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Long bone growth and evolution revealed by three-dimensional imaging

JORDI ESTEFA

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Abstract
Estefa, J. 2020. Long bone growth and evolution revealed by three-dimensional imaging. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1910. 52 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0885-2.

Propagation phase-contrast synchrotron radiation microtomography is a non-destructive method used for studying histology in three dimensions (3D). Using it, the 3D organization of the diaphyseal cortical vascularization in the humerus of two seymouriamorphs was analyzed in this thesis. Their vascularization suggests a combination of active growth and a long pre-reproductive period, an intermediate condition between that of Devonian tetrapods and early amniotes, reflecting a gradual change in evolution. The focus of the thesis then shifts to the metaphysis of long bones. The latter possesses complex 3D structures difficult to capture in 2D images. Observations in extant tetrapods have shown that hematopoiesis in long-bones requires the presence of tubular marrow processes opening onto an open medullary cavity with a centralized vascular system. A network of tubular marrow processes was found in connection with interconnected small cavities in the metaphyses of seymouriamorphs which may have acted as open spaces containing a centralized vascular mesh. Based on this interpretation, the long-bone marrow cavity of the Permian stem-amniotes studied here could have been the oldest evidence of possible hematopoiesis among tetrapods. As a third focus, both computer simulations (Finite Element Analysis) and empirical experiments were conducted to investigate the role of Secondary Ossification Centers (SOCs) within the epiphyses of mammals. The results indicate that the presence of a SOC protects the growth plate from mechanical stresses, allowing the cells there to withstand six times more stress. Finally, the 3D microanatomy of the metaphyses and epiphyses in the humeri of monotreme, marsupial and placental extant mammals were investigated at different developmental stages. The data were used to produce a nomenclature based on the degree of epiphyseal ossification encompassing the entire development of all the condyles within a single epiphysis. This nomenclature was used to describe the epiphyseal development in a large group of mammals and highlight differences in ossification timing between groups. These results offer a unique glimpse into the development and evolution of long-bones. They highlight the value of visualizing long-bone microstructure in both 2D and 3D, and the need to develop new nomenclatures that reflect the 3D nature of the data.

Keywords: 3D histology, hematopoiesis, humerus, tetrapods

Jordi Estefa, Department of Organismal Biology, Evolution and Developmental Biology, Norbyv 18 A, Uppsala University, SE-75236 Uppsala, Sweden.

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Als meus pares

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Estefa, J.**, Klembara, J., Tafforeau, P., Sanchez, S. Limb-bone development of seymouriamorphs: implications for the evolution of growth strategy in stem amniotes. *In review in Frontiers in Earth Science*.

II **Estefa, J.**, Tafforeau, P., Clement, A.M., Klembara, J., Niedźwiedzki, G., Berruyer, C., Sanchez, S. New lights on the early evolution of limb-bone growth plate and bone marrow. *In review in eLife.*

III Xie, M., Gol'din, P., Nele Herdina, A., **Estefa, J.**, Medvedeva, E.V., Li, L., Newton, P.T., Kotova, S., Shavkuta, B., Saxena, A., Shumate, L.T., Metscher, B., Großschmidt, K., Nishimori, S., Akovantseva, A., Linares Arregui, I., Tafforeau, P., Fried, K., Carlström, M., Simon, A., Gasser, C., Kronenberg, H.M., Bastepe, M., Cooper, K.L., Timashev, P., Sanchez, S., Adameyko, I., Eriksson, A., Chagin, A.S. Secondary ossification center induces and protects growth plate structure. *In review in eLife.*

IV **Estefa, J.**, Bijl, S., Ponstein, J., Fernandez, V., Chinsamy, A., Tafforeau, P., Sanchez, S. Development of long-bone epiphyses in mammals. *Manuscript*.

Contents

Introduction

The bones of vertebrates provide the body with support and protection for internal organs. Bones also act as anchor points for the musculature and function as levers to facilitate locomotion and other activities. Whether fish fins, limbs, or wings, vertebrate appendages are essential for locomotion, as well as many other functions. Limb bones ossify very early in development and therefore record one of the most complete growth histories for the animal (Castanet et al.,1993). In addition, it has been demonstrated that skeletal elements greatly adapt to environmental conditions in order, for example, to resist biomechanical constraints (Castanet et al.,1993; Lieberman et al., 2001; deMargerie et al., 2005). Based on our knowledge of extant-vertebrate skeletal biology, the morphology, histology and microanatomy of fossil limb bones can be used to infer the life history, physiology, biology and lifestyle of fossil taxa (e.g., Botha, 2003; Germain & Laurin, 2005; Köhler et al., 2012; Köhler & Moyà-Solà, 2009; Voeten et al., 2018).

Long bones are those limb bones that are longer than they are wide. They are divided along the long axis into three parts, two of which are paired: a single diaphysis in the middle, flanked by a pair of metaphyses, which are flanked in turn by a pair of epiphyses that form the ends of the bone. The diaphysis forms the shaft of the bone (Francillon-Vieillot et al., 1990), and it is characterized by an exterior tubular cortical bone surrounding an inner marrow cavity. The metaphyses include growth plates, responsible for the elongation of the bone (Anderson & Shapiro, 2010; Nilsson, 2007). They are associated with trabecular bone, where red blood cells are produced (Wilson & Trumpp, 2006). The epiphyses usually constitute the articulations (Francillon-Vieillot et al., 1990), see “Anatomy of Long-bones”. They remain cartilaginous in most groups, but can ossify during the development of lizards, birds and mammals by means of a Secondary Ossification Center (SOC), (Haines, 1938, 1942).

Two types of growth take place in long bones, thickening and elongation. Thickening of the bone occurs by circumferential bone deposition, initially on top of existing cartilage and later on existing cortical bone (Francillon-Vieillot et al., 1990). Elongation of the bone takes place in the metaphysis through a process called endochondral ossification in which cartilage is replaced by bone (Amizuka et al., 2012; Mackie et al., 2008; Mackie et al., 2011). The ossification of the bone begins in the diaphysis (Francillon-Vieillot et al., 1990; Maes & Kronenberg, 2015). This region therefore rec-

ords the largest amount of cortical deposit (Castanet et al., 1993). For this reason, the thickening of the bone is commonly studied by means of cross-sections in the diaphysis (very often at mid-shaft, e.g., Castanet, 1985; Cubo et al., 2008; Montes et al., 2010; Sanchez et al., 2008). Diaphyseal histology and microanatomy are highly informative and can be used to determine not only the age and growth rate of the sample using skeletochronology (Castanet, 1985), but also aspects of the animal's lifestyle (Laurin et al., 2011).

Epiphyses and metaphyses however display many complex morphologies, even between proximal and distal ends from the same bone. They may also go through major modifications during ontogeny (see e.g., Watanabe & Matsuoka, 2013). All this variability requires more than just a single transverse section to compare even homologous bones from different species. In addition, the information retrieved by serial thin-sectioning is limited by the loss of material between sections, deformations during sectioning and, most importantly, shows only a fixed plane of view (Sutton et al., 2014). For all these reasons, the elongation of long bones has proved difficult to study by traditional sectioning techniques, and has not received a great deal of attention.

Propagation-based phase-contrast synchrotron radiation microtomography (PPC-SRμCT) is a relatively new technique developed for the study of histology in three-dimensions (Tafforeau et al., 2006). This method is non-destructive for fossils at the morphological and histological level, although it can degrade ancient biomolecules. Furthermore, it allows for the visualization of microanatomical structures in detail from every angle, both as virtual thin sections and as 3D models. It has been used to study different aspects of paleohistology, including long bone microstructure (Sanchez et al., 2012; Tafforeau et al., 2006; Tafforeau & Smith, 2008).

In this thesis these methods are used to investigate the diaphyses of rare or exceptionally preserved fossils in order to reconstruct their growth histories and processes (**Papers 1 and 2**). The use of PPC-SRμCT also provides new insights on the relationship between the elongation process and the development of bone marrow by visualizing metaphyseal 3D structures (**Paper 2**). Lastly, the role and development of the epiphyses, and particularly the centers of ossification within them, are investigated in **Papers 3 and 4**. This thesis therefore offers a unique glimpse into the development and evolution of long bones.

Phylogenetic context

The first four-limbed vertebrates (early tetrapods) evolved within lobe-finned fish (sarcopterygians) during the Devonian, more than 395 million years ago (Ma) (Ahlberg, 2019; Clack, 2012). Early tetrapods gradually adapted to more terrestrial lifestyles, and since then, the posture and gait of tetrapods has diversified in response to various biomechanical constraints and other evolutionary pressures. Today, tetrapods include amphibians and amniotes (reptiles, birds and mammals) (Figure 1).

Amphibians evolved from early tetrapods during the Lower Carboniferous (332 Ma) and rapidly diversified (Clack, 2012). Temnospondyls are a group of fossil amphibians that lived between the Carboniferous and the Cretaceous (approximately 332 to 120 Ma). Most temnospondyls were aquatic, but some of them were terrestrial (e.g., Reisz et al., 2009). Temnospondyls form a paraphyletic group, i.e., it includes their most recent common ancestor and part but not all of its descendants, in this case the crown amphibians (Anderson, 2008; Milner, 1988; Pardo et al., 2017; Ruta & Coates, 2007; Schoch & Milner, 2004; Schoch, 2018; Sigurdsen & Green, 2011; Trueb & Cloutier, 1991), but see Vallin & Laurin (2004) for an alternative view).

Crown amphibians, also called Lissamphibians, include modern amphibians, which are divided into three monophyletic groups: Anura (frogs and toads), Urodela (salamanders and newts) and Apoda (caecilians). As a group, crown amphibians are monophyletic (i.e., a group composed of all descendants of one common ancestor, also called a *clade*) in most phylogenies (e.g., Ruta & Coates, 2007; Vallin & Laurin, 2004), but some authors place Apoda outside of that clade (Anderson, 2007). Caecilians are characterized by the absence of limbs, and because limb bones are the focus of this thesis, their exact phylogenetic position is not relevant for the discussions presented here. For this reason, we use the term 'crown amphibians' interchangeably with 'batrachians', the clade that includes anurans and urodeles but not apodes, to refer to anurans and urodeles (**Paper 2**). Amphibians have often been used as examples of transitional forms between early aquatic and fully terrestrial tetrapods, largely due to their dependency on water for breeding and during their early stages of life (Duellman & Trueb, 1994). However, amphibians have many derived characters and one should take caution when using them as proxy models for understanding fossil tetrapods (see **Paper 2**).

Amphibians and amniotes branched around 332 Ma, during the Carboniferous (Benton & Donoghue, 2007). Amniotes gradually evolved a series of traits that made them less dependent on water compared to amphibians, including highly keratinized skin to protect from desiccation and allow them to occupy dryer areas; a stronger bite to feed on prey with strong skin; internal fertilization that could take place on land; more efficient kidneys that re-

duced water waste by excreting concentrated urine, or uric acid; and the production of eggs with a shell and an amnion that can be laid on land (Hickman et al., 2002). Some amniotes would later adapt secondarily to the aquatic environment (e.g., cetaceans and ichthyosaurs).

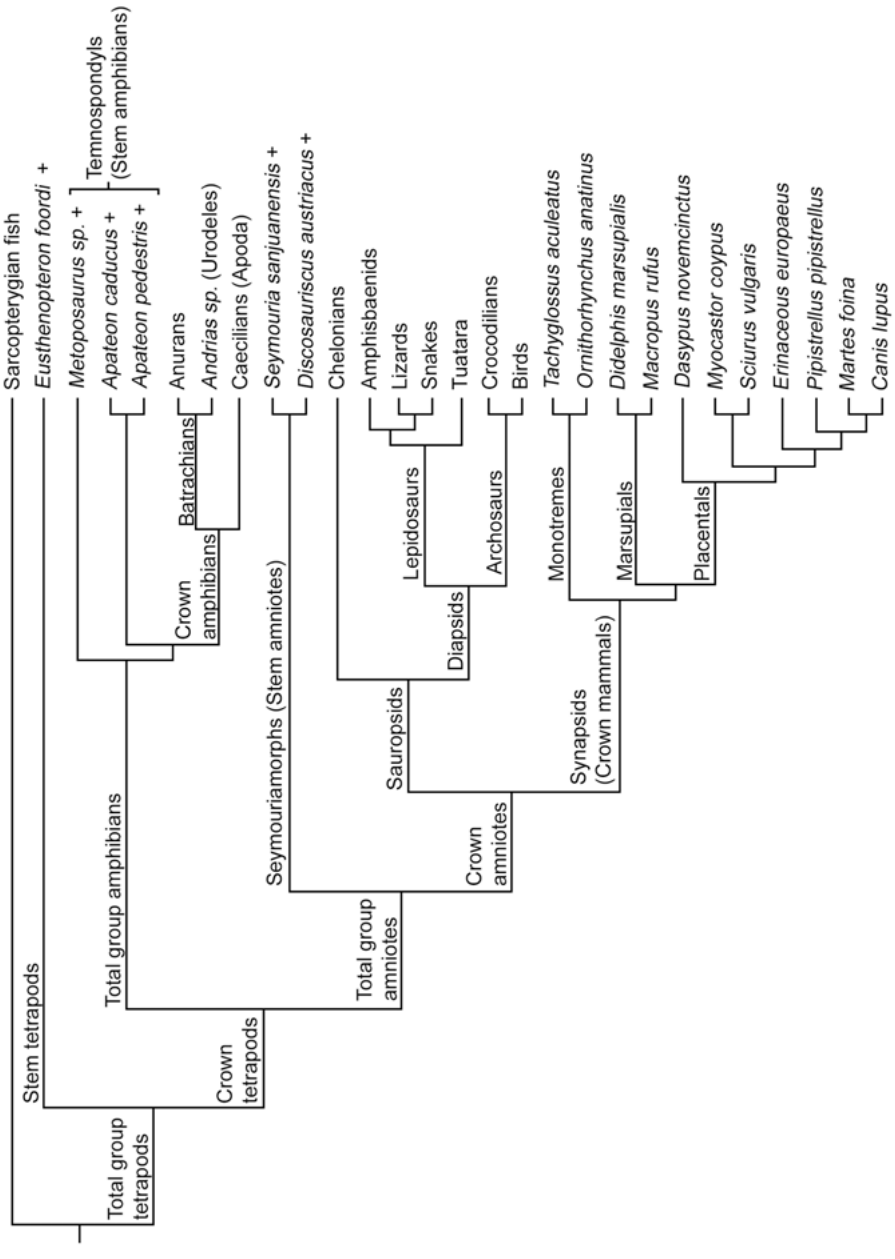

Figure 1. Phylogeny of tetrapods showing the species studied in this thesis. (Based on (Kemp, 2005; Ruta & Coates, 2007; Zheng & Wiens, 2016).

Seymouriamorphs were a group of stem amniotes (Anderson, 2008; Ruta & Coates, 2007; Sigurdsen & Green, 2011; but see Marjanovi & Laurin, 2013; Vallin & Laurin, 2004 for an alternative view) that lived during the Permian (252-298 Ma; Bulanov, 2003). A stem group consists of all fossil taxa that are more closely related to a crown group, of which they are not part, than to any other living species. Despite being closely related to crown amniotes, seymouriamorphs developed through an aquatic larval stage, like amphibians (Berman & Martens 1993; Klembara et al., 2001; Klembara et al., 2007; Klembara, 2009; Sanchez et al., 2008; White 1939), even in terrestrial species.

Early in their evolution, amniotes diverged into two main groups: the sauropsids (which includes reptiles and birds) and the synapsids (which includes mammals) (Hickman et al., 2002). The traditional view, based on morphological characters and used in Figure 1, states that sauropsids diverged during the Carboniferous (about 312 Ma) into what later evolved to become the chelonians (turtles and "tortoises") and the diapsids (Hickman et al., 2002). Molecular analyses, however, place Chelonia as sister group to Archosauria (Crawford et al., 2015). Despite being an ancient lineage, the characteristic shell of chelonians did not appear until the Upper Triassic (about 200 Ma) (Hickman et al., 2002). Diapsids include lepidosaurs (amphisbaenids, lizards, snakes and the tuatara) and archosaurs (crocodilians and birds), which diverged during the Permian (around 259-299 Ma) (Hickman et al., 2002).

Synapsids were predominant during the Permian and Triassic (Kemp, 2005). However, the only clade that survived until today are the mammals (Kemp, 2005). Modern mammals are divided into three groups: monotremes, marsupials and placentals. All mammals have hair, warm blooded metabolism, three bones in their middle ear and other derived traits, but their main apomorphy is that females feed their young with milk produced in mammary glands (Hickman et al., 2002).

There are only three genera of extant monotremes, one for the platypus (*Ornithorhynchus*) and two for the echidnas (*Tachyglossus* and *Zaglossus*), which are only found in Australia and New Guinea (Woodburne, 1984). Monotremes are the only mammals that lay eggs, while the rest are viviparous (embryonic development occurs inside the parent). Marsupials are characterized by giving birth to relatively less developed young and by having an abdominal pouch where they keep them as long as they are breastfeeding (Hickman et al., 2002). Marsupials are currently only found in North and South America and Australia (Woodburne, 1984).

The embryos of placentals develop in the uterus of the mother and are born more fully formed than those of marsupials (Hickman et al., 2002). This character has been traditionally thought to confer placental mammals an evolutionary advantage compared to marsupials and monotremes, which allowed them to spread all around the world (Hickman et al., 2002; Sears, 2004), but see Kirsch (1977). Despite forming two independent clades, pla-

centals and marsupials have evolved numerous traits in parallel (i.e., homoplasy or evolutionary convergence), such as dental replacement (Gomes Rodrigues et al., 2017), organization of the neocortex (Karlen & Krubitzer, 2007), gliding flight (Flaherty et al., 2008), and other specialized behaviors (Forget & Vander Wall, 2001). This makes them good comparative models for understanding the causes of their evolution.

Anatomy of long bones

Limbs are divided into three parts from their proximal to their distal end: stylopod, including the femur/humerus; zeugopod, including the tibia/radius and fibula/ulna; and autopod, including all the bones of the feet/hands (Francillon-Vieillot et al., 1990). The bones within each of these parts are subject to different stresses, and despite their morphological and developmental similarities, forelimbs and hindlimbs are not identical structures. Thus, one should be careful when comparing, for example, a humerus with a femur, even though both are stylopod elements.

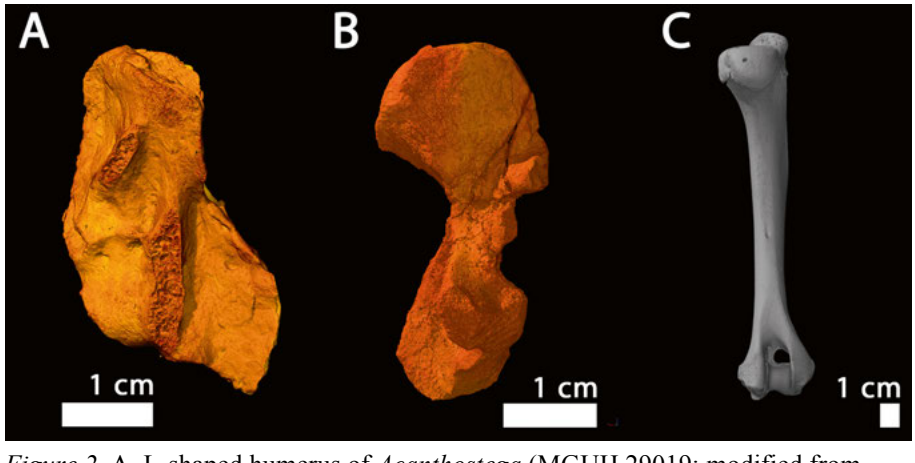

Figure 2. A, L-shaped humerus of *Acanthostega* (MGUH 29019; modified from Sanchez et al., 2016); B, Tetrahedral-shaped humerus of *Seymouria* (CM 28597); C, Tubular humerus of *Canis familiaris* (Dr. Paul Tafforeau's collection).

The general morphology of the humerus has changed greatly over time. The humeral morphology in the earliest tetrapods like *Acanthostega*, and even in later stem tetrapods is "L-shaped" (Figure 2A; Hall, 2006; Sanchez et al., 2016). After the water-to-land transition, the first truly terrestrial tetrapods evolved a "tetrahedral-shaped" humerus consisting of two roughly fan-like flat surfaces connected to each other at their "heads" with a rotation of 45-50° angle (Figure 2B). This morphology allows for greater flexion of the

elbow and situate zeugopod and stylopod elements at approximately 90° angle to each other (Hall, 2006). Tetrahedral-shaped humeri are present in the stem groups of amphibians (e.g., *Eryops*; Miner, 1925) and amniotes (seymouriamorphs; Klembara & Bartik, 1999; Klembara et al., 2001) (**Papers 1 and 2**). Both amphibians and amniotes convergently evolved more tubular humeri in later forms (Figure 2C), although they may further derive in a different morphology, as can be seen in echidnas (Jenkins, 1970) and turtles (Nakajima et al., 2014).

Regardless of their shape, long bones have three main parts mirrored from midshaft in their longitudinal axis: the diaphysis, the metaphyses, and the epiphyses (Francillon-Vieillot et al., 1990). The diaphysis constitutes the largest and central region of a long bone, and is composed by the medullary cavity and the cortex (Figure 3A). The cortex, or cortical bone, refers to the perichondral and periosteal bone deposit forming the outer layer of bone (Francillon-Vieillot et al., 1990). Cortical bone is commonly studied through transversal sections, where a type of growth marks called Lines of Arrested Growth (LAGs) may be visible (Castanet et al., 1993; Francillon-Vieillot et al., 1990). LAGs form a series of concentric rings that mark complete pauses in the thickening of the bone (Castanet et al., 1993; Francillon-Vieillot et al., 1990). Fluctuations in growth rates in which growth does not fully stop produce thicker growth marks: zones and annuli. These areas are distinguished by the organization of the bone matrix fibers, with zones having woven, or parallel fibers, and annuli being made of lamellar bone (Castanet et al., 1993).

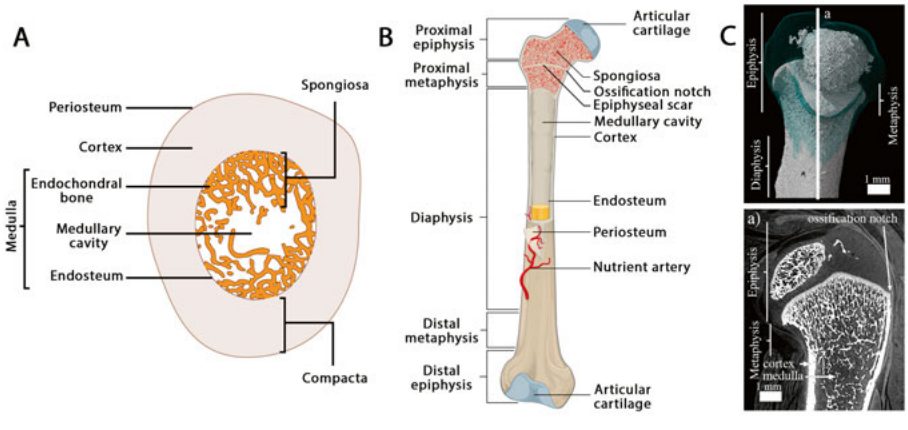

Figure 3. Anatomy of a long bone in, A, transversal section and, B, longitudinal section. C, shows the proximal humeral epiphyses of a nine-banded armadillo (*Dasypus novemcinctus*) in 3D (top) and virtual thin section (bottom) (see Paper 4). B is modified from OpenStax College CC BY 3.0 https://creativecommons.org/licenses/by/3.0/deed.en.

The cortex progressively becomes thinner towards the epiphysis, up to the point of the ossification notch, the limit where the cortical bone ends between the shaft and the epiphysis (Francillon-Vieillot et al., 1990) (Figure 3B and C). A network of vascular canals extends within the cortical bone where it plays a major role during the bone growth (Francillon-Vieillot et al., 1990). The medullary cavity is the inner space of the bone, which is in-filled with bone tissue, including endochondral and endosteal bone (Francillon-Vieillot et al., 1990). Compacta and spongiosa refer to the general porosity of the bone (Francillon-Vieillot et al., 1990; Parfitt, 1983) (Figure 3A and C). When the bony tissue occupies more volume than the pores, we describe the tissue as compacta, and when the volume occupied by the pores exceeds the volume occupied by the bony tissue, we refer to it as spongiosa (Parfitt, 1983).

The metaphyses are located at each end of the shaft (Figure 3B and C). They are responsible for the elongation of the bone and also host Hematopoietic Stem Cells (HSC) for the production of blood cells (hematopoiesis) (Wilson & Trumpp, 2006). The elongation process and the histology of the metaphyses are explained in more detail in the section "Long-bone development" and in **Paper 2**. Hematopoiesis takes place in the bone marrow of most tetrapods, but not in aquatic vertebrates (Kapp et al., 2018), where it remains in the thymus, liver, spleen and kidney (Akiyoshi & Inoue, 2012; Hightower & Pierre, 1971). Sanchez et al. (2014) interpreted a series of longitudinally oriented tubes in the medullary cavity of the humerus of *Eusthenopteron*, a fish member of the tetrapod stem group, as evidence for bone marrow processes. Based on this, it has been hypothesized that the migration of hematopoiesis into the bone marrow happened previous to the water-to-land transition (Kapp et al., 2018). In this light, bone would protect hematopoietic cells from pronounced temperature changes (Weiss & Wislocki, 1956) or mutations caused by UV light radiation (Kapp et al., 2018), or it would increase the efficiency of red blood cell production to cope with the higher physiological demands of aerial respiration (Tanaka, 1976).

The epiphyses occupy the extremities of the bone beyond the metaphyses, (Francillon-Vieillot et al., 1990) and comprise one or more condyles in the case of convex articulations (see **Paper 4**). Usually the epiphyses are part of the articulations, but that is not always the case. For example, the proximal epiphysis of the ulna of the short-beaked echidna constitutes the tip of the olecranon, while the articulation with the distal epiphysis of the humerus occurs at the shaft (Figure 4). The epiphyses of adults may ossify or remain cartilaginous depending on the species (Haines, 1938, 1942). When articular epiphyses ossify, they are capped by an articular cartilage (Francillon-Vieillot et al., 1990). In fossils, cartilaginous epiphyses are not preserved and thus the ends of a fossilized long bone may not correspond to the ends of the bone when the animal was alive, but instead to the ossification front of the shaft (Tsai & Holliday, 2015).

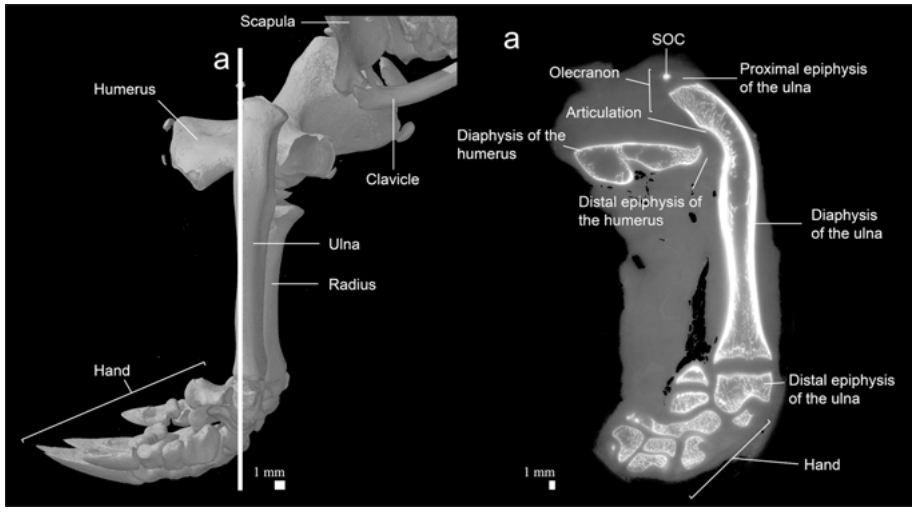

Figure 4. Virtual reconstruction (left) and thin section (right) of the right arm of a short-beaked echidna (*Tachyglossus aculeatus*).

Long bone development

Standard (mammalian) model

Due to its relevance in medicine, the study of the long bone development has focused in great part on the mammalian skeleton. Most descriptions have been made for medical purposes (e.g., Galić et al., 2016; Koshino, 1975; O'Connor et al., 2008; Serrat et al., 2007; Stevenson, 1924), although others have focused in commercial or conservational purposes (e.g., Carden & Hayden 2006; Breugelmans et al., 2007; Zeder et al., 2015). The prevalence of the mammalian model in the literature has made it the default and standard model of long bone development (e.g., Hickman et al., 2002; Kardong, 1998; Romer & Parsons, 1977).

At early ontogenetic stages the entire skeleton is cartilaginous, and at this point long bones are just rod-shaped anlagen yet to be ossified (Mackie et al., 2008; Maes & Kronenberg, 2015; Pazzaglia et al., 2011; Rivas & Shapiro, 2002) (Figure 5A). The ossification of a rod begins with deposition of bone tissue around the mid-shaft (Figure 5B). This first perichondral deposition (bone tissue on top of cartilage) is immediately followed by periosteal deposition (Mackie et al., 2008; Maes & Kronenberg, 2015). Periosteal deposition produces the thickening of the bone and typically continues until late ontogenetic stages (Mackie et al., 2008; Maes & Kronenberg, 2015), to accommodate for the increase in size of the individual. This layer of bone is

the cortex, while the inner space is delimited by what will become the medullary cavity (Francillon-Vieillot et al., 1990).

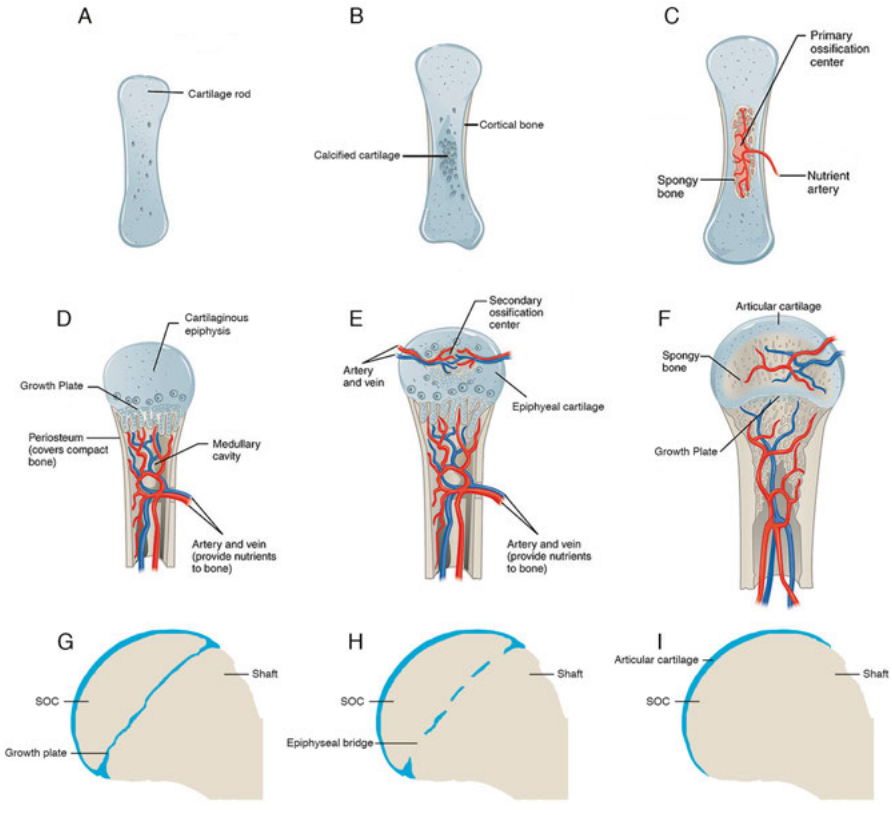

Figure 5. Cortical and endochondral ossification sequence of an idealized mammalian long bone. (A-F have been modified from OpenStax College CC BY 3.0 https://creativecommons.org/licenses/by/3.0/deed.en.).

After perichondral ossification begins, the cartilage in the core of the rod calcifies (Mackie et al., 2008; Maes & Kronenberg, 2015) (Figure 5B). Blood vessels then invade the cartilage at mid-shaft (Mackie et al., 2008; Maes & Kronenberg, 2015) (Figure 5C). The blood vessels become embedded in the cortical bone as it thickens, but are distinguishable from cortical vascularization because they pierce the cortex completely, connecting the inner and outer parts of the bone (Mackie et al., 2008; Maes & Kronenberg, 2015). The first and biggest of these canals is called the nutrient foramen, and contains the nutrient artery, which brings cells into the rod that will erode the cartilage (i.e., chondroclasts) and deposit bone tissue on the surface of the cartilage remnants (i.e., osteoblasts) (Mackie et al., 2008; Maes & Kronenberg, 2015).

The initial bony structure formed through this ossification process is called the Primary Ossification Center (POC; Figure 5C) (Mackie et al., 2008; Maes & Kronenberg, 2015). As the POC expands, a growth plate (or physis) forms at each of its extremities, separating the diaphysis and metaphyses from the epiphyses (Figure 5D; Mackie et al., 2008; Maes & Kronenberg, 2015). The ossification notch, however, does not necessarily correspond with the extent of the ossification front at the center growth plate (see **Paper 4**). Each growth plate is composed of a series of layers of chondrocytes (embedded cartilage cells) organized by degree of maturity (Nilsson, 2007; Anderson & Shapiro, 2010), which are responsible for the endochondral ossification and elongation occurring at the end of the shaft.

A growth plate is divided into three zones based on the type of the cells present: the resting zone, the proliferative zone and the hypertrophic zone (Figure 6; Anderson & Shapiro, 2010; Nilsson, 2007). Closest to the epiphysis, there is the resting zone, where mesenchymal stem cells produce chondrocytes. These chondrocytes accumulate organized into columns, forming the proliferative zone. The bone elongates as a result of the rapid multiplication of the stacked chondrocytes pushing the epiphysis away from the diaphysis.

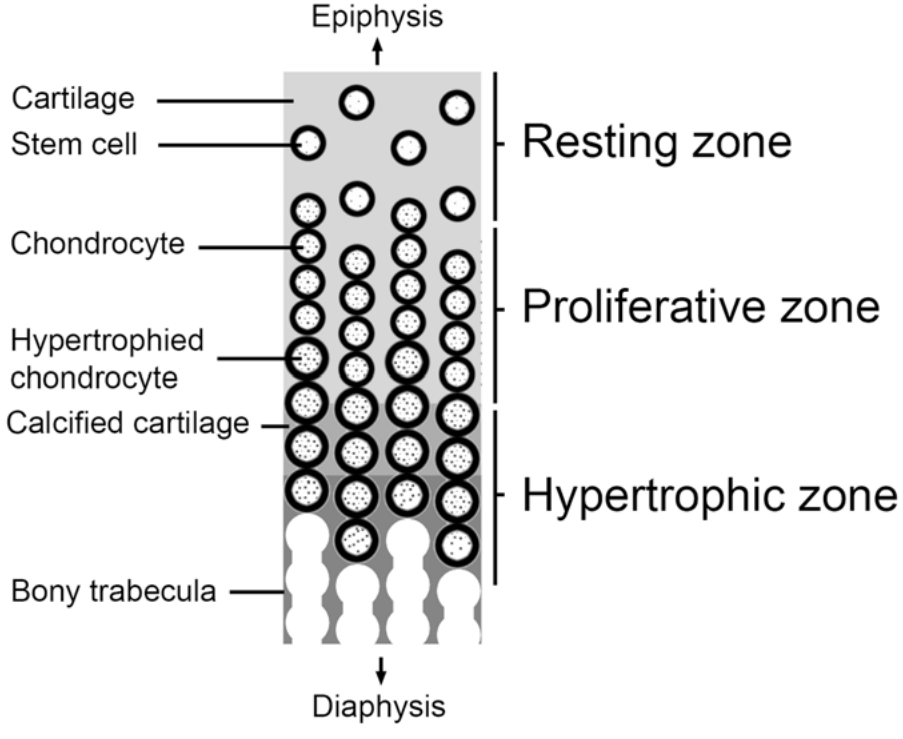

Figure 6. Zones of the growth plate.

The oldest chondrocytes of the proliferative zone secrete cartilage matrix. During this process they hypertrophy by gradually increasing in volume, marking the beginning of the hypertrophic zone. The cartilage matrix closer to the diaphysis calcifies and is later eroded and substituted by bone. The bone deposition forms trabeculae in the longitudinal orientation of the hypertrophied chondrocyte columns. The first deposits of bone form a network of trabeculae known as "primary spongiosa", while the new trabeculae formed within the marrow cavity through remodeling processes constitute the "secondary spongiosa" (Francillon-Vieillot et al., 1990).

In mammals, the epiphyses ossify by the formation of Secondary Ossification Centers (SOCs) within the epiphyseal cartilage (Figure 5E). The cells of SOCs and POCs follow the same series of transformations but the trabecular mesh of SOCs exhibit a reticular organization, due to the lack of a clear columnar organization of the chondrocytes (Byers & Brown 2006; Pazzaglia et al., 2011). A SOC within an epiphysis expands centrifugally until it occupies the entire volume of the epiphysis, except by the articular cartilage (Figures 5F, G). A single epiphysis may develop one or more SOC, typically one for each condyle, that later in development will merge into a single unit (see **Paper 4**).

When the cells of the resting zone exhaust their capacity to form new chondrocytes the elongation of the bone stops (Roach et al., 2003). Once the growth plate ceases its activity, the SOC fuses gradually with the shaft (Parfitt, 2002). The fusion between the SOCs and the shaft begins with the creation of "epiphyseal bridges" of bone scattered along the growth plate (Haines, 1975) (Figure 5H). These bridges are bone depositions connecting epiphyseal to metaphyseal trabeculae. The bridges expand and eventually the totality of the space between the SOC and the shaft is substituted by transversally oriented trabeculae, forming a visible line called "epiphyseal scar" (Haines, 1975). In some groups, remodeling processes occurring later in ontogeny may remove any trace of the epiphyseal scar, thus erasing the evidence of the growth plate (Figure 5I).

Deviations in other groups

The shaft of ray-finned fishes (actinopterygians) grows by perichondral deposition and their cartilage anlage is substituted via endochondral ossification in the absence of a growth plate (Sanchez et al., 2014; Witten & Huysseune, 2007). In adult ray-finned fishes the diaphyseal cartilage can be eroded in such a way that the endochondral bone is organized in a ladder-like configuration (Sanchez et al., 2014). On the other hand, the Devonian lobe-finned fish *Eusthenopteron*, a member of the tetrapod stem group, shows longitudinally-oriented trabeculae along the shaft which suggests that it already had a growth plate similar to the one of modern taxa (Sanchez et al., 2014). The

growth plate then, seems to have appeared within the evolution of sarcopterygians.

There exist a number of variations in the development of the growth plate between the different groups of tetrapods. In amphibians the hypertrophied chondrocytes of the growth plate do not organize into columns (Castanet et al., 2003). Instead, the chondrocytes organize in scattered bundles, so the ossification process produces a reticular mesh of bony trabeculae leaving behind large empty cavities as a byproduct (de Ricqlès, 1964, 1965; Quilhac et al., 2014). This type of mineralization pattern is called globular calcification as is characterized by the formation of *Globuli ossei* (Francillon-Vieillot et al., 1990). *Globuli ossei* are spherical masses of mineralized tissue formed either by modified hypertrophied chondrocytes or by marrow cells that migrate into spaces previously occupied by now dead hypertrophied chondrocytes (Quilhac et al., 2014).

In chelonians, the cartilage rod first calcifies locally at the limiting zone between the periosteal bone and the cartilaginous epiphysis (Haines, 1938). There, canals pierce the cortex and form accessory erosion rooms, where endochondral bone is formed. In crocodiles, the calcification and later endochondral ossification starts in cavities originated from the diaphysis around the cartilage cone (remnants of the original cartilaginous anlage in the metaphyses), not from canals piercing the cortex (Haines, 1938).

In stem mammals like *Dicynodon* (Haines, 1938) the trabeculae at the metaphysis are oriented radially suggesting the presence of a growth plate in the living animal. Later in ontogeny, in most species, this trabecular organization is remodeled, thereby causing the disappearance of the longitudinal arrangement of the spongy network.

The epiphyses remain cartilaginous in chelonians, crocodilians, urodeles, actinopterygians, Devonian fish and stem mammals (Haines, 1938, 1942; Sanchez et al., 2014). The epiphysis of urodeles remain cartilaginous, but in anurans calcifies in later ontogenetic stages, doing so in a characteristic match-head configuration (Haines, 1942). The epiphysis of the temnospondyl *Apateon* resembles those of urodeles (Sanchez et al., 2010a). SOCs can be found at the epiphyses of mammals, lepidosaurs and birds (Haines, 1942; Hogg, 1980). SOCs seem to have evolved independently in the three groups (Figure 7), as Paleozoic taxa also lack ossified epiphyses (as seen in e.g., Haines, 1938; Liebe & Hurum, 2012; Rogers et al., 2016).

In birds, epiphyseal ossification in most bones occurs by the gradual invasion of the growth plate into the space previously occupied by the epiphysis (see images in Breugelmans et al., 2007). The epiphysis is technically reduced to a layer of articular cartilage, but the mature condition is morphologically equivalent to that achieved through a SOC. The only real SOCs in birds are found exclusively at the proximal epiphysis of the tibia (**Paper 3**, Hogg, 1980). Bird epiphyses are pierced by numerous long and longitudinally oriented cartilage canals going from the perichondrium to the marrow

cavity (**Paper 3**). The endochondral ossification in the proximal epiphysis of the tibia starts around those cartilage canals (Haines, 1969).

Within lepidosaurs, the tuatara (genus *Sphenodon*) does not develop a SOC, but instead the cartilage of the epiphysis calcifies internally forming a center of calcification (Haines, 1939). At the adult stage, the calcified cartilage can be destroyed and replaced by extension of the marrow cavity (Haines, 1939). The epiphyses of lizards retain some similarity to those of the tuatara but their calcified cartilage is eventually replaced by endochondral bone. Endochondral ossification occurs around cartilage canals connected to the perichondrium (Haines, 1941).

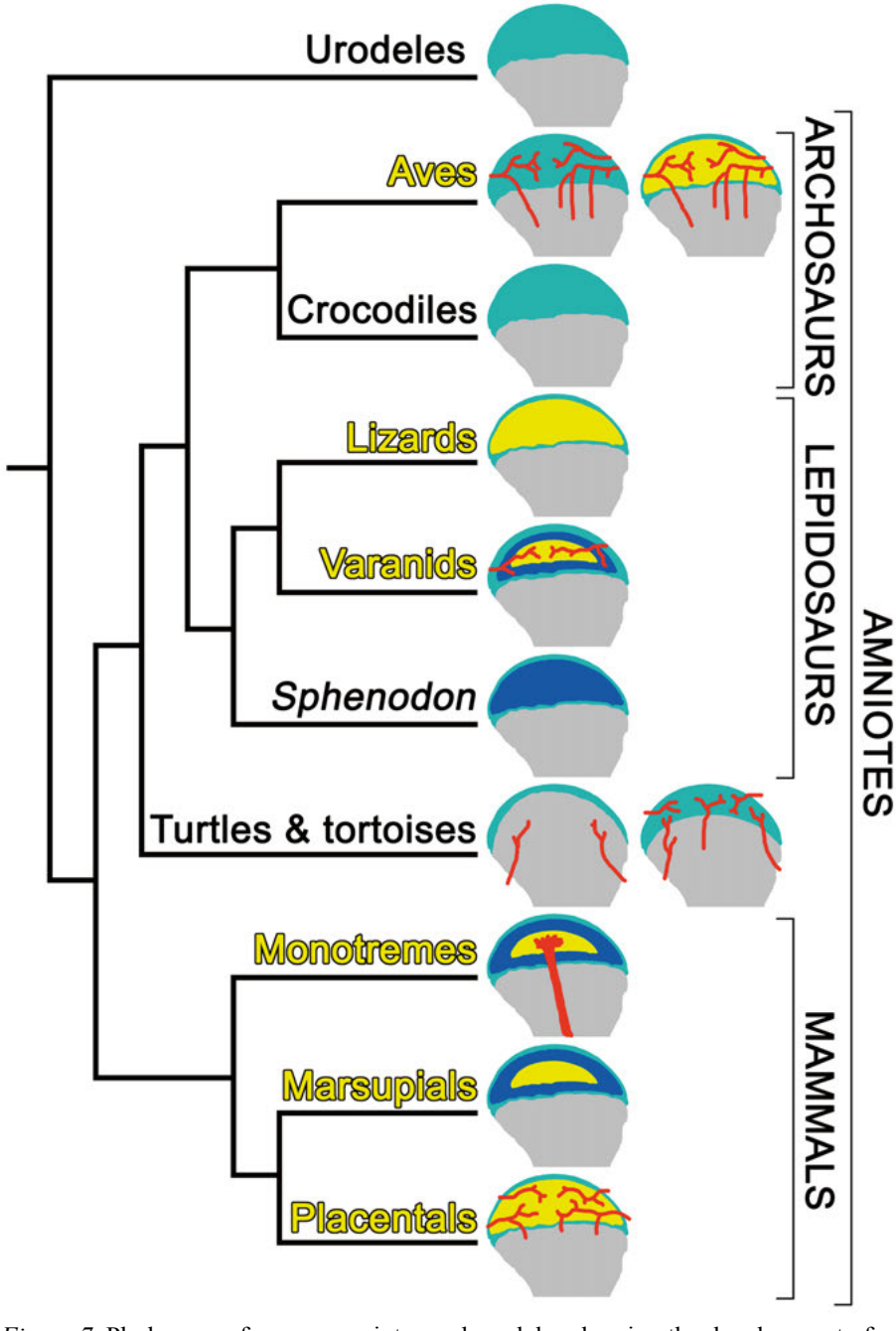

Figure 7. Phylogeny of crown amniotes and urodeles showing the development of the epiphysis. The names of the groups that do not ossify their epiphyses are in black and the ones that develop SOCs are in yellow. Cartilage is shown in light blue, calcified cartilage in deep blue, the metaphysis is shown in gray, SOCs are shown in yellow, and the vascularization is shown in red.

Aims of research

The overall aim of this thesis is to expand our current knowledge of long bones during the evolution of terrestrial tetrapods.

Paper 1 provides new three-dimensional (3D) descriptions of the diaphyseal histology of two species of stem amniotes (seymouriamorphs) in order to investigate if the changes in developmental strategies in juvenile tetrapods occurred abruptly or gradually during terrestrialization.

Paper 2 provides descriptions of the metaphyseal histology and microanatomy of stem amniotes (seymouriamorphs) and stem amphibians (temnospondyls) to understand the evolution of the growth plate in tetrapods. It also investigates the evolution of hematopoiesis in long bones.

Paper 3 tests the hypothesis that SOCs evolved with a protective role towards the growth plate and discusses its implications for the evolution of amniote locomotion outside of water.

Paper 4 presents a new nomenclature for standardizing the different developmental stages of an ossifying epiphysis based on the descriptions of new 3D models of the metaphyses and epiphyses of eleven species of mammals.

Materials

Below is a summary of the living and fossil tetrapod taxa presented in this thesis that I have worked with. Their phylogenetic relationships are shown in Figure 1.

Sarcopterygian (Paper 3)

The humerus of *Eusthenopteron* NRM P248d (adult), from the Late-Devonian locality of Miguasha, Quebec, Canada. It belongs to the collection of Naturhistoriska Riksmuseet in Stockholm.

Temnospondyls (Paper 2)

The humerus, ulna and radius of two specimens of *Apateon caducus*, GPIM-N 1297 (juvenile; ulna and radius) and GPIM-N 1572 (adult; humerus), from the Carboniferous-Permian locality of Erdesbach, Saar Nahe Basin, Germany. They belong to the collections of the Staatliches Museum für Naturkunde (Stuttgart, Germany).

The humerus, ulna and radius of two adult specimens of *Apateon pedestris* (SMNS 54981 and SMNS 54988), from the Carboniferous-Permian locality of Odernheim, Saar Nahe Basin, Germany. They belong to the collections of the Staatliches Museum für Naturkunde (Stuttgart, Germany).

The humerus of an adult specimen of *Metoposaurus sp*. (unregistered), from the Late Triassic locality of Skarszyny in southern Poland. The specimen belongs to the collections of the Institute of Paleobiology, Polish Academy of Sciences (Warsaw, Poland).

Seymouriamorphs (Papers 1, 2 and 3)

The humeri of two specimens of *Seymouria sanjuanensis*, MNG 7747 (juvenile) and CM 28597 (adult). MNG 7747 from the Tambach Formation, Bromacker locality, Lower Permian of Central Germany, belongs to the collections of the Museum der Natur (Gotha, Germany); and CM 28597 from the Cutler Formation, Lower Permian of North-Central New Mexico, USA, belongs to the Carnegie Museum of Natural History (Pittsburgh, USA).

The humerus of a subadult specimen of *Discosauriscus austriacus*, SNM Z 15568, from the Lower Permian of Kochov-Horka in the Czech Republic,

belongs to the collections of the Slovak National Museum in Bratislava (Bratislava, Slovakia).

Mammals (Papers 3 and 4)

Two specimens of short-beaked echidna (*Tachyglossus aculeatus* MNHN 1901-379 and MNHN 1903-537), a platypus (*Ornithorhynchus anatinus* MNHN 1884-1119), two specimens of common opossum (*Didelphis marsupialis* MNHN 1992-398 and MNHN 2000-218), and four specimens of nine-banded armadillo (*Dasypus novemcinctus* MNHN1992-6 (1), MNHN1992-6 (2), MNHN2001-1530 and MNHN1998-2255), belong to the vertebrate collection of the Muséum national d'Histoire naturelle of Paris (MNHN).

The humerus of a red kangaroo (*Macropus rufus*), a coypu (*Myocastor coypus*), a red squirrel (*Sciurus vulgaris*), a European hedgehog (*Erinaceous europaeus*), a common pipistrelle (*Pipistrellus pipistrellus*), a beech marten (*Martes foina*), a dog (*Canis lupus familiaris*) and a Eurasian wolf (*Canis lupus lupus*), belong to the private collection of Dr. P. Tafforeau.

Methods

2D histology

Classical thin sectioning and optical microscopy allow for the study of histological and microanatomical structures (Castanet et al., 1993; Francillon-Vieillot et al., 1990). Thin-sectioning was used on the bones of the *Apateon* specimens in **Paper 2** and on mice and rats in **Paper 3**. The main advantages of using thin sections are its relative cheapness (although valuable samples are cut), high-resolution (limited by optics and wavelength), and the possibility of using different types of light (through the use of different filters, light sources, optics and light direction) to highlight different structures within the sample, for example, by using the refringent property of LAGs to visualize them with polarized light. Although in some cases especially thick sections can reflect the 3D organization of some tissues, for the most part, thin sections suffer the major disadvantage of being limited to two-dimensional information and overall 3D patterns must be inferred and contextualized from multiple sections.

3D histology

Researchers first tried to overcome the limitations of classic histology by reconstructing 3D models from stacked serialized 2D images (tomograms). Initially, this was achieved by physical-optical tomography, a time consuming and destructive technique that is limited in its resolution by the thickness of the sections (Sutton et al., 2014). The need for a non-destructive technique that allows the visualization of the internal anatomy of a given sample led to the eventual development of X-ray computed tomography (X-ray CT). This technique uses X-rays to map differences in density within the scanned sample (Sutton et al., 2014). Tafforeau et al. (2006, 2007) and Tafforeau & Smith (2008) developed propagation-based phase-contrast synchrotron-radiation microtomography (PPC-SRμCT) for its application in paleontology and dental paleohistology at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. Virtual bone paleohistology based on PPC-SRμCT was developed thereafter (Sanchez et al., 2012). This technique was used to produce the 3D data in the four papers included in this thesis.

A synchrotron is a particle accelerator wherein the electric field accelerating the electrons and the magnetic field used to keep them in a closed loop are synchronized with the moving particles (Sutton et al., 2014). This process generates high-energy X-rays used to scan samples at high-resolution (Sutton et al., 2014). Furthermore, the contrast between the different structures scanned increases with the propagation distance of the beam, which can be used to highlight differences between the rock matrix and the bone in fossil samples (**Papers 1, 2 and 3**; Tafforeau et al., 2006). Higher resolutions imply smaller fields of view, which in turn limits the size of the samples scanned. While it is possible to concatenate several scans done at small fields of view, in practice they will take too much time for covering a large sample such as an echidna humerus for example. To solve this issue, we employed a multiscale approach (Tafforeau et al., 2007; Sanchez et al., 2012) consisting in scanning the whole sample at relatively low-resolution (voxel size: 7-25 μm) and using that scan to detect optimal locations to scan at higher resolutions (voxel size: 0.7-3 μm). This approach was used to study the cortical vascularization of both specimens of *Seymouria sanjuanensis* (**Papers 1 and 2**).

The X-ray tomogram series produced are converted into 3D datasets that are segmented and analyzed using a specialized software called Volume Graphics Studio Max (VGStudio Max v2.4, Volume Graphics, Germany). The data were segmented into regions of interest based on a given range of grey values for structures with clear boundaries, including the cortex and trabeculae of bony tissue. Some structures were more difficult to segment (e.g., vascular canals and cartilage tissue) and in these instances boundaries were drawn freehand. This type of segmentation introduces errors by making surfaced appear striated from the parallel strokes used to hand-select them. As such, this technique was only used for structures where relative position was important, not surface morphology.

Other techniques

Paper 3 is a collaborative effort between almost thirty people from different institutions, as such I did not perform many of the experiments presented on **Paper 3**. To maintain the focus on my contributions I centered this thesis on 3D virtual bone histology. Some other of the more complex techniques used are briefly summarized here for easier understanding of **Paper 3**.

Atomic Force Microscopy (AFM) combs the surface of the sample with a mechanical probe, reaching resolutions much higher than those obtained with optical methods. This technique was used to measure the stiffness of the hypertrophied chondrocytes of the growth plate.

Axitinib is an inhibitor of the vascular endothelial growth factor (VEGF) receptor that delays the development of SOCs. We used it to do the pharmacological and genetic manipulations to test the role of SOCs *in vivo*.

Finite Element Analysis (FEA) is a mathematical method used to solve problems in engineering by reducing a complex geometry into a finite number of elements with simple geometries. FEA was employed in **Paper 3** to simulate the distribution of stresses along 2D models of four epiphyseal conditions.

TUNEL detects DNA fragmentation from apoptotic cells. It was used to test whether the death of chondrocytes was due to mechanical damages or induced apoptosis.

Results and discussion

Limb-bone development of seymouriamorphs: implications for the evolution of growth strategy in stem amniotes (Paper 1)

Paper 1 investigated the 3D histology of three specimens of seymouriamorphs from two different species: *Discosauriscus austriacus* and *Seymouria sanjuanensis*. *Discosauriscus austriacus* has been interpreted as an aquatic animal during its juvenile stage, but it becomes more terrestrial in adulthood (Klembara et al., 2001; Klembara, 2009; Sanchez et al., 2008). *Seymouria sanjuanensis*, on the other hand, is considered one of the earliest fully terrestrial tetrapods, probably born in an aquatic environment but being already independent of it in juveniles (Berman & Martens, 1993; Klembara et al., 2007; White, 1939).

The humeri of *S. sanjuanensis* develop through endochondral ossification in the manner of extant amniotes, as indicated by the presence of a compact cortex, longitudinally-oriented trabeculae and Liesegang's rings (indicating remnants of calcified cartilage). The remnants of calcified cartilage, although present in both specimens, are more numerous in the juvenile than in the adult, where the trabeculae are oriented in a more reticular manner, suggesting active remodeling. The cortical thickness remains constant through ontogeny, which is explained by a combination of fast periosteal deposition with intense erosion. This active growth dynamic does not allow for LAGs to be preserved, making it impossible to perform a skeletochronological study. This turned our attention towards the cortical vascularization, composed of numerous canals.

Discosauriscus austriacus has a thick cortex with numerous LAGs and no Liesegang's rings in the spongiosa. Although its outermost cortical layer is not very vascularized, the innermost cortex exhibits a large volume of vascularization and round-shaped bone cell lacunae. All these characteristics support the conclusion that *D. austriacus* had faster bone deposition rate than previously thought.

When put in an evolutionary context and compared to Devonian stem tetrapods and Paleozoic temnospondyls of similar size, all with slow bone deposition patterns, seymouriamorphs seem to represent a first step towards the

faster bone growth found in early and modern amniotes. However, the LAGs of *D. austriacus* show a long pre-reproductive period, unlike modern amniotes (Sanchez et al., 2008) but similarly to the Devonian stem tetrapods (Sanchez et al., 2014, 2016) and Permian temnospondyls (Ricklefs, 2010; Sanchez et al., 2010a, 2010b). This is interpreted as an indication that the transition towards the amniote-like fast development strategy was not a drastic event but happened gradually along the evolution of amniotes.

New lights on the early evolution of limb-bone growth-plate and bone marrow (Paper 2)

Hematopoiesis in aquatic vertebrates takes place in the thymus, liver, spleen and kidney (Akiyoshi & Inoue, 2012; Hightower & Pierre, 1971; Kapp et al., 2018). However, in terrestrial tetrapod groups the hematopoietic function has moved, at least in part, to the bone marrow of long bones (Kapp et al., 2018; Tanaka, 1976; Weiss & Wislocki, 1956). This shift is believed to have taken place as an adaptation to the water-to-land transition (Kapp et al., 2018). It has been proposed that the migration of hematopoiesis into long-bones was necessary to cope with the increased blood production required for the more metabolically demanding aerial respiration during terriestrial locomotion (Tanaka, 1976). Others think that it evolved to protect hematopoietic stem cells from UV light (Kapp et al., 2018) and/or changes in temperature (Weiss & Wislocki, 1956). Sanchez et al. (2014) described a series of longitudinal tubes crossing the entire marrow cavity of the humerus in *Eusthenopteron*, a fish member of the tetrapod stem group, and identified them as marrow processes. Based on the assumption that these marrow processes could have potential hematopoietic function, Kapp et al. (2018) concluded that hematopoiesis in long bones was an exaptation (i.e., a trait that changes function during evolution) of the marrow processes to terrestriality.

In order to further understand the evolution of long bone hematopoiesis, we identified, segmented and compared the marrow processes within the metaphyseal trabecular mesh of two stem amphibians (*Apateon* and *Metoposaurus*) and the two stem amniotes described in **Paper 1** (*Seymouria* and *Discosauriscus*). Observations of the bone marrow and humeral microarchitecture in extant-tetrapod long bones showed that a compartmentalization of the trabecular mesh favours a regionalization of the vascular network that prevents hematopoiesis in the medullary cavity of long bones whereas an open medullary cavity with a centralized vascular mesh permits hematopoiesis (Tanaka, 1976). Our observations of the 3D humeral microarchitecture of stem tetrapods, batrachians and amniotes show that Permian tetrapods (such as *Seymouria* and *Discosauriscus*) seem to be among the oldest tetrapods to

exhibit a centralized marrow organization allowing hematopoiesis as in extant amniotes.

Secondary ossification center induces and protects growth plate structure (Paper 3)

For this ambitious project we sought to understand the role of Secondary Ossification Centers (SOCs) in relation to the growth plate. My contribution was to provide evolutionary context and information on fossil specimens, and to help develop prerequisite conditions for Finite Element Analysis (FEA) simulations.

Development of SOCs in long bones of jerboa, mice and rats coincides with their transition from crawling to walking, a time when the epiphyses are subject to increasingly higher mechanical stresses. Similarly, some bats develop the SOCs of their thumb and foot phalange earlier than those of other bones, coinciding with their need to cling to their mother or the roost. The inverse process, but at an evolutionary scale, can be observed in cetaceans during their secondary adaptation to water: as they become less subject to gravity, the SOCs decrease in size. These observations suggest that biomechanical constraints seem to determine the expression of SOCs.

In order to test this hypothesis, FEA was performed in four theoretical 2D models of epiphyses from tetrapods that included, (1) an epiphysis with no SOC and non-oriented growth-plate organization, like in fetal amniotes and urodeles; (2), an epiphysis without SOCs but with metaphyseal longitudinal trabeculae, like in stem tetrapods, chelonians and crocodilians; (3), an epiphysis with no SOCs but with a longitudinal trabecular mesh invading part of the epiphyseal cartilage, like in birds and non-avian dinosaurs; and, (4), an epiphysis with SOCs, like in synapsids, including mammals.

FEA was performed in these models based on two load configurations: from the top, mimicking a static stance and from the side, mimicking locomotion. The results show that the presence of a SOC (model 4) would add stiffness to the epiphysis and reduce the stress on the growth plate compared to a fully cartilaginous model (model 1). The presence of marrow processes and an extended trabecular mesh (model 3) reduced the stresses on the growth plate as well, albeit to a lesser degree. This suggests that SOCs and trabecular protrusions could be two evolutionary strategies to reduce stress in the growth plate.

To empirically demonstrate how the SOC protects the growth plate from stress, we checked the effect of mechanical loading of tibias *ex vivo* and *in vitro* from 10-day-old rats and 30-day-old mice. At those ages the two rodents have tibias of similar size and shape, but only mice has already devel-

oped a SOC. Staining for dead cells and the reduced expression of typical chondrocyte markers suggest that the chondrocytes of mice (with a SOC) endured higher loads than those of rats (without a SOC). TUNEL assay showed that cell death occurred through caspase-dependent apoptosis probably via the YAPp73 signaling pathway, not by mechanical damage. We also used genetic and pharmacological manipulation of the SOC *in vivo* in order to remove interspecific differences, with similar results. The most affected layer of the growth plate was the hypertrophic zone. Measurements taken with AFM showed that hypertrophied chondrocytes are less stiff than other columnar chondrocytes. The results indicated that the presence of a SOC allows the growth-plate chondrocytes to support six times more stress than without a SOC, before activating cell destruction through caspase-dependent apoptosis probably via the YAPp73 signaling pathway.

From an evolutionary perspective, the appearance of SOCs would have been crucial to allow the elongation of the bone in terrestrial tetrapods under the biomechanical constraints produced by new evolving postures and terrestrial lifestyles. The absence of SOCs in crocodilians and chelonians could be explained by an early development of limb bones in a non-constrained environment (e.g., aquatic environment) in combination to reduced levels of stress applied to the epiphyses as a result of a more sprawling gait. Lizards would develop SOCs, despite having a sprawling gait, due to their terrestrial lifestyle since their birth, as it involves adopting bipedal or semi-erect gaits to avoid predation. In dinosaurs, including birds, the production of a longitudinal trabecular mesh in their metaphyses partly compensate for the absence of SOCs, and may be further compensated in birds by the numerous bone fusions. The tibiotarsus and the proximal tarsometatarsus of birds present SOC or SOC-like bone fusions, which could be explained as adaptations to reinforce the protection of the growth plate in response to constant bipedalism.

Development of long bone epiphyses in mammals (Paper 4)

For this study, the proximal humeral epiphyses from eleven species of crown mammals were scanned at different ontogenetic stages to study the development of Secondary Ossification Centers (SOCs). The data showed a great variation between their morphology, vascularization, microanatomy, degree of ossification and relative position of SOCs. Furthermore, the 3D histology showed the presence of up to three SOCs within the same epiphysis, one for each condyle (the articulation and the lesser and greater tubercles). A comparative study of these skeletal features permitted to create a nomenclature

characterizing the main discriminant steps of the SOC development within the proximal humeral epiphysis. This nomenclature comprises up to seven stages that reflect the degree of ossification and fusion of the SOCs. This nomenclature includes for the first time stages encompassing the entire ontogeny of the animal, i.e., from fully cartilaginous to fully ossified and remodeled epiphyses, unlike those proposed in previous studies (Cameriere et al., 2012; Ebeye et al., 2016; Galić et al., 2016; O'Connor et al., 2008).

Using this system, we can now compare equivalent stages of epiphyseal development in monotreme, marsupial and placental mammals to check whether they show similar or different developmental trajectories. The SOC located within the greater tubercle is the first to begin ossifying during ontogeny in all mammals. In placentals the articular condyle and the lesser tubercle begin ossifying later but reach higher degrees of ossification than the greater tubercle. In placental mammals, the SOCs at the lesser tubercle and the articulation are the first to merge, while monotremes merge the articulation with the greater tubercle first. The first merging event in marsupials remains unknown due to lack of data, but both lesser and greater tubercles begin ossifying before the articulation, which may or may not be the case in the other two groups. The condyles of monotremes and marsupials first develop centers of calcification before ossifying them into SOCs. This condition is different in placental mammals, where calcified cartilage is almost immediately eroded and substituted by bone. This nomenclature shows a great potential for increasing the resolution of studies on comparative developmental timing to allow for better assessment of age, population/taxon differences, or even diagnosing pathologies.

Concluding remarks and future perspectives

The main goal of this thesis research was to provide a better understanding of the evolution of long bones by analyzing the three-dimensional histology and microanatomy of long bones from both fossil and extant tetrapods. For this, we investigated the diaphysis and epiphysis from stem amniotes and compared it to those of temnospondyls in order to present a clearer picture of the way they evolved. Next, we performed computer simulations and empirical experiments to investigate the role of SOCs. And finally, we studied the epiphyses of several species from the three main mammalian groups and proposed a new nomenclature for the description of the epiphyses through ontogeny. This research was able to show that:

- The slow growth pattern typical of Devonian tetrapods, temnospondyls and extant amphibians evolved gradually into the fast growth of amniotes during the Paleozoic, as suggested by the dynamic bone growth pattern and long pre-reproductive period of stem amniotes (**Paper 1**).
- Developmental strategies (growth rate, pre-reproductive period) and type of bone elongation pattern (longitudinal trabeculae) are independent of each other, contrary to previous assumptions (**Paper 1**).
- Studies on a large range of tetrapods (including finned stem tetrapods) suggest that the hematopoietic function in long bones did not migrate into marrow cavities before the water-to-land transition (**Paper 2**).
- SOCs protect the hypertrophied chondrocytes of the growth plate from mechanical stresses that may trigger their apoptosis (**Paper 3**).
- A nomenclature for ossification staging that takes in account the whole development of the epiphyses can be used for comparisons between ontogenetic stages and species (**Paper 4**).
- The individual ossification stage of each of the developing SOCs within a single epiphysis increases the resolution of developmental timing studies and highlights previously missed differences between mammalian groups (**Paper 4**).

This research brings a new perspective on the microstructural organization of long bones by approaching it with 3D histological methods, which reflect closer the reality of the samples. It also highlights the importance of revisiting studied material using different techniques, like PPC-SRμCT, to allow the visualization of well-preserved material.

I have shown here that metaphyses and epiphyses can be just as informative about evolutionary aspects as diaphyses. Future studies should focus as much attention to metaphyses and epiphyses as past studies have paid to diaphyses. So far the histology from different bones has been compared assuming none or little variability between epiphyses and thus there has been little consistency in the bones selected. To be able to determine how representative our ideas are about the epiphyses, future studies should assess the variability of epiphyses between species, bones, ends and ontogenetic stages.

Based on the overall shape of their humerus, the locomotion of Paleozoic tetrapods may have been based in the rotation of the stylopod, as is the case in modern echidnas (Gambaryan & Kuznetsov, 2013; Jenkins, 1970; Pridmore, 1985). The results of **Paper 3** indicate that the evolution of terrestrial locomotion with movement of the limb based on the translation of the humerus, like that of species with tubular morphologies, may have been limited by cartilaginous epiphyses not being able to protect the growth plate. Future research should test whether locomotion types involving prolonged periods of translational movement of the limbs evolved as consequence of the appearance of SOCs, allowing the diversification into the more efficient semi-erect and erect postures that we find in most modern tetrapods.

As a follow up to the 3D segmentation and description of the mammalian epiphyses in **Paper 4**, we plan to use different parameters (e.g., cartilage volume, surface of the growth plate, diameter of the canals) to perform multivariate analyses that may reveal any possible hidden correlations with lifestyle, posture, activity, growth rate, and other traits of the animals. Correlations between the different parameters could orient future studies testing evolutionary causes.

Crocodilians are the extant group whose long bone development seems to resemble most that of stem amniotes. The marrow processes of the growth plate of crocodilians are very obvious in cross sections (Haines, 1938), but they have never been studied in 3D, so it is still not possible to assess how well they reflect the ancestral character state.

Most epiphyses in birds develop similarly to the ones of crocodilians and chelonians, but they end up ossifying their epiphyses in three different ways (personal observation). In most bones, the growth plate advances through the epiphyseal cartilage without elongating the bone, which leaves a thin layer of epiphyses acting as articular cartilage (see Breugelmans et al., 2007). In the proximal epiphysis of the tibiotarsus birds develop a SOC in the same way as mammals do (**Paper 3**). The carpals and the tarsals, however, seem to develop within the epiphyseal cartilage of the adjacent long bones, in a

way similar to the development of SOCs (personal observation and see Ossa-Fuentes et al., 2015). Whether or not carpal and tarsal bones act as protection for the hypertrophied chondrocytes in the growth plate, in the same way as a mammal SOC does, is yet to be tested (**Paper 3**). The study of these three parallel types of epiphyseal ossification promise new clues about the origin of SOCs in birds.

As shown in **Paper 4**, marsupials and monotremes form a block of calcified cartilage within the epiphysis before the formation of SOCs. In placental mammals the transition between calcified cartilage and bony tissue is much faster. A block of cartilage also forms before ossification in the epiphyses of lepidosaurs (lizards and the tuatara) and during the development of the POC at early ontogenetic stages. To further investigate these similarities could be key for determining the primitive state of SOCs.

My work clearly shows that amphibians do not reflect primitive states and are therefore not a good proxy for the histology of stem tetrapods. The description of extant taxa is key to understanding developmental processes and provides a framework to interpret the fossil record correctly, but without the study of fossils it would be practically impossible to reconstruct and understand the evolutionary steps that have led to modern forms. The groups of crown amniotes that have evolved SOCs have done so independently. Only the study of their stem groups will bring the context in which that happened.

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Svensk sammanfattning

Ryggradsdjurens skelett ger kroppen stadga, form och skydd för de inre organen. Skelettbenen fungerar också som fästpunkter för muskulaturen och som hävstänger vilka tillåter djuret att förflytta sig. Oavsett om de har formen av fenor, ben eller vingar har ryggradsdjurens extremiteter stor betydelse för deras rörelseförmåga. Denna koppling, och det faktum att extremitetsbenen lagrar biologisk information om t.ex. tillväxthastighet under djurets livstid, gör att vi kan dra slutsatser om utdöda djurs livsstil och evolutionshistoria genom att studera dessa bens morfologi, mikroanatomi och histologi.

Extremitetsben vars längd är större än diametern kalls för rörben. Ett rörben kan delas upp i tre olika sektioner: diafys, metafys och epifys, symmetriskt arrangerade kring benets mittpunkt. Diafysen bildar rörbenets skaft och karaktäriseras av en rörformad struktur med kompakt kortikal benvävnad kring en central märghåla. I ändarna på diafysen ansluter metafyserna, vilka innehåller tillväxtplattorna och märgvävnad som producerar röda blodkroppar. Epifyserna ligger utanför metafyserna och utgör benets ändar. Normalt bildar de också ledytorna. Hos ödlor, fåglar och däggdjur förbenas epifyserna under utvecklingen av ett så kallat sekundärt förbeningscentrum, som ligger utanför tillväxtplattan.

Rörben växer på två sätt, genom förtjockning och förlängning. Förtjockning sker när ny kortikal benvävnad avlagras på benets utsida. Förlängning sker vid tillväxtplattorna genom såkallad endokondral benbildning, där brosk bryts ned och ersätts av ben. Förbeningen av ett rörben börjar i mitten av skaftet, så förtjockningen har pågått längst där och kortikalbenet är därför tjockast i den regionen. Av det skälet studeras oftast benets förtjockningstillväxt just i mitten av diafysen. Detta kan göras ganska enkelt med klassiska histologiska metoder där benet snittas och snittet tunnslipas tills det blir halvgenomskinligt och kan studeras under mikroskop.

Synkrotronmikrotomografi med faskontrast (Propogation-based phase-contrast synchrotron radiation microtomography, PPC-SRμCT) är en relativt ny teknik som används för att studera histologi i tre dimensioner. Eftersom tekniken inte skadar studieobjektet kan den användas på unika och exceptionellt välbevarade fossil, och den producerar mer pålitliga data för vetenskaplig analys än traditionella tekniker (**Artikel 1 och 2**). Särskilt metafyserna har en komplex tredimensionell struktur som är på gränsen till omöjlig att visualisera med tvådimensionella snittbilder. Nya insikter om förläng-

ningsprocessen och dess relation till produktionen av röda blodkroppar har uppnåtts genom att använda PPC-SRμCT för att visualisera metafysernas tredimensionella struktur (**Artikel 2 och 4**). Epifysernas roll och utveckling, framförallt deras sekundära förbeningscentra, undersöktes i **Artikel 3 och 4**.

I **Artikel 1** studerade vi diafysens mikronatomi och histologi hos två stamgrupps-amnioter, fossila djur nära besläktade med amnioterna (d.v.s. däggdjuren, reptilerna och fåglarna). För att utröna arternas tillväxtstrategier fokuserade vi på det tredimensionella nätverket av blodkärlskanaler i kortikalbenet, eftersom en av arterna saknar "Lines of Arrested Growth" (LAGs), det vill säga de "årsringar" som normalt markerar benvävnadens tillväxthastighet. Stamgrupps-amnioterna förefaller ha kombinerat snabb tillväxt med en lång "barndom" innan de uppnådde reproduktiv ålder, en tillväxtstrategi som kan tolkas som ett mellansteg mellan den hos tidiga tetrapoder och amfibier (långsam tillväxt, lång "barndom") å den ena sidan och den hos amnioter (snabb tillväxt, kort "barndom") å den andra. Det verkar alltså som om omställningen av tillväxtstrategin under amnioternas evolution skedde gradvis.

I **Artikel 2** fokuserade vi på metafyserna hos stamgrupps-amfibier och hos de stamgrupps-amnioter vi studerat i **Artikel 1**. Vi lyckades för första gången särskilja två typer av strukturer: märghålor och märgprocesser. På basis av studier av tidiga tetrapoder och fossila kvastfeniga fiskar har vi tidigare trott att produktionen av röda blodkroppar (hematopoes) i rörben utvecklats i märgprocesserna redan innan övergången från vatten till land. Våra observationer på stamgrupps-amnioter tyder emellertid på att rörbenshematopoes i själva verket utvecklades i märghålorna efter anpassningen till landliv. Resultaten kommer att påverka den framtida tolkningen så väl av fossil som av de evolutionära krafter vilka ledde till att hematopoes etablerades i rörbenen.

Observationer på förbeningsprocessen i sekundära förbeningscentra hos nulevande däggdjur pekar på ett sammanhang mellan den mekaniska belastning som benet utsätts för och dess förbeningsgrad. Ben som utsätts för stora påfrestningar förefaller förbenas tidigare i livet. I **Artikel 3** använder vi datorsimuleringar och empiriska experiment för att utforska de sekundära förbeningscentrens funktion. Våra resultat tyder på att sekundära förbeningscentra skyddar den underliggande tillväxtplattan från mekaniska belastningar så att den klarar sex gånger högre belastning än en oskyddad platta.

I **Artikel 4** studerar vi metafysernas och epifysernas tredimensionella mikroanatomi under olika utvecklingsstadier hos tio arter av moderna däggdjur från de tre stora huvudgrupperna: kloakdjur, pungdjur och moderkaksdjur. Vi använde våra data för att skapa en sammanhängande nomenklatur för förbeningen av epifyserna som sträcker sig över hela utvecklingsprocessen av alla kondylerna i en epifys, från broskstadiet till helt förbenade och remodellerade. Med hjälp av detta system beskriver vi epifysernas utveckling hos däggdjur och synliggör skillnader i förbeningsprocessens timing hos

olika arter. Den nya nomenklaturen kommer att göra framtida utvecklingsstudier mer precisa och hjälpa oss att upptäcka tidigare förbisedda skillnader mellan grupper.

Avhandlingen ger en unik glimt av rörbenens evolution och utveckling genom att tillhandahålla en ny tredimensionell kontext för tidigare kända element. Den innehåller studier på rörbenens tre regioner – diafys, metafys och epifys – och påvisar hur värdefullt det är att kunna studera dessa strukturer i tre dimensioner så väl som i två när deras funktioner skall utforskas.

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