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, the most prominent of which are the osteocytes. These cells are thought to play a fundamental role in bone physiology, 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 at clarifying 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 ray-finned fish species, fossil and extant 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 (though rarely), and cellular bone was reacquired several times independently within euteleosts, in salmons and relatives, tunas and the opah. 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.

Keywords: osteocyte, acellular bone, anosteocytic bone, Actinopterygii, Teleostei, Salmoniformes, Scombridae, ancestral state reconstruction, bone remodelling, endothermy
I. INTRODUCTION
(1) General introduction
Vertebrate bone is a living tissue that, besides its mineralized extracellular component, comprises cells of three different types. Surface-based osteoblasts and osteoclasts synthesize and resorb bone, respectively, and osteocytes are more versatile cells that fulfil various functions (Francillon-Vieillot et al., 1990; 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, Hall, & Witten, 2006) and communicate with each other through a network of canaliculi (Cao et al., 2011).

Osteocytes play a key role in bone physiology: (1) they act as mechanical sensors detecting changes in bone strain; (2) they guide bone remodelling by activating or deactivating the osteoclasts they communicate with; (3) 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, 1961a; 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; Meunier & Huysseune, 1992; 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 already 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 undergo 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, 1961a, 1961b, 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: (1) the distribution of bone type within teleosts (does it follow ecological, physiological or phylogenetic patterns?), (2) the origin of acellular bone (does it have a unique origin, or multiple convergent appearances?), and (3) 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 understand the evolution of bone within vertebrates as a whole, and the role of osteocytes in bone physiology (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 (1961a), researchers attempted to explain the distributions of osteocytic and anosteocytic 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 of using bone as an additional source of metabolic minerals (Moss, 1961a, 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 all 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: (1) that cellular bone is the plesiomorphic condition for teleosts, actinopterygians and osteichthyans in general (Ørvig, 1951, 1967, Moss, 1961a, 1963); and (2) that acellular bone is found in ‘advanced’ or ‘higher’ teleost groups (Moss, 1961a, 1963, Meunier, 1987, 1989; Ricqlès et al., 1991; Meunier & Huysseune, 1992; Witten et al., 2004). As noted by the past 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 on an explicit phylogenetic framework based on character analysis.

Several authors used cellularity as a phylogenetic character: acellular bone is proposed as a synapomorphy uniting (1) Osmeriformes (true smelts) and Neoteleostei (the clade including spiny-rayed fishes, amongst others) by Rosen (1985); (2) Esociformes (pikes and mudminnows), Osmeriformes and Neoteleostei by Parenti (1986); (3) 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: (1) 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); (2) Neoteleostei sensu Rosen (1973, 1985) is not a monophyletic group, with Stomiiformes (viperfishes and allies) now considered sister to Osmeriformes (Li et al., 2010; Near et al., 2012; Betancur-R. et al., 2013, 2017).
Aim of the review

Most research on acellular teleost bone has been focused on its structure, development and function (Moss, 1961b; Meunier, 1989; Meunier & Huysseune, 1992; Cohen et al., 2012; Dean & Shahar, 2012; Shahar & Dean, 2013), but the evolutionary origin and phylogenetic distribution of this bone type has not been the focus of recent research.

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 database results from a review of the literature, plus some of our own observations, bringing together most of the data published so far on actinopterygian bone to constitute a dataset covering the whole diversity of the group. Including this data for the first time into an explicit phylogenetic framework finally allows us to draw a possible historical scenario for the loss of osteocytes in teleosts.

II. TELEOST ACCELLULAR BONE: STRUCTURE AND FUNCTION

(1) Structure and development

In teleosts, bone is found in the endoskeleton (the skeleton proper, i.e., cranial, axial and appendicular skeleton; Fig. 1A, C-F) and in the dermal exoskeleton, including 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, teleost acellular bone is considered true bone because it shares its developmental origin and main characteristics with every other vertebrate bone tissue (Moss, 1961a; Witten & Huysseune, 2009; Dean & Shahar, 2012): (1) it is composed of hydroxyapatite crystals in a mesh of type I collagen fibres; (2) it has the same functional properties as other bone tissues (muscle insertion and organ support); (3) its extracellular matrix is secreted by osteoblasts and resorbed by osteoclasts; (4) 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 lacunocanicular 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 lacunocanicular 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). Acellular bone can be
vascular or avascular, osteoblastic canaliculi being more numerous in avascular than in
vascular acellular bone (Francillon-Vieillot et al., 1990).

In cellular bone, osteocytes originate from osteoblasts that get 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 toward 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, 1961b) has been rejected in 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 higher than in cellular bone
(Meunier, 1984a; Cohen et al., 2012), hydroxyapatite crystals are smaller and the
organic fraction is larger as well (Moss & Posner, 1960). This higher mineral content,
along with the reduction in porosity associated with 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 unique collagen
fibre structure in acellular teleost bone gives it an increased toughness compared to
tetrapod (e.g., human) cellular bone (Atkins et al., 2015b).

(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) as well as induction of osteoclasts from implanted
mammalian tissue (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 lack the ruffled border at the level of the resorption
surface, and 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 correspond to a non-activated state,
in 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 (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). 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
**Figure 1.** Examples of cellular (A-D) and acellular bone (E-F) 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. (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. (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 well visible. (D) ‘Virtual thin section’ obtained by stacking PPC 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 canaliculi of Williamson in cross-section (white arrowheads). (E) Thin section through acellular bone in the rib of an acanthomorph, the sea bass *Dicentrarchus labrax*, showing numerous radially-arranged osteoblastic canaliculi. (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). Images modified from (A) Meunier, Otero, & Laurin (2018), (C, F) Meunier & Herbin (2014).

requirement, as it occurs conspicuously in certain diadromous teleost species (e.g., European eel, salmoniforms) before and during migration (Kacem & Meunier, 2000, 2003; Sbaihi *et al.*, 2007). In teleosts with acellular bone, osteocytic osteolysis is impossible, making calcium and phosphorus seemingly 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).

(a) Remodelling

Teleost bony tissues consist mainly of primary bone in most species (Meunier, 1987) and bone remodelling appears to be less abundant in teleosts than in tetrapods – it was even long thought to be absent (Moss, 1961b). 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 & Ricqlès, 1986; Witten & Huysseune, 2009; Dean & Shahar, 2012; Shahar & Dean, 2013; Atkins *et al.*, 2014, 2015a; 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, Kaufman, & Glowacki, 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 a very intense remodelling activity as a response to fracture and load (Amprino & Godina, 1956; Poplin, Poplin, & Ricqlès, 1976; Castanet & 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, 2015a).

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
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 of 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).

Figure 2. Distribution of cellular and acellular bone in vertebrate phylogeny (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 (2014a, 2014b).
(2) 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 of actinopterygians, including modern coelacanths, modern lungfishes, modern tetrapods (lissamphibians, mammals, diapsids) and fossil taxa falling on their respective stem groups (Meunier, Cupello, & Clément, in press; Sire et al., 2009; Zylberberg, Meunier, & Laurin, 2010; Schultz, 2016). 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 or perichondral 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 surveys by Kölliker (1859) and Moss (1961a, 1965), to which we added data from various fossil and extant species where required to better resolve the phylogenetic and temporal distribution (see 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-µCT) 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 µCT scans have been done at the ID-19 (microtomography) beamline of the European Synchrotron Radiation Facility (ESRF), using a white beam with energy levels comprised between 35 and 105 keV, obtaining a voxel size of 0.72 µm.

For all extant and fossil taxa, we used the dentary as a 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 genuinely reflect 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 endoskeleton and scales of † Cheirolepis (Zylberberg, Meunier, &
Laurin, 2016) and †Moythomasia (Sire et al., 2009; Schultzze, 2016) and in the scales of †Mimipiscis (Richter & Smith, 1995). Endoskeletal bone and scales are always cellular in non-teleost actinopterygians, for example in bichirs (Polypteriformes), bowfins and gars (Holostei) (Kölliker, 1859; Moss, 1961a; 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 endoskeleton, but it is nonetheless composed of cellular bone (e.g., Kölliker, 1859; Stéphan, 1900; 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), †pholidophorids (Meunier & Brito, 2004). Our SRµCT data bring 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 seventeen Jurassic and Cretaceous taxa (Tables 1, S1) interpreted as stem-group teleosts (e.g., Arratia, 2015).

(c) Elopomorpha

Within Elopomorpha, cellular bone is found in tarpons and their allies (Elopiformes), in both scales and endoskeletal elements (Kölliker, 1859; Meunier & Brito, 2004). Several eels (Anguilliformes) are described as acellular by Kölliker (1859). However, they all seem to pertain to an outdated taxonomy that treated leptocephalus larvae as separate taxa (Table S1). For example, he reports cellular bone in the sorcerer eel Nettastoma melanurum and acellular bone in †Hyoprorus messanensis, corresponding to the larva of N. melanurum (Eschmeyer, Fricke, & van der Laan, 2018). Although Moss (1961a) 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, 1961a), but this is contradicted by our observations (described below). Finally, our SRµCT data reveals cellular bone in several fossil albuliforms (e.g., †Istieus, †Lebonichthys), elopiforms (e.g., †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 endoskeleton and scales (Kölliker, 1859; Meunier & Brito, 2004; Meunier, Brito, & Leal, 2013a; Meunier, Dutheil, & Brito, 2013b). 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
are consistently recovered as sister to Tharrias araripes Knightia, including cellular s
Gonorhynchiformes), Phareodus A, 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 allies (Clupeomorpha) appear to have cellular bone (Kölliker, 1859; Moss, 1961a, 1965). Although Moss (1961a) reported acellular bone in the anchovy Anchoviella sp. and the American shad Alosa sapidissima (Moss, 1961a), 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 namournensis and †Ellumichthys 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 (1961a, 1965), extensively sampled the considerable diversity of the primarily 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 observe 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 gonorynchiform †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 (Weigle & Franz-Odendaal, 2016). In conclusion, cellular bone is present in all ostariophysans surveyed so far, with the notable exceptions of Trichomycterus. In addition, slickheads (Alopecophaliformes) are consistently recovered as sister to ostariophysans in molecular phylogenies (Lavoué et al., 2008; Near et al., 2012; Betancur-R. et al., 2013; Straube et al., 2018; Hughes et al., 2018). The only species surveyed from the group, Alopecophalus 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, 1961a, 1965), smelts (Osmeridae; Moss, 1961a, 1965) and viperfishes and their allies (Stomiiformes; Kölliker, 1859; Germain, Schnell, & Meunier, in press).

Conversely, cellular bone is found in *Argentia 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 allies (Salmoniformes) are generally described as having cellular bone (Kölliker, 1859; Moss, 1961a, 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 appears to always 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 genus of uncertain placement †*Ctenothrissa vexillifer* (Kölliker, 1859; Moss, 1961a; Davesne *et al*., 2018). Spiny-rayed fishes (Acanthomorpha) contribute the greatest fraction of neoteleost species diversity. Amongst the approximately 17 000 acanthonmorph species (more than 300 being surveyed in the present study), acellular bone is virtually universal (Kölliker, 1859; Moss, 1961a, 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 (Ekanyake & Hall, 1987; Cohen *et al*., 2012). Within acanthonmorphs, cellular bone is only known conclusively in two relatively species-poor lineages: the ‘true’ tunas *Auxis, Euthynnus, Katsuwonus* and *Thunnus* (Kölliker, 1859; Stéphan, 1900; Ampriro & Godina, 1956; Moss, 1961a; 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 endoskeletal elements, but also in scales and in fin rays and spines (Meunier *et al*., 2008a; Wainwright, Ingersoll, & Lauder, 2018; Santamaria *et al*., 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, endoskeleton and exoskeletal elements such as fin rays and spines (Kölliker, 1859; Moss, 1961a, 1963; Meunier & Huysseune, 1992).
elasmoid scales of most modern teleosts are an exception: they are often acellular when
the rest of the skeleton is cellular (see below, ‘Phylogenetic distribution of acellular bone
in actinopterygian scales’). Moss (1961a) reported that the bonefish *Albula vulpes*
displayed 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’ (1961a) statement.
Weigele and 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 (i.e., 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 observed with SRµCT osteocytes in the frontal
bone of the carp *Cyprinus carpio*, a closely related cyprinid. 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
clarifying whether this pattern of mixed bone types is widespread in teleosts, or specific
to *D. rerio*.

(b) Alleged osteocytes in tubular and hyperostotic bone

The presence of 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
canalici 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 russelli* (Paig-Tran, Barrios, & Ferry, 2016), two acanthomorphs that have
otherwise acellular bone. However, such osteocytes do not appear to be systematically
present 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 argenteus* 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, Meunier, & Kacem, 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 in 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, 1987; Meunier & Sire, 2004; 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; Meunier et al., 2016; Brito, Meunier, & Gayet, 2000) 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, 1984b; Meunier et al., 2008b; 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, 1984b, 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, 1984b; Meunier & Brito, 2004). It is, however, acellular in other taxa with cellular bone including clupeomorphs, ostariophysans and salmoniforms (Meunier & Brito, 2004; Meunier, Sorba, & Béarez, 2004; Sire et al., 2009). Taxa with acellular bone seem to always 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 dataset of 677 fossil and extant actinopterygians, we scored the presence of cellular or acellular bone for endoskeletal elements and scales (Table S1). When bone lacks osteocytes only in certain skeletal elements (e.g., some elasmoid scales in teleosts) or ontogenetic stages (e.g., in larval anguilliforms) we scored its status as ‘cellular’.

This dataset was mapped on three time-calibrated trees stemming from three recent multi-locus or phylogenomic studies of actinopterygian intrarelationships. Topology #1 (T1) is obtained in an analysis of nine nuclear protein-coding loci including 232 taxa, all extant (Nar 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., Ortí, & Pyron, 2015); Topology #3 (T3) is 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 + Stomiformes 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 these topologies and in our cellularity dataset. When two different species of the same genus were present in two different datasets, we considered the genus as a whole, since no case of variability of cell type 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 (Lepidogalaxiformes) and the jellynose fishes (Ateleopodiformes). Ancestral states at the nodes were reconstructed with the ace function of the APE package in R (Paradis, Claude, & Strimmer, 2004). Two models of ancestral character state estimations were tested: an ‘all rates different’ 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 the transition frequencies was very low even with the ARD model, but the Akaike information criterion 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 (Fig. 3). 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 (Figs. 3, S2). The reconstructed ancestral state for Euteleostei is ambiguous and varies from one topology to the other.
Figure 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 from Iglésias (2014b).

With T1, the ancestral state for Euteleostei is equivocal. The likelihoods of the ancestral state being ‘cellular’ or ‘acellular’ are comprised 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 datasets 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; Straube et al., 2018; Hughes 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 of all other euteleosts (Li et al., 2010; Near et al., 2012; Betancur-R. et al., 2013; Campbell et al., 2017; Straube et al., 2018; Hughes et al., 2018). As long as the phylogeny of euteleosts is not stabilised, and the osteohistology of more taxa not 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 clad 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 the fossil 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, S1-3). Two distinct neoteleost lineages are reconstructed as having reacquired cellular bone independently: (1) the ‘true’ tunas Auxis, Katsuwonus, Euthynnus and Thunnus, forming the probably monophyletic tribe Thunnini within Scombridae; (2) the opah Lampris in the monotypic Lamprididae (Fig. 3).

In conclusion, the clad in which acellular bone appears is equivocal with our ancestral state reconstructions (Fig. 4). Two hypotheses are supported by different topologies, as follows: (1) acellular bone appears in Euteleostei, as supported by T2 and T3; (2) acellular bone appears independently in Esociformes and in the clad that unites Stomiati, Galaxiiformes and Neoteleostei, as supported by T1. Since T1 barely supports the second hypothesis over the first, we would tend to consider the first hypothesis (an appearance of acellular bone in Euteleostei) to be more likely.

In any case, acellular bone is almost entirely absent outside of Euteleostei, being only described so far in: (1) some larval anguilliforms, (2) the clupeiform Anchovielia sp., (3) certain cranial dermal bones of the cypriniform Danio rerio, and (4) the siluriform Trichomycterus sp. (see above, ‘Phylogenetic distribution of acellular bone in teleosts and other actinopterygians’; 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 has never been properly established, but cannot be excluded in this context (see above, ‘Occurrence of mixed bone types’, 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 suitably test this hypothesis.

(3) Secondary reacquisition of cellular bone

(a) Probable occurrence in salmoniforms

Our ancestral state reconstructions suggest that cellular bone was secondarily reacquired in salmonins, trouts and their allies (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 on it 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): (1) in the grayling *Thymallus thymallus* (Thymallinae), we did not observe osteocytes conclusively; (2) in the shortnose cisco *Coregonus reighardi* (Coregoninae), osteocytes are present, but scarcely distributed within bone; (3) 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.

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 (Near et al., 2012; Crête-Lafrenière, Weir, & Bernatchez, 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 an 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 halastasy (e.g., 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.
T1
- Argentina silus
- Umbra limi
- Esox lucius
- Esox americanus
- Coregonus sp.
- Thymallus sp.
- Parahucho perryi
- Salvelinus sp.
- Stomias boa
- Argyropelecus sp.
- Hypomesus pretiosus
- Osmerus mordax
- Thaleichthys pacificus

T2
- Argentina sp.
- Galaxias sp.
- Umbra limi
- Esox americanus
- Esox lucius
- Coregonus sp.
- Thymallus sp.
- Oncorhynchus sp.
- Salvelinus sp.
- Parahucho perryi
- Salmo salar
- Hypomesus pretiosus
- Thaleichthys pacificus
- Osmerus mordax
- Gonostoma sp.
- Argyropelecus sp.
- Chaenodorus sloani
- Stomias boa
- Borostomias sp.

T3
- Umbra sp.
- Esox lucius
- Thymallus thymallus
- Coregonus sp.
- Salmo salar
- Salvelinus fontinalis
- Oncorhynchus mykiss
- Argentina sp.
- Galaxias sp.
- Osmerus sp.
- Borostomias sp.

- Argentiniformes
- Esociformes
- Salmoniformes
- Stomiiformes
- Osmeriformes
- Galaxiiformes
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 of 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 halastasy 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 reliably place into the clade Perlagaria, itself included in the ultradiverse acanthomorph clade Percomorpha (Betancur-R. et al., 2013, 2017; Near et al., 2013; Miya et al., 2013; Alfaro et al., 2018). The ‘true’ tunas (Thunnini) consist in 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 percomorphs 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 been known for long in tunas (Kölliker, 1859; Stéphan, 1900; Amprino & Godina, 1956; Moss, 1961a), fewer 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, 1961a). 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 supports that acellular bone is plesiomorphic for lampridiforms (Davesne et al., 2018). Thus, Lampris sp. 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 of them do not correlate with the presence of osteocytes. (1) 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, 1961a), billfishes (Kölliker, 1859; Amprino & Godina, 1956; Moss, 1961a; Atkins et al., 2014) and many scombrids outside of ‘true’ tunas. (2) 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 wahoo Acanthocybium solandri both commonly exceed 1000 mm in total length (Collette & Nauen, 1983) and are anosteocytic (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. (3) Finally, the reacquisition of osteocytes does not seem to be linked with structural homeostasis: bone in tunas, opah and billfishes appears to have an active, intense and sustained resorption and remodelling activity (Fig. 5B,D-E) evidenced by the extensive presence of secondary bone (Amprino & Godina, 1956; Poplin et al., 1976; Castanet & 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; 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).
Figure 5. (A) Phylogenetic distribution of bone type in endothermic acanthomorph teleost 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 (2014b) 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). Image courtesy A. Atkins. (C) PPC synchrotron tomographic slice in a rib of the butterfly kingfish Gasterochisma melampus, a scombrid (AMNH I-93480 SD). Bone is acellular. (D) PPC synchrotron tomographic slice in a rib of the dogtooth ‘tuna’ Gymnosarda uniclor, a scombrid (MNHN.ICOS.00492). Bone is acellular. Note secondary bone deposition around the blood vessels, delimited by resorption lines (white arrowheads). (E) PPC 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).

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 exterior and retained within the body thanks 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 non-Thunnini scambroid (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, 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 billfishes (Atkins et al., 2014), and our SRμCT data failed to find them in G. melampus (Table 1, Fig. 5B,C), implying that cranial endotherms, unlike red-muscle endotherms, have acellular bone.

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 both 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 to both hunt prey and produce heat via the myotomal or pectoral red muscles. Since muscles are important consumers of calcium, an element primarily found in bony tissues, osteolytic 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 a 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), the spindle-shaped osteocytes seem to be 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 do not seem to be connect to each other at all (Totland et al., 2011). Moreover, their morphology does not seem to change significantly between primary and remodeled bone, for example in Lampris (Davesne et al., 2018).

Figure 6. Osteocyte morphology in taxa that retain the ancestral cellular bone (A) or that secondarily reacquired it from acellular ancestors (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 canaluli branching in all directions (arrows). (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). (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). (D) 'Spindle-shaped' osteocytes in the rib of the opah Lampris sp. (MNHN-ZA-AC-A-7506), an acanthomorph. Note the few canaluli, all pointing in the same direction (arrows). Images modified from (A) Meunier & Huysseune (1992), (D) 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. Weigle & Franz-Odendaal (2016) proposed that both types of osteocytes have different developmental origins, and that the elongate, spindle-shaped osteocytes would derive from the elongate 'osteoblastic-like' cells that line up 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 possibly lost at the euteleost node. Structural similarities in osteocyte morphology appear to further 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 above, “Functional properties of acellular bone”). 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, 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 is available from 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 could hypothesised to 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 above). Other mechanisms may have been involved, such as developmental heterochrony (e.g., Parenti, 1986).

Tunas, opahs and potentially salmoniforms all reacquired osteocytes secondarily (see above). 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 using bone as a major source of calcium and phosphorus, which would constitute a key adaptive advantage in an organism experiencing increased pressure on maintaining an efficient muscle activity. Mineral homeostasis is then proposed to have played a major role in the evolution of acellular bone in teleost fishes.

VI. CONCLUSION

1. According to our ancestral state reconstructions (Figs. 3,4), 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 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 could be due to their small 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 suggest 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 as 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, probably 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 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 of using 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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Table 1. Bone type (presence or absence of osteocytes) in the taxa surveyed by our SR-µCT data. C = cellular bone; A = acellular 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 (UMMZ); Université de Poitiers, France.

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