Electronic Supplemental Material

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

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This document includes:

- complete Material and Methods (detailed description of the sample collection and experimental procedures, as well as specimen preparation and analytical methods used),
- supplemental Figures, and
- supplemental Tables.

Material and Methods

European eels

To address the question of bone loss, a total of 30 male and female eels were investigated. Nine of them were young yellow eels (4 male, 5 female), 10 were silver eels (5 male, 5 female) and 11 (6 male, 5 female) eels were artificially matured adults (AM). The eels were anaesthetized prior to the hormone injections (benzocain, 80 ppm). Yellow and silver eels were caught in coastal waters, whereas the last developmental stage before spawning was accomplished by hormone injections. Artificial maturation was performed in males by weekly injections of human chorionic gonadotropin (hCG, Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands, 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 BV, s-Hertogenbosch, The Netherlands, 20 mg per week) for 8-13 weeks and then, within 18 hours after the last CPE injection, with 17,20β-dihydroxy-4-pregnen-3-one (DHP, Sigma Aldrich Chemie BV, 2 µg g^{-1} body weight) injection into the ovary to induce ovulation [8,27-29]. According to their natural behaviour in the open sea, hormone-treated eels were starved 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 (PSU)). Eye index as a parameter for sexual maturity was calculated according to Pankhurst including the horizontal and vertical eye diameter and total body length [30]. The fish were euthanized by an overdose of the anaesthetic MS-222. Animal care and experimental procedures were performed with approval from the environmental authority of Schleswig-Holstein (91-6/12).

Blood analysis

Blood samples of 1-2 ml from all experimental groups were collected from the caudal vein using heparinised syringes (Henke Sass Wolf, Tuttlingen, Germany) with a disposable hypodermic needle (0.6 9 30 mm; B. Braun, Emmenbrücke, Germany), followed by high-speed centrifugation and plasma storage at -80°C for further analysis. Plasma was thawed on ice before the amount of phosphate and calcium was determined by the GPO-PAP method using enzymatic colorimetric kits (Greiner, Frickenhausen, Germany). Cortisol was determined by enzyme immunoassay (REF RE 52611, IBL International GmbH, Hamburg, Germany). All colorimetric assays were performed in duplicate with a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland) and calculated from a standard dilution series to compensate for run-to-run variation. Recoveries were determined by spike experiments and >90% recovery rate for all plasma parameters were analysed.

Preparation of bone tissue

In order to create histological sections, bone specimens were fixed in 3.7% formaldehyde for 3 days, dehydrated, embedded in methyl-methacrylate, and cut on a Microtec rotation microtome (Techno-Med GmbH, Munich, Germany). Afterwards the 5 µm sections were stained by toluidine blue, trichrome Mason-Goldner and von Kossa according to previous studies.

Histology

Von Kossa-stained sections were used to quantify bone volume per tissue volume (BV/TV) with the OsteoMeasure histomorphometry system (OsteoMetrics, Atlanta, GA, USA) following the American Society for Bone and Mineral Research (ASBMR)

guidelines of Dempster et al. [31]. In the vertebrae, two opposing vertebral bodies were analysed. The anterior and posterior endplates of vertebral bodies were analysed for vertebral body endplate thickness (Vert. Thickness). Skull bone mass (BV/TV) was quantified in standardized areas around the skull base. Ventral and dorsal sutures could be identified as landmarks for the appropriate regions of interests. Toluidine blue and Mason-Goldner trichrome stained sections were used to study the morphology of the bone as well as the type of bone cells such as osteoblasts, osteoclasts, and osteocytes. Here, osteoclast surface per bone surface (Oc.S./BS, %), and osteoclast number per bone perimeter (N.Oc/B.Pm., 1/mm), as well as eroded surface per bone surface (ES/BS, %) were quantified with the OsteoMeasure system in line with ASBMR guidelines as well. Polarized light microscopy was performed to visualize collagen fibrils and their orientation in the bone matrix.

Bone mineral density distribution

Ground sections of embedded vertebrae and skulls were polished and sputter-coated with carbon for bone mineral density distribution analysis. Quantitative backscattered electron imaging (qBEI) was used to assess the degree of mineralisation of the specimens (BSE Detector, Type 202; K.E. Developments Ltd., Cambridge, England) [32]. The scanning electron microscope (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, England) was operated at 20 kV and 680 pA at a constant working distance. The electron beam was kept constant at 680 pA using a Faraday cup (MAC Consultants Ltd., UK). The signal amplification (brightness and contrast) was calibrated during the entire procedure by keeping measurements of carbon and aluminum standards (MAC Consultants Ltd., UK). The

gray level histograms of bone were standardized using a threshold routine (Image J 1.42, National Institutes of Health, USA). The obtained gray values were transformed into calcium weight percentages as described previously [32-34]. Images were subsequently thresholded with ImageJ software in order to quantify the osteocyte lacunar number (Tt.Lc.N/B.Ar, 1/mm^2) and osteocyte lacunar area (Lc.Ar, μm^2). 200-400 lacunae per specimen were measured.

FTIR

Fourier transform infrared (FTIR) spectroscopy was performed to further evaluate the characteristics of the bone matrix, such as the amount of mineral present per matrix, carbonate (carbonate substitution for hydroxyl and phosphate groups within hydroxyapatite) and amide (protein – primary type I collagen) [40]. The FTIR spectra were acquired with a Universal ATR sampling accessory connected to a Frontier FTIR spectrometer (Perkin Elmer, Waltham, MA, USA) over a spectral range of 570-4000 cm^{-1} at a spectral resolution of 4 cm^{-1} and 8 scans per pixel (pixel-size = 1.56 µm). The software 'SpectrumIMAGE R 1.7.1.0401' was used to analyse the spectral maps with an implemented atmospheric correction. The mineral:matrix ratio (MMR) was calculated by taking the area ratio of the phosphate peak at 1178-900 cm^{-1} to the amide I peak at 1700-1585 cm^{-1}. The carbonate:phosphate ratio (CPR) was calculated by taking the area ratio of the carbonate peak at 890-850 cm^{-1} to the phosphate peak [35]. Furthermore, the proteoglycan content (PGC) was measured in decalcified specimens by division of the integrated areas of the carbohydrate peak by the amide I peak at 1140-900 cm^{-1}.

High-resolution peripheral quantitative computed tomography (HR-pQCT) and micro-computed tomography (μ-CT)

To evaluate the bone microarchitecture, bone specimens were assessed by HR-pQCT and μ-CT. Skulls and vertebrae were dissected and fixed in 3.7% formaldehyde for 3 days. HR-pQCT (Xtreme-CT®, Scanco Medical, Bruettisellen, Switzerland) was performed on skulls at 60 kV and 900 μA with a resolution of 82 μm. μCT scanning (μCT42; Scanco Medical, Bruettisellen, Switzerland) was performed at 40 kV (114 mA) in skulls and vertebral bodies. The generated raw data were manually segmented for analyses with the μCT Evaluation Program V6.0 (Scanco Medical) and displayed in μCT Ray V3.8 for visualization (Scanco Medical). The 3D bone structure was characterized by means of tissue mineral density (TMD, mg HA/cm^3) and orbital cavity distance in the cranial skeleton. Orbital cavity distance was measured in the images using ImageJ.

Acid etching and analysis of canalicular connections

Visualization of osteocyte lacunae and their canalicular network was performed by acid etching on non-demineralised vertebral bodies and skull bones [36]. Acid etching can be used to visualize osteocytes lacunae and their canalicular network. This technique allows quasi-three-dimensional observation of the nanoscale canaliculi across a two-dimensional plane [36]. The specimens were embedded in methyl-methacrylate blocks and polished to ensure a flat coplanar surface using an automatic grinding system (Exakt, Germany) as described previously [36,50]. The acid etching procedure was performed following the protocol by Milovanovic et et al. [36]. Polished samples were submerged in 9% phosphoric acid for 20 seconds followed by a short rinse in deionized water (1-2 s). They were then put into 5%

sodium hypochlorite for 5 minutes and rinsed in deionized water again. The specimens were allowed to dry at room temperature without the use of a heating cabinet. Thus, high temperature was avoided to ensure a mild drying process that limited the development of vapor- and surface tension [36].

In order to guarantee high-resolution SEM images, the surface of the specimens was sputter-coated with gold using a sputter coater (Cressington 108, Cressington Sc. Instr. Ltd., Watford, UK). Subsequently, the specimens were analysed in the SEM (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, England). The microscope was operated at 20 kV and 100 pA at a constant working distance.

Second harmonic generation (SHG) microscopy

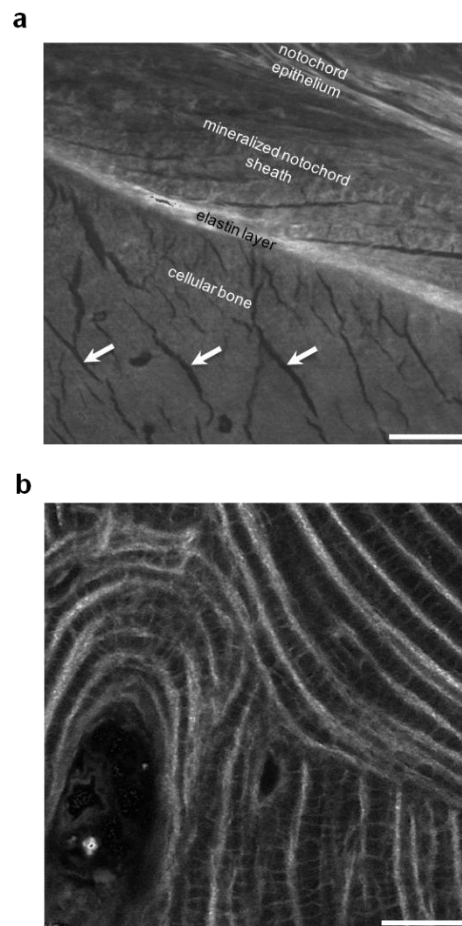
SHG microscopy was used to further visualize the collagen orientation and its pervasion by osteocyte canalicular connections in eel bone compared to human osteonal bone. Non-centrosymmetric materials such as collagens produce a SHG signal. Skeletal imaging of teleost bone by means of SHG have been previously reported to offer additional insight [18]. Unstained sections were examined under a modified Olympus FV1200MPE Multiphoton Laser Scanning Microscope system (Olympus Co., Shinjuku-ku, Tokyo, Japan) equipped with a tuneable Spectra-Physics MaiTai laser source that has a wavelength of 900 nm as described elsewhere [37,38].

Statistical analysis

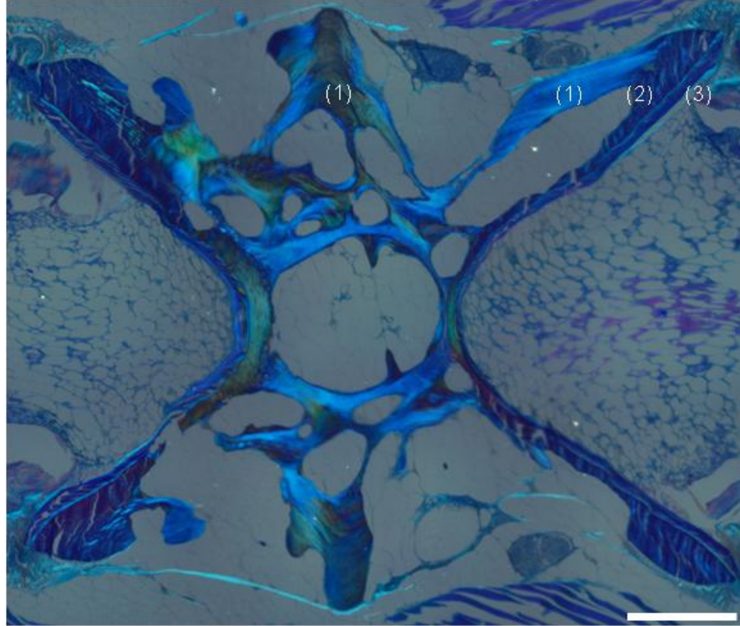
The SPSS statistical program (version 22, IBM Corporation) was used for statistical analysis. The Kolmogorov-Smirnov test was used to check for normal distribution of

the measured parameters. Results were presented as mean values ± SEM. Inter-group comparisons were performed using analysis of variance (ANOVA), with post-hoc pairwise comparisons between individual groups under Bonferroni correction. p-values under 0.05 were considered statistically significant.

Supplemental Figures



Supplemental Fig. 1: *(a) Second harmonic generation (SHG) microscopy shows the different zones of the European eel's vertebral bone, while no typical lamellar bone structure could be detected. The cellular bone contains Sharpey fibres (white arrows) that are orientated perpendicularly to the osteocyte lacunae. In between the cellular bone and the mineralised notochord sheath there is a thin layer with a strong signal intensity, which might act as arrestor for osteoclast attachment. No osteocyte canaliculi could be observed in the osteocytic bone concordantly to qBEI and acid etching techniques. (b) Human cortical bone shows haversian canals, typically oriented collagen fibres and existence of canaliculi. Scale bar 20 µm.*



Supplemental Fig. 2: *One complete vertebral body of a European yellow eel is shown to clarify the bone structure with its different units. (1) There is secondary lamellar bone, which contains osteocytes. (2) Primary bone is of woven composition and contains osteocytes as well. (3) The mineralised acellular notochord sheath represents the most inner layer and is not accessible for resorption in European eels. Toluidine blue stain imaged in polarized light. Scale bar 500 µm.*

Supplemental Tables

Supplemental Table 1: *Group characteristics of shape, weight, plasma ion and cortisol levels and structural indices obtained by HR-pQCT data. Significant changes (p<0.05) between artificially matured (AM) and yellow (*). AM and silver (#), and silver and yellow eels (†).*

	-m a l e s-			-f e m a l e s-		
	Yellow (n=4)	Silver (n=5)	AM (n=6)	Yellow (n=5)	Silver (n=5)	AM (n=5)
Length (cm)	36.7 ± 2.6	35.8 ± 1.3	34.5 ± 1.3	73.8 ± 4.8	88.3 ± 8.9†	78.0 ± 9.5
Weight (g)	79.0 ± 19.6	84.7 ± 9.8	79.8 ± 13.0	74.9 ± 10.4	173.1 ± 63.9†	64.3 ± 34.7$^{\#}$
Calcium (mg/dl)	54.0 ± 5.6	41.0 ± 5.8	47.6 ± 17.3	46.2 ± 3.1	53.7 ± 0.4†	98.9 ± 4.0$^{*\#}$
Phosphate (mg/dl)	99.3 ± 9.2	83.0 ± 21.5	50.2 ± 14.5$^{*\#}$	65.5 ± 8.3	69.0 ± 7.5	129.6 ±13.6$^{*\#}$
Cortisol (mg/dl)	63.3 ± 19.2	48.3 ± 22.3	12.2 ± 5.1$^{*\#}$	25.9 ± 8.1	11.30 ± 7.9†	19.3 ± 8.7
Orb.Cav.Dist (mm)	23 ± 1.4	25.3 ± 1.1†	27.5 ± 1.6$^{*\#}$	22.5 ± 1.9	25.7 ± 4.2	31.5 ± 0.7$^{*\#}$
Eye index	5.3 ± 0.2	8.1 ± 1.3†	11.1 ± 1.0$^{*\#}$	8.3 ± 2.3	12.5 ± 1.1†	14.7 ± 2.6$^{*\#}$

Supplemental Table 2: *Evaluation of bone volume per tissue volume (BV/TV) and vertebral thickness for both sexes separately. (*) indicates significant changes between AM and yellow eels, (#) between AM and silver eels (p<0.05).*

	-m a l e s-			-f e m a l e s-		
	Yellow	Silver	AM	Yellow	Silver	AM
BV/TV vertebra (%)	17.0 ± 5.6	14.8 ± 4.1	6.5 ± 1.3*#	18.4 ± 3.3	15.3 ± 2.9	8.0 ± 2.6*#
BV/TV skull (%)	21.6 ± 2.3	14.5 ± 6.1	5.5 ± 2.2*#	18.9 ± 5.8	13.7 ± 4.7	4.6 ± 1.9*#
Vert. Thickness (µm)	132.5 ± 23.8	115 ± 52.8	47.9 ± 15.7*#	179.9 ± 72.9	200.5 ± 40.7	102.5 ± 35.1*#