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The phylogenetic origin and evolution of acellular bone in teleost fishes: insights into osteocyte function in bone metabolism

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ABSTRACT

Vertebrate bone is composed of three main cell types: osteoblasts, osteoclasts and osteocytes, the latter being by far the most numerous. Osteocytes are thought to play a fundamental role in bone physiology and homeostasis, however they are entirely absent in most extant species of teleosts, a group that comprises the vast majority of bony 'fishes', and approximately half of vertebrates. Understanding how this acellular (anosteocytic) bone appeared and was maintained in such an important vertebrate group has important implications for our understanding of the function and evolution of osteocytes. Nevertheless, although it is clear that cellular bone is ancestral for teleosts, it has not been clear in which specific subgroup the osteocytes were lost. This review aims to clarify the phylogenetic distribution of cellular and acellular bone in teleosts, to identify its precise origin, reversals to cellularity, and their implications. We surveyed the bone type for more than 600 fossil and extant ray-finned fish species and optimised the results on recent large-scale molecular phylogenetic trees, estimating ancestral states. We find that acellular bone is a probable synapomorphy of Euteleostei, a group uniting approximately two-thirds of teleost species. We also confirm homoplasy in these traits: acellular bone occurs in some non-euteleosts (although rarely), and cellular bone was reacquired several times independently within euteleosts, in salmons and relatives, tunas and the opah (*Lampris* sp.). The occurrence of peculiar ecological (e.g. anadromous migration) and physiological (e.g. red-muscle endothermy) strategies in these lineages might explain the reacquisition of osteocytes. Our review supports that the main contribution of osteocytes in teleost bone is to mineral homeostasis (*via* osteocytic osteolysis) and not to strain detection or bone remodelling, helping to clarify their role in bone physiology.

Key words: osteocyte, acellular bone, anosteocytic bone, Actinopterygii, Teleostei, Salmoniformes, Scombridae, ancestral state reconstruction, bone remodelling, endothermy.

CONTENTS

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I. INTRODUCTION

(1) General introduction

Vertebrate bone is a living tissue that, besides its mineralised extracellular component, comprises cells of three different types. Surface-based osteoblasts and osteoclasts synthesise and resorb bone, respectively, and osteocytes are more versatile cells that fulfil various functions (Francillon-Vieillot *et al.*, 1990; de Ricqlès *et al.*, 1991; Bonewald, 2011; Shahar & Dean, 2013; Hall, 2015). Osteocytes are by far the dominant cellular component, constituting up to 95% of bone cells in mammals. They derive from osteoblasts of the bone surfaces that become embedded into the bone matrix in cavities called osteocyte lacunae (Franz-Odendaal *et al.*, 2006) and communicate with each other through a network of canaliculi (Cao *et al.*, 2011).

Osteocytes play a key role in bone physiology: (i) they act as mechanical sensors detecting changes in bone strain; (ii) they guide bone remodelling by activating or deactivating the osteoclasts they communicate with; (iii) and they are involved in calcium and phosphorus metabolic regulation through direct resorption of the bone around their lacunae (Witten & Huysseune, 2009; Rochefort, Pallu & Benhamou, 2010; Bonewald, 2011; Wysolmerski, 2012; Shahar & Dean, 2013). This double role in mineral and mechanical homeostasis would suggest that osteocytes are indispensable for bone to function normally (Moss, 1961*b*; Shahar & Dean, 2013). However, bone is entirely devoid of osteocytes in most teleosts, (Kölliker, 1859; Stéphan, 1900; Enlow & Brown, 1956; Moss, 1963; Meunier, 1987, 1989; de Ricqlès *et al.*, 1991; Meunier & Huysseune, 1992; Huysseune, 2000; Witten *et al.*, 2004; Shahar & Dean, 2013) a group of ray-finned fishes that comprises more than half of modern vertebrate species.

Nineteenth century histologists noted the absence of 'bone corpuscles' (i.e. osteocyte lacunae) in the bone of some teleosts (Williamson, 1851; Gegenbaur, Kölliker & Müller, 1853; Mettenheimer, 1854; Quekett, 1855). This inspired Kölliker (1859) to undertake a remarkable survey of more than 250 ray-finned fish species, distinguishing those with acellular bone (improperly named 'osteoid' at the time) from those with cellular bone. Moss and colleagues later described the structure, mineral composition and development of teleost acellular bone, confirming its nature as true bone (Moss & Posner, 1960; Moss, 1961*a*,*b*, 1962, 1963, 1965; Moss & Freilich, 1963). Later, Weiss & Watabe (1979) proposed the term 'anosteocytic bone', which is more precise because this tissue still bears other cell types (osteoblasts and osteoclasts) on its surface. Nevertheless, the term 'acellular bone' remains widely used in modern literature, and we apply that term here.

That bone is acellular in such a large and ecologically important group as teleosts raises numerous questions pertaining to: (i) the distribution of bone type within teleosts (does it follow ecological, physiological or phylogenetic patterns?), (ii) the origin of acellular bone (does it have a unique origin, or multiple convergent appearances?), and (iii) the function of such a bone type (does the absence of osteocytes impact bone structure, function and homeostasis?). Addressing these questions has critical implications to understanding the evolution of bone within vertebrates as a whole, and the role of osteocytes in bone physiology (Huysseune, 2000; Witten *et al.*, 2004; Shahar & Dean, 2013; Currey, Dean & Shahar, 2017).

(2) The evolution of acellular bone: state of the art

Following the surveys of Kölliker (1859) and Moss (1961*b*), researchers attempted to explain the distributions of cellular and acellular bone among teleost species. For example, an early hypothesis proposed that acellular bone occurs because marine environments are richer in dissolved calcium, decreasing the need to use bone as an additional source of metabolic minerals (Moss, 1961*b*, 1963). However, acellular bone is also present in freshwater teleost taxa such as esocids (pikes), centrarchids (sunfishes), percids ('true' perches), and cichlids (Moss, 1965). In virtually every teleost species, the entire skeleton seems to be composed exclusively of either cellular or acellular bone, and closely related species mostly seem to share the same bone type (Kölliker, 1859). Following these observations, cellularity was quickly recognised as a potentially significant phylogenetic character (e.g. Kölliker, 1859; Berg, 1947). Indeed, at least two studies have used the presence or absence of osteocytes to discuss the systematic position of enigmatic fossil taxa (Gaudant & Meunier, 2004; Mayrinck *et al.*, 2017).

Deep divergences in teleost phylogeny have been poorly resolved until recently, meaning that the phylogenetic distribution of cellularity has not been clear. Nevertheless, there is broad consensus on two statements: (i) that cellular bone is the plesiomorphic condition for teleosts, actinopterygians and osteichthyans in general (Ørvig, 1951, 1967; Moss, 1961*b*, 1963); and (ii) that acellular bone is found in 'advanced' or 'higher' teleost groups (Moss, 1961*b*, 1963; Meunier, 1987, 1989; de Ricqlès *et al.*, 1991; Meunier & Huysseune, 1992; Witten *et al.*, 2004). As noted by previous authors themselves, these propositions are imprecise and potentially misleading. Indeed, the pattern appears to be much more complex: for example, acellular bone is found in certain 'lower' teleosts such as pikes and cellular bone is found in some 'higher' taxa such as tunas (Amprino & Godina, 1956; Moss, 1963; Meunier, 1989; Meunier & Huysseune, 1992). Moreover, the systematic distributions of both bone types have been described using subjective and poorly defined systematic categories (e.g. 'advanced teleosts'), not from an explicit phylogenetic framework based on character analysis.

Several authors used cellularity as a phylogenetic character: acellular bone is proposed as a synapomorphy uniting (i) Osmeriformes (true smelts) and Neoteleostei (the clade including spiny-rayed fishes, amongst others) by Rosen (1985); (ii) Esociformes (pikes and mudminnows), Osmeriformes and Neoteleostei by Parenti (1986); (iii) Esociformes and Neoteleostei by Johnson & Patterson (1996), the latter being the only phylogeny based on the analysis of a character matrix. However, the usefulness of this previous work is limited because the underlying phylogenetic frameworks have been superseded by more recent classifications based on molecular phylogenies that extensively sample both taxa and loci (e.g. Near *et al.*, 2012; Betancur-R *et al.*, 2013, 2017). The most relevant changes relative to anatomical hypotheses include: (i) Esociformes do not form an exclusive clade with Neoteleostei, but instead consistently appear to be sister to Salmoniformes (Ramsden *et al.*, 2003; Wilson & Williams, 2010; Near *et al.*, 2012; Betancur-R. *et al.*, 2013; Campbell *et al.*, 2013); (ii) Neoteleostei *sensu* Rosen (1973, 1985) is not a monophyletic group, with Stomiiformes (viperfishes and relatives) now considered sister to Osmeriformes (Li *et al.*, 2010; Near *et al.*, 2012; Betancur-R. *et al.*, 2013, 2017).

(3) Aim of this review

While most research on acellular teleost bone has been focused on its structure, development and function (Moss, 1961*a*; Meunier, 1989; Meunier & Huysseune, 1992; Cohen *et al.*, 2012; Dean & Shahar, 2012; Shahar & Dean, 2013), the evolutionary origin and phylogenetic distribution of this bone type has not been studied in detail.

Explaining the evolutionary origins of acellular bone requires an explicitly phylogenetic approach that can distinguish the role of adaptation from that of phylogenetic history in the distribution of bone types among species. This review aims to clarify the distribution of cellular and acellular bone in teleosts within a phylogenetic context that is now available thanks to an array of recently published large-scale molecular analyses (e.g. Near *et al.*, 2012; Betancur-R. *et al.*, 2013; Hughes *et al.*, 2018). We also review the structure of acellular bone, emphasising its functional similarity to cellular bone (Witten & Huysseune, 2009; Cohen *et al.*, 2012; Shahar & Dean, 2013; Currey *et al.*, 2017). Our review of the existing literature, complemented by our own observations, brings together most of the data published to date on actinopterygian bone to constitute a data set covering the whole diversity of the group. Including this data into an explicit phylogenetic framework for the first time, finally allows us to draw a possible historical scenario for the loss of osteocytes in teleosts.

II. TELEOST ACELLULAR BONE: STRUCTURE AND FUNCTION

(1) Structure and development

In teleosts, bone is found in the cranial, axial and appendicular skeleton (Fig. 1A, C–F) and in scales (Fig. 1B), lepidotrichia (fin rays) and the tissues that derive from them (Patterson, 1977; Schaeffer, 1977; Francillon-Vieillot *et al.*,

1990). Despite its structural peculiarities that led historical authors to improperly designate it under other names [e.g. 'osteoid', (Kölliker, 1859)], teleost acellular bone is considered true bone because it shares its developmental origin and main characteristics with every other vertebrate bone tissue (Moss, 1961*b*; Witten & Huysseune, 2009; Dean & Shahar, 2012): (i) it is composed of hydroxyapatite crystals in a mesh of type I collagen fibres; (ii) it has the same functional properties as other bone tissues (muscle insertion and organ support); (iii) its extracellular matrix is secreted by osteoblasts and resorbed by osteoclasts; (iv) it can be submitted to active remodelling.

Typical cellular bone contains numerous mature osteocytes that, despite being completely surrounded by mineralised tissue, communicate with each other and with the bone surface *via* a network of canaliculi containing cytoplasmic processes (Fig. 1C, D). This lacunocanalicular system permeates bone and gives osteocytes their characteristic star-shaped appearance (Meunier, 1987; Cao *et al.*, 2011). It is however not clear whether osteocytes form a proper lacunocanalicular network in all teleosts with cellular bone (Fiaz, van Leeuwen & Kranenbarg, 2010; Totland *et al.*, 2011). In acellular bone, on the other hand, there are no osteocytes or lacunae within the bone mineral matrix (Fig. 1E, F), but it is sometimes penetrated by osteoblastic canaliculi from the bone surface (Francillon-Vieillot *et al.*, 1990; Sire & Meunier, 1994, 2017). In the 'tubular acellular bone' of a few taxa, tubules containing a bundle of collagen fibres and numerous osteoblastic cytoplasmic processes permeate acellular primary bone (Hughes, Bassett & Moffat, 1994; Sire & Meunier, 2017; Meunier & Béarez, 2019). These tubules are superficially similar, but structurally distinct from the canals of Williamson (Fig. 1D) that are known only from the cellular bone of holosteans and fossil stem teleosts (Williamson, 1849; Ørvig, 1951; Sire & Meunier, 1994; Meunier & Brito, 2004). Acellular bone can be vascular or avascular, osteoblastic canaliculi being more numerous in avascular acellular bone than in vascular acellular bone (Francillon-Vieillot *et al.*, 1990).

In cellular bone, osteocytes originate from osteoblasts that become surrounded by the mineral matrix they secreted (Franz-Odendaal *et al.*, 2006). Conversely, in acellular bone osteoblasts remain on the outer surface and secrete extracellular matrix exclusively towards the interior of bone, never ending up surrounded by bone to turn into osteocytes (Weiss & Watabe, 1979; Ekanayake & Hall, 1987, 1988). The hypothesis that acellular bone could form through intracellular mineralisation of osteocytes that are already entrapped in bone (Moss, 1961*a*) has been rejected since a study on the medaka *Oryzias latipes* (Ekanayake & Hall, 1987).

(2) Functional properties of acellular bone

(*a*) *Mechanical properties*

The mineral fraction in acellular bone is proportionally slightly higher than in cellular bone (Meunier, 1984*a*; Cohen *et al.*, 2012). This higher mineral content, along with the reduction in porosity associated with the absence of osteocytes have been hypothesised to increase the stiffness of acellular bone (Horton & Summers, 2009). However, comparative studies of structural stress have suggested that acellular and cellular bone have equivalent stiffness (Horton & Summers, 2009; Cohen *et al.*, 2012; Dean & Shahar, 2012; Currey & Shahar, 2013). On the other hand, the collagen fibre ultrastructure in acellular teleost bone gives it an increased toughness compared to tetrapod (e.g. human) cellular bone (Atkins *et al.*, 2015*b*).

(*b*) *Resorption*

Osteoclasts, the cells primarily responsible for bone resorption, were long thought to be absent from acellular teleost bone, although resorption was still observed (Blanc, 1953; Moss, 1963; Weiss & Watabe, 1979; Glowacki *et al.*, 1986). Indeed, osteoclasts in acellular bone are structurally different from the 'typical' osteoclasts found in cellular bone, explaining why they long went undetected: they are generally mononucleated instead of multinucleated as in cellular bone (Sire, Huysseune & Meunier, 1990; Witten, 1997; Witten & Villwock, 1997; Witten & Huysseune, 2009). This structural difference may be explained by the absence of osteocytes, which promote the growth of multinucleated osteoclasts (Witten & Huysseune, 2009, 2010).

(*c*) *Mineral metabolism*

Bone plays a crucial role in calcium metabolism in vertebrates, both as a consumer and as a source of calcium. However, this role seems less critical in teleosts than in terrestrial vertebrates since, as aquatic animals, teleosts can mobilise calcium and other elements directly from the ambient water *via* their gills and/or digestive system (Takagi & Yamada, 1992; Witten & Huysseune, 2009; Shahar & Dean, 2013). Phosphorus availability appears to be more critical than that of calcium for healthy growth in both marine and freshwater teleosts (Witten & Huysseune, 2009; Shahar & Dean, 2013), and bone does not seem to mineralise when phosphorus is absent from the diet (Witten *et al.*, 2016, 2019). Nevertheless, a specific type of bone resorption (osteocytic osteolysis) is undertaken by the osteocytes themselves and may be linked to periods of increased metabolic calcium and/or phosphorus requirement, as it occurs conspicuously in certain diadromous teleost species [e.g. European eel (*Anguilla anguilla*), salmoniforms] before and during migration (Kacem & Meunier, 2000, 2003; Sbaihi *et al.*, 2007). In teleosts with acellular bone, osteocytic osteolysis is impossible, potentially making calcium and phosphorus more difficult to mobilise from and into the skeleton than in those with cellular bone (Moss, 1962; Simmons, Simmons & Marshall, 1970; Witten, 1997; Witten & Huysseune, 2009).

(*d*) *Remodelling*

Teleost bony tissues consist mainly of primary bone in most species (Meunier, 1987) and bone remodelling may be

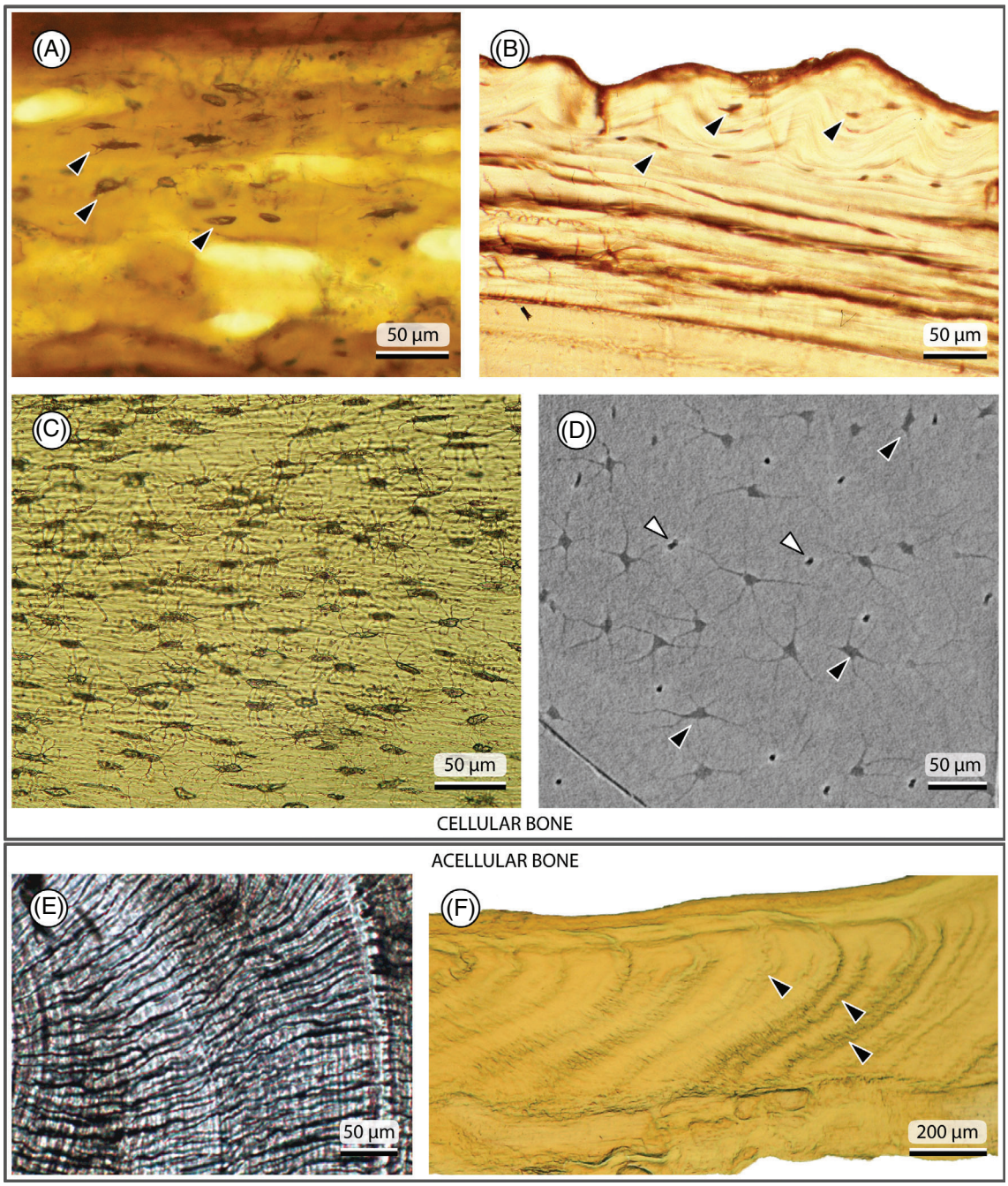

Fig. 1. Examples of cellular (A–D) and acellular (E, F) bone in teleosts and close relatives. (A) Ground section through cellular bone in the jaw of the Devonian actinopterygian †*Cheirolepis canadensis* (MHNM 05-340), observed in transmitted natural light. Osteocyte lacunae are marked with black arrowheads. Modified from Meunier, Otero & Laurin (2018*c*). (B) Thin section through a scale of an osteoglossomorph, the arowana *Osteoglossum bicirrhosum*, observed in transmitted natural light. Osteocyte lacunae are visible in the superficial bony layer, and marked with black arrowheads. Photograph by F. J. Meunier. (C) Thin section through cellular bone in the rib of an ostariophysan, the barbel *Barbus barbus*, observed in transmitted natural light. Osteocyte lacunae and their associated lacunocanalicular network are clearly visible. Modified from Meunier & Herbin (2014). (D) 'Virtual thin section' obtained by stacking synchrotron tomographic slices of the dentary of the Jurassic stem teleost †*Dorsetichthys bechei* (OUMNH J.3369). Star-shaped osteocyte lacunae and their canaliculi are visible (black arrowheads), as well as canals of Williamson in cross-section (white arrowheads). Image produced by D. Davesne and A. D. Schmitt. (E) Thin section through acellular bone in the rib of an acanthomorph, the sea bass *Dicentrarchus labrax*, showing numerous radially arranged osteoblastic canaliculi. Photograph by D. Davesne. (F) Thin section through acellular bone in the vertebra of an acanthomorph, the anglerfish *Lophius* sp. Bone is relatively featureless, apart from visible successive growth marks (black arrowheads). Photograph by F. J. Meunier.

less abundant in teleosts than in tetrapods – it was even long thought to be absent (Moss, 1961*a*). Nevertheless, bone remodelling occurs in teleosts, in taxa with both cellular (Witten, Hansen & Hall, 2001; Witten & Hall, 2003; Nemoto *et al.*, 2007; Witten & Huysseune, 2009) and acellular bone (Castanet & de Ricqlès, 1986; Witten & Huysseune, 2009; Dean & Shahar, 2012; Shahar & Dean, 2013; Atkins *et al.*, 2014, 2015*a*; Currey *et al.*, 2017). For instance, hyperostoses are widespread in teleosts with acellular bone, and their formation requires an important remodelling activity (Meunier & Desse, 1986; Smith-Vaniz *et al.*, 1995). In billfishes (Istiophoriformes), that lack osteocytes, bone in the rostrum is riddled with secondary osteons overlapping primary osteons, akin to what is found in the haversian bone of tetrapods and suggesting very intense remodelling activity as a response to fracture and load (Amprino & Godina, 1956; Poplin, Poplin & de Ricqlès, 1976; Castanet & de Ricqlès, 1986; Atkins *et al.*, 2014). These examples suggest that, in the absence of osteocytes as sensors, acellular bone is nevertheless capable of detecting strain and damage by some mechanism that is yet not fully understood (Kranenbarg *et al.*, 2005; Witten & Huysseune, 2009; Fiaz *et al.*, 2010; Dean & Shahar, 2012; Shahar & Dean, 2013; Atkins *et al.*, 2014, 2015*a*). In its general structure, biomechanics, and mechanisms of bone resorption and remodelling, acellular teleost bone then appears to be functionally very similar to cellular teleost bone. This suggests that the presence of osteocytes is not strictly necessary to achieve these functions. This leaves osteocytic osteolysis, a potentially important mechanism involved in calcium and/or phosphorus metabolism (Witten & Huysseune, 2009; Cohen *et al.*, 2012; Shahar & Dean, 2013; Doherty, Ghalambor & Donahue, 2015), as the main function entirely lacking in acellular bone.

III. PHYLOGENETIC DISTRIBUTION OF ACELLULAR BONE

(1) Acellular bone outside of actinopterygians

(*a*) *Palaeozoic jawless vertebrates*

A peculiar bone-like tissue devoid of osteocytes, called aspidin, has long been known in the dermal skeleton of †heterostracans, a group of Palaeozoic jawless vertebrates (Gross, 1930; Halstead, 1969). Similar tissues were later described in other early jawless stem gnathostome lineages, such as †anaspids, †thelodonts and †galeaspids (Stensiö, 1958; Sire, Donoghue & Vickaryous, 2009; Keating & Donoghue, 2016). Aspidin appears to be structurally very similar to teleost acellular bone, with probable collagen bundles (akin to the 'tubules' of teleosts) penetrating the mineralised tissue (Keating *et al.*, 2018). The occurrence of either cellular or acellular bone in various early vertebrate lineages (Fig. 2) led to a debate over which one was phylogenetically older (Ørvig, 1951; Denison, 1963; Halstead, 1963; Smith & Hall, 1990). The earliest vertebrates with cellular bone are the jawless †osteostracans that appear in the Silurian (Stensiö, 1958; Smith & Hall, 1990; Donoghue & Sansom, 2002), although osteocytes have also been described in the dermal bone of a late Ordovician †arandaspid (Sansom *et al.*, 2013). Abundant evidence supports the placement of †osteostracans as the sister group to gnathostomes (jawed vertebrates): it seems likely that cellular bone would then be a synapomorphy of the clade uniting †osteostracans and gnathostomes (Donoghue & Sansom, 2002; Brazeau & Friedman, 2014), with a potential convergent appearance in †arandaspids (Fig. 2). This would imply that bone in †anaspids, †thelodonts, †heterostracans and †galeaspids is primitively devoid of osteocytes, making acellular bone the plesiomorphic state for skeletonising vertebrates (Denison, 1963; Halstead, 1963, 1969; Donoghue & Sansom, 2002; Keating *et al.*, 2018).

(*b*) *Jawed vertebrates*

As a plesiomorphic character for gnathostomes (Fig. 2), cellular bone is found in Palaeozoic jawed stem gnathostomes such as †'placoderms' (Ørvig, 1951; Downs & Donoghue, 2009; Sire *et al.*, 2009; Giles, Rücklin & Donoghue, 2013) and in fossils interpreted as stem osteichthyans, such as †*Andreolepis*, †*Lophosteus* and †*Psarolepis* (Jerve *et al.*, 2016; Qu *et al.*, 2017). Bone is cellular in sarcopterygians, the sister group to actinopterygians, including modern coelacanths, modern lungfishes, modern tetrapods (lissamphibians, mammals, diapsids) and fossil taxa falling on their respective stems (Sire *et al.*, 2009; Zylberberg, Meunier & Laurin, 2010; Schultze, 2016; Meunier, Cupello & Clément, 2019).

On the other hand, acellular bone also occurs in different gnathostome lineages. A prominent example is the basal bone layer in the odontodes of various chondrichthyans (cartilaginous fishes) and their close relatives, including Palaeozoic †'acanthodians' (Sire *et al.*, 2009; Chevrinais, Sire & Cloutier, 2017). Acellular perichondral bone is also found in the modified dorsal fin of the Palaeozoic stem holocephalan †*Akmonistion* (Coates *et al.*, 1998), while the fin rays of the African lungfish *Protopterus* are composed of acellular dermal bone (Géraudie & Meunier, 1984). Finally, acellular bone is found in very localised zones of specialised tissues in a few tetrapods, for example in cranial bones and sutures of †pachycephalosaurid and †ceratopsian dinosaurs (Goodwin & Horner, 2004; Bailleul & Horner, 2016). In all these taxa, acellular bone is found exclusively in dermal bone, leaving teleosts as the only known vertebrates with occurrence of acellular endochondral bone.

(2) Phylogenetic distribution of acellular bone in teleosts and other actinopterygians

(*a*) *Material of study*

To evaluate the phylogenetic distribution of cellular and acellular bone in actinopterygians, we reviewed more than 150 years of literature on ray-finned fish bone. The most comprehensive sources of information were the extensive

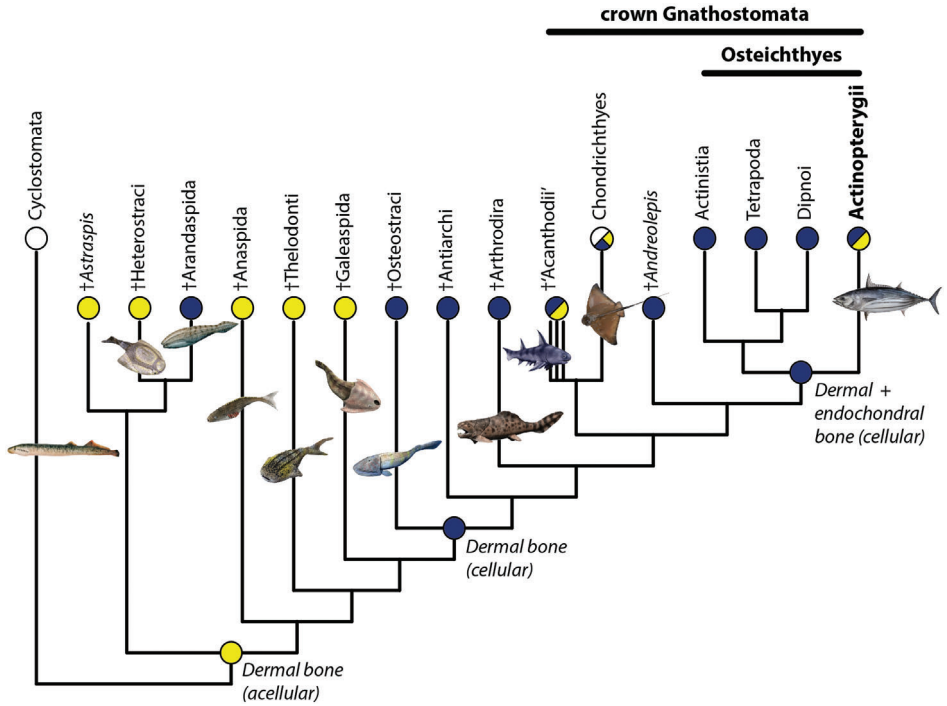

Fig. 2. Distribution of cellular and acellular bone in the phylogeny of vertebrates (modified from Keating *et al.*, 2018). The coloured circles at the tip of branches reflect bone type in the clade: acellular (yellow), cellular (dark blue), or bone absent (white). Taxon pictures from N. Tamura, and Iglésias (2014*a*,*b*).

surveys by Kölliker (1859) and Moss (1961*b*, 1965), to which we added data from various fossil and extant species where required to better resolve the phylogenetic and temporal distribution (see online Supporting information, Table S1, for details on these sources). In total, our database includes 677 fossil and extant taxa. In addition, we obtained propagation phase contrast synchrotron microtomography (PPC-SRμCT) data from museum specimens of 108 extant and fossil species (Table 1, Table S1), bringing new information or corroborating our knowledge on the presence or absence of osteocytes in their bones. The SRμCT scans were carried out at the ID-19 (microtomography) beamline of the European Synchrotron Radiation Facility (ESRF), using a filtered white beam with energy levels between 35 and 105 keV, obtaining a voxel size of 0.72 μm.

For all extant and fossil taxa, we used the dentary as the bone of study (and in some cases, a rib). This bone appears to be cellular, even when both bone types coexist in the skeleton (Weigele & Franz-Odendaal, 2016). We then consider that the lack of osteocytes in the dentary is likely to reflect genuine acellularity in a given taxon.

(*b*) *Non-teleost actinopterygians*

Cellular bone is present in the earliest actinopterygians from the Devonian (Table S1): for example, in the bones and scales of †*Cheirolepis* (Zylberberg *et al.*, 2016) and †*Moythomasia* (Sire *et al.*, 2009; Schultze, 2016) and in the scales of †*Mimipiscis* (Richter & Smith, 1995). Bone and scales are always cellular in modern non-teleost actinopterygians, for example in bichirs (Polypteriformes), bowfins and gars (Holostei) (Kölliker, 1859; Moss, 1961*b*; Sire & Meunier, 1994; Daget *et al.*, 2001; Sire *et al.*, 2009) and in their Mesozoic fossil relatives (Goodrich, 1907; Ørvig, 1978; Gayet & Meunier, 1992; Meunier & Brito, 2004; Meunier *et al.*, 2016). Sturgeons and paddlefishes (Acipenseriformes) have a poorly mineralised skeleton, but it is nonetheless composed of cellular bone (e.g. Kölliker, 1859; Stéphan, 1900; de Buffrénil *et al.*, 2016; Leprévost *et al.*, 2017). Finally, many clades of extinct Mesozoic actinopterygians have been surveyed histologically and show cellular bone, for example: †saurichthyids (Scheyer *et al.*, 2014), †aspidorhynchids (Brito & Meunier, 2000), †pachycormids (Meunier & Brito, 2004; Liston *et al.*, 2013), and †pholidophorids (Meunier & Brito, 2004). Our SRμCT data provide additional information on a series of fossil non-teleost actinopterygians, revealing the presence of cellular bone in the Jurassic stem chondrostean †*Chondrosteus acipenseroides*, the Jurassic †pycnodontiform †*Proscinetes elegans*, the Jurassic †dapediid †*Dapedium* sp., the Triassic holosteans †*Heterolepidotus dorsalis* and †*Eoeugnathus megalepis* and the Jurassic stem bowfin †*Caturus furcatus*. These data also confirm the presence of cellular bone in 17 Jurassic and Cretaceous taxa (Tables S1, Figure S1) interpreted as stem teleosts (e.g. Arratia, 2015).

(*c*) *Elopomorpha*

Within Elopomorpha, cellular bone is found in tarpons and their relatives (Elopiformes), including in scales (Kölliker, 1859; Meunier & Brito, 2004). Several eels (Anguilliformes)

Table 1. Bone type (presence or absence of osteocytes) in the taxa surveyed by our synchrotron microtomography (SR-μCT) data.

Taxon		Species name	Vernacular name	Type	Bone sampled	Specimen used
†Cheirolepidiformes	†Cheirolepididae	†*Cheirolepis canadensis*	–	C	dentary	UMMP 3453
Polypteriformes	Polypteridae	*Erpetoichthys calabaricus*	reedfish	C	dentary	Université de Poitiers, uncat.
		Polypterus delhezi	barred bichir	C	dentary	Université de Poitiers, uncat.
Incertae sedis	*Incertae sedis*	†*Birgeria stensioei*	–	C	dentary	PIMUZ T2188
Chondrostei	†Chondrosteidae	†*Chondrosteus acipenseroides*	–	C	dentary	NHMUK PV P 2261a
	Aipenseridae	*Acipenser gueldenstaedtii*	Danube sturgeon	C	dentary	MNHN.ICOS.01529
†Pycnodontiformes	†Pycnodontidae	†*Proscinetes elegans*	–	C	dentary	NHMUK PV P 1626
†Dapediiformes	†Dapediidae	†*Dapedium* sp.	–	C	dentary	OUMNH J.3041
Holostei	*Incertae sedis*	†*Eoeugnathus megalepis*	–	C	dentary	PIMUZ T344
		†*Heterolepidotus dorsalis*	–	C	dentary	NHMUK PV P 10290
		†*Hulettia americana*	–	C	dentary	UMMP 11217
	Amiidae	*Amia calva*	bowfin	C	dentary	OUMNH 21648
	†Caturidae	†*Caturus furcatus*	–	C	dentary	private collection
	Lepisosteidae	*Atractosteus tropicus*	tropical gar	C	dentary	MNHN.ICOS. PB-901
		Lepisosteus oculatus	spotted gar	C	dentary	UMMZ 178806/S
	†Semionotidae	†*Semionotus elegans*	–	C	dentary	UMMP 13664
†Aspidorhynchiformes	†Aspidorhynchidae	†*Aspidorhynchus* cf. *eodus*	–	C	dentary	private collection
		†*Vinctifer comptoni*	–	C	dentary	UMMP 101950
†Pachycormiformes	†Pachycormidae	†*Euthynotus incognitus*	–	C	dentary, rib	NHMUK PV P 2044
		†*Hypsocormus* sp.	–	C	dentary	private collection
		†*Leedsichthys problematicus*	–	C	gill raker	private collection
		†*Pachycormus macropterus*	–	C	dentary	MNHN.F.JRE87
†Pholidophoriformes	*Incertae sedis*	†*Pholidophoroides crenulata*	–	C	dentary	NHMUK PV OR 36313
		†*Pholidophoropsis caudalis*	–	C	dentary	OUMNH J.3363
†Dorsetichthyiformes	†Dorsetichthyidae	†*Dorsetichthys bechei*	–	C	dentary	OUMNH J.3369
†Leptolepidiformes	†Ascalaboidae	†*Ascalabos voithii*	–	C	dentary	NHMUK PV P 3673a
		†*Tharsis dubius*	–	C	dentary	NHMUK PV OR 37852b
	†Leptolepididae	†*Leptolepis macrophthalmus*	–	C	dentary	private collection
†Ichthyodectiformes	*Incertae sedis*	†*Allothrissops regleyi*	–	C	dentary, rib	NHMUK PV P 921
		†*Pachythrissops laevis*	–	C	dentary	NHMUK PV P 41859
		†*Thrissops formosus*	–	C	dentary, rib	NHMUK PV OR 35013
	†Ichthyodectidae	†*Ichthyodectes* cf.*ctenodon*	–	C	dentary	UMMP V56318
		†*Xiphactinus* cf.*audax*	–	C	dentary	UMMP 11003
†Crossognathiformes	†Crossognathidae	†*Rhacolepis buccalis*	–	C	dentary	UMMP 101952
Elopomorpha	*Incertae sedis*	†*Osmeroides* sp.	–	C	dentary	OUMNH K.64151
		†*Urenchelys germanus*	–	C	dentary	NHMUK PV P 62726
	Albulidae	*Albula vulpes*	bonefish	C	rib, opercle, ceratobranchial	UMMZ 186965/S
		†*Istieus grandis*	–	C	dentary	NHMUK PV P 3886
	Elopidae	†*Anaethalion angustus*	–	C	dentary, rib	NHMUK PV OR 37926
		†*Davichthys gardineri*	–	C	dentary	NHMUK PV P 63231
		Elops saurus	ladyfish	C	dentary, rib	UMMZ 189366/S

Table 1. Continued

Taxon		Species name	Vernacular name	Type	Bone sampled	Specimen used
	Megalopidae	†*Flindersichthys denmaedi*	–	C	dentary	NHMUK PV P 59694
		Megalops cyprinoides	Indo-Pacific tarpon	C	dentary, rib	MNHN.ICOS.00987
	Anguillidae	*Anguilla anguilla*	European eel	C	dentary	MNHN.ICOS.PB-D-35
	Congridae	*Conger conger*	European conger	C	dentary, rib	MNHN.ICOS.PB-SP-24
	Muraenesocidae	*Muraenesox cinereus*	daggertooth pike-conger	C	dentary	MNHN.ICOS.00286
	Muraenidae	*Gymnothorax moringa*	spotted moray	C	dentary, rib	UMMZ 173403/S
		Muraena helena	Mediterranean moray	C	dentary	MNHN.ICOS.01039
Osteoglossomorpha	Hiodontidae	†*Eohiodon falcatus*	–	C	dentary	NHMUK PV P 61245
		Hiodon alosoides	goldeye	C	dentary	UMMZ 189540/S
		Hiodon tergisus	mooneye	C	rib	UMMZ 180315/S
	Notopteridae	*Chitala chitala*	giant featherback	C	dentary, rib	UMMZ 193675/S
	Osteoglossidae	*Arapaima gigas*	arapaima	C	dentary, rib	MNHN.ICOS.PB-557
		†*Brychaetus muelleri*	–	C	dentary	NHMUK PV OR 28424
		Osteoglossum bicirrhosum	silver arowana	C	dentary, rib	MNHN.ICOS.00630
		†*Phareodus encaustus*	–	C	dentary	NHMUK PV P 64636I
Clupeomorpha	*Incertae sedis*	†*Knightia* sp.	–	C	dentary	UMMP Tmp-1008
	†Armigatidae	†*Armigatus namourensis*	–	C	dentary, rib	NHMUK PV P 63151a
	†Ellimmichthyidae	†*Ellimmichthys longicostatus*	–	C	dentary, rib	NHMUK PV P 9855
	Chirocentridae	*Chirocentrus dorab*	wolf-herring	C	dentary, rib	UMMZ 220543/S
	Clupeidae	*Alosa pseudoharengus*	alewife	C	dentary, rib	UMMZ 187300/S
		Sardinops sagax	Pacific sardine	C	dentary	MNHN.ICOS.PB-5036
Gonorhynchiformes	Chanidae	†*Tharrias araripes*	–	C	dentary, rib	NHMUK PV P 54675b
Cypriniformes	Catostomidae	*Catostomus commersonii*	white sucker	C	dentary, rib	UMMZ 178869/S
	Cyprinidae	*Cyprinus carpio*	common carp	C	various bones[1]	MNHN.ICOS.00610
	Leuciscidae	*Abramis brama*	freshwater bream	C	dentary	MNHN.ICOS.00756
	Tincidae	*Tinca tinca*	tench	C	dentary	MNHN.ICOS.00585
Characiformes	Cynodontidae	*Hydrolycus scomberoides*	payara	C	dentary	MNHN.ICOS.01021
	Erythrinidae	*Hoplias malabaricus*	trahira	C	dentary, rib	MNHN.ICOS.00631
	Serrasalmidae	*Serrasalmus spilopleura*	speckled piranha	C	dentary, rib	MNHN.ICOS.01027
Siluriformes	Ariidae	*Ariopsis felis*	hardhead sea catfish	C	dentary, rib	UMMZ 223241/S
		Galeichthys feliceps	white barbel	C	dentary	MNHN.ICOS.00875
	Heptapteridae	*Pimelodella gracilis*	graceful pimelodella	C	dentary, rib	UMMZ 204550/S
	Trichomycteridae	*Trichomycterus* sp.	pencil catfish	A	dentary	MNHN.ICOS.00887
Gymnotiformes	Gymnotidae	*Gymnotus carapo*	banded knifefish	C	dentary, rib	UMMZ 207893/S
Euteleostei	*Incertae sedis*	†*Spaniodon elongatus*	–	C	dentary, rib	NHMUK PV OR 44831
Salmoniformes	Salmonidae	*Coregonus reighardi*	shortnose cisco	C	dentary, rib	UMMZ 172476/S
		Oncorhynchus clarkii	cutthroat trout	C	dentary	UMMZ 191615/S
		†*Oncorhynchus lacustris*	–	C	dentary	UMMP 47839
		Oncorhynchus mykiss	rainbow trout	C	dentary	UMMZ uncat.
		Oncorhynchus tshawytscha	Chinook salmon	C	dentary	UMMZ uncat.
		†*Paleolox larsoni*	–	C	dentary	UMMP 50352
		Parahucho perryi	Japanese huchen	C	dentary	UMMZ 187612
		†*Prosopium prolixus*	–	C	dentary	UMMP 21728

Table 1. continued

Taxon		Species name	Vernacular name	Type	Bone sampled	Specimen used
		Prosopium williamsoni	mountain whitefish	C	dentary	UMMZ 182503/S
		Salmo salar	Atlantic salmon	C	dentary	MNHN.ICOS.00619
		Salmo trutta	sea trout	C	dentary	UMMZ uncat.
		Salvelinus confluentus	bull trout	C	dentary	UMMZ uncat.
		Salvelinus fontinalis	brook trout	C	dentary	UMMZ uncat.
		Salvelinus namaycush	lake trout	C	dentary	UMMZ 177542
		Stenodus leucichthys	inconnu	C	dentary, rib	UMMZ 187119/S
		Thymallus thymallus	grayling	A	rib	MNHN.ICOS.00626
Acanthomorpha	Veliferidae	*Metavelifer multiradiatus*	spinyfin velifer	A	rib	AMNH I-91798 SD
	Lampridiidae	*Lampris* sp.	opah	C	sclerotic ossicle	AMNH I-21766 SD
	Polymixiidae	*Polymixia nobilis*	stout beardfish	A	rib	AMNH I-210677 SD
	Coryphaenidae	*Coryphaena hippurus*	common dolphinfish	A	rib	MNHN.ICOS.00189
	Carangidae	*Trachurus trachurus*	horse mackerel	A	rib	MNHN.ICOS.PB-A-14
	Xiphiidae	*Xiphias gladius*	swordfish	A	rib, sclerotic ossicle	MNHN.ICOS.PB-6988, AMNH I-15658 SD
	Istiophoridae	*Kajikia albida*	Atlantic white marlin	A	rib, sclerotic ossicle	UMMZ 198674/S
	Moronidae	*Dicentrarchus labrax*	European seabass	A	rib	private collection
	Scaridae	*Chlorurus microrhinos*	steephead parrotfish	A	rib	MNHN.ICOS.00912
	Scombridae	*Acanthocybium solandri*	wahoo	A	rib	MNHN.ICOS.01010
		Euthynnus affinis	little tunny	C	rib	AMNH I-56274 SD
		Gasterochisma melampus	butterfly kingfish	A	rib, sclerotic ossicle	AMNH I-93480 SD
		Gymnosarda unicolor	dogtooth 'tuna'	A	rib	MNHN.ICOS.00492
		Sarda orientalis	striped bonito	A	rib	MNHN.ICOS.00954
		Scomber australasicus	blue mackerel	A	rib	MNHN.ICOS.00254
		Thunnus obesus	bigeye tuna	C	rib	MNHN.ICOS.00374

C, cellular bone; A, acellular bone.

[1]Bones sampled for *Cyprinus carpio*: frontal, maxilla, dentary, pharyngobranchial, opercle, abdominal vertebra, rib, dorsal-fin spine, cleithrum, pelvic bone. Specimens were obtained from private collectors and from the following natural history collections: American Museum of Natural History, New York City, USA (AMNH); Muséum national d'Histoire naturelle, Paris, France (MNHN); Natural History Museum, London, UK (NHMUK); Oxford University Museum of Natural History, Oxford, UK (OUMNH); Paleontological Institute and Museum, Zurich, Switzerland (PIMUZ); University of Michigan Museum of Paleontology (UMMP) and of Zoology, Ann Arbor, USA (UMMZ); Université de Poitiers, France.

are described as having acellular bone by Kölliker (1859). However, they all seem to pertain to an outdated taxonomy that treated leptocephalus larvae as separate taxa (Table S1). For example, Kölliker (1859) reports cellular bone in the sorcerer eel *Nettastoma melanurum* and acellular bone in '*Hyoprorus messanensis*', corresponding to the larva of *N. melanurum* (Eschmeyer *et al.*, 2019). Although Moss (1961*b*) reports acellular bone in the moray eel *Gymnothorax moringa*, we confirm the presence of osteocytes in this species, as well as in the adults of every other anguilliform surveyed, including the freshwater eels *Anguilla anguilla* and *A. rostrata* (Stéphan, 1900; Moss, 1965; Lopez, 1970), the conger eel *Conger conger* and the pike conger *Muraenesox cinereus* (Table 1). The bonefish *Albula vulpes* was described as having a mix of cellular and acellular bone (Moss, 1961*b*), but this is contradicted by our observations (see Section III.3*a*). Finally, our SRμCT data reveal cellular bone in several fossil albuliforms (†*Istieus*, †*Lebonichthys*), elopiforms (†*Ichthyemidion*, †*Anaethalion*, †*Flindersichthys*) and anguilliforms (†*Urenchelys*). In conclusion, we find that cellular bone is present in post-larval individuals of all elopomorphs surveyed so far.

(*d*) *Osteoglossomorpha*

Fossil and extant bony-tongue fishes (Osteoglossomorpha) have cellular bone in their skeleton, including scales (Kölliker, 1859; Meunier & Brito, 2004; Meunier, Brito & Leal, 2013*a*; Meunier, Dutheil & Brito, 2013*b*). Moss (1965) reported acellular bone in the two modern mooneye (Hiodontidae) species, *Hiodon alosoides* and *H. tergisus*. However, Kölliker (1859) described cellular bone in '*Hyodon claudulus*', that could be synonymised with *H. alosoides* (Eschmeyer *et al.*, 2019). We resolved this uncertainty using unambiguous observations of osteocyte lacunae in SRμCT images of dentaries and/or ribs from *H. alosoides*, *H. tergisus* and their Eocene close relative †*Eohiodon falcatus*, confirming the presence of cellular bone in hiodontids. We also find cellular bone in *Arapaima gigas*, in the arowana *Osteoglossum bicirrhosum* and its extinct Eocene relatives †*Brychaetus muelleri* and †*Phareodus encaustus*, as well as in the featherback *Chitala chitala* (Table 1). In conclusion, it is likely that cellular bone is present in all osteoglossomorphs (Table S1).

(*e*) *Clupeomorpha*

Herrings and their relatives (Clupeomorpha) appear to have cellular bone (Kölliker, 1859; Moss, 1961*b*, 1965). Although Moss (1961*b*) reported acellular bone in the anchovy *Anchoviella* sp. and the American shad *Alosa sapidissima*, he later updated this observation by reporting cellular bone in *A. sapidissima* and three other *Alosa* species (Moss, 1965). Our SRμCT data reveal cellular bone in all clupeomorphs surveyed (Table 1), including the Cretaceous †*Armigatus namourensis* and †*Ellimmichthys longicostatus* and the Eocene †*Knightia* sp., as well as the extant wolf-herring *Chirocentrus dorab*, the Pacific sardine *Sardinops sagax* and the alewife *Alosa pseudoharengus*. In conclusion, it is likely that cellular bone is present in all clupeomorphs (Table S1), with the possible exception of *Anchoviella* that needs further appraisal.

(*f*) *Ostariophysi*

Kölliker (1859) and Moss (1961*b*, 1965), extensively sampled the considerable diversity of the mostly freshwater ostariophysans, including milkfishes (Gonorhynchiformes), carps and relatives (Cypriniformes), characins and relatives (Characiformes), catfishes (Siluriformes) and electric 'eels' (Gymnotiformes). Their surveys totalled 115 species, virtually all of which appear to have cellular bone (Table S1). We also observed cellular bone in our SRμCT images of the carp *Cyprinus carpio*, the tench *Tinca tinca*, the bream *Abramis brama* (Cypriniformes), the trahira *Hoplias malabaricus*, the payara *Hydrolycus scomberoides*, the piranha *Serrasalmus spilopleura* (Characiformes), the catfishes *Ariopsis felis*, *Galeichthys feliceps* and *Pimelodella gracilis* (Siluriformes), and the banded knifefish *Gymnotus carapo* (Gymnotiformes), as well as in the Early Cretaceous gonorhynchiform †*Tharrias araripes* (Table 1). Acellular bone is only described in two ostariophysan species (Table S1): in the diminutive pencil catfish *Trichomycterus punctulatus* (Kölliker, 1859),which is confirmed by our SRμCT data from the dentary of another *Trichomycterus* species, and in some cranial dermal bones of the zebrafish *Danio rerio* (Weigele & Franz-Odendaal, 2016). In conclusion, cellular bone is present in all ostariophysans surveyed so far, with the notable exceptions of *Trichomycterus*. In addition, slickheads (Alepocephaliformes) are consistently recovered as sister to ostariophysans in molecular phylogenies (Lavoué *et al.*, 2008; Near *et al.*, 2012; Betancur-R *et al.*, 2013; Hughes *et al.*, 2018; Straube *et al.*, 2018). The only species surveyed from the group, *Alepocephalus rostratus*, has cellular bone (Kölliker, 1859).

(*g*) *Non-neoteleost Euteleostei*

Bone type is variable amongst Euteleostei, but generally homogeneous within a given lineage (Table S1). Acellular bone is found in galaxiids (but only two species of *Galaxias* have been surveyed), pikes and mudminnows (Esociformes; Kölliker, 1859; Moss, 1961*b*, 1965), smelts (Osmeridae; Moss, 1961*b*, 1965) and viperfishes and their relatives (Stomiiformes; Kölliker, 1859; Germain *et al.*, 2019). Conversely, cellular bone is found in *Argentina silus* (the only member of Argentiniformes that was sampled) and we observe it in the Late Cretaceous †*Spaniodon elongatus*, a taxon whose phylogenetic position within euteleosts is uncertain (e.g. Taverne & Filleul, 2003). Salmons, trouts and their relatives (Salmoniformes) are generally described as having cellular bone (Kölliker, 1859; Moss, 1961*b*, 1965; Hughes *et al.*, 1994; Witten & Hall, 2002; Totland *et al.*, 2011), but our extensive SRμCT sampling within the group complicates this pattern (Table 1). Bone always appears to be cellular in the 'typical' trouts and salmons (Salmoninae). We confirm this for extant and fossil representatives of *Oncorhynchus*, *Salmo*, *Salvelinus* and *Parahucho*. The whitefishes *Coregonus reighardi*, *Prosopium williamsoni* and *Stenodus leucichthys*

(Coregoninae) also seem to have osteocytes, but they are much scarcer than in salmonines, and irregularly distributed inside of bone. This is consistent with the observation of Moss (1965), who described variation in osteocyte abundance within the skeleton in some salmoniforms. Finally, in the grayling *Thymallus thymallus* (Thymallinae), bone seems to be acellular.

(*h*) *Neoteleostei, including Acanthomorpha*

Within the euteleost subclade Neoteleostei (*sensu* Betancur-R *et al.*, 2017), acellular bone is found in various lizardfishes (Aulopiformes), including the Late Cretaceous †*Eurypholis* sp., in the lanternfish (Myctophiformes) *Notoscopelus elongatus* and in the Cretaceous taxon of uncertain placement †*Ctenothrissa vexillifer* (Kölliker, 1859; Moss, 1961*b*; Davesne *et al.*, 2018). Spiny-rayed fishes (Acanthomorpha) contribute the greatest fraction of neoteleost species diversity. Amongst the approximately 17,000 acanthomorph species (more than 300 being surveyed in the present study), acellular bone is virtually universal (Kölliker, 1859; Moss, 1961*b*, 1965) and is found throughout taxa displaying a broad range of morphologies and ecologies (Table S1), from marine benthic taxa such as toadfishes and sculpins (Simmons *et al.*, 1970; Horton & Summers, 2009), to pelagic fast-swimming taxa like jacks and billfishes (Smith-Vaniz *et al.*, 1995; Atkins *et al.*, 2014), deep-sea eelpouts (Meunier & Arnulf, 2018), or freshwater ricefishes and tilapias (Ekanayake & Hall, 1987; Cohen *et al.*, 2012). Within acanthomorphs, cellular bone is only known conclusively in two relatively species-poor lineages: the 'true' tunas *Auxis*, *Euthynnus*, *Katsuwonus* and *Thunnus* (Kölliker, 1859; Stéphan, 1900; Amprino & Godina, 1956; Moss, 1961*b*; Meunier & Huysseune, 1992; Santamaria *et al.*, 2018) and the opah *Lampris* (Davesne *et al.*, 2018). At least in tunas, osteocytes are present not only in bones, but also in scales, fin rays and spines (Meunier *et al.*, 2008*a*; Santamaria *et al.*, 2018; Wainwright, Ingersoll & Lauder, 2018).

(3) Intra-specific and intra-individual variation

(*a*) *Occurrence of mixed bone types*

Comparative literature generally states that when cellular or acellular bone is found, it occurs throughout the whole skeleton, including dermal and endochondral bone, fin rays and spines (Kölliker, 1859; Moss, 1961*b*, 1963; Meunier & Huysseune, 1992). The incompletely mineralised elasmoid scales of most modern teleosts are an exception: they are often acellular when the rest of the skeleton is cellular (see Section III.4). Moss (1961*b*) reported that the bonefish *Albula vulpes* displays a mix of cellular and acellular bone, with the latter being found in the operculum and gill arches. However, our SRμCT data including the operculum and gill arches show osteocytes in all of these elements. These observations suggest that the whole skeleton of *A. vulpes* is cellular, contradicting Moss' (1961*b*) statement.

Weigele & Franz-Odendaal (2016) showed that in the zebrafish *Danio rerio*, bones with and without osteocytes coexist within the cranial skeleton of a given individual. Both dermal and endochondral bones can be cellular or acellular, but dermal intramembranous bones of the neurocranium seem more likely to be acellular, while endochondral bones of the splanchnocranium (e.g. palatoquadrate, hyoid and branchial arches) are all cellular. These results imply that using only the dermal neurocranium to describe bone type in a teleost species can potentially be misleading. Conversely, jaw bones (such as the dentary) and the postcranium (vertebrae excepted) are all cellular in *D. rerio* (Weigele & Franz-Odendaal, 2016). This suggests that our SRμCT data (Table 1), which rely on dentaries and/or ribs, accurately reflect cellularity: if acellular bone is found in these elements it is most likely to reflect the rest of the skeleton.

A possibility is that this pattern of mixed bone types stems from the very small adult body size of *D. rerio*. In very thin bones, there might not be enough bone matrix for osteoblasts to become entrapped and turn into osteocytes. For instance, some of the acellular bones observed in *D. rerio* are approximately 10 μm thick (Weigele & Franz-Odendaal, 2016), in an animal which rarely exceeds 40 mm in total adult length (Spence *et al.*, 2008). While the frontal bone is described as acellular in *D. rerio* (Weigele & Franz-Odendaal, 2016), we observe with SRμCT osteocytes in the frontal bone of the carp *Cyprinus carpio*, a closely related cypriniform. Since this observation comes from a carp of 452 mm in total length, it would corroborate our hypothesis of a size-related acellularity in *D. rerio*, and potentially other teleosts. Surveying various cranial bones in other teleost taxa and on specimens of various sizes would help clarify whether this pattern of mixed bone types is widespread in teleosts, or specific to *D. rerio*. Observations based on ontogenetic series of other taxa also corroborate that the absence of osteocytes might be explained by the size of the bone. For example, Huysseune (2000) reports that very young individuals of teleosts with cellular bone often lack osteocytes, which appear once bone becomes thicker. This would also explain Kölliker's (1859) observations of acellular bone in larval anguilliforms (see Section III.2*c*).

(*b*) *Alleged osteocytes in tubular and hyperostotic bone*

The presence of a few osteocytes in very localised zones of otherwise acellular bone has been suggested for some species, relying upon two specific cases. In the first case, osteocytes were detected in tubules containing collagen bundles and osteoblastic canaliculi in three species of sparids (sea breams), an acanthomorph family otherwise characterised by acellular bone (Hughes *et al.*, 1994). However these results are seemingly contradicted by more recent data (Sire & Meunier, 2017): at least in the case of *Sparus aurata* these tubules do not appear to contain osteocyte nuclei.

In the second case, osteocytes were described within areas of hyperostosis in the cleithrum of the jack *Caranx latus* (Smith-Vaniz *et al.*, 1995) and in dorsal pterygiophores of the oarfish *Regalecus russellii* (Paig-Tran *et al.*, 2016),

two acanthomorphs that otherwise have acellular bone. However, such osteocytes do not appear to be present systematically in acanthomorph hyperostotic bone: they are absent from the hyperostoses of the scabbardfish *Trichiurus lepturus*, the jack mackerel *Trachurus trachurus*, the sicklefish *Drepane africana*, the grunt *Pomadasys kaakan* and the searobin *Prionotus stephanophrys* (Desse *et al.*, 1981; Meunier & Desse, 1994; Meunier, Béarez & Francillon-Vieillot, 1999; Meunier, Gaudant & Bonelli, 2010). The black skipjack tuna *Euthynnus lineatus* has cellular bone in its hyperostotic vertebrae (Béarez *et al.*, 2005), however this is consistent with the presence of cellular bone throughout the rest of its skeleton. The occurrence of osteocytes in hyperostotic regions of an otherwise acellular skeleton then appears to be the exception rather than the rule; it nevertheless requires explanation.

A possibility is that these localised osteocytes could form *via* an accidental incorporation of osteoblasts during the exceptionally rapid growth of hyperostotic bone. This arrangement may be temporary and accidental, and would differ from 'true' cellular bone. Determining whether these osteocytes are present in all hyperostotic individuals of a given species, for example, would help to assess the nature of this phenomenon.

(4) Phylogenetic distribution of acellular bone in actinopterygian scales

The phylogenetic distribution of osteocytes in actinopterygian scales (Table S1) has been less studied than in the rest of the skeleton (Parenti, 1986). Scales in actinopterygians primitively consist of a bony basal plate covered by dentine and ganoine (an enamel-like tissue). The bony component remains as a thin external layer in the elasmoid scales of most teleosts (Francillon-Vieillot *et al.*, 1990; Meunier & Huysseune, 1992; Sire *et al.*, 2009). In ganoid scales, bone is always cellular, as shown in early actinopterygians (Richter & Smith, 1995; Sire *et al.*, 2009; Zylberberg *et al.*, 2016), bichirs (Daget *et al.*, 2001; Sire *et al.*, 2009), holosteans (Meunier, François & Castanet, 1978; Brito, Meunier & Gayet, 2000; Meunier *et al.*, 2016) and stem teleosts (Brito & Meunier, 2000; Meunier & Brito, 2004). In elasmoid scales, found in all teleosts but also in amiids and the extant coelacanth *Latimeria* (Smith, Hobdell & Miller, 1972; Meunier, 1984*b*; Meunier *et al.*, 2008*b*; Sire *et al.*, 2009), the situation is more complex. In this type of scales, the basal layer develops into an incompletely mineralised plywood-like structure called elasmodine (previously described as isopedine). The basal layer in the scales in amiids and some teleosts (e.g. *Megalops*, *Hiodon*, *Arapaima*, *Chanos*) incorporates cells superficially similar to osteocytes, called elasmocytes (Meunier, 1984*b*, 1987; Meunier & Brito, 2004). The bony layer is cellular in the elasmoid scales of amiids (Meunier & Poplin, 1995), elopomorphs (e.g. *Megalops*, *Elops*, *Albula*) and at least some osteoglossomorphs (Meunier, 1984*b*; Meunier & Brito, 2004). It is, however, acellular in other taxa with cellular bone including clupeomorphs, ostariophysans and salmoniforms (Meunier, 1987; Meunier & Brito, 2004; Meunier *et al.*, 2004; Sire *et al.*, 2009). Taxa with acellular bone always seem to have acellular scales as well (Kölliker, 1859). In the tunas *Thunnus alalunga* and *T. obesus* scales are composed of cellular bone (Meunier & Sire, 1981; Wainwright *et al.*, 2018), in agreement with the rest of the skeleton. Since many teleosts with cellular bone lack osteocytes in their scales, it then seems that acellularisation in scales phylogenetically precedes that of the rest of the skeleton (Kölliker, 1859; Meunier, 1987; Meunier & Huysseune, 1992).

IV. PHYLOGENETIC ORIGIN AND EVOLUTION OF ACELLULAR BONE

(1) Ancestral character state reconstruction

For our entire data set of 677 fossil and extant actinopterygians, we scored the presence of cellular or acellular bone (Table S1; scales scored separately). When bone lacks osteocytes only in certain skeletal elements (e.g. teleosts with cellular bone but acellular scales) or ontogenetic stages (e.g. in larval anguilliforms) we scored its status as 'cellular'. This data set was mapped onto three time-calibrated trees stemming from three recent multi-locus or phylogenomic studies of actinopterygian intra-relationships. Topology #1 (T1) was obtained in an analysis of nine nuclear protein-coding loci including 232 taxa, all extant (Near *et al.*, 2012); Topology #2 (T2) is based on an analysis (Betancur-R *et al.*, 2013) of 20 nuclear and one mitochondrial loci including 1582 extant taxa, to which 240 fossil taxa were added based on previously argued phylogenetic placements (Betancur-R *et al.*, 2015); Topology #3 (T3) was obtained from a transcriptomic analysis of 1721 exons (Hughes *et al.*, 2018). In order to achieve consistency in clade names, we relied on the phylogenetic classification proposed by Betancur-R *et al.* (2017), itself based on the molecular phylogeny that yielded T2.

All three topologies mostly differ at the level of the first dichotomies within Euteleostei. They all recover an Osmeriformes + Stomiiformes clade (Stomiati) and a Salmoniformes + Esociformes clade, but Galaxiiformes are sister to Neoteleostei *sensu stricto* in T1, to Salmoniformes + Esociformes in T2, and to Stomiati in T3. Similarly, Argentiniformes are sister to Salmoniformes + Esociformes in T1, to this clade + Galaxiiformes in T2, and to Galaxiiformes + Stomiati in T3.

We used a sub-sample of taxa that are included in both our cellularity data set and at least one of the topologies. When two different species of the same genus were used in two different data sets, we considered the genus as a whole, since no case of cell type variability between species of the same genus is known. This sub-sample retains 100 extant taxa for T1, 292 taxa including 26 fossils for T2, and 121 extant taxa for T3. Every major actinopterygian lineage is present in the resulting trees with a few exceptions for which osteohistological data are lacking entirely, such as the salamanderfish (Lepidogalaxiiformes)

and the jellynose fishes (Ateleopodiformes). Ancestral states at the nodes were reconstructed with the *ace* function of the APE package in R (Paradis *et al.*, 2004). Two models of ancestral character state estimations were tested: an 'all rates different' (ARD) model (that allows transitions from cellular to acellular and from acellular to cellular to have different frequencies) and a 'symmetrical' model (that constrains transition frequencies to be equal). The difference between transition frequencies was very low even with the ARD model, but the Akaike information criterion (AIC) very slightly favoured the symmetrical model, leading us to apply the latter to our analyses.

(2) Reconstructed origin of acellular bone

Results from all topologies recover cellular bone as the plesiomorphic state for actinopterygians, teleosts and every other node outside of Euteleostei, with a very high likelihood of 0.99 (Figs 3, S1–S3). T2 includes fossil taxa but they did not affect the ancestral state reconstructions, since those that were sampled all possess cellular bone in a region of the tree where it is also found in extant taxa (Fig. 3).

The reconstructed ancestral state for Euteleostei is ambiguous and varies from one topology to the other. With T1, the ancestral state for Euteleostei is equivocal. The likelihoods of the ancestral state being 'cellular' or 'acellular' are between 0.45 and 0.55 for three clades: Euteleostei, Argentiniformes+(Esociformes+Salmoniformes) and Esociformes + Salmoniformes (Figs 4, S1). In this scenario, whether cellular bone in argentiniforms and salmoniforms is a secondary reacquisition or the retention of an ancestral state is unclear.

With T2, the ancestral state for Euteleostei is acellular bone with a very high likelihood of 0.95 (Figs 3, 4, S2), implying that argentiniforms and salmoniforms both reacquired cellular bone secondarily and separately. T3 also implies an ancestral acellular bone for Euteleostei (and a secondary reacquisition of cellular bone in argentiniforms and salmoniforms), albeit with a slightly lower likelihood of 0.89 (Figs 4, S3).

T1 and T2 were both produced with similar methods involving multi-locus molecular data sets adequately covering actinopterygian diversity, and it is difficult to establish whether one is more credible than the other. Phylogenetic resolution at the base of the euteleost tree is poor due to conflict between molecular markers and sparse taxon sampling, and remains a point of contention in the literature (Campbell *et al.*, 2017; Hughes *et al.*, 2018; Straube *et al.*, 2018).

Bone histology of the salamanderfish *Lepidogalaxias* has never been studied, but could be critical to accurately reconstruct the ancestral euteleostean state, since it is consistently recovered by molecular studies as the sister group to all other euteleosts (Li *et al.*, 2010; Near *et al.*, 2012; Betancur-R *et al.*, 2013; Campbell *et al.*, 2017; Hughes *et al.*, 2018; Straube *et al.*, 2018). As long as the phylogeny of euteleosts is not stabilised, and the osteohistology of more taxa not is sampled (e.g. other argentiniforms and galaxiids, *Lepidogalaxias*), ambiguity concerning the exact phylogenetic origin of acellular bone will remain. Certain early fossil euteleosts, such as the Late Cretaceous †*Spaniodon* (that has cellular bone) could also potentially play a key role in elucidating this character's evolution. However, their usefulness is hampered by even greater phylogenetic uncertainty than that for living lineages. For example †*Spaniodon* was included in a clade grouping esociforms, salmoniforms and osmeriforms in a phylogenetic analysis (Taverne & Filleul, 2003), but this topology is rejected by modern molecular phylogenies, leaving the position of this fossil taxon unknown.

The megadiverse Neoteleostei (more than 18,000 extant species) are reconstructed as having acellular bone ancestrally with all three topologies (likelihood = 0.99; Figs 3, 4, S1–S3). Two distinct neoteleost lineages are reconstructed as having reacquired cellular bone independently: (i) the 'true' tunas *Auxis*, *Katsuwonus*, *Euthynnus* and *Thunnus*, forming the probably monophyletic Thunnini within Scombridae; (ii) the opah *Lampris* in the monotypic Lamprididae (Fig. 3).

In conclusion, the clade in which acellular bone appears is equivocal with our ancestral state reconstructions. T2 and T3 clearly support that acellular bone appears in Euteleostei, while the ancestral state for Euteleostei is equivocal with T1, leaving open the possibility of an independent appearance of acellular bone in Esociformes and in the clade that unites Stomiati, Galaxiiformes and Neoteleostei (Figs 4, S3).

In any case, acellular bone is almost entirely absent outside of Euteleostei, being notably described in: (i) some larval anguilliforms, (ii) the clupeiform *Anchoviella* sp., (iii) certain cranial dermal bones of the cypriniform *Danio rerio*, and (iv) the siluriform *Trichomycterus* sp. (see Section III.2; Fig 3). It is noteworthy that all these occurrences correspond to either larvae or to taxa with characteristically small adult body sizes. A size-related explanation for the absence of osteocytes cannot be excluded in this context (see Section III.3*a* for an exploration in the case of *D. rerio*).

Acellular bone seems to appear phylogenetically earlier in scales than in the rest of the skeleton (Meunier, 1987; Meunier & Huysseune, 1992). Since acellular scales are described in clupeomorphs, ostariophysans and every euteleost with the exception of tunas (Table S1), we hypothesise that acellular scales are a character state of the clade Clupeocephala (i.e. all modern teleosts but elopomorphs and osteoglossomorphs). A systematic review of the histology of teleost scales is needed to test this hypothesis suitably. The nature of the external layer of teleost scales is controversial, and some authors have proposed that it has a different evolutionary origin to bone (e.g. Sire *et al.*, 2009), potentially explaining why cellularity is lost earlier in this tissue than in 'true' bone.

(3) Secondary reacquisition of cellular bone

(*a*) *Probable occurrence in salmoniforms*

Our ancestral state reconstructions suggest that cellular bone was secondarily reacquired in salmons, trouts and

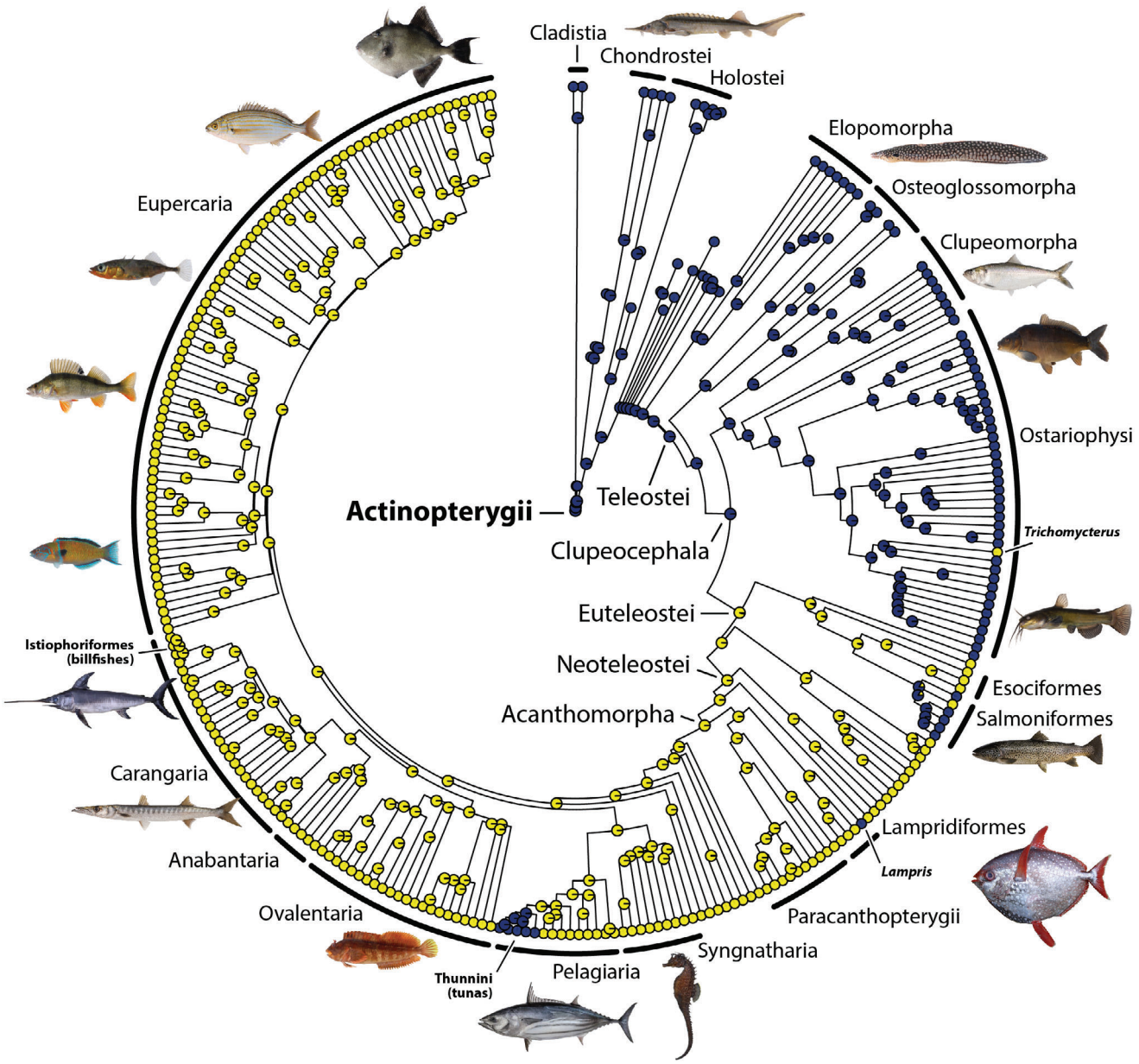

Fig. 3. Time-calibrated multilocus tree of actinopterygians (ray-finned fishes), obtained from the optimisation of the character states 'cellular bone' (in dark blue) and 'acellular bone' in (yellow) on the topology T2 (Betancur-R *et al.*, 2015). Character states for coded species are at the tips, and the reconstructed ancestral states at the nodes. A few key taxa, discussed in the text, are signalled in bold case. Taxon pictures are from Iglésias (2014*b*).

their relatives (Salmoniformes), but this is equivocal due to topological uncertainty at the base of the euteleost tree (Fig 4). The same reconstructions also equivocally support a separate secondary reacquisition of osteocytes in argentiniforms. However, since our data only rely on one species (*Argentina silus*) and the phylogenetic position of argentiniformes is highly uncertain, we refrain from commenting until more observations are available.

As described above, cellular bone does not seem to be distributed uniformly within salmoniforms, according to our SRμCT data (Tables 1, S1): (i) in the grayling *Thymallus thymallus* (Thymallinae), we did not observe osteocytes conclusively; (ii) in the shortnose cisco *Coregonus reighardi* (Coregoninae), osteocytes are present, but sparsely distributed within bone; (iii) in *Stenodus leucichthys* (Coregoninae) and all observed Salmoninae, osteocytes are present, and uniformly distributed inside bone. Moss (1965) already noted that osteocyte abundance varies within bone elements in at least some salmoniforms, which has been interpreted by Parenti (1986) as a possible 'intermediate' stage between cellular and acellular bone.

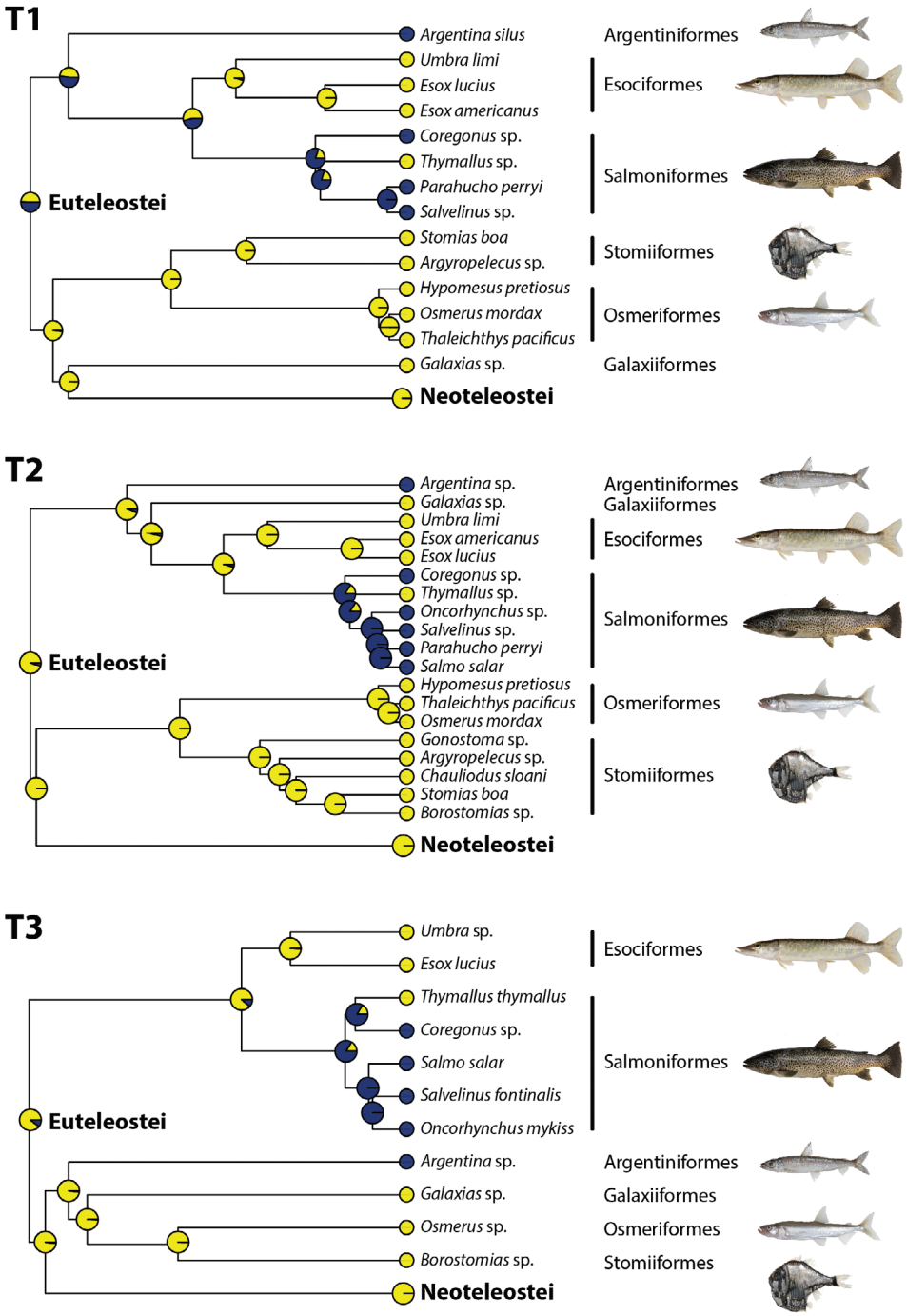

Fig. 4. Sections of the time-calibrated multilocus trees obtained from the optimisation of the character states 'cellular bone' (in dark blue) and 'acellular bone' in (yellow) on topologies T1 (Near *et al.*, 2012), T2 (Betancur-R *et al.*, 2015) and T3 (Hughes *et al.*, 2018), highlighting divergences at the level of the euteleost clade. Character states for coded species are at the tips, and the reconstructed ancestral states at the nodes. Taxon pictures are from Iglésias (2014*b*).

Salmoniform phylogeny is currently disputed, particularly in the relationships between thymallines, coregonines and salmonines. Recent molecular studies have recovered three different topologies: Coregoninae + Salmoninae (Alexandrou *et al.*, 2013; Horreo, 2017), Thymallinae + Salmoninae (Crête-Lafrenière, Weir & Bernatchez, 2012; Near *et al.*, 2012; Betancur-R *et al.*, 2013), and Coregoninae + Thymallinae (Campbell *et al.*, 2013; Macqueen & Johnston, 2014; Hughes *et al.*, 2018), also affecting the three topologies we used in our analyses. These competing phylogenies mean that the pattern of evolution of cellular bone in salmoniforms as a whole is uncertain.

Many salmoniforms are anadromous, meaning that sexually mature individuals migrate upstream over sometimes long distances. This behaviour involves intense and sustained swimming activity, which is likely to affect physiology and metabolism. How it influences bone growth and structure is not fully understood, but it appears that bone responds adaptively to the anadromous lifestyle. In the Atlantic salmon (*Salmo salar*), bones undergo halastasis (a diffuse demineralisation without degradation of the organic matrix) during spawning migration (Kacem & Meunier, 2003, 2009). In addition, *S. salar* shows a prominent increase in the volume of osteocyte lacunae in adult specimens compared to juveniles, which is probably explained by osteocytic osteolysis (Kacem & Meunier, 2000). Moreover, bone in salmons exposed to sustained swimming shows increases in osteocyte abundance (Totland *et al.*, 2011). These observations support the hypothesis that osteocytes play an important role in resorbing salmon bone during anadromous migration. Anadromy is likely to be a trait that evolved multiple times in various lineages within salmoniforms from strictly freshwater ancestors (McDowall, 1997, 2001; Alexandrou *et al.*, 2013). Anadromy is widespread in salmonines (especially in the clade formed by *Salmo*, *Oncorhynchus* and *Salvelinus*), and in most species of *Coregonus* (Alexandrou *et al.*, 2013). Osteocytes are also observed in all of these taxa, while they seem to be absent in the non-migrating freshwater *Thymallus* and in esociforms, the probable sister group to salmoniforms. The occurrence of cellular bone then roughly follows that of anadromy in this particular teleost clade. A notable exception occurs in the genus *Prosopium*, a non-migrating taxon that possesses cellular bone. Nevertheless, it is possible that the hypothesised reacquisition of cellular bone in at least some salmoniforms would have allowed or facilitated the evolution of anadromy in these animals, using a combination of halastasis and osteocytic osteolysis to function as a source of calcium and/or phosphorus for metabolism and muscle activity. A more extensive survey of bone histology in salmoniforms, especially for taxa that have not been studied so far (such as the non-migrating salmonines *Hucho* and *Brachymystax*), and in anadromous euteleosts outside of salmoniforms, is necessary to investigate the potential coevolution of bone cellularity with anadromous habits.

(*b*) *Convergent occurrences in red-muscle endotherms*

Unlike salmoniforms, there is no ambiguity that osteocytes were reacquired secondarily in two acanthomorph lineages (Figs 3, 5A): tunas and the opah (Davesne *et al.*, 2018). Tunas are scombrids, a family that molecular analyses place reliably into the clade Pelagiaria, itself included in the ultradiverse acanthomorph clade Percomorpha (Betancur-R *et al.*, 2013, 2017; Miya *et al.*, 2013; Near *et al.*, 2013; Alfaro *et al.*, 2018). The 'true' tunas (Thunnini) consist of five genera (*Allothunnus*, *Auxis*, *Euthynnus*, *Katsuwonus* and *Thunnus*), and their monophyly is supported by morphological (Collette *et al.*, 1984; Carpenter, Collette & Russo, 1995) and most molecular phylogenies (Block *et al.*, 1993; Betancur-R *et al.*, 2013; Miya *et al.*, 2013). The opah (*Lampris* sp.) is a lampridiform, a clade whose phylogenetic position within acanthomorphs is uncertain, but that branches outside of Percomorpha in any case (Betancur-R *et al.*, 2013; Near *et al.*, 2013; Davesne *et al.*, 2014, 2016; Alfaro *et al.*, 2018). There is then clear evidence that the secondary reacquisition of osteocytes occurred independently in both lineages (Davesne *et al.*, 2018).

While cellular bone has long been known in tunas (Kölliker, 1859; Stéphan, 1900; Amprino & Godina, 1956; Moss, 1961*b*), less data was available on other scombrid taxa and acellular bone was known only from the Atlantic mackerel *Scomber scombrus* and the Spanish mackerel *Scomberomorus maculatus* (Kölliker, 1859; Amprino & Godina, 1956; Moss, 1961*b*). Our SRμCT data allow us to confirm the absence of osteocytes from the ribs of a larger sample of scombrids: the butterfly kingfish *Gasterochisma melampus*, the blue mackerel *Scomber australasicus*, the wahoo *Acanthocybium solandri*, the bonito *Sarda orientalis* and the dogtooth 'tuna' *Gymnosarda unicolor* (Table 1, Fig. 5C, D). *Sarda* and *Gymnosarda* are particularly relevant because they probably constitute the sister group to Thunnini (Collette *et al.*, 1984; Block *et al.*, 1993; Miya *et al.*, 2013). All of these taxa are outside of Thunnini, supporting that 'true' tunas are the only scombrids with cellular bone (Fig. 5A, E).

Within lampridiforms, acellular bone has been described in the ribbonfishes *Trachipterus trachypterus* and *Zu cristatus* (Kölliker, 1859), in the oarfish *Regalecus russelii* (Paig-Tran *et al.*, 2016) and in the veliferid *Velifer hypselopterus* (Davesne *et al.*, 2018). Our SRμCT data show that the veliferid *Metavelifer multiradiatus* also lacks osteocytes (Table 1), and veliferids are probably sister to all other lampridiforms (Olney, Johnson & Baldwin, 1993; Wiley, Johnson & Dimmick, 1998; Davesne *et al.*, 2014). The absence of osteocytes in veliferids, and in the Cretaceous stem lampridiform †'*Aipichthys*' *velifer* (Davesne *et al.*, 2014; Delbarre *et al.*, 2016) supports that acellular bone is plesiomorphic for lampridiforms (Davesne *et al.*, 2018). Thus, the opah is secondarily cellular within lampridiforms, akin to 'true' tunas within scombrids (Fig. 5A).

Tunas and the opah share many life-history traits, to which the reappearance of osteocytes could potentially be imputed. However, a closer examination of these traits across acanthomorph diversity reveals that most do not correlate with the presence of osteocytes. (i) Sustained, active swimming is also found in other large-bodied pelagic predators with acellular bone, such as carangids (Smith-Vaniz *et al.*, 1995), the dolphinfish *Coryphaena hippurus* (Moss, 1961*b*), billfishes (Kölliker, 1859; Amprino & Godina, 1956; Moss, 1961*b*; Atkins *et al.*, 2014) and several scombrids outside of 'true' tunas (Fig. 5A, C, D). (ii) A large body size does not seem to be a factor either: within scombrids, the osteocytic bullet tuna *Auxis rochei* rarely exceeds 350 mm in total length as an adult (Collette & Nauen, 1983), while the dogtooth 'tuna' *Gymnosarda unicolor* and the wahoo *Acanthocybium solandri* both commonly exceed 1000 mm in total length (Collette & Nauen, 1983) and are anosteocytic

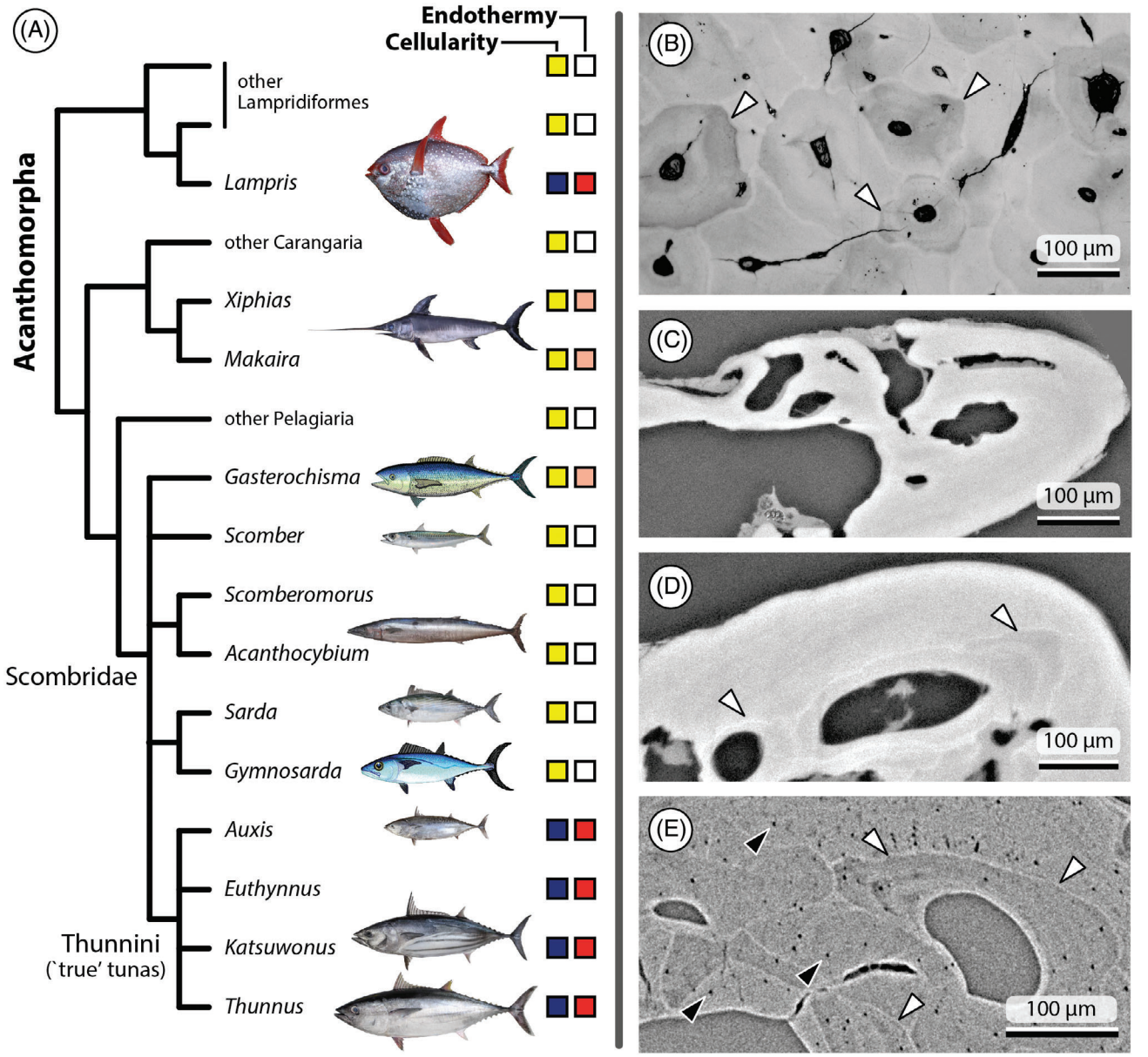

Fig. 5. (A) Phylogenetic distribution of bone type in endothermic acanthomorph teleosts and their close relatives (modified from Davesne *et al.*, 2018). The squares represent bone type (acellular in yellow, cellular in dark blue) and thermal physiology (ectothermy in white, cranial endothermy in salmon pink, red-muscle endothermy in red). Taxon pictures from Iglésias (2014*b*) and R. N. Cada (www.fishbase.org). (B) Thin section through the rostrum of the marlin *Makaira nigricans*, a billfish. Bone is acellular, but shows secondary osteons delimited by resorption lines (white arrowheads). Photograph courtesy of A. Atkins. (C) Synchrotron tomographic slice in a rib of the butterfly kingfish *Gasterochisma melampus*, a scombrid (AMNH I-93480 SD). Bone is acellular. (D) Synchrotron tomographic slice in a rib of the dogtooth 'tuna' *Gymnosarda unicolor*, a scombrid (MNHN.ICOS.00492). Bone is acellular. Note secondary bone deposition around the blood vessels, delimited by resorption lines (white arrowheads). (E) Synchrotron tomographic slice in a rib of the 'true' tuna *Euthynnus affinis* (AMNH I-56274 SD). Bone is cellular (osteocytes marked with black arrowheads), with extensive deposition of secondary bone delimited by resorption lines (white arrowheads). (C–E) Images produced by D. Davesne.

(Fig. 5D). Other very large pelagic acanthomorphs such as the oarfish *Regalecus* sp., billfishes, or the oceanic sunfish *Mola mola* (Kölliker, 1859) all have acellular bone as well. (iii) Finally, the reacquisition of osteocytes does not seem to be linked with bone remodelling activity: bone in tunas, opah and billfishes appears to have active, intense and sustained resorption and remodelling activities (Fig. 5B, D, E) evidenced by the extensive presence of secondary bone (Amprino & Godina, 1956; Poplin *et al.*, 1976; Castanet & de Ricqlès, 1986; Atkins *et al.*, 2014; Davesne *et al.*, 2018). However, bone in billfishes is acellular (Fig. 5B), confirming that this intense remodelling activity does not require the

presence of osteocytes (Atkins *et al.*, 2014; Currey *et al.*, 2017).

Conversely, a correlation between cellular bone and endothermy in acanthomorphs appears to be more substantiated (Meunier, 1987; de Ricqlès *et al.*, 1991; Meunier & Huysseune, 1992; Davesne *et al.*, 2018). Our new SRμCT data confirm that cellular bone co-occurs with a modification in the distribution and position of the lateral aerobic red muscles (Fig. 5A), that concentrate in the anterior portion of the body and become internalised within myotomes, coming closer to the axial skeleton; this configuration is unique to 'true' tunas amongst scombrids (Graham, Koehrn & Dickson, 1983; Block *et al.*, 1993; Graham & Dickson, 2000, 2004). This configuration is thought to be associated with heat production and retention (i.e. endothermy): the heat that is produced by muscle activity during swimming is insulated from the outside and retained within the body due to a network of specialised blood vessels, named retia (Graham *et al.*, 1983; Graham & Dickson, 2001; Katz, 2002). This peculiar configuration has been called 'red-muscle endothermy' by various authors (Block *et al.*, 1993; Dickson & Graham, 2004; Watanabe *et al.*, 2015). The opah developed a distinct form of red-muscle endothermy in which the red pectoral-fin muscles produce most of the heat, are insulated from the outside by a thick fatty layer, and the heat is kept and redistributed *via* retia located within the gills (Wegner *et al.*, 2015). A form of endothermy is also found in two other acanthomorph lineages: billfishes and the butterfly kingfish *Gasterochisma melampus*, a scombrid that is not a 'true' tuna (Fig. 5A). In these cases, heat is produced by specialised modified ocular muscles (the superior rectus in billfishes and the lateral rectus in *G. melampus*) that lost their contractile activity and cycle calcium ions between the cytoplasm and sarcoplasmic reticulum (Carey, 1982; Block, 1986; Block, 1994; Dickson & Graham, 2004). Since it only warms the brain and the eyes, this configuration is often called 'cranial endothermy' (Dickson & Graham, 2004). Osteocytes are absent in the bill and ribs of billfishes (Atkins *et al.*, 2014), and our SRμCT data failed to find them in a rib of *G. melampus* (Table 1, Fig. 5B, C), implying that cranial endotherms, unlike red-muscle endotherms, have acellular bone. We also observe acellular bone in the sclerotic ossicles of *G. melampus* and of the billfishes *Kajikia albida* and *Xiphias gladius* (Table 1), confirming that the cellularity of a bone is not affected by its proximity to the heat-generating muscles. In the opah, the sclerotic ossicles are made of cellular bone like the rest of the skeleton (Table 1).

Heat production by red muscles involved in swimming (rather than modified ocular muscles) and redistribution in a large proportion of the body (rather than in the brain region only) is the key distinction between red-muscle and cranial endothermy. Given that both acanthomorph lineages that developed red-muscle endothermy are also the only ones that reacquired osteocytes, a correlation between these characters is likely (Davesne *et al.*, 2018). As for salmoniforms, we can hypothesise that the correlation stems from an intense muscular activity associated with sustained swimming. The latter is necessary both to hunt prey and to produce heat *via* the myotomal or pectoral red muscles. Since muscles are important consumers of calcium, an element primarily found in bony tissues, osteocytic osteolysis potentially played an important role in the appearance of red-muscle endothermic strategies. Whether the reacquisition of osteocytes facilitated the evolution of red-muscle endothermy, or both characters coevolved under a common selective pressure is unclear.

(c) *Structural evidence for osteocyte re-acquisition in salmoniforms, tunas and opahs*

While osteocyte morphology is very diverse in vertebrate bone in general, two main morphologies seem to occur in teleost cellular bone (Fig. 6). In the first type, osteocytes have a rounded or irregular cell body, and show numerous, thin cytoplasmic processes that branch into canaliculi in all directions. This gives these osteocytes a typically 'star-shaped' morphology (Fig. 6A). In the second type, osteocytes are much more elongate ('spindle-shaped') and orientate in a preferential direction, presumably following the collagen lamellae of the extracellular matrix (Kerschnitzki *et al.*, 2011). Their cell bodies are more regular in shape, and they have only two cytoplasmic processes that are located at the extremities of the cell body, aligning with its long axis. They also have very few, non-branching canaliculi, that tend to orientate in preferential directions (Fig. 6B, C).

Both osteocyte types seem to coexist within teleost cellular bone, for example in *D. rerio* (Weigele & Franz-Odendaal, 2016). Conversely, in the bone of salmoniforms and 'true' tunas (Stéphan, 1900; Meunier & Huysseune, 1992; Totland *et al.*, 2011; Davesne *et al.*, 2018), spindle-shaped osteocytes seem to be the only type that is present (Fig. 6B,C). In the opah, osteocytes are close to the 'spindle-shaped' morphology, since they have very few cytoplasmic processes and canaliculi that all orientate in a preferential direction, but they are not located at the extremities of the cell body like in tunas and salmoniforms (Fig. 6D). It is not clear whether this second type of osteocytes forms a connected canalicular system; at least in salmons they might not be connected to each other at all (Totland *et al.*, 2011). Moreover, their morphology does not seem to change significantly between primary and remodelled bone, for example in the opah (Davesne *et al.*, 2018).

It appears that the three lineages that have in common an inferred or likely reacquisition of cellular bone share these structural similarities in osteocyte morphology. This suggests that their peculiar morphology might be linked with the evolutionary reacquisition of osteocytes from an ancestral acellular bone. Weigele & Franz-Odendaal (2016) proposed that these types of osteocytes have different developmental origins, and that the elongate, spindle-shaped osteocytes are derived from the elongate 'osteoblast-like' cells that line the bone. It is possible that all secondarily reacquired osteocytes share this unique developmental origin, and that the other, 'typical' osteocytes derive from a mode of formation that does not occur in salmoniforms, tunas and opahs and was

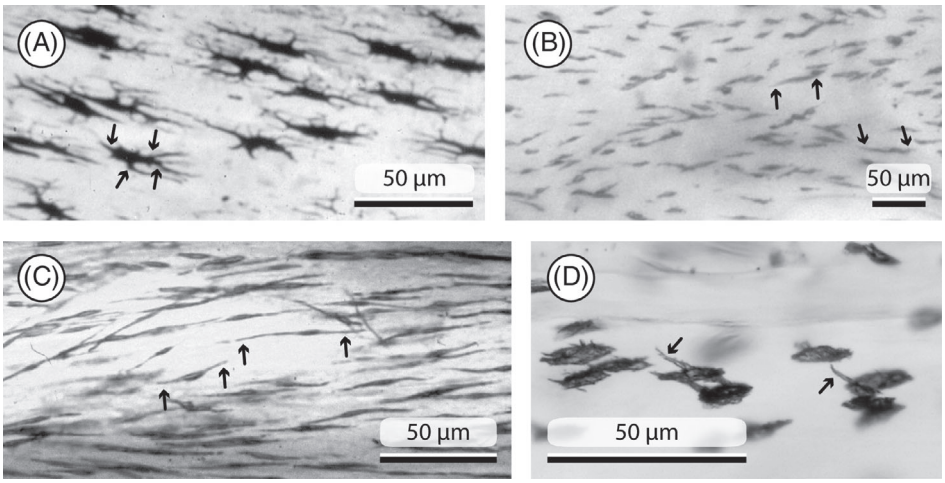

Fig. 6. Osteocyte morphology in taxa that retain the ancestral cellular bone (A) or that secondarily reacquired it from ancestors with acellular bone (B–D). (A) 'Star-shaped' osteocytes in the dorsal-fin spine of the carp *Cyprinus carpio*, an ostariophysan. Note the irregular shape of the lacunae, and the numerous cytoplasmic processes ending in canaliculi branching in all directions (arrows). Modified from Meunier & Huysseune (1992). (B) 'Spindle-shaped' osteocytes in the coracoid of the salmon *Salmo salar* (NHMUK, uncatalogued), a salmoniform. Note the two cytoplasmic processes located at both extremities of the cell axis (arrows). Photograph by D. Davesne and A. D. Schmitt. (C) 'Spindle-shaped' osteocytes in the dorsal-fin spine of the tuna *Katsuwonus pelamis*, an acanthomorph. Note the two cytoplasmic processes located at both extremities of the cell axis (arrows). Photograph by F. J. Meunier. (D) 'Spindle-shaped' osteocytes in the rib of the opah *Lampris* sp. (MNHN-ZA-AC-A-7506), an acanthomorph. Note the few canaliculi, all pointing in the same direction (arrows). Modified from Davesne *et al.* (2018).

possibly lost at the euteleost node. Structural similarities in osteocyte morphology appear further to support that their reacquisition is underlined by shared, and not fully understood, mechanisms.

V. THE ROLE OF MINERAL HOMEOSTASIS IN THE LOSS AND REACQUISITION OF OSTEOCYTES

Of the main functions of bone, those related to mechanical homeostasis (e.g. strain detection and bone remodelling) function in the absence of osteocytes (see Section II.2). Mineral homeostasis, on the other hand, relies on a variety of mechanisms including halastasis, i.e. a diffuse demineralisation of the bone without affecting its organic matrix (Lopez, 1976; Kacem & Meunier, 2003; Sbaihi *et al.*, 2007), osteoblast-mediated bone resorption (Francillon-Vieillot *et al.*, 1990; de Ricqlès *et al.*, 1991), and osteocyte-mediated bone resorption (osteocytic osteolysis). Halastasis has only been observed so far in taxa with cellular bone, and evidently osteocytic osteolysis is lacking in acellular bone. This suggests that acellular bone is less efficient than cellular bone in regulating mineral content in the body. In aquatic animals like teleosts, however, it is likely that enough calcium and phosphorus is available from the diet and ambient water to compensate the less-efficient mineral homeostasis (Witten & Huysseune, 2009; Cohen *et al.*, 2012; Shahar & Dean, 2013; Doherty *et al.*, 2015). Therefore, it is possible that osteocytes are not required either for mechanical or mineral homeostasis in teleosts because both functions can be achieved by other means (Dean & Shahar, 2012). In that context, the disappearance of osteocytes in at least some euteleosts may be due to a relaxed selective pressure that does not compensate the cost of maintaining them (Shahar & Dean, 2013; Doherty *et al.*, 2015). However, this hypothesis alone clearly does not explain the phylogenetic distribution of acellular bone: if a low selective pressure was not preventing the loss of osteocytes, we would expect this phenomenon to be widespread in teleosts and other aquatic vertebrates. Our data support the contrary: probably just a single main disappearance of cellular bone, potentially in euteleosts, along with other, extremely rare losses in species-poor lineages (at least in *Trichomycterus* sp. and some bones of *Danio rerio*) that could be size-related (see Section III.3*a*). Other mechanisms may have been involved, such as heterochrony (e.g. Parenti, 1986).

Tunas, opahs and potentially salmoniforms all reacquired osteocytes secondarily (see Section IV.3). They also share specific adaptations that lead to increased and sustained muscular activity: an anadromous migrating behaviour in salmoniforms, and specialised red muscles involved in heat production in tunas and opahs. At least in these taxa, the main function of osteocytes could be that of osteocytic osteolysis, as has been proposed for teleosts as a whole by previous authors (e.g. Cohen *et al.*, 2012). Reacquiring osteocytes would allow the use of bone as a major source of calcium and phosphorus, which would constitute a key adaptive advantage in an organism experiencing increased pressure on maintaining efficient muscle activity. Mineral homeostasis is then proposed to have played a major role in the evolution of acellular bone in teleost fishes.

VI. CONCLUSIONS

(1) According to our ancestral state reconstructions (Figs 3 and 4), acellular bone is a synapomorphy of either Euteleostei (as supported by two out of three tree topologies), or of a smaller clade consisting of Stomiati, Galaxiiformes and Neoteleostei (as supported by one tree topology). New analyses incorporating histological information on more euteleost taxa (e.g. *Lepidogalaxias*, more argentiniforms and galaxiiforms) including early fossil representatives, and a stabilisation of the euteleost phylogeny, are both necessary to clarify the ambiguity on the exact clade in which acellular bone evolved. Given the equivocal support for the euteleost ancestral state in T1 (Fig. 4), and considering other lines of evidence (such as structural similarities between osteocytes in salmoniforms and tunas; Fig. 6), we consider it to be more likely that osteocytes were lost in Euteleostei, with a secondary reacquisition in salmoniforms.

(2) Scales became acellular earlier than the rest of the skeleton in teleost phylogeny, probably in the clade Clupeocephala, which includes clupeomorphs, ostariophysans and euteleosts. More comparative data are needed to confirm this hypothesis.

(3) Scales aside, acellular bone appears to be almost absent outside of Euteleostei (Fig. 3). We reject its occurrence in the bonefish *Albula vulpes* and the mooneyes *Hiodon* sp. The catfish *Trichomycterus* sp. appears to be acellular and the zebrafish *Danio rerio* has both cellular and acellular bone in its cranial skeleton, but the occurrence of acellular bone in both may be explained by their small adult body sizes. More comparative data encompassing multiple bones in multiple teleost species will be necessary to support whether these are isolated or more widespread occurrences.

(4) Within spiny-rayed teleosts (Acanthomorpha), osteocytes have been secondarily reacquired in tunas (Thunnini) and in the opah *Lampris* sp. The exact co-occurrence of osteocytes with that of an endothermic physiology based on red muscle activity (Fig. 5) strongly suggests that these traits are correlated in acanthomorph teleosts. Other traits shared by tunas and the opah are also present in some acanthomorphs with acellular bone (e.g. large body size, cranial endothermy, intense bone remodelling), and so are less plausible explanations of the evolutionary reacquisition of osteocytes.

(5) Acellular teleost bone can perform every structural and mechanical function of cellular bone (e.g. detection of strains and constraints, adaptive remodelling) and both have very similar mechanical properties. However, acellular bone seems to be less efficient in terms of mineral homeostasis, potentially because it lacks the possibility to perform osteocytic osteolysis. Osteocytes are secondarily reacquired in lineages that may have increased requirements for minerals, mostly to support an intense and sustained muscular activity: the red-muscle endotherms and (potentially) the anadromous salmoniforms. This pattern seems to support the hypothesis that the most fundamental role of osteocytes in teleost bone physiology is that of mineral, rather than mechanical homeostasis.

(6) Our review of the available evidence with the addition of new data allowed us to establish for the first time a detailed phylogenetic hypothesis for the evolution of osteocytes in teleosts. Acellular bone is a fundamental model to understand bone function, because it lacks a cell type that is classically thought to play a major role in the structure and maintenance of bony tissues. This review highlights the need to use large-scale comparative histological data, backed by a rigorous phylogenetic framework, to address fundamental questions on the interplay of bone structure, function and physiology.

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IX. SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Time-calibrated multilocus tree of extant actinopterygians (ray-finned fishes), obtained from the optimisation of the character states 'cellular bone' (in dark blue) and 'acellular bone' (in yellow) on the topology T1 (Near *et al.*, 2012).

Figure S2. Time-calibrated multilocus tree of extant and fossil actinopterygians (ray-finned fishes), obtained from the optimisation of the character states 'cellular bone' (in dark blue) and 'acellular bone' (in yellow) on the topology T2 (Betancur-R. *et al.*, 2015).

Figure S3. Time-calibrated multilocus tree of extant actinopterygians (ray-finned fishes), obtained from the optimisation of the character states 'cellular bone' (in dark blue) and 'acellular bone' (in yellow) on the topology T3 (Hughes *et al.*, 2018).

Table S1. Complete list of actinopterygian (ray-finned fish) taxa surveyed by our literature review, including additional species obtained with our synchrotron microtomography (SRμCT) data.

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