

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 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 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 salmon 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.

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 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; 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-Odenaal, 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, 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; 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: (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 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

46
47 Following the surveys of Kölliker (1859) and Moss (1961*b*), researchers attempted to
48 explain the distributions of cellular and acellular bone among teleost species. For
49 example, an early hypothesis proposed that acellular bone occurs because marine
50 environments are richer in dissolved calcium, decreasing the need to use bone as an
51 additional source of metabolic minerals (Moss, 1961*b*, 1963). However, acellular bone is
52 also present in freshwater teleost taxa such as esocids (pikes), centrarchids (sunfishes),
53 percids ('true' perches), and cichlids (Moss, 1965). In virtually all species, the entire
54 skeleton seems to be composed exclusively of either cellular or acellular bone, and
55 closely related species mostly seem to share the same bone type (Kölliker, 1859).
56 Following these observations, cellularity was quickly recognised as a potentially
57 significant phylogenetic character (e.g. Kölliker, 1859; Berg, 1947). Indeed, at least two
58 studies have used the presence or absence of osteocytes to discuss the systematic
59 position of enigmatic fossil taxa (Gaudant & Meunier, 2004; Mayrinck *et al.*, 2017).

60 Deep divergences in teleost phylogeny have been poorly resolved until recently,
61 meaning that the phylogenetic distribution of cellularity has not been clear. Nevertheless,
62 there is broad consensus on two statements: (1) that cellular bone is the plesiomorphic
63 condition for teleosts, actinopterygians and osteichthyans in general (Ørvig, 1951, 1967;
64 Moss, 1961*b*, 1963); and (2) that acellular bone is found in 'advanced' or 'higher' teleost
65 groups (Moss, 1961*b*, 1963; Meunier, 1987, 1989; Ricqlès *et al.*, 1991; Meunier &
66 Huysseune, 1992; Witten *et al.*, 2004). As noted by the past authors themselves, these
67 propositions are imprecise and potentially misleading. Indeed, the pattern appears to be
68 much more complex: for example, acellular bone is found in certain 'lower' teleosts such
69 as pikes and cellular bone is found in some 'higher' taxa such as tunas (Amprino &
70 Godina, 1956; Moss, 1963; Meunier, 1989; Meunier & Huysseune, 1992). Moreover, the
71 systematic distributions of both bone types have been described using subjective and
72 poorly defined systematic categories (e.g. 'advanced teleosts'), not on an explicit
73 phylogenetic framework based on character analysis.

74 Several authors used cellularity as a phylogenetic character: acellular bone is proposed
75 as a synapomorphy uniting (1) Osmeriformes (true smelts) and Neoteleostei (the clade
76 including spiny-rayed fishes, amongst others) by Rosen (1985); (2) Esociformes (pikes
77 and mudminnows), Osmeriformes and Neoteleostei by Parenti (1986); (3) Esociformes
78 and Neoteleostei by Johnson & Patterson (1996), the latter being the only phylogeny
79 based on the analysis of a character matrix. However, the usefulness of this previous
80 work is limited because the underlying phylogenetic frameworks have been superseded
81 by more recent classifications based on molecular phylogenies that extensively sample
82 both taxa and loci (e.g. Near *et al.*, 2012; Betancur-R. *et al.*, 2013, 2017). The most
83 relevant changes relative to anatomical hypotheses include: (1) Esociformes do not form
84 an exclusive clade with Neoteleostei, but instead consistently appear to be sister to
85 Salmoniformes (Ramsden *et al.*, 2003; Wilson & Williams, 2010; Near *et al.*, 2012;
86 Betancur-R. *et al.*, 2013; Campbell *et al.*, 2013); (2) Neoteleostei *sensu* Rosen (1973,
87 1985) is not a monophyletic group, with Stomiiformes (viperfishes and relatives) now
88 considered sister to Osmeriformes (Li *et al.*, 2010; Near *et al.*, 2012; Betancur-R. *et al.*,
89 2013, 2017).

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93 (3) Aim of this review

94 While most research on acellular teleost bone has been focused on its structure,
95 development and function (Moss, 1961a; Meunier, 1989; Meunier & Huisseune, 1992;
96 Cohen *et al.*, 2012; Dean & Shahar, 2012; Shahar & Dean, 2013), the evolutionary
97 origin and phylogenetic distribution of this bone type has not been studied in detail.
98 Explaining the evolutionary origins of acellular bone requires an explicitly phylogenetic
99 approach that can distinguish the role of adaptation from that of phylogenetic history in
100 the distribution of bone types among species. This review aims to clarify the distribution
101 of cellular and acellular bone in teleosts within a phylogenetic context that is now
102 available thanks to an array of recently published large-scale molecular analyses (e.g.
103 Near *et al.*, 2012; Betancur-R. *et al.*, 2013; Hughes *et al.*, 2018). We also review the
104 structure of acellular bone, emphasising its functional similarity to cellular bone (Witten &
105 Huisseune, 2009; Cohen *et al.*, 2012; Shahar & Dean, 2013; Currey *et al.*, 2017). Our
106 review of the existing literature, complemented by our own observations, brings together
107 most of the data published to date on actinopterygian bone to constitute a data set
108 covering the whole diversity of the group. Including this data into an explicit phylogenetic
109 framework for the first time, finally allows us to draw a possible historical scenario for the
110 loss of osteocytes in teleosts.

112 II. TELEOST ACELLULAR BONE: STRUCTURE AND FUNCTION

113 (1) Structure and development

114 In teleosts, bone is found in the endoskeleton (the skeleton proper, i.e., cranial, axial and
115 appendicular skeleton; Fig. 1A, C-F) and in the dermal exoskeleton, including scales In
116 teleosts, bone is found in the cranial, axial and appendicular skeleton (Fig. 1A, C-F) and
117 in scales (Fig. 1B), lepidotrichia (fin rays) and the tissues that derive from them
118 (Patterson, 1977; Schaeffer, 1977; Francillon-Vieillot *et al.*, 1990). Despite its structural
119 peculiarities that led historical authors to improperly designate it under other names (e.g.
120 'osteoid', Kölliker, 1859), teleost acellular bone is considered true bone because it
121 shares its developmental origin and main characteristics with every other vertebrate
122 bone tissue (Moss, 1961b; Witten & Huisseune, 2009; Dean & Shahar, 2012): (1) it is
123 composed of hydroxyapatite crystals in a mesh of type I collagen fibres; (2) it has the
124 same functional properties as other bone tissues (muscle insertion and organ support);
125 (3) its extracellular matrix is secreted by osteoblasts and resorbed by osteoclasts; (4) it
126 can be submitted to active remodelling.

127 Typical cellular bone contains numerous mature osteocytes that, despite being
128 completely surrounded by mineralised tissue, communicate with each other and with the
129 bone surface *via* a network of canaliculi containing cytoplasmic processes (Fig. 1C, D).
130 This lacunocanicular system permeates bone and gives osteocytes their characteristic
131 star-shaped appearance (Meunier, 1987; Cao *et al.*, 2011). It is however not clear
132 whether osteocytes form a proper lacunocanicular network in all teleosts with cellular
133 bone (Fiaz, van Leeuwen & Kranenbarg, 2010; Totland *et al.*, 2011). In acellular bone,
134 on the other hand, there are no osteocytes or lacunae within the bone mineral matrix
135 (Fig. 1E, F), but it is sometimes penetrated by osteoblastic canaliculi from the bone
136 surface (Francillon-Vieillot *et al.*, 1990; Sire & Meunier, 1994, 2017). In the 'tubular
137 acellular bone' of a few taxa, tubules containing a bundle of collagen fibres and
138 numerous osteoblastic cytoplasmic processes permeate acellular primary bone
139 (Hughes, Bassett, & Moffat, 1994; Sire & Meunier, 2017; Meunier & Béarez, 2019).

140 These tubules are superficially similar, but structurally distinct from the canals of
141 Williamson (Fig. 1D) that are known only from the cellular bone of holosteans and fossil
142 stem teleosts (Williamson, 1849; Ørvig, 1951; Sire & Meunier, 1994; Meunier & Brito,
143 2004). Acellular bone can be vascular or avascular, osteoblastic canaliculi being more
144 numerous in avascular acellular bone than in vascular acellular bone (Francillon-Vieillot
145 *et al.*, 1990).

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

154 (2) Functional properties of acellular bone

155 (a) Mechanical properties

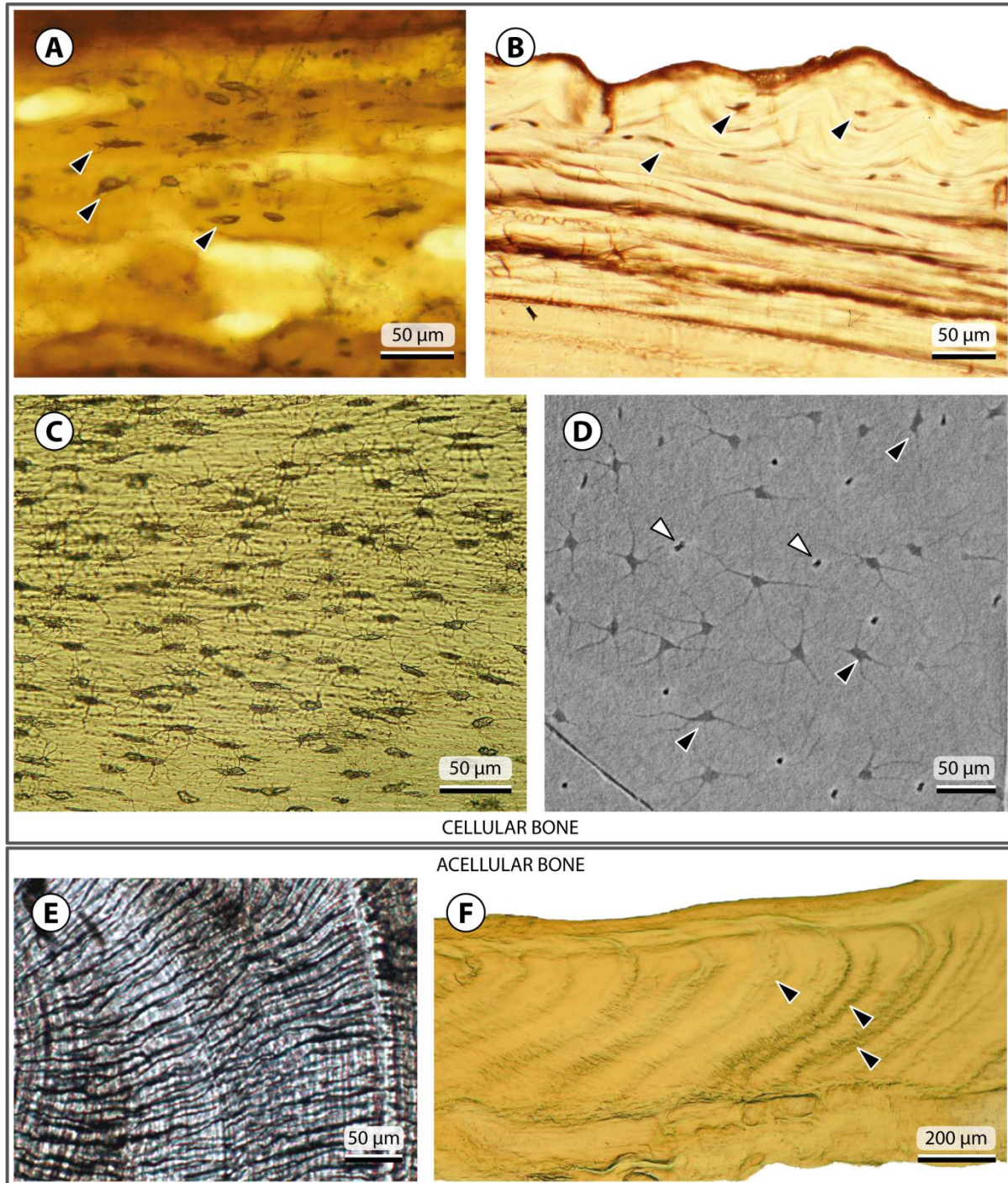
156 The mineral fraction in acellular bone is proportionally slightly higher than in cellular
157 bone (Meunier, 1984a; Cohen *et al.*, 2012). This higher mineral content, along with the
158 reduction in porosity associated with the absence of osteocytes have been hypothesised
159 to increase the stiffness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

166 (b) Resorption

167 Osteoclasts, the cells primarily responsible for bone resorption, were long thought to be
168 absent from acellular teleost bone, although resorption was still observed (Blanc, 1953;
169 Moss, 1963; Weiss & Watabe, 1979; Glowacki *et al.*, 1986). Indeed, osteoclasts in
170 acellular bone are structurally different from the 'typical' osteoclasts found in cellular
171 bone, explaining why they long went undetected: they are generally mononucleated
172 instead of multinucleated as in cellular bone (Sire, Huysseune, & Meunier, 1990; Witten,
173 1997; Witten & Villwock, 1997; Witten & Huysseune, 2009). This structural difference
174 may be explained by the absence of osteocytes, which promote the growth of
175 multinucleated osteoclasts (Witten & Huysseune, 2009, 2010).

177 (c) Mineral metabolism

178 Bone plays a crucial role in calcium metabolism in vertebrates, both as a consumer and
179 as a source of calcium. However, this role seems less critical in teleosts than in
180 terrestrial vertebrates since, as aquatic animals, teleosts can mobilise calcium and other
181 elements directly from the ambient water *via* their gills and/or digestive system (Takagi &
182 Yamada, 1992; Witten & Huysseune, 2009; Shahar & Dean, 2013). Phosphorus
183 availability appears to be more critical than that of calcium for healthy growth in both
184 marine and freshwater teleosts (Witten & Huysseune, 2009; Shahar & Dean, 2013), and
185 bone does not seem to mineralise when phosphorus is absent from the diet
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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 *et al.* (2018c). (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

198 tomographic slices of the dentary of the Jurassic stem teleost †*Dorsetichthys bechei* (OUMNH J.3369).
199 Star-shaped osteocyte lacunae and their canaliculi are visible (black arrowheads), as well as canals of
200 Williamson in cross-section (white arrowheads). Image produced by D. Davesne and A. D. Schmitt. (E)
201 Thin section through acellular bone in the rib of an acanthomorph, the sea bass *Dicentrarchus labrax*,
202 showing numerous radially arranged osteoblastic canaliculi. Photograph by D. Davesne. (F) Thin section
203 through acellular bone in the vertebra of an acanthomorph, the anglerfish *Lophius* sp. Bone is relatively
204 featureless, apart from visible successive growth marks (black arrowheads). Photograph by F. J. Meunier.
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209 (Witten *et al.*, 2016, 2018). Nevertheless, a specific type of bone resorption (osteocytic
210 osteolysis) is undertaken by the osteocytes themselves and may be linked to periods of
211 increased metabolic calcium and/or phosphorus requirement, as it occurs conspicuously
212 in certain diadromous teleost species [e.g. European eel (*Anguilla anguilla*),
213 salmoniforms] before and during migration (Kacem & Meunier, 2000, 2003; Sbaihi *et al.*,
214 2007). In teleosts with acellular bone, osteocytic osteolysis is impossible, potentially
215 making calcium and phosphorus more difficult to mobilise from and into the skeleton
216 than in those with cellular bone (Moss, 1962; Simmons, Simmons & Marshall, 1970;
217 Witten, 1997; Witten & Huysseune, 2009).
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219 (d) Remodelling

220 Teleost bony tissues consist mainly of primary bone in most species (Meunier, 1987)
221 and bone remodelling appears to be less abundant in teleosts than in tetrapods – it was
222 even long thought to be absent (Moss, 1961a). Nevertheless, bone remodelling occurs
223 in teleosts, in taxa with both cellular (Witten, Hansen, & Hall, 2001; Witten & Hall, 2003;
224 Nemoto *et al.*, 2007; Witten & Huysseune, 2009) and acellular bone (Castanet &
225 Ricqlès, 1986; Witten & Huysseune, 2009; Dean & Shahar, 2012; Shahar & Dean, 2013;
226 Atkins *et al.*, 2014, 2015a; Currey *et al.*, 2017). For instance, hyperostoses are
227 widespread in teleosts with acellular bone, and their formation requires an important
228 remodelling activity (Meunier & Desse, 1986; Smith-Vaniz, Kaufman & Glowacki, 1995).
229 In billfishes (Istiophoriformes), that lack osteocytes, bone in the rostrum is riddled with
230 secondary osteons overlapping primary osteons, akin to what is found in the haversian
231 bone of tetrapods and suggesting very intense remodelling activity as a response to
232 fracture and load (Amprino & Godina, 1956; Poplin, Poplin & Ricqlès, 1976; Castanet &
233 Ricqlès, 1986; Atkins *et al.*, 2014). These examples suggest that, in the absence of
234 osteocytes as sensors, acellular bone is nevertheless capable of detecting strain and
235 damage by some mechanism that is yet not fully understood (Kranenbarg *et al.*, 2005;
236 Witten & Huysseune, 2009; Fiaz *et al.*, 2010; Dean & Shahar, 2012; Shahar & Dean,
237 2013; Atkins *et al.*, 2014, 2015a).

238 In its general structure, biomechanics, and mechanisms of bone resorption and
239 remodelling, acellular teleost bone then appears to be functionally very similar to cellular
240 teleost bone. This suggests that the presence of osteocytes is not strictly necessary to
241 achieve these functions. This leaves osteocytic osteolysis, a potentially important
242 mechanism involved in calcium and/or phosphorus metabolism (Witten & Huysseune,
243 2009; Cohen *et al.*, 2012; Shahar & Dean, 2013; Doherty, Ghalambor & Donahue,
244 2015), as the main function entirely lacking in acellular bone.
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246 III. PHYLOGENETIC DISTRIBUTION OF ACELLULAR BONE

247 (1) Acellular bone outside of actinopterygians

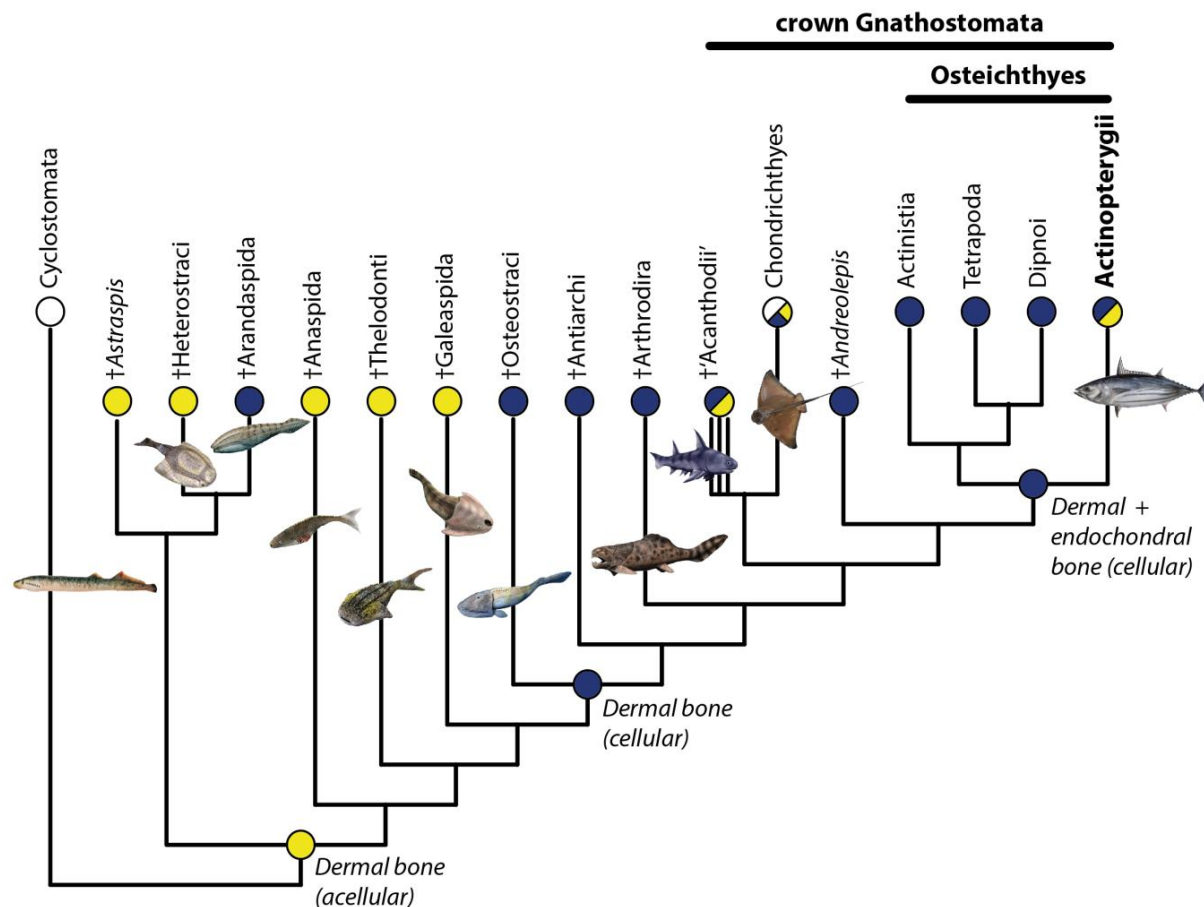
248 (a) Palaeozoic jawless vertebrates

249 A peculiar bone-like tissue devoid of osteocytes, called aspidin, has long been known in
250 the dermal skeleton of †heterostracans, a group of Palaeozoic jawless vertebrates
251 (Gross, 1930; Halstead, 1969). Similar tissues were later described in other early
252 jawless stem gnathostome lineages, such as †anaspids, †thelodonts and †galeaspids
253 (Stensiö, 1958; Sire, Donoghue & Vickaryous, 2009; Keating & Donoghue, 2016).
254 Aspidin appears to be structurally very similar to teleost acellular bone, with probable
255 collagen bundles (akin to the ‘tubules’ of teleosts) penetrating the mineralised tissue
256 (Keating *et al.*, 2018). The occurrence of either cellular or acellular bone in various early
257 vertebrate lineages (Fig. 2) led to a debate over which one was phylogenetically older
258 (Ørvig, 1951; Denison, 1963; Halstead, 1963; Smith & Hall, 1990). The earliest
259 vertebrates with cellular bone are the jawless †osteostracans that appear in the Silurian
260 (Stensiö, 1958; Smith & Hall, 1990; Donoghue & Sansom, 2002), although osteocytes
261 have also been described in the dermal bone of a late Ordovician †arandaspid (Sansom
262 *et al.*, 2013). Abundant evidence supports the placement of †osteostracans as the sister
263 group to gnathostomes (jawed vertebrates): it seems likely that cellular bone would then
264 be a synapomorphy of the clade uniting †osteostracans and gnathostomes (Donoghue
265 & Sansom, 2002; Brazeau & Friedman, 2014), with a potential convergent appearance
266 in †arandaspids (Fig. 2). This would imply that bone in †anaspids, †thelodonts,
267 †heterostracans and †galeaspids is primitively devoid of osteocytes, making acellular
268 bone the plesiomorphic state for skeletonising vertebrates (Denison, 1963; Halstead,
269 1963, 1969; Donoghue & Sansom, 2002; Keating *et al.*, 2018).

270 (b) Jawed vertebrates

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

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



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

298 (2) Phylogenetic distribution of acellular bone in teleosts and other 299 actinopterygians

300 (a) Material of study

301 To evaluate the phylogenetic distribution of cellular and acellular bone in
302 actinopterygians, we reviewed more than 150 years of literature on ray-finned fish bone.
303 The most comprehensive sources of information were the extensive surveys by Kölliker
304 (1859) and Moss (1961*b*, 1965), to which we added data from various fossil and extant
305 species where required to better resolve the phylogenetic and temporal distribution (see
306 online Supporting information, Table S1, for details on these sources). In total, our
307 database includes 677 fossil and extant taxa. In addition, we obtained propagation
308 phase contrast synchrotron microtomography (PPC-SR μ CT) data from museum
309 specimens of 108 extant and fossil species (Table 1, Table S1), bringing new
310 information or corroborating our knowledge on the presence or absence of osteocytes in
311 their bones. The SR μ CT scans were carried out at the ID-19 (microtomography)
312 beamline of the European Synchrotron Radiation Facility (ESRF), using a white beam
313 with energy levels between 35 and 105 keV, obtaining a voxel size of 0.72 μ m.

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

320 Cellular bone is present in the earliest actinopterygians from the Devonian (Table S1):
321 for example, in the bones and scales of †*Cheirolepis* (Zylberberg, Meunier & Laurin,
322 2016) and †*Moythomasia* (Sire *et al.*, 2009; Schultze, 2016) and in the scales of
323 †*Mimipiscis* (Richter & Smith, 1995). Bone and scales are always cellular in modern
324 non-teleost actinopterygians, for example in bichirs (Polypteriformes), bowfins and gars
325 (Holostei) (Kölliker, 1859; Moss, 1961*b*; Sire & Meunier, 1994; Daget *et al.*, 2001; Sire *et*
326 *al.*, 2009) and in their Mesozoic fossil relatives (Goodrich, 1907; Ørvig, 1978; Gayet &
327 Meunier, 1992; Meunier & Brito, 2004; Meunier *et al.*, 2016). Sturgeons and
328 paddlefishes (Acipenseriformes) have a poorly mineralised skeleton, but it is
329 nonetheless composed of cellular bone (e.g. Kölliker, 1859; Stéphan, 1900; Buffrénil *et*
330 *al.*, 2016; Leprévost *et al.*, 2017). Finally, many clades of extinct Mesozoic
331 actinopterygians have been surveyed histologically and show cellular bone, for example:
332 †saurichthyids (Scheyer *et al.*, 2014), †aspidorhynchids (Brito & Meunier, 2000),
333 †pachycormids (Meunier & Brito, 2004; Liston *et al.*, 2013), †pholidophorids (Meunier &
334 Brito, 2004). Our SR μ CT data provide additional information on a series of fossil non-
335 teleost actinopterygians, revealing the presence of cellular bone in the Jurassic stem
336 chondrosteian †*Chondrosteus acipenseroides*, the Jurassic †pseudodontiform
337 †*Proscinetes elegans*, the Jurassic †dapediid †*Dapedium* sp., the Triassic holosteans
338 †*Heterolepidotus dorsalis* and †*Eoeugnathus megalepis* and the Jurassic stem bowfin
339 †*Caturus furcatus*. These data also confirm the presence of cellular bone in 17 Jurassic
340 and Cretaceous taxa (Tables 1, S1) interpreted as stem-group teleosts (e.g. Arratia,
341 2015).
342

343 (c) *Elopomorpha*

344 Within Elopomorpha, cellular bone is found in tarpons and their relatives (Elopiformes),
345 including in scales (Kölliker, 1859; Meunier & Brito, 2004). Several eels (Anguilliformes)
346 are described as having acellular bone by Kölliker (1859). However, they all seem to
347 pertain to an outdated taxonomy that treated leptocephalus larvae as separate taxa
348 (Table S1). For example, Kölliker (1859) reports cellular bone in the sorcerer eel
349 *Nettastoma melanurum* and acellular bone in '*Hyoprorus messanensis*', corresponding
350 to the larva of *N. melanurum* (Eschmeyer, Fricke & van der Laan, 2018). Although Moss
351 (1961*b*) reports acellular bone in the moray eel *Gymnothorax moringa*, we confirm the
352 presence of osteocytes in this species, as well as in the adults of every other
353 anguilliform surveyed, including the freshwater eels *Anguilla anguilla* and *A. rostrata*
354 (Stéphan, 1900; Moss, 1965; Lopez, 1970), the conger eel *Conger conger* and the pike
355 conger *Muraenesox cinereus* (Table 1). The bonefish *Albula vulpes* was described as
356 having a mix of cellular and acellular bone (Moss, 1961*b*), but this is contradicted by our
357 observations (see Section III.3a). Finally, our SR μ CT data reveal cellular bone in several
358 fossil albuliforms (e.g. †*Istieus*, †*Lebonichthys*), elopiforms (e.g. †*Ichthyemidion*,
359 †*Anaethalion*, †*Flindersichthys*) and anguilliforms (†*Urenchelys*). In conclusion, we find
360 that cellular bone is present in post-larval individuals of all elopomorphs surveyed so far.

361
362 (d) *Osteoglossomorpha*
363 Fossil and extant bony-tongue fishes (Osteoglossomorpha) have cellular bone in their
364 skeleton, including scales (Kölliker, 1859; Meunier & Brito, 2004; Meunier, Brito & Leal,
365 2013a; Meunier, Dutheil & Brito, 2013b). Moss (1965) reported acellular bone in the two
366 modern mooneye (Hiodontidae) species, *Hiodon alosoides* and *H. tergisus*. However,
367 Kölliker (1859) described cellular bone in '*Hyodon claudulus*' that could be synonymised
368 with *H. alosoides* (Eschmeyer *et al.*, 2018). We resolved this uncertainty using
369 unambiguous observations of osteocyte lacunae in SR μ CT images of dentaries and/or
370 ribs from *H. alosoides*, *H. tergisus* and their Eocene close relative †*Eohiodon falcatus*,
371 confirming the presence of cellular bone in hiodontids. We also find cellular bone in
372 *Arapaima gigas*, in the arowana *Osteoglossum bicirrhosum* and its extinct Eocene
373 relatives †*Brychaetus muelleri* and †*Phareodus encaustus*, as well as in the featherback
374 *Chitala chitala* (Table 1). In conclusion, it is likely that cellular bone is present in all
375 osteoglossomorphs (Table S1).

376
377 (e) *Clupeomorpha*
378 Herrings and their relatives (Clupeomorpha) appear to have cellular bone (Kölliker,
379 1859; Moss, 1961b, 1965). Although Moss (1961b) reported acellular bone in the
380 anchovy *Anchoviella* sp. and the American shad *Alosa sapidissima*, he later updated this
381 observation by reporting cellular bone in *A. sapidissima* and three other *Alosa* species
382 (Moss, 1965). Our SR μ CT data reveal cellular bone in all clupeomorphs surveyed (Table
383 1), including the Cretaceous †*Armigatus namourensis* and †*Ellimmichthys longicostatus*
384 and the Eocene †*Knightsia* sp., as well as the extant wolf-herring *Chirocentrus dorab*, the
385 Pacific sardine *Sardinops sagax* and the alewife *Alosa pseudoharengus*. In conclusion, it
386 is likely that cellular bone is present in all clupeomorphs (Table S1), with the possible
387 exception of *Anchoviella* that needs further appraisal.

388
389 (f) *Ostariophysii*
390 Kölliker (1859) and Moss (1961b, 1965), extensively sampled the considerable diversity
391 of the mostly freshwater ostariophysans, including milkfishes (Gonorhynchiformes),
392 carps and relatives (Cypriniformes), characins and relatives (Characiformes), catfishes
393 (Siluriformes) and electric 'eels' (Gymnotiformes). Their surveys totalled 115 species,
394 virtually all of which appear to have cellular bone (Table S1). We also observed cellular
395 bone in our SR μ CT images of the carp *Cyprinus carpio*, the tench *Tinca tinca*, the bream
396 *Abramis brama* (Cypriniformes), the trahira *Hoplias malabaricus*, the payara *Hydrolycus*
397 *scomberoides*, the piranha *Serrasalmus spilopleura* (Characiformes), the catfishes
398 *Ariopsis felis*, *Galeichthys feliceps* and *Pimelodella gracilis* (Siluriformes), and the
399 banded knifefish *Gymnotus carapo* (Gymnotiformes), as well as in the Early Cretaceous
400 gonorhynchiform †*Tharrias araripes* (Table 1). Acellular bone is only described in two
401 ostariophysan species (Table S1): in the diminutive pencil catfish *Trichomycterus*
402 *punctulatus* (Kölliker, 1859), which is confirmed by our SR μ CT data from the dentary of
403 another *Trichomycterus* species, and in some cranial dermal bones of the zebrafish
404 *Danio rerio* (Weigele & Franz-Odenaal, 2016). In conclusion, cellular bone is present in
405 all ostariophysans surveyed so far, with the notable exceptions of *Trichomycterus*. In
406 addition, slickheads (Alepocephaliformes) are consistently recovered as sister to
407 ostariophysans in molecular phylogenies (Lavoué *et al.*, 2008; Near *et al.*, 2012;

408 Betancur-R. *et al.*, 2013; Straube *et al.*, 2018; Hughes *et al.*, 2018). The only species
409 surveyed from the group, *Alepocephalus rostratus*, has cellular bone (Kölliker, 1859).

410
411 (g) *Non-neoteleost Euteleostei*
412 Bone type is variable amongst Euteleostei, but generally homogeneous within a given
413 lineage (Table S1). Acellular bone is found in galaxiids (but only two species of *Galaxias*
414 have been surveyed), pikes and mudminnows (Esociformes; Kölliker, 1859; Moss,
415 1961*b*, 1965), smelts (Osmeridae; Moss, 1961*b*, 1965) and viperfishes and their
416 relatives (Stomiiformes; Kölliker, 1859; Germain, Schnell & Meunier, in press).
417 Conversely, cellular bone is found in *Argentina silus* (the only member of
418 Argentiniformes that was sampled) and we observe it in the Late Cretaceous
419 †*Spaniodon elongatus*, a taxon whose phylogenetic position within euteleosts is
420 uncertain (e.g. Taverne & Filleul, 2003). Salmon, trouts and their relatives
421 (Salmoniformes) are generally described as having cellular bone (Kölliker, 1859; Moss,
422 1961*b*, 1965; Hughes *et al.*, 1994; Witten & Hall, 2002; Totland *et al.*, 2011), but our
423 extensive SR μ CT sampling within the group complicates this pattern (Table 1). Bone
424 appears always to be cellular in the 'typical' trouts and salmon (Salmoninae). We
425 confirm this for extant and fossil representatives of *Oncorhynchus*, *Salmo*, *Salvelinus*
426 and *Parahucho*. The whitefishes *Coregonus reighardi*, *Prosopium williamsoni* and
427 *Stenodus leucichthys* (Coregoninae) also seem to have osteocytes, but they are much
428 scarcer than in salmonines, and irregularly distributed inside of bone. This is consistent
429 with the observation of Moss (1965), who described variation in osteocyte abundance
430 within the skeleton in some salmoniforms. Finally, in the grayling *Thymallus thymallus*
431 (Thymallinae), bone seems to be acellular.

432
433
434 (h) *Neoteleostei, including Acanthomorpha*
435 Within the euteleost subclade Neoteleostei (*sensu* Betancur-R. *et al.*, 2017), acellular
436 bone is found in various lizardfishes (Aulopiformes), including the Late Cretaceous
437 †*Eurypholis* sp., in the lanternfish (Myctophiformes) *Notoscopelus elongatus* and in the
438 Cretaceous genus of uncertain placement †*Ctenothrissa vexillifer* (Kölliker, 1859; Moss,
439 1961*b*; Davesne *et al.*, 2018). Spiny-rayed fishes (Acanthomorpha) contribute the
440 greatest fraction of neoteleost species diversity. Amongst the approximately 17,000
441 acanthomorph species (more than 300 being surveyed in the present study), acellular
442 bone is virtually universal (Kölliker, 1859; Moss, 1961*b*, 1965) and is found throughout
443 taxa displaying a broad range of morphologies and ecologies (Table S1), from marine
444 benthic taxa such as toadfishes and sculpins (Simmons *et al.*, 1970; Horton & Summers,
445 2009), to pelagic fast-swimming taxa like jacks and billfishes (Smith-Vaniz *et al.*, 1995;
446 Atkins *et al.*, 2014), deep-sea eelpouts (Meunier & Arnulf, 2018), or freshwater ricefishes
447 and tilapias (Ekanayake & Hall, 1987; Cohen *et al.*, 2012). Within acanthomorphs,
448 cellular bone is only known conclusively in two relatively species-poor lineages: the 'true'
449 tunas *Auxis*, *Euthynnus*, *Katsuwonus* and *Thunnus* (Kölliker, 1859; Stéphan, 1900;
450 Amprino & Godina, 1956; Moss, 1961*b*; Meunier & Huysseune, 1992; Santamaria *et al.*,
451 2018) and the opah *Lampris* (Davesne *et al.*, 2018). At least in tunas, osteocytes are
452 present not only in bones, but also in scales, fin rays and spines (Meunier *et al.*, 2008a;
453 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, 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* displayed a mix of cellular and acellular bone, with the latter being found in the operculum and gill arches. However, our SR μ CT 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-Odenaal (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-Odenaal, 2016). This suggests that our SR μ CT 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 μ m thick (Weigele & Franz-Odenaal, 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-Odenaal, 2016), we observe with SR μ CT 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 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

501 characterised by acellular bone (Hughes *et al.*, 1994). However these results are
502 seemingly contradicted by more recent data (Sire & Meunier, 2017): at least in the case
503 of *Sparus aurata* these tubules do not appear to contain osteocyte nuclei. In the second
504 case, osteocytes were described within areas of hyperostosis in the cleithrum of the jack
505 *Caranx latus* (Smith-Vaniz *et al.*, 1995) and in dorsal pterygiophores of the oarfish
506 *Regalecus russellii* (Paig-Tran, Barrios & Ferry, 2016), two acanthomorphs that
507 otherwise have acellular bone. However, such osteocytes do not appear to be present
508 systematically in acanthomorph hyperostotic bone: they are absent from the
509 hyperostoses of the scabbardfish *Trichiurus lepturus*, the jack mackerel *Trachurus*
510 *trachurus*, the sicklefish *Drepane africana*, the grunt *Pomadasys kaakan* and the
511 searobin *Prionotus stephanophrys* (Desse *et al.*, 1981; Meunier & Desse, 1994;
512 Meunier, Béarez & Francillon-Vieillot, 1999; Meunier, Gaudant & Bonelli, 2010). The
513 black skipjack tuna *Euthynnus lineatus* has cellular bone in its hyperostotic vertebrae
514 (Béarez, Meunier & Kacem, 2005), however this is consistent with the presence of
515 cellular bone throughout the rest of its skeleton. The occurrence of osteocytes in
516 hyperostotic regions of an otherwise acellular skeleton then appears to be the exception
517 rather than the rule; it nevertheless requires explanation.

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

523

524 (4) Phylogenetic distribution of acellular bone in actinopterygian scales

525 The phylogenetic distribution of osteocytes in actinopterygian scales (Table S1) has
526 been less studied than in the rest of the skeleton (Parenti, 1986). Scales in
527 actinopterygians primitively consist of a bony basal plate covered by dentine and
528 ganoine (an enamel-like tissue). The bony component remains as a thin external layer in
529 the elasmoid scales of most teleosts (Francillon-Vieillot *et al.*, 1990; Meunier &
530 Huisseune, 1992; Sire *et al.*, 2009). In ganoid scales, bone is always cellular, as shown
531 in early actinopterygians (Richter & Smith, 1995; Sire *et al.*, 2009; Zylberberg *et al.*,
532 2016), bichirs (Daget *et al.*, 2001; Sire *et al.*, 2009), holosteans (Meunier, François &
533 Castanet, 1978; Meunier *et al.*, 2016; Brito, Meunier & Gayet, 2000) and stem teleosts
534 (BrITO & Meunier, 2000; Meunier & Brito, 2004). In elasmoid scales, found in all teleosts
535 but also in amiids and the extant coelacanth *Latimeria* (Smith, Hobdell & Miller, 1972;
536 Meunier, 1984*b*; Meunier *et al.*, 2008*b*; Sire *et al.*, 2009), the situation is more complex.
537 In this type of scales, the basal layer develops into an incompletely mineralised plywood-
538 like structure called elasmordine (previously described as isopedine). The basal layer in
539 the scales in amiids and some teleosts (e.g. *Megalops*, *Hiodon*, *Arapaima*, *Chanos*)
540 incorporates cells superficially similar to osteocytes, called elasmocytes (Meunier,
541 1984*b*, 1987; Meunier & Brito, 2004). The bony layer is cellular in the elasmoid scales of
542 amiids (Meunier & Poplin, 1995), elopomorphs (e.g. *Megalops*, *Elops*, *Albula*) and at
543 least some osteoglossomorphs (Meunier, 1984*b*; Meunier & Brito, 2004). It is, however,
544 acellular in other taxa with cellular bone including clupeomorphs, ostariophysans and
545 salmoniforms (Meunier, 1987; Meunier & Brito, 2004; Meunier, Sorba & Béarez, 2004;
546 Sire *et al.*, 2009). Taxa with acellular bone always seem to have acellular scales as well
547 (Kölliker, 1859). In the tunas *Thunnus alalunga* and *T. obesus* scales are composed of

548 cellular bone (Meunier & Sire, 1981; Wainwright *et al.*, 2018), in agreement with the rest
549 of the skeleton. Since many teleosts with cellular bone lack osteocytes in their scales, it
550 then seems that acellularisation in scales phylogenetically precedes that of the rest of
551 the skeleton (Kölliker, 1859; Meunier, 1987; Meunier & Huysseune, 1992).
552

553 **IV. PHYLOGENETIC ORIGIN AND EVOLUTION OF ACELLULAR BONE**

554 **(1) Ancestral character state reconstruction**

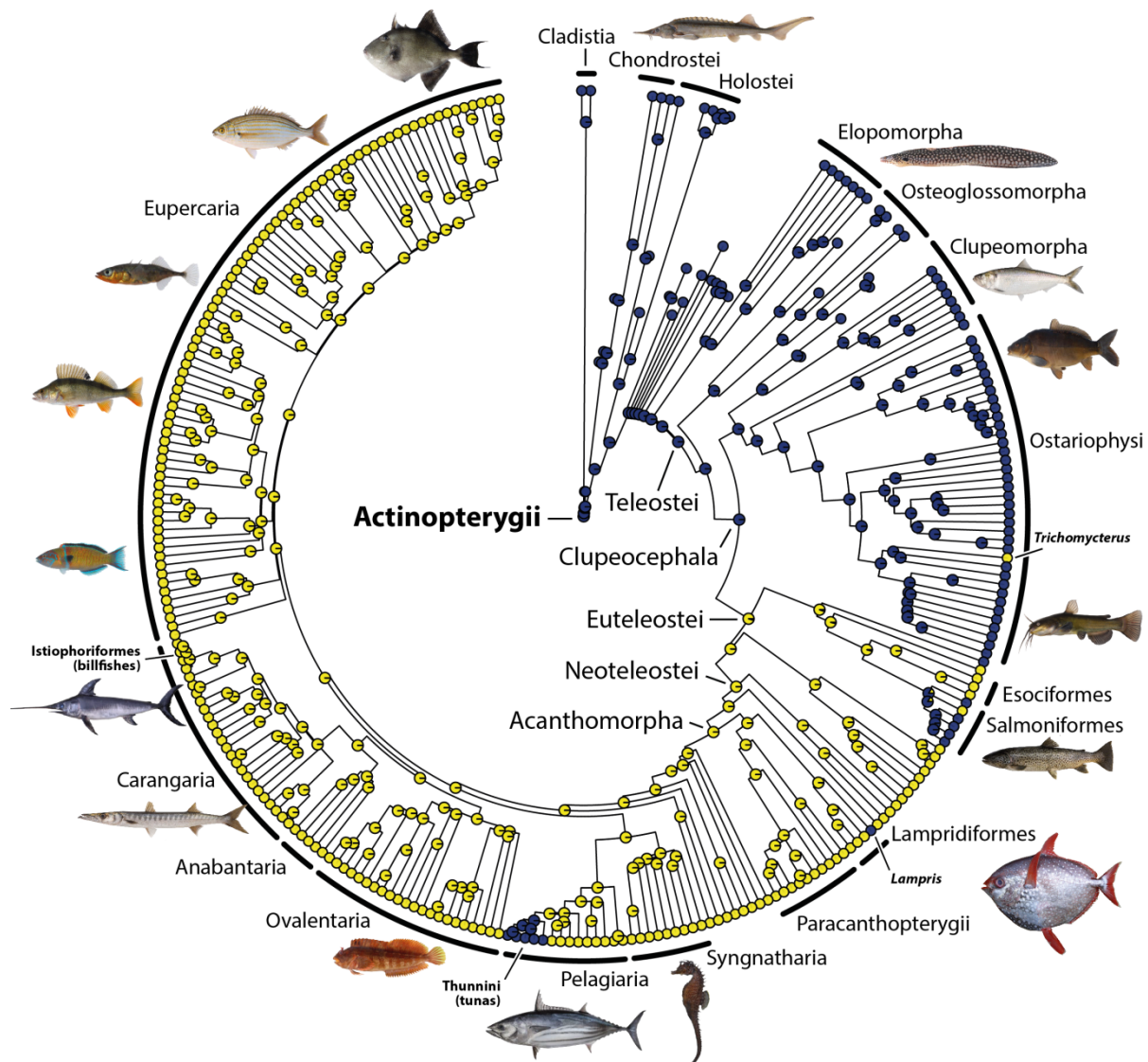
555

556 For our entire data set of 677 fossil and extant actinopterygians, we scored the presence
557 of cellular or acellular bone (Table S1; scales scored separately). When bone lacks
558 osteocytes only in certain skeletal elements (e.g. teleosts with cellular bone but acellular
559 scales) or ontogenetic stages (e.g. in larval anguilliforms) we scored its status as
560 'cellular'.

561 This data set was mapped onto three time-calibrated trees stemming from three recent
562 multi-locus or phylogenomic studies of actinopterygian intra-relationships. Topology #1
563 (T1) was obtained in an analysis of nine nuclear protein-coding loci including 232 taxa,
564 all extant (Near *et al.*, 2012); Topology #2 (T2) is based on an analysis (Betancur-R. *et*
565 *al.*, 2013) of 20 nuclear and one mitochondrial loci including 1582 extant taxa, to which
566 240 fossil taxa were added based on previously argued phylogenetic placements
567 (Betancur-R., Ortí & Pyron, 2015); Topology #3 (T3) was obtained from a transcriptomic
568 analysis of 1721 exons (Hughes *et al.*, 2018). In order to achieve consistency in clade
569 names, we relied on the phylogenetic classification proposed by Betancur-R. *et al.*
570 (2017), itself based on the molecular phylogeny that yielded T2.

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

577 We used a sub-sample of taxa that are included in both our cellularity data set and at
578 least one of the topologies. When two different species of the same genus were used in
579 two different data sets, we considered the genus as a whole, since no case of variability
580 of cell type between species of the same genus is known. This sub-sample retains 100
581 extant taxa for T1, 292 taxa including 26 fossils for T2, and 121 extant taxa for T3. Every
582 major actinopterygian lineage is present in the resulting trees with a few exceptions for
583 which osteohistological data are lacking entirely, such as the salamanderfish
584 (Lepidogalaxiiformes) and the jellynose fishes (Atelepodiformes). Ancestral states at
585 the nodes were reconstructed with the *ace* function of the APE package in R (Paradis,
586 Claude & Strimmer, 2004). Two models of ancestral character state estimations were
587 tested: an 'all rates different' (ARD) model (that allows transitions from cellular to
588 acellular and from acellular to cellular to have different frequencies) and a 'symmetrical'
589 model (that constrains transition frequencies to be equal). The difference between the
590 transition frequencies was very low even with the ARD model, but the Akaike information
591 criterion (AIC) very slightly favoured the symmetrical model, leading us to apply the latter
592 to our analyses.



593
 594 **Figure 3.** Time-calibrated multilocus tree of actinopterygians (ray-finned fishes), obtained from the
 595 optimisation of the character states 'cellular bone' (in dark blue) and 'acellular bone' in (yellow) on the
 596 topology T2 (Betancur-R. *et al.*, 2015). Character states for coded species are at the tips, and the
 597 reconstructed ancestral states at the nodes. A few key taxa, discussed in the text, are signalled in bold
 598 case. Taxon pictures are from Iglésias (2014b).
 599

600 (2) Reconstructed origin of acellular bone

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

606 The reconstructed ancestral state for Euteleostei is ambiguous and varies from one
 607 topology to the other. With T1, the ancestral state for Euteleostei is equivocal. The
 608 likelihoods of the ancestral state being 'cellular' or 'acellular' are between 0.45 and 0.55

609 for three clades: Euteleostei, Argentiniformes + (Esociformes + Salmoniformes) and
610 Esociformes + Salmoniformes (Figs 4, S1). In this scenario, whether cellular bone in
611 argentiniforms and salmoniforms is a secondary reacquisition or the retention of an
612 ancestral state is unclear.

613 With T2, the ancestral state for Euteleostei is acellular bone with a very high likelihood of
614 0.95 (Figs 3, 4, S2), implying that argentiniforms and salmoniforms both reacquired
615 cellular bone secondarily and separately. T3 also implies an ancestral acellular bone for
616 Euteleostei (and a secondary reacquisition of cellular bone in argentiniforms and
617 salmoniforms), albeit with a slightly lower likelihood of 0.89 (Figs. 4, S3).

618 T1 and T2 were both produced with similar methods involving multi-locus molecular data
619 sets adequately covering actinopterygian diversity, and it is difficult to establish whether
620 one is more credible than the other. Phylogenetic resolution at the base of the euteleost
621 tree is poor due to conflict between molecular markers and sparse taxon sampling, and
622 remains a point of contention in the literature (Campbell *et al.*, 2017; Straube *et al.*,
623 2018; Hughes *et al.*, 2018).

624 Bone histology of the salamanderfish *Lepidogalaxias* has never been studied, but could
625 be critical to accurately reconstruct the ancestral euteleostean state, since it is
626 consistently recovered by molecular studies as the sister group to all other euteleosts (Li
627 *et al.*, 2010; Near *et al.*, 2012; Betancur-R. *et al.*, 2013; Campbell *et al.*, 2017; Straube *et al.*,
628 2018; Hughes *et al.*, 2018). As long as the phylogeny of euteleosts is not stabilised,
629 and the osteohistology of more taxa not sampled (e.g. other argentiniforms and
630 galaxiids, *Lepidogalaxias*), ambiguity concerning the exact phylogenetic origin of
631 acellular bone will remain. Certain early fossil euteleosts, such as the Late Cretaceous
632 †*Spaniodon* (that has cellular bone) could also potentially play a key role in elucidating
633 this character's evolution. However, their usefulness is hampered by even greater
634 phylogenetic uncertainty than that for living lineages. For example †*Spaniodon* was
635 included in a clade grouping esociforms, salmoniforms and osmeriforms in a
636 phylogenetic analysis (Taverne & Filleul, 2003), but this topology is rejected by modern
637 molecular phylogenies, leaving the position of this fossil taxon unknown.
638 The megadiverse Neoteleostei (more than 18,000 extant species) are reconstructed as
639 having acellular bone ancestrally with all three topologies (likelihood = 0.99; Figs 3, 4,
640 S1–S3). Two distinct neoteleost lineages are reconstructed as having reacquired cellular
641 bone independently: (1) the 'true' tunas *Auxis*, *Katsuwonus*, *Euthynnus* and *Thunnus*,
642 forming the probably monophyletic tribe Thunnini within Scombridae; (2) the opah
643 *Lampris* in the monotypic Lamprididae (Fig. 3).

644 In conclusion, the clade in which acellular bone appears is equivocal with our ancestral
645 state reconstructions. T2 and T3 clearly support that acellular bone appears in
646 Euteleostei, while the ancestral state for Euteleostei is equivocal with T1, leaving open
647 the possibility of an independent appearance of acellular bone in Esociformes and in the
648 clade that unites Stomiati, Galaxiiformes and Neoteleostei (Figs 4, S1–S3).

649 In any case, acellular bone is almost entirely absent outside of Euteleostei, being
650 notably described in: (1) some larval anguilliforms, (2) the clupeiform *Anchoviella* sp., (3)
651 certain cranial dermal bones of the cypriniform *Danio rerio*, and (4) the siluriform
652 *Trichomycterus* sp. (see Section III.2; Fig. 3). It is noteworthy that all these occurrences
653 correspond to either larvae or to taxa with characteristically small adult body sizes. A
654 size-related explanation for the absence of osteocytes cannot be excluded in this
655 context (see Section III.3a for an exploration in the case of *D. rerio*).

656 Acellular bone seems to appear phylogenetically earlier in scales than in the rest of the
657 skeleton (Meunier, 1987; Meunier & Huysseune, 1992). Since acellular scales are
658 described in clupeomorphs, ostariophysans and every euteleost with the exception of
659 tunas (Table S1), we hypothesise that acellular scales are a character state of the clade
660 Clupeocephala (i.e. all modern teleosts but elopomorphs and osteoglossomorphs). A
661 systematic review of the histology of teleost scales is needed to test this hypothesis
662 suitably. The nature of the external layer of teleost scales is controversial, and some
663 authors have proposed that it has a different evolutionary origin to bone (e.g. Sire *et al.*,
664 2009), potentially explaining why cellularity is lost earlier in this tissue than in 'true' bone.
665

666 (3) Secondary reacquisition of cellular bone

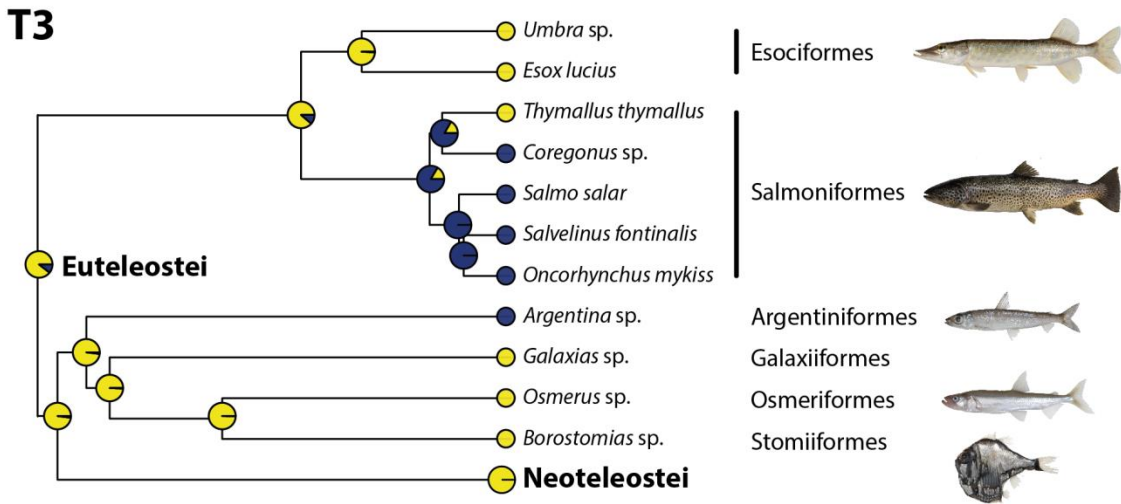
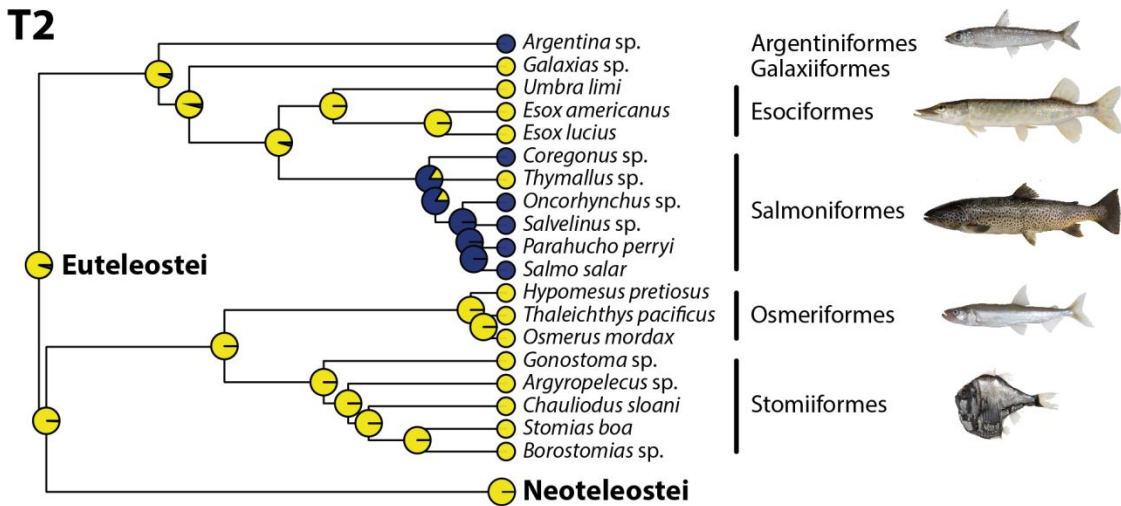
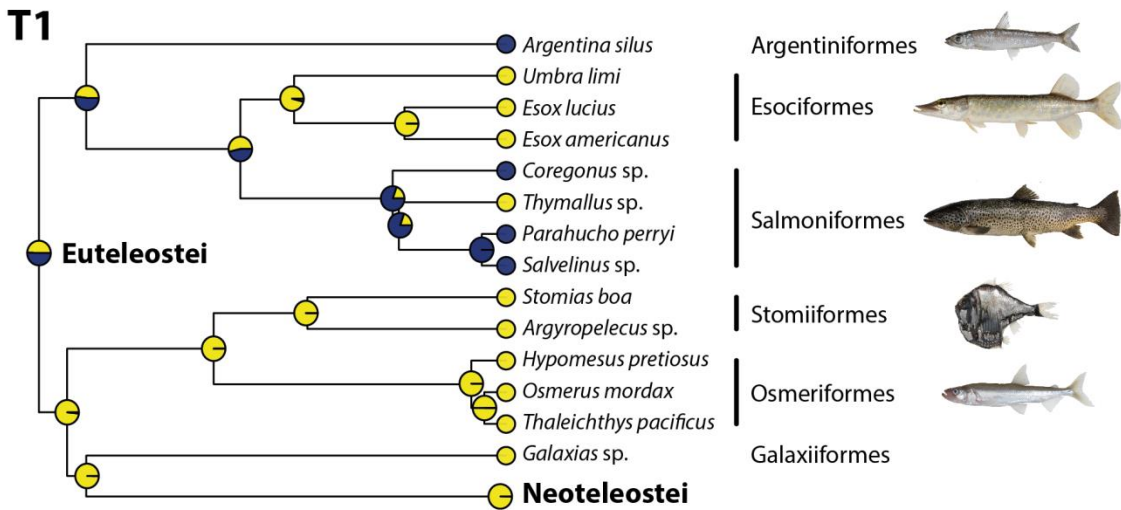
667 (a) Probable occurrence in salmoniforms

668 Our ancestral state reconstructions suggest that cellular bone was secondarily
669 reacquired in salmon, trouts and their relatives (Salmoniformes), but this is equivocal
670 due to topological uncertainty at the base of the euteleost tree (Fig. 4). The same
671 reconstructions also equivocally support a separate secondary reacquisition of
672 osteocytes in argentiniforms. However, since our data only rely on one species
673 (*Argentina silus*) and the phylogenetic position of argentiniformes is highly uncertain, we
674 refrain from commenting until more observations are available.

675 As described above, cellular bone does not seem to be distributed uniformly within
676 salmoniforms, according to our SR μ CT data (Tables 1, S1): (1) in the grayling *Thymallus*
677 *thymallus* (Thymallinae), we did not observe osteocytes conclusively; (2) in the
678 shortnose cisco *Coregonus reighardi* (Coregoninae), osteocytes are present, but
679 sparsely distributed within bone; (3) in *Stenodus leucichthys* (Coregoninae) and all
680 observed Salmoninae, osteocytes are present, and uniformly distributed inside bone.
681 Moss (1965) already noted that osteocyte abundance varies within bone elements in at
682 least some salmoniforms, which has been interpreted by Parenti (1986) as a possible
683 'intermediate' stage between cellular and acellular bone.

684 Salmoniform phylogeny is currently disputed, particularly in the relationships between
685 thymallines, coregonines and salmonines. Recent molecular studies have recovered
686 three different topologies: Coregoninae + Salmoninae (Alexandrou *et al.*, 2013; Horreo,
687 2017), Thymallinae + Salmoninae (Near *et al.*, 2012; Crête-Lafrenière, Weir &
688 Bernatchez, 2012; Betancur-R. *et al.*, 2013), and Coregoninae + Thymallinae (Campbell
689 *et al.*, 2013; Macqueen & Johnston, 2014; Hughes *et al.*, 2018), also affecting the three
690 topologies we used in our analyses. These competing phylogenies mean that the pattern
691 of evolution of cellular bone in salmoniforms as a whole is uncertain.

692 Many salmoniforms are anadromous, meaning that sexually mature individuals migrate
693 upstream over sometimes long distances. This behaviour involves intense and sustained
694 swimming activity, which is likely to affect physiology and metabolism. How it influences
695 bone growth and structure is not fully understood, but it appears that bone responds
696 adaptively to the anadromous lifestyle. In the Atlantic salmon (*Salmo salar*), bones
697 undergo halastasis (a diffuse demineralisation without degradation of the organic matrix)
698 during spawning migration (Kacem & Meunier, 2003, 2009). In addition, *S. salar* shows a
699 prominent increase in the volume of osteocyte lacunae in adult specimens compared to
700 juveniles, which is probably explained by osteocytic osteolysis (Kacem & Meunier,
701 2000). Moreover, bone in salmon exposed to sustained swimming shows increases in



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

708
709 osteocyte abundance (Totland *et al.*, 2011). These observations support the hypothesis
710 that osteocytes play an important role in resorbing salmon bone during anadromous
711 migration.

712 Anadromy is likely to be a trait that evolved multiple times in various lineages within
713 salmoniforms from strictly freshwater ancestors (McDowall, 1997, 2001; Alexandrou *et al.*, 2013).
714 Anadromy is widespread in salmonines (especially in the clade formed by
715 *Salmo*, *Oncorhynchus* and *Salvelinus*), and in most species of *Coregonus* (Alexandrou
716 *et al.*, 2013). Osteocytes are also observed in all of these taxa, while they seem to be
717 absent in the non-migrating freshwater *Thymallus* and in esociforms, the probable sister
718 group to salmoniforms. The occurrence of cellular bone then roughly follows that of
719 anadromy in this particular teleost clade. A notable exception occurs in the genus
720 *Prosopium*, a non-migrating taxon that possesses cellular bone. Nevertheless, it is
721 possible that the hypothesised reacquisition of cellular bone in at least some
722 salmoniforms would have allowed or facilitated the evolution of anadromy in these
723 animals, using a combination of halastasis and osteocytic osteolysis to function as a
724 source of calcium and/or phosphorus for metabolism and muscle activity. A more
725 extensive survey of bone histology in salmoniforms, especially for taxa that have not
726 been studied so far (such as the non-migrating salmonines *Hucho* and *Brachymystax*),
727 and in anadromous euteleosts outside of salmoniforms, is necessary to investigate the
728 potential coevolution of bone cellularity with anadromous habits.

729
730 *(b) Convergent occurrences in red-muscle endotherms*

731 Unlike salmoniforms, there is no ambiguity that osteocytes were reacquired secondarily
732 in two acanthomorph lineages (Figs 3, 5A): tunas and the opah (Davesne *et al.*, 2018).
733 Tunas are scombrids, a family that molecular analyses place reliably into the clade
734 Pelagiaria, itself included in the ultradiverse acanthomorph clade Percomorpha
735 (Betancur-R. *et al.*, 2013, 2017; Near *et al.*, 2013; Miya *et al.*, 2013; Alfaro *et al.*, 2018).
736 The 'true' tunas (Thunnini) consist of five genera (*Allothunnus*, *Auxis*, *Euthynnus*,
737 *Katsuwonus* and *Thunnus*), and their monophyly is supported by morphological (Collette
738 *et al.*, 1984; Carpenter, Collette & Russo, 1995) and most molecular phylogenies (Block
739 *et al.*, 1993; Betancur-R. *et al.*, 2013; Miya *et al.*, 2013). The opah (*Lampris* sp.) is a
740 lampridiform, a clade whose phylogenetic position within acanthomorphs is uncertain,
741 but that branches outside of Percomorpha in any case (Betancur-R. *et al.*, 2013; Near *et al.*,
742 2013; Davesne *et al.*, 2014, 2016; Alfaro *et al.*, 2018). There is then clear evidence
743 that the secondary reacquisition of osteocytes occurred independently in both lineages
744 (Davesne *et al.*, 2018).

745 While cellular bone has long been known in tunas (Kölliker, 1859; Stéphan, 1900;
746 Amprino & Godina, 1956; Moss, 1961b), fewer data were available on other scombrid
747 taxa and acellular bone was known only from the Atlantic mackerel *Scomber scombrus*
748 and the Spanish mackerel *Scomberomorus maculatus* (Kölliker, 1859; Amprino &
749 Godina, 1956; Moss, 1961b). Our SR μ CT data allow us to confirm the absence of

750 osteocytes from the ribs of a larger sample of scombrids: the butterfly kingfish
751 *Gasterochisma melampus*, the blue mackerel *Scomber australasicus*, the wahoo
752 *Acanthocybium solandri*, the bonito *Sarda orientalis* and the dogtooth 'tuna'
753 *Gymnosarda unicolor* (Table 1, Fig. 5C, D). *Sarda* and *Gymnosarda* are particularly
754 relevant because they probably constitute the sister group to Thunnini (Collette *et al.*,
755 1984; Block *et al.*, 1993; Miya *et al.*, 2013). All of these taxa are outside of Thunnini,
756 supporting that 'true' tunas are the only scombrids with cellular bone (Fig. 5A, E).
757 Within lampridiforms, acellular bone has been described in the ribbonfishes *Trachipterus*
758 *trachipterus* and *Zu cristatus* (Kölliker, 1859), in the oarfish *Regalecus russelii* (Paig-
759 Tran *et al.*, 2016) and in the veliferid *Velifer hypselopterus* (Davesne *et al.*, 2018). Our
760 SR μ CT data show that the veliferid *Metavelifer multiradiatus* also lacks osteocytes
761 (Table 1), and veliferids are probably sister to all other lampridiforms (Olney, Johnson &
762 Baldwin, 1993; Wiley, Johnson & Dimmick, 1998; Davesne *et al.*, 2014). The absence of
763 osteocytes in veliferids, and in the Cretaceous stem lampridiform †*Aipichthys* *velifer*
764 supports that acellular bone is plesiomorphic for lampridiforms (Davesne *et al.*, 2018).
765 Thus, the opah is secondarily cellular within lampridiforms, akin to 'true' tunas within
766 scombrids (Fig. 5A).
767 Tunas and the opah share many life-history traits, to which the reappearance of
768 osteocytes could potentially be imputed. However, a closer examination of these traits
769 across acanthomorph diversity reveals that most do not correlate with the presence of
770 osteocytes. (1) Sustained, active swimming is also found in other large-bodied pelagic
771 predators with acellular bone, such as carangids (Smith-Vaniz *et al.*, 1995), the
772 dolphinfish *Coryphaena hippurus* (Moss, 1961*b*), billfishes (Kölliker, 1859; Amprino &
773 Godina, 1956; Moss, 1961*b*; Atkins *et al.*, 2014) and several scombrids outside of 'true'
774 tunas (Fig. 5A, C, D). (2) A large body size does not seem to be a factor either: within
775 scombrids, the osteocytic bullet tuna *Auxis rochei* rarely exceeds 350 mm in total length
776 as an adult (Collette & Nauen, 1983), while the dogtooth 'tuna' *Gymnosarda unicolor*
777 and wahoo *Acanthocybium solandri* both commonly exceed 1000 mm in total length
778 (Collette & Nauen, 1983) and are anosteocytic (Fig. 5D). Other very large pelagic
779 acanthomorphs such as the oarfish *Regalecus* sp., billfishes, or the oceanic sunfish
780 *Mola mola* (Kölliker, 1859) all have acellular bone as well. (3) Finally, the reacquisition of
781 osteocytes does not seem to be linked with structural homeostasis: bone in tunas, opah
782 and billfishes appears to have active, intense and sustained resorption and remodelling
783 activities (Fig. 5B, D, E) evidenced by the extensive presence of secondary bone
784 (Amprino & Godina, 1956; Poplin *et al.*, 1976; Castanet & Ricqlès, 1986; Atkins *et al.*,
785 2014; Davesne *et al.*, 2018). However, bone in billfishes is acellular (Fig. 5B), confirming
786 that this intense remodelling activity does not require the presence of osteocytes (Atkins
787 *et al.*, 2014; Currey *et al.*, 2017).
788 Conversely, a correlation between cellular bone and endothermy in acanthomorphs
789 appears to be more substantiated (Meunier, 1987; Ricqlès *et al.*, 1991; Meunier &
790 Huisseune, 1992; Davesne *et al.*, 2018). Our new SR μ CT data confirm that cellular
791 bone co-occurs with a modification in the distribution and position of the lateral aerobic
792 red muscles (Fig. 5A), that concentrate in the anterior portion of the body and become
793 internalised within myotomes, coming closer to the axial skeleton; this configuration is
794 unique to 'true' tunas amongst scombrids (Graham, Koehn, & Dickson, 1983; Block *et*
795 *al.*, 1993; Graham & Dickson, 2000, 2004).
796

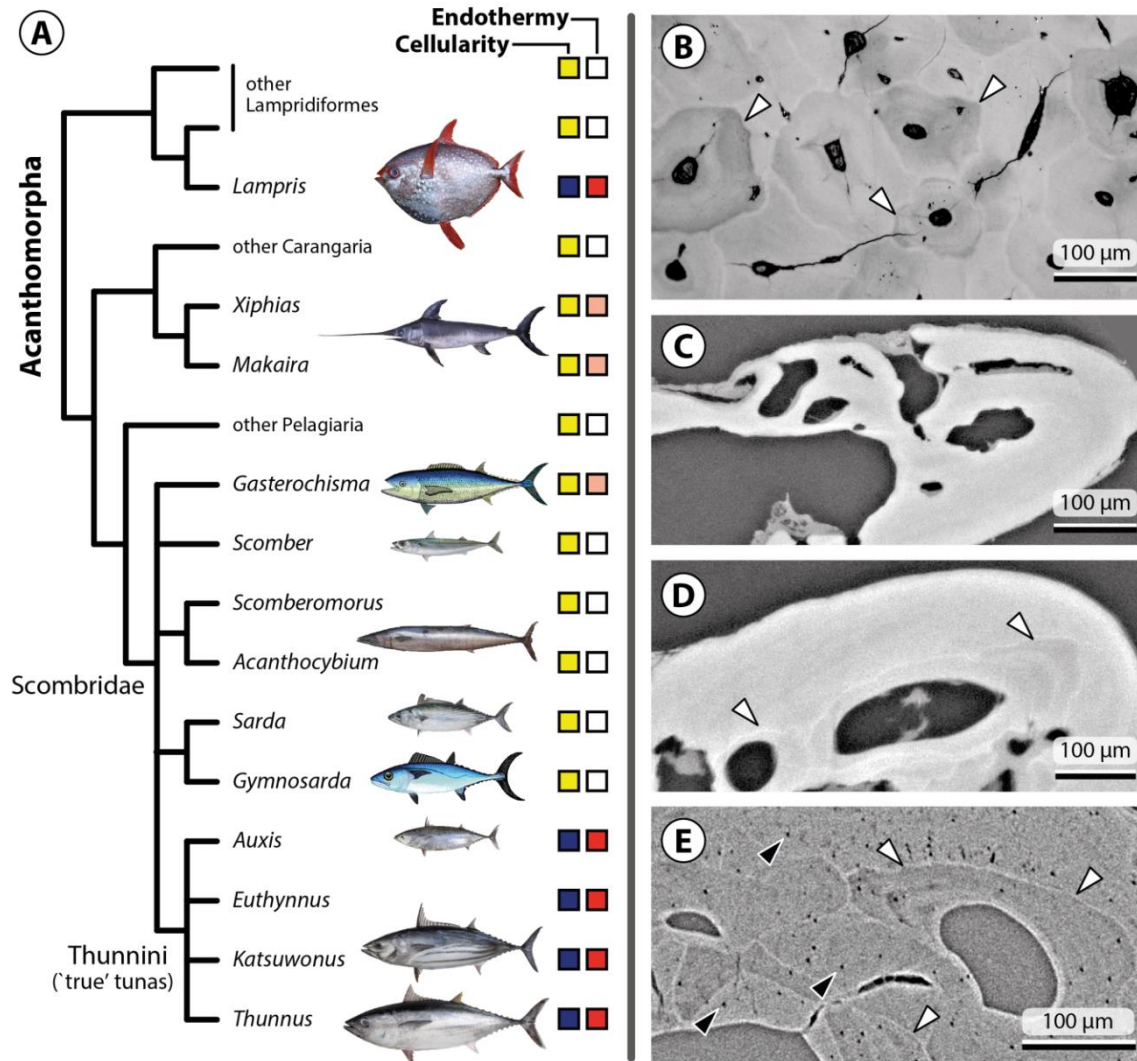


Figure 5. (A) Phylogenetic distribution of bone type in endothermic acanthomorph teleosts and their close relatives (modified from Davesne *et al.*, 2018). The squares represent bone type (acellular in yellow, cellular in dark blue) and thermal physiology (ectothermy in white, cranial endothermy in salmon pink, red-muscle endothermy in red). Taxon pictures from Iglésias (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). 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.

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 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

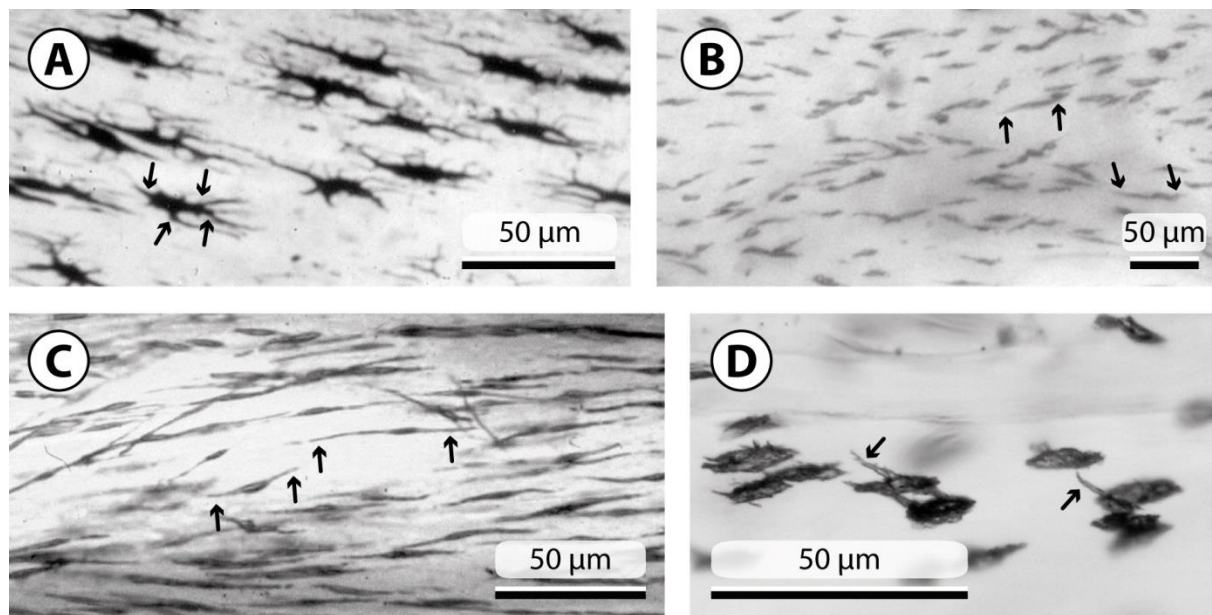
817 (Block *et al.*, 1993; Dickson & Graham, 2004; Watanabe *et al.*, 2015). The opah
818 developed a distinct form of red-muscle endothermy in which the red pectoral-fin
819 muscles produce most of the heat, are insulated from the outside by a thick fatty layer,
820 and the heat is kept and redistributed *via* retia located within the gills (Wegner *et al.*,
821 2015). A form of endothermy is also found in two other acanthomorph lineages: billfishes
822 and the butterfly kingfish *Gasterochisma melampus*, a non-Thunnini scombrid (Fig. 5A).
823 In these cases, heat is produced by specialised modified ocular muscles (the superior
824 rectus in billfishes and the lateral rectus in *G. melampus*) that lost their contractile
825 activity and cycle calcium ions between the cytoplasm and sarcoplasmic reticulum
826 (Carey, 1982; Block, 1986, 1994; Dickson & Graham, 2004). Since it only warms the
827 brain and the eyes, this configuration is often called 'cranial endothermy' (Dickson &
828 Graham, 2004). Osteocytes are absent in the bill and ribs of billfishes (Atkins *et al.*,
829 2014), and our SR μ CT data failed to find them in a rib of *G. melampus* (Table 1, Fig.
830 5B,C), implying that cranial endotherms, unlike red-muscle endotherms, have acellular
831 bone. We also observe acellular bone in the sclerotic ossicles of *G. melampus* and of
832 the billfishes *Kajikia albida* and *Xiphias gladius* (Table 1), confirming that the cellularity
833 of a bone is not affected by its proximity to the heat-generating muscles. In the opah, the
834 sclerotic ossicles have cellular bone like the rest of the skeleton (Table 1).
835 Heat production by red muscles involved in swimming (rather than modified ocular
836 muscles) and redistribution in a large proportion of the body (rather than in the brain
837 region only) is the key distinction between red-muscle and cranial endothermy. Given
838 that both acanthomorph lineages that developed red-muscle endothermy are also the
839 only ones that reacquired osteocytes, a correlation between these characters is likely
840 (Davesne *et al.*, 2018). As for salmoniforms, we can hypothesise that the correlation
841 stems from an intense muscular activity associated with sustained swimming. The latter
842 is necessary both to hunt prey and to produce heat *via* the myotomal or pectoral red
843 muscles. Since muscles are important consumers of calcium, an element primarily found
844 in bony tissues, osteolytic osteolysis potentially played an important role in the
845 appearance of red-muscle endothermic strategies. Whether the reacquisition of
846 osteocytes facilitated the evolution of red-muscle endothermy, or both characters
847 coevolved under a common selective pressure is unclear.

848
849 (c) *Structural evidence for a re-acquisition in salmoniforms, tunas and opahs*

850 While osteocyte morphology is very diverse in vertebrate bone in general, two main
851 morphologies seem to occur in teleost cellular bone (Fig. 6). In the first type, osteocytes
852 have a rounded or irregular cell body, and show numerous, thin cytoplasmic processes
853 that branch into canaliculi in all directions. This gives these osteocytes a typically 'star-
854 shaped' morphology (Fig. 6A). In the second type, osteocytes are much more elongate
855 ('spindle-shaped') and orientate in a preferential direction, presumably following the
856 collagen lamellae of the extracellular matrix (Kerschnitzki *et al.*, 2011). Their cell bodies
857 are more regular in shape, and they have only two cytoplasmic processes that are
858 located at the extremities of the cell body, aligning with its long axis. They also have very
859 few, non-branching canaliculi, that tend to orientate in preferential directions (Fig. 6B, C).
860 Both osteocyte types seem to coexist within teleost cellular bone, for example in *D. rerio*
861 (Weigle & Franz-Odenaal, 2016). Conversely, in the bone of salmoniforms and 'true'
862 tunas (Stéphan, 1900; Meunier & Huysseune, 1992; Totland *et al.*, 2011; Davesne *et al.*,
863 2018), the spindle-shaped osteocytes seem to be the only type that is present (Fig.

864 6B,C). In the opah, osteocytes are close to the 'spindle-shaped' morphology, since they
 865 have very few cytoplasmic processes and canaliculi that all orientate in a preferential
 866 direction, but they are not located at the extremities of the cell body like in tunas and
 867 salmoniforms (Fig. 6D). It is not clear whether this second type of osteocytes forms a
 868 connected canalicular system; at least in salmonids they might not be connected to each
 869 other at all (Totland *et al.*, 2011). Moreover, their morphology does not seem to change
 870 significantly between primary and remodelled bone, for example in the opah (Davesne *et*
 871 *al.*, 2018).

872 It appears that the three lineages that have in common an inferred or likely reacquisition
 873 of cellular bone share these structural similarities in osteocyte morphology. This
 874 suggests that their peculiar morphology might be linked with the evolutionary
 875 reacquisition of osteocytes from an ancestral acellular bone. Weigle & Franz-Odenaal
 876 (2016) proposed that these types of osteocytes have different developmental origins,
 877 and that the elongate, spindle-shaped osteocytes are derived from the elongate
 878 'osteoblast-like' cells that line the bone. It is possible that all secondarily reacquired
 879 osteocytes share this unique developmental origin, and that the other, 'typical'
 880 osteocytes derive from a mode of formation that does not occur in salmoniforms, tunas
 881 and opahs and was possibly lost at the euteleost node. Structural similarities in
 882 osteocyte morphology appear further to support that their reacquisition is underlined by
 883 shared, and not fully understood, mechanisms.
 884



885
 886
 887 **Figure 6.** Osteocyte morphology in taxa that retain the ancestral cellular bone (A) or that secondarily
 888 reacquired it from acellular ancestors (B–D). (A) 'Star-shaped' osteocytes in the dorsal-fin spine of the
 889 carp *Cyprinus carpio*, an ostariophysan. Note the irregular shape of the lacunae, and the numerous
 890 cytoplasmic processes ending in canaliculi branching in all directions (arrows). Modified from Meunier &
 891 Huyseune (1992). (B) 'Spindle-shaped' osteocytes in the coracoid of the salmon *Salmo salar* (NHMUK,
 892 uncatalogued), a salmoniform. Note the two cytoplasmic processes located at both extremities of the cell
 893 axis (arrows). Photograph by D. Davesne and A. D. Schmitt. (C) 'Spindle-shaped' osteocytes in the
 894 dorsal-fin spine of the tuna *Katsuwonus pelamis*, an acanthomorph. Note the two cytoplasmic processes
 895 located at both extremities of the cell axis (arrows). Photograph by F. J. Meunier. (D) 'Spindle-shaped'

896 osteocytes in the rib of the opah *Lampris* sp. (MNHN-ZA-AC-A-7506), an acanthomorph. Note the few
897 canaliculi, all pointing in the same direction (arrows). Modified from Davesne *et al.* (2018).

898 899 **V. THE ROLE OF MINERAL HOMEOSTASIS IN THE LOSS AND** 900 **REACQUISITION OF OSTEOCYTES**

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

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

937 938 **VI. CONCLUSION**

939 (1) According to our ancestral state reconstructions (Figs. 3,4), acellular bone is a
940 synapomorphy of either Euteleostei (as supported by two out of three tree topologies), or
941 of a smaller clade consisting of Stomiati, Galaxiiformes and Neoteleostei (as supported
942 by one tree topology). New analyses incorporating histological information on more

943 euteleost taxa (e.g. *Lepidogalaxias*, more argentiniforms and galaxiiforms) including
944 early fossil representatives, and a stabilisation of the euteleost phylogeny, are both
945 necessary to clarify the ambiguity on the exact clade in which acellular bone evolved.
946 Given the equivocal support for the euteleost ancestral state in T1 (Fig. 4), and
947 considering other lines of evidence (such as structural similarities between osteocytes in
948 salmoniforms and tunas; Fig. 6), we consider it to be more likely that osteocytes were
949 lost in Euteleostei, with a secondary reacquisition in salmoniforms.

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

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

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

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

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

985

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1647 **IX. SUPPORTING INFORMATION**

1648 Additional supporting information may be found in the online version of this article.

1649 **Table S1.** Complete list of actinopterygian (ray-finned fish) taxa surveyed by our
1650 literature review, including additional species obtained with our synchrotron
1651 microtomography (SR μ CT) data.

1652 The supra-specific taxonomy follows Betancur-R. *et al.* (2017). In the case of older
1653 literature, species names were often outdated. We used FishBase (Froese & Pauly,
1654 2019) and the Catalog of Fishes (Eschmeyer *et al.*, 2019) to identify the corresponding
1655 valid names.

1656 Key for the 'Notes' column: ¹leptocephalus larva; ²possible error in identifying bone type;
1657 ³localised cellular bone in hyperostoses; ⁴acellular hyperostotic bone; ⁵alleged
1658 osteocytes in cytoplasmic tubules.

1659 **Fig. S1.** Time-calibrated multilocus tree of extant actinopterygians (ray-finned fishes),
1660 obtained from the optimisation of the character states 'cellular bone' (in dark blue) and
1661 'acellular bone' (in yellow) on the topology T1 (Near *et al.*, 2012).

1662 **Fig. S2.** Time-calibrated multilocus tree of extant and fossil actinopterygians (ray-finned
1663 fishes), obtained from the optimisation of the character states 'cellular bone' (in dark
1664 blue) and 'acellular bone' (in yellow) on the topology T2 (Betancur-R. *et al.*, 2015).

1665 **Fig. S3.** Time-calibrated multilocus tree of extant actinopterygians (ray-finned fishes),
1666 obtained from the optimisation of the character states 'cellular bone' (in dark blue) and
1667 'acellular bone' (in yellow) on the topology T3 (Hughes *et al.*, 2018).
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Table 1. Bone type (presence or absence of osteocytes) in the taxa surveyed by our synchrotron microtomography (SR- μ CT) data. C = cellular bone; A = acellular bone. ¹Bones 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 (UMMZ); Université de Poitiers, France.

Taxon	Species name	Vernacular name	Type	Bone sampled	Specimen used		
†Cheirolepidiformes	†Cheirolepididae	† <i>Cheirolepis canadensis</i>	-	C	dentary	UMMP 3453	
Polypteriformes	Polypteridae	<i>Erpetoichthys calabaricus</i>	reedfish	C	dentary	Université de Poitiers, uncat.	
		<i>Polypterus delhezi</i>	barred bichir	C	dentary	Université de Poitiers, uncat.	
<i>Incertae sedis</i>	<i>Incertae sedis</i>	† <i>Birgeria stensioei</i>	-	C	dentary	PIMUZ T2188	
Chondrostei	†Chondrosteidae	† <i>Chondrosteus acipenseroides</i>	-	C	dentary	NHMUK PV P 2261a	
	Aipenseridae	<i>Acipenser gueldenstaedtii</i>	Danube sturgeon	C	dentary	MNHN.ICOS.01529	
†Pycnodontiformes	†Pycnodontidae	† <i>Proscinetes elegans</i>	-	C	dentary	NHMUK PV P 1626	
†Dapediiformes	†Dapediidae	† <i>Dapedium</i> sp.	-	C	dentary	OUMNH J.3041	
Holostei	<i>Incertae sedis</i>	† <i>Eoeugnathus megalepis</i>	-	C	dentary	PIMUZ T344	
		† <i>Heterolepidotus dorsalis</i>	-	C	dentary	NHMUK PV P 10290	
		† <i>Hulettia americana</i>	-	C	dentary	UMMP 11217	
	Amiidae	<i>Amia calva</i>	bowfin	C	dentary	OUMNH 21648	
	†Caturidae	† <i>Caturus furcatus</i>	-	C	dentary	private collection	
	Lepisosteidae	<i>Atractosteus tropicus</i>	tropical gar	C	dentary	MNHN.ICOS. PB-901	
		<i>Lepisosteus oculatus</i>	spotted gar	C	dentary	UMMZ 178806/S	
	†Semionotidae	† <i>Semionotus elegans</i>	-	C	dentary	UMMP 13664	
	†Aspidorhynchiformes	†Aspidorhynchidae	† <i>Aspidorhynchus cf.eodus</i>	-	C	dentary	private collection
			† <i>Vinctifer comptoni</i>	-	C	dentary	UMMP 101950
†Pachycormiformes	†Pachycormidae	† <i>Euthynotus incognitus</i>	-	C	dentary, rib	NHMUK PV P 2044	
		† <i>Hypsocormus</i> sp.	-	C	dentary	private collection	
		† <i>Leedsichthys problematicus</i>	-	C	gill raker	private collection	
		† <i>Pachycormus macropterus</i>	-	C	dentary	MNHN.F.JRE87	
†Pholidophoriformes	<i>Incertae sedis</i>	† <i>Pholidophoroides crenulata</i>	-	C	dentary	NHMUK PV OR 36313	

		† <i>Pholidophoropsis caudalis</i>	-	C	dentary	OUMNH J.3363
†Dorsetichthyiformes	†Dorsetichthyidae	† <i>Dorsetichthys bechei</i>	-	C	dentary	OUMNH J.3369
†Leptolepidiformes	†Ascalaboidae	† <i>Ascalabos voithii</i>	-	C	dentary	NHMUK PV P 3673a
		† <i>Tharsis dubius</i>	-	C	dentary	NHMUK PV OR 37852b
	†Leptolepididae	† <i>Leptolepis macrophthalmus</i>	-	C	dentary	private collection
†Ichthyodectiformes	<i>Incertae sedis</i>	† <i>Allothrissops regleyi</i>	-	C	dentary, rib	NHMUK PV P 921
		† <i>Pachythrissops laevis</i>	-	C	dentary	NHMUK PV P 41859
		† <i>Thrissops formosus</i>	-	C	dentary, rib	NHMUK PV OR 35013
	†Ichthyodectidae	† <i>Ichthyodectes cf. ctenodon</i>	-	C	dentary	UMMP V56318
		† <i>Xiphactinus cf. audax</i>	-	C	dentary	UMMP 11003
†Crossognathiformes	†Crossognathidae	† <i>Rhacolepis buccalis</i>	-	C	dentary	UMMP 101952
Elopomorpha	<i>Incertae sedis</i>	† <i>Osmeroides</i> sp.	-	C	dentary	OUMNH K.64151
		† <i>Urenchelys germanus</i>	-	C	dentary	NHMUK PV P 62726
	Albulidae	<i>Albula vulpes</i>	bonefish	C	rib, opercle, ceratobranchial	UMMZ 186965/S
		† <i>Istieus grandis</i>	-	C	dentary	NHMUK PV P 3886
	Elopidae	† <i>Anaethalion angustus</i>	-	C	dentary, rib	NHMUK PV OR 37926
		† <i>Davichthys gardineri</i>	-	C	dentary	NHMUK PV P 63231
		<i>Elops saurus</i>	ladyfish	C	dentary, rib	UMMZ 189366/S
	Megalopidae	† <i>Flindersichthys denmaedi</i>	-	C	dentary	NHMUK PV P 59694
		<i>Megalops cyprinoides</i>	Indo-Pacific tarpon	C	dentary, rib	MNHN.ICOS.00987
	Anguillidae	<i>Anguilla anguilla</i>	European eel	C	dentary	MNHN.ICOS. D-35
	Congridae	<i>Conger conger</i>	European conger	C	dentary, rib	MNHN.ICOS.SP-24
	Muraenesocidae	<i>Muraenesox cinereus</i>	daggertooth pike-conger	C	dentary	MNHN.ICOS.00286
	Muraenidae	<i>Gymnothorax moringa</i>	spotted moray	C	dentary, rib	UMMZ 173403/S
		<i>Muraena helena</i>	Mediterranean moray	C	dentary	MNHN.ICOS.01039
Osteoglossomorpha	Hiodontidae	† <i>Eohiodon falcatus</i>	-	C	dentary	NHMUK PV P 61245
		<i>Hiodon alosoides</i>	goldeye	C	dentary	UMMZ 189540/S
		<i>Hiodon tergisus</i>	mooneye	C	rib	UMMZ 180315/S
	Notopteridae	<i>Chitala chitala</i>	giant featherback	C	dentary, rib	UMMZ 193675/S

	Osteoglossidae	<i>Arapaima gigas</i>	arapaima	C	dentary, rib	MNHN.ICOS.00557
		† <i>Brychaetus muelleri</i>	-	C	dentary	NHMUK PV OR 28424
		<i>Osteoglossum bicirrhosum</i>	silver arowana	C	dentary, rib	MNHN.ICOS.00630
		† <i>Phareodus encaustus</i>	-	C	dentary	NHMUK PV P 646361
Clupeomorpha	<i>Incertae sedis</i>	† <i>Knightia</i> sp.	-	C	dentary	UMMP Tmp-1008
	†Armigatidae	† <i>Armigatus namourensis</i>	-	C	dentary, rib	NHMUK PV P 63151a
	†Ellimmichthyidae	† <i>Ellimmichthys longicostatus</i>	-	C	dentary, rib	NHMUK PV P 9855
	Chirocentridae	<i>Chirocentrus dorab</i>	wolf-herring	C	dentary, rib	UMMZ 220543/S
	Clupeidae	<i>Alosa pseudoharengus</i>	alewife	C	dentary, rib	UMMZ 187300/S
		<i>Sardinops sagax</i>	Pacific sardine	C	dentary	MNHN.ICOS.5036
Gonorhynchiformes	Chanidae	† <i>Tharrias araripes</i>	-	C	dentary, rib	NHMUK PV P 54675b
Cypriniformes	Catostomidae	<i>Catostomus commersonii</i>	white sucker	C	dentary, rib	UMMZ 178869/S
	Leuciscidae	<i>Abramis brama</i>	freshwater bream	C	dentary	MNHN.ICOS.00756
	Cyprinidae	<i>Cyprinus carpio</i>	common carp	C	various bones ¹	MNHN.ICOS.00610
	Tincidae	<i>Tinca tinca</i>	tench	C	dentary	MNHN.ICOS.00585
Characiformes	Cynodontidae	<i>Hydrolycus scomberoides</i>	payara	C	dentary	MNHN.ICOS.01021
	Erythrinidae	<i>Hoplias malabaricus</i>	trahira	C	dentary, rib	MNHN.ICOS.00631
	Serrasalminidae	<i>Serrasalmus spilopleura</i>	speckled piranha	C	dentary, rib	MNHN.ICOS.01027
Siluriformes	Ariidae	<i>Ariopsis felis</i>	hardhead sea catfish	C	dentary, rib	UMMZ 223241/S
		<i>Galeichthys feliceps</i>	white barbel	C	dentary	MNHN.ICOS.00875
	Heptapteridae	<i>Pimelodella gracilis</i>	graceful pimelodella	C	dentary, rib	UMMZ 204550/S
	Trichomycteridae	<i>Trichomycterus</i> sp.	pencil catfish	A	dentary	MNHN.ICOS.00887
Gymnotiformes	Gymnotidae	<i>Gymnotus carapo</i>	banded knifefish	C	dentary, rib	UMMZ 207893/S
Euteleostei	<i>Incertae sedis</i>	† <i>Spaniodon elongatus</i>	-	C	dentary, rib	NHMUK PV OR 44831
Salmoniformes	Salmonidae	<i>Coregonus reighardi</i>	shortnose cisco	C	dentary, rib	UMMZ 172476/S
		<i>Oncorhynchus clarkii</i>	cutthroat trout	C	dentary	UMMZ 191615/S
		† <i>Oncorhynchus lacustris</i>	-	C	dentary	UMMP 47839
		<i>Oncorhynchus mykiss</i>	rainbow trout	C	dentary	UMMZ uncat.
		<i>Oncorhynchus tshawytscha</i>	Chinook salmon	C	dentary	UMMZ uncat.

		† <i>Paleolox larsoni</i>	-	C	dentary	UMMP 50352
		<i>Parahucho perryi</i>	Japanese huchen	C	dentary	UMMZ 187612
		† <i>Prosopium prolixus</i>	-	C	dentary	UMMP 21728
		<i>Prosopium williamsoni</i>	mountain whitefish	C	dentary	UMMZ 182503/S
		<i>Salmo salar</i>	Atlantic salmon	C	dentary	MNHN.ICOS.00619
		<i>Salmo trutta</i>	sea trout	C	dentary	UMMZ uncat.
		<i>Salvelinus confluentus</i>	bull trout	C	dentary	UMMZ uncat.
		<i>Salvelinus fontinalis</i>	brook trout	C	dentary	UMMZ uncat.
		<i>Salvelinus namaycush</i>	lake trout	C	dentary	UMMZ 177542
		<i>Stenodus leucichthys</i>	inconnu	C	dentary, rib	UMMZ 187119/S
		<i>Thymallus thymallus</i>	grayling	A	rib	MNHN.ICOS.00626
Acanthomorpha	Veliferidae	<i>Metavelifer multiradiatus</i>	spinyfin velifer	A	rib	AMNH I-91798 SD
	Lamprididae	<i>Lampris</i> sp.	opah	C	sclerotic ossicle	AMNH I-21766 SD
	Polymixiidae	<i>Polymixia nobilis</i>	stout beardfish	A	rib	AMNH I-210677 SD
	Coryphaenidae	<i>Coryphaena hippurus</i>	common dolphinfish	A	rib	MNHN.ICOS.00189
	Carangidae	<i>Trachurus trachurus</i>	horse mackerel	A	rib	MNHN.ICOS.A-14
	Xiphiidae	<i>Xiphias gladius</i>	swordfish	A	rib, sclerotic ossicle	MNHN.ICOS.6988, AMNH I-15658 SD
	Istiophoridae	<i>Kajikia albida</i>	Atlantic white marlin	A	rib, sclerotic ossicle	UMMZ 198674/S
	Moronidae	<i>Dicentrarchus labrax</i>	European seabass	A	rib	private collection
	Scaridae	<i>Chlorurus microrhinos</i>	steephead parrotfish	A	rib	MNHN.ICOS.00912
	Scombridae	<i>Acanthocybium solandri</i>	wahoo	A	rib	MNHN.ICOS.01010
		<i>Euthynnus affinis</i>	little tunny	C	rib	AMNH I-56274 SD
		<i>Gasterochisma melampus</i>	butterfly kingfish	A	rib, sclerotic ossicle	AMNH I-93480 SD
		<i>Gymnosarda unicolor</i>	dogtooth 'tuna'	A	rib	MNHN.ICOS.00492
		<i>Sarda orientalis</i>	striped bonito	A	rib	MNHN.ICOS.00954
		<i>Scomber australasicus</i>	blue mackerel	A	rib	MNHN.ICOS.00254
		<i>Thunnus obesus</i>	bigeye tuna	C	rib	MNHN.ICOS.00374