# Evolution of posture in amniotes-Diving into the trabecular architecture of the femoral head 

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#### Abstract

Extant amniotes show remarkable postural diversity. Broadly speaking, limbs with erect (strongly adducted, more vertically oriented) posture are found in mammals that are particularly heavy (graviportal) or show good running skills (cursorial), while crouched (highly flexed) limbs are found in taxa with more generalized locomotion. In Reptilia, crocodylians have a "semi-erect" (somewhat adducted) posture, birds have more crouched limbs and lepidosaurs have sprawling (well-abducted) limbs. Both synapsids and reptiles underwent a postural transition from sprawling to more erect limbs during the Mesozoic Era. In Reptilia, this postural change is prominent among archosauriforms in the Triassic Period. However, limb posture in many key Triassic taxa remains poorly known. In Synapsida, the chronology of this transition is less clear, and competing hypotheses exist. On land, the limb bones are subject to various stresses related to body support that partly shape their external and internal morphology. Indeed, bone trabeculae (lattice-like bony struts that form the spongy bone tissue) tend to orient themselves along lines of force. Here, we study the link between femoral posture and the femoral trabecular architecture using phylogenetic generalized least squares. We show that microanatomical parameters measured on bone cubes extracted from the femoral head of a sample of amniote femora depend strongly on body mass, but not on femoral posture or lifestyle. We reconstruct ancestral states of femoral posture and various microanatomical parameters to study the "sprawling-to-erect" transition in reptiles and synapsids, and obtain conflicting results. We tentatively infer femoral posture in several hypothetical ancestors using phylogenetic flexible discriminant analysis from maximum likelihood estimates of the microanatomical parameters. In general, the trabecular network of the femoral head is not a good indicator of femoral posture. However, ancestral state reconstruction methods hold great promise for advancing our understanding of the evolution of posture in amniotes.


## KEYWORDS

Amniota, ancestral state reconstruction, bone cube, femur, limb posture, microanatomy, palaeobiology, Reptilia, Synapsida, trabecular bone

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## 1 | INTRODUCTION

Limbs first evolved during the Devonian Period in aquatic organisms, such as Acanthostega, and presumably were first used for locomotion in shallow waters and to rest on the bottom (Coates, 1996; Laurin, 2010; Molnar et al., 2021). In later organisms, these primordial limbs experienced new functional constraints inherent to land environments, related in particular to the support of body weight. These constraints shaped the limbs' external and internal morphology, and thereby, the first land vertebrates developed novel locomotor and postural strategies favouring access to various ecological niches and contributing to the evolutionary success of tetrapods. The first terrestrial vertebrates were quadrupedal with a sprawling limb posture, that is, the stylopod was held horizontally with the distal end pointing laterally (Bakker, 1971; Charig et al., 1972). A recent study combining palaeoichnology (the study of ancient tracks) and robotics supported this inference with quantitative methods for the first time in an early stem amniote (Nyakatura et al., 2019). Today, extant amniote taxa (reptiles and mammals) present a great diversity of postures and locomotor modes associated with a morphological and microanatomical disparity.

Both reptiles and synapsids underwent a postural transition during the Mesozoic Era. The first parasagittally locomoting erect bipedal amniotes seem to have evolved convergently during the Triassic in the archosaurian clades Avemetatarsalia and Pseudosuchia (Cuff et al., 2022; Hutchinson, 2006; Kubo \& Benton, 2009; Sereno, 1991; Sullivan, 2015). Parasagittally locomoting erect bipedalism is often cited as a key element in the success of Avemetatarsalia (e.g. Kubo \& Kubo, 2012). However, the steps that led to this bipedal state remain enigmatic and more conclusively determining the locomotion and posture adopted by many Triassic taxa, such as the archosauriform Euparkeria, involve considerable obstacles and ambiguities (e.g. Bishop et al., 2020). Although it is accepted that synapsids experienced a postural transition from approximately transversely oriented to more parasagittally oriented limbs, the timing of this transition has been widely debated without reaching a consensus. Some authors (Jenkins, 1973; Jenkins \& Parrington, 1976; Pridmore, 1985; Sereno, 2006) have argued that early mammals had already acquired a more parasagittal limb posture and gait by the Late Triassic/Early Jurassic based on anatomical evidence, while others (Gambaryan \& Kielan-Jaworowska, 1997; Kielan-Jaworowska \& Hurum, 2006) favoured the hypothesis of a later acquisition in early therians based on both anatomy and ichnology. More erect limbs may have existed as early as the Permian. Indeed, several lineages of Permo-Triassic therapsids, such as Anomodontia and Cynodontia, have been described as having had a "semi-erect" posture based on anatomical and biomechanical evidence (Blob, 2001; Fahn-Lai et al., 2018; Fröbisch, 2006). Today, posture in mammals and in older stem taxa, such as Dimetrodon, still raises many questions, triggering numerous studies that enrich our knowledge of the evolution of locomotion in synapsids (Brocklehurst et al., 2022; Jones et al., 2021; Regnault et al., 2020).

Limb bones support the weight of the body and are therefore subject to various forces that partly shape their external and internal form during ontogeny. Yet bone trabeculae tend to orient themselves along the lines of force: this is known as Wolff's law, or the trajectorial theory (Wolff, 1893). Before this law was formulated, von Meyer (1867) had interpreted the spongy structures of the human femoral head in the light of Culmann's remarks, who had noted a certain similarity with the internal tension and compression lines of a crane. Since these early observations, the functional role of trabecular bone, that is, its ability to distribute mechanical stresses to improve strength (Currey, 2013), has received increasing attention. Indeed, numerous studies have demonstrated the effect of different factors, such as allometry, locomotion or lifestyle, on trabecular bone in various skeletal elements (limb bones, vertebrae, etc.) in both reptiles and mammals (Amson et al., 2017; Biewener et al., 1996; Bishop et al., 2018; Doube et al., 2011; Hildebrand et al., 1999; Hollister et al., 1994; Kivell et al., 2018; Plasse et al., 2019; Pontzer et al., 2006; Ryan \& Ketcham, 2002; Ryan \& van Rietbergen, 2005; Smit et al., 1997; Tsegai et al., 2013). These studies have used a range of methods, from computer simulations including finite element analyses (FEA), to in vivo experiments, to the study of bone cubes or spheres, also known as VOIs (volume of interest) or ROIs (region of interest), which are defined within a bone to virtually subsample a region to be analysed. Beyond functional adaptations, trabecular bone, like any biological structure, is impacted by various factors (ontogeny, metabolism, phylogeny, etc.) whose influence remains enigmatic (Currey, 2013; Kivell, 2016).

Methods of ancestral state reconstruction aim to infer the characteristics of ancestral taxa from the characteristics of their descendants using models of character evolution (Pagel, 1999). They have been extensively used to study vertebrate evolution: from inference of metabolic rate (Benton, 2021; Legendre et al., 2016) and lifestyle (Canoville \& Laurin, 2010) to "resurrection" of genetic sequences (Chang et al., 2002; Thornton, 2004), diet reconstruction (Brocklehurst, 2016) and soft tissue studies (Campione et al., 2020; Tsai et al., 2018). The use of these methods for postural issues is rarer (Buchwitz et al., 2021; Grinham et al., 2019), and, to our knowledge, they have never been applied to the femoral trabecular architecture in the context of the postural transitions in amniotes during the Mesozoic.

In this study, we use phylogenetic comparative methods, such as phylogenetic generalized least squares (PGLS), to better characterize the relationship between femoral posture and the femoral head trabecular architecture in amniotes. Given previous studies highlighting that larger taxa tend to have greater bone volume and thicker trabeculae (Doube et al., 2011; Houssaye et al., 2016), we expect to find similar scaling relationships with our sample. Also, larger taxa exhibit more erect (adducted, upright) limbs, which reduces weight-related stresses (Biewener, 1990), and this could reduce anisotropy (Doube et al., 2011). As our sample contains taxa with a variety of femoral postures (from erect to sprawling), we expect to find differences in anisotropy between postural groups.

We use ancestral state reconstruction methods in a novel manner to infer the ancestral condition of various microanatomical parameters measured on bone cubes extracted from the femoral head of a sample of extant amniote taxa, but also to more directly infer ancestral posture at nodes of interest in the context of the "sprawling-to-erect" transitions in reptiles and synapsids. This study not only sheds light on the relationship between posture and microanatomy in amniotes but also aims to demonstrate the relevance of ancestral state reconstruction approaches to postural issues in vertebrates.

## 2 | MATERIALS AND METHODS

## 2.1 | Biological sample

To conduct the statistical analyses in this study, we compiled a set of microanatomical data measured on bone cubes extracted from the femoral head of a large number of amniote taxa. We retrieved the list of taxa from Doube et al. (2011) and enriched it with new taxa, notably squamates and turtles, which were not previously included. However, we did not retain all mammal taxa, as they were overrepresented in the study by Doube et al. (2011). Indeed, if we consider extant species diversity, there are about 5000 extant species of mammals (Upham et al., 2019), compared to about 15000 extant species of reptiles: 10000 species of birds (Jetz et al., 2012), about 5000 species of limbed squamates (Brandley et al., 2008), about 350 species of turtles (Thomson et al., 2021) and about 25 species of crocodylians (Brochu, 2003). Our sample is composed of 93 amniote species for which femoral posture is known (Table 1). These include 57 mammal species and 36 reptile species ( 24 birds, 6 squamates, 3 crocodylians and 3 turtles). Each species is represented by one individual. We tried to build our sample to be as representative as possible of the taxonomic and postural diversity of amniotes. Our sample contains three extinct taxa: Raphus cucullatus, Pezophaps solitaria and Dinornis sp. The latter became extinct recently (within the last five centuries).

## 2.2 | Postural categories

We defined four postural categories: sprawling, crouched, erect and "semi-erect." In sprawling taxa (lepidosaurs, turtles, but also monotremes, such as Ornithorhynchus), the femur extends laterally, while in crouched taxa (small mammals and small birds), it points more or less anteriorly. In erect taxa (large mammals and large birds), the femur is held more vertically under the body. Finally, the "semierect" posture of crocodylians can be regarded as intermediate between sprawling and erect. We are aware of the limitations of such a classification. For example, that limb posture in amniotes is more of a continuum than well-defined postural categories, or that the term "semi-erect" is evolutionarily and functionally ambiguous, but these
categories remain practical in the framework of comparative phylogenetic studies.

## 2.3 | Data acquisition

We strictly followed the protocol by Doube et al. (2011) for extracting new bone cubes in order to obtain comparable data. To validate the protocol, we retrieved some of the bone cubes from Doube et al. (2011) available online. The bone cubes were extracted and processed in ImageJ (Abràmoff et al., 2004) to reproduce some of the trabecular measurements provided in the electronic supplementary material of Doube et al. (2011). CT data for the new taxa in the sample were obtained either from the literature or by scanning the femora on the AST-RX platform of the Muséum national d'histoire naturelle and on the MRI platform of the university of Montpellier. Bone cubes were extracted in ImageJ with BoneJ 1.4.3 (Doube et al., 2010) following the fit sphere routine (Doube et al., 2011). We extracted the largest cube that could be contained in a sphere fitted by least squares in the femoral head (Figure 1). First, the scans of the femur were resliced in ImageJ to position the bone vertically. Secondly, we placed six points to delineate the volume of the femoral head: two points to delineate the upper and lower parts of the femoral head, and four additional points (anterior, posterior, medial and lateral) on the slice halfway between the two slices comprising the first two points. Once extracted, the bone cubes were binarized using IsoData thresholding, purified (with BoneJ), eroded (in ImageJ: Process > Binary), purified again and dilated (in ImageJ: Process > Binary). For a detailed description of the procedure for extracting and processing bone cube data, see Doube et al. (2010). We then measured six parameters with BoneJ (Figure 1): BV/TV, the bone volume fraction, corresponding to the number of bone voxels divided by the total number of voxels in the cube; BS/TV, the bone surface area per unit volume, defined as the bone surface area, obtained by summing the surface area of all the triangles constituting a 3D mesh of the trabecular network, divided by the total volume of the cube; Tb.Th, the mean trabecular thickness; Tb.Sp, the mean trabecular spacing; Conn.D, the connectivity density, which corresponds to the number of trabeculae divided by the total volume of the cube; DA, the degree of anisotropy, reflecting a more or less pronounced trabecular orientation ( $0<\mathrm{DA}<1$; 0 indicating no orientation and 1 parallel trabeculae). The new unprocessed bone cubes are publicly available at https:// doi.org/10.5061/dryad.83bk3j9x2.

Ketcham and Ryan (2004) noted that texture orientations could be over-represented towards the edges and corners of a cubic volume. However, our study is not affected by this "edge and corner bias." Indeed, in BoneJ 1.4.3, anisotropy is calculated using the mean intercept length (MIL) method from sampling spheres randomly distributed inside the image stack (see Doube et al., 2010). These spheres are never closer to the sides of the image than their radius (M. Doube, personal communication).
TABLE 1 List of the amniote taxa analysed in this study.

| Taxon |  | Collection number | Femoral posture | Lifestyle | Body mass | BV/TV | BS/TV | Tb.Th | Tb.Sp | Conn.D | DA |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Afrosoricida | Chrysochloris asiatica | MNHN-ZM-MO-1991-626 | C | Fo | 36.93 | 0.447 | 10.583 | 0.134 | 0.249 | 81.741 | 0.737 |
| Artiodactyla | Bos taurus | RVC cow2 | E | Te | 618642.42 | 0.498 | 4.043 | 0.34 | 0.57 | 3.862 | 0.502 |
|  | Capra hircus | RVC goat1 | E | Te | 60500 | 0.368 | 4.518 | 0.228 | 0.581 | 5.502 | 0.545 |
|  | Cervus elaphus | IC ic_deer1 | E | Te | 160166.665 | 0.408 | 4.943 | 0.207 | 0.569 | 4.846 | 0.4 |
|  | Giraffa camelopardalis | RVC giraffe2 | E | Te | 800000 | 0.559 | 4.006 | 0.328 | 0.525 | 2.522 | 0.497 |
|  | Madoqua saltiana | UMZC H22281 | E | Te | 3337.235 | 0.472 | 6.379 | 0.194 | 0.336 | 6.054 | 0.628 |
|  | Ovis aries | RVC sheep2 | E | Te | 110000 | 0.436 | 4.144 | 0.288 | 0.595 | 3.602 | 0.347 |
|  | Sus scrofa | RVC pig1 | E | Te | 135000 | 0.461 | 7.423 | 0.161 | 0.288 | 27.745 | 0.524 |
|  | Tragulus javanicus | UMZC H15013 | E | Te | 1753.965 | 0.324 | 6.415 | 0.144 | 0.455 | 12.754 | 0.551 |
|  | Tragulus kanchil | UMZC H15052 | E | Te | 3850 | 0.398 | 5.547 | 0.201 | 0.483 | 2.556 | 0.481 |
|  | Tragulus napu | UMZC H14975 | E | Te | 3850 | 0.478 | 5.916 | 0.215 | 0.373 | 7.115 | 0.613 |
|  | Vicugna pacos | RVC alpaca1 | E | Te | 50000 | 0.416 | 5.55 | 0.202 | 0.489 | 8.691 | 0.348 |
| Carnivora | Acinonyx jubatus | RVC kent_cheetah | E | Te | 50577.92 | 0.569 | 5.11 | 0.291 | 0.408 | 7.39 | 0.485 |
|  | Canis lupus | RVC wolf1 | E | Te | 29190.755 | 0.425 | 5.183 | 0.23 | 0.464 | 8.155 | 0.541 |
|  | Felis catus | RVC RVCfcatus21 | E | Te | 4573.08 | 0.456 | 6.176 | 0.202 | 0.401 | 9.766 | 0.569 |
|  | Felis silvestris | UFGK unnumbered specimen | E | Te | 5036.54 | 0.481 | 3.581 | 0.383 | 0.596 | 2.134 | 0.746 |
|  | Herpestes ichneumon | UMZC K4695 | C | Te | 2950.01 | 0.481 | 7.849 | 0.165 | 0.278 | 26.325 | 0.462 |
|  | Mustela putorius | UMZC K2361 | C | Te | 809 | 0.401 | 8.804 | 0.129 | 0.26 | 51.729 | 0.486 |
|  | Nandinia binotata | UMZC K4493 | E | Ar | 2167.2 | 0.344 | 7.663 | 0.125 | 0.324 | 29.087 | 0.655 |
|  | Panthera tigris | RVC tiger_2 | E | Te | 128800 | 0.459 | 3.518 | 0.365 | 0.648 | 3.372 | 0.473 |
|  | Paradoxurus hermaphroditus | NHM 1855.12.8.4 | C | Ar | 3180 | 0.355 | 6.427 | 0.152 | 0.495 | 26.3 | 0.378 |
|  | Prionailurus bengalensis | NHM 1989.251 | E | Te | 4150 | 0.531 | 6.317 | 0.228 | 0.327 | 12.471 | 0.615 |
|  | Prionailurus viverrinus | NHM 1860.7.22.22 | E | Te | 10650 | 0.423 | 5.324 | 0.221 | 0.432 | 9.618 | 0.601 |
|  | Pseudalopex fulvipes | NHM 1851.11.8.4 | E | Te | 3990 | 0.503 | 6.501 | 0.215 | 0.315 | 15.732 | 0.599 |
|  | Ursus americanus | MNHN-ZM-MO-1902-1415 | E | Te | 132405 | 0.565 | 2.869 | 0.451 | 0.515 | 2.942 | 0.379 |
|  | Vulpes lagopus | UMZC K3668 | E | Te | 3600 | 0.34 | 5.789 | 0.169 | 0.463 | 6.684 | 0.664 |
|  | Vulpes vulpes | UMZC K3585 | E | Te | 4580.3 | 0.464 | 6.809 | 0.192 | 0.31 | 25.128 | 0.61 |
|  | Vulpes zerda | UMZC K3782 | E | Te | 1207.5 | 0.421 | 8.53 | 0.133 | 0.281 | 32.716 | 0.591 |
| Dasyuromorphia | Dasyurus viverrinus | UMZC A6.11/1 | C | Te | 982 | 0.366 | 7.51 | 0.142 | 0.296 | 19.655 | 0.818 |
| Diprotodontia | Macropus rufogriseus | Ker wallaby1 | C | Te | 16525 | 0.566 | 4.54 | 0.326 | 0.438 | 5.816 | 0.723 |
|  | Potorous tridactylus | UMZC A12.84/2 | C | Fo | 1054.67 | 0.387 | 5.777 | 0.179 | 0.407 | 7.168 | 0.701 |
|  | Trichosurus vulpecula | UMZC A9.16/14 | C | Ar | 2005 | 0.409 | 6.424 | 0.169 | 0.347 | 13.67 | 0.749 |

TABLE 1 (Continued)

| Taxon |  | Collection number | Femoral posture | Lifestyle | Body mass | BV/TV | BS/TV | Tb.Th | Tb.Sp | Conn.D | DA |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Eulipotyphla | Erinaceus europaeus | UMZC E5131J | C | Te | 777.95 | 0.407 | 11.396 | 0.106 | 0.194 | 119.732 | 0.515 |
|  | Talpa europaea | NHM 1972.929 | C | Fo | 110.415 | 0.377 | 13.741 | 0.091 | 0.215 | 187.636 | 0.568 |
| Lagomorpha | Lepus europaeus | RVC hare1 | C | Te | 3673.33 | 0.347 | 6.919 | 0.145 | 0.431 | 11.679 | 0.585 |
|  | Ochotona rufescens | NHM 1874.11.21.32 | C | Fo | 250 | 0.443 | 11.111 | 0.121 | 0.232 | 70.915 | 0.29 |
| Monotremata | Ornithorhynchus anatinus | MNHN-ZM-AC-1906-484 | S | Aq | 1225 | 0.397 | 5.933 | 0.206 | 0.376 | 18.132 | 0.754 |
|  | Tachyglossus aculeatus | MNHN-ZM-AC-1884-1125 | C | Fo | 3169.5 | 0.295 | 7.361 | 0.139 | 0.34 | 40.012 | 0.66 |
| Peramelemorphia | Perameles gunnii | UMZC A7.17/1 | C | Te | 801.5 | 0.465 | 6.818 | 0.189 | 0.349 | 7.312 | 0.71 |
| Perissodactyla | Ceratotherium simum | RVC french_rhino | E | Te | 2230000 | 0.422 | 4.62 | 0.247 | 0.493 | 4.177 | 0.559 |
|  | Equus caballus | RVC prz_horse1 | E | Te | 250000 | 0.21 | 2.433 | 0.279 | 0.964 | 3.48 | 0.657 |
| Pholidota | Smutsia temminckii | MNHN-ZM-AC-1897-134 | C | Te | 9586.87 | 0.408 | 4.419 | 0.22 | 0.361 | 21.281 | 0.412 |
| Primates | Callithrix jacchus | UMZC E7901.E | C | Ar | 255.2 | 0.368 | 10.401 | 0.105 | 0.238 | 84.836 | 0.518 |
|  | Cheirogaleus major | NHM 1872.8.19.11 | C | Ar | 420.11 | 0.392 | 8.578 | 0.124 | 0.29 | 86.92 | 0.506 |
|  | Galagoides thomasi | NHM 1982.484 | C | Ar | 63.8 | 0.231 | 10.052 | 0.07 | 0.334 | 53.105 | 0.553 |
|  | Lemur catta | MNHN-ZM-AC-1910-101 | C | Ar | 2555 | 0.459 | 5.791 | 0.207 | 0.372 | 13.945 | 0.463 |
|  | Macaca fascicularis | UMZC E7454.B | E | Ar | 4569.32 | 0.366 | 7.037 | 0.148 | 0.362 | 21.152 | 0.512 |
|  | Saimiri sciureus | NHM 1989.291 | C | Ar | 836.7 | 0.25 | 6.645 | 0.114 | 0.445 | 34.081 | 0.4 |
| Proboscidea | Elephas maximus | RVC gita | E | Te | 3220000 | 0.479 | 2.749 | 0.511 | 0.851 | 1.655 | 0.459 |
| Rodentia | Jaculus orientalis | UMZC E3172 | C | Fo | 136.13 | 0.364 | 10.233 | 0.106 | 0.283 | 94.439 | 0.576 |
|  | Mus musculus | IC Mouse1 | C | Fo | 20.5 | 0.501 | 17.661 | 0.095 | 0.146 | 150.237 | 0.572 |
|  | Myocastor coypus | UMZC E3369 | C | Aq | 7150 | 0.437 | 6.685 | 0.195 | 0.314 | 25.846 | 0.679 |
|  | Pedetes capensis | UMZC E1445 | C | Fo | 2774.625 | 0.44 | 5.61 | 0.216 | 0.413 | 7.34 | 0.506 |
|  | Sciurus vulgaris | RVC squirrel1 | C | Ar | 328.875 | 0.447 | 10.181 | 0.139 | 0.224 | 45.399 | 0.714 |
| Scandentia | Tupaia belangeri | UMZC E4061C | C | Ar | 200 | 0.446 | 11.656 | 0.107 | 0.209 | 56.559 | 0.646 |
| Soricomorpha | Solenodon paradoxus | MNHN-ZM-MO-1980-237 | C | Fo | 900 | 0.611 | 9.635 | 0.162 | 0.165 | 38.787 | 0.795 |
|  | Suncus etruscus | NHM 1965.3735 | C | Te | 2.33 | 0.291 | 21.371 | 0.051 | 0.149 | 699.902 | 0.691 |
| Accipitriformes | Gypaetus barbatus | MNHN-ZO-AC-1993-52 | C | Te | 5606.042 | 0.324 | 2.782 | 0.325 | 1.077 | 0.39 | 0.637 |

TABLE 1 (Continued)

| Taxon |  | Collection number | Femoral posture | Lifestyle | Body mass | BV/TV | BS/TV | Tb.Th | Tb.Sp | Conn.D | DA |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Anseriformes | Anas superciliosa | UMZC 222.a | C | Aq | 981 | 0.206 | 4.02 | 0.135 | 1.043 | 5.392 | 0.432 |
|  | Anser albifrons | UMZC 242.E | C | Aq | 2387.5 | 0.346 | 8.427 | 0.112 | 0.305 | 25.647 | 0.403 |
|  | Branta bernicla | UMZC 246.F | C | Aq | 1347.25 | 0.11 | 2.723 | 0.108 | 1.378 | 5.728 | 0.62 |
|  | Cereopsis novaehollandiae | UMZC 242.AA | C | Aq | 3770 | 0.163 | 2.089 | 0.213 | 1.746 | 0.85 | 0.482 |
|  | Chenonetta jubata | UMZC 246.G | C | Aq | 812.5 | 0.153 | 3.326 | 0.128 | 1.06 | 7.96 | 0.489 |
|  | Cygnus olor | RVC swan1 | E | Aq | 10230 | 0.301 | 3.365 | 0.24 | 1.013 | 0.73 | 0.392 |
|  | Somateria mollissima | UMZC 704 | C | Aq | 2092 | 0.164 | 3.664 | 0.129 | 0.916 | 3.801 | 0.577 |
| Apterygiformes | Apteryx haastii | UMZC 378.p | C | Te | 2409 | 0.396 | 5.643 | 0.19 | 0.453 | 5.901 | 0.505 |
|  | Apteryx owenii | UMZC 378.iii | C | Te | 1200 | 0.393 | 5.467 | 0.204 | 0.487 | 7.41 | 0.577 |
| Casuariiformes | Casuarius casuarius | MNHN-ZO-AC-1946-72 | E | Te | 44000 | 0.705 | 2.115 | 0.731 | 0.441 | 1.693 | 0.75 |
|  | Dromaius novaehollandiae | RVC emu_1 | E | Te | 36200 | 0.328 | 2.496 | 0.366 | 1.173 | 0.799 | 0.675 |
| Columbiformes | Columba livia | RVC racepigeon | C | Te | 320 | 0.235 | 6.149 | 0.109 | 0.523 | 14.391 | 0.348 |
|  | Pezophaps solitaria $\dagger$ | YPM 1154 | E | Te | 14000 | 0.51 | 1.732 | 0.957 | 1.079 | 0.256 | 0.83 |
|  | Raphus cucullatus $\dagger$ | YPM 2064 | E | Te | 14000 | 0.449 | 1.626 | 0.94 | 1.437 | 0.145 | 0.751 |
| Crocodylia | Alligator mississippiensis | MNHN-ZA-AC-1945-54 | SE | Aq | 62000 | 0.362 | 3.985 | 0.262 | 0.477 | 7.402 | 0.583 |
|  | Caiman crocodilus | MNHN-ZA-AC-1910-87 | SE | Aq | 10900 | 0.359 | 6.977 | 0.16 | 0.291 | 30.367 | 0.722 |
|  | Crocodylus niloticus | RVC crocodile1 | SE | Aq | 94200 | 0.368 | 4.777 | 0.231 | 0.45 | 11.161 | 0.598 |
| Cuculiformes | Geococcyx californianus | UMZC 429.p | C | Te | 376 | 0.244 | 4.892 | 0.156 | 0.725 | 8.167 | 0.519 |
| Dinornithiformes $\dagger$ | Dinornis sp. $\dagger$ | YPM 421 | E | Te | 173500 | 0.491 | 1.137 | 1.209 | 1.518 | 0.077 | 0.802 |
| Galliformes | Coturnix ypsilophora | UMZC 405.A | C | Te | 107.5 | 0.218 | 9.166 | 0.071 | 0.388 | 247.161 | 0.435 |
|  | Meleagris gallopavo | RVC turkey1 | C | Te | 5811 | 0.274 | 5.777 | 0.124 | 0.625 | 22.641 | 0.428 |
|  | Numida meleagris | RVC guineafowl1 | C | Te | 1375 | 0.362 | 4.627 | 0.216 | 0.66 | 3.804 | 0.361 |
| Passeriformes | Pica pica | RVC magpie1 | C | Te | 213.375 | 0.363 | 9.63 | 0.107 | 0.345 | 26.451 | 0.494 |
| Rheiformes | Rhea americana | MNHN-ZO-AC-1876-730 | E | Te | 23000 | 0.423 | 3.435 | 0.309 | 0.6 | 2.665 | 0.72 |
| Squamata | Basiliscus basiliscus | MNHN-ZA-AC-1888-124 | S | Ar | 225 | 0.305 | 9.414 | 0.108 | 0.317 | 47.759 | 0.522 |
|  | Basiliscus vittatus | MNHN-ZA-AC-1883-1830 | S | Ar | 60.87 | 0.303 | 9.237 | 0.095 | 0.326 | 37.012 | 0.813 |
|  | Cyclura cornuta | MNHN-ZA-AC-1907-107 | S | Te | 16578.115 | 0.311 | 5.149 | 0.187 | 0.463 | 14.267 | 0.617 |
|  | Iguana iguana | MNHN-ZA-AC-1974-129 | S | Ar | 1530 | 0.322 | 5.275 | 0.163 | 0.498 | 6.181 | 0.719 |
|  | Phrynosoma cornutum | MNHN-ZA-AC-1893-662 | S | Te | 27.335 | 0.253 | 12.817 | 0.065 | 0.25 | 140.609 | 0.697 |
|  | Tiliqua scincoides | MNHN-ZA-AC-1898-285 | S | Te | 496.4 | 0.272 | 9.067 | 0.084 | 0.35 | 41.424 | 0.632 |
| Struthioniformes | Struthio camelus | RVC ostrich1 | E | Te | 109250 | 0.254 | 1.547 | 0.424 | 2.355 | 1.78 | 0.694 |
| Tinamiformes | Eudromia elegans | UMZC 404.E | C | Te | 678 | 0.336 | 6.424 | 0.145 | 0.512 | 18.974 | 0.754 |

TABLE 1 (Continued)

| Taxon |  | Collection number | Femoral posture | Lifestyle | Body mass | BV/TV | BS/TV | Tb.Th | Tb.Sp | Conn.D | DA |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Testudines | Astrochelys radiata | MNHN-ZA-AC-1891-1151 | S | Te | 10217 | 0.314 | 4.787 | 0.177 | 0.46 | 19.203 | 0.575 |
|  | Chelonoidis carbonarius | MNHN-ZA-AC-1877-404 | S | Te | 2000 | 0.332 | 6.226 | 0.146 | 0.382 | 34.228 | 0.574 |
|  | Chelydra serpentina | MNHN-ZA-AC-1897-255 | S | Aq | 5170 | 0.267 | 5.189 | 0.124 | 0.293 | 44.525 | 0.517 |

Note: For each taxon, the reported body mass value (in grams) is the median (Myhrvold et al., 2015).
 E, erect; Fo, fossorial; S, sprawling; SE, "semi-erect"; Tb.Sp, the mean trabecular spacing; Tb.Th, the mean trabecular thickness; Te, terrestrial.
 London, UK; UFGK, Institut für Ur- und Frühgeschichte, Cologne, Germany; UMZC, University Museum of Zoology, Cambridge, UK; YPM, Yale Peabody Museum, New Haven, USA.

## 2.4 | Principal component analysis

We performed a principal component analysis (PCA) on the microanatomical parameters. PCA creates new uncorrelated variables from the original variables and projects them into a space whose dimensions, called principal components (PCs), successively maximize the variance (Jolliffe, 2022). This analysis was performed using the PCA function in the $R$ package FactorMineR (Lê et al., 2008).

## 2.5 | Building reference time-calibrated phylogenies

In this study, we carried out several phylogenetically informed statistical analyses. Therefore, we needed time-calibrated phylogenetic trees comprising the amniote taxa in our sample. We constructed a set of 100 time trees to account for phylogenetic uncertainties. We retrieved 100 mammal, bird and squamate trees from tree distributions on VertLife: mammal trees were extracted from 10000 trees (node-dated, 5911 species) from Upham et al. (2019), bird trees (including Tinamus major) were sampled from 10000 trees (Hackett All Species) from Jetz et al. (2012), and squamate trees from 10000 trees from Tonini et al. (2016). For crocodylians and turtles, we built a unique tree (1 for crocodylians and 1 for turtles) directly in R (R Core Team, 2013). The trees were assembled in R using the phytools package (Revell, 2012) and TreePar (Stadler, 2011). We set the divergence between mammals and reptiles at 330 Myr based on Didier and Laurin (2020). For later divergence times, we used the online resource TimeTree (Kumar et al., 2017). We set the divergence between Lepidosauria and other reptiles at 281 Myr. We considered turtles as the sister taxon to Archosauria (birds and crocodylians), as suggested by recent molecular studies (Chiari et al., 2012; Irisarri et al., 2017), and set the age of the turtle+archosaur clade (Archelosauria) at 261 Myr . We set the divergence between Astrochelys radiata and Chelonoidis carbonaria (Testudinidae) and between Chelydra serpentina and Testudinidae at 48 and 105 Myr (adjusted time), respectively. The divergence between birds and crocodylians (Archosauria) was set at 245 Myr. Raphus cucullatus, Pezophaps solitaria and Dinornis were absent from the bird trees of Jetz et al. (2012), so we had to add them. The divergence between Raphus and Pezophaps and between Columba livia and the Raphus+Pezophaps clade was set at 13.1 and 58 Myr (adjusted time), respectively. The divergence between Dinornis and Tinamidae (Tinamus major+Eudromia elegans) was initially set at 77 Myr (adjusted time), but this was older than the age of the Palaeognathae node for some of the trees in our phylogenetic tree set. We therefore decided to branch Dinornis halfway between the Palaeognathae and Tinamidae nodes for each tree. We then removed Tinamus major from our trees as it was not part of our analysed amniote sample. Finally, we set the divergence between Alligator mississippiensis and Caiman crocodilus (Alligatoridae), and between Crocodylus niloticus and Alligatoridae at 68 and 87 Myr,

$\mathrm{BV} / \mathrm{TV}=$ Bone volume/Total volume
BS/TV = Bone surface/Total volume
Tb.Th = Trabecular thickness
$\mathrm{Tb} . \mathrm{Sp}=$ Trabecular spacing
Conn.D $=$ Connectivity density (number of trabeculae)
DA = Degree of anisotropy (trabecular orientation)

FIGURE 1 Proximal femur of Gypaetus barbatus (MNHN-ZO-AC-1993-52). Bone cubes were extracted following the fit sphere routine (Doube et al., 2011). We extracted the largest cube that could be contained in a sphere fitted by least squares in the femoral head. The microanatomical parameters were measured in ImageJ with BoneJ 1.4.3 (Doube et al., 2010).
respectively. The trees in Newick tree format are available as supporting information (Appendix S1).

## 2.6 | Phylogenetic signal

We tested the phylogenetic signal in femoral posture. This was done using the delta statistic (Borges et al., 2019), which is based on the uncertainty associated with ancestral state reconstruction. The delta statistic is proportional to the phylogenetic signal and inversely proportional to the uncertainty at the nodes. We tested the phylogenetic signal in femoral posture with our 100 trees and calculated a $p$-value each time based on 10 random permutations of femoral posture at the tips of the tree branches. We also searched for a phylogenetic signal in the aforementioned microanatomical parameters using the phylosig function of the R package phytools (Revell, 2012), which computes the K-statistic (Blomberg et al., 2003). A strong phylogenetic signal, implying that closely related species are more similar to each other than would be expected under a Brownian model of evolution, is indicated by a K-statistic greater than 1. A weaker phylogenetic signal than that expected under a Brownian model of evolution is indicated by a $K$-statistic less than 1. The function also performs a randomization test to derive a $p$-value (1000 randomizations). We calculated $K$ for the 100 trees in our phylogenetic tree set.

## 2.7 | Phylogenetic generalized least squares

All the microanatomical parameters presented above have been previously associated with femoral posture, body mass or lifestyle in several amniote clades (Bishop et al., 2018; Doube et al., 2011; Mielke et al., 2018; Plasse et al., 2019). As mentioned above, the taxa in our sample show a diversity of femoral postures, as well as a
variety of lifestyles (semi-aquatic, arboreal, fossorial and terrestrial; see Table 1), but also differ greatly in terms of body mass (from 2.33 g in Suncus etruscus to 3.22 t in Elaphas maximus). To explore these relationships with our sample, we designed several linear models in R using the gls function from the package nlme (Pinheiro et al., 2021). The function fits a linear model using generalized least squares (GLS). It allows the model errors to be correlated and/or have unequal variances. It is especially appropriate in the case of phylogenetic dependence. Here the expected covariance between two taxa for a given trait is the evolution of that trait under a Brownian model during the time between the root and their last common ancestor. PGLS was conducted with all 100 phylogenetic trees.

## 2.8 | Ancestral state reconstruction

We used the ace and fastAnc functions in the $R$ packages ape (Paradis \& Schliep, 2019) and phytools (Revell, 2012) respectively to reconstruct the ancestral states of femoral posture and of the microanatomical parameters. For femoral posture (discrete), inferred states are empirical Bayesian posterior probabilities under an equal rate Markov model. For the microanatomical parameters (continuous), inferred values are maximum likelihood estimates. Several nodes were of particular interest to us (Figure 2). In reptiles, the Triassic divergence between avemetatarsalians and pseudosuchians (Archosauria) also corresponds to the appearance of the first erect forms within these two clades (Hutchinson, 2006). In synapsids, the divergence between monotremes and therians (Mammalia) in the Early Jurassic, and that between metatherians and eutherians (Theria) in the Late Jurassic, are important because they represent two hypothetical origins of more parasagittal limbs in this clade (Kielan-Jaworowska \& Hurum, 2006; Pridmore, 1985). For the microanatomical parameters, we constructed a distance matrix to


FIGURE 2 Simplified cladogram showing the relationships between the main amniote taxa studied. Stars indicate nodes of interest for ancestral reconstruction: 1, Archosauria; 2, Mammalia; 3, Theria.
compare the inferred values at the nodes of interest to our extant sample. Node states and values were reconstructed for the 100 phylogenetic trees at our disposal.

## 2.9 | Phylogenetic flexible discriminant analysis

We used phylogenetic flexible discriminant analysis (PFDA) to discriminate femoral posture based on the microanatomical parameters and to infer posture in the hypothetical last common ancestors of Archosauria, Mammalia and Theria while accounting for the phylogeny. In practice, PFDA consists of a combination of linear regressions where a categorical dependent variable is recoded into dummy variables to be treated as a continuous variable (Motani \& Schmitz, 2011). PFDA includes a phylogenetic variance-covariance matrix whose terms reflect the shared evolutionary time between two given taxa. The matrix is multiplied by lambda (Pagel, 1999), which is optimized to minimize the error of the model. We performed leave-one-out cross-validation with our 100 phylogenetic trees to identify the combination of microanatomical variables that best explains femoral posture. PFDA cannot make inferences at nodes. Instead, we inferred femoral posture in three hypothetical common ancestors to which we assigned the values of the microanatomical parameters derived from the ancestral state reconstruction and which we branched 0.1 Myr before each node of interest (branch length $=0.1$ Myr; see Appendix S1).

## 3 | RESULTS

## 3.1 | Principal component analysis

Principal component analysis is moderately effective in separating femoral postures based on the microanatomical parameters. The first two PCs account for almost 70\% of the variance. Crouched species occupy the centre-left of the graph, while erect species are found in the centre-right (Figure 3). Sprawling and "semi-erect" species are all in the middle of the graph and overlap with crouched and erect species. In general, crouched mammals occupy the upper part of the graph, while crouched reptiles are found in the lower part. Crouched mammals and reptiles show a similar dispersion. Conversely, erect mammals are concentrated in the middle of the graph, while erect reptiles (birds) are more dispersed and confined to the right side. BS/TV, Tb.Th, Tb.Sp and Conn.D are well represented on the first PC and are the main contributors to the latter, while BV/TV and DA are better represented and contribute more to the second PC. BV/TV and DA are positively correlated, as are BS.TV and Conn.D. The latter two are also negatively correlated with Tb.Sp. PCA plots showing the first and third axes and the second and third axes are available as supporting information (Appendix S2: Figures S1 and S2), along with the numerical values of the PCA (Appendix S2: Tables S2-S4).

## 3.2 | Phylogenetic signal

The delta statistic for femoral posture ranges from 3.428 to 26.676 and is always significantly higher than the randomized deltas ( $p$-values $<0.001$ ), indicating that femoral posture conveys a strong phylogenetic signal (Table 2). The K-statistics for the microanatomical parameters are all significantly different from those with a random distribution (Table 2). However, $K$ is always less than 1, indicating that closely related species are more distinct from each other than would be expected with a Brownian model of evolution.

## 3.3 | Interaction with microanatomy, posture, body mass and lifestyle

The allometric relationships of the microanatomical parameters, as shown by PGLS with our amniote sample (Table 3), are the following: the bone volume fraction (BV/TV) does not vary with body mass, unlike all other parameters. Indeed, the trabecular thickness (Tb.Th) and the trabecular spacing (Tb.Sp) increase with body mass, while the bone area per unit volume (BS/TV), the connectivity density (Conn.D) and the degree of anisotropy (DA) decrease (Appendix S2: Table S5). However, none of the microanatomical parameters are associated with lifestyle or femoral posture (Table 3).


FIGURE 3 Principal component analysis showing the variation between the taxa in our sample. Eigenvalues and contribution of each microanatomical variable to the first two PCs are shown. BS/TV, bone surface/total volume; BV/TV, bone volume/total volume; Conn.D, connectivity density (number of trabeculae); DA, degree of anisotropy (trabecular orientation); Tb.Sp, trabecular spacing; Tb.Th, trabecular thickness.

TABLE 2 Phylogenetic signal in the data.

| Trait | Mean delta statistic (min-max) | Mean $K$-statistic (min-max) | Mean $p$-value (min-max) |
| :--- | :--- | :--- | :---: |
| Femoral posture | $14.525(3.428-26.676)$ | $<0.001^{* * *}(<0.001-<0.001)$ |  |
| BV/TV |  | $0.141(0.099-0.173)$ | $0.002^{* *}(0.001-0.013)$ |
| BS/TV | $0.209(0.158-0.247)$ | $0.001^{* *}(0.001-0.002)$ |  |
| Tb.Th | $0.22(0.149-0.283)$ | $0.009^{* *}(0.001-0.055)$ |  |
| Tb.Sp | $0.186(0.119-0.238)$ | $0.009^{* *}(0.001-0.09)$ |  |
| Conn.D | $0.286(0.242-0.328)$ | $0.02^{*}(0.006-0.045)$ |  |
| DA | $0.128(0.076-0.159)$ | $0.005^{* *}(0.001-0.043)$ |  |

Note: Values are means obtained from 100 phylogenetic trees. Asterisks indicate mean $p$-values that are statistically significant: one asterisk (*) indicates a mean $p$-value that is below or equal to 0.05 ; two asterisks $\left({ }^{* *}\right)$ indicate a mean $p$-value that is below or equal to 0.01 ; three asterisks ( ${ }^{* * *}$ ) indicate a mean $p$-value that is below or equal to 0.001 .

## 3.4 | Ancestral state reconstruction

A sprawling posture (Figure 4) is the most probable at the Archosauria node (mean probability $=0.487$ ), followed by a crouched posture (0.341). "Semi-erect" or erect postures are the least probable ( 0.131 and 0.04 , respectively). A crouched posture is the most probable at the Mammalia node (0.886). Sprawling, "semi-erect" and erect postures all have a probability of less than 0.1 ( $0.094,0.011$ and 0.009 , respectively). A crouched posture is even more likely at the Theria node (0.974). A sprawling posture has a probability of 0.02 , while "semi-erect" or erect postures have a probability of only 0.003 . We also reconstructed the ancestral values for the microanatomical parameters. For the hypothetical ancestor of archosaurs, the most similar extant descendant according to the phenotypic distance matrix is Caiman crocodilus. The most similar extant descendant to the ancestor of mammals and therians is Tiliqua scincoides and Basiliscus basiliscus,
respectively. Graphs with all reconstructed states and numerical values for nodes of interest are available as supporting information (Appendix S2: Figures S3-S9; Tables S6 and S7).

## 3.5 | Postural inferences at nodes

We do not present here the best PFDA model because the crossvalidation results associated with it are very unbalanced between the postural categories (Appendix S2: Table S8). Instead, we show the model with the most balanced cross-validation results for each femoral posture (Table 4). It is based on the following combination of microanatomical parameters: BV/TV, BS/TV, and Conn.D.

In all cases, PFDA is only moderately successful in discriminating femoral posture (mean model rate of correct classification: 62\%; Figure 5; Table 4). Although crouched species are reasonably recovered by the analysis (73\%), both erect and sprawling species hardly

TABLE 3 Effect of body mass, lifestyle and femoral posture on the microanatomical parameters.

| PGLS model formula | Independent variable | Mean chi-square (min-max) | Mean $p$-value (min-max) |
| :---: | :---: | :---: | :---: |
| $\frac{\mathrm{BV}}{\mathrm{TV}} \sim \mathrm{BM}+\mathrm{LS}+\mathrm{FP}$ | BM | 0.812 (<0.001-2.428) | 0.436 (0.123-0.991) |
|  | LS | 1.059 (0.767-1.531) | 0.787 (0.675-0.857) |
|  | FP | 1.498 (0.655-3.255) | 0.687 (0.354-0.884) |
| $\frac{\mathrm{BS}}{\mathrm{TV}} \sim \mathrm{BM}+\mathrm{LS}+\mathrm{FP}$ | BM | 42.653 (18.355-55.78) | <0.001*** (<0.001-<0.001) |
|  | LS | 1.162 (0.727-1.532) | 0.762 (0.675-0.867) |
|  | FP | 0.473 (0.216-2.8) | 0.924 (0.424-0.975) |
| Tb. Th $\sim \mathrm{BM}+\mathrm{LS}+\mathrm{FP}$ | BM | 13.567 (3.973-18.544) | $0.002^{* *}(<0.001-0.049)$ |
|  | LS | 0.231 (0.119-0.435) | 0.972 (0.933-0.99) |
|  | FP | 4.858 (2.403-9.646) | 0.197 (0.022-0.493) |
| Tb. $S p^{\sim}$ BM + LS + FP | BM | 7.236 (1.074-10.341) | 0.015* (0.002-0.303) |
|  | LS | 0.408 (0.145-0.665) | 0.938 (0.882-0.986) |
|  | FP | 0.497 (0.154-2.456) | 0.917 (0.483-0.985) |
| Conn. ${ }^{\sim}$ BM + LS + FP | BM | 25.184 (18.105-29.566) | $<0.001^{* * *}(<0.001-<0.001)$ |
|  | LS | 3.44 (2.524-4.577) | 0.332 (0.206-0.471) |
|  | FP | 0.35 (0.22-0.69) | 0.95 (0.875-0.974) |
| $D A \sim B M+L S+F P$ | BM | 11.352 (6.555-51.044) | $0.003 * *(<0.001-0.012)$ |
|  | LS | 0.782 (0.427-2.149) | 0.853 (0.542-0.935) |
|  | FP | 3.716 (0.932-11.108) | 0.336 (0.011-0.818) |

Note: Body mass is log10 transformed. Asterisks indicate mean $p$-values that are statistically significant: one asterisk (*) indicates a mean $p$-value that is below or equal to 0.05 ; two asterisks $\left({ }^{(* *)}\right.$ indicate a mean $p$-value that is below or equal to 0.01 ; three asterisks $\left({ }^{(* * *)}\right.$ indicate a mean $p$-value that is below or equal to 0.001 .
Abbreviations: BM, body mass; FP, femoral posture; LS, lifestyle.


FIGURE 4 Posterior probabilities of femoral posture at nodes of interest (bottom to top: Archosauria; Mammalia; Theria) derived from ancestral state reconstruction with our sample of extant taxa.
pass $50 \%$ of correct classification (57\% and 50\%, respectively). "Semi-erect" species are always misclassified. We were still able to make postural inferences for the hypothetical ancestral taxa. All hypothetical ancestors are always inferred to be sprawlers (Figure 5), regardless of lambda value ( $0.25<$ lambda $<0.28$; mean $=0.27$ ), and even with the best PFDA model. All posterior probabilities for each ancestral taxon are available as supporting information (Appendix S2: Tables S9 and S10).

## 4 | DISCUSSION

## 4.1 | Femoral microanatomy vs. phylogeny, body mass and posture

BS/TV, Tb.Th, Tb.Sp and Conn.D are the main contributors to the first axis of the PCA. Crouched and erect species are better separated along this axis, with crouched and erect taxa having lower and higher values, respectively (Figure 3). However, PGLS do not reveal a significant association between these microanatomical parameters and femoral posture (Table 3). This may be due to the fact that PCA does not take phylogeny into account, unlike PGLS. Indeed, femoral posture carries a strong phylogenetic signal (Table 2). Furthermore, all these parameters are significantly associated with body mass (Table 3). Indeed, the trabecular thickness (Tb.Th) and the trabecular spacing (Tb.Sp) increase with body mass, while the bone area per

TABLE 4 Success rate of the PFDA inference model of femoral posture assessed by cross-validation.

| PFDA model formula | Mean model RCC (min-max) | Femoral posture | Mean group RCC (min-max) |
| :--- | :--- | :--- | :--- |
| FP $\sim \frac{B V}{T V}+\frac{\text { BS }}{\text { TV }}+$ Conn.D | $0.618(0.591-0.645)$ | Crouched | $0.731(0.705-0.773)$ |
|  |  | Erect | $0.567(0.5-0.639)$ |
| $0.0 .495(0.4-0.5)$ |  |  |  |

Abbreviations: FP, femoral posture; PFDA, phylogenetic flexible discriminant analysis; RCC, rate of correct classification.


FIGURE 5 Discrimination of femoral posture within our amniote sample, as shown by phylogenetic flexible discriminant analysis (PFDA) on the microanatomical parameters with tree 1.
unit volume (BS/TV), the number of trabeculae in the bone cube (Conn.D) and the degree of anisotropy (DA) decrease (Appendix S2: Table S5). Previous studies identified similar scaling patterns within mammals and birds (Doube et al., 2011), but also reptiles (Plasse et al., 2019). Here we show that these allometric relationships appear to hold when considering amniotes as a whole. However, this is not surprising since we partially reused data from Doube et al. (2011). Thus, the postural patterns revealed by PCA could be spurious and reflect both the effect of the phylogeny and body mass. Therefore, the microanatomical parameters measured in 3D at the femoral head may not be appropriate proxies to characterize femoral posture once phylogeny and body mass are taken into account, at least with our sample and methods. This may be related to the location where the bone cubes were extracted, that is, the centre of the femoral head. Indeed, several studies of primates have shown that the core of an epiphysis carries less functional signal than the peripheral (subchondral) areas (Cazenave et al., 2021; Georgiou et al., 2020). This could also be related to the presence of a secondary ossification centre in the femoral head of mammals and lepidosaurs (Carter et al., 1998; Xie et al., 2020). It would be interesting in the future to test for a functional signal with bone volumes from other locations (e.g. metaphysis). In addition, it should be mentioned that trabecular bone in the proximal femur depends, among other things, on the loading conditions at the hip. These vary according to locomotion or posture, but can also vary between taxa within the same postural
group (Christen et al., 2014; Ryan \& Ketcham, 2005). How this may affect our results requires further investigation.

Principal component analysis reveals that Conn.D and BS/TV are positively correlated, which is not surprising. Indeed, if the number of trabeculae increases, this logically leads to an increase in bone surface. Conn.D and BS/TV are also both negatively correlated with Tb.Sp, which is not surprising either. Indeed, if the number of trabeculae increases, the trabecular spacing mechanically decreases. The positive correlation between BV/TV and DA is more difficult to explain. However, this apparent correlation might be an artefact. Indeed, if the cube extracted from the femoral head contains little bone, it becomes more difficult to detect a potential trabecular orientation (Plasse et al., 2019).

## 4.2 | Palaeobiological implications

A sprawling posture is the most probable at the Archosauria node based on ancestral state reconstruction (Figure 4; Appendix S2: Table S6). This is consistent with the reconstructed values of the microanatomical parameters at this node that place the ancestor of archosaurs close to Caiman crocodilus (Appendix S2: Table S7), although there is no significant association between the microanatomical parameters and femoral posture (Table 3). However, the ancestor of crocodylians and birds lived in the Triassic, a period
of postural transition for archosaurs (Cuff et al., 2022; Kubo \& Benton, 2009; Sereno, 1991; Sullivan, 2015). Archosauria may have exhibited a mosaic of characters, as is the case for earlier Triassic taxa, such as Euparkeria capensis, an archosauriform (Demuth et al., 2020), and as posterior probabilities seem to suggest (a crouched posture is the second most probable posture at this node; see Appendix S2: Figure S4 and Table S6). A key issue is that we reconstructed ancestral states and values from extant and recently extinct species only. Crocodylians and birds in particular are very different from what the ancestral archosaur probably was like. Thus, our results may be partially subject to a "pull of the recent" bias. However, the inclusion of fossils with "known" posture in the sample could help to alleviate this issue. Indeed, the posture of some extinct species can be reliably estimated, or "known" well enough (i.e. general consensus in the field, based on good evidence, even if indirect), to accept them as useful "facts" (data) for further analyses. For example, we now "know" that non-avian theropod dinosaurs had more erect limbs than birds (Gatesy, 1991). Similarly, we "know" that sauropod dinosaurs had columnar limbs to reduce weight constraints (Hutchinson, 2021). Including such taxa in the models would certainly help to refine the reconstructions. The most probable posture at the Mammalia and Theria nodes is crouched, implying that more parasagittal limbs appeared early in the evolution of synapsids, with the first mammals and potentially before. However, these results are in complete contradiction with the maximum likelihood estimates of the microanatomical parameters at these nodes. The latter place the bone microarchitecture of the ancestor of mammals closer to Tiliqua scincoides and the ancestor of therians closer to Basiliscus basiliscus, two squamates with sprawling limbs (Appendix S2: Table S7). In addition to supporting a late origin of parasagittal gait, this implies a potential convergence in the acquisition of more parasagittal femora. However, insofar as we were unable to demonstrate an association between the bone microarchitecture and femoral posture with our amniote sample, we consider that functional hypotheses based on the maximum likelihood estimates of the microanatomical parameters are less reliable than nodal estimates based on observed femoral posture of extant taxa. Be that as it may, it is interesting to compare our findings with previous hypotheses. Among the proponents of an early origin of parasagittalism, Sereno (2006), on the basis of morphological evidence, argued that multituberculates had parasagittal forelimbs and that parasagittal gait evolved once in mammals, sometime before the Late Jurassic. Pridmore (1985), following Jenkins and Parrington (1976), who pointed out the similarities between the postcranial skeletons of Late Triassic mammaliaforms and later therian mammals, argued that parasagittal gait had an Early Triassic origin. Among the supporters of the late parasagittal gait hypothesis, Gambaryan and Kielan-Jaworowska (1997), and later Kielan-Jaworowska and Hurum (2006), provided morphological and ichnological arguments for a therian origin of parasagittal gait. This needs to be studied in more detail. Hopefully, further research, building on recent biomechanical work (e.g. Brocklehurst et al., 2022), will shed light on this issue.

## 4.3 | Inference model

Phylogenetic flexible discriminant analysis does not perform better than PCA in discriminating between femoral postures. The best combination of microanatomical parameters achieved by cross-validation yields a model rate of correct classification of about $65 \%$, but very contrasting results between the postural categories (Appendix S2: Table S8). The main source of error is that some erect species are modelled as crouched. The model presented in the Results section reduces this problem somewhat, but an imbalance persists (Table 4). A larger sample size could probably reduce the residual error further, but it also appears that the postural signal is weak for the 3D microanatomical parameters. Postural inferences with hypothetical ancestral taxa are consistent with the reconstructed ancestral microanatomical parameters and support a late postural transition in mammals. However, these results should be viewed with caution with regard to the low robustness of the model, which is most likely due to the lack of association between the microanatomical parameters and femoral posture (Table 3).

## 5 | CONCLUSIONS

None of the microanatomical parameters measured on 3D bone cubes extracted from the femoral head of a sample of amniote taxa, that is, the bone volume fraction ( $\mathrm{BV} / \mathrm{TV}$ ), the bone surface area (BS/TV), the mean trabecular thickness (Tb.Th), the mean trabecular spacing (Tb.Sp), the connectivity density (Conn.D) and the degree of anisotropy (DA), are significantly associated with femoral posture, nor with lifestyle, as shown by phylogenetic generalized least squares. On the contrary, we show that all microanatomical parameters, except BV/TV, are significantly associated with body mass. In addition, we identify that femoral posture carries a phylogenetic signal. A different sampling area for the bone cubes, for example in the metaphysis, which potentially carries a stronger functional signal, could lead to different results. It would also be interesting to apply the method to other bones of the appendicular skeleton, such as the humerus, to see if a similar pattern is found.

Reconstruction of the ancestral femoral posture based on nodal reconstructions and a sample of extant taxa suggests that the most probable posture at the Archosauria node is sprawling, consistently with the maximum likelihood estimates of the microanatomical parameters at this node. However, the reconstructions could suffer from a "pull of the recent" bias, as crocodylians and birds differ from the probable ancestral archosaurian condition. A crouched posture is the most likely posture at the Mammalia and Theria nodes, implying an early postural transition in Synapsida, towards the Late Triassic/Early Jurassic. However, the reconstructed ancestral microanatomical parameters give opposite results, except for the Archosauria node. Indeed, the reconstructed parameters indicate a more sprawling posture at the Mammalia and Theria nodes, suggesting a late postural transition. The addition of new taxa to the reconstructions, particularly extinct species with "known" posture
that are anatomically closer to the ancestral condition of archosaurs, mammals and therians, could refine the inferences at these nodes. Overall, the results obtained from the maximum likelihood estimates of the microanatomical parameters are less reliable than those obtained from the observed femoral posture, as we fail to find a significant association between the microanatomical parameters and femoral posture in our sample.

Phylogenetic flexible discriminant analysis based on the microanatomical parameters does not perform better than PCA in separating the femoral postures. The rate of correct classification is highly unbalanced between postural categories. Adding taxa to the sample might help to overcome this problem, but considering the PFDA and PCA results together, it appears that, in amniotes, the trabecular architecture of the femoral articular head does not carry a particularly strong postural signal, at least with our sample. Nevertheless, we tentatively infer the posture of the hypothetical ancestor of archosaurs, mammals and therians. The model infers a sprawling posture in all cases and thus supports a late postural transition in Synapsida. However, these results should be taken with caution given the lack of a statistically-validated relationship between the microanatomical data and femoral posture.

The growing interest in postural issues in extant and extinct animals in recent decades has improved our knowledge of vertebrate evolution and augurs exciting future discoveries. In this paper, we show that, despite the weak association between femoral posture and the trabecular architecture of the femoral head in amniotes, ancestral state reconstruction methods applied to postural problems are promising. They deserve a more prominent place in the study of postural transitions, especially in the case of Mesozoic amniotes.

## AUTHOR CONTRIBUTIONS

Jordan Gônet: Conceptualization (lead); data curation (lead); formal analysis (lead); writing - original draft (lead); writing - review and editing (equal). Michel Laurin: Conceptualization (supporting); data curation (supporting); formal analysis (supporting); writing - original draft (supporting); writing - review and editing (equal). John R. Hutchinson: Conceptualization (supporting); data curation (supporting); formal analysis (supporting); writing - original draft (supporting); writing - review and editing (equal).

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are publicly available on the Dryad Digital Repository: https://doi.org/10.5061/ dryad.83bk3j9x2.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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