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How the European eel (*Anguilla anguilla*) loses its skeletal framework across lifetime

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European eels (*Anguilla anguilla*) undertake an impressive 5 000 km long migration from European fresh waters through the North Atlantic Ocean to the Sargasso Sea. Along with sexual maturation, the eel skeleton undergoes a remarkable morphological transformation during migration, where a hitherto completely obscure bone loss phenomenon occurs. To unravel mechanisms of the maturation-related decay of the skeleton, we performed a multiscale assessment of eels' bones at different life-cycle stages. Accordingly, the skeleton reflects extensive bone loss that is mediated via multinucleated bone-resorbing osteoclasts, while other resorption mechanisms such as osteocytic osteolysis or matrix demineralization were not observed. Preserving mechanical stability and releasing minerals for energy metabolism are two mutually exclusive functions of the skeleton that are orchestrated in eels through the presence of two spatially segregated hard tissues: cellular bone and acellular notochord. The cellular bone serves as a source of mineral release following osteoclastic resorption, whereas the mineralized notochord sheath, which is inaccessible for resorption processes due to an unmineralized cover layer, ensures sufficient mechanical stability as a part of the notochord sheath. Clearly, an eel's skeleton is structurally optimized to meet the metabolic challenge of fasting and simultaneous sexual development during an exhausting journey to spawning areas, while the function of the vertebral column is maintained to achieve this goal.

1. Background

European eels (*Anguilla anguilla*) undertake a 5 000 km long spawning migration from European fresh waters through the ocean to the Sargasso Sea [1]. The complex life cycle of eels starts with larvae called leptocephali [2] that are carried back by currents to European coasts where they metamorphose into glass eels. Entering fresh water, the glass eels develop into sequential morphological stages known as 'elvers' and 'yellow eels'. Eels remain in fresh water for 5–20 years until they become 'silver eels'. These eels then enter marine waters again to migrate to spawning areas (figure 1*a*,*b*). During the long and energy-consuming journey, silver eels complete gonad maturation [2] and entirely abstain from food intake [3]. Furthermore, eels not only deplete their energy reserves, they also lose substantial amounts of bone volume during the spawning migration [4]. The specific

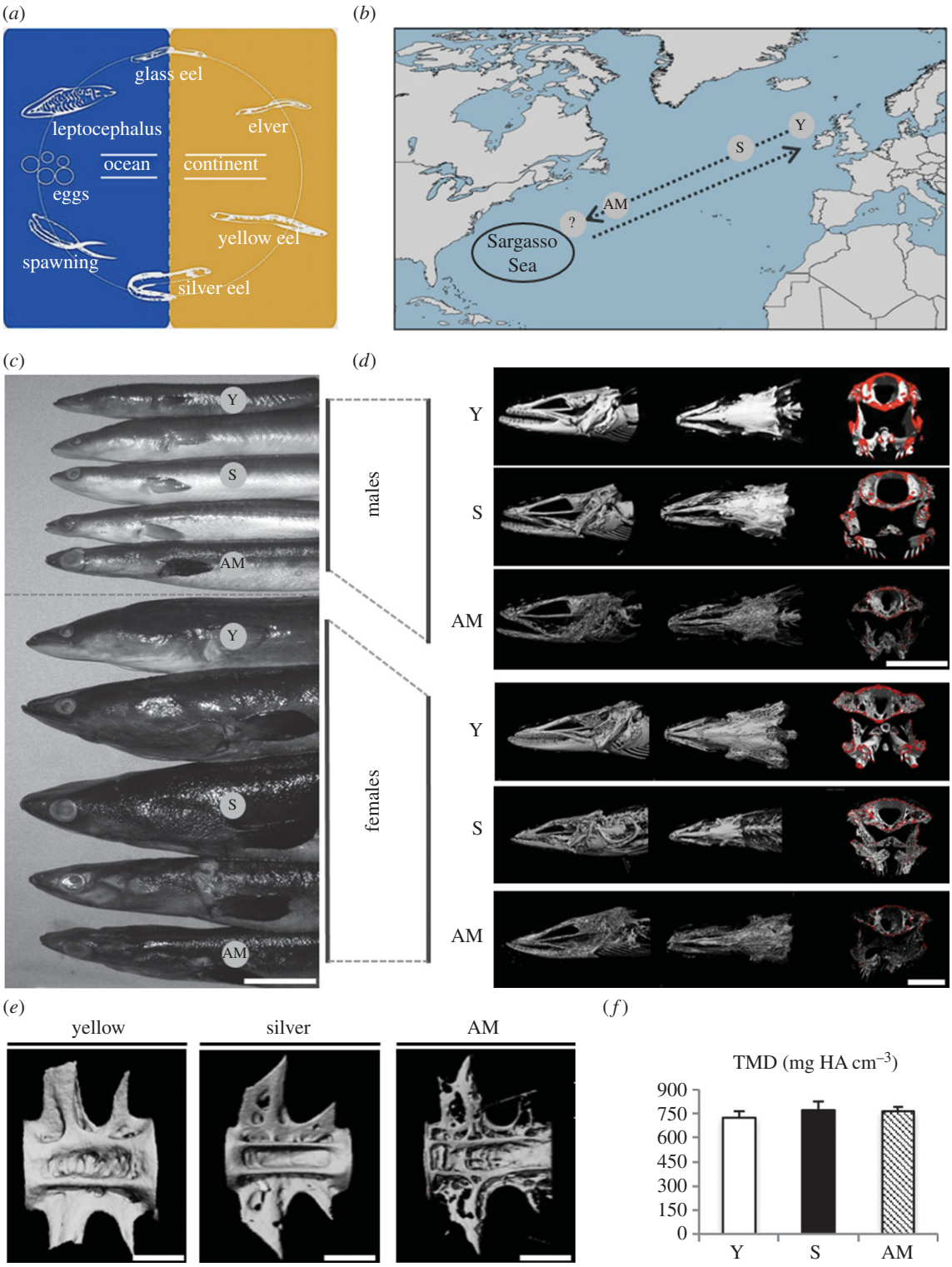

Figure 1. Life cycle of the European eel *Anguilla anguilla*. (*a*,*b*) Silver eels travel to the Sargasso Sea to spawn. Larvae (leptocephalus) are transported back to European fresh water where they develop into glass eels and eventually yellow eels. (*c*) Photographs of the eels show the morphological change during maturation. Note the increase in eye size. Scale bar, 2 cm. (*d*) Images obtained by microcomputed tomography (μ-CT) point out severe bone loss in skulls during maturation among yellow (Y), silver (S), and artificially matured eels (AM) in both sexes. Red colouring indicates the cutting plane for histologic analysis of the skull base. Scale bar, 1 cm. (*e*) Representative three-dimensional reconstructions of μ-CT scans equally show the dramatic bone loss in vertebral bodies among the three groups. Scale bar, 1 mm. (*f*) Overall mineralization (tissue mineral density, TMD) obtained by μ-CT did not change significantly with maturation. (Online version in colour.)

factors driving this bone loss as well as the exact mechanisms of bone loss are not yet well understood.

Since eel migration takes place in the deep sea, and tag sizes in satellite tracking exclude small-sized animals like eels [5], it is not possible to trace the animals or to obtain samples directly from migrating eels. Therefore, studies investigating the effect of maturation on the animals' skeleton require experimentally induced maturation through hormone injections [2,6]. Moreover, eels are an endangered species, which is why an artificial maturation procedure has been developed as an experimental attempt of artificial fertilization and reproduction [7]. The artificial maturation method as applied here is based on previously reported protocols used to induce maturing of males and females for controlled reproduction in aquaculture [7,8].

Although much is still unknown about precise hormonal regulation of bone metabolism in many groups of teleost fish

[9], it has been shown that eel bone responds to injections of 1,25-dihydroxycholecalciferol, calcitonin, and thyroid hormone [10–12]. In particular, cortisol can trigger bone resorption in eels, suggesting that cortisol is a conserved endocrine regulator of bone loss in vertebrates [13].

The vertebrate skeleton has a variety of functions. It gives protection and support for internal organs and provides stability and mobility together with the muscular system. The skeleton is also an important storage organ for minerals and it participates in regulation of plasma mineral homeostasis [4]. In tetrapods, the skeleton is mainly involved in the animal's calcium metabolism, whereas in teleost fish, the skeleton is primary involved in phosphorous metabolism [9,14,15]. Clearly, some skeletal functions mutually compete, such as using the mineralized bone to ensure mechanical stability versus using the bone as a reservoir of calcium and phosphorus. The comparative structural and compositional analyses of bone tissue in different species can help to gain insights into these two major roles of the skeleton, specifically into the different pathways in which the vertebrate skeleton responds to internal and external challenges.

Within the teleost group there is an evolutionary trend towards the loss of osteocytes (acellular bone) [9,16]. By contrast, in vertebrates with cellular bone, osteocytes are the most numerous bone cells and are central regulators of bone remodelling in response to bone mineral metabolism and mechanical load [17]. Some small teleost fish such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), which are established models for biomedical bone research [18], are deprived of osteocytes in the early stages of life, while medaka do not feature osteocytes at all [9,19,20]. Therefore, studying teleost fish models can reveal conserved and alternative pathways for bone adaptation and metabolism [15,21–24]. Furthermore, analyses of bone metabolism in various species are essential for clarifying the evolutionary physiology of bone [15,25].

Eels belong to a basal teleost group whose skeleton contains osteocytes [26], as is the case in all tetrapods including humans. It is, therefore, essential to understand how the bone tissue of eels is organized and for which of the functions (mechanical stabilizer and mineral storage) it is optimized. In this study, the European eel was used as a model to study the mechanism of bone resorption and mineral homeostasis, as these processes seem to be part of their natural biology. Osteocytic pathways that are known to orchestrate bone remodelling in mammals are examined for their involvement in eel bone resorption, which is triggered by artificial maturation with hormones in male and female eels prior to the start of their spawning migration. This is the first study that investigates the skeleton of adult, sexually mature eels in both sexes. The skeleton of matured eels is compared with the skeleton of earlier life stages: juvenile yellow eels and sexually immature silver eels. In-depth understanding of the structural organization of eel bones as well as the mechanisms driving bone resorption in maturation contribute to the understanding of the opposing forces that maintain the mechanical stability of the skeleton and that exploit the skeleton as a mineral resource.

2. Material and methods

A detailed description of the applied procedures and methods is given in the electronic supplementary material.

(a) European eels

A total of 30 male and female eels were investigated (nine yellow, 10 silver, and 11 artificially matured-AM eels). Yellow and silver eels were caught in coastal waters, whereas the last developmental stage before spawning was accomplished by hormone injections. Here, artificial maturation was performed in males by weekly injections of human chorionic gonadotropin (hCG, Sigma Aldrich, 1 IU g^{-1} body weight) for 12–14 weeks following a standardized protocol [8]. In females, maturation was performed with carp pituitary extract (CPE, Catvis, 20 mg $week^{-1}$) for 8–13 weeks and then, within 18 h after the last CPE injection, with an injection of 17,20β-dihydroxy-4-pregnen-3-one (DHP, Sigma Aldrich, 2 μg g^{-1} body weight) into the ovary to induce ovulation [8,27–29]. According to their natural behaviour in open sea, hormone-treated eels fasted from their first injection. The maturation process was performed in a water recirculation system saturated with sea salt (18°C, [30–35] Practical Salinity Unit). Eye index—a parameter for sexual maturity was calculated [30]. Blood levels of calcium, phosphorus, and cortisol were measured.

(b) Histology

von Kossa-stained bone sections were used to quantify bone volume per tissue volume (BV/TV) [31]. Toluidine blue and Mason-Goldner trichrome stained sections were used to study the morphology of bone and resorption parameters (osteoclast surface per bone surface, Oc.S/BS; osteoclast number per bone perimeter, N.Oc/B.Pm; eroded surface per bone surface, ES/BS). Collagen fibril orientation in the bone matrix was visualized via polarized light microscopy.

(c) Mineralization analysis

Quantitative backscattered-electron imaging (qBEI) was used to assess the degree of mineralization [32] based on the relationship between calcium weight percentage and qBEI signal intensities [32–34]. qBEI images were also used to quantify the osteocyte lacunar number (Tt.Lc.N/B.Ar, 1 mm^{-2}) and area (Lc.Ar, μm^2).

(d) Fourier transform infrared spectroscopy

FTIR further evaluated the bone matrix characteristics: mineral : matrix ratio (amount of mineral per organic matrix), carbonate : phosphate ratio (degree of carbonate substitution for hydroxyl and phosphate groups within hydroxyapatite), and proteoglycan content [35].

(e) High-resolution computed tomographic imaging

HR-pQCT and μ-CT were applied to assess bone microarchitecture, tissue mineral density (TMD, mgHA cm^{-3}), and orbital cavity distance.

(f) Acid etching and analysis of canalicular connections

The osteocyte lacunocanalicular network was visualized via scanning electron microscopy on acid-etched methylmethacrylate-embedded bone samples, according to the established protocol [36].

(g) Second harmonic generation microscopy

SHG microscopy [18,37,38] was used to further visualize the collagen orientation and its pervasion by osteocyte canalicular connections.

(h) Statistical analysis

The Kolmogorov–Smirnov test was used for verifying normal distribution of the measured parameters. Inter-group comparisons were performed using ANOVA with Bonferroni's post hoc

comparisons. The analyses were performed using SPSS v. 22 (IBM Corporation) at a 0.05 significance level.

3. Results

In this study, European eels were investigated as an experimental model of severe bone loss in vertebrae and skull bones related to the sexual maturation of female and male eels prior to spawning (figures 1*a–e* and 2*a*,*b*).

Laboratory tests showed that plasma calcium levels were maintained in silver eels and hormone-treated eels, but plasma phosphate decreased significantly in hormone-treated male eels compared with yellow and silver eels, whereas it increased in female eels. Also, cortisol levels dropped significantly in the hormone-treated male group to a level of 12.2 ± 5.1 mg dl^{-1} (electronic supplementary material, table S1). Total body length and weight did not differ significantly between the male groups but increased significantly in female silver eels (electronic supplementary material, table S1).

There are various morphological differences between mature males and mature females, however, an enlargement of eye size (eye index) could be identified in both sexes (figure 1*c*; electronic supplementary material, table S1). Bone loss was detected in mature males and females to a similar degree (figures 1*d*,*e* and 2*a*,*b*). Specifically, the thickness of the vertebral end plates was significantly reduced in artificially matured eels (73 ± 38 µm) compared with 164 ± 63 µm in yellow eels ($p < 0.001$) and 151 ± 60 µm in silver eels ($p < 0.001$). Furthermore, the thinning of the skull's cortical bone was accompanied by a significant increase of the Euclidean distance between the orbital cavities (electronic supplementary material, table S1).

Vertebral bone volume/tissue volume (BV/TV) was significantly decreased in artificially matured eels ($7.2 \pm 2.1\%$) compared with yellow eels ($17.9 \pm 3.9\%$, $p < 0.001$) and silver eels ($15 \pm 3.5\%$, $p < 0.001$) in both sexes, however, the transition from yellow to silver eels was not accompanied by a significant decline in BV/TV ($p > 0.05$). In the skull base, BV/TV decreased significantly with maturation ($p < 0.001$), declining already at the stage of silver eels versus yellow eels ($14.3 \pm 5.1\%$ versus $19.8 \pm 4.9\%$, $p = 0.023$) and further to $5.1 \pm 2.1\%$ in artificially matured eels ($p < 0.001$, figure 2*b*). The decline of bone volume and vertebral thickness measured separately for both sexes is shown in the electronic supplementary material, table S2.

Microstructural analysis of skull bones confirmed the presence of osteocytes. By contrast, the vertebral bone of all three groups showed the typical composition of a vertebral body of a basal teleost fish with two types of mineralized tissue: an inner layer of the acellular (anosteocytic) mineralized notochord sheath and an outer layer of cellular (osteocytic) woven and lamellar bone (figure 2*c*; electronic supplementary material, figure S2). The cellular bone showed clear signs of osteoclastic resorption in terms of eroded surfaces, whereas the mineralized notochord sheath was not affected by osteoclastic resorption, maintaining its volume fraction among all three investigated groups of eels (figures 2*d* and 4*e*,*f*).

The resorption process was not accompanied by a decreasing calcium content in the remaining mineralized bone (i.e. demineralization) (figures 1*e*,*f* and 3*a–c*). In fact, qBEI analysis revealed no changes in overall mineralization of the remaining matrix in vertebral bodies and skull bones of artificially matured eels. However, in hormone-treated eels, qBEI showed a peak shift towards higher matrix mineralization (figure 3*d*) similar to the mineral content of the acellular notochord sheath when measured separately (figure 2*e*).

Measurements of the osteocyte number per bone area showed a declining number of osteocyte lacunae in cellular bone (N.Ot.Lc/B.Ar = 287.8 ± 109.5 versus 201.3 ± 50.3 versus 133.7 ± 46.3 mm^{-2}) but no significant enlargement of osteocyte lacunae during artificial maturation (figure 3*e–g*). qBEI and acid-etching techniques, as well as SHG microscopy, revealed no clear signs of existing osteocyte canaliculi in eel bone, apart from scarce extensions at some lacunar walls. The line-like connections that are seen in the mineralized notochord sheath are direct extensions of the fibrous notochord sheath that connects the vertebral bodies (figure 4*h*, black arrows). Furthermore, there are abundant collagen fibre bundles anchored into osteocytic bone that forms around the notochord sheath (figure 4*h*, white arrows; electronic supplementary material, figure S1, white arrows). These are most likely Sharpey's fibres.

FTIR spectroscopy indicated a higher mineral-to-matrix ratio in the mineralized part of the notochord sheath (8.18 ± 1.78) than in cellular bone (6.58 ± 1.37), which supports the qBEI data (figure 4*b*). Between these two layers there was a thin layer that showed a very low mineral : matrix ratio (5.54 ± 0.47) indicating a low mineral content, which was also seen in toluidine blue staining, where this layer was stained light blue (figure 4*a*). Furthermore, the carbonate : phosphate ratio was lower in the osteocytic bone (0.0061 ± 0.0011 versus 0.00727 ± 0.00089) exposed to resorption (figure 4*c*). The proteoglycan content of the non-mineralized inner layer of the notochord sheath was very high, whereas the mineralized part of the notochord sheath showed lower proteoglycan content than the adjacent osteocytic bone (0.142 ± 0.019 versus 0.154 ± 0.015; figure 4*d*).

The induction of sexual maturation caused an increase in resorption-related structural changes (figure 4*e*,*f*). Identified osteoclasts were multinucleated and were located in typical resorption lacunae at the surface of the osteocyte-containing bone (figure 4*g*). Namely, in mature eels, an increased eroded surface per bone surface (ES/BS) in comparison with yellow and silver eels was evident ($p < 0.001$, $p < 0.001$). The number of multinucleated osteoclasts per bone perimeter (N.Oc/B.Pm) was notably increased in comparison with yellow eels ($p < 0.001$) and silver eels ($p = 0.012$). The osteoclast surface per bone surface (Oc.S/BS) in mature eels was substantially higher than in yellow eels ($p = 0.011$) and tended to be higher than in silver eels (figure 4*i–k*).

4. Discussion

In general, three mechanisms of bone resorption have been observed in teleost fish in response to metabolic demands or for adaptation to mechanical load: osteoclastic resorption, osteocyte-mediated resorption (osteocytic osteolysis), and halastatic demineralization [9]. The latter describes the removal of mineral substance with no degradation of the organic matrix of the bone [39]. Previous studies suggest that these processes may also occur in eels [26], but it is still unknown what mechanism triggers bone resorption in eels in connection with sexual maturation.

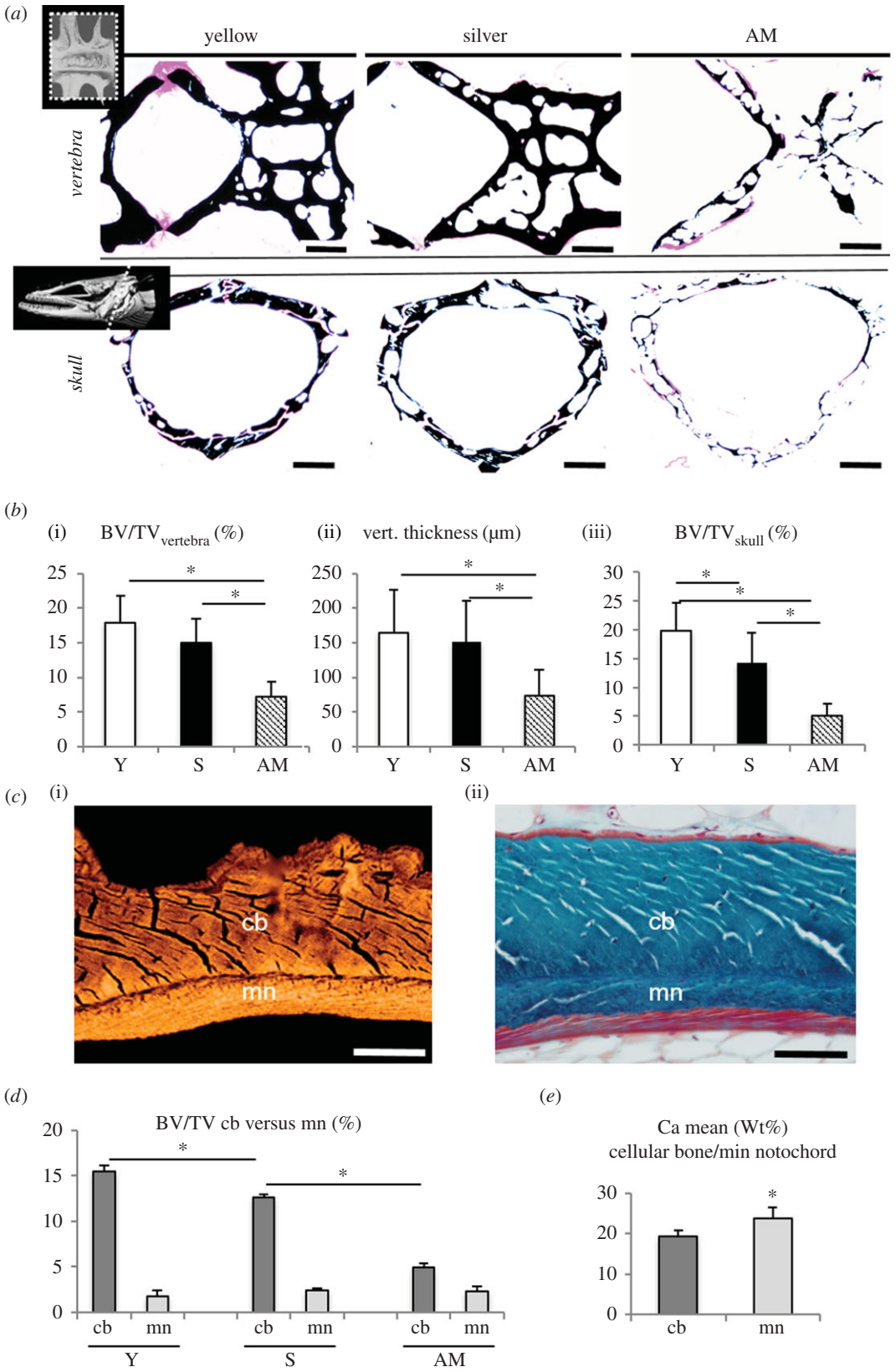

Figure 2. Massive bone loss during maturation. (*a*) Images of von Kossa-stained sections show the bone loss during maturation in the vertebra (above) and skull base (below). Scale bar, 200 μm. (*b*) Bar graphs indicate decreasing bone volume per tissue volume (BV/TV) in the (i) vertebra and (iii) skull, as well as decreasing (ii) thickness of the vertebral endplates. (*c*) Higher magnification reveals that vertebral bone is divided into two compartments. There are two types of mineralized tissue: cellular (osteocytic) bone (cb) and a mineralized notochord sheath (mn), (i) image obtained by qBEI and (ii) trichrome Goldner staining. Scale bar, 50 μm. (*d*) Quantification of osteocytic bone and acellular notochord bone separately showed that only osteocytic bone was resorbed, whereas the mineralized notochord sheath persists in artificially matured eels. * denotes the significant decrease of cellular bone among the groups. (*e*) The mineralized part of the notochord shows higher mineralization (Ca mean) than the cellular bone when measured separately. *$p < 0.05$.

In mammals, bone is an important reservoir of calcium ions that can be deposited or released depending on the current needs of tightly regulated plasma calcium levels [40]. Teleost fish maintain similar tightly regulated plasma calcium levels but they can take in calcium from the water and release excess calcium via the gills [9,16]. Thus, teleosts need not involve

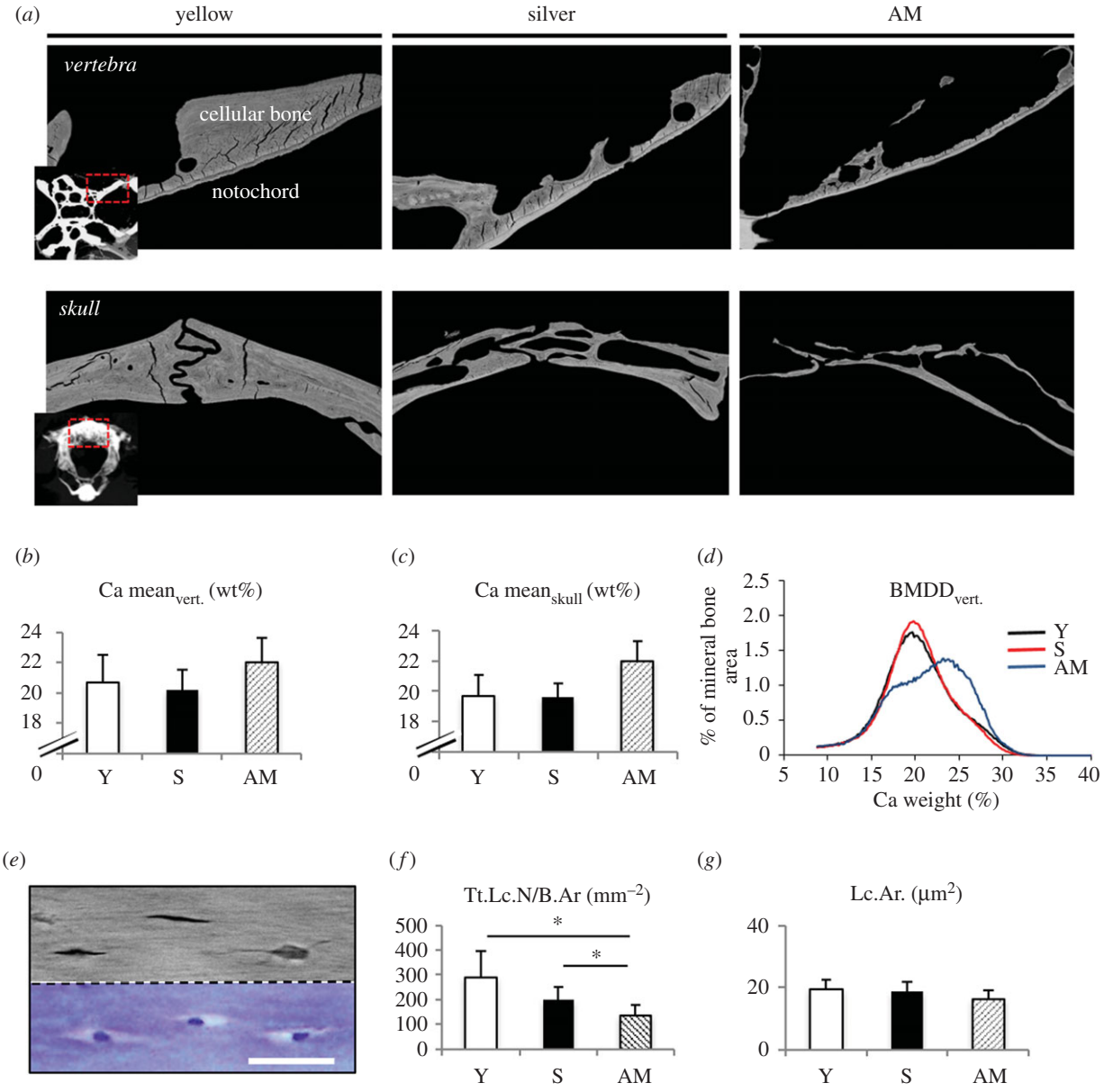

Figure 3. Bone mineralization and osteocyte parameters during maturation. (*a*) qBEI images confirm the massive bone loss in vertebra and skull. Scale bar, 100 µm. (*b*,*c*) Ca mean values did not differ significantly in any of the three groups although there was a trend towards higher values in AM. (*d*) Ca weight analysis shows that this is due to a higher proportion of high-mineralized tissue in AM. The second peak represents the remaining high-mineralized notochord and conforms to the results when analysing mineralized notochord alone. (BMDD = bone mineral density distribution.) (*e*) Observing corresponding areas within the vertebra, osteocyte lacunae identified by qBEI clearly show a cell nucleus in corresponding histology (toluidine blue staining). Scale bar, 20 µm. (*f*) The number of osteocyte lacunae was measured per cellular bone area (Tt.Lc.N./B.Ar.). There is a decreased number of osteocyte lacunae between artificially matured and yellow eels and matured and silver eels (*$p < 0.05$). (*g*) Lacunar area did not change significantly among the three groups. (Online version in colour.)

bone in their calcium metabolism. By contrast, phosphorus cannot be obtained from the water through the gills and, therefore, teleost fish rely on dietary phosphorous intake [9,41]. As phosphorus is an essential factor for eel metabolism, sexual maturation, and primary yolk synthesis [4], eels that stop feeding prior to spawning have to find an alternative source of phosphorus. Hence, it was suggested that they might mobilize phosphorus from the endoskeleton through bone resorption [4].

Moreover, it has not been clarified whether calcium or phosphate deficiency may be an initiating stimulus for bone resorption. Our findings revealed that the plasma calcium concentration remains unchanged during artificial sexual maturation. Furthermore, in contrast with previous suggestions [13] we showed that the mechanism of increased bone resorption in sexually mature eels was obviously cortisol independent, given that measurements of blood cortisol levels showed considerably low values in mature eels. In addition, excessive bone resorption liberates a large amount of organic material (mainly collagen), which under certain circumstances may mainly contribute as an additional source for energy production, given that protein oxidation was suggested to be an equally important process during starvation in eels [42,43]. Thus, bone loss in fasting eels may have a partly similar function to the bone resorption occurring during hibernation in some animals, another condition involving prolonged fasting [44,45]. Nevertheless, spawning migration in eels relies heavily on extensive fat reserves that are mostly found in muscles, skin, and liver [46,47].

We observed abundant multinucleated osteoclasts in both female and male eels. Similar observations have been made in mature and starving male Atlantic salmon where the extension of the lower jaw (kype) is resorbed after spawning by giant

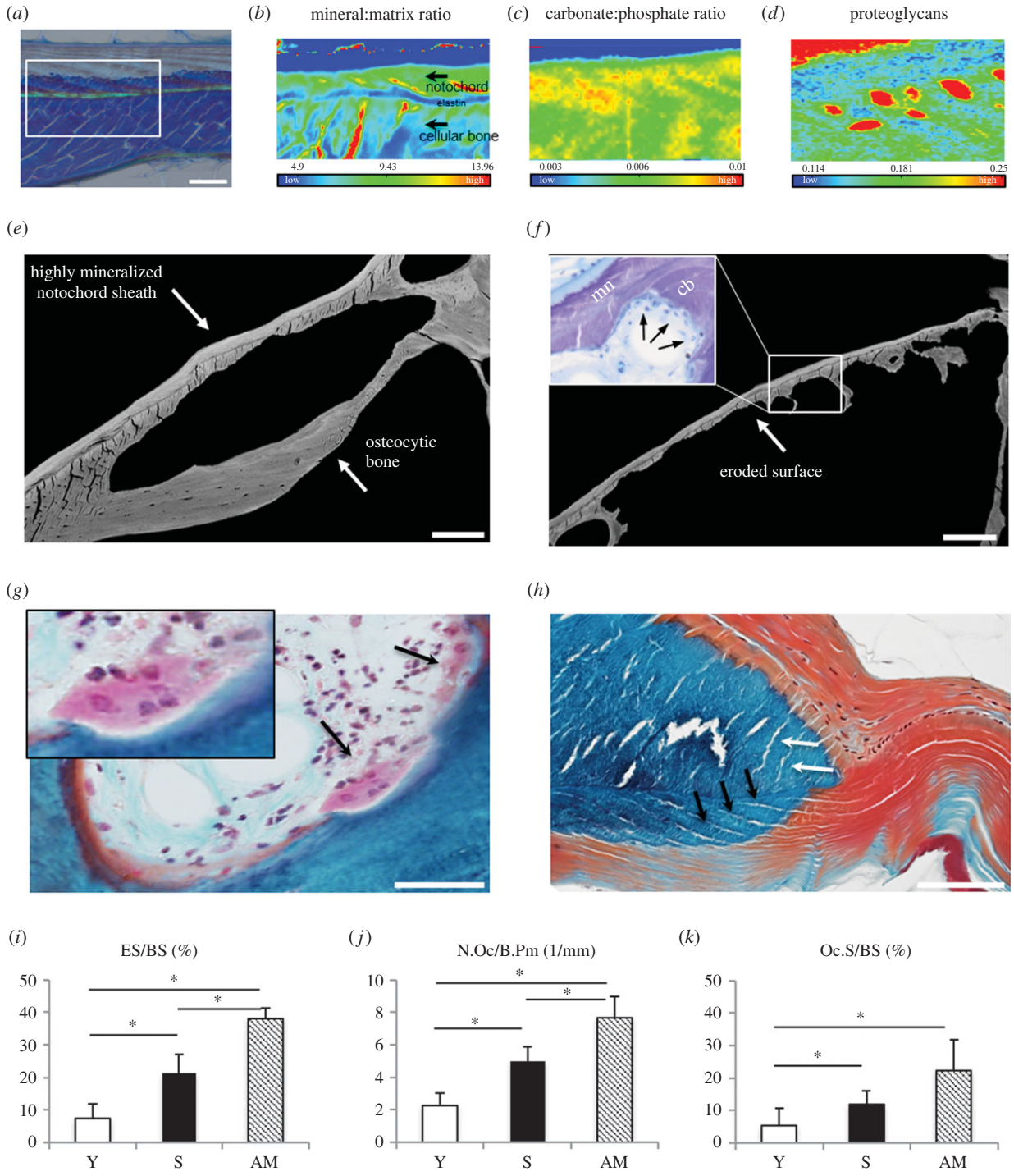

Figure 4. (*a–d*) Fourier transform infrared (FTIR) spectroscopy in the eel's vertebral endplates. (*a*) Toluidine blue staining signifies the scanning area for FTIR. Scale bar, 50 µm. (*b*) The mineral : matrix ratio was higher in the mineralized notochord sheath. Note the prominent non-mineralized elastin layer in between the cellular bone and the notochord. (*c*) The increased carbonate : phosphate ratio is either due to higher carbonate or lower phosphate contents in the mineralized notochord compared with the cellular bone. (*d*) The proteoglycan content was substantially lower in the mineralized notochord sheath, which reveals that its structural composition is different from the cellular bone matrix (red patches are due to cutting artefacts). (*e–k*) Mechanism of osteoclastic resorption. (*e*) The qBEI of a silver eel in higher magnification shows mineralized notochord and cellular bone where only cellular bone can be resorbed. Scale bar, 100 µm. (*f*) Hormone-treated eels present large eroded surfaces. The cellular bone was almost completely resorbed. Scale bar, 100 µm. (*g*) Histology of a trichrome Goldner stained specimen shows multinucleated osteoclasts in Howship's lacuna. Scale bar, 50 µm. (*h*) Fibres of the enlarged notochord sheath in the intervertebral space continue in the mineralized part of the sheath (black arrows). In the outer bone layer there are equivalent structures that have been identified as Sharpey's fibres (white arrows). Scale bar, 100 µm. (*i–k*) Resorption parameters such as eroded surface per bone surface (ES/BS), number of osteoclasts per bone perimeter (N.Oc./B.Pm), and osteoclast surface per bone surface (Oc.S/BS) increased significantly among the groups. $*p < 0.05$.

multinucleated osteoclasts [48]. As has been done for Atlantic salmon it can be speculated that multinuclear osteoclasts are needed for fast extensive resorption [49], a mode of resorption that is characterized by the presence of deep Howship lacunae and extended areas of eroded bone surface. The fact that osteoclasts in sexually mature eels are multinucleated and are located at the surface of the osteocyte-containing bone lends support to the general notion that there is a possible link between the presence of osteocytes and multinucleated osteoclasts as a primary osteoclast type [9]. However, revealing the

potential role of osteocytes in triggering fast and extensive bone resorption requires further investigation.

In this study, enlarged osteocyte lacunae associated with osteocytic osteolysis were not observed. While resorption-related enlargement of osteocyte lacunae by approximately 20–40% was previously found in Japanese eels [12,13], our experiments provide no evidence that European eels' osteocytes are potent contributors of mineral release at this stage of life. Osteocyte lacunar quantification revealed that, with a density of approximately 200 mm^{-2} and an area approximately 20 μm^2, they are less densely packed and much smaller in eels than in mammals, such as humans [50] or mice [51], and also than in other teleost fish (approx. 400 mm^{-2}, 40 μm^2) [16]. Osteocyte canaliculi were found to be scarce or absent (figure 3*e*; electronic supplementary material, figure S1), as described for several teleost species. Given that other members of the order Anguilliformes such as the spotted moray (*Gymnothorax moringa*) have acellular bone [52], the reduction of canaliculi in *A. anguilla* could perhaps represent a bone type that is intermediate between cellular and acellular bone.

Bone resorption was indeed evident at the osteocyte-containing bony areas; yet, there are several examples of anosteocytic tissues, including teleost bone, being remodelled and resorbed by (mostly mononucleated) osteoclasts [53–57]. Our experiments question if/how the small and not densely packed osteocytes are involved in signalling bone resorption in eels, especially considering that they show almost no canalicular connections. Furthermore, osteocyte lacunar numbers further decreased during maturation, which is an effect similar to ageing human bone or osteoporosis [50]. However, the reduction of osteocytes also has anatomical reasons unrelated to ageing. Namely, the first layer of bone around the notochord is woven bone with low numbers of osteocytes and abundant fibres. The built second layer is lamellar bone with higher numbers of osteocytes (electronic supplementary material, figure S2). When the first layer of lamellar bone is resorbed by osteoclasts, woven bone with lower osteocyte numbers remains. Further research is needed to clarify the osteocytes' role in signalling bone resorption in eels.

A shift in the histogram to higher calcium values in the bone matrix of hormone-treated eels was evident because of the resorbed lower mineralized cellular bone portions, while the highly mineralized notochord sheath remained (figures 2*d*,*e* and 4*d*). FTIR spectroscopy confirmed the findings of higher mineralization and lower proteoglycan content in the mineralized notochord in comparison with the cellular bone matrix. A lower carbonate : phosphate ratio, as present in osteocytic bone, highlights younger tissue age [35,58,59] and reflects a low carbonate and/or a high phosphate content. Low proteoglycan content in the acellular mineralized notochord sheath reflects that its basic structure is related to a different embryologic tissue origin than the adjacent osteocytic bone.

Bone development in teleost fish and in mammals requires bone resorption and remodelling at an early stage [20]. By contrast, teleost vertebral bodies develop within and grow around the notochord sheath, in a process that initially does not involve bone resorption or bone remodelling [9]. The notochord sheath of teleost fish is resorbed and penetrated by osteoclasts under special conditions, such as after upregulation of RANKL (receptor activator of nuclear factor kappa-B ligand) in transgenic medaka models and is connected to skeletal malformations in non-genetically manipulated animals [22,23,60–62]. However, the mineralized notochord sheath was not resorbed in mature eels, most likely due to specific anatomical organization that features a non-mineralized layer between the mineralized notochord and the osteocytic bone (figure 4*a*,*b*; electronic supplementary material, figure S1*a*). This non-mineralized area has been found to be composed of elastin [62] and, in contrast with some other species where it becomes fragmented allowing invasion of the notochord [63], the elastin layer was apparently intact in mature eels and served as an unmineralized barrier to osteoclastic attachment. Namely, osteoclasts need a mineralized substrate and cannot attach to non-mineralized surfaces [59,64,65]. Similarly, in salmon with severe phosphorus deficiency and an enormous amount of non-mineralized bone, bone and the notochord were not resorbed as they were protected from resorption by the non-mineralized bone layer [41].

In summary, although eels present two types of mineralized tissue (cellular bone and the mineralized notochord sheath), only the osteocytic bone was subject to osteoclastic resorption to release mineral (and organic components) for metabolic functions needed during sexual maturation and spawning migration. By contrast, the elastin layer protects the notochord from resorption. This reveals structural optimization of the eel skeleton to fulfil two potentially conflicting functions of mineralized tissue (preserving mechanical strength and releasing minerals when necessary). Specifically, these functions are spatially segregated to two different hard tissue compartments: cellular bone that may function as a reservoir of minerals, especially phosphorus, and the acellular mineralized notochord sheath that is unaffected by osteoclastic actions and, therefore, ensures enough stability even under phosphorus-deficient conditions. The notochord alone, without surrounding bone from vertebral bodies, is a main contributor to a stable axial skeleton even in very large bony fish such as sturgeons, coelacanths, and lungfish [41]. The notochord as a hydroskeleton is under high pressure [66], such that fenestration of the notochord sheath by osteoclasts would be dangerous.

5. Conclusion

During the spawning migration to the Sargasso Sea, European eels abstain from food and lose substantial amounts of bone. Here, we show not only the degree of skeletal decay, but also the mechanisms responsible for bone degradation in eels. While eels clearly show two types of mineralized tissues, exclusively cellular bone is subject to osteoclastic resorption and required mineral release. By contrast, the mineralized notochord sheath remains structurally intact ensuring sufficient mechanical stability of the skeleton during the exhausting migration. Studying marine vertebrates highlights conserved and alternative pathways for bone adaptation to mechanical and metabolic challenges in an evolutionary context. In-depth characterization of bone homeostatic mechanisms in teleosts may open avenues for the prevention and treatment of human bone loss syndromes.

Ethics. Animal care and experimental procedures were performed with approval from the environmental authority of Schleswig-Holstein (91-6/12).

Data accessibility. Detailed data are available in the electronic supplementary material.

Authors' contributions. T.R., F.N., M.A., and B.B. designed the study. T.R., F.N., S.W., R.P.M., A.J., F.N.S., M.H., and B.B. performed the

experiments. T.R., F.N., P.M., R.P.M., P.E.W., M.A., and B.B. analysed the data. T.R., P.M., and B.B. drafted the manuscript. T.R., P.M., P.E.W., and B.B. revised the manuscript content. All authors approved the final version of manuscript. T.R. and B.B. take responsibility for the integrity of the data analysis.

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