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Effects of scaling on microstructural features of the osteochondral unit: a comparative analysis of 38 mammalian species

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Since Galileo’s days the effect of size on the anatomical characteristics of the structural elements of the body has been a subject of interest. However, the effects of scaling at tissue level have received little interest and virtually no data exist on the subject with respect to the osteochondral unit in the joint, despite this being one of the most lesion-prone and clinically relevant parts of the musculoskeletal system.

Imaging techniques, including FTIR imaging, polarized light microscopy and micro computed tomography, were combined to study the response to increasing body mass of the osteochondral unit. We analyzed the effect of scaling on structural characteristics of articular cartilage, subchondral plate and the supporting trabecular bone, across a wide range of mammals at microscopic level.

We demonstrated that, while total cartilage thickness scales to body mass in a negative allometric fashion, thickness of different cartilage layers did not. Cartilage tissue layers were found to adapt to increasing loads principally in the deep zone with the superficial layers becoming relatively thinner. While subchondral plate thickness was found to have no correlation to body mass, bone volume fraction correlated negatively to body mass ($r= -0.41$, $p<0.01$). This implies that heavier animals will have a relatively more porous subchondral plate; possibly to allow a more homogeneous transmission of forces from the subchondral bone towards the thicker cartilage layer. The underlying trabecular bone was found to have thicker trabeculae ($r=0.59$, $p<0.001$), as expected since this structure carries most loads and plays a role in force mitigation.

List of abbreviations

BM – Body mass
PGs - Proteoglycans
CCZ – Calcified cartilage zone
ROI – Region of interest
FTIRI – Fourier transfer infrared imaging
PLM – Polarized light microscopy
microCT – Micro computed tomography
SC Th – Subchondral plate thickness
SC BV/TV – Subchondral bone volume fraction
Tb Th – Trabecular thickness
Tb BV/TV – Trabecular bone volume fraction

Keywords

Osteochondral unit; trabecular bone scaling; cartilage thickness scaling; osteochondral comparative analysis; cartilage imaging; bone imaging.
1. Introduction

Almost 400 years ago, after visiting Venice’s Arsenal, Galileo laid the foundations to modern deformable body mechanics, by starting a discussion about scaling [1]. He took inspiration from shipbuilding and wondered how structural components would scale in bigger ships to avoid collapsing under excessive weight. This led to the formulation of the Square-Cube law that Galileo formulated as “the ratio of two volumes is greater than the ratio of their surfaces” [1]. In other words, when an object undergoes a proportional increase in size, its surface area is proportional to the square of the multiplier, while its volume is proportional to the cube of the multiplier. In addition, it also means that the stress on a larger cube is greater than the stress on a smaller cube due to its own weight [2]. Later, in the Discourses and Mathematical Demonstrations Relating to Two New Sciences, Galileo applied the law to living beings and deduced that animals could not be simply scaled up, or their bones would break under excessive weight [1]. Since then, macroscopic scaling of limbs and their components has been discussed extensively in literature [3-6]. Nevertheless, investigations on the microscopic level have been limited [7-10]. In particular, the adaptations to loading of the ensemble of the articular components (i.e. subchondral bone, cartilage and their interface) at microscopic level have never been analyzed in a comparative fashion across a large range of species.

The general structure and organization of diarthrodial joints is similar in all mammalian species. The function of these diarthrodial joints is both to allow almost frictionless motion of the articulating bony components of the skeleton and to accommodate and mitigate the substantial biomechanical forces that are generated by locomotion. Hence, joint function requires its elements to provide excellent lubrication between articulating surfaces, allow force transmission and absorption, to mitigate the effects of acceleration, vibrations and peak forces generated by locomotion. To accomplish these tasks, joint components work in synergy and should be considered as a unit [11, 12], composed of articular cartilage, the subchondral bone plate and trabecular bone rather than as individual components.

Basic biochemistry, biomechanics and morphological characteristics of the major components of diarthrodial joints (i.e. hyaline articular cartilage and bone) have been studied frequently in relation to pathological
changes and effectiveness of different treatments [12-15]. These studies, however, are usually focused on humans and animal species that are of interest as models for orthopedic research in a translational sense [15, 16]. In nature, the spectrum of sizes and body weights in mammals is much wider than in the few species used as animal models for musculo-skeletal diseases [7, 17]. We have previously shown in a study over a wide range of species that articular width in the stifle (knee) joint scales isometrically (a=0.33) with body mass [7]. If we assume that joint form is not essentially influenced by size, joint surface will do the same [18]. If then the microscopic configuration of the osteochondral unit would remain the same, the stress in the unit would increase linearly with weight given the Square-Cube law. Both articular cartilage and bone increase in size with body mass, and isolated studies on these two tissues have shown that they do not scale isometrically, but have a negative allometric relationship with increasing body mass [7-9] and therefore do not fully compensate for increasing body mass. In theory, an increase in loading can also be compensated for by changes in composition of the constituting elements of the osteochondral unit (that would possibly influence strength of the structure). However, previous studies comparing articular cartilage biochemical composition across a variety of mammalian species covering a range of body masses revealed that gross biochemical composition was constant (6, 12). The composition and structure of articular cartilage, however, does change with depth, so that three layers (superficial, middle and deep) can be identified, based on compositional characteristics like proteoglycans and collagen content, and structural characteristics like collagen orientation. In this last case, fibrils are oriented parallel to the articular surface in the superficial zones, and transition through a random orientation to the deep zone in which they are oriented perpendicular to the subchondral bone [19].

The current study aims to comprehensively investigate the microstructural and compositional features of the osteochondral unit (across a wide range of terrestrial mammals) and their relationship to each other and to body mass (BM). This will reveal where the adaptations to increasing loads (and BM) [20] reside and will determine which microscopic features follow isometric scaling and which do not.

It was hypothesized that in articular cartilage all layers would scale with negative allometry, as found earlier for total thickness (2), and that increased load would be accommodated by either structural adaptations in the
subchondral plate or the trabecular subchondral bone, or by adaptations of the components in one or more of these layers.

2. Materials and methods

To investigate spatial biochemical composition of single layers of cartilage, Fourier-Transform Infrared Imaging (FTIRI) [21-23] was employed, allowing to determine relative content of proteoglycans (PG) and collagen by measuring absorption of specific peaks [24]. To evaluate the orientation and distribution of the cartilaginous collagen network, Polarized Light Microscopy (PLM) was chosen for its capacity to visualize the orientation of anisotropic materials [25-27]. Finally, for the detailed analysis of the microstructural features of the subchondral and trabecular bone, Micro-Computed Tomography (micro-CT) was selected for the accurate measurement of micron-sized structures that constitute the bony tissue [28, 29].

2.1 Collection of materials and tissue harvest

Osteochondral tissue cylinders of 6 mm in diameter were harvested post-mortem from the weight bearing central area of the medial femoral condyles of adult animals sent for autopsy to the Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. Animal species, age and body mass were recorded, and macroscopic pictures of the joints were taken. Joints demonstrating macroscopic or microscopic signs of cartilage degeneration were excluded; animals displaying signs of incomplete endochondral ossification were identified as immature and excluded as well. Human tissue samples were obtained from the Department of Pathology, University Medical Center Utrecht, The Netherlands, with approval of the local ethical committee, in line with the Dutch code of conduct for “Proper Secondary Use of Human Tissue”.

In total 81 tissue samples (39 for histology, FTIR and PLM, and 42 for micro-CT) were harvested from mammals belonging to 38 different species (Table 1); samples for histology were fixed in formalin 4%, while samples for micro-CT analysis were stored in 70% ethanol; all samples were stored at room temperature until further use.

2.2 Histological preparation and analysis
Samples were decalcified using Luthra solution (3.2% 11M HCl, 10 % formic acid in distilled water), dehydrated, cleared in xylene, embedded in paraffin and cut to yield 5 μm sections. Sections were stained with fast green and Safranin-O for measurements of cartilage thickness (distance from the surface to the interface with the subchondral bone), and of the calcified cartilage zone (from tidemark to bone surface). Digital images were analyzed using Cell^F software (Olympus, USA). Average thickness of cartilage and calcified cartilage zone (CCZ) for each sample was determined by averaging 4 measurements per image taken from different locations of the section.

2.3 Fourier-Transform Infrared Imaging (FTIRI) and Polarized Light Microscopy (PLM)

Unstained histological sections were inserted in a controlled atmosphere chamber without humidity and specific regions of interest (ROIs) (Fig. 1A) were measured using a FTIR imaging system (PerkinElmer Spectrum Spotlight 300-system). The absorption spectrum of a pixel (25 x 25 μm²) was translated to relative values (Fig. 1B). Collagen content was estimated with amide I (1585-1720 cm⁻¹) absorption and PGs with absorption at carbohydrate region 984-1140 cm⁻¹ [23, 24] (Fig. 1C). The values obtained for each pixel within the ROI constituted a matrix with information on PG and collagen contents, which was subsequently used for detailed analysis of the zonal structure of cartilage tissue (Fig. 1B).

Collagen fiber orientation was visualized using PLM, and used for the classification of the superficial, middle and deep layer of cartilage [25, 30] (Fig 2). An Abrio PLM system (Cri Inc., Woburn, MA, USA) mounted on a light microscope (Nikon Diaphot TMD, Nikon Inc.) was used for the PLM measurements. The area with the minimum birefringence value was assumed to be the border between the superficial and the middle zones, whereas the deep zone was considered to begin when the orientation angle values reached a plateau (typically close to 90 degrees with respect to cartilage surface) [31, 32] (Fig. 2D).

The combination of this information with the quantitative data contained in the matrix obtained with FTIRI allowed for relative quantification of proteoglycans and collagen in selected regions, and layer by layer. The PLM allowed also quantifying of individual cartilage layer thickness (superficial, middle and deep).
2.4 Micro-CT

Micro Computed Tomography (micro-CT) images of the osteochondral cores were obtained with a micro-CT scanner (Quantum FX, Perkin Elmer, USA, voxel size = 20 μm³). The automatically reconstructed micro-CT images were subsequently converted to series of 2D TIFF images and were binarized using local thresholding (Bernsen technique). BoneJ software [33] was used to determine thickness (SC Th) and bone volume fraction (SC BV/TV) of the subchondral plate/calcified cartilage with manual selection of the ROI (Fig. 3A); trabecular thickness (Tb Th) and bone volume fraction (Tb BV/TV) of the underlying trabecular bone were also determined with the same methodology. To obtain real subchondral plate thickness, CCZ thickness was measured by light microscopy (Fig. 3B), and its value was subtracted from the CT measurements to obtain real SC Th (Fig. 3C).

2.5 Statistics

Initial statistical analysis was performed with R software 3.5.0 [34], to obtain a correlation matrix with Pearson coefficients [35, 36]. For correlations between body mass and thickness of different osteochondral features, a regression analysis using a power curve fit was performed. Statistical comparison of the obtained power coefficients with the theoretical coefficient of 0.33 (isometric scaling) was performed using a one-sample T-test. Limit of statistical significance was set at p<0.05.

3. Results

3.1 Depth-wise architecture of articular cartilage is preserved across species

Thickness of the cartilage layer (calcified plus non-calcified) varied widely between species, ranging from 64.6 μm in the mouse to 3.25 mm in the African elephant. Calcified cartilage thickness ranged from 46.4 μm in the mouse to 310 μm in the African elephant. Total cartilage thickness correlated with body mass (BM) with a negative allometric relationship ($R^2$=0.83, $a = 0.29$), in line with previous findings of Malda et al. [7].

Layer thickness of each layer was normalized to superficial layer thickness to show how layers relate to each other (Fig. 4A). Relative layer thickness was expressed for each layer as a percentage of total thickness, and
correlated to BM. The relative thickness of the deep zone of the articular cartilage showed an increasing trend in relation to body mass, whereas the relative thicknesses of both the superficial and the middle zone showed a tendency to decrease proportionally in relation to body size (Fig. 4B). Absolute values for the thickness of cartilage layers showed no clear correlation to body mass, and seemed to scale independently of BM (Fig. 4C-4E).

Combination of FTIR and PLM techniques allowed for relative quantification and comparison (dimensionless numbers) of spatial collagen and PG content across species. Relative total collagen and PG content, when measured over the total thickness of the cartilage, showed no dependency to body mass (Fig. 5A, 5B), with a small variation in composition across all species when considering content on the whole thickness of the tissue. Layer-by-layer analysis (superficial, middle, deep) showed this was true in all cartilage layers for both collagen and proteoglycans (Fig. 5C, 5D). Normalizing the contents of collagen and PG to superficial layer content, deep layer content was highest, and superficial layer was lowest for both collagen and PG (Fig. 5E, 5F).

**3.2 Subchondral bone transmits and trabecular bone carries the load**

The thickness of the subchondral plate ranged from 67.25 μm in the mouse to 1.13 mm in the horse. Mean subchondral plate thickness (SC Th) was highest in the equine specie (901 ± 344 μm, n=3), while in the elephants the thickness was 149 ± 59 μm (n=3). Subchondral bone volume fraction (SC BV/TV) showed a negative correlation to body mass (r= -0.41, p<0.01), and a positive correlation to SC Th (r = 0.53, p<0.001) while SC Th showed no correlation to body mass (Fig.6A).

Trabecular thickness (Tb Th) ranged from 66.6 μm in the mouse to 545.8 μm in the elephant. Trabecular thickness (Tb Th) correlated with body mass (BM) in a negative allometric relationship ($R^2=0.81, \alpha = 0.14$) (Fig. 6B). Trabecular bone fraction (Tb BV/TV) showed a positive correlation with body mass (r=0.59, p<0.001), and trabecular thickness and trabecular BV/TV also showed a strong correlation between each other (r=0.88, p<0.001).

An analysis of the log ratio of thickness of cartilage, subchondral bone and trabecular bone, was performed to visualize differences between animals at the extremities of our BM range (*i.e.* belonging to the Muridae...
family and to the Elephantidae family), however statistical analysis revealed no significant differences (Fig. 7).

4. Discussion

A comprehensive analysis of how the osteochondral unit changes with body mass was performed using a combination of different techniques for measuring the microstructural features. Overall articular cartilage thickness scaled in a negative allometric fashion, confirming earlier findings [7].

The current study showed that, contrary to our first hypothesis, this is attributable to the scaling behavior of the deep layer, where both superficial and intermediate layers became relatively thinner. The explanation may be that the deep zone is richest in PGs and hence thought to be primary responsible of transmission of load to the underlying bone [37-39]. It is also in line with the role that is attributed to the superficial layer[40, 41], which is thought to have a major role in the homogeneous distribution of the impact forces and loads away from directly-loaded regions [42, 43], more than in load attenuation and with the fact that in physics, as one scales down, forces like viscous drag become more important than weight [1, 44].

The biochemical composition of articular cartilage was remarkably consistent across species in both absolute and relative terms. This suggests that evolutionary pressure has led to the best possible combination of PG and collagen to effectuate the duty of shock-absorption and transfer of forces to the subchondral and trabecular bone in terrestrial locomotion. The normalization of content to the superficial layer confirmed layer dependency, as was expected from previous studies on selected species [45]. However, as remarkable as it seems that there are no substantial variations in the major structural components of cartilage from mouse to elephant, there may be some in characteristics of those components that were not specifically measured, such as the post-translational modifications of collagen of which cross-links are the most likely candidates.

Benninghoff first described the arching structure formed by the collagen fibers of articular cartilage that run from their anchoring site in the calcified zone first through the deep zone, directed perpendicularly to the subchondral plate, to then describe an arch at the beginning of the transitional zone, with the keystone of the arch in or near the superficial zone where the fiber runs tangential to the cartilage surface before starting its
return journey back to the subchondral bone, forming the second pillar of the arch [46]. If we assume the arching parts to be semi-circular, the thickness of the middle zone is theoretically given by the radius of the arches. The constant thickness of the superficial and middle layers and the increasing thickness of the deep layer with increasing total cartilage thickness suggest that the radius of the arches remains constant with their pillars becoming longer. That would mean that the adaptation of the collagen architecture to scaling would consist of the arches becoming more slender with increasing cartilage thickness; and not proportionally increase in size. In this case, the relative number of arches per unit of (subchondral bone plate) surface would remain constant and, given the constant ratio of total collagen to total mass of cartilage, the ratio of collagen fibril thickness to total cartilage thickness would not increase isometrically with the pillars of the arches becoming relatively thinner. However, verification of this theory would require large numbers of samples from differently sized animals from the same species, as there are relatively large differences in configuration of the collagen arches over the species[15].

The response of the subchondral unit to increasing body mass is more complex. Although it was hypothesized that the subchondral bone plate thickness would scale with body mass as well, this was not the case. Unexpectedly, the subchondral bone volume fraction (and subsequently porosity) did show a negative correlation with BM. This is of interest, because it suggests that the porosity of the interface between cartilage and bone increases in larger animals. The reason may be that a less dense structure may be better able to effectively distribute and transmit forces homogeneously to the underlying trabecular bone. It might possibly decrease brittleness, which could be a relevant issue in larger species [47].

The trabecular bone itself features thicker trabeculae and is denser with increasing body mass to accommodate the higher forces. This could permit accommodation of higher forces and is thus an adaptation to increased body mass. The relatively thicker trabeculae are able to withstand higher loads, facilitated by the even distribution through the subchondral plate. A relatively less dense structure may also allow better nutrition efficiency towards the cartilage by facilitating diffusion from the subchondral bone. However, this should be confirmed by an analysis of micro- and nano- porosity, which would require imaging with a higher resolution than possible with light microscopy. There is in fact evidence in literature that, in an experimental setting, there is exchange of nutrients at the interface of bone and cartilage, although of minimal order
compared to the nutrient exchange with the synovial fluid [48]. In fact, Arkill et al. reported that areas of
direct contact of non-calcified cartilage with the subchondral bone allow for a five-fold solute exchange
compared to calcified regions [49]. Furthermore they showed that even calcified cartilage is permeable to
small solutes so that the subchondral circulation may indeed have a significant role in nutrition of the deep
cartilage layer [49].

The scaling of the trabecular bone features confirmed our hypothesis of negative allometry, with a slope
value ($\alpha = 0.14$) in line with previous studies [9]. Interestingly, this relationship was shown to change to
isometry when comparing only primates, as demonstrated by a meta-analysis conducted on over 30 primate
species by Ryan et al. [50]. The analysis of the relative thickness of cartilage, subchondral plate and
trabeculae (Fig. 7) suggests that size may impose different rules at the extremities of the weight spectrum.
The relationship seems to be rather similar amongst most species and sizes, but very small and very large
animals appear to have their own ratios when it comes to scaling. Some of these exceptions have been
reported in literature, mostly with respect to the smaller species such as mice and rats in which trabecular
bone features [9] and cartilage cellularity [7] were shown not to follow the general patterns. Data on the
other end of the spectrum are virtually lacking. A much higher sample pool would be needed for statistical
confirmation of these trends.

While offering new insights into the variation in the osteochondral unit structure across species, this study
has some limitations. Traditional biochemistry still remains the gold standard for the characterization of
extracellular matrix composition, but was replaced by FTIR analysis in this study. This was done because
traditional biochemical analysis of the different cartilage layers would have been very difficult and subject to
various sources of error [24]. The use of FTIR imaging has previously been applied for spatial analysis of the
main ECM components of cartilage (i.e. collagen and proteoglycans) [21-23], but is performed under
controlled atmospheric conditions, in absence of water and on dehydrated sections [21]. This may affect
thickness measurements due to shrinkage of samples related to fixation for paraffin embedding; however, it
should be noted that the order of shrinkage is largely dependent on water content [51], and is common to all
morphological studies performed using fixated tissue. Further, the distribution of components should remain
relatively unaltered [52] and in the current study the overall biochemical composition of cartilage was found to be similar to earlier reports using classic biochemical analysis methods [7].

Lastly, standard osteochondral units of 6 mm diameter were harvested and analyzed in all species. These were taken from a weight bearing area of the medial condyle of the femur. In the mouse this means that the sample consisted of virtually the entire condyle, while in the elephant it represented a small portion on the articular surface of the medial condyle. Since it is known that there is topographical heterogeneity in cartilage composition related to weight-bearing [38, 53], this means that in the larger species variability may be a little larger depending on exact sample location. However, this effect was most likely limited, as samples were in all cases taken from a load-bearing area.

5. Conclusions

Articular cartilage is a tissue with a high degree of specialization that, in a functional sense, can only be appreciated in the wider context of the osteochondral unit. Our findings suggest that the tissue’s structure has remained remarkably preserved across mammalian species during evolution, and that the trabecular and subchondral bone -in particular- adapt to the increasing body mass. The natural constancy and apparent immutability of the cartilage should be considered when designing strategies for regeneration and/or functional repair.

Declarations of interest

Irina A.D. Mancini, Lassi Rieppo, Behdad Pouran, Isaac O. Afara, Filipe M. Serra Braganca, Mattie H.P. van Rijen, Marja Kik, Harrie Weinans, Juha Toyras, René van Weeren and Jos Malda declare that they have no conflict of interest.

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Bibliography

FIG. 1. FTIR imaging methodology. ROIs were selected using as reference the margin of cartilage interfacing with the synovial joint space, and the interface with the subchondral bone plate (A). The ROI was divided in pixels of 25 x 25 μm² (B, black square), and infrared absorption spectra were recorded for each pixel (C). The absorption for specific intervals was calculated to obtain relative contents of collagen and proteoglycans. Collagen content was estimated with amide I (1585-1720 cm⁻¹) absorption and PGs with absorption at carbohydrate region (984-1140 cm⁻¹). The values obtained for each pixel within the ROI constituted a matrix with information on PG and collagen contents, which was subsequently used for detailed analysis of the zonal structure of cartilage tissue.

FIG. 2. Osteochondral microscopic images of Thompson's gazelle (20 kg, Top) and Giraffe (609.5 kg, bottom). Left images show sections stained for glycosaminoglycans with safranin-O (2A, 2C). On the right are polarized light microscopy images (2B, 2D). Blue colour indicates that fibres are parallel to the surface, while red indicates fibres having perpendicular orientation. The use of PLM allows determination of collagen fibres orientation, and consequently of the transition from superficial, to middle to deep layer of cartilage (Fig 2D).
FIG. 3. Micro-computed tomography (micro-CT) images of the osteochondral cores were obtained with a micro-CT scanner (Quantum FX, Perkin Elmer, USA, resolution 20 µm). ROIs were selected manually (Fig. 3A, white), and used to determine subchondral plate thickness with BoneJ software. As microCT cannot discriminate between calcified cartilage and subchondral bone, CCZ thickness was measured using light microscopy (Fig. 3B, black), and its value was subtracted from the CT measurements (Fig. 3C, white selection) to obtain SC Th.

FIG. 4. Summary of cartilage scaling along body mass. Thickness of each layer was normalized to superficial layer thickness to show layers’ relation to each other (Fig. 4A). Relative layer thickness was represented for each layer as expression of percentage of total thickness, and correlated to BM (Fig. 4B). Relative deep zone thickness (yellow) showed an increasing trend in relation to BM whereas relative superficial (green) and middle (blue) zone thickness showed a tendency to decrease along increasing BM (Fig. 4B). Absolute values for the thickness of the different single layers showed no clear correlation to body mass, and seemed to scale independently of BM (Fig. 4C-4E).
FIG. 5. Relative collagen and proteoglycan contents in cartilage, measured over total thickness (Fig. 5A-5B respectively), and layer per layer (Fig. 5C-5D). Overall collagen and proteoglycan contents showed no dependency to body mass (Fig. 5A collagen, 5B proteoglycans), with a small variation in cartilage composition across all species. Layer per layer analysis (superficial, middle and deep, respectively in green, blue and yellow) showed this was also true for both collagen and proteoglycans within each specific layer across species (Fig. 5C, 5D). Normalizing the content of collagen and PG to superficial layer content, deep layer content was highest, and superficial layer was lowest for both collagen and PG (Fig. 5E, 5F).
FIG. 6. Scaling of subchondral plate and trabecular thickness. Subchondral plate thickness (SC Th) showed no correlation to body mass (Fig. 6A). Images obtained with microCT of rat (Fig. 6C) and elephant (Fig. 6D) show small differences in subchondral plate (red square) thickness between animals of very different sizes. Trabecular thickness (Tb Th) correlated to body mass (BM), with a negative allometric relationship to body mass ($R^2=0.81$, $\alpha = 0.14$) (Fig. 6B). Images obtained with microCT rat (Fig. 6E) and elephant (Fig. 6F) show increase in trabecular bone (red square) thickness along BM.

FIG. 7. Analysis of the log ratios of cartilage, subchondral plate and trabecular bone thicknesses, was performed to visualize possible differences between animals at the extremities of BM range (i.e. belonging to the Muridae family and to the Elephantidae family), however statistical analysis could not highlight any significant differences.
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<td>Cheetah (Acinonyx jubatus)</td>
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<td>Impala (Aepyceros melampus)</td>
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<tr>
<td>Red kangaroo (Macropus rufus)</td>
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<tr>
<td>Human (Homo sapiens)</td>
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<tr>
<td>Fallow deer (Dama dama)</td>
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<td>Gorilla (Gorilla gorilla)</td>
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<td>Siberian tiger (Panthera tigris)</td>
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<td>Reindeer (Rangifer tarandus)</td>
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<td>Lion (Panthera leo)</td>
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<td>Greater Kudu (Tragelaphus strepsiceros)</td>
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<td>Shetland pony (Equus ferus caballus)</td>
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<td>South American tapir (Tapirus terrestris)</td>
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<td>Watussi (Bos taurus taurus watussi)</td>
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<td>Dairy cow (Bos taurus)</td>
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<td>Rothschild’s giraffe (Giraffa camelopardalis)</td>
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<td>Horse (Equus ferus caballus)</td>
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<td>Banteng (Bos javanicus)</td>
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<td>White rhinoceros (Ceratotherium simum)</td>
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<td>Asian elephant (Elephas maximus)</td>
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<td>African elephant (Loxodonta africana)</td>
<td>4000</td>
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</table>

**TAB. 1.** Numbers of animals per species included in this study.
Contribution of each author

I.A.D. Mancini — investigation, formal analysis, visualization, writing - original draft, writing – review and editing, conceptualization, project administration

L. Rieppo — software, methodology, validation, visualization, writing – review and editing, formal analysis,

B. Pouran – software, writing – review and editing, investigation

I. O. Afara – methodology, software, writing – review and editing,

F. M. Serra Braganca – visualization, writing – review and editing, validation

M.H.P. van Rijen – investigation, writing – review and editing

M. Kik – investigation, resources, writing – review and editing

H. Weinans – methodology, resources

J. Toyras – methodology, writing – review and editing, resources, supervision

P. R. van Weeren – conceptualization, writing -original draft, writing – review and editing, resources, funding acquisition, supervision

J. Malda – conceptualization, writing -original draft, writing – review and editing, resources, funding acquisition, supervision
Graphical abstract

- Relatively thicker superficial layer
- Thinner trabeculae
- Lower bone volume fraction

Increasing body mass

- Relatively thinner superficial layer
- Thicker trabeculae
- Higher bone volume fraction
Highlights

- The deep zone is the cartilage layer that adapts to increasing body mass through isometric scaling.
- The superficial and middle cartilage layers become relatively thinner in larger animals.
- Subchondral bone plate characteristics did not change with body mass.
- As body mass increases, trabecular bone compensates with thicker trabeculae.