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Bone mineral density and microarchitecture change during skeletal growth in harbor seals (*Phoca vitulina*) from the German coast

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Across species, the skeletal system shares mutual functions, including the protection of inner organs, structural basis for locomotion, and acting as an endocrine organ, thus being of pivotal importance for survival. However, insights into skeletal characteristics of marine mammals are limited, especially in the growing skeleton. Harbor seals (*Phoca vitulina*) are common marine mammals in the North and Baltic Seas and are suitable indicators of the condition of their ecosystem. Here, we analyzed whole-body areal bone mineral density (aBMD) by dual-energy X-ray absorptiometry (DXA) and lumbar vertebrae by high-resolution peripheral quantitative computed tomography (HR-pQCT) in neonate, juvenile, and adult harbor seals. Along skeletal growth, an increase in two-dimensional aBMD by DXA was paralleled by three-dimensional volumetric BMD by HR-pQCT, which could be attributed to an increasing trabecular thickness while trabecular number remained constant. Strong associations were observed between body dimensions (weight and length) and aBMD and trabecular microarchitecture ($R^2 = 0.71\text{--}0.92$, all $p < 0.001$). To validate the results of the DXA measurement (i.e., the standard method used worldwide to diagnose osteoporosis in humans), we performed linear regression analyses with the three-dimensional measurements from the HR-pQCT method, which revealed strong associations between the two imaging techniques (e.g., aBMD and Tb.Th: $R^2 = 0.96$, $p < 0.0001$). Taken together, our findings highlight the importance of systematic skeletal investigations in marine mammals during growth, illustrating the high accuracy of DXA in this context. Regardless of the limited sample size, the observed trabecular thickening is likely to represent a distinct pattern of vertebral bone maturation. As differences in nutritional status, among other factors, are likely to affect skeletal health, it appears essential to routinely perform skeletal assessments in marine mammals. Placing the results in the context of environmental exposures may allow effective measures to protect their populations.

Harbor seals (*Phoca vitulina*) are common marine mammals along the German coast and are found in the North and Baltic Seas. As they are apex predators and long-living marine mammals (20–25 years for males and 30–35 years for females), and have a small home range, they are a suitable indicator of the condition of their ecosystem^{1–3}. Multiple studies report on different anthropogenic impacts and their effects on the immune, endocrine, and reproduction system of harbor seals in the North and Baltic Sea^{2,4–6}. However, only a limited number of studies have investigated the skeletal system and potential changes due to various stressors^{7,8}.

Despite fundamental environmental differences, such as between sea and land, the skeletal system shares similar functions across species. At least five shared characteristics of the skeleton can be defined, including

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providing the structural basis for locomotion, protection of inner organs, the main storage for calcium, bone as an endocrine organ, and sound transmission during hearing^{9,10}. Bone is remodeled lifelong through a balance of bone formation and resorption, which is tightly coupled and carried out by two different bone cell types, namely bone-forming osteoblasts and bone-resorbing osteoclasts¹¹. Next to these two cell types (i.e., osteoblasts and osteoclasts), matrix-embedded mechanosensitive osteocytes represent the most abundant cell type, orchestrating the remodeling process by various processes, including the production of signaling proteins and hormones^{12,13}. Specific alterations in osteocyte characteristics, namely a lower number of osteocyte lacunae per bone area as a lower lacunar area, have previously been shown for sperm whales in comparison to other whale species, as deep diving may be associated with reduced osteocyte viability¹⁴. The remodeling process is very sensitive to environmental changes and, for this reason, also influenced by multiple factors, such as vitamins, hormones, nutrients, and pollutants, among others^{15–17}. Besides, disturbances or alterations in bone quality can have significant impacts on the overall health or physiology of an individual¹⁸.

In human medicine, bone densitometry is used for diagnostics and treatment monitoring of bone diseases in routine clinical practice, with dual-energy X-ray absorptiometry (DXA) representing the gold standard method¹⁹. Areal bone mineral density (aBMD) is used in humans for the definition of osteoporosis and assessment of fracture risk²⁰. However, a relevant proportion of individuals with fragility fractures characteristic of osteoporosis are not identified by DXA, but by evaluation of bone microarchitecture using high-resolution peripheral quantitative computed tomography (HR-pQCT)²¹. HR-pQCT has been developed as an in vivo successor to micro-CT²² to circumvent its analysis capability limited to smaller ex vivo specimens and has since demonstrated substantial added value in routine clinical practice and skeletal research²³. In this sense, three-dimensional HR-pQCT analyses allow the assessment of bone microarchitecture parameters such as trabecular thickness (Tb.Th) or three-dimensional trabecular bone mineral density (Tb.BMD), which was previously limited to ex vivo evaluations such as bone histomorphometry or quantitative backscattered electron imaging (qBEI).

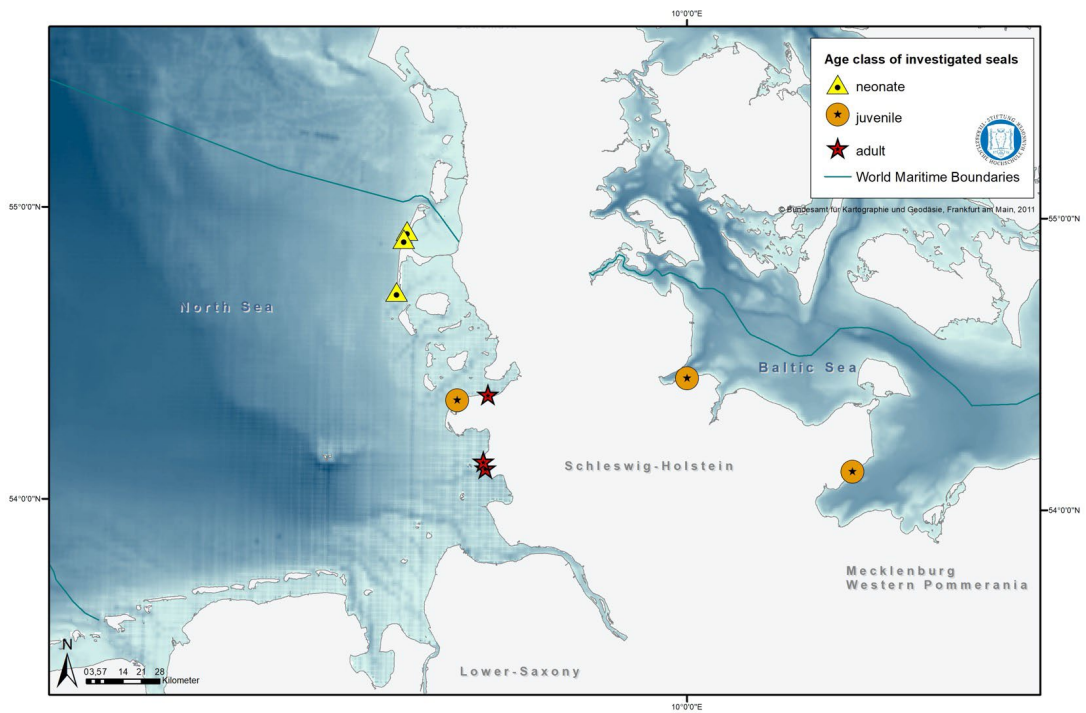
Wildlife studies on the skeleton are comparably limited, but in the last years, an increase in the number of reports has been noticeable^{8,15,24,25}. One marine species is the common bottlenose dolphin (*Tursiops truncatus*), measuring aBMD at different ages in this dolphin^{17,23}. The authors showed that aBMD of common bottlenose dolphins increases with age, although a considerable variance of aBMD was found²³. DXA has been mainly used to measure aBMD in different animal species and investigate distinct bone conditions^{15,26,27}, as this method is available worldwide and has excellent validity. Such investigations are important, as multiple different environmental and anthropogenic stressors (e.g., pollutants or overfishing) influence the health status of marine mammals. A study of ringed seals (*Pusa hispida*) demonstrated critical effects of contaminants on aBMD¹⁴. Marine mammals are at the end of the food chain and therefore can also indicate possible health effects for humans. Additionally, marine mammals are key species for their ecosystems, because they play an important role in structuring the ecosystem they live in. These combined aspects make them good health indicators^{1,28}. The skeleton, in particular, can be analyzed to derive relevant information about growth, blood production, reproduction, and mineral and energy storage²⁹. In that sense, previous bone examinations have shown critical information about individuals, such as decreased bone mass or pathological changes like increased porosity in east Greenland polar bears, baltic gray seals, and Florida pumas^{27,30–32} or even traumatic alterations such as fractures in harbor porpoises³³. However, the diagnosis of osteoporosis has not yet been standardized in marine mammals (including harbor seals). Therefore, it appears of pivotal importance to transfer those technologies to wildlife research and conservation.

As the skeleton undergoes a remarkable development during growth, including rapid growth in the first years of the life of mammals³⁴, defining age-specific characteristics of harbor seals may be of central relevance. Whereas one study investigated age-associated changes in the microarchitecture of the mandible of harbor seals on the German coast⁸, no reports of vertebral aBMD or bone microarchitecture parameters are available. As the spine is of importance for the functional integrity of the skeleton and thus the survival of the animal, in this study we examined nine harbor seals of three different age groups using DXA and HR-pQCT to determine the skeletal features and their association with age.

Material and methods

Samples and necropsy. The samples originate from harbor seals, which were found dead, bycaught or euthanized by hunters at the German coasts due to conditions incompatible with survival, including completely apathetic, mild to severe cough and breathing sounds, sometimes bloody nose (Fig. 1A). Five of the nine investigated harbor seals were found dead, while the other mammals had to be euthanized (Table 1). To prevent sex bias on skeletal parameters¹⁵, only female animals were included. A sample of nine harbor seals was examined each three for one age group. Length and body weight including blubber were measured in the animals and their nutritional status was determined based on blubber thickness, measured at the neck (dorsal and ventral) and on the state of the muscles^{35,36}. In that sense, it was defined as poor (5–20 mm), moderate (21–30 mm), or good (31–50 mm). This way, they were classified as neonates (≤ 8 weeks of postnatal age), juveniles (8 weeks–5 years of age) or adults (beyond 5 years of age). The necropsies of seals based on the international guidelines for cetacean³⁶. Seven of the nine animals were collected from the North Sea, two from the Baltic Sea. All animals were collected between 2018 and 2021. The animals were found all year round, including breeding and molting season. The exact age of five animals was determined by the growth layers counted in the cementum of the canine teeth as described in Lockyer et al.³⁷, as animals younger than 1.5 years do not show easily the growth layers in the cementum. The age determination of neonate harbor seals was based on external characteristics including the time of the year it was found and the known time of birth. The classification of the age classes (neonate, juvenile and adult) based on body length³⁶. No living animals were used in this study.

A



B

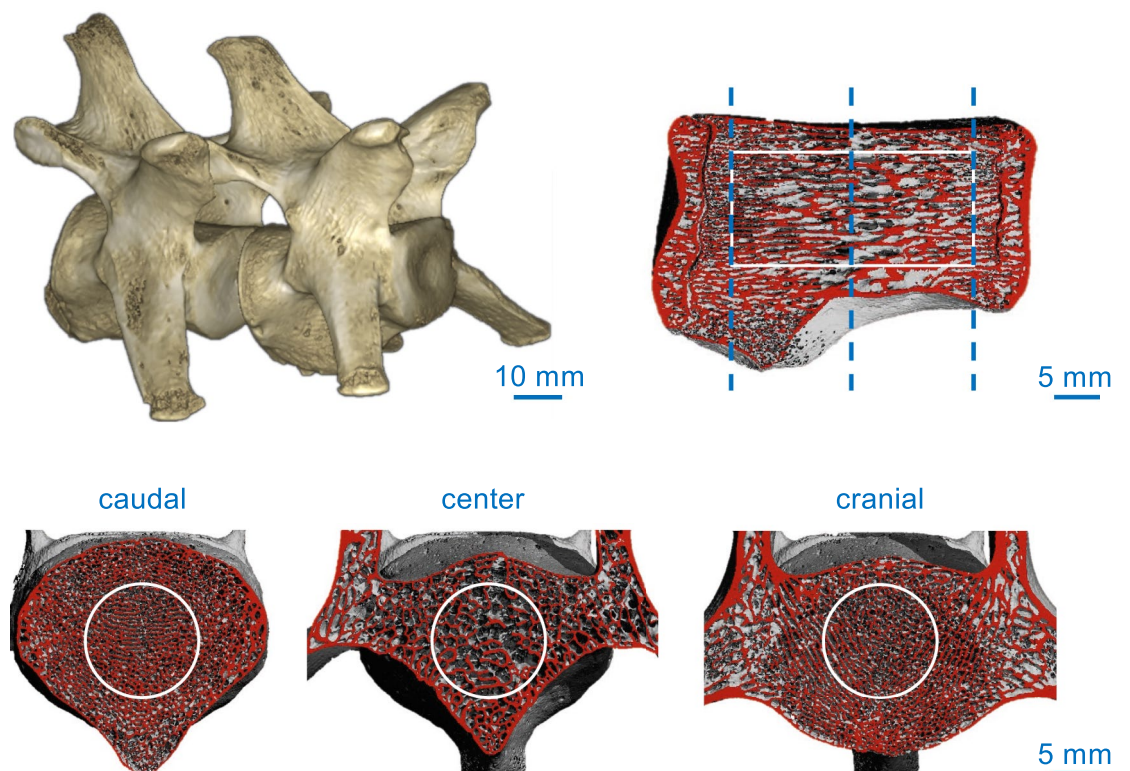


Figure 1. Map of the investigated harbor seals and imaging methodology. (A) The triangle shows the neonates, the circles show the juveniles, and the stars are the adult harbor seals. The map was created using ArcGIS software (ArcMap Version 10.6.1). (B) Three-dimensional reconstruction of segmented lumbar vertebrae (upper left panel) scanned by HR-pQCT and a sagittal view to indicate the scanned volume of interest (VOI, contoured in white).

| Animal number | Age group | Age | Water | Date | Nutritional status | Cause of death |
|---------------|-----------|----------|------------|------------|--------------------|--|
| P.v. 1 | Neo | 1–2 mo | North Sea | 02.08.2019 | Poor | Cachexia |
| P.v. 2 | Neo | 1–2 mo | North Sea | 18.08.2019 | Poor | Cachexia |
| P.v. 3 | Neo | 1–2 mo | North Sea | 02.08.2019 | Poor | Cachexia |
| P.v. 4 | Juv | < 1 year | North Sea | 14.02.2019 | Moderate | Bronchopneumonia with endoparasitosis |
| P.v. 5 | Juv | n.d | Baltic Sea | 12.06.2020 | Good | n.d |
| P.v. 6 | Juv | 3 years | Baltic Sea | 04.02.2021 | Moderate | n.d |
| P.v. 7 | Adu | 17 years | North Sea | 30.12.2018 | Good | Abortion with a uterine rupture and internal bleeding |
| P.v. 8 | Adu | 11 years | North Sea | 25.05.2020 | Good | Intestinal displacement |
| P.v. 9 | Adu | 17 years | North Sea | 04.02.2020 | Good | Gastritis with a high grade parasitosis of the stomach |

Table 1. Demographic data of the investigated harbor seals (*Phoca vitulina*) from the German coast. *Neo*, neonate, *mo.* months, *Juv.* juvenile, *Adu.* adult, *n.d.* not determinable. Poor: blubber thickness 5–20 mm; moderate: blubber thickness 21–30 mm; good: blubber thickness 31–50 mm.

Areal bone mineral density (aBMD). To evaluate aBMD, dual-energy X-ray absorptiometry (DXA, Lunar Prodigy iDXA; GE Healthcare, Madison, WI, USA) was performed. The DXA device was daily calibrated using a phantom provided by the manufacturer to ensure scan validity. For the analysis, the entire animal was placed on the table of the scanner, and a whole-body scan was conducted. Subsequently, aBMD was calculated using the manufacturer's software (enCORE-software v15-GE Healthcare Lunar, Buckinghamshire, UK), which determined the BMD in grams per square centimeter (g/cm^2)¹⁵. Each scan was checked manually for correct bone segmentation. Total body aBMD values were used for subsequent analyses.

High-resolution peripheral quantitative computed tomography (HR-pQCT). After necropsy³⁵, the lumbar vertebrae were fixed in 3.7% formaldehyde. For the assessment of the trabecular bone microarchitecture, HR-pQCT measurements (XtremeCT II[†], Scanco Medical AG, Brüttisellen, Switzerland) were performed of the entire vertebra using an ex vivo protocol provided from the manufacturer (60 kVp, 900 μA , 100 ms integration time, voxel size of 42 μm , Fig. 1B). Data consistency were ensured by the daily use of the calibration phantom. During the scan, the specimens were fixed within special casts. Within a standardized cylindrical volume of interest (VOI) in-between the epiphyses of the vertebrae (Fig. 1B), a three-dimensional microarchitectural dataset was generated and trabecular parameters calculated, including trabecular volumetric bone mineral density (Tb.BMD, mgHA/cm^3), bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th, mm), and trabecular number (Tb.N, 1/mm)²³.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 8.4.0, GraphPad Software, Inc., La Jolla, CA, USA). Normality-distribution of the data were tested using Shapiro–Wilk test. As all data were normally distributed, one-way ANOVA and repeated measures with Tukey correction was carried out for the comparison of the three groups. For the analysis of an association between DXA values, body length or weight and bone microarchitectural parameters, linear regression analyses were performed and the coefficient of determination R^2 and the regression slopes with confidence intervals (CIs) were calculated. Results are given as absolute values or the mean \pm standard deviation (SD). The dashed lines of the truncated violin plots represent the median and quartiles. The level of significance was defined as $p < 0.05$. Exact p -values are reported unless $p < 0.0001$.

Ethic statement. The Institute for terrestrial and aquatic wildlife research (TAW) has been authorized by MEKUN (Ministry for Energy Transition, Climate Protection, Environment and Nature) to perform health monitoring for dead stranded or stranded animals and has all the necessary permits required for this.

Results

The nutritional status varied among the mammals. All neonates were diagnosed with emaciation (i.e., poor nutritional status), while the nutritional status was classified as moderate in two of the three young animals and good in the remaining animals. All neonate harbor seals died because of cachexia due to separation from the mother. One of the juvenile mammals had a bronchopneumonia with endoparasitosis, whereas for the other two juveniles the reason of death could not be clearly defined (Table 1). The three adults died due to different diseases: One animal had a gastritis with a high grade parasitosis of the stomach, one animal was found with an intestinal displacement and the last one died due to an abortion with a uterine rupture and internal bleeding, causing a sepsis and an associated shock.

For the evaluation of skeletal characteristics, we evaluated the DXA measurements by age group. Notably, an increase in aBMD with age was observed with significant differences between adults and neonates as well as between adults and juveniles but not between neonates and juveniles (Fig. 2A). Likewise, HR-pQCT-derived Tb.BMD was significantly higher in adults compared to neonates and juveniles (Fig. 2B). When analyzing three-dimensional bone microarchitecture parameters, significant differences of BV/TV (Fig. 2C) were observed

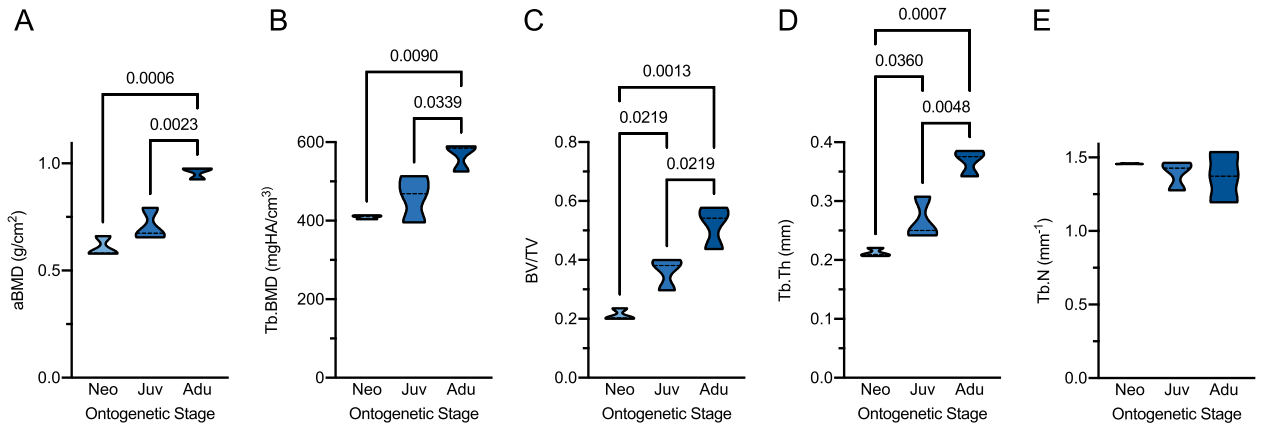


Figure 2. Skeletal parameters between the different age-groups. (A) DXA-derived areal bone mineral density (aBMD) and (B–E) HR-pQCT-derived trabecular bone microarchitecture including (B) trabecular BMD (Tb.BMD), (C) bone volume per total volume (BV/TV), (D) trabecular thickness (Tb.Th), and (E) trabecular number (Tb.N). Only significant p-values (<0.05) are given for the pairwise comparisons. *Neo* neonate, *Juv* juvenile, *Adu* adult.

between each of the age groups, which could be attributed to an increase in Tb.Th (Fig. 2D). No significant differences were observed for Tb.N between the age groups ($p = 0.247$, Fig. 2E).

When evaluating the association between weight and densitometric or microarchitectural parameters, strong associations were observed for all parameters (Fig. 3A,B). Specifically, the strongest regressions were shown between body weight and aBMD as well as Tb.Th ($R^2 = 0.92$, $p < 0.0001$ and $R^2 = 0.87$, $p = 0.0003$, respectively). Comparable strong associations were detected between body length and aBMD as BV/TV, whereas three-dimensional Tb.BMD showed moderate to strong associations with body weight ($R^2 = 0.75$, $p = 0.003$) and length

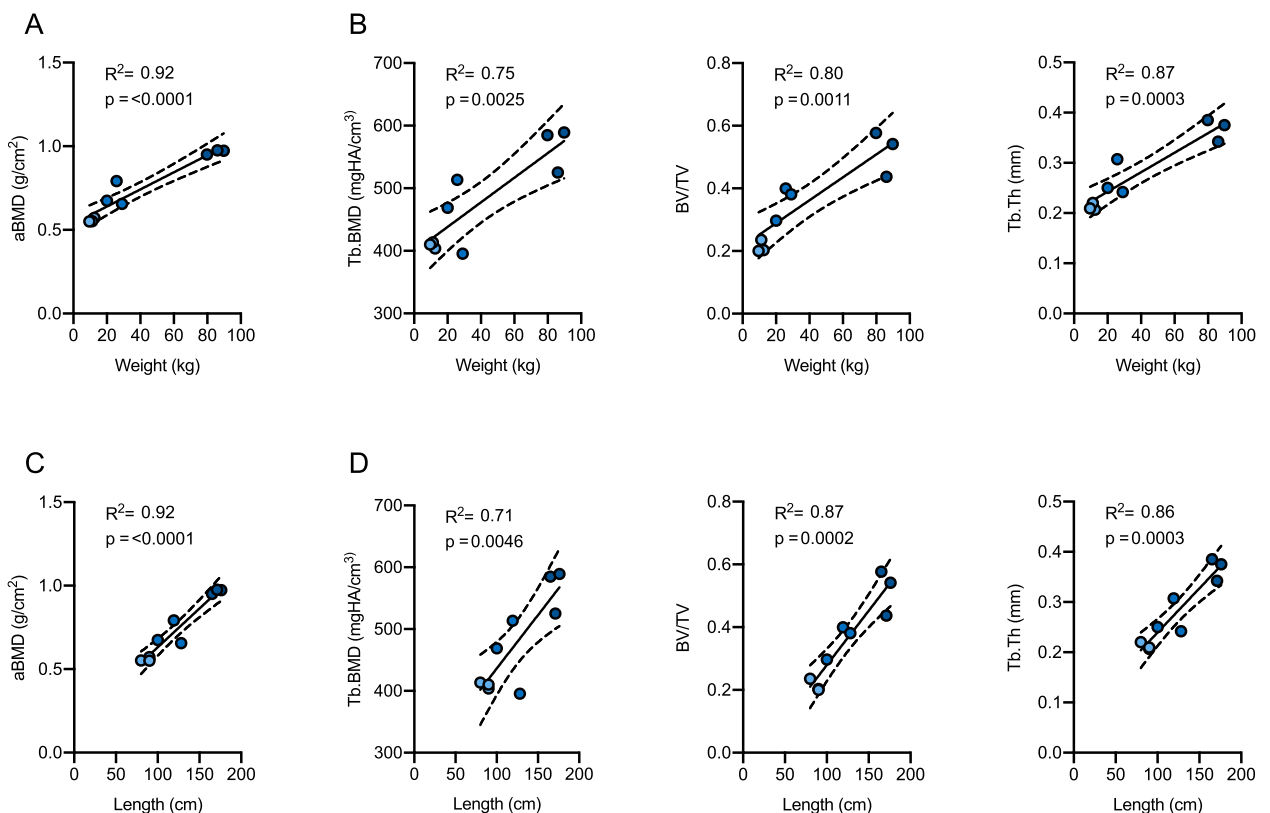


Figure 3. Association between body weight or length and skeletal parameters. Linear regression analysis of body weight and (A) DXA-derived areal bone mineral density (aBMD) and (B) HR-pQCT-derived trabecular bone microarchitecture including trabecular BMD (Tb.BMD), bone volume per total volume (BV/TV) and trabecular thickness (Tb.Th). Linear regression analysis of body length and (C) aBMD and (D) trabecular bone microarchitecture.

($R^2 = 0.71$, $p = 0.005$, Fig. 3C,D). Interestingly, no association with body weight and length was observed for Tb.N ($R^2 = 0.028$, $p = 0.669$ and $R^2 = 0.099$, $p = 0.408$, respectively).

Lastly, as HR-pQCT analysis is not as globally available as DXA, we aimed to compare the findings of DXA and HR-pQCT measurements to answer the question of transferability for clinical routine. Of note, strong positive associations between two-dimensional aBMD and most of the three-dimensional bone microarchitecture parameters were observed between the two imaging modalities (Fig. 4). In that sense, aBMD and volumetric BMD (i.e., Tb.BMD) showed strong associations ($R^2 = 0.87$, $p = 0.0002$), comparable to BV/TV ($R^2 = 0.86$, $p = 0.0003$), which was surpassed by Tb.Th ($R^2 = 0.96$, $p < 0.0001$). Interestingly, no association was observed between aBMD and Tb.N, which remained stable regardless of the increase in aBMD ($R^2 = 0.047$, $p = 0.577$).

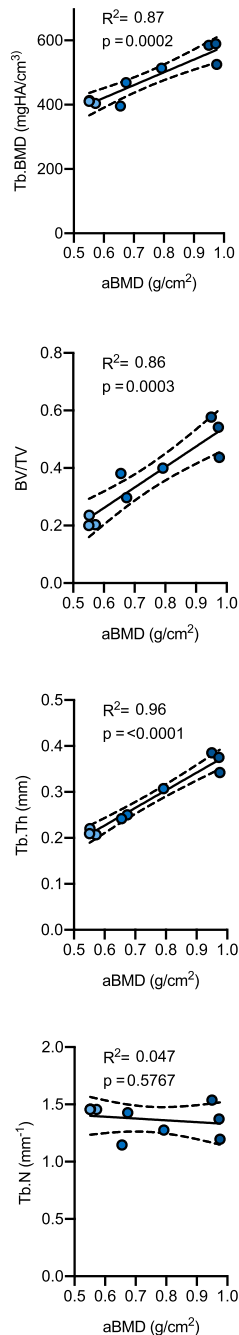


Figure 4. Association between skeletal parameters derived from DXA and HR-pQCT measurements. *aBMD* areal bone mineral density, *Tb.BMD* trabecular bone mineral density, *BV/TV* bone volume per total volume, *Tb.Th* trabecular thickness, *Tb.N* trabecular number.

Discussion

Despite the importance of skeletal health for the survival of marine mammals, insights into the characteristics of bone mineral density and especially bone microarchitecture during growth are scarce. As harbor seals (*Phoca vitulina*) are common along the German coast with a comparably long lifespan, they have been used as a suitable indicator for the respective ecosystem before². The findings of the necropsies showed different conditions leading to death in the nine harbor seals, varying between the age groups as nutritional status³⁶. All identified causes of death found are occurring frequently in this species in German waters and are therefore representative findings.

As the spine is of pivotal importance for the functional integrity of the skeleton and thus the survival of the animal³⁸, extending the knowledge of the individual properties in different species exposed to varying environmental conditions and ways of locomotion appears of great scientific and clinical relevance. Previously, in-depth skeletal examinations of specific anatomic sites in harbor seals were limited to the temporomandibular joint^{39,40}. Specifically, one report using HR-pQCT showed an increase in cortical thickness but a decrease in BV/TV and Tb.Th during growth for a portion of the mandibular corpus⁸. However, little is known about age-related changes in vertebral aBMD and the microarchitecture of harbor seals. Assessment of vertebral aBMD corresponds to the clinical standard site in human medicine and might be the most appropriate place to make relevant deductions such as fracture risk. Moreover, previous studies in marine mammals investigating aBMD characteristics focused predominantly on the effects of environmental pollutants^{17,32} but not on age-group associated changes.

With this first study of its kind in harbor seals, we were able to show that, except for Tb.N, vertebral parameters of aBMD and bone microarchitecture change with ontogenetic skeletal growth. These findings suggest that the basis for the osseous scaffold or skeleton is developed at a very young age, marked by a comparably high number of trabeculae, becoming increasingly mineralized and increasing in thickness as development progresses. In this regard, thickening of the trabeculae was also observed in human children with increasing age⁴¹. However, it must be noted that both DXA and HR-pQCT are not able to detect unmineralized bone matrix (i.e., osteoid), likely resulting in low structural parameters at a young age.

Because it is accepted that lower mechanical load is associated with a decline in skeletal characteristics⁴² and, especially, cachexia secondary to poor nutritional status is likely to affect bone mineral density^{43,44}, the measured parameters of such neonates are prone to be conditionally inferior compared to harbor seals of the same age but in good nutritional status. This also applies, to a smaller degree, to the juvenile seals, of whom two showed a moderate nutritional status. Therefore, future studies investigating aBMD in different age groups are needed to place these results in the context of healthy animals. Despite the potential influence of nutritional status, it appears likely that the high association between demographic and skeletal parameters would remain and possibly only the magnitude of the relationship would change. In the long term, we plan to examine more animals by DXA and establish reference values by routine measurement to allow a standardized diagnosis of osteoporosis. Furthermore, an evaluation of skeletal features in animals with cachexia compared to those without appears to be of further interest.

Regardless of the small number of samples, the evaluation of an association between body weight or length and skeletal parameters showed remarkable positive linear associations for aBMD, Tb.BMD, and Tb.Th, representing the hallmark of osseous development along with increasing osseous mineralization. This trabecular thickening along increasing age was also observed in undecalcified histomorphometry. Interestingly, Tb.N did show a trend towards a negative association with age, supporting the hypothesized increase in mineralization of early-formed trabeculae. Likewise, a lower number of trabeculae during skeletal growth had also been reported for humans before⁴¹.

Besides the novelty of this study, some limitations need to be mentioned such as the limited sample size. As we analyzed data of deceased animals, as mentioned above, the nutritional status in the neonate mammals potentially impacted the bone quality measured in our study. Therefore, attention needs to be paid when comparing the presented findings to healthy animals of the same age. Moreover, no standardized evaluation of chemical pollutants was conducted, representing an open question worth investigating in future studies. Additionally, only females were included to avoid sex bias on skeletal parameters. The individuals were found all year round, including breeding and molting season. Therefore, the animals should give a good overview of the complete year with different nutritional status and possible differences in BMD during breeding and molting season. However, inclusion of males could provide additional comparative data as the bone architecture of females might also change due to their breeding status. This should be addressed by future studies. Lastly, a specification of the mammals' age is not feasible for marine mammals, especially for such of very young age. Therefore, objective data were available for body length and weight only. However, as the age-determination is primarily based upon these variables, we think that it is reasonable to speculate that the findings represent age-specific characteristics.

In conclusion, we here demonstrated the age-specific characteristics of whole-body aBMD derived by DXA and vertebral bone microarchitecture by HR-pQCT in harbor seals from the Baltic and the North Seas. By combining the two techniques, a distinct pattern of vertebral bone maturation marked by trabecular thickening was highlighted. Considering the high association of DXA parameters and bone microarchitecture by HR-pQCT, a routine high-throughput skeletal examination via DXA should be performed in marine mammals to expand the knowledge of skeletal characteristics and health and disease, and specifically to explore the influence of environmental factors and different nutritional states.

Data availability

Data are shown in, and can be extracted from, graphs in Figs. 2, 3 and 4 and Table 1.

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Author contributions

J.D., B.S., R.O., T.R., and U.S. generated and collected the data and performed the analysis. J.D., B.S., and T.R. drafted the manuscript. R.O., M.A., and U.S. made substantial conceptual and editorial contributions to this manuscript. All authors reviewed the manuscript.

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Additional information

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