skates, and holocephalans, such as chimaeras) to understand their evolution. For example, tiles of mineralized polygonal structures called tesserae occur at cartilage surfaces in chondrichthyans, but recent studies showing trabecular mineralization at elasmobranch cartilage surfaces suggest that tesserae are not as common as previously thought. Also, while areolar mineralized tissue in elasmobranchs is generally considered a unique, shared chondrichthyan feature, some chondrichthyan species demonstrate bone-like tissues in both a specific region of tesserae termed the cap zone and continuous (not tiled) mineralized neural arches. To clarify the distribution of specific endoskeletal features among extant chondrichthyans, adult skeletal tissues in a holocephalan chimaera (spotted ratfish) and two elasmobranchs (small-spotted catshark and little skate) were characterized using synchrotron radiation and desktop micro-CT imaging, and histological and immunofluorescent assays. Endoskeletal mineralization in the ratfish, catshark, and little skate varied both quantitively in tissue mineral density (TMD), and qualitatively in the morphology and localization of mineralized structures and tissues. For example, TMD of several skeletal elements was significantly lower in ratfish, compared to catshark and little skate. Trabecular and areolar mineralization were shared among these extant chondrichthyan species, but tesserae and

bone-like tissues were not. Interestingly, three separate analyses argued that the adult chimaera endoskeleton has features of the embryonic little skate endoskeleton. Generally, this study proposes specific terminology for character states of the extant chondrichthyan endoskeleton and infers those states in ancestral chondrichthyans with reference to fossil data.

Introduction

The skeleton of chondrichthyans, or cartilaginous fishes, has long fascinated evolutionary biologists (Daniel, 1934; Janvier, 1996; Kölliker, 1860; Maisey, 2013; Ørvig, 1951; Smith and Hall, 1990; Wurmbach, 1932). However, only in the past two decades has an active research community revealed the histological and molecular complexity of extant chondrichthyan skeletal tissues. Even so, these studies are limited in scope, focussing mostly on specific skeletal features of the Elasmobranchii subclass of extant Chondrichthyes, such as sharks, skates, and rays (Atake et al., 2019; Dean et al., 2017; Eames et al., 2007; Johanson et al., 2012; Kemp and Westrin, 1979; Omelon et al., 2014; Porter et al., 2006; Seidel et al., 2016), with only a few including the Holocephali subclass, such as chimaera (Gillis et al., 2011; Herbert et al., 2022; Pears et al., 2020; Seidel et al., 2020; Smith et al., 2020). Unfortunately, an in-depth description of the extant holocephalan endoskeleton is lacking, leading to a deficit in understanding skeletal tissue character states across extant chondrichthyans.

Like most vertebrates, skeletal mineralization in extant chondrichthyans occurs by incorporation of biominerals into extracellular compartments of skeletal tissues (Golub, 2009; Lowenstam, 1981; Omelon et al., 2014), giving rise to diverse mineralized tissue types in several anatomical sites of the chondrichthyan endoskeleton (**Table 1**). Traditionally recognized as a defining endoskeletal feature of Chondrichthyes, tesserae form a mineralization pattern consisting of an array of distinct, polygonal units at the surface of cartilages (Kemp and Westrin, 1979; Maisey, 2013; Maisey et al., 2021). In skates, however, a recently characterized trabecular mineralization pattern lies directly underneath the polygonal pattern of tesserae (Atake et al., 2019). In fact, the cartilage surface in several endoskeletal regions of skates and rays only contains the trabecular mineralization pattern (Atake et al., 2019; Jayasankar et al., 2020), suggesting that polygonal tesserae may not be as common as traditionally thought. Like polygonal tesserae, the little skate trabecular mineralization also forms an arrayed pattern, but the repeating units are in the form of trabecular struts interspersed by unmineralized regions, leading us to call them trabecular tesserae (Atake et al., 2019; Atake and Eames, 2021). Descriptions of a trabecular mineralization pattern in the endoskeleton of sharks or chimaeras are currently lacking, leaving open the possibility that this is a phenotype specific to the Batoidea superorder (including skates and rays) of the subclass Elasmobranchii. In some chimaeras, the surfaces of cartilage in the anterior fused vertebrae (synarcual) and chondrocranium have sheets of mineralization that abut one another (Pears et al., 2020; Seidel et al., 2020), but these do not have an arrayed pattern of repeating units, so perhaps they should not be called tesserae.

Recent evidence suggests that histological features of tesserae may vary according to the mineralization pattern. Traditional polygonal tesserae have distinct histological zones, with a cap zone superficial to a body zone near the surface of chondrichthyan cartilage (Atake et al., 2019; Berio et al., 2021; Kemp and Westrin, 1979; Seidel et al., 2016). While skates have polygonal tesserae, trabecular tesserae in little skate have very small or even absent cap zones overlying extensive body zones (Atake et al., 2019). Additionally, specific regions of tesserae termed spokes are hypermineralized and often devoid of cells, and spokes appear to be a common feature of tesserae regardless of their mineralization pattern (Atake et al., 2019; Pears et al., 2020; Seidel et al., 2020; Seidel et al., 2016). Careful 3D spatial analyses are needed to correlate histological zones and mineralization patterns of tesserae.

Table 1.

Character states of the mineralized chondrichthyan endoskeleton.

Traits do not apply to all regions of a given fish's skeleton.

Character	State	Description
mineralization patterns	polygonal tesserae (PT)	array of distinct polygonal units between the perichondrium and trabecular tesserae
	trabecular tesserae (TT)	array of distinct trabecular struts, often with hypermineralized spokes, below the perichondrium
	non-tesseral trabeculae (NTT)	non-arrayed, distinct trabecular struts, often with hypermineralized spokes, below the perichondrium
	areolar (AR)	continuous, compact sheets or rings in the notochordal sheath
	bone-like tissue (BLT)	continuous, compact regions between the perichondrium and a cartilaginous core, often in neural arches and cap zones
histological features	body zone (BZ)	distinct region of mineralized tissue below the perichondrium, containing Col2, rounded cell lacunae, and low-cellularity spokes
	cap zone (CZ)	distinct region of mineralized tissue between the perichondrium and body zone, containing tightly-wound Col1 fibers and elongated cell lacunae
	bone-like tissue (BLT)	mineralized tissue of tightly-wound Col1 fibres and elongated cell lacunae connected by canaliculi-like channels

Some chondrichthyans have mineralized endoskeletal tissues that have been characterized as bone-like (Atake and Eames, 2021). For example, the cap zone of tesserae displays bony features, like elongate cell lacunae in a mineralized ECM of densely packed type I collagen (Col1) fibers (Atake et al., 2019; Kemp and Westrin, 1979; Seidel et al., 2017; Seidel et al., 2016). Unlike the cap zone, the body zone displays typical features of hyaline cartilage, which is the main cartilage type in vertebrates (Goldring et al., 2006). The body zone consists of round chondrocyte lacunae embedded in a mineralized matrix abundant in loosely packed type II collagen (Col2) fibers and sulfated glycosaminoglycans (Enault et al., 2015; Seidel et al., 2017). As another example of bone-like tissue, several elasmobranch species have a non-tiled, mineralized perichondral tissue in their neural arches (Atake et al., 2019; Berio et al., 2021; Bordat, 1987; Eames et al., 2007; Kemp and Westrin, 1979; Seidel et al., 2016). The neural arch bone-like tissue demonstrates morphological and histological features of perichondral bone, including a compact mineralization pattern, elongate cell lacunae, canaliculi-like channels connecting adjacent lacunae, and tightly packed Col1 fibers (Bordat, 1987; Eames et al., 2007; Kemp and Westrin, 1979; Peignoux-Deville et al., 1982; Rossert and de Crombrugghe, 2002; Seidel et al., 2017). Bone-like tissues might be a defining feature of extant chondrichthyans, but relevant data from chimaeras are needed.

Finally, areolar mineralized tissue is generally considered a unique feature of the extant chondrichthyan endoskeleton, characterized mostly in elasmobranchs as large sheets of mineralization in the notochordal sheath of the vertebral body (centrum) (Didier, 1995; Ridewood and MacBride, 1921). Areolar mineralized tissue typically contains concentric lamellae of elongate lacunae in a fibrocartilage-like extracellular matrix and a compact mineralization pattern (Atake et al., 2019; Criswell et al., 2017; Dean and Summers, 2006; Eames et al., 2007). Mineralized centra are not thought to be common in chimaeras, but when present, they can appear as multiple mineralized rings in each vertebral segment, which contrasts with the grossly biconcave morphology of segmented centra in elasmobranchs (Gadow and Abbott, 1895).

With hopes of revealing shared and clade-specific features among chondrichthyans, here we clarify many of the unresolved issues highlighted above, proposing specific terminology for character states of the extant chondrichthyan endoskeleton (**Table 1**). Testing the hypothesis that extant chondrichthyans have homologous histological and morphological traits of endoskeletal mineralized tissues, adult tissues in several endoskeletal regions of the spotted ratfish *Hydrolagus colliei* (chimaera), small-spotted catshark *Scyliorhinus canicula* (shark), and little skate *Leucoraja erinacea* (skate) were characterized using synchrotron radiation and desktop micro-CT imaging, and histological and immunofluorescent assays. Tesseral mineralization, cap zones, and neural arch bone-like tissues were absent in the ratfish, but present in these elasmobranchs. Three separate analyses argued that the endoskeleton of this chimaera displays histological and morphological features of the embryonic little skate endoskeleton. While additional chondrichthyan species can bolster these findings, the data argue that trabecular and areolar mineralization patterns, but not tesserae, are shared among extant chondrichthyans. Considering fossil outgroups, we finish by inferring ancestral and derived character states of the chondrichthyan endoskeleton.

Results

Adult mineralized tissues in spotted ratfish had lower tissue mineral density (TMD) than small-spotted catshark or little skate

To assess whether chimaeras demonstrate skeletal mineralization in anatomical sites that are homologous to those of elasmobranchs, 3D rendering of micro-CT images of anterior regions of adult ratfish were qualitatively and quantitatively analyzed (**Fig. 1A,B**). Micro-CT images

revealed mineralized tissues in several endoskeletal regions, including neural arches and centra of segmented vertebrae (**Fig. 1C,D**), skeletal elements in the pharyngeal skeleton (**Fig. 1E**), and the fused vertebral synarcual (**Fig. 1F,G**). Although elasmobranchs mineralize neural arches in their precaudal and caudal vertebrae (Atake et al., 2019; Berio et al., 2021; Eames et al., 2007), mineralized neural arches were present mainly in precaudal vertebrae of ratfish (**Fig. 1C,D**; data not shown). Mineralized skeletal elements in the pharyngeal skeleton included the basihyal, Meckel's, ceratohyal, basibranchial, hypobranchials, and ceratobranchial (**Fig. 1E**). Spinal nerve foramina are common in the synarcual of cartilaginous fishes and were present in the ventral sides of the ratfish synarcual (**Fig. 1F**; Claeson, 2011; Johanson et al., 2015; Pears et al., 2020). Unlike the synarcual in skates and other batoids, where mineralized centra are not discernible (Atake et al., 2019; Claeson, 2011), the synarcual in ratfish had distinct mineralized centra for its entire length (**Fig. 1G**).

TMDs of the synarcual, neural arches, and centra in ratfish, catshark, and little skate were quantitated and compared, where possible, since sharks do not have a synarcual (**Fig. 1H**). Because little skate does not have centra within the synarcual, centra in the ratfish synarcual were digitally segmented and excluded for synarcual TMD data. The average TMD of the ratfish synarcual was 0.31± 0.11 gHA/cm^3, significantly lower than the TMD of the little skate synarcual, which was 0.94 ± 0.04 gHA/cm^3 (**Fig. 1H**; p = 6.5×10^{-4}). Similarly, the TMD of ratfish neural arches (0.40 ± 0.05 gHA/cm^3) was significantly lower than the TMD of neural arches of either catshark (0.81 ± 0.04 gHA/cm^3; p =9 x10^{-5}) or little skate (0.96 ± 0.04 gHA/cm^3; p =4.5×10^{-10}; **Fig. 1H**). The average TMD of ratfish centra (0.42 ± 0.10 gHA/cm^3) was also significantly lower than the TMD of centra of either catshark (0.84 ± 0.02 gHA/cm^3; p =1.1 x10^{-4}) or little skate (0.88 ± 0.05 gHA/cm^3; p =3.4 x10^{-5}; **Fig. 1H**). In ratfish, TMD of segmented centra within the synarcual was similar to that of centra in segmented vertebrae (0.46 ± 0.11 gHA/cm^5). These analyses showed that the synarcual, neural arches, and centra in ratfish had significantly lower TMDs compared to homologous elements in catshark and little skate.

Neural arches in ratfish lacked bone-like tissue

To clarify whether chimaeras have bone-like tissue like elasmobranchs, micro-CT and histological analyses of neural arches in segmented precaudal vertebrae of ratfish were compared to neural arches in segmented precaudal vertebrae of catshark and little skate. Mineralization of neural arches in ratfish was organized in a non-continuous pattern of small compact and trabecular regions (**Fig. 2A**). This mineralization pattern in spotted ratfish contrasted with the continuous mineralization pattern displayed by the compact, bone-like tissue in catshark and little skate (**Fig. 2B,C**; Atake et al., 2019; Berio et al., 2021). Furthermore, cross-section views demonstrated that mineralized tissues in ratfish were restricted to the medial and lateral periphery of the neural arches (**Fig. 2D**). By contrast, mineralization of bone-like tissues in catshark and little skate extended much deeper in the neural arch (**Fig. 2E,F**).

Histologically, Alizarin red staining of calcium showed that mineralized tissues in ratfish neural arches were much thinner and not continuous like bone-like tissues in catshark and little skate neural arches (**Fig. 2G-I**). Alcian blue staining of sulfated proteoglycans marked cartilage in most neural arch regions in ratfish, while only the cartilaginous core regions of catshark and little skate neural arches were Alcian blue-positive (**Fig. 2G-I**). The neural arch core in catshark was mostly unmineralized cartilage, while that of little skate was mineralized cartilage (**Fig. 2H,I**). Birefringence of picrosirius-stained, tightly packed collagen fibers (likely reticular) were observed in muscles lateral to neural arches in all three species (**Fig. 2J-L**). Birefringence was not observed in ratfish neural arches, whereas birefringence in neural arch bone-like tissues was observed in catshark and little skate (**Fig. 2K,L**). Most regions (including unmineralized cartilage and mineralized tissue) in ratfish neural arches demonstrated Col2 immunofluorescence (**Fig. 2J**). Only the cartilaginous core in neural arches of catshark and little skate demonstrated Col2

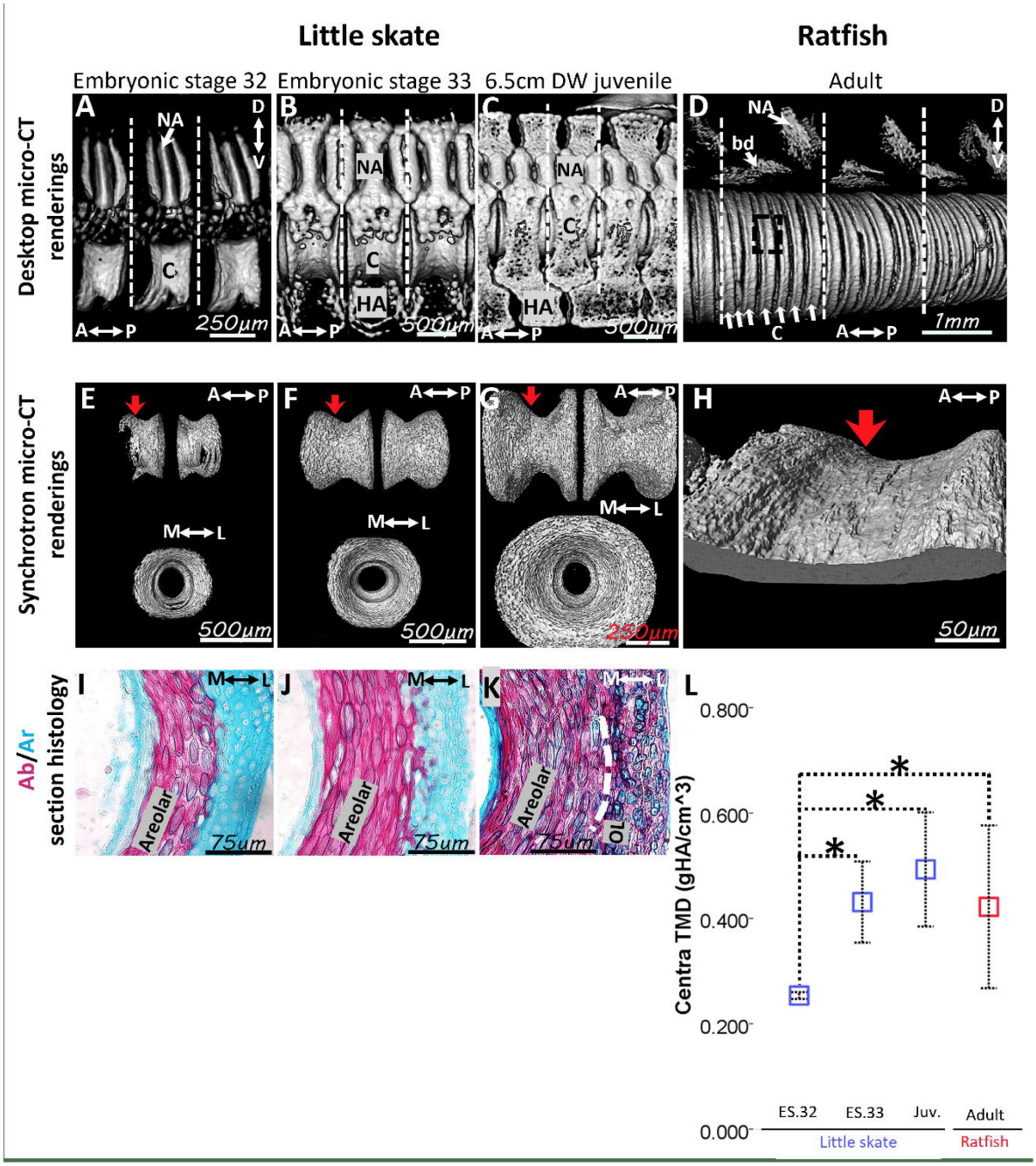


Figure 1.

Mineralized tissues were abundant in the spotted ratfish, but they had lower tissue mineral density (TMD) compared to the little skate and the small-spotted catshark.

(**A**) Photograph of the ratfish *Hydrolagus colliei* illustrating the anterior region that was micro-CT imaged. (**B-G**) Micro-CT 3D renderings of the anterior region of ratfish (B) showed mineralized tissues in neural arches, basidorsals, and centra in segmented vertebrae (C,D), in the ceratohyal and other skeletal elements in the pharyngeal skeleton (E), and in the fused vertebral synarcual (F,G). (**H**) Comparative quantitative analyses showed that TMD of the synarcual, neural arch, and centra in ratfish were significantly lower than TMDs of same regions in little skate and catshark. Abbreviations: A, anterior; bb, basibranchial; bd, basidorsal; bh, basihyal; c, centrum cb, ceratobranchial; ch, ceratohyal; D, dorsal; hbs, hypobranchials; L, left; MK, Meckel's; na, neural arch; P, posterior; R, right; snf, spinal nerve foramina; V, ventral. * indicates statistically significant comparison (p<0.05). Scale bars: as indicated.

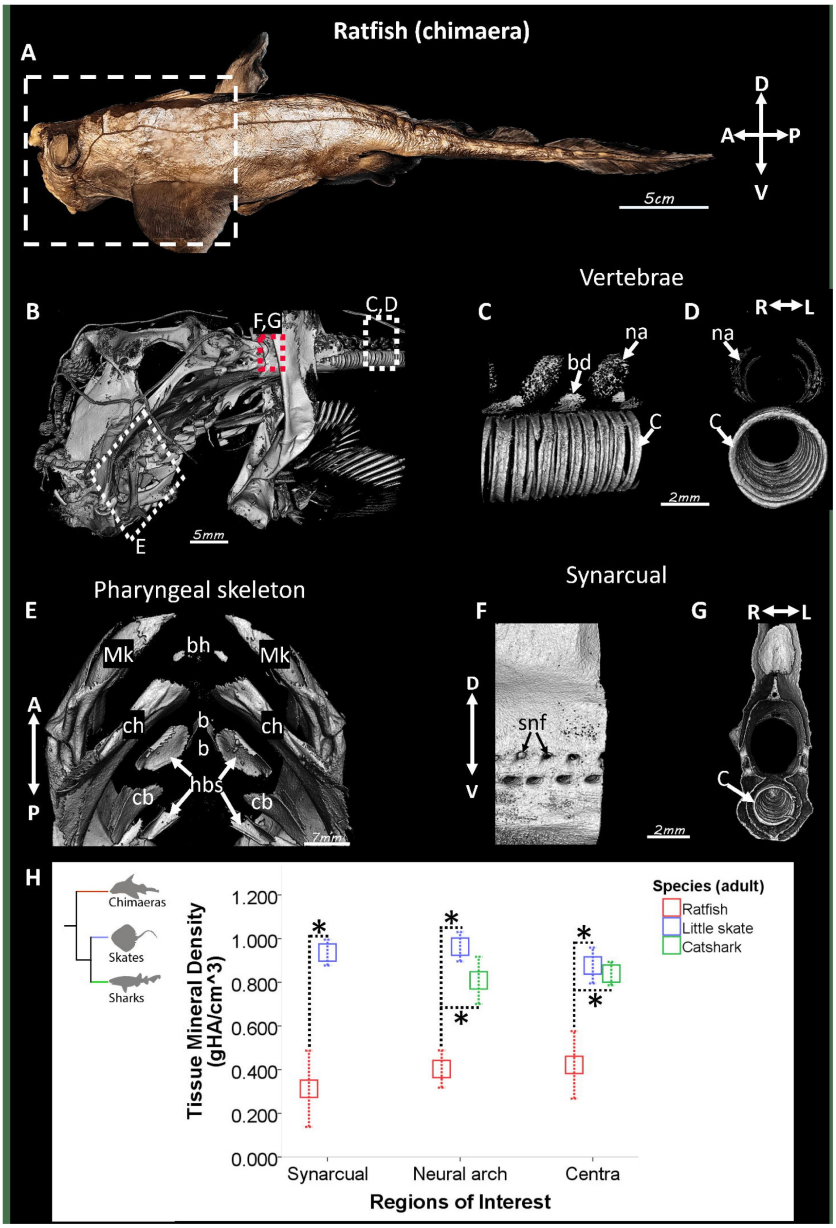


Figure 2.

Ratfish lack neural arch bone-like tissue.

(**A-C**) Micro-CT 3D renderings showed a non-continuous pattern of mineralized tissues in neural arches of segmented vertebrae in ratfish (A), compared to the continuous mineralization pattern of compact, bone-like tissue in catshark (B) and little skate (C). (**D-F**) Micro-CT 2D virtual slices showed that mineralized tissues (arrows) in ratfish neural arches were restricted to medial and lateral peripheries (D), whereas the bone-like tissue in catshark and little skate extended much deeper in the neural arch (E,F). (**G-I**) Alizarin red staining of histological sections confirmed micro-CT representation of mineralization patterns in ratfish (G), catshark (H), and little skate (I) neural arches, and Alcian blue revealed cartilaginous regions. (**J-L**) Picrosirius red staining did not reveal birefringent collagen fibers in ratfish neural arches (J), but such staining was abundant in bone-like tissues in catshark (K) and little skate (L). Birefringent fibers (likely reticular) were present in muscles (vertical arrows) on the lateral sides of neural arches in all species. Col2 immunostaining confirmed Alcian blue staining, identifying cartilage regions in all species. The neural arch core in catshark (K) and little skate (L) consisted of mostly unmineralized cartilage and mineralized cartilage, respectively. Abbreviations: 2D, two-dimensional; 3D, three-dimensional; Ab, Alcian blue; Ar, Alizarin red; BLT, bone-like tissue; c, cartilage; Col2, collagen type 2; D, dorsal; IF, immunofluorescence; L, lateral; M, medial; MC, mineralized cartilage; PSR, picrosirius red; V, ventral. Scale bars: as indicated.

immunofluorescence (**Fig. 2K,L**). These results showed that bone-like tissue was absent in neural arches of ratfish, and the non-continuous pattern of mineralized tissue in ratfish neural arches was mineralized cartilage.

Novel characterization of non-tesseral trabecular mineralization in ratfish and catshark showed the absence of a bone-like cap zone

To clarify whether tesserae are defining endoskeletal features of extant chondrichthyans, morphological patterns and histological features of mineralized tissues were characterized in ceratohyals of ratfish, caudal vertebrae of catshark, and precaudal vertebrae and hyomandibulae of little skate. Desktop micro-CT renderings of ratfish ceratohyals and catshark haemal arches revealed mineralized tissues with a trabecular pattern like that of trabecular tesserae in the vertebral neural spine of little skate (**Fig. 3A-G**). However, the trabecular pattern in ratfish and catshark was not as clearly organized into arrays of repeating trabecular units like that of trabecular tesserae in little skate, and for this reason, trabecular patterns in ratfish and catshark were characterized as non-tesseral. In the hyomandibulae and other skeletal elements of little skate, including synarcual, propterygium, and vertebral transverse processes, a trabecular mineralization pattern occurred underneath the superficial polygonal tesseral pattern (**Fig. 3D,H**,H'; Atake et al., 2019). Notably, many regions in ratfish (vertebrae, pharyngeal skeleton, and the synarcual) and catshark (vertebrae and ceratohyals) did not exhibit the polygonal mineralization pattern typical of tesserae (Atake et al., 2019; Kemp and Westrin, 1979; Maisey, 2013; Marramà et al., 2019; Seidel et al., 2016; Wilga and Ferry, 2015).

To assess whether trabecular mineralized tissues in ratfish, catshark, and little skate exhibit similar histological features, cross sections of tissues with non-tesseral patterns were compared to those with tesseral patterns. Non-tesseral mineralized tissues in Alcian blue-positive perichondral regions in ratfish ceratohyals and catshark haemal arches stained with Alizarin red (**Fig. 3I,J**; perichondrium marked by straight dashed line). The discrete organization of mineralized tissues and Alcian blue and Alizarin red staining patterns of non-tesseral mineralized tissues in ratfish and catshark were similar to the tesseral body zone in little skate (**Fig. 3I-L**). However, Alcian blue-positive cartilage separated Alizarin red-positive non-tesseral mineralized tissues from overlying perichondrium in ratfish and catshark (**Fig. 3I,J**). A bone-like cap zone just below the perichondrium was only present in tesseral mineralized tissues in little skate (**Fig. 3K,L**; perichondrium marked by straight dashed line). The bone-like cap zone stained more intensely than the body zone with Alizarin red and was relatively smaller and larger in trabecular tesserae and polygonal tesserae, respectively (**Fig. 3K,L**). Perichondrium of both non-tesseral and tesseral mineralized tissues stained with the general connective tissue dye fast green (**Fig. 3M-P**). Matrix of the little skate bone-like cap zone was fast green-positive and had birefringent collagen fibers, but these staining patterns were not observed in non-tesseral mineralized tissues in ratfish and catshark (**Fig. 3M-T**). Instead, regions just below the perichondrium of ratfish ceratohyals and catshark haemal arches stained for sulfated proteoglycans by Safranin O and were Col2-positive cartilage (**Fig. 3M,N**,Q,R). Staining patterns of non-tesseral mineralized tissues in ratfish and catshark were generally consistent with those of mineralized cartilage in the tesseral body zone of little skate, although chondrocyte lacunae in these regions were not obvious in ratfish (**Fig. 3I-T**). Therefore, histological features of trabecular mineralized tissues in ratfish ceratohyals and catshark haemal arches were similar to only the body zones of tesserae in little skate, which consisted of mineralized cartilage.

Spatial organization of histological features correlated with trabecular and polygonal mineralization patterns

Given that trabecular mineralized tissues typically have small or even absent cap zones, and polygonal tesserae typically have large cap zones, histology and micro-CT segmentation were used to see whether there was a correlation between histological features and mineralization patterns

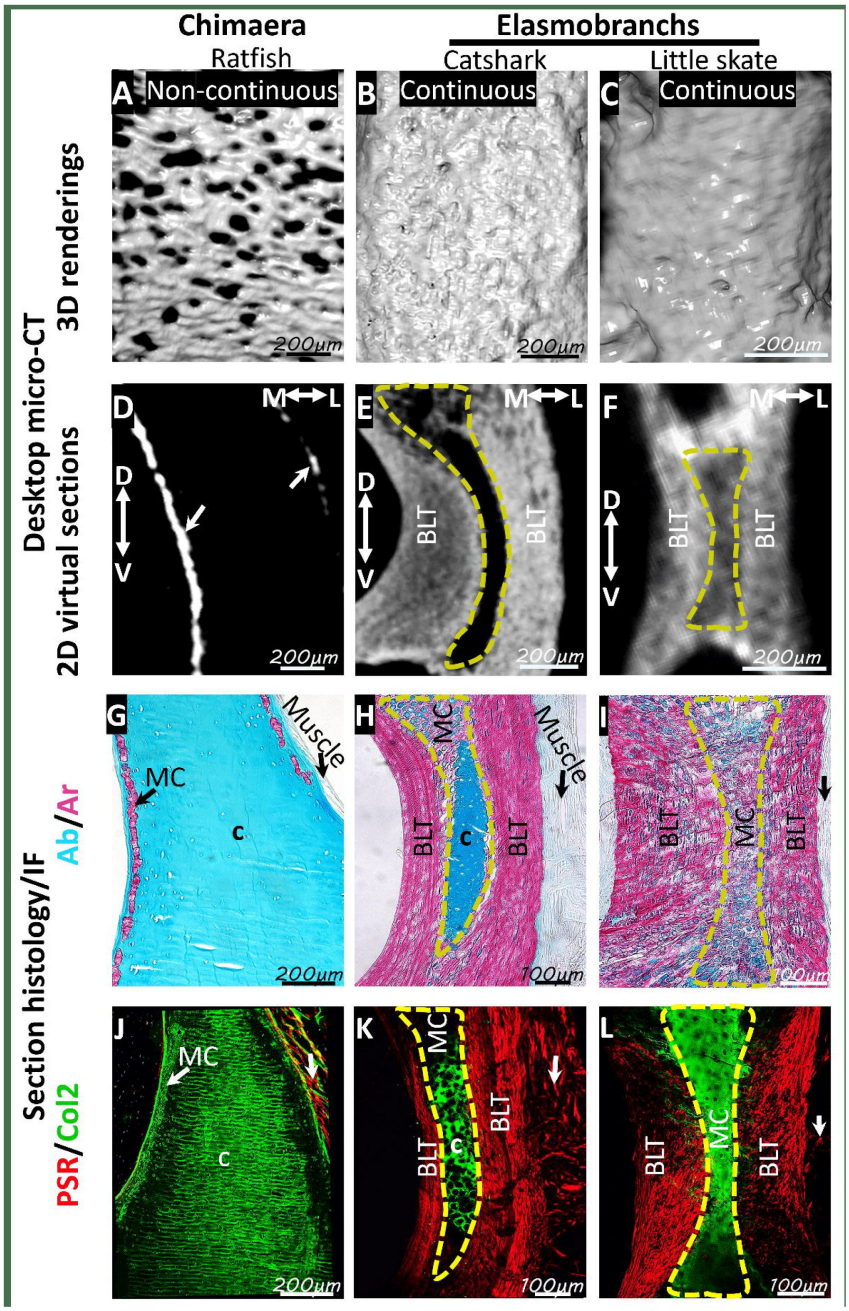


Figure 3.

Ratfish, catshark, and little skate all showed trabecular mineralization, but ratfish and catshark did not have a bone-like cap zone.

(**A-H**) Micro-CT renderings of the ratfish ceratohyal (A,E) and catshark caudal vertebra (B,F) demonstrated non-tesseral trabecular mineralization, while little skate hyomandibula had tesseral trabecular mineralization (C,G). Little skate precaudal vertebra had tesseral polygonal mineralization (D,H) overlying tesseral trabecular mineralization (H'). Regions for hi-mag views in panels E-H' are indicated in corresponding panels of A-D. (**I-P**) Alcian blue/Alizarin red and Safranin O/fast green staining of histological sections demonstrated that the body zone of non-tesseral or tesseral trabecular mineralized tissues in ratfish (I,M), catshark (J,N), and little skate (K,L,O,P) was mineralized cartilage, while the cap zone of tesseral polygonal mineralization was bone-like (K,L,O,P). (**Q-T**) Col2 immunostaining of adjacent sections to panels I-P confirmed cartilage regions in all species, while picrosirius red staining confirmed the bone-like tesseral cap zone in little skate. Abbreviations: Ab, Alcian blue; Ar, Alizarin red; Bz, body zone; c, cartilage; Col2, collagen type 2; Cz, cap zone; D, dorsal; IF, immunofluorescence; L, lateral; M, medial; PSR, picrosirius red; NA, neural arch; NS, neural spine; V, ventral. Scale bars: as indicated.

in ratfish and little skate. After demineralization, non-tesseral tissues in ratfish and tesseral tissues in little skate exhibited spokes, which are regions of very low cellularity in the body zone that often appeared as tears or sectioning artifacts and did not stain strongly with any Trichrome dye (**Fig. 4A-C**). Non-spoke regions of the body zone stained either slightly blue for collagen fibers with Trichrome's aniline blue or magenta with Trichrome's acid fuchsin, and these staining patterns were consistent in non-tesseral and tesseral tissues. Tightly-wound collagen fibers were marked by Trichrome's acid fuchsin, but only in the larger and smaller cap zones of little skate polygonal and trabecular tesserae, respectively (**Fig. 4A-C**). Also, the presence of elongate lacunae distinguished the cap zone from other tesseral regions (**Fig. 4A-C**).

Enough histological features were also visible in synchrotron radiation micro-CT renderings (**Fig. 4D-F**), making it possible to segment and analyze the spatial correlation between mineralization patterns and histological features, such as cap zone and spokes (red and blue, respectively, in **Fig. 4G-I**). Segmentation of 3D renderings of deep surface views showed that hypermineralized spokes were localized to non-tesseral and tesseral trabecular mineralization patterns in ratfish and little skate, respectively (blue in **Fig. 4J-L**). Unlike the well-organized spokes seen in trabecular patterns of little skate tesserae, spokes in ratfish trabecular mineralized tissues were patchy and less-organized. Therefore, both non-tesseral and tesseral trabecular mineralization patterns correlated with, and appeared to derive from, spokes and non-spoke regions within body zones.

Unlike little skate polygonal tesserae, trabecular mineralized tissues in ratfish and trabecular tesserae in skate both displayed identical trabecular mineralization patterns whether viewed from the superficial or deep surfaces of the cartilage (**Fig. 4J,K**,M,N). The smaller cap zones in trabecular tesserae were discernible on the superficial surface, but they were not as laterally extensive as cap zones of polygonal tesserae (**Fig. 4H,I**,N,O). Thus, larger and laterally extensive cap zones appeared to be the reason why polygonal tesserae displayed a superficial polygonal mineralization pattern.

Ratfish centra contained areolar mineralized tissue

To clarify whether ratfish, catshark, and little skate have areolar mineralized tissue, morphological and histological features of centra were revealed by desktop micro-CT and section histology. Precaudal vertebrae in ratfish had multiple rings of centra per vertebral segment (i.e., unit with paired neural arches and basidorsals; **Fig. 5A**). Each centrum in ratfish precaudal vertebrae demonstrated a compact mineralization pattern like that of centra in the catshark and little skate (**Fig. 5A-C**). However, no ratfish centrum displayed the typical biconcave gross morphology associated with elasmobranch centra (**Fig. 5A-C**; (Atake et al., 2019; Clement, 1992; Criswell et al., 2017).

Alizarin red and Alcian blue section histology showed that centra in ratfish, catshark, and little skate comprised of compact mineralized tissue spanning different numbers of centrum layers. The ratfish centrum only had mineralized tissue in the middle layer, but an outer layer of the catshark centrum had mineralized cartilage, while inner and outer layers of the little skate centrum contained mineralized cartilage (**Fig. 5D-I**). Areolar mineralized tissues in the middle layer of the centrum of ratfish, catshark, and little skate had elongate cell lacunae organized in concentric lamellae (inserts in **Fig. 5G-I**; Atake et al., 2019; Criswell et al., 2017; Eames et al., 2007), and they were birefringent (data not shown). Markers of cartilage differed in areolar mineralized tissue among ratfish, catshark, and little skate (**Fig. 5J-O**). In ratfish, areolar mineralized tissue had relatively strong Safranin O staining and Col2 immunofluorescence (**Fig. 5J,M**). Areolar mineralized tissue in catshark demonstrated weak Safranin O staining and Col2 immunofluorescence (**Fig. 5K,N**). By contrast, areolar mineralized tissue in little skate did not display Safranin O staining or Col2 immunofluorescence (**Fig. 5L,O**). Overall, these data suggested that ratfish have areolar mineralized tissue.

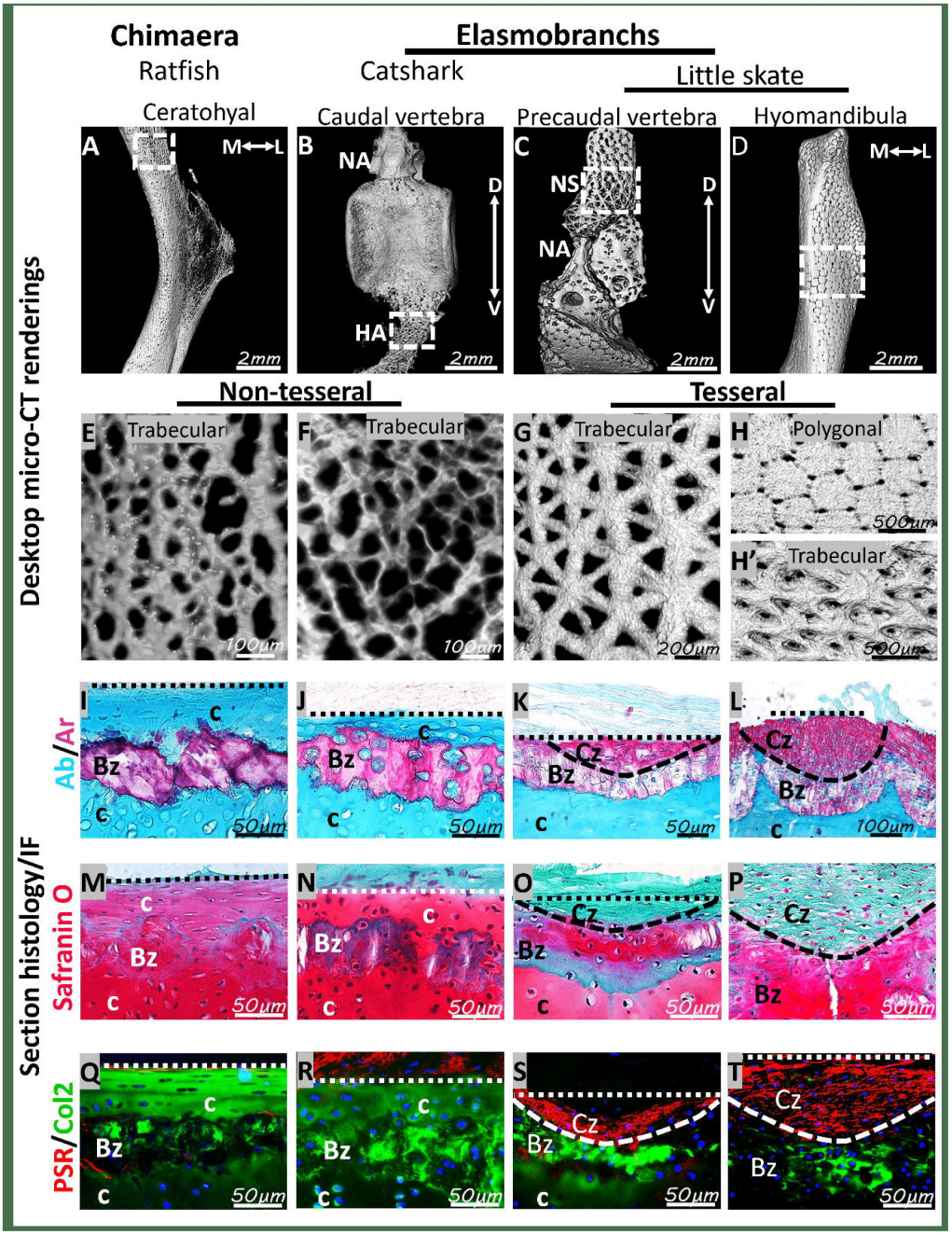


Figure 4.

Micro-CT segmentation clearly correlated histological features and mineralization patterns.

(**A-C**) Trichrome staining showed that spokes in the body zone of ratfish non-tesseral trabeculae (A) or little skate trabecular (B) or polygonal (C) tesserae had low cellularity and were unstained, while intense acid fuchsin staining marked the smaller and larger cap zones of little skate trabecular (B) and polygonal (C) tesserae, respectively. (**D-I**) 3D renderings of synchrotron micro-CT data showed that hypermineralized spokes (*, segmented in blue) were in the body zone of ratfish non-tesseral trabeculae (D,G) or little skate trabecular (E,H) or polygonal (F,I) tesserae. Tesseral cap zones could also be segmented clearly (red) in little skate (E,F,H,I). (**J-O**) Segmented 3D renderings demonstrated that spokes in ratfish were patchy in deep (J) or superficial (M) views, while little skate spokes displayed a radiating pattern traversing most of the trabecular patterns in either trabecular (K,N) or polygonal (L,O) tesserae. The smaller cap zone in trabecular tesserae (N) was not laterally extensive enough to display a superficial polygonal pattern like that of polygonal tesserae (O). Abbreviations: Bz, body zone; Cz, cap zone. Scale bars: as indicated.

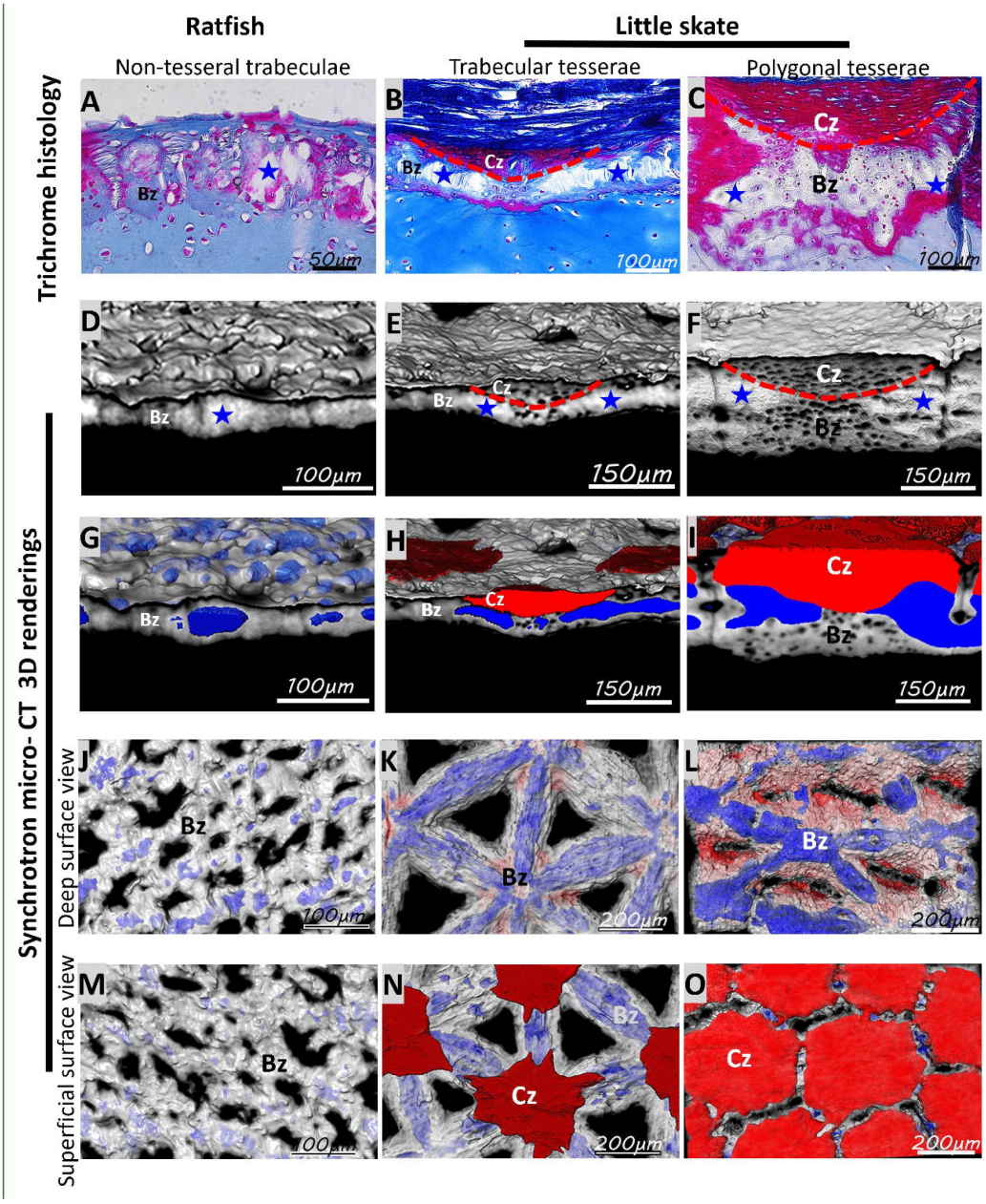


Figure 5.

Ratfish centra had the same features as areolar mineralized tissues in little skate and catshark.

(**A-C**) Micro-CT renderings demonstrated that centra in ratfish (A), catshark (B), and little skate (C) all had a compact mineralization, but ratfish centra were organized in multiple rings and did not display the gross biconcave morphology seen in catshark or little skate. (**D-O**) Alcian blue/Alizarin red and Safranin O section histology and Col2 immunostaining demonstrated that mineralized centra in ratfish (D,G,J), catshark (E,H,K), and little skate (F,I,L) all comprised a middle layer with elongate lacunae in concentric lamellae, but in ratfish this was mineralized cartilage, while catshark and little skate had little to no Safranin O or Col2 staining, respectively, in this region. Catshark mineralized centra also comprised the outer layer, while little skate mineralized centra also comprised both inner and outer layers. These additional centra layers had rounded cells embedded in matrix of weak Safranin O staining and Col2 immunofluorescence. Regions for hi-mag views in panels G-O are indicated in corresponding panels of D-F; regions for hi-mag inserts in panels G-I are indicated in those panels. Abbreviations: A, anterior; Ab, Alcian blue; Ar, Alizarin red; c, cartilage; Col2, collagen type 2; D, dorsal; IF, immunofluorescence; IL, inner layer; L, lateral; M, medial, OL, outer layer; P, posterior; V, ventral. Scale bars: as indicated; inserts in G-I are 20 µm.

Qualitative and quantitative features of centra in the adult ratfish were similar to those of little skate embryos

The biconcave centra morphology is thought to be acquired in elasmobranchs during development when the perichordal sheath becomes constricted by mesenchymal cells (Arratia et al., 2001; Gadow and Abbott, 1895; Kölliker, 1860). To shed light on differences in adult centra morphology, morphological and histological features of centra in a developmental series of little skate were compared to those of adult ratfish. In little skate embryos and hatched juveniles, desktop micro-CT renderings showed mineralization in the centrum and pairs of neural arches, basidorsals, and haemal arches of each vertebral segment (**Fig. 6A-C**). Expanding upon recent work showing that anterior and posterior segments of somites fuse during skate vertebral development, similar to tetrapods (Criswell and Gillis, 2020), independent anterior and posterior mineralizations of each interneural appeared to later fuse during development. For example, independent mineralization of the posterior and anterior interneurals were visible in stage 32 little skate embryos, whereas the dorsal and ventral aspects of each appeared fused by stage 33, and they were fully fused in a 6.5cm DW juvenile (**Fig. 6A-C**). In adult ratfish, each vertebral segment of paired neural arches and basidorsals had an average of six centra (**Fig. 6D**). Digital segmentation of synchrotron radiation (SR) micro-CT renderings showed that centra in little skate stage 32 embryos were slightly constricted in the middle of each element's anterior-posterior axis, and the extent of this constriction progressed through 6.5 cm disc width (DW) juveniles, when the classic biconcave morphology was present (**Fig. 6E-G**). SR micro-CT renderings showed that adult ratfish centra were constricted to a similar extent as younger centra in little skate embryos (**Fig. 6E,F**,H). In little skate embryos, Alcian blue and Alizarin red section histology showed that the areolar mineralized tissue initiated in the middle layer of the centrum and had elongate cell lacunae organized in concentric lamellae, similar to that observed in adult ratfish (**Fig. 5D**; **Fig. 6I,J**). At later developmental stages of little skate, such as 6.5 cm DW juveniles, the outer layer of the centrum also demonstrated mineralized cartilage (**Fig. 6K**).

To further confirm these morphological and histological similarities of centra in little skate embryos and adult ratfish, TMDs of centra in the developmental series of little skate were compared to that of adult ratfish. TMD of little skate centra increased significantly from stage 32 to stage 33 embryos (p = 9.5 x10^{-3}), while TMD of 6.5 cm DW juveniles was only significantly higher than TMD of stage 32 embryos (**Fig. 6L**; p =9.6 x10). TMD of adult ratfish centra was significantly higher than TMD of centra in little skate stage 32 embryos, but it was statistically indistinguishable from TMD of centra in little skate stage 33 embryos and 6.5 cm DW juveniles (**Fig. 6L**). Therefore, adult ratfish centra had morphological, histological, and TMD similarities with centra from little skate embryos, particularly those at stage 33.

Discussion

Which tissues are common in the extant chondrichthyan endoskeleton? Bone-like and areolar tissues have been characterized in several members of the chondrichthyan subclass Elasmobranchii, such as sharks, skates, and rays (Atake et al., 2019; Berio et al., 2021; Bordat, 1987; Kemp and Westrin, 1979; Ørvig, 1951; Peignoux-Deville et al., 1982; Seidel et al., 2016). In addition, polygonal tesserae are traditionally associated with chondrichthyans (Atake et al., 2019; Kemp and Westrin, 1979; Maisey, 2013; Marramà et al., 2019; Seidel et al., 2016; Wilga and Ferry, 2015), but trabecular tesserae were recently characterized in skates and rays (Atake et al., 2019; Jayasankar et al., 2020).

Two main qualities of this paper go a long way in revealing features of the ancestral chondrichthyan endoskeleton. First, we explicitly identifed discrete character states of extant chondrichthyan endoskeletons (**Table 1**), helping to unify terminology of important features for

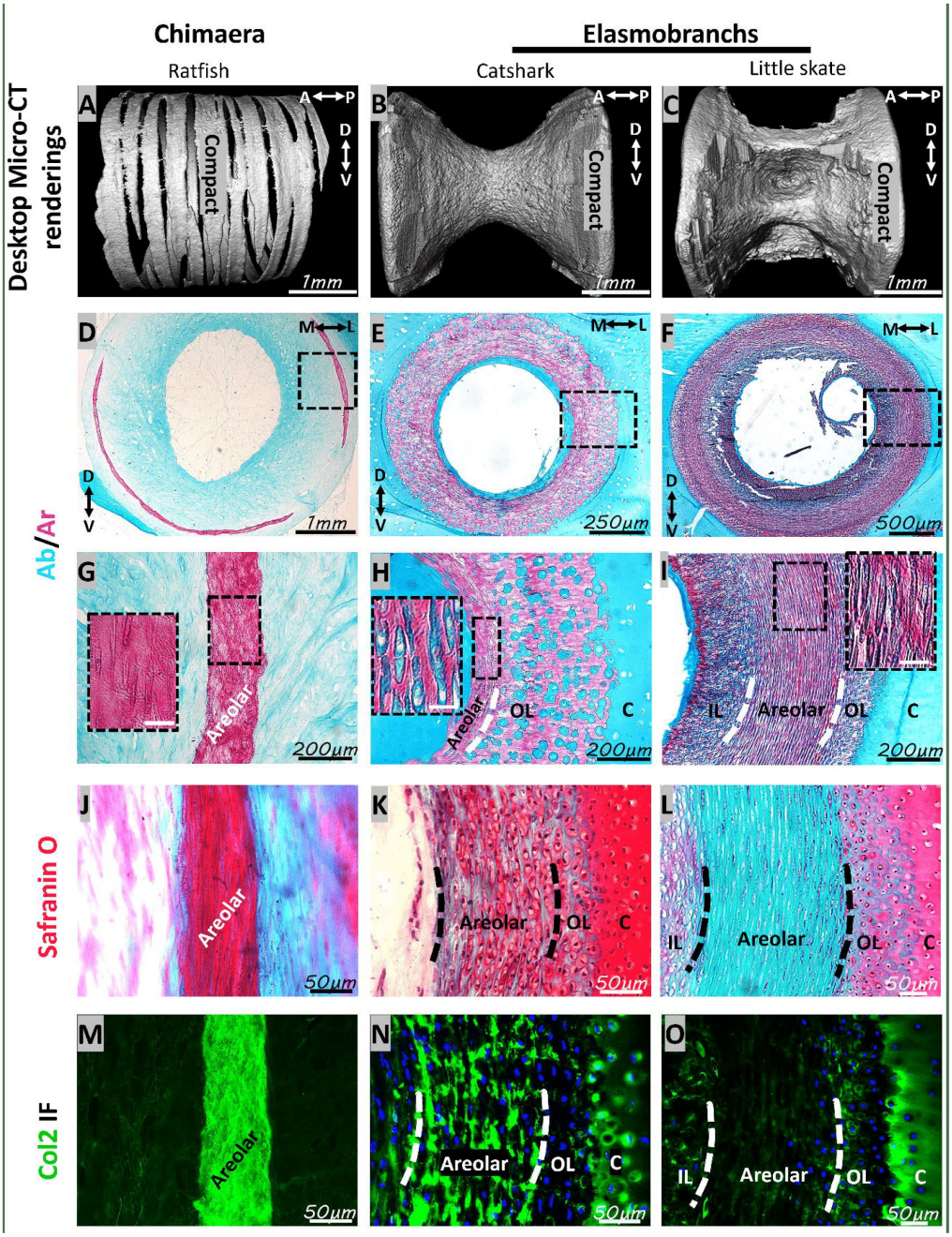


Figure 6.

Morphological and histological features of centra were shared between little skate embryos and juveniles and adult ratfish.

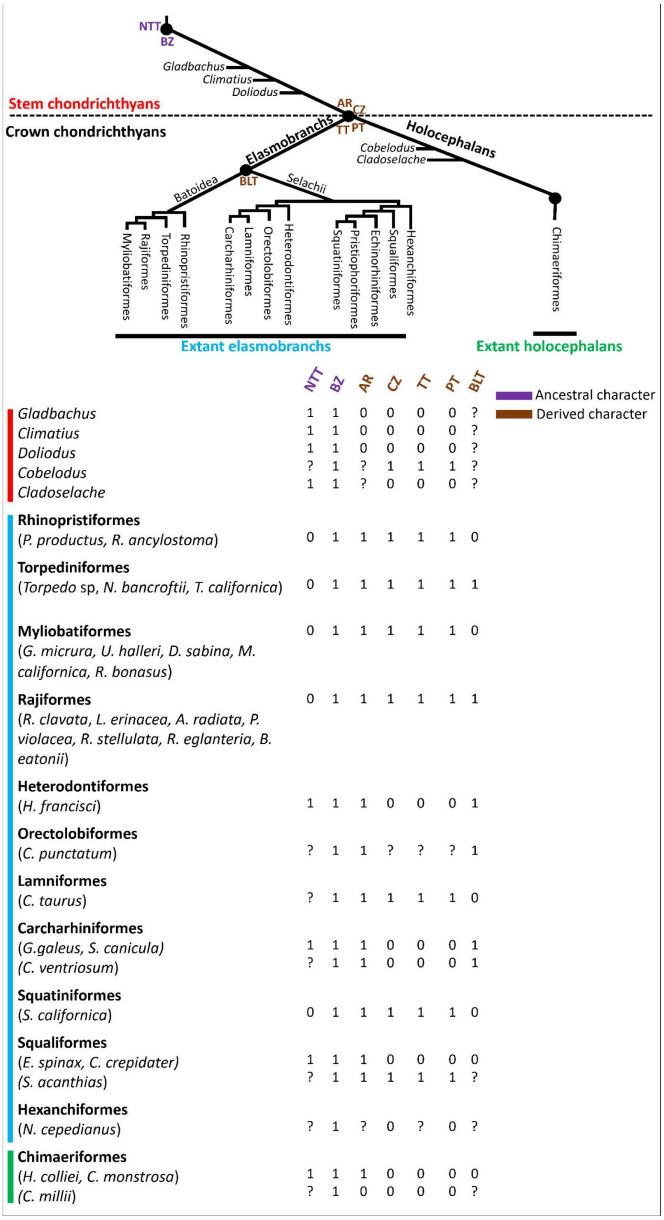
(**A-D**) Desktop micro-CT renderings showed centra and other mineralized structures in vertebral segments (dashed lines) of little skate embryos and juveniles (A-C) and adult ratfish (D). (**E-H**) Synchrotron micro-CT renderings of centra in little skate embryos and juveniles (E-G) and adult ratfish (H) showed that centra in the ratfish were slightly constricted, like those of little skate embryos (red arrows). (**I-K**) Alcian blue/Alizarin red section histology showed that areolar mineralized tissue constituted the only centra layer in little skate embryos (I,J), whereas an outer mineralized centra layer was present in 6.5 cm DW juveniles (K). (**L**) Tissue mineral density (TMD) of centra in ratfish was similar to those of stage 33 embryos and 6.5 cm DW juveniles of little skate. Abbreviation: A, anterior; Ab, Alcian blue; Ar, Alizarin red; bd, basidorsal; C, centrum; HA, haemal arch; L, lateral; M, medial; NA, neural arch; OL, outer layer; P, posterior.* indicates statistically significant comparison (p<0.05). Scale bars: as indicated.

the growing chondrichthyan research community. Second, we presented extensive, side-by-side comparative data across various extant chondrichthyans, two elasmobranch species (small-spotted catshark and little skate) and one chimaera/holocephalan (spotted ratfish). Including ratfish is another critical aspect of this paper, because holocephalans are generally understudied, but must be considered when inferring ancestral chondrichthyan traits. Importantly, previous characterization of skeletal mineralization in other adult chimaeridae neither focused on segmented neural arches to analyze bone-like tissues or areolar mineralization, nor clarified histological zones of tesserae (Berio et al., 2021; Debiais-Thibaud, 2018; Pears et al., 2020; Seidel et al., 2020)

Our new data and other published data across various extant and fossil chondrichthyan species can be compiled to compare character states of the extant endoskeleton and infer the ancestral condition (**Fig. 7**). In what appears to be a novel character trait of crown chondrichthyans, trabecular mineralization occurs in many endoskeletal regions in several elasmobranch and chimaera species, including some fossil chondrichthyans (Atake et al., 2019; Coates et al., 2018; Jayasankar et al., 2020; Maisey et al., 2021; Ørvig, 1951). Among extant chondrichthyans, trabecular mineralization always forms near the surface of cartilage, but the exact mineralization pattern varies. Little skate, along with all other batoids and some selachians examined, have arrayed patterns of discrete trabecular units, similar to the repeating polygonal units of traditional tesserae, supporting their description as trabecular tesserae. On the other hand, a non-arrayed trabecular mineralization pattern, not emphasized in chondrichthyans before, appears to be present in catshark and some other selachians and in ratfish and all other chimaeras examined, including *Callorhinchus milii* and *Chimaera monstrosa* (Pears et al., 2020; Seidel et al., 2020). To distinguish this pattern from the regular, arrayed patterns of polygonal and trabecular tesserae, we propose the term “non-tesseral trabecular mineralization”. Given that some fossil chondrichthyans, such as *Gladbachus*, *Climatius*, *Doliodus*, and *Cobelodus*, also appear to display this mineralization pattern in their endoskeletons (Coates et al., 2018; Maisey et al., 2021; Ørvig, 1951), non-tesseral trabecular mineralization might be a new plesiomorphic (ancestral) character of chondrichthyans (Maisey et al., 2021).

Bone-like tissues do not appear to be shared among extant chondrichthyans, while areolar mineralization does seem to be shared (**Fig. 7**). Morphological and histological features of bone-like tissues in neural arches or tesseral cap zones are present in catshark, little skate, and many other elasmobranch species, but absent in ratfish and *C. monstrosa* (Atake et al., 2019; Berio et al., 2021; Eames et al., 2007; Kemp and Westrin, 1979; Seidel et al., 2020; Seidel et al., 2017; Seidel et al., 2016; Wurmbach, 1932). While these data refute the hypothesis that bone-like tissues generally are a symplesiomorphic (shared ancestral) feature of extant chondrichthyans, they further support the hypothesis that neural arch perichondral bone-like tissue is a synapomorphic (shared derived) character of elasmobranchs, but subsequently lost in several lineages (Atake et al., 2019; Berio et al., 2021; Maisey et al., 2021). On the other hand, similar features of centra are observed in ratfish, catshark, little skate, and many other extant chondrichthyans examined (Atake et al., 2019; Criswell et al., 2017; Dean and Summers, 2006; Debiais-Thibaud, 2019; Eames et al., 2007; Porter et al., 2006). Since most data on areolar mineralization focussed on elasmobranchs (Criswell et al., 2017; Eames et al., 2007; Ridewood and MacBride, 1921), histological features of multiple-ringed centra in other chimaera should be determined (Didier, 1995). Stem chondrichthyans did not appear to mineralize their centra, so data from extant chondrichthyans argue that areolar mineralized tissue is likely a synapomorphic character of crown chondrichthyans (Frey et al., 2019; Maisey et al., 2021; Miles, 1970).

Cap and body zones are histological terms that traditionally describe chondrichthyan tesserae (Dean and Summers, 2006; Kemp and Westrin, 1979), but data from adult samples of ratfish, catshark, little skate, and several other species argue that the body zone is the only histological zone of endoskeletal mineralization shared by extant chondrichthyans (**Fig. 7**). Recent



	NTT	BZ	AR	CZ	TT	PT	BLT
Gladbachus	1	1	0	0	0	0	?
Climatius	1	1	0	0	0	0	?
Doliodus	1	1	0	0	0	0	?
Cobelodus	?	1	?	1	1	1	?
Cladoselache	1	1	?	0	0	0	?
Rhinopristiformes (*P. productus, R. ancylostoma*)	0	1	1	1	1	1	0
Torpediniformes (*Torpedo* sp, *N. bancroftii, T. californica*)	0	1	1	1	1	1	1
Myliobatiformes (*G. micrura, U. halleri, D. sabina, M. californica, R. bonasus*)	0	1	1	1	1	1	0
Rajiformes (*R. clavata, L. erinacea, A. radiata, P. violacea, R. stellulata, R. eglanteria, B. eatonii*)	0	1	1	1	1	1	1
Heterodontiformes (*H. francisci*)	1	1	1	0	0	0	1
Orectolobiformes (*C. punctatum*)	?	1	1	?	?	?	1
Lamniformes (*C. taurus*)	?	1	1	1	1	1	0
Carcharhiniformes (*G.galeus, S. canicula*)	1	1	1	0	0	0	1
(*C. ventriosum*)	?	1	1	0	0	0	1
Squatiniformes (*S. californica*)	0	1	1	1	1	1	0
Squaliformes (*E. spinax, C. crepidater*)	1	1	1	0	0	0	0
(*S. acanthias*)	?	1	1	1	1	1	?
Hexanchiformes (*N. cepedianus*)	?	1	?	0	?	0	?
Chimaeriformes (*H. colliei, C. monstrosa*)	1	1	1	0	0	0	0
(*C. millii*)	?	1	0	0	0	0	?

Figure 7.

Character states of mineralized tissues in various extant and fossil chondrichthyan species suggest shared and derived features.

Non-tesseral trabecular mineralization (NTT) is likely a symplesiomorphic (shared ancestral) character of stem and crown chondrichthyans, because it is pervasive in fossil and extant chondrichthyans. Polygonal (PT) and trabecular (TT) tesseral mineralization patterns appear to be absent in extant holocephalans and stem chondrichthyans, but their presence in some extant elasmobranchs and a fossil holocephalan suggest that these tesseral mineralizations are synapomorphic (shared derived) characters of crown chondrichthyans, subsequently lost in extant holocephalans. Areolar (AR) mineralized tissue is present in extant elasmobranchs and holocephalans, but its absence in stem chondrichthyans suggests that it is a synapomorphic character of crown chondrichthyans. The absence of neural arch bone-like tissue (BLT) in holocephalans further supports the conclusion that it is a synapomorphic character of elasmobranchs. The presence of a histological body zone (BZ) in either tesseral or non-tesseral mineralized tissues of stem and crown chondrichthyans suggests that the body zone is a symplesiomorphic character of all chondrichthyans. The histological cap zone (CZ) in some elasmobranchs, along with the inference that polygonal tesserae in a fossil holocephalan means that they had cap zones, suggests that the cap zone is also a synapomorphic character of crown chondrichthyans. 0, 1, andindicate absence, presence, and unknown, respectively, of characters for the listed species. See Supplemental Table 2 for downloadable character matrix.

histological data of tesserae are few and limited to sharks, skates, and rays, where cap and body zones have been reported (Atake et al., 2019; Berio et al., 2021; Eames et al., 2007; Enault et al., 2015; Kemp and Westrin, 1979; Seidel et al., 2017). While birefringent fibers marked the cap zones in both polygonal and trabecular tesserae in little skate, non-tesseral trabeculae in catshark and ratfish did not have discernible cap zones. In contrast to commonly-accepted knowledge, these catshark data suggest that even sharks do not always have the cap zone. Taken alongside recent published data (Maisey et al., 2021; Pears et al., 2020; Seidel et al., 2020), no living species of chimaera likely has cap zones. On the other hand, Safranin O staining and Col2 immunofluorescence showed that histological body zones generated the mineralized endoskeletal cartilage of ratfish, catshark, and little skate. In total, these data suggest that the ancestral stem chondrichthyan generated most of its endoskeletal mineralization with histological body zones, although as the next paragraph highlights, cap zones might have been lost secondarily in chimaera and some shark species.

Digitally segmented 3D regions of micro-CT renderings showed, for the first time, exact spatial correlations between histological zones and mineralization patterns, potentially impacting histological interpretations of fossils. Strikingly, large and laterally extensive cap zones were the basis of the traditional polygonal tesseral mineralization pattern in little skate. Accordingly, similar polygonal tesserae recently described in fossil holocephalans likely have cap zones in addition to body zones and a trabecular tesseral mineralization underlying the polygonal mineralization pattern, and these might have been lost secondarily during holocephalan evolution (**Fig. 7**; Atake et al., 2019; Pears et al., 2020). Increased descriptive power from synchrotron imaging of polygonal tesserae in fossil elasmobranchs and holocephalans can clarify this point. Two other correlations were identified here in body zones. First, spokes were located in body zones. Second, spoke and non-spoke regions of the body zone comprised the trabecular mineralization patterns, regardless of whether these were organized as arrayed tesserae or not. Spokes appeared much more spatially organized in polygonal tesserae and trabecular tesserae than in the non-tesseral trabecular mineralization pattern. By extension of the body zone correlation with trabecular mineralization, trabecular mineralization of some fossil elasmobranchs and holocephalans like *Palaeobates polaris* and *Cladoselache* might derive solely from body zones (Maisey et al., 2021; Ørvig, 1951). Given such dramatic variation in extant and fossil chondrichthyans, cellular and molecular mechanisms underlying the evolution of tesseral and non-tesseral mineralization patterns is a wide open field of future study.

Finally, three independent features suggested that the adult ratfish endoskeleton shared features of the embryonic little skate endoskeleton. First, tissue mineral density (TMD) was significantly lower in a number of adult ratfish endoskeletal elements, compared to adult catshark and little skate. In fact, the TMD of adult ratfish centra was indistinguishable from the TMD of embryonic little skate centra. Second, in contrast to previous assertions that mineralized centra in chimaeras were unconstricted (Didier, 1995; Gadow and Abbott, 1895), micro-CT renderings of ratfish centra showed slight constriction, which was like those of centra in little skate embryos. Third, even histological similarities of centra in little skate embryos and adult ratfish were discovered. Both had elongate cell lacunae organized in concentric lamellae only in the mineralized middle layer of the centrum.

A tantalizing possibility is that the similarities between adult ratfish and embryonic little skate endoskeletons represents the effects of a phenomenon termed paedomorphism, or the derived condition of an adult species retaining embryonic traits of an ancestor (Garstang, 1922; Liem, 2001). Given lower TMD in many adult ratfish endoskeletal elements, the ratfish might generally have paedomorphic endoskeletal development, because as mineralized skeletal tissues develop, their TMD tends to increase (Forbes, 1976). The genetic basis for reduced skeletal mineralization in chimaeras should be investigated further, especially since the only published full chondrichthyan genome was from *Callorhinchus milii* (Callorhinchidae) (Venkatesh et al., 2014), and Callorhinchidae is the only chimaera family, for example, that does not mineralize their

centra (**Fig. 7**; Didier, 1995; Gadow and Abbott, 1895). Our findings on slight constriction of centra in both adult ratfish and embryonic little skate independently supported a previous assertion that the perichordal sheath in adult chimaeras remained at a stage corresponding to that of embryonic elasmobranchs (Gadow and Abbott, 1895). Additional future discoveries, however, would be needed to support the idea that the endoskeleton of ratfish (perhaps even extant holocephalans generally) is paedomorphic with respect to that of the common ancestor of holocephalans and elasmobranchs. First, these traits (lower endoskeletal TMD, slight morphological constriction of centra, and elongated lacunae in the only mineralized (middle) layer of centra) must be present in this last common ancestor. Second, primitive holocephalans must have altered these traits. Hopefully, paleontologists can resolve these issues.

In summary, analyses of extant and fossil data suggest different features of the chondrichthyan endoskeleton appeared (and disappeared) at different times during evolution of this fascinating clade (**Fig. 7**). The widespread distribution of non-tesseral trabecular mineralization among fossil stem and extant crown chondrichthyans argue that this is a symplesiomorphic chondrichthyan character. Areolar mineralization is widespread among extant chondrichthyan species, but absent in early stem chondrichthyans, suggesting it evolved in a later stem chondrichthyan that gave rise to holocephalans and elasmobranchs. The presence of tesserae in extant elasmobranchs and the fossil holocephalan *Cobelodus* argues that this trait was lost secondarily in extant holocephalans. Bone-like tissues in cap zones or neural arches appears restricted to extant elasmobranchs. Extant species have correlations between the histological body zone and any form of trabecular mineralization on the one hand, and the cap zone and polygonal tesserae on the other. Given these correlations, the body zone might also be symplesiomorphic for all chondrichthyans, while the cap zone might be synapomorphic for crown chondrichthyans, secondarily lost in extant holocephalans.

Materials and Methods

Specimens

All samples were approved for use under the University of Saskatchewan ethical protocol (AUP 20130092). Vertebrae (precaudal and caudal) from three samples of adult small-spotted catshark with total lengths of 31 cm were provided by the University of Montpellier Aquarium (Planet Ocean Montpellier). Adult, juvenile, and embryonic samples of the little skate were obtained from Marine Biological Labs (Falmouth, MA, USA). Four samples of adult little skate with total lengths (TL) ranging from 43.5 cm – 47.5 cm were sampled (**Supplemental Table 1**). Little skate juveniles (n=7) were staged by measuring their TL and disc widths (DW) and ranged from 5.5 cm DW, 10 cm TL to 6.5 cm DW, 11 cm TL (**Supplemental Table 1**; Stehmann, 2002). The average DW and TL of stage 32 embryos (n=6) and stage 33 embryos (n=6) were 3.2 cm DW, 7.5 cm TL and 3.5 cm DW, 8 cm TL, respectively (Supplemental Table 1; Maxwell et al., 2008; Vazquez et al., 2020). Five samples of adult spotted ratfish with total lengths ranging from 28.5 cm to 45 cm were captured by deep-water trawl in the San Juan Islands, WA, frozen within one or two hours of catch, and kept frozen for several years until thawed for experiments (Supplemental Table 1).

Regions of interest, such as ceratohyal, synarcual, and precaudal and caudal vertebrae, were dissected from the little skate, catshark, and ratfish samples (Supplemental Table 1). Dissected tissues were preserved by fixation in 4% paraformaldehyde in PBS (pH 7.4) and dehydrated through a graded ethanol series.

Micro-CT imaging and data processing

Desktop micro-CT imaging of ratfish ROIs, little skate ROIs, and catshark precaudal vertebrae was done using SkyScan 1172 desktop microtomograph (Bruker SkyScan, Kontich, Belgium). Desktop micro-CT projections were acquired using a 0.5 mm aluminium filter at 40 kV and 250 µA, 100 ms

exposure time, and 10 µm voxel resolution, and reconstructed using NRecon (Bruker SkyScan, Kontich, Belgium). A lower resolution projection (i.e., 26 µm voxel, 700 ms exposure time) of an anterior region of the ratfish was acquired in parts (**Fig. 1A**). Scans from the different parts were later stitched together using Dragonfly v2021.1 (Object Research Systems, Canada). Desktop imaging of catshark caudal vertebrae was performed using CT scanner: EasyTom 150 scanner at 70 kV, 80 µA, 1.43 s exposure time, and 6 µm voxel resolution. Reconstructions were done with Xact software (v11025) images were rendered with Avizo Lite software (v2019.3). Tissue mineral density (TMD) of ratfish, little skate, and catshark ROIs was quantitated on SkyScan 1172 desktop micro-CT images using CTAnalyzer v1.16 (CTAn, Bruker SkyScan, Kontich, Belgium) as previously described (Atake et al., 2019).

Synchrotron radiation (SR)-based micro-CT imaging of ratfish and little skate was done on the Biomedical Imaging and Therapy-Insertion Device 05ID-2 (BMIT-ID) line at the Canadian Light Source. SR micro-CT projections were acquired with a 28 keV photon energy, 1.44 µm voxel resolution, 1s exposure time, and a sample to detector distance of 9 cm. Reconstruction of SR micro-CT projections and phase retrieval was done using UFO-KIT software (*https://github.com/ufo-kit/ufo-core.git*). A delta/beta ratio of 200 was applied during phase retrieval. 3D rendered volumes and 2D virtual slices of reconstructed data were generated with Amira 6.0 (FEI Group, USA). 3D segmentation and color-coding of morphological features were done using segmentation editor tools in Amira.

Histological and immunofluorescence assays

Tissues for section histology were not demineralized before sectioning. Tissues were embedded in optimum cutting temperature compound (Tissue Tek, Torrance, CA, United States), and serial tissue sections of 10 µm thickness were made with a Cryostar NX50 cryostat (Fisher Scientific, United States).

Tissue sections were stained as described with Safranin O (Ferguson et al., 1998), picrosirius red (Junqueira et al., 1978), or Milligan's Trichrome (Ashique et al., 2022). Alcian blue/Alizarin red staining was done using a modified acid-free protocol (Eames et al., 2011). Briefly, tissues sections were washed with 100 mM Tris pH 7.5/10 mM $MgCl_2$, stained with 0.04% Alcian blue/70%EtOH/10mM $MgCl_2$ pH 7.5, taken through graded EtOH series (80% EtOH/100 mM Tris pH 7.5/10 mM MgCl2; 50% EtOH/100 mM Tris pH 7.5; 25% EtOH/100 mM Tris pH 7.5), stained with 0.1% Alizarin red/0.1 % KOH pH 7.5, de-stained with two washes of 0.1% KOH, and dehydrated through 25% EtOH, 50% EtOH, 80% EtOH, and 100% EtOH series before coverslipping. Tissue sections were demineralized using 5% EDTA for 10 mins before staining with Safranin O to improve staining of mineralized cartilage.

To minimize the chances of tissues falling off slides, tissue sections for immunofluorescence were incubated at 55°C for 15 mins before treatments and then washed with PBST (1xPBS/0.5x triton-X) between subsequent treatments. Tissues were treated separately with trypsin (0.1% in 5%EDTA/1xPBS) and hyaluronidase (0.5% in 1xPBS/0.5x triton-X) at 37°C for 15 mins each. The tissues were blocked with 4% goat serum/2% sheep/1xPBS for 1 hr, and then incubated overnight at 4°C with either Col2 antibody (1:100, II-II6B3, Hybridoma Bank) or blocking solution (negative control). Secondary antibody labelling was done using 488-conjugated goat anti-mouse antibody (1:1000, A32723 ThermoFisher Scientific) for 3 hours.

Quantitative and statistical analyses

Statistical analyses of TMDs were performed using SPSS V.22 (SPSS). Shapiro–Wilk test was used to test for normal distribution of data. To assess differences among means, one-way analysis of variance (ANOVA) was used followed by Tukey HSD or Games-Howell post hoc analyses, depending upon whether homogeneity of variance assumption (Levene's test) was met or not, respectively.

Data availability

Raw data from microCT projections and quantitative measurements have been deposited at Dryad (DOI: *10.5061/dryad.crjdfn3b9*).

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Additional files

Supplemental Table 1

Supplemental Table 2

Additional information

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References

1. Arratia G., Schultze H.P., Casciotta J (2001) **Vertebral column and associated elements in dipnoans and comparison with other fishes: development and homology** *J Morphol* **250**:101–172 https://doi.org/10.1002/jmor.1062 | Google Scholar

2. Ashique A.M., Atake O.J., Ovens K., Guo R., Pratt I.V., Detrich H.W., Cooper D.M., Desvignes T., Postlethwait J.H., Eames B.F (2022) **Bone microstructure and bone mineral density are not systemically different in Antarctic icefishes and related Antarctic notothenioids** *Journal of Anatomy* **240**:34–49 https://doi.org/10.1111/joa.13537 | Google Scholar

3. Atake O.J., Cooper D.M.L., Eames B.F (2019) **Bone-like features in skate suggest a novel elasmobranch synapomorphy and deep homology of trabecular mineralization patterns** *Acta Biomater* **84**:424–436 https://doi.org/10.1016/j.actbio.2018.11.047 | Google Scholar

4. Atake O.J., Eames B.F (2021) **Mineralized Cartilage and Bone-Like Tissues in Chondrichthyans Offer Potential Insights Into the Evolution and Development of Mineralized Tissues in the Vertebrate Endoskeleton** *Frontiers in Genetics* **12** https://doi.org/10.3389/fgene.2021.762042 | Google Scholar

5. Berio F., Broyon M., Enault S., Pirot N., López-Romero F.A., Debiais-Thibaud M (2021) **Diversity and Evolution of Mineralized Skeletal Tissues in Chondrichthyans** *Frontiers in Ecology and Evolution* **9**:223 https://doi.org/10.3389/fevo.2021.660767 | Google Scholar

6. Bordat C (1987) **Étude ultrastructurale de l'os des vertèbres du Sélacien Scyliorhinus canicula L** *Canadian Journal of Zoology* **65**:1435–1444 https://doi.org/10.1139/z87-226 | Google Scholar

7. Claeson K.M (2011) **The synarcual cartilage of batoids with emphasis on the synarcual of Rajidae** *J Morphol* **272**:1444–1463 https://doi.org/10.1002/jmor.10996 | Google Scholar

8. Clement J (1992) **Re-examination of the fine structure of endoskeletal mineralization in chondrichthyans: implications for growth, ageing and calcium homeostasis** *Marine and Freshwater Research* **43**:157–181 Google Scholar

9. Coates M.I., Finarelli J.A., Sansom I.J., Andreev P.S., Criswell K.E., Tietjen K., Rivers M.L., La Riviere P.J. (2018) **An early chondrichthyan and the evolutionary assembly of a shark body plan** *Proc Biol Sci* **285**:20172418 https://doi.org/10.1098/rspb.2017.2418 | Google Scholar

10. Criswell K.E., Coates M.I., Gillis J.A (2017) **Embryonic development of the axial column in the little skate, Leucoraja erinacea** *J Morphol* **278**:300–320 https://doi.org/10.1002/jmor.20637 | Google Scholar

11. Criswell K.E., Gillis J.A (2020) **Resegmentation is an ancestral feature of the gnathostome vertebral skeleton** *eLife* **9**:e51696 https://doi.org/10.7554/eLife.51696 | Google Scholar

12. Daniel J.F (1934) **The elasmobranch fishes** Berkeley, California: University of California press Google Scholar

13. Dean M.N., Bizzarro J.J., Clark B., Underwood C.J., Johanson Z (2017) **Large batoid fishes frequently consume stingrays despite skeletal damage** *Royal Society Open Science* **4**:170674 https://doi.org/10.1098/rsos.170674 | Google Scholar

14. Dean M.N., Summers A.P (2006) **Mineralized cartilage in the skeleton of chondrichthyan fishes** *Zoology (Jena)* **109**:164–168 https://doi.org/10.1016/j.zool.2006.03.002 | Google Scholar

15. Debiais-Thibaud M (2018) **The Evolution of Endoskeletal Mineralisation in Chondrichthyan Fish, Evolution and development of fishes** Cambridge University Press pp. 110–125 Google Scholar

16. Debiais-Thibaud M (2019) **The Evolution of Endoskeletal Mineralisation in Chondrichthyan Fish: Development, Cells and Molecules** In: Underwood C., Richter M., Johanson Z, editors. *Evolution and Development of Fishes* Cambridge: Cambridge University Press pp. 110–125 Google Scholar

17. Didier D.A (1995) **Phylogenetic systematics of extant chimaeroid fishes (Holocephali, Chimaeroidei). American Museum of Natural History** *New York* Google Scholar

18. Eames B.F., Allen N., Young J., Kaplan A., Helms J.A., Schneider R.A (2007) **Skeletogenesis in the swell shark Cephaloscyllium ventriosum** *J Anat* **210**:542–554 https://doi.org/10.1111/j.1469-7580.2007.00723.x | Google Scholar

19. Eames B.F., Yan Y.-L., Swartz M.E., Levic D.S., Knapik E.W., Postlethwait J.H., Kimmel C.B (2011) **Mutations in fam20b and xylt1 reveal that cartilage matrix controls timing of endochondral ossification by inhibiting chondrocyte maturation** *PLoS Genetics* **7**:e1002246 https://doi.org/10.1371/journal.pgen.1002246 | Google Scholar

20. Enault S., Munoz D.N., Silva W.T., Borday-Birraux V., Bonade M., Oulion S., Venteo S., Marcellini S., Debiais-Thibaud M (2015) **Molecular footprinting of skeletal tissues in the catshark Scyliorhinus canicula and the clawed frog Xenopus tropicalis identifies conserved and derived features of vertebrate calcification** *Front Genet* **6**:283 https://doi.org/10.3389/fgene.2015.00283 | Google Scholar

21. Ferguson C.M., Miclau T., Hu D., Alpern E., Helms J.A (1998) **Common molecular pathways in skeletal morphogenesis and repair** *Ann N Y Acad Sci* **857**:33–42 https://doi.org/10.1111/j.1749-6632.1998.tb10105.x | Google Scholar

22. Forbes G.B (1976) **Calcium accumulation by the human fetus** *Pediatrics* **57**:976–977 https://doi.org/10.1542/peds.57.6.976 | Google Scholar

23. Frey L., Coates M., Ginter M., Hairapetian V., Rucklin M., Jerjen I., Klug C (2019) **The early elasmobranch Phoebodus: phylogenetic relationships, ecomorphology and a new time-scale for shark evolution** *Proc Biol Sci* **286**:20191336 https://doi.org/10.1098/rspb.2019.1336 | Google Scholar

24. Gadow H.F., Abbott E (1895) **IV. On the evolution of the vertebral column of fishes** . *Philosophical Transactions of the Royal Society of London. (B.)* **186**:163–221 https://doi.org/10.1098/rstb.1895.0004 | Google Scholar

25. Garstang W (1922) **The Theory of Recapitulation: A Critical Re-statement of the Biogenetic Law** *Zoological Journal of the Linnean Society* **35**:81–101 https://doi.org/10.1111/j.1096-3642.1922.tb00464.x | Google Scholar

26. Gillis J.A., Rawlinson K.A., Bell J., Lyon W.S., Baker C.V., Shubin N.H (2011) **Holocephalan embryos provide evidence for gill arch appendage reduction and opercular evolution in cartilaginous fishes** *Proc Natl Acad Sci U S A* **108**:1507–1512 https://doi.org/10.1073/pnas.1012968108 | Google Scholar

27. Goldring M.B., Tsuchimochi K., Ijiri K (2006) **The control of chondrogenesis** *J Cell Biochem* **97**:33–44 https://doi.org/10.1002/jcb.20652 | Google Scholar

28. Golub E.E (2009) **Role of matrix vesicles in biomineralization** *Biochim Biophys Acta* **1790**:1592–1598 https://doi.org/10.1016/j.bbagen.2009.09.006 | Google Scholar

29. Herbert A.M., Dean M.N., Summers A.P., Wilga C.D (2022) **Biomechanics of the jaws of spotted ratfish** *Journal of Experimental Biology* **225** https://doi.org/10.1242/jeb.243748 | Google Scholar

30. Janvier P (1996) **Early vertebrates** Oxford University Press Google Scholar

31. Jayasankar A.K., Seidel R., Hosny A., Weaver J.C., Fratzl P., Chen J., Dean M.N (2020) **Multi-scale modeling and mechanical performance characterization of stingray skeleton-inspired tessellations** *Journal of the Mechanics and Physics of Solids* **138**:103906 https://doi.org/10.1016/j.jmps.2020.103906 | Google Scholar

32. Johanson Z., Boisvert C., Maksimenko A., Currie P., Trinajstic K (2015) **Development of the Synarcual in the Elephant Sharks (Holocephali; Chondrichthyes): Implications for Vertebral Formation and Fusion** *PLoS One* **10**:e0135138 https://doi.org/10.1371/journal.pone.0135138 | Google Scholar

33. Johanson Z., Trinajstic K., Carr R., Ritchie A (2012) **Evolution and development of the synarcual in early vertebrates** *Zoomorphology* **132**:95–110 https://doi.org/10.1007/s00435-012-0169-9 | Google Scholar

34. Junqueira L.C., Cossermelli W., Brentani R (1978) **Differential staining of collagens type I, II and III by Sirius Red and polarization microscopy** *Arch Histol Jpn* **41**:267–274 https://doi.org/10.1679/aohc1950.41.267 | Google Scholar

35. Kemp N.E., Westrin S.K (1979) **Ultrastructure of calcified cartilage in the endoskeletal tesserae of sharks** *J Morphol* **160**:75–109 https://doi.org/10.1002/jmor.1051600106 | Google Scholar

36. Kölliker A (1860) **Über das Ende der Wirbelsäule der Ganoiden und einiger Teleostier** W. Engelmann Google Scholar

37. Liem K.F., Bemis Willam E, Walker Warren F, Grande Lance (2001) **Functional anatomy of the vertebrates: an evolutionary perspective, Third ed** Belmont, CA: Brooks/Cole Google Scholar

38. Lowenstam H.A (1981) **Minerals formed by organisms** *Science* **211**:1126–1131 https://doi.org/10.1126/science.7008198 | Google Scholar

39. Maisey J.G (2013) **The diversity of tessellated calcification in modern and extinct chondrichthyans** *Revue de Paléobiologie* **32**:355–371 Google Scholar

40. Maisey J.G., Denton J.S.S., Burrow C., Pradel A (2021) **Architectural and ultrastructural features of tessellated calcified cartilage in modern and extinct chondrichthyan fishes** *J Fish Biol* **98**:919–941 https://doi.org/10.1111/jfb.14376 | Google Scholar

41. Marramà G., Schultz O., Kriwet J (2019) **A new Miocene skate from the Central Paratethys (Upper Austria): the first unambiguous skeletal record for the Rajiformes (Chondrichthyes: Batomorphii)** *J Syst Palaeontol* **17**:937–960 https://doi.org/10.1080/14772019.2018.1486336 | Google Scholar

42. Maxwell E.E., Fröbisch N.B., Heppleston A.C (2008) **Variability and Conservation in Late Chondrichthyan Development: Ontogeny of the Winter Skate (Leucoraja ocellata)** *The Anatomical Record* **291**:1079–1087 https://doi.org/10.1002/ar.20719 | Google Scholar

43. Miles R.S (1970) **Remarks on the Vertebral Column and Caudal Fin of Acanthodian Fishes** *Lethaia* **3**:343–362 https://doi.org/10.1111/j.1502-3931.1970.tb00828.x | Google Scholar

44. Omelon S., Georgiou J., Variola F., Dean M.N (2014) **Colocation and role of polyphosphates and alkaline phosphatase in apatite biomineralization of elasmobranch tesserae** *Acta Biomater* **10**:3899–3910 https://doi.org/10.1016/j.actbio.2014.06.008 | Google Scholar

45. Ørvig T (1951) **The endoskeleton, with remarks on the hard tissues of lower vertebrates in general, Histologic studies of Placoderms and fossil Elasmobranchs. Almqvist & Wiksell** *Stockholm* :321–454 Google Scholar

46. Pears J.B., Johanson Z., Trinajstic K., Dean M.N., Boisvert C.A (2020) **Mineralization of the Callorhinchus Vertebral Column (Holocephali; Chondrichthyes)** *Front Genet* **11**:571694 https://doi.org/10.3389/fgene.2020.571694 | Google Scholar

47. Peignoux-Deville J., Lallier F., Vidal B (1982) **Evidence for the presence of osseous tissue in dogfish vertebrae** *Cell Tissue Res* **222**:605–614 https://doi.org/10.1007/BF00213858 | Google Scholar

48. Porter M.E., Beltrán J.L., Koob T.J., Summers A.P (2006) **Material properties and biochemical composition of mineralized vertebral cartilage in seven elasmobranch species (Chondrichthyes)** *J Exp Biol* **209**:2920–2928 https://doi.org/10.1242/jeb.02325 | Google Scholar

49. Ridewood W., MacBride E.W (1921) **VIII.—On the calcification of the vertebral centra in sharks and rays. Philosophical Transactions of the Royal Society of London. Series B** *Containing Papers of a Biological Character* **210**:311–407 https://doi.org/10.1098/rstb.1921.0008 | Google Scholar

50. Rossert J., de Crombrugghe B. (2002) **Type I collagen: structure, synthesis, and regulation** In: Bilezikian J.P., Raisz L.G., Rodan G.A., editors. *Principles of bone biology* Elsevier pp. 189–XVIII Google Scholar

51. Seidel R., Blumer M., Chaumel J., Amini S., Dean M.N (2020) **Endoskeletal mineralization in chimaera and a comparative guide to tessellated cartilage in chondrichthyan fishes (sharks, rays and chimaera)** *J R Soc Interface* **17**:20200474 https://doi.org/10.1098/rsif.2020.0474 | Google Scholar

52. Seidel R., Blumer M., Pechriggl E.J., Lyons K., Hall B.K., Fratzl P., Weaver J.C., Dean M.N (2017) **Calcified cartilage or bone? Collagens in the tessellated endoskeletons of cartilaginous fish (sharks and rays)** *J Struct Biol* **200**:54–71 https://doi.org/10.1016/j.jsb.2017.09.005 | Google Scholar

53. Seidel R., Lyons K., Blumer M., Zaslansky P., Fratzl P., Weaver J.C., Dean M.N (2016) **Ultrastructural and developmental features of the tessellated endoskeleton of elasmobranchs (sharks and rays)** *J Anat* **229**:681–702 https://doi.org/10.1111/joa.12508 | Google Scholar

54. Smith M., Manzanares E., Underwood C., Healy C., Clark B., Johanson Z (2020) **Holocephalan (Chondrichthyes) dental plates with hypermineralized dentine as a substitute for missing teeth through developmental plasticity** *J Fish Biol* **97**:16–27 https://doi.org/10.1111/jfb.14302 | Google Scholar

55. Smith M.M., Hall B.K (1990) **Development and evolutionary origins of vertebrate skeletogenic and odontogenic tissues** *Biol Rev Camb Philos Soc* **65**:277–373 https://doi.org/10.1111/j.1469-185x.1990.tb01427.x | Google Scholar

56. Stehmann M.F (2002) **Proposal of a maturity stages scale for oviparous and viviparous cartilaginous fishes (Pisces, Chondrichthyes)** *Archive of Fishery and Marine research* **50**:23–48 Google Scholar

57. Vazquez D.M., Díaz de Astarloa J.M., Gabbanelli V., Mabragaña E. (2020) **Comparative embryonic development patterns in three deep-water skates from the southwest Atlantic** *Deep Sea Research Part I: Oceanographic Research Papers* **161**:103301 https://doi.org/10.1016/j.dsr.2020.103301 | Google Scholar

58. Venkatesh B., Lee A.P., Ravi V., Maurya A.K., Lian M.M., Swann J.B., Ohta Y., Flajnik M.F., Sutoh Y., Kasahara M., Hoon S., Gangu V., Roy S.W., Irimia M., Korzh V., Kondrychyn I., Lim Z.W., Tay B.-H., Tohari S., Kong K.W., Ho S., Lorente-Galdos B., Quilez J., Marques-Bonet T., Raney B.J., Ingham P.W., Tay A., Hillier L.W., Minx P., Boehm T., Wilson R.K., Brenner S., Warren W.C (2014) **Elephant shark genome provides unique insights into gnathostome evolution** *Nature* **505**:174–179 https://doi.org/10.1038/nature12826 | Google Scholar

59. Wilga C.A.D., Ferry L.A (2015) **4 – Functional Anatomy and Biomechanics of Feeding in Elasmobranchs** In: Shadwick R.E., Farrell A.P., Brauner C.J., editors. *Fish Physiology* Academic Press pp. 153–187 Google Scholar

60. Wurmbach H (1932) **Das Wachstum des Selachierwirbels und seiner Gewebe** *Zool Jahrb (Abt Anat Ont Tiere)* **55**:1–136 Google Scholar

Author information

Oghenevwogaga J Atake
Department of Anatomy, Physiology, and Pharmacology, University of Saskatchewan, Saskatoon, Canada
ORCID iD: 0000-0003-2803-0183

Fidji Berio
Institut des Sciences de l'Evolution, University of Montpellier, CNRS, IRD, EPHE, Montpellier, France
ORCID iD: 0000-0003-0810-9783

Melanie Debiais-Thibaud
Institut des Sciences de l'Evolution, University of Montpellier, CNRS, IRD, EPHE, Montpellier, France
ORCID iD: 0000-0002-1377-2515

B Frank Eames
Department of Anatomy, Physiology, and Pharmacology, University of Saskatchewan, Saskatoon, Canada

For correspondence: b.frank@usask.ca

Editors

Reviewer #2 (Public review):

General comment:

This is a very valuable and unique comparative study. An excellent combination of scanning and histological data from three different species is presented. Obtaining the material for such a comparative study is never trivial. The study presents new data and thus provides the basis for an in-depth discussion about chondrichthyan mineralised skeletal tissues.

Comments on previous revisions:

The manuscript has been revised and improved and can be published. A very nice manuscript, indeed. My only recommendation (point of discussion, not a requirement) would still be to think about the claim of paedomorphosis in a holocephalan.

Within the chondrichthyes, how distant holocephali are in relation to elasmobranchii remains uncertain, holocephali are quite a specialised group. Holocephali are also older than Batoidea and Selachii. As paedomorphosis is a derived character, I imagine it is difficult to establish that development in an extant holocephalan is derived compared to development in elasmobranchii. If this type of development would have been typical for the "older" holocephali it would not be paedomorphic. Also, the uncertainty how distant holocephali are from elasmobranchii makes it difficult to identify paedomorphosis with reference to chondrichthyes.

[Editors note: the authors have made further revisions in response to the previous reviews.]

https://doi.org/10.7554/eLife.94900.3.sa1

Author response:

The following is the authors' response to the previous reviews

> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *It seems as if the main point of the paper is about the new data related to rat fish although your title is describing it as extant cartilaginous fishes and you bounce around between the little skate and ratfish. So here's an opportunity for you to adjust the title to emphasize ratfish is given the fact that leader you describe how this is your significant new data contribution. Either way, the organization of the paper can be adjusted so that the reader can follow along the same order for all sections so that it's very clear for comparative purposes of new data and what they mean. My opinion is that I want to read, for each subheading in the results, about the the ratfish first because this is your most interesting novel data. Then I want to know any confirmation about morphology in little skate. And then I want to know about any gaps you fill with the cat shark. (It is ok if you keep the order of "skate, ratfish, then shark, but I think it undersells the new data).*

The main points of the paper are 1) to define terms for chondrichthyan skeletal features in order to unify research questions in the field, and 2) add novel data on how these features might be distributed among chondrichthyan clades. However, we agree with the reviewer that many readers might be more interested in the ratfish data, so we have adjusted the order of presentation to emphasize ratfish throughout the manuscript.

> *Strengths:*
>
> *The imagery and new data availability for ratfish are valuable and may help to determine new phylogenetically informative characters for understanding the evolution of cartilaginous fishes. You also allude to the fossil record.*

Thank you for the nice feedback.

> *Opportunities:*
>
> *I am concerned about the statement of ratfish paedomorphism because stage 32 and 33 were not statistically significantly different from one another (figure and prior sentences). So, these ratfish TMDs overlap the range of both 32 and 33. I think you need more specimens and stages to state this definitely based on TMD. What else leads you to think these are paedomorphic? Right now they are different, but it's unclear why. You need more outgroups.*

Sorry, but we had reported that the TMD of centra from little skate did significantly increase between stage 32 and 33. Supporting our argument that ratfish had features of little skate embryos, TMD of adult ratfish centra was significantly lower than TMD of adult skate centra (Fig1). Also, it was significantly higher than stage 33 skate centra, but it was statistically indistinguishable from that of stage 33 and juvenile stages of skate centra. While we do agree that more samples from these and additional groups would bolster these data, we feel they are sufficiently powered to support our conclusions for this current paper.

> *Your headings for the results subsection and figures are nice snapshots of your interpretations of the results and I think they would be better repurposed in your abstract, which needs more depth.*

We have included more data summarized in results sub-heading in the abstract as suggested (lines 32-37).

> *Historical literature is more abundant than what you've listed. Your first sentence describes a long fascination and only goes back to 1990. But there are authors that have had this fascination for centuries and so I think you'll benefit from looking back. Especially because several of them have looked into histology and development of these fishes.*
>
> *I agree that in the past 15 years or so a lot more work has been done because it can be done using newer technologies and I don't think your list is exhaustive. You need to expand this list and history which will help with your ultimate comparative analysis without you needed to sample too many new data yourself.*

We have added additional recent and older references: Kölliker, 1860; Daniel, 1934; Wurmbach, 1932; Liem, 2001; Arratia et al., 2001.

> *I'd like to see modifications to figure 7 so that you can add more continuity between the characters, illustrated in figure 7 and the body of the text.*

We address a similar comment from this reviewer in more detail below, hoping that any concerns about continuity have been addressed with inclusion of a summary of proposed characters in a new Table 1, re-writing of the Discussion, and modified Fig7 and re-written Fig7 legend.

> *Generally Holocephalans are the outgroup to elasmobranchs - right now they are presented as sister taxa with no ability to indicate derivation. Why isn't the catshark included in this diagram?*

While a little unclear exactly what was requested, we restructured the branches to indicate that holocephalans diverged earlier from the ancestors that led to elasmobranchs. Also in response to this comment, we added catshark (*S. canicula*) and little skate (*L. erinacea*) specifically to the character matrix.

> *In the last paragraph of the introduction, you say that "the data argue" and I admit, I am confused. Whose data? Is this a prediction or results or summary of other people's work? Either way, could be clarified to emphasize the contribution you are about to present.*

Sorry for this lack of clarity, and we have changed the wording in this revision to hopefully avoid this misunderstanding.

> ***Reviewer #2 (Public Review):***
>
> *General comment:*
>
> *This is a very valuable and unique comparative study. An excellent combination of scanning and histological data from three different species is presented. Obtaining the material for such a comparative study is never trivial. The study presents new data and thus provides the basis for an in-depth discussion about chondrichthyan mineralised skeletal tissues.*

many thanks for the kind words

> *I have, however, some comments. Some information is lacking and should be added to the manuscript text. I also suggest changes in the result and the discussion section of the manuscript.*
>
> *Introduction:*
>
> *The reader gets the impression almost no research on chondrichthyan skeletal tissues was done before the 2010 ("last 15 years", L45). I suggest to correct that and to cite also previous studies on chondrichthyan skeletal tissues, this includes studies from before 1900.*

We have added additional older references, as detailed above.

> *Material and Methods:*
>
> *Please complete L473-492: Three different Micro-CT scanners were used for three different species? ScyScan 117 for the skate samples. Catshark different scanner, please provide full details. Chimera Scncrotron Scan? Please provide full details for all scanning protocols.*

We clarified exact scanners and settings for each micro-CT experiment in the Methods (lines 476-497).

> *TMD is established in the same way in all three scanners? Actually not possible. Or, all specimens were scanned with the same scanner to establish TMD? If so please provide the protocol.*

Indeed, the same scanner was used for TMD comparisons, and we included exact details on how TMD was established and compared with internal controls in the Methods. (lines 486-488)

> *Please complete L494 ff: Tissue embedding medium and embedding protocol is missing. Specimens have been decalcified, if yes how? Have specimens been sectioned non-decalcified or decalcified?*
>
> *Please complete L506 ff: Tissue embedding medium and embedding protocol is missing. Description of controls are missing.*

Methods were updated to include these details (lines 500-503).

> *Results:*
>
> *L147: It is valuable and interesting to compare the degree of mineralisation in individuals from the three different species. It appears, however, not possible to provide numerical data for Tissue Mineral Density (TMD). First requirement, all specimens must be scanned with the same scanner and the same calibration values. This in not stated in the M&M section. But even if this was the case, all specimens derive from different sample locations and have, been preserved differently. Type of fixation, extension of fixation time in formalin, frozen, unfrozen, conditions of sample storage, age of the samples, and many more parameters, all influence TMD values. Likewise the relative age of the animals (adult is not the same as adult) influences TMD. One must assume different sampling and storage conditions and different types of progression into adulthood. Thus, the observation of different degrees of mineralisation is very interesting but I suggest not to link this observation to numerical values.*

These are very good points, but for the following reasons we feel that they were not sufficiently relevant to our study, so the quantitative data for TMD remain scientifically valid and critical for the field moving forward. Critically, 1) all of the samples used for TMD calculations underwent the same fixation protocols, and 2) most importantly, all samples for TMD were scanned on the same micro-CT scanner using the same calibration phantoms for each scanning session. Finally, while the exact age of each adult was not specified, we note for Fig1 that clear statistically significant differences in TMD were observed among various skeletal elements from ratfish, shark, and skate. Indeed, ratfish TMD was considerably lower than TMD reported for a variety of fishes and tetrapods (summarized in our paper about icefish skeletons, who actually have similar TMD to ratfish: https://doi.org/10.1111/joa.13537).

In , however, we added a caveat to the paper's Methods (lines 466-469), stating that adult ratfish were frozen within 1 or 2 hours of collection from the wild, staying frozen for several years prior to thawing and immediate fixation.

> *Parts of the results are mixed with discussion. Sometimes, a result chapter also needs a few references but this result chapter is full of references.*

As mentioned above, we reduced background-style writing and citations in each Results section.

> *Based on different protocols, the staining characteristics of the tissue are analysed. This is very good and provides valuable additional data. The authors should inform the not only about the staining (positive of negative) abut also about the histochemical characters of the staining. L218: "fast green positive" means what? L234: "marked by Trichrome acid fuchsin" means what? And so on, see also L237, L289, L291*

We included more details throughout the Results upon each dye's first description on what is generally reflected by the specific dyes of the staining protocols. (lines 178, 180, 184, 223, 227, and 243-244)

> *Discussion*
>
> *Please completely remove figure 7, please adjust and severely downsize the discussion related to figure 7. It is very interesting and valuable to compare three species from three different groups of elasmobranchs. Results of this comparison also validate an interesting discussion about possible phylogenetic aspects. This is, however, not the basis for claims about the skeletal tissue organisation of all extinct and extant members of the groups to which the three species belong. The discussion refers to "selected representatives" (L364), but how representative are the selected species? Can there be a extant species that represents the entire large group, all sharks, rays or chimeras? Are the three selected species basal representatives with a generalist life style?*

These are good points, and yes, we certainly appreciate that the limited sampling in our data might lead to faulty general conclusions about these clades. In fact, we stated this limitation clearly in the Introduction (lines 126-128), and we removed "representative" from this revision. We also replaced general reference to chondrichthyans in the Title by listing the specific species sampled. However, in the Discussion, we also compare our data with previously published additional species evaluated with similar assays, which confirms the trend that we are concluding. We look forward to future papers specifically testing the hypotheses generated by our conclusions in this paper, which serves as a benchmark for identifying shared and derived features of the chondrichthyan endoskeleton.

> *Please completely remove the discussion about paedomorphosis in chimeras (already in the result section). This discussion is based on a wrong idea about the definition of paedomorphosis. Paedomorphosis can occur in members of the same group. Humans have paedormorphic characters within the primates, Ambystoma mexicanum is paedormorphic within the urodeals. Paedomorphosis does not extend to members of different vertebrate branches. That elasmobranchs have a developmental stage that resembles chimera vertebra mineralisation does not define chimera vertebra centra as paedomorphic. Teleost have a herocercal caudal fin anlage during development, that does not mean the heterocercal fins in sturgeons or elasmobranchs are paedomorphic characters.*

We agree with the reviewer that discussion of paedomorphosis should apply to members of the same group. In our paper, we are examining paedomorphosis in a holocephalan, relative to elasmobranch fishes in the same group (Chrondrichthyes), so this is an appropriate application of paedomorphosis. In response to this comment, we clarified that our statement of paedomorphosis in ratfish was made with respect to elasmobranchs (lines 37-39; 418-420).

> *L432-435: In times of Gadow & Abott (1895) science had completely wrong ideas bout the phylogenic position of chondrichthyans within the gnathostomes. It is curious that Gadow & Abott (1895) are being cited in support of the paedomorphosis claim.*

If paedomorphosis is being examined within Chondrichthyes, such as in our paper and in the Gadow and Abbott paper, then it is an appropriate reference, even if Gadow and Abbott (and many others) got the relative position of Chondrichthyes among other vertebrates incorrect.

> *The SCPP part of the discussion is unrelated to the data obtained by this study. Kawaki & WEISS (2003) describe a gene family (called SCPP) that control Ca-binding extracellular phosphoproteins in enamel, in bone and dentine, in saliva and in milk. It evolved by gene duplication and differentiation. They date it back to a first enamel matrix protein in conodonts (Reif 2006). Conodonts, a group of enigmatic invertebrates have mineralised structures but these structure are neither bone nor mineralised cartilage. Cat fish (6 % of all vertebrate species) on the other hand, have bone but do not have SCPP genes (Lui et al. 206). Other calcium binding proteins, such as osteocalcin, were initially believed to be required for mineralisation. It turned out that osteocalcin is rather a mineralisation inhibitor, at best it regulates the arrangement collagen fiber bundles. The osteocalcin -/- mouse has fully mineralised bone. As the function of the SCPP gene product for bone formation is unknown, there is no need to discuss SCPP genes. It would perhaps be better to finish the manuscript with summery that focuses on the subject and the methodology of this nice study.*

We completely agree with the reviewer that many papers claim to associate the functions of SCPP genes with bone formation, or even mineralization generally. The Science paper with the elephant shark genome made it very popular to associate SCPP genes with bone formation, but we feel that this was a false comparison (for many reasons)! In response to the reviewer's comments, however, we removed the SCPP discussion points, moving the previous general sentence about the genetic basis for reduced skeletal mineralization to the end of the previous paragraph (lines 435-439). We also added another brief Discussion paragraph afterwards, ending as suggested with a summary of our proposed shared and derived chondrichthyan endoskeletal traits (lines 440-453).

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Further Strengths and Opportunities:*

> *Your headings for the results subsection and figures are nice snapshots of your interpretations of the results and I think they would be better repurposed in your abstract, which needs more depth. It's a little unusual to try and state an interpretation of results as the heading title in a results section and the figures so it feels out of place. You could also use the headings as the last statement of each section, after you've presented the results. In order I would change these results subheadings to:*
>
> *Tissue Mineral Density (TMD)*
>
> *Tissue Properties of Neural Arches*
>
> *Trabecular mineralization*
>
> *Cap zone and Body zone Mineralization Patterns*
>
> *Areolar mineralization*
>
> *Developmental Variation*

Sorry, but we feel that summary Results sub-headings are the best way to effectively communicate to readers the story that the data tell, and this style has been consistently used in our previous publications. No changes were made.

> *You allude to the fossil record and that is great. That said historical literature is more abundant than what you've listed. Your first sentence describes a long fascination and only goes back to 1990. But there are authors that have had this fascination for centuries and so I think you'll benefit from looking back. Especially because several of them have looked into histology of these fishes. You even have one sentence citing Coates et al. 2018, Frey et al., 2019 and ørvig 1951 to talk about the potential that fossils displayed trabecular mineralization. That feels like you are burying the lead and may have actually been part of the story for where you came up with your hypothesis in the beginning... or the next step in future research. I feel like this is really worth spending some more time on in the intro and/or the discussion.*

We've added older REFs as pointed out above. Regarding fossil evidence for trabecular mineralization, no, those studies did not lead to our research question. But after we discovered how widespread trabecular mineralization was in extant samples, we consulted these papers, which did not focus on the mineralization patterns per se, but certainly led us to emphasize how those patterns fit in the context of chondrichthyan evolution, which is how we discussed them.

> *I agree that in the past 15 years or so a lot more work has been done because it can be done using newer technologies. That said there's a lot more work by Mason Dean's lab starting in 2010 that you should take a look at related to tesserae structure... they're looking at additional taxa than what you did as well. It will be valuable for than you to be able to make any sort of phylogenetic inference as part of your discussion and enhance the info your present in figure 7. Go further back in time... For example:*
>
> *de Beer, G. R. 1932. On the skeleton of the hyoid arch in rays and skates. Quarterly*
>
> *Journal of Microscopical Science. 75: 307-319, pls. 19-21.*
>
> *de Beer, G. R. 1937. The Development of the Vertebrate Skull. The University Press,Oxford.*

Indeed, we have read all of Mason's work, citing 9 of his papers, and where possible, we have incorporated their data on different species into our Discussion and Fig7. Thanks for the de

Beer REFs. While they contain histology of developing chondrichthyan elements, they appear to refer principally to gross anatomical features, so were not included in our Intro/Discussion.

> *Most sections with in the results, read more like a discussion than a presentation of the new data and you jump directly into using an argument of those data too early. Go back in and remove the references or save those paragraphs for the discussion section. Particularly because this journal has you skip the method section until the end, I think it's important to set up this section with a little bit more brevity and conciseness. For instance, in the first section about tissue mineral density, change that subheading to just say tissue mineral density. Then you can go into the presentation of what you see in the ratfish, and then what you see in the little skate, and then that's it. You save the discussion about what other elasmobranch's or mineralizing their neural arches, etc. for another section.*

We dramatically reduced background-style writing and citations in each Results section (other than the first section of minor points about general features of the ratfish, compared to catshark and little skate), keeping only a few to briefly remind the general reader of the context of these skeletal features.

> *I like that your first sentence in the paragraph is describing why you are doing. a particular method and comparison because it shows me (the reader) where you're sampling from. Something else is that maybe as part of the first figure rather than having just each with the graph have a small sketch for little skate and catch shark to show where you sampled from for comparative purposes. That would relate back, then to clarifying other figures as well.*

done (also adding a phylogenetic tree).

> *Second instance is your section on trabecular mineralization. This has so many references in it. It does not read like results at all. It looks like a discussion. However, the trabecular mineralization is one of the most interesting aspect of this paper, and how you are describing it as a unique feature. I really just want a very clear description of what the definition of this trabecular mineralization is going to be.*

In addition to adding Table 1 to define each proposed endoskeletal character state, we have changed the structure of this section and hope it better communicates our novel trabecular mineralization results. We also moved the topic of trabecular mineralization to the first detailed Discussion point (lines 347-363) to better emphasize this specific topic.

> *Carry this reformatting through for all subsections of the results.*

As mentioned above, we significantly reduced background-style writing and citations in each Results section.

> *I'd like to see modifications to figure 7 so that you can add more continuity between the characters, illustrated in figure 7 and the body of the text. I think you can give the characters a number so that you can actually refer to them in each subsection of the results. They can even be numbered sequentially so that they are presented in a standard character matrix format, that future researchers can add directly to their own character matrices. You could actually turn it into a separate table so it doesn't taking up that entire space of the figure, because there need to be additional taxa referred to on the diagram. Namely, you don't have any out groups in figure 7 so it's hard to describe any state specifically as ancestral and wor derived. Generally Holocephalans are the*

> *outgroup to elasmobranchs - right now they are presented as sister taxa with no ability to indicate derivation. Why isn't the catshark included in this diagram?*

The character matrix is a fantastic idea, and we should have included it in the first place! We created Table 1 summarizing the traits and terminology at the end of the Introduction, also adding the character matrix in Fig7 as suggested, including specific fossil and extant species. For the Fig7 branching and catshark inclusion, please see above.

> *You can repurpose the figure captions as narrative body text. Use less narrative in the figure captions. These are your results actually, so move that text to the results section as a way to truncate and get to the point faster.*

By figure captions, we assume the reviewer refers to figure legends. We like to explain figures to some degree of sufficiency in the legends, since some people do not read the main text and simply skim a manuscript's abstract, figures, and figure legends. That said, we did reduce the wording, as requested.

> *More specific comments about semantics are listed here:*
>
> *The abstract starts negative and doesn't state a question although one is referenced. Potential revision - "Comprehensive examination of mineralized endoskeletal tissues warranted further exploration to understand the diversity of chondrichthyans... Evidence suggests for instance that trabecular structures are not common, however, this may be due to sampling (bring up fossil record.) We expand our understanding by characterizing the skate, cat shark, and ratfish... (Then add your current headings of the results section to the abstract, because those are the relevant takeaways.)"*

We re-wrote much of the abstract, hoping that the points come across more effectively. For example, we started with "Specific character traits of mineralized endoskeletal tissues need to be clearly defined and comprehensively examined among extant chondrichthyans (elasmobranchs, such as sharks and skates, and holocephalans, such as chimaeras) to understand their evolution". We also stated an objective for the experiments presented in the paper: "To clarify the distribution of specific endoskeletal features among extant chondrichthyans".

> *In the last paragraph of the introduction, you say that "the data argue" and I admit, I am confused. Whose data? Is this a prediction or results or summary of other people's work? Either way, could be clarified to emphasize the contribution you are about to present.*

Sorry for this lack of clarity, and we have changed the wording in this revision to hopefully avoid this misunderstanding.

> *In the second paragraph of the TMD section, you mention the synarcual comparison. I'm not sure I follow. These are results, not methods. Tell me what you are comparing directly. The non-centrum part of the synarcual separate from the centrum? They both have both parts... did you mean the comparison of those both to the cat shark? Just be specific about which taxon, which region, and which density. No need to go into reasons why you chose those regions here.. Put into methods and discussion for interpretation.*

We hope that we have now clarified wording of that section.

> *Label the spokes somehow either in caption or on figure direction. I think I see it as part of figure 4E, I, and J, but maybe I'm misinterpreting.*

Based upon histological features (e.g., regions of very low cellularity with Trichrome unstained matrix) and hypermineralization, spokes in Fig4 are labelled with * and segmented in blue. We detailed how spokes were identified in main text (lines 241-243; 252-254) and figure legend (lines 597-603).

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Other comments*
>
> *L40: remove paedomorphism*

no change; see above

> *L53: down tune languish, remove "severely" and "major"*

done (lines 57-59)

> *L86: provide species and endoskeletal elements that are mineralized*

no change; this paragraph was written generally, because the papers cited looked at cap zones of many different skeletal elements and neural arches in many different species

> *L130: remove TMD, replace by relative, descriptive, values*

no change; see above

> *L135: What are "segmented vertebral neural arches and centra" ?*

changed to “neural arches and centra of segmented vertebrae” (lines 140-141)

> *L166: L168 "compact" vs. "irregular". Partial mineralisation is not necessarily irregular.*

thanks for pointing out this issue; we changed wording, instead contrasting “non-continuous” and “continuous” mineralization patterns (lines 171-174)

> *L192: "several endoskeletal regions". Provide all regions*

all regions provided (lines 198-199)

> *L269: "has never been carefully characterized in chimeras". Carefully means what? Here, also only one chimera is analyses, not several species.*

sentence removed

> *302: Can't believe there is no better citation for elasmobranch vertebral centra development than Gadow and Abott (1895)*

added Arriata and Kolliker REFs here (lines 293-295)

> *L318 ff: remove discussion from result chapter*

references to paedomorphism were removed from this Results section

> *L342: refer to the species studied, not to the entire group.*

sorry, the line numbering for the reviewer and our original manuscript have been a little off for some reason, and we were unclear exactly to which line of text this comment referred. Generally in this revision, however, we have tried to restrict our direct analyses to the species analyzed, but in the Discussion we do extrapolate a bit from our data when considering relevant published papers of other species.

> *346: "selected representative". Selection criteria are missing*

“selected representative” removed

> *L348: down tune, remove "critical"*

Done

> *L351: down tune, remove "critical"*

done

> *L 364: "Since stem chondrichthyans did not typically mineralize their centra". Means there are fossil stem chondrichthyans with full mineralised centra?*

Re-worded to “Stem chondrichthyans did not appear to mineralize their centra” (lines 379)

> *L379: down tune and change to: "we propose the term "non-tesseral trabecular mineralization. Possibly a plesiomorphic (ancestral) character of chondrichthyans"*

no change; sorry, but we feel this character state needs to be emphasized as we wrote in this paper, so that its evolutionary relationship to other chondrichthyan endoskeletal features, such as tesserae, can be clarified.

> *L407: suggests so far palaeontologist have not been "careful" enough?*

apologies; sentence re-worded, emphasizing that synchrotron imaging might increase details of these descriptions (lines 406-408)

> *414: down tune, remove "we propose". Replace by "possibly" or "it can be discussed if"*

sentence re-worded and “we propose” removed (lines 412-415)

> *L420: remove paragraph*

no action; see above

> *L436: remove paragraph*

no action; see above

> *L450: perhaps add summery of the discussion. A summery that focuses on the subject and the methodology of this nice study.*

yes, in response to the reviewer’s comment, we finished the discussion with a summary of the current study. (lines 440-453)

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