

# Heterochrony and post-natal growth in mammals – an examination of growth plates in limbs

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## Abstract

Mammals display a broad spectrum of limb specializations coupled with different locomotor strategies and habitat occupation. This anatomical diversity reflects different patterns of development and growth, including the timing of epiphyseal growth plate closure in the long bones of the skeleton. We investigated the sequence of union in 15 growth plates in the limbs of about 400 specimens, representing 58 mammalian species: 34 placentals, 23 marsupials and one monotreme. We found a common general pattern of growth plate closure sequence, but one that is universal neither between species nor in higher-order taxa. Locomotor habitat has no detectable correlation with the growth plate closure sequence, but observed patterns indicate that growth plate closure sequence is determined more strongly through phylogenetic factors. For example, the girdle elements (acetabulum and coracoid process) always ossify first in marsupials, whereas the distal humerus is fused before the girdle elements in some placentals. We also found that heterochronic shifts (changes in timing) in the growth plate closure sequence of marsupials occur with a higher rate than in placentals. This presents a contrast with the more limited variation in timing and morphospace occupation typical for marsupial development. Moreover, unlike placentals, marsupials maintain many epiphyses separated throughout life. However, as complete union of all epiphyseal growth plates is recorded in monotremes, the marsupial condition might represent the derived state.

## Introduction

Mammals display a broad spectrum of limb morphology coupled with different locomotor strategies and habitat occupation (Polly, 2007). Structural differences in form between species often reflect functional specializations (Farnum, 2007; Polly, 2007). The individual limb phenotype is formed during ontogeny, and as selection can act on any level of biological organization and at any time during ontogeny, the possibilities for creating different limb morphologies are numerous (Farnum, 2007). One important aspect in limb evolution that leads to interspecific structural difference is the change

of timing (i.e. heterochrony) of individual events in skeletal ontogeny (Richardson *et al.*, 2009). Heterochronic shifts were found to be responsible for disparity in body proportions not only in prenatal development, but also during post-natal growth (Raff & Wray, 1989; Farnum, 2007). For example, it has been shown that allometric growth and growth heterochrony in limbs of different primates lead to proportion differences that are potential adaptations to different locomotor behaviours (Shea, 1993).

One aspect of post-natal long bone growth includes elongation by increment at their cartilaginous ends (Hall, 2005; Farnum, 2007). Mammals build a secondary ossification centre within this cartilage, and longitudinal growth is restricted to the cartilaginous growth plate between the bony metaphysis and the secondary ossification centre (Hall, 2005; Farnum, 2007). This cartilaginous growth plate grows by division and vol-

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ume increase in the cartilage cells before it is replaced by bone (endochondral ossification) (Hall, 2005; Farnum, 2007). When the finite proliferative capacity of the chondrocytes is exhausted, longitudinal bone growth ceases and bony union occurs (Parfitt, 2002; Nilsson & Baron, 2004; Hall, 2005). The growth plates of all long bones of a skeleton fuse at different times, resulting in a sequence of growth plate union. Variation in this sequence among different mammalian clades remains understudied, mainly due to a widespread view that post-cranial growth plate closure is evolutionary conserved across Mammalia (King *et al.*, 2001). Stevenson (1924, p. 89) stated that the particular sequence of epiphyseal union reported in his study 'may be considered a general mammalian trait'. Further evidence for this hypothesis was then provided by Dawson (1925), Todd (1930a,b), Koch (1935) and Todd & Todd (1938). Contrarily, other analyses have demonstrated a variation in growth plate closure sequences among mammalian species (Washburn, 1946; Shigehara, 1980).

To summarize, it is not clear whether there is disparity in epiphyseal growth plate closure sequence and whether heterochronic shifts – if they exist – influence limb proportions and hence functional adaptations. In our study, we approached this problem by asking to what extent the epiphyseal growth plate closure sequence is influenced by intrinsic (phylogenetic relationships) (Weisbecker *et al.*, 2008) and extrinsic (different biomechanical loadings through different locomotor habits) (Carter *et al.*, 1991) factors. This is the first study that investigates this issue in a wide spectrum of species that are representatives of all major mammalian clades.

We expect that influences of phylogeny may cause the most heterochronic changes in higher-order taxa because of their greater amount of morphological and physiological discrepancies. Examples are the marsupial–placental dichotomy (Sánchez-Villagra, 2002; Weisbecker *et al.*, 2008), the 'Atlantogenata'–Boreoeutheria dichotomy and the Euarchontoglires–Laurasiatheria dichotomy (Asher *et al.*, 2009). The functional aspects that may lead to heterochronic shifts in growth plate closure sequence include the function of single joints adjacent to the growth plates. This aspect is important regarding mechanical loadings. We therefore expect that the growth plate closure sequences of members of one locomotor group are more similar to one another than they are to sequences of taxa from other locomotor groups.

In this study, we investigated the sequence of growth plate closure in mammals. First, we evaluated whether growth plate closure patterns are conserved among the studied mammalian species and display the patterns originally found by Stevenson (1924). Second, we examined whether heterochronic shifts and peculiarities in growth plate closure sequence characterize major mammalian clades. Third, we investigated whether adaptations to dif-

ferent locomotor styles include heterochronic shifts in growth plate closure sequences. Further, we studied growth plate closure in relation to relative age.

## Materials and methods

### Specimens

We examined a cross-sectional ontogenetic sample comprising 395 skeletons representing 34 genera and 58 species: 34 placentals, 23 marsupials and 1 monotreme (Table 1, Table S1). A list of the investigated specimens is available from the Dryad Digital Repository. In nine genera, more than one species per genus was analysed (Fig. 1, Table S1). The maximum number of species included in one genus is seven (*Macropus* spp.). On average, genera included 11.6 specimens (max. 29 specimens; min. four specimens) (Table 1). Taxa were selected based on the availability of specimens representing multiple ontogenetic stages and to represent ecological and phylogenetic breadth (Fig. 1). Captive and wild-caught animals were sampled, and both sexes were used. Domesticated species (e.g. *Felis catus*, *Equus caballus caballus/Equus ferus caballus*) and skeletons exhibiting pathologies were excluded from the study. Also excluded were specimens with all growth plates open or all growth plates closed.

Data were collected from specimens at the following institutions: AIM, Anthropologisches Institut und Museum der Universität Zürich; MNHN, Muséum national d'Histoire naturelle, Paris; NHM, The Natural History Museum, London; NMB, Naturhistorisches Museum Basel; NMBE, Naturhistorisches Museum Bern; NRM, Naturhistoriska Riksmuseet, Stockholm; PIMUZ, Paläontologisches Institut und Museum der Universität Zürich; SZ, Zoologische Schausammlung der Universität Tübingen; UMZC, University Museum of Zoology, Cambridge University; ZMM\_MAM, Museum für Naturkunde, Berlin; ZMUZH, Zoologisches Museum der Universität Zürich; and ZSM, Zoologische Staatssammlung München.

### Data collection

The closure states in 15 growth plates in the fore- and hindlimbs were scored: coracoid process of the scapula; proximal humerus; distal humerus; proximal ulna; distal ulna; proximal radius; distal radius; acetabulum of the pelvis; head of the femur; greater trochanter of the femur; distal femur; proximal tibia; distal tibia; proximal fibula; and distal fibula. In *Capreolus capreolus* and *Equus* spp., only 13 and 12 growth plate sutures, respectively, could be scored, because of an extensive reduction in the fibula in both genera and absence of the distal ulna in *Equus* spp. (Schmid, 1972). Only specimens with a complete record of closure stages in all growth plates were used for the analyses. Growth

**Table 1** Genus and species names (Wilson & Reeder, 2005), taxonomic designation (Wilson & Reeder, 2005) and colloquial names (Nowak, 1999; Wilson & Reeder, 2005); number of specimens per genus/species examined (*n*) and locomotor category (Nowak, 1999) of investigated taxa.

Taxa	Colloquial name	<i>n</i>	Locomotor category
<b>Monotremata</b>			
<i>Tachyglossus aculeatus</i>	Short-nosed echidna	7	Terrestrial
<b>Marsupialia</b>			
<b>Didelphimorphia</b>			
<i>Didelphis</i> spp.	Large American opossum	11	Terrestrial
<b>Diprotodontia</b>			
<i>Dorcopsis muelleri</i>	Brown dorcopsis	6	Bipedal saltatorial
<i>Macropus</i> spp.	Wallaby/wallaroo /kangaroo	22	Bipedal saltatorial
<i>Phascolarctos cinereus</i>	Koala	4	Arboreal
<b>Dasyuromorphia</b>			
<i>Thylacinus cynocephalus</i>	Thylacine/Tasmanian tiger	9	Cursorial
<i>Sarcophilus harrisi</i>	Tasmanian devil	8	Terrestrial
<i>Dasyurus</i> spp.	Native 'cat'/tiger 'cat'/quoll	10	Terrestrial /scansorial
<b>Peramelemorphia</b>			
<i>Perameles</i> spp.	Long-nosed bandicoot	6	Terrestrial
<i>Isodon</i> spp.	Short-nosed bandicoot	4	Terrestrial
<b>Placentalia</b>			
<b>Cingulata</b>			
<i>Dasybus</i> spp.	Long-nosed armadillo	5	Semi-fossorial
<b>Pilosa</b>			
<i>Myrmecophaga tridactyla</i>	Giant anteater	5	Terrestrial
<b>Hyracoidea</b>			
<i>Heterohyrax brucei</i>	Gray hyrax/yellow-spotted hyrax	7	Scansorial
<i>Procavia capensis</i>	Rock dassie/rock hyrax	13	Scansorial
<b>Tubulidentata</b>			
<i>Orycteropus afer</i>	Aardvark	6	Semi-fossorial
<b>Afrosoricida</b>			
<i>Tenrec ecaudatus</i>	Tenrec	6	Terrestrial
<b>Erinaceomorpha</b>			
<i>Erinaceus europaeus</i>	West European hedgehog	21	Terrestrial
<b>Artiodactyla</b>			
<i>Capreolus capreolus</i>	European roe deer	14	Cursorial
<b>Perissodactyla</b>			
<i>Equus</i> spp.	Horse, zebra, and ass	12	Cursorial
<b>Primates</b>			
<i>Perodicticus potto</i>	Potto	16	Arboreal
<i>Chlorocebus aethiops</i>	Grivet	15	Scansorial

**Table 1** (Continued)

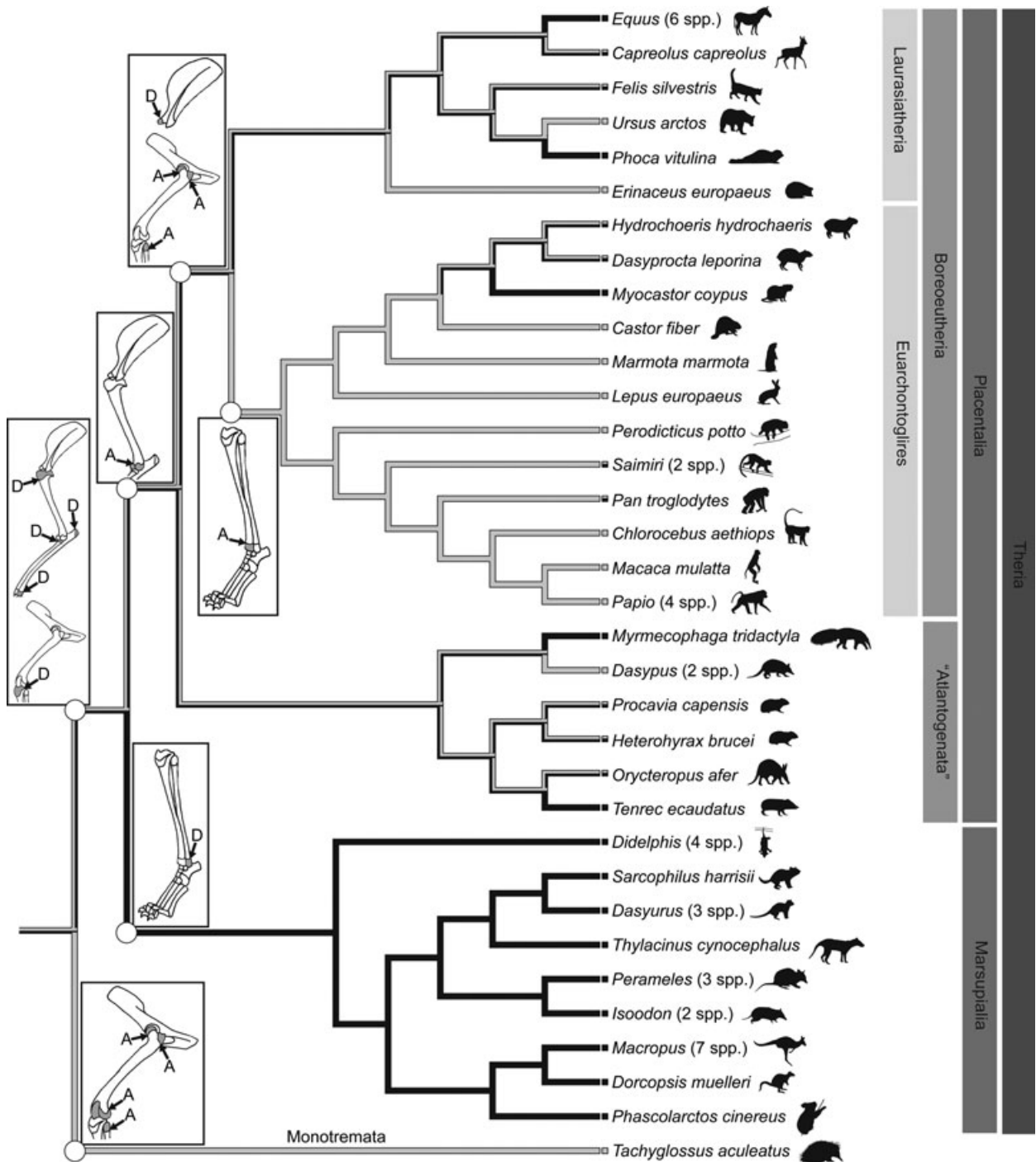
Taxa	Colloquial name	<i>n</i>	Locomotor category
<i>Macaca mulatta</i>	Rhesus monkey	25	Scansorial
<i>Papio</i> spp.	Baboon	19	Terrestrial
<i>Pan troglodytes</i>	Common chimpanzee	5	Scansorial
<i>Saimiri</i> spp.	Squirrel monkey	11	Arboreal
<b>Rodentia</b>			
<i>Marmota marmota</i>	Alpine marmot	9	Semi-fossorial
<i>Castor fiber</i>	Eurasian beaver	21	Semi-aquatic
<i>Myocastor coypus</i>	Nutria/coypu	11	Semi-aquatic
<i>Hydrochoeris hydrochaeris</i>	Capybara	5	Semi-aquatic
<i>Dasyprocta leporina</i>	Red-rumped agouti	7	Cursorial
<b>Lagomorpha</b>			
<i>Lepus europaeus</i>	European hare	20	Cursorial
<b>Carnivora</b>			
<i>Phoca vitulina</i>	Harbor seal /common seal	29	Aquatic
<i>Ursus arctos</i>	Brown bear	15	Terrestrial
<i>Felis silvestris</i>	Wildcat	11	Scansorial

plates from the left and the right sides of the body were used.

Growth plates were scored as either open or closed. A suture was considered as closed if the outer surface of the growth plate suture was partly or completely obliterated by bone. Epiphyseal union begins in the central parts of the growth plate and subsequently extends to the outer surface (Todd, 1930a). Therefore, obliteration of the outer surface of the growth plate suture signifies the latest possible point in time during ontogeny when longitudinal growth stops. All closure stages of every investigated specimen are available from the Dryad Digital Repository. Additionally, well-documented and comprehensive literature data were considered in the analysis.

### Tooth wear and locomotor groups

Generalized individual dental age stages (IDAS; Anders *et al.*, 2011) were used to document the degree of tooth eruption and wear and hence individual age class: IDAS 1, infant (period from birth to the complete eruption and beginning wear of the first molar); IDAS 2, juvenile (covers the time until the entire permanent dentition is fully erupted); IDAS 3, adult (covers the time until the loss of the inner profile in the first molar, that is, no enamel, except at the rim, is left on the occlusal surface); IDAS 4, late adult (covers the time until the complete loss of the inner profile in the second molar); IDAS 5, senile (the occlusal surface of the entire dentition is heavily worn, with the loss of functional structures and breakdown of the dentition). *Phoca vituli-*



**Fig. 1** Compound phylogeny featuring species and genera investigated (for references see text): 24 placentals, nine marsupials and one monotreme as outgroup. Taxonomic names of groups are based on Asher & Helgen (2010). Different shades of branches illustrate traced character history (most parsimonious solution) of the anatomical region that is fused first. Grey: an element of the elbow is fused first, black: an element of the girdles is fused first (for categorization of anatomical regions, see Fig. 2). Polymorphic branches (shown with both shades) indicate terminal taxa in which both conditions can be found. Squares at the tips of the branches indicate the conditions that were found in the investigated specimens. Boxes illustrate heterochronic changes in growth plate fusion that were detected using Parsimov-based genetic inference (PGI) (Harrison & Larsson, 2008). Branch lengths do not reflect those in the analysis. A: acceleration; D: delay.

*na* was excluded from the tooth wear analysis because of the homodont post-canines. Data about the IDAS of the investigated specimens are available from the Dryad Digital Repository. We acknowledge the biases introduced by the fact that the species studied exhibit different dental replacement patterns (van Nievelt & Smith, 2005) and that the wear stages in the similar loci are not fully comparable. However, these wear stages provide an age estimation that serves to examine the general pattern we wish to investigate.

Species were assigned to locomotor categories, and almost all investigated species of a genus perform the same mode of locomotion (Nowak, 1999) (Table 2). The exception is *Dasyurus*, which was categorized as terrestrial/scansorial because *Dasyurus maculatus* is scansorial, whereas the other species of the genus analysed are terrestrial. *Thylacinus cynocephalus* and *Dasyprocta leporina* are less cursorial than *Equus* spp. and *Capreolus* spp., although they are classified in the same group following the literature (Nowak, 1999). *Lepus europaeus* is a quadrupedal saltator. This is a specialization of the cursorial lifestyle and different from the bipedal saltatorial locomotion of *Macropus* spp. (Polly, 2007). *Dorcopsis* spp. shows fewer specializations associated with bipedal saltatorial locomotion than *Macropus* spp., although they are classified under the same category. Even though in the same locomotor group of semi-fossoriality, *Marmota marmota* exhibits only a few structural specializations for digging, whereas *Orycteropus afer* and *Dasyypus* spp. are highly specialized. However, all three taxa are scratch diggers (Polly, 2007).

**Table 2** Locomotor categories used in the analyses and their definitions (parts of definitions are modified from Samuels & Van Valkenburgh, 2008).

Locomotor category	Definition
Terrestrial	Ground dwelling. Perhaps able to run, swim, dig and climb, but none of these activities is performed extensively
Scansorial	Good capability to climb. Foraging and escape on the ground as well as in trees and/or on outcrops
Arboreal	Living in trees. Grasping capability. The ground is frequented only occasionally. Foraging and escape mostly off the ground
Cursorial	Capability to reach high speed, for example, for escape
Semi-aquatic	Swim for dispersal, escape, or foraging, but maintaining the ability to disperse across or acquire food on land
Aquatic	Swim for dispersal, escape, or forage. Do not usually disperse across or acquire food on land
Semi-fossorial	Digging to build burrows for shelter, but no foraging underground
Bipedal saltatorial	Capable of jumping behaviour characterized by simultaneous use of the hindlimbs and the tail

## Phylogenetic framework

For heterochrony analyses, we used the phylogenetic tree of Meredith *et al.* (2011) for the major clades. The phylogenetic position of xenarthrans and thus the monophyly of 'Atlantogenata' are subjects of controversy. Meredith *et al.* (2011) supported the monophyly of 'Atlantogenata', although the position of the placental root is still debated and it is suggested that either afrotherians, xenarthrans, or boreoeutherians are basal to the placental mammalian tree (Murphy *et al.*, 2007; Nishihara *et al.*, 2009; O'Leary *et al.*, 2013). Therefore, 'Atlantogenata' is referred here under quotation marks. The position of Perissodactyla within Laurasiatheria is also under debate, and it is suggested that Perissodactyla is either the sister group of Artiodactyla or Ferae (Carnivora and Pholidota) (Asher *et al.*, 2009). Hallström *et al.* (2011) suggested that the genome evolution of Laurasiatheria was network-like, resulting in a hard polytomy between Perissodactyla, Artiodactyla, Carnivora, and Chiroptera. In this study, we used the phylogenetic relationships suggested by Asher (2007), Prasad *et al.* (2008), Meredith *et al.* (2011) and Zhou *et al.* (2012), which considered Artiodactyla as being the sister group of Perissodactyla. Within Rodentia and Caviomorpha, the studies by Blanga-Kanfi *et al.* (2009) and Fabre *et al.* (2012), respectively, were used. The relationships within Cercopithecidae are according to Perelman *et al.* (2011). *Thylacinus cynocephalus* was positioned basal to the Dasyuomorpha as proposed by Miller *et al.* (2009). The resulting, compound phylogenetic tree is shown in Fig. 1. To estimate the evolutionary rates of the growth plate closure sequences and to correct the adjusted closure scores for influences of different degrees of phylogenetic relatedness of the terminal taxa, we used the timetree provided by dos Reis *et al.* (2012), which is based on 36 nuclear and 274 mitochondrial genomes, combined with fossil calibrations. In those cases, where the terminal taxa from dos Reis *et al.* (2012) were not congruent with our terminal taxa, the branch length estimates of the closest relative were used. In cases where dos Reis *et al.* (2012) provided estimates for a major clade, but not for taxa within that clade, we used the known divergence time of that major clade and evenly distributed the branch lengths between the nodes of unknown age within that major clade.

## Growth plate closure pattern conservation and variation

The sequence of growth plate closure was determined for every species or genus, when appropriate. The stage of closure of each growth plate ('open' = 1 or 'close' = 0) was summed up per species or genus. Following the assumption that growth plates that close early in life would be found to be closed in many specimens within an ontogenetic series, we assumed that

high numbers resulting from the sums for a particular growth plate indicate early closure of that growth plate.

To compare the sequences and to examine the rank variation in a particular closure event, each rank was adjusted following Laurin & Germain (2011):

$$r_s = (r - r_{\min}) / (r_{\max} - r_{\min}),$$

where  $r_s$  is the adjusted value of a rank,  $r$  is the absolute rank of a given closure event,  $r_{\min}$  is the lowest observed number of  $r$  ( $r_{\min} = 1$  in this study), and  $r_{\max}$  is the highest observed number of ranks for this taxon. This normalization was used because it diminishes the impact of differing numbers of ranks and hence different resolution (Koyabu *et al.*, 2011). One-way ANOVA and Tukey's *b post hoc* tests were performed to compare the means of adjusted ranks per growth plate within marsupials and placentals, 'Atlantogenata' and Boreoeutheria, as well as Laurasiatheria and Euarchontoglires separately. Additionally, one-way ANOVA was performed to investigate the sequences between major groups. This analysis was performed a second time using adjusted closure scores that were previously corrected for influences of different degrees of phylogenetic relatedness of the terminal taxa. This adjustment was computed as described in Meslin *et al.* (2012). We calculated the 78% confidence intervals of the inferred ancestral value of the common ancestor of marsupials and of the common ancestor of placentals using the PDAP:PDTree package, version 1.16 (Midford *et al.*, 2011), and Mesquite, version 2.75, respectively (Maddison & Maddison, 2011). Using this calculation, it was possible to assess whether the differences in the ancestral values of each clade are statistically significant.

Adjusted sequences were compared for higher-order clades and locomotor groups using Kendall's coefficient of concordance (Kendall's  $W$ ), a nonparametric algorithm that is used to measure the agreement between several sequences (Field, 2005). Values for  $W$  can vary between 0 (no agreement) and 1 (perfect agreement). This analysis was repeated four times with sequence data from all 15 growth plates, with data from anatomical regions (Fig. 2), and separately 'pressure epiphyses' (positioned perpendicular to the bone axis and compressed by the gravitational force and reaction force of the adjacent diaphysis) and 'traction epiphyses' (provide attachment for tendons and muscles and are subject to lateral stresses and tension) (Parsons, 1904; Serrano *et al.*, 2011) (Fig. 2).

The human closure sequence found by Stevenson (1924) was considered to be a universal pattern in mammals (Todd, 1930b). To test this hypothesis, we computed Kendall's tau ( $\tau$ ) (Kendall, 1955) for our adjusted sequences and the sequence found by Stevenson (1924). Kendall's  $\tau$  measures the cross-tabulation correspondence between two rankings. Values for  $\tau$  can vary between 1 (perfect agreement between the rankings)

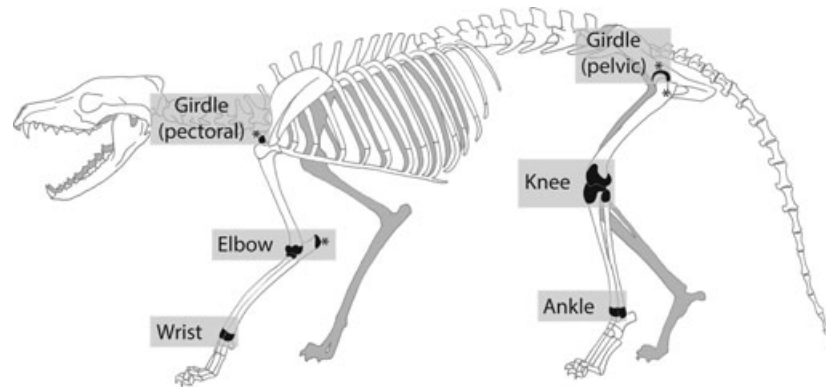
and  $-1$  (perfect disagreement). A value of 0 indicates independence. We chose Kendall's tau-b statistic to enable adjustments for ties within the rankings.

Second, we compared the taxa that were investigated by other authors and by us. There are important methodological problems in comparing growth plate closure sequences of different authors. First, closure information might differ greatly when looking at dry bones, X-rays, or cleared and stained wet specimens (Bull & Payne, 1982). Second, the definition of the moment of closure varies considerably among authors, and it is difficult to find agreements among the authors or the definition is omitted (Bull & Payne, 1982). Moreover, sequences are studied at different taxonomic levels (species, genera, families). Consequently, data obtained from the literature were not included in all our analyses and were used to address in general the issue of variability among clades and to establish whether differences in data acquisition have a significant influence on the computed closure sequence. The taxa chosen for this comparison were *Papio* spp., *Macaca mulatta*, *Pan troglodytes* and *Saimiri sciureus*. Moreover, we compared some sequences from the literature between each other using Kendall's  $\tau$ . When possible, we calculated the sequences of fusion with the data provided by the literature, but considering our definition for the fusion stage. If this was not possible, the sequences were used as provided. All sequences obtained from other authors were adjusted as already described, and missing growth plates were removed from the analysis.

Statistical analyses were conducted with version 20.0.0; IBM SPSS statistics (Armonk, NY, USA). Clades with reduced bones (e.g. *C. capreolus* and *Equus* spp.) were excluded from Kendall's  $\tau$ , Kendall's  $W$  and ANOVA. Due to the great number of statistical tests in this study, we applied the false discovery rate procedure (FDR) (Benjamini & Hochberg, 1995; Curran-Everett, 2000) to resulting p-values of Kendall's  $\tau$ , Kendall's  $W$  and ANOVA using R, version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria). This procedure was applied because multiple comparisons can lead to a higher rate of false-positive results, that is, statistically significant differences that are actually not different. The FDR analysis is a method to discover these false positives.

## Heterochrony analysis

We performed Parsimov-based genetic inference (PGi) (Harrison & Larsson, 2008) to analyse heterochronic changes in growth plate closure sequences. PGi examines the sequence as one single, complex character and uses the Parsimov algorithm as an edit-cost function to optimize ancestral states and sequence heterochronies. PGi was computed using R, version 2.15.1, and the package *pgi* 2.0. The analysis included all 15 growth plates and 34 genera. The parameters (Harrison & Larsson, 2008) used for the analysis were as follows: number of



**Fig. 2** Schematic drawing of the skeleton of a *Thylacinus cynocephalus*. Epiphyses and growth plates (black) investigated here and their correspondence with anatomical regions are indicated. Pectoral girdle: coracoid process of the scapula; pelvic girdle: acetabulum of the pelvis; elbow: distal humerus, proximal ulna and proximal radius; wrist: distal radius and distal ulna; knee: distal femur, proximal tibia and proximal fibula; ankle: distal tibia and distal fibula. Asterisks indicate 'traction epiphyses' (Parsons, 1904; Serrano *et al.*, 2011): coracoid of the scapula, proximal ulna, acetabulum of the pelvis and greater trochanter of the femur. All other epiphyses are 'pressure epiphyses' (Parsons, 1904; Serrano *et al.*, 2011).

sequences per cycle: 150; number of cycles: 150; and number of sequences retained at each node: 150. Semi-exhaustive search with a limited number of permutations per cycle (max. 10 000) was performed. The PGi analysis was computed eight times independently, and subsequently, the shortest tree, which is assumed to recover the most probable heterochronies, was chosen. Further, event pairing and Parsimov (Jeffery *et al.*, 2005) as described by Koyabu *et al.* (2011) and a continuous analysis (Germain & Laurin, 2009) were conducted to compare the results. Event pairing was computed using R, version 2.15.1, and the continuous analysis was computed using Mesquite, version 2.75 (Maddison & Maddison, 2011), and the PDAP:PDTREE module, version 1.16 (Midford *et al.*, 2011). The phylogeny and branch length estimates were computed as described above.

### Growth plate closure and relative age

To track growth plate closure throughout life history, the relative closure state of every specimen with known IDAS was calculated. IDAS was used as a proxy for relative individual age. The relative closure state per specimen was computed as the number of closed growth plates divided by the total number of scored growth plates in that specimen (12, 13 or 15, see above). One-way ANOVA was performed, and IDAS was used as the independent variable.

## Results

### General patterns of fusion

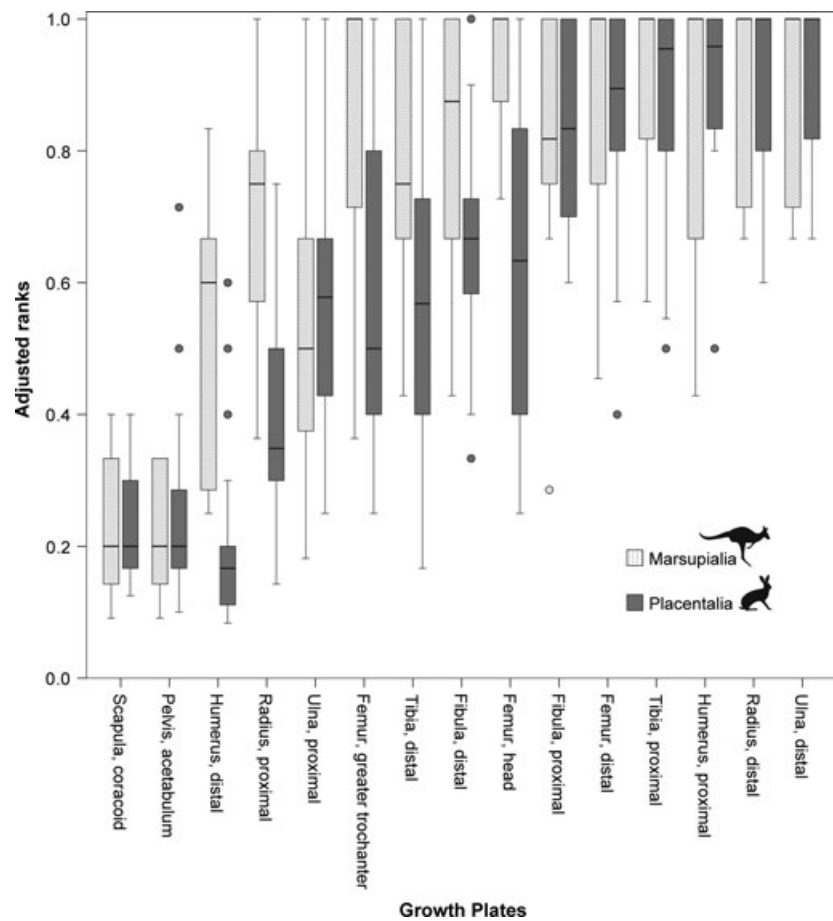
Examining the raw closure sequences for each main mammalian group, we found that in marsupials, the closure of the sutures of the girdles (i.e. coracoid

process of the pectoral girdle and acetabulum of the pelvic girdle) occurs in all species examined before the closure of the growth plates at the elbow (i.e. distal humerus, proximal ulna and radius) (Table S2). *Tachyglossus aculeatus* has the opposite sequence (Table S2). In placentals, the position of the girdles within the closure sequence is variable (Table S2). However, it is always an element of the girdles or the elbow that is ossified first. In placentals, 20.83% of the taxa (distributed randomly) have a girdle element ossifying first; 41.67% (distributed randomly) has an elbow element ossifying first; in the remaining 37.5%, both elements ossify first, which could be by simultaneous closure or unresolved because of the coarseness of data.

In about 56% of investigated taxa (distributed randomly), all elbow elements fuse before any knee element. In the remaining 44% of taxa, there is a partial overlap of fusion events in the elbow and the knee. However, in none of the taxa do any knee elements fuse before an elbow element (Table S2). A similar pattern exists at the ankle and the wrist. In 62.5% of investigated taxa (distributed randomly), excluding *Equus* spp. and *C. capreolus*, all ankle elements fuse before the wrist elements. In the remaining taxa, there is an overlap of fusion events in the ankle and the wrist. In none of the taxa do the wrist elements fuse before the ankle elements (Table S3). Moreover, wrist and ankle elements generally fuse relatively late in the ontogeny. The position of 'tension epiphyses' and 'pressure epiphyses' within the sequences is variable (Tables S2 and S3).

One-way ANOVA of adjusted ranks of all taxa showed that the mean relative closure scores are significantly different among the growth plates examined ( $F_{14,479} = 52.629$ ,  $P < 0.0001$ ) (Fig. 3). This result indicates that approximately the same growth plates are closed early or late in all mammals examined, that is,

**Fig. 3** Adjusted rank ranges ( $r/r_{\max}$ ) of single growth plates across species and genera for marsupials (dotted) and placentals (grey). Ranks range from 0 (fusing first) to 1 (fusing last).



the growth plate closure sequences are rather similar. One-way ANOVA between major clades detected significant differences in the closure scores of the distal humerus, proximal radius, femoral head and femoral greater trochanter in placentals compared with marsupials (Table 3). The same conclusion was obtained after the FDR analysis. Differences in closure scores were also reported in major clades within placentals (Table 3). However, after applying FDR analyses, the only difference that remained statistically significant was the closure score of the greater trochanter of the femur in the comparison of 'Atlantogenata' with Boreoeutheria. In a second step, the confidence intervals of the adjusted closure scores at the nodal values of the last common ancestors of marsupials and placentals were compared to correct the scores for phylogenetic relatedness of the terminal clades (Meslin *et al.*, 2012). The mean relative closure scores remained significantly different among the growth plates examined using one-way ANOVA ( $F_{14,59} = 5.419$ ,  $P < 0.0001$ ). However, two-tailed t-tests (similar variance) detected no significant differences in the closure scores of growth plates between marsupials and placentals.

There are also significant differences in adjusted ranks within clades, often taking together growth plates that are included in one joint and comprise anatomical regions (Washburn, 1946) (Table 4). Girdle elements ossify simultaneously and at a different point in time than all other growth plates in all investigated major mammalian clades (Table 4). Growth plates of the elbow ossify simultaneously in marsupials, but not in placentals (Table 4). In addition, the growth plates of the elbow in marsupials, and the wrist, proximal femur, knee and ankle in marsupials and placentals fuse simultaneously, but they also fuse at a similar time as the growth plates of other anatomical regions. For example, elements of the elbow in marsupials fuse at the same time, but also at the same time as elements of the ankle (Table 4). The sequence of fusion of anatomical regions in marsupials and placentals is different. The simultaneous fusion of growth plates included in an anatomical region was also found in the subgroups (Boreoeutheria, 'Atlantogenata', Laurasiatheria, Euarchontoglires) in approximately the same sequence. Only in 'Atlantogenata' do the ankle elements not fuse simultaneously.



### Conservation of growth plate closure sequences

The sequences are different as shown by the analysis of the sequences across clades with Kendall's W (Table 5): the values for W are relatively low and variable, even though many comparisons of sequence commonality within clades are statistically significantly similar. The most significant similarities were found when the

**Table 3** Differences in adjusted growth plate closure scores between clades. Results from one-way ANOVA are shown. As a comparison, heterochronic changes computed with Parsimov-based genetic inference (PGi) (Harrison & Larsson, 2008) are given. In contrast to ANOVA, PGi gives hypotheses about the most probable evolutionary direction of heterochronic changes.

	$F_{1,30}$	$P$	$\rho$ (FDR)	Direction (PGi)
Marsupialia and Placentalia				
HD	28.299	< <b>0.001</b>	< <b>0.001</b>	Early in placentals
RP	21.618	< <b>0.001</b>	< <b>0.001</b>	No shift
FeH	15.785	< <b>0.001</b>	< <b>0.001</b>	No shift
FeG	7.009	<b>0.012</b>	0.045	No shift
'Atlantogenata' and Boreoeutheria				
HD	7.875	<b>0.011</b>	0.083	No shift
UP	4.736	<b>0.042</b>	0.158	No shift
FeH	6.064	<b>0.023</b>	0.115	Early in Boreoeutheria
FeG	21.249	< <b>0.001</b>	< <b>0.001</b>	Early in Boreoeutheria
Laurasiatheria and Euarchontoglires				
RP	7.975	<b>0.014</b>	0.210	No shift

FDR, false discovery rate; FeG, femur, greater trochanter; FeH, femur, head; HD, humerus, distal; RP, radius, proximal; UP, ulna, proximal; PGi, Parsimov-based genetic inference. Significantly similar results (on the basis of a 0.95 confidence interval) are indicated with bold font.

**Table 4** Growth plates grouped according to their timing of closure through Tukey's *b post-hoc* tests (on the basis of a 0.95 confidence interval) revealed that the mean adjusted ranks often group growth plates (indicated in grey) that are included in one joint and thus comprise anatomical regions (Fig. 2).

Marsupialia, $n = 9$	1	2	3	4	Placentalia, $n = 22$	1	2	3	4
PA	0.022				HD	0.064			
SC	0.028				SC	0.104			
HD		0.405			PA	0.120			
UP		0.448	0.448		RP		0.280		
RP		0.660	0.660	0.660	UP			0.493	
TD			0.733	0.733	FeG			0.509	
FD			0.766	0.766	FeH			0.565	
FP			0.748	0.748	TD			0.524	
TP				0.844	FD			0.618	
FeD				0.818	FP				0.825
HP				0.815	FeD				0.830
FeG				0.799	TP				0.859
FeH				0.932	HP				0.887
RD				0.863	RD				0.885
UD				0.852	UD				0.897

$n$ , number of genera; FD, fibula, distal; FP, fibula, proximal; FeD, femur, distal; FeG, femur, greater trochanter; FeH, femur, head; HD, humerus, distal; HP, humerus, proximal; PA, pelvis, acetabulum; RD, radius, distal; RP, radius, proximal; SC, scapula, coracoid; TD, tibia, distal; TP, tibia, proximal; UD, ulna, distal; UP, ulna, proximal.

sequences of all 15 growth plates were used to calculate the sequence commonality within clades (Table 5). Except for one comparison ('pressure epiphyses' in Euarchontoglires), all comparisons remained statistically significant after applying FDR analyses.

We summarized literature data about epiphyseal closure sequence from 34 studies (Tables S4 and S5). Comparing data from humans (Stevenson, 1924) to our sequence data for all investigated mammals, most sequences across the mammalian clade – and especially primates – are strongly correlated with the sequence found by Stevenson (1924), although no perfect correlation ( $\tau = 1$ ) was detected (Table 6). All initially statistically significant results stayed significant after applying FDR analyses. The results from other comparisons using Kendall's  $\tau$  are summarized in Table 7. Using one-way ANOVA and Tukey's *b post hoc* tests, we found that the values for  $\tau$  that resulted from different comparisons of the sequences from the literature (a–e in Table 7) were not significantly different from each other ( $F_{4,65} = 1.830$ ,  $P = 0.082$ ).

### Heterochrony analysis

The eight PGi consensus trees had similar lengths of 194, 196 ( $\times 2$ ), 197 ( $\times 2$ ) and 198 ( $\times 3$ ) steps, indicating appropriateness of the used parameters. The shortest tree (194 steps) was chosen as best reflecting possible trends in the closure sequence of growth plates throughout mammalian evolution. Heterochronic shifts in major mammalian clades are shown in Fig. 1. They are partially consistent with findings from the analysis of adjusted closure scores with one-way ANOVA. The

**Table 5** Concordance between growth plate closure sequences within clades and locomotor groups using Kendall's W.

	All growth plates ( <i>n</i> = 15)			Anatomical regions ( <i>n</i> = 5)		'Pressure epiphyses' ( <i>n</i> = 11)		'Traction epiphyses' ( <i>n</i> = 4)	
	<i>N</i>	Kendall's		<i>W</i>	<i>P</i> (p(FDR))	Kendall's		Kendall's	
		<i>W</i>	<i>P</i> (p(FDR))			<i>W</i>	<i>P</i> (p(FDR))	<i>W</i>	<i>P</i> (p(FDR))
<b>Clades</b>									
Mammalia	32	0.268	<b>&lt; 0.001 (&lt; 0.001)</b>	0.145	0.869 (1.000)	0.295	<b>&lt; 0.001 (&lt; 0.001)</b>	0.165	0.926 (1.000)
Placentalia	22	0.248	<b>&lt; 0.001 (&lt; 0.001)</b>	0.191	0.519 (1.000)	0.248	<b>&lt; 0.001 (&lt; 0.001)</b>	0.127	0.969 (1.000)
Marsupialia	9	0.436	<b>&lt; 0.001 (&lt; 0.001)</b>	0.305	0.142 (0.887)	0.456	<b>&lt; 0.001 (&lt; 0.001)</b>	0.394	0.126 (0.608)
'Atlantogenata'	6	0.275	<b>0.001 (0.002)</b>	0.248	0.286 (1.000)	0.143	0.165 (0.186)	0.441	0.117 (0.608)
Boreoeutheria	16	0.196	<b>&lt; 0.001 (&lt; 0.001)</b>	0.148	0.744 (1.000)	0.216	<b>0.002 (0.007)</b>	0.07	0.997 (1.000)
Laurasiatheria	4	0.081	0.301 (0.319)	0.105	0.665 (1.000)	0.23	0.055 (0.010)	0.056	0.879 (1.000)
Euarchontoglires	12	0.211	<b>&lt; 0.001 (&lt; 0.001)</b>	0.106	0.885 (1.000)	0.175	<b>0.031 (0.080)</b>	0.077	0.985 (1.000)
Carnivora	3	0.043	0.526 (0.526)	0.013	0.939 (1.000)	0.261	0.057 (0.010)	0.083	0.717 (1.000)
Rodentia	5	0.263	<b>0.003 (0.006)</b>	0.000	1.000 (1.000)	0.161	0.132 (0.158)	0.165	0.62 (1.000)
Primates	6	0.200	<b>0.010 (0.015)</b>	0.131	0.656 (1.000)	0.176	0.085 (0.118)	0.051	0.962 (1.000)
Dasyurumorphia	3	0.186	0.061 (0.084)	0.015	0.926 (1.000)	0.115	0.281 (0.281)	0.500	0.135 (0.608)
Diprodontia	3	0.318	<b>0.008 (0.014)</b>	0.380	0.15 (0.887)	0.519	<b>0.003 (0.009)</b>	1	<b>&lt; 0.001 (&lt; 0.001)</b>
<b>Locomotor groups</b>									
Semi-aquatic	3	0.308	<b>0.01 (0.015)</b>	0.000	1.000 (1.000)	0.121	0.264 (0.280)	0.175	0.497 (1.000)
Terrestrial	10	0.365	<b>&lt; 0.001 (&lt; 0.001)</b>	0.146	0.682 (1.000)	0.322	<b>&lt; 0.001 (&lt; 0.001)</b>	0.183	0.678 (1.000)
Cursorial	3	0.502	<b>0.001 (0.002)</b>	0.013	0.939 (1.000)	0.246	0.067 (0.101)	< 0.001	1.000 (1.000)
Arboreal	3	0.130	0.143 (0.171)	0.108	0.584 (1.000)	0.255	0.061 (0.010)	0.083	0.717 (1.000)
Semi-fossorial	3	0.180	0.067 (0.086)	0.450	0.105 (0.887)	0.209	0.100 (0.129)	< 0.001	1.000 (1.000)
Scansorial	6	0.081	0.301 (0.319)	0.293	0.197 (0.887)	0.196	0.056 (0.010)	0.114	0.810 (1.000)

FDR, false discovery rate; *N*, number of compared clades/group; *n*, number of compared growth plates. Significantly similar results (on the basis of a 0.95 confidence interval) are indicated with bold font.

complete set of shifts is shown in Fig. S1. Parsimov did not detect any shifts in deep nodes that represent major clades (results not shown).

The continuous analysis detected only few marsupial and placental taxa that fall outside the 95% confidence interval of Theria. No clade, including major clades within Placentalia, is represented by an outstanding number of species that deviate from the 95% confidence interval of Theria. The evolutionary rates of the growth plates of Marsupialia and Placentalia are shown in Table 8.

### Growth plate closure and relative age

When comparing the relative number of closed growth plates in marsupials and placentals, the differences in the mean values are significantly different among the IDAS in placentals ( $F_{3,136} = 6.25$ ,  $P = 0.001$ ), but not in marsupials ( $F_{3,48} = 2.591$ ,  $P = 0.064$ ) (Fig. 4). In 'Atlantogenata' ( $F_{2,16} = 0.682$ ,  $P = 0.522$ ) and Laurasiatheria ( $F_{1,14} = 2.958$ ,  $P = 1.109$ ), there is no trend, whereas in Euarchontoglires ( $F_{3,104} = 7.207$ ,  $P < 0.0001$ ) and Boreoeutheria ( $F_{3,119} = 7.820$ ,  $P < 0.0001$ ), there is a significant positive trend. The positive trend in placentals might even be underestimated because we did not score specimens with all growth plates closed. This would have resulted in an increased number of high values for relative number of closed growth plates in late adult or senile specimens and probably a greater positive

trend. The positive trend in placentals indicates that when increasing the relative age, more growth plates are closed. Unexpectedly, there is no such trend in marsupials. Moreover, with the exception of one single specimen of *T. cynocephalus* (NHM 1963.8.30.1), no marsupial specimen has all the growth plates closed in our sample, even in late adult or senile stages. In contrast, we observed while sampling that closure of all growth plates (100% relative closure) can be observed in numerous placental specimens (e.g. various specimens of *Dendrohyrax* spp., *Myrmecophaga* spp., *O. afer*, *Equus* spp., *C. capreolus*, *Felis silvestris*, *Castor fiber*, *D. leporina*, *M. marmota*, *P. troglodytes*, *Papio* spp.). However, the marsupial pattern is not homogeneous: different growth plates were found with obliterated or lapsed (never completed) union (Table S6). Growth plates that close completely in all investigated species are the coracoid of the scapula, the acetabulum of the pelvis, the proximal radius and the distal humerus (Table S6).

### Discussion

In this study, we investigated the sequence of epiphyseal growth plate union in the limb bones of diverse mammalian taxa. The influence of phylogenetic relationships and biomechanical factors on the commonality of sequences was analysed, and the relation of growth plate closure to relative age was established. Although the

**Table 6** Concordance in growth plate closure sequences of taxa investigated in this study compared with the human sequence by Stevenson (1924) using Kendall's  $\tau$ .

Genera	Kendall's $\tau$	<i>P</i>	p (FDR)
<i>Papio</i> spp.	0.943	< <b>0.001</b>	< <b>0.001</b>
<i>Saimiri</i> spp.	0.876	< <b>0.001</b>	< <b>0.001</b>
<i>Pan troglodytes</i>	0.856	< <b>0.001</b>	< <b>0.001</b>
<i>Perodicticus potto</i>	0.845	< <b>0.001</b>	< <b>0.001</b>
<i>Macaca mulatta</i>	0.841	< <b>0.001</b>	< <b>0.001</b>
<i>Felis silvestris</i>	0.838	< <b>0.001</b>	< <b>0.001</b>
<i>Chlorocebus aethiops</i>	0.826	< <b>0.001</b>	< <b>0.001</b>
<i>Heterohyrax brucei</i>	0.807	< <b>0.001</b>	< <b>0.001</b>
<i>Erinaceus europaeus</i>	0.780	< <b>0.001</b>	< <b>0.001</b>
<i>Dasyurus</i> spp.	0.757	< <b>0.001</b>	< <b>0.001</b>
<i>Lepus europaeus</i>	0.742	< <b>0.001</b>	< <b>0.001</b>
<i>Procyon capensis</i>	0.735	<b>0.001</b>	<b>0.002</b>
<i>Hydrochoeris hydrochaeris</i>	0.723	<b>0.001</b>	<b>0.002</b>
<i>Dorcopsis muelleri</i>	0.716	<b>0.001</b>	<b>0.002</b>
<i>Castor fiber</i>	0.713	<b>0.001</b>	<b>0.002</b>
<i>Capreolus capreolus</i>	0.701	<b>0.002</b>	<b>0.003</b>
<i>Tenrec ecaudatus</i>	0.688	<b>0.002</b>	<b>0.003</b>
<i>Dasyprocta leporina</i>	0.686	<b>0.001</b>	<b>0.002</b>
<i>Ursus arctos</i>	0.681	<b>0.001</b>	<b>0.002</b>
<i>Marmota marmota</i>	0.674	<b>0.002</b>	<b>0.003</b>
<i>Dasyus</i> spp.	0.658	<b>0.003</b>	<b>0.004</b>
<i>Macropus</i> spp.	0.636	<b>0.002</b>	<b>0.003</b>
<i>Myocastor coypus</i>	0.622	<b>0.003</b>	<b>0.004</b>
<i>Didelphis</i> spp.	0.613	<b>0.005</b>	<b>0.006</b>
<i>Orycteropus afer</i>	0.592	<b>0.005</b>	<b>0.006</b>
<i>Isoodon macrourus</i>	0.578	<b>0.013</b>	<b>0.017</b>
<i>Myrmecophaga tridactyla</i>	0.483	<b>0.026</b>	<b>0.032</b>
<i>Perameles</i> spp.	0.478	<b>0.033</b>	<b>0.036</b>
<i>Thylacinus cynocephalus</i>	0.467	<b>0.028</b>	<b>0.033</b>
<i>Phoca vitulina</i>	0.442	<b>0.031</b>	<b>0.035</b>
<i>Sarcophilus harrisii</i>	0.368	0.079	0.084
<i>Tachyglossus aculeatus</i>	0.283	0.178	0.184
<i>Phascolarctos cinereus</i>	0.056	0.806	0.806

FDR, false discovery rate.

Significant results are indicated with bold font.

sequence of growth plate closure is not conserved across mammals, we identified conserved closure patterns and similar timing of closure in anatomical regions. First, few heterochronic shifts (change of timing) characterize major mammalian clades. Second, biomechanical factors (locomotor group affiliation) have no coherent influence on the sequence of closure. Third, most examined growth plates of marsupials have a higher evolutionary rate than the same growth plates in placentals. Fourth, marsupials keep some of their epiphyses separated during their entire life, in contrast to placentals.

### Growth plate closure sequences in a phylogenetic context

Stevenson (1924), as reported also by many other authors (Dawson, 1925; Todd, 1930a,b; Koch, 1935; Todd & Todd, 1938), claimed that the sequence of

**Table 7** Comparisons (a–e) of own sequence data and sequence data from the literature using Kendall's  $\tau$ .

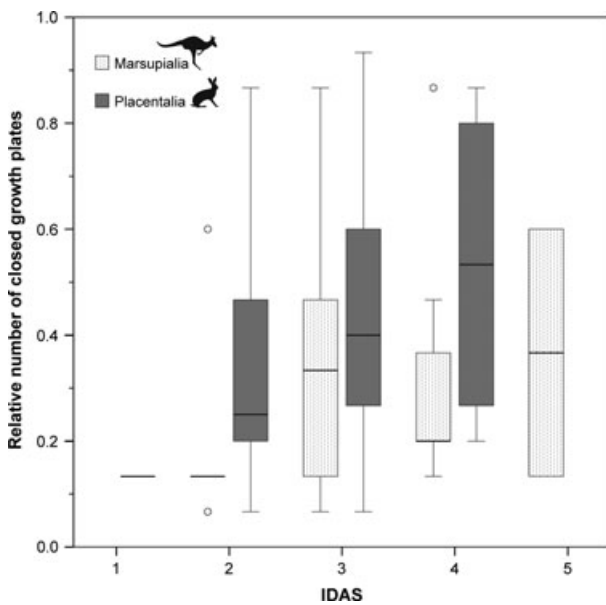
Comparisons	Range of Kendall's $\tau$ (mean)
(a) Taxa from the present study in comparison with Stevenson's (1924) data for humans (Table 6)	0.056–0.943 (0.658)
(b) Same taxon from the present study and the literature	0.568–0.933 (0.769)
(c) Different sexes from the same taxon (literature)	0.490–0.982 (0.732)
(d) Taxa from the literature in comparison with Stevenson's (1924) data for humans: authors who claim that the closure sequence is a mammalian universal	0.742–0.876 (0.812)
(e) Taxa from the literature in comparison with Stevenson's (1924) data for humans: authors who claim that the closure sequence is variable	0.245–0.846 (0.612)

**Table 8** Evolutionary rates (changes per million years) of the timing of closure of different growth plates in the limb bones of marsupials and placentals.

Growth plates	Marsupialia	Placentalia
PA	$8.17 \times 10^{-05}$	$3.53 \times 10^{-04}$
SST	$2.95 \times 10^{-04}$	$2.04 \times 10^{-04}$
FeH	$4.97 \times 10^{-04}$	$1.27 \times 10^{-03}$
RD	$8.09 \times 10^{-04}$	$4.34 \times 10^{-04}$
DU	$8.16 \times 10^{-04}$	$3.22 \times 10^{-04}$
HD	$8.75 \times 10^{-04}$	$2.09 \times 10^{-04}$
TP	$1.17 \times 10^{-03}$	$4.72 \times 10^{-04}$
TD	$1.19 \times 10^{-03}$	$1.27 \times 10^{-03}$
UP	$1.25 \times 10^{-03}$	$4.44 \times 10^{-04}$
RP	$1.45 \times 10^{-03}$	$6.21 \times 10^{-04}$
FP	$1.56 \times 10^{-03}$	$5.83 \times 10^{-04}$
FD	$1.86 \times 10^{-03}$	$7.06 \times 10^{-04}$
FeD	$2.45 \times 10^{-03}$	$7.81 \times 10^{-04}$
HP	$2.78 \times 10^{-03}$	$3.54 \times 10^{-04}$
FeG	$2.84 \times 10^{-03}$	$9.24 \times 10^{-04}$

FD, fibula, distal; FP, fibula, proximal; FeD, femur, distal; FeG, femur, greater trochanter; FeH, femur, head; HD, humerus, distal; HP, humerus, proximal; PA, pelvis, acetabulum; RD, radius, distal; RP, radius, proximal; SC, scapula, coracoid; TD, tibia, distal; TP, tibia, proximal; UD, ulna, distal; UP, ulna, proximal.

epiphyseal union was a general and uniform mammalian trait. We tested this statement by examining raw closure sequences and using Kendall's  $\tau$  and Kendall's *W*. Some patterns of closure were similar in all investigated species and genera (Fig. 3, Tables S2 and S3). The sequence commonality (using Kendall's *W*) ranged from almost no correlation to very strong correlation, whereas the mean correlation was medium to strong (Table 5). Similar values of correlation (medium to very



**Fig. 4** Relative number of closed growth plates in marsupials (dotted) and placentals (grey) of different individual age, indicated as individual dental age stages (IDAS) (generalized individual dental age stages) (Anders *et al.*, 2011). IDAS 2, juvenile; IDAS 3, adult; IDAS 4, late adult; IDAS 5, senile. Notice that we reported no marsupials with IDAS 1 and no placentals with IDAS 5.

strong) resulted from comparing between the literature data and our data against the sequence found by Stevenson (1924) using Kendall's  $\tau$  (Table 6, a in Table 7). However, the values for  $\tau$  in the sequences found by the authors who claim that the growth plate closure sequence is a mammalian universal (d in Table 7) are not more similar to Stevenson's (1924) sequence than the sequences found by authors who propose that there is variability across Mammalia (e in Table 7). Moreover, the differences between sexes can be greater than the differences between taxa (c in Table 7). The comparisons between same taxa from the present study (b in Table 7) and the literature resulted in similar values for  $\tau$  when comparing different sexes or taxa to one another, suggesting that possible errors due to different observers and definitions of growth plate closure do not result in greater sequence differences than can be observed between different sexes or taxa. Therefore, the comparisons between the different literature sources are feasible.

Concluding, our sequences and those from the literature suggest that growth plate closure sequence is not a mammalian universal, even though some consistencies exist. In particular, the strong correlation of Kendall's  $\tau$  between humans (Stevenson, 1924) and the primates investigated in this study (Table 6), as well as the strong correlation of Kendall's  $W$  within major mammalian clades (Table 5), suggests phylogenetic influence on growth plate union. Fossil evidence fur-

ther confirms that growth plate closure sequences did evolve considerably through time. The growth plates that connect the coracoid to the scapula as well as ilium, ischium, and pubis together are unfused in the Jurassic dryolestoid *Henkelotherium guimarotae*, whereas almost all growth plates of the long bones, with the exception of the distal tibia and fibula, are completely fused (Krebs, 1991). This is a remarkable deviation from the general early fusion of growth plates of the girdles in the extant mammals investigated here.

The heterochrony method PGI has revealed many shifts in young taxa, whereas there are only few changes in deeper nodes (Fig. 1, Fig. S1). Additionally, the continuous analysis detected only few marsupials and placentals outside the 95% confidence interval of Theria. These taxa represent different clades and therefore indicate that heterochronic shifts do not characterize major clades, but rather young taxa. Germain & Laurin (2009) explained similar findings from other authors as a methodological artefact; they detected that type I error (false-positive) rates on terminal branches are higher than on internal branches when using heterochrony analyses such as Parsimov or continuous analysis. Therefore, it is pointless to speculate about possible adaptive significance of all these changes in all investigated taxa, and we will restrict this discussion to the heterochronic shifts in major mammalian clades. However, most differences in adjusted closure scores between major clades disappeared when we corrected the adjusted closure scores for the phylogenetic relatedness of the terminal taxa, that is, when comparing the confidence intervals of the last common ancestor of marsupials and the last common ancestor of placentals. The observed changes between clades in the data that were not corrected for phylogenetic relationship do therefore probably not characterize deep nodes but rather 'families', genera or species. This would imply that the observed pattern is not a methodological artefact, but rather a biological phenomenon. This finding is consistent with the hypothesis that modifications during early development, such as cartilage formation, lead to major phenotype alterations, for example, digit reduction, whereas modifications in the later development and growth, such as ossification, lead to differences in skeletal features between species of the same genus or closely related genera (Farnum, 2007).

Differing from the universal altriciality of marsupials, placentals show an extensive range of neonatal maturity and gestation length that expose the growing organism to differing selection pressures. These variations in life-history traits often coincide with heterochrony. In marsupials, the forelimbs are more developed than the hindlimbs by the time of birth, resulting from an acceleration in early development of the former and a delay in early development and ossification of the latter (Weisbecker *et al.*, 2008; Sears, 2009; Sears *et al.*, 2012). This phenomenon is

crucial for the young being able to crawl to the pouch of the mother and is probably caused by 'trade-offs' in energy allocation (Weisbecker *et al.*, 2008). Moreover, it has traditionally been presumed and empirically tested (Sears, 2004; Bennett & Goswami, 2013) that the developmental constraints due to the specialized and conserved marsupial mode of reproduction lead to relatively low anatomical diversity in comparison with placentals. In contrast, Sánchez-Villagra (2013) argued that these developmental biases can be – and have often been observed to actually be – circumvented. The observed smaller morphospace and lower taxonomical diversity in marsupials might be more a reflection of a lack of ecological opportunity and a multitude of factors involving geographical and physiological variables (Sánchez-Villagra, 2013). In fact, we are not able to recognize developmental constraints with our data. Instead, we found that in marsupials, 12 of the 15 growth plates investigated have higher evolutionary rates of heterochronic shifts than in placentals, whereas only three growth plates have a higher rate in placentals (Table 8).

We expected that the peculiar mode of prenatal development of marsupials might have also consequences for the post-natal growth: in marsupials, growth plates in the hindlimbs might stay open for a longer period of time than in the forelimbs to allow subsequent growth. However, the only heterochronic shift that concerns the hindlimbs in marsupials is a delay in the closure of the distal fibula (Fig. 1). This delay could be the result of the hindlimbs growing for a longer period of time than the forelimbs to catch up with the already highly developed forelimbs. However, the distal growth plate of the tibia does not shift in a similar direction. Significant results from ANOVA (not detected using PGI) indicated possible heterochronic shifts in growth plates of the proximal femur (Table 3). However, the direction of the shift and the clade in which the shifts most probably occurred are not known. We conclude that the heterochronic shifts in growth plate closure is not a function of the onset of ossification. There is no causal relationship between the two. Moreover, the detected higher evolutionary rate in marsupials, together with the hypothesis that shifts in the growth plate closure sequence are characteristic for young taxa rather than for major clades, indicates that discrepancies between the life history of marsupials and placentals are not mirrored by the sequence of epiphyseal growth plate closure.

The placental groups Afrotheria and Xenarthra (together 'Atlantogenata') are characterized by ontogenetic peculiarities not found in boreoeutherians, for example, late dental eruption, unusually rapid growth to adult size and unusually variable vertebral formulae (Asher *et al.*, 2009). Therefore, we wondered whether members of this clade might also share a peculiar pattern of growth plate closure. Even though we

detected several changes using ANOVA, PGI showed that these changes resulted from changes within Boreoeutheria (Table 3). Shifts in the fore- and hindlimbs are on the branch leading to Boreoeutheria and within Boreoeutheria to Euarchontoglires (Fig. 1). No shifts characterize 'Atlantogenata' and Laurasiatheria (Fig. 1). As the terminal taxa within Boreoeutheria and 'Atlantogenata' are highly diverse, we can think of no adaptive hypothesis explaining these shifts.

### Growth plate closure sequences in a biomechanical context

The functional aspects that may lead to heterochronic shifts in the growth plate closure sequence include the function of single joints adjacent to the growth plates. This aspect is important regarding mechanical loadings. Stresses are assumed to strongly influence musculoskeletal growth and cause the skeleton to alter its shape to cope with physical demands (Carter *et al.*, 1991). The different epiphyses and growth plates of bones are discriminated in 'traction epiphyses' and 'pressure epiphyses' (Parsons, 1904; Serrano *et al.*, 2011) (Fig. 2). 'Pressure epiphyses' are positioned perpendicular to the bone axis and are compressed by the gravitational force and reaction force of the adjacent diaphysis (Serrano *et al.*, 2011). Due to this compression and the movement of the joints, 'pressure epiphyses' are under hydrostatic and shear stresses (Serrano *et al.*, 2011). Arkin & Katz (1956), Carter & Wong (1988), Carter *et al.* (1991, 1998) found that these stresses decrease the rate of epiphyseal cartilage growth. 'Traction epiphyses' provide attachment for tendons and muscles and are subject to lateral stresses and tension (Parsons, 1904; Serrano *et al.*, 2011). These lateral forces cause high shear stresses, which are assumed to promote ossification (Arkin & Katz, 1956; Porter, 1978; Breburda *et al.*, 2001).

Because of these different influences of stress components on cartilage maintenance, growth and ossification, it seems likely that the growth plate closure sequences of members of one locomotor group are more similar to one another than they are to sequences of taxa from other locomotor groups. Moreover, the sequences of closure might be more similar when examining these two growth plate types separately (Serrano *et al.*, 2011).

We found no significant greater concordance in growth plate closure sequences within locomotor groups (Tables 2 and 5) than within clades (Table 5). Investigating the special case of bipedal locomotion in which the forelimb and all its epiphyses are free from gravitational pressure, we failed to find high accordance in the sequences of the bipedal genera (*Dorcopsis* spp. and *Macropus* spp.) and the sequence found in humans (Stevenson, 1924) (Table 6). Lack of accordance might be coupled with the 'five-footed' gait in *Macropus* spp.

during grazing or browsing: they balance on tail and forelegs while swinging their hindlimbs forward (Nowak, 1999). This peculiar mode of locomotion loads forelimbs and tail with gravitational pressure. Growth plates of human arms, on the other hand, are constantly under tension due to their own weight and carrying items. However, we did not find heterochronic changes in growth plate fusion associated with biomechanical stresses due to bipedality.

Comparing the sequences of 'tension epiphyses' and 'traction epiphyses' separately does not result in more concordance among clades and locomotor groups than when comparing all growth plates together (Table 5). Our result contradicts the findings by Serrano *et al.* (2011) who reported perfect concordance in growth plate closure sequence in different artiodactyls when distinguishing between these two types of epiphyses.

### Anatomical regions

Using ANOVA and post-hoc tests, we found that the growth plates of anatomical regions (girdles, elbow, wrist, proximal femur, knee, ankle) (Fig. 2) are more integrated than two growth plates of the same bone (Table 4). We confirm previous findings about single clades (Washburn, 1946; Hofer *et al.*, 1956; Tappen & Severson, 1971; Cheverud, 1981). However, as stated for New World monkeys by Tappen & Severson (1971), there appears to be overlap of the sequence of union between regions.

### Complete growth plate closure and lapsed union

Whereas complete closure of all growth plates in late adult and senile specimens could be observed in many placental species, this is not the case for marsupials. Additionally, increasing age involves an increased number of closed growth plates in placentals, whereas no correlation was found in marsupials (Fig. 4). Lacking of growth plate union in marsupials has previously been reported by Washburn (1946) and Lowrance (1949). Incomplete fusion of growth plates in placentals is known for some species, including rat (Dawson, 1925, 1934; Mullender *et al.*, 1996; Roach *et al.*, 2003) and mouse (Dawson, 1935). There is further some evidence for incomplete fusion of growth plates in the xenarthran *Dasyopus hybridus* (Ciancio *et al.*, 2012) and in other members of 'Atlantogenata' (S. Werning, personal communication). These findings suggest that marsupials might continue growing throughout life, a hypothesis that is supported by Lowrance (1949). This author found that the variability in the bone lengths of the opossum exceeds that of placentals and suggested that certain parts of the opossum skeleton continue growing throughout life. Furthermore, histological data suggest that some marsupials do not generally reach skeletal maturity before death (S. Werning, personal communi-

cation). Contrastingly, longitudinal bone growth stops in rats, even though the cartilaginous growth plate endures until late adulthood (Roach *et al.*, 2003). These findings raise the question whether the condition found in marsupials and some placentals is primitive or derived.

The secondary ossification centres are usually tied to the evolution of determinate growth. Calcified and bony epiphyses evolved several times independently in many vertebrate lineages: Teleostei, Anura, Lepidosauria, Theria and possibly Aves (Haines, 1942; Carter *et al.*, 1998). Although bony epiphyses have been reported in some kannemeyeriid dicynodonts, a group of nonmammaliaform synapsids (Walter, 1985), the lack of secondary ossification centres was hypothesized to be the plesiomorphic condition in cynodonts and mammaliaforms (Luo *et al.*, 2007). Martin (2005) studied several long bones with different sizes of the basal mammaliaform *Haldanodon expectatus* (Docodonta). This author reported the absence of secondary ossification centres and suggested that this finding might indicate indeterminate growth. Martin (2005) also reported open sutures between the scapula and the coracoid in *Haldanodon*. Although we analysed the growth plates in the long bones together with the sutures within the girdles (i.e. between scapula and coracoid as well as between pubis, ilium, and ischium), they have a different origin. The girdle sutures connect primary ossification centres, whereas the growth plates in the long bones connect primary and secondary ossification centres. Thus, the question about the evolution of the secondary ossification centres remains unresolved when the sutures of the girdles are analysed.

Secondary ossification centres and growth plates have been found in the long bones of several Mesozoic mammals: several multituberculate species (Kielan-Jaworowska & Gambaryan, 1994; Hurum & Kielan-Jaworowska, 2008), the spalacotheriid *Zhangheotherium quinquecuspidens* (Luo & Ji, 2005), the dryolestoid *H. guimarotae* (Krebs, 1991) and *Vincelestes neuquenianus*, a close relative of Theria (Rougier, 1993). Moreover, secondary ossification centres are known from extant monotremes. All specimens show different levels of fusion, including the complete fusion stage. These data suggest that secondary ossification centres may have been present in the last common ancestor of crown mammals.

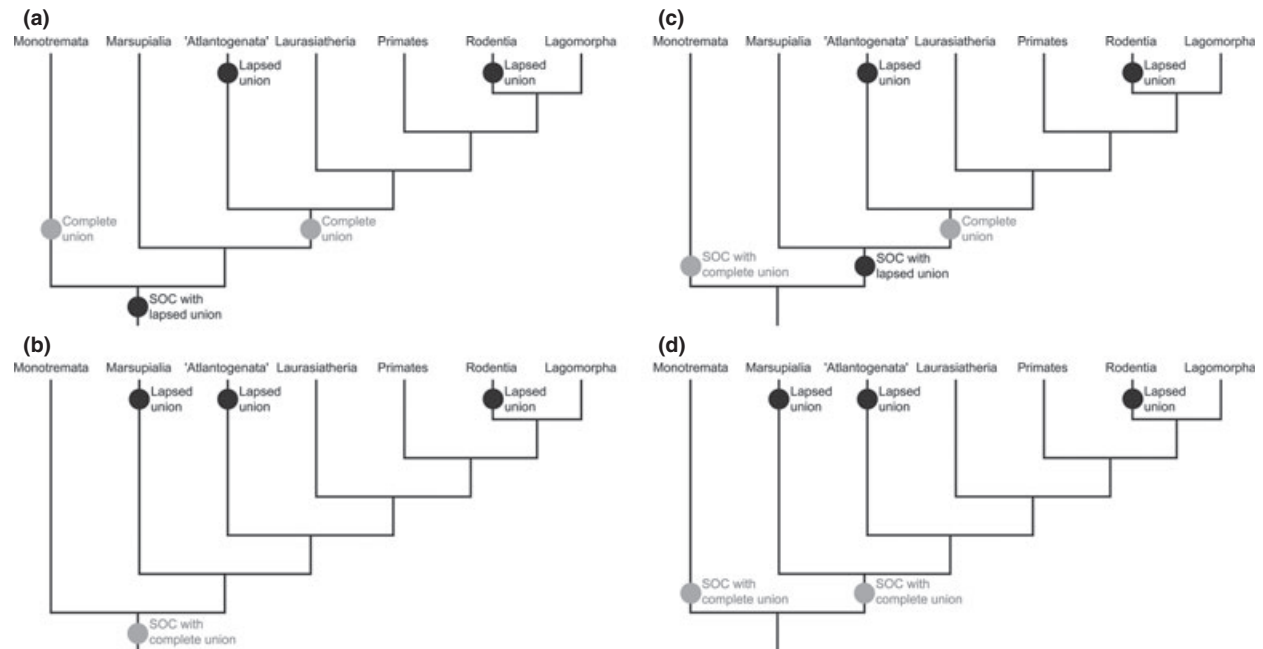
Alternatively, the absence of secondary ossification centres in *Jeholodens jenkinsi* (Triconodonta) (Ji *et al.*, 1999), *Yanoconodon allini* (Triconodonta) (Luo *et al.*, 2007), *Gobiconodon ostromi* (Triconodonta) (Jenkins & Schaff, 1988), *Volaticotherium antiquum* (Meng *et al.*, 2006), and *Akidolestes cifellii* (Spalacotherioidea) (Li & Luo, 2006) might indicate either skeletal maturity of the specimens and determinate growth, or the complete lack of secondary ossification centres, as suggested by Luo *et al.* (2007). The second possibility implies that

secondary ossification centres have evolved twice in crown mammals: in Monotremata and Theria.

Regarding Monotremata, we reported two *T. aculeatus* specimens (NRM A875308 and ZMM MAM 35998), in which all growth plates were closed. Taking this finding into account, there are four possibilities to explain the evolution of secondary ossification centres and determinate growth in extant mammals (Fig. 5a–d). We first report the hypothesis that secondary ossification centres evolved only once in crown mammals (Fig. 5a,b). If the last common ancestor of all living mammals showed lapsed union, complete union might have evolved twice within crown mammals: first, on the lineage leading to modern monotremes, and second, on the lineage leading to Placentalia (Fig. 5a). If so, lapsed union must have reversed among placentals (Fig. 5a). Alternatively, the finding of complete union in monotremes and some extinct mammals suggests that this could represent the primitive mammalian condition, and lapsed union might be a newly evolved trait in marsupials and some placentals (Fig. 5b). The second scenario (Fig. 5b) requires only three steps to explain the observed patterns and is more parsimonious than the first scenario, which requires four steps (Fig. 5a). If secondary ossification centres evolved separately in the lineage leading to extant monotremes and in therians, there is no most parsimonious solution for this problem, either complete or lapsed union could be the ancestral state in Placentalia (Fig. 5c,d). The evidence about the ancestral condition in mammals, that is, complete or incomplete growth plate closure, is not conclusive.

## Conclusion

This study shows that there are common patterns of growth plate closure across the mammalian clades, but that there is no single growth plate closure sequence that is uniform in all mammals. Differences in mechanical stresses that act on growth plates have no or only minor influences on the sequences. These findings indicate that growth plate closure sequence is determined more strongly by intrinsic (phylogenetic) factors than by extrinsic (biomechanical) factors. We detected a large number of heterochronic shifts, but they seem to occur in patterns that are not explained by simple functional correlates. We also found that heterochronic shifts in the growth plates of marsupials occur with a higher rate than in placentals. This presents a contrast with the more limited variation in timing and morphospace occupation typical for marsupial development (Sears, 2004; Bennett & Goswami, 2013). Moreover, we found no correspondence between patterns of pre-natal and post-natal limb development and growth: one could interpret the heterochronic shifts in post-natal growth in marsupials, in contrast to placentals, as a reaction of post-natal growth otherwise present in pre-natal phases of development, as measured in different aspects of organogenesis, chondrogenesis and osteogenesis (Weisbecker *et al.*, 2008; Sears, 2009; Sears *et al.*, 2012). However, we would like to point out that this pattern of developmental morphospace occupation does not necessarily reflect a mechanistic or causal mechanism. Instead, it may just be a result of evolutionary



**Fig. 5** Four scenarios (a–d) can explain observations of the evolution of secondary ossification centres as well as complete and lapsed growth plate union in mammals.

pathways determined by ecological opportunity (Sánchez-Villagra, 2013).

Even marsupials that are senile have some open growth plates, and there is evidence that marsupials continue growing throughout life. Apart from that, a likely reason for our findings is a decoupling of longitudinal growth from growth plate fusion, as hypothesized for humans by Stevenson (1924), which is probably a widespread if not universal phenomenon in mammals. Recent evidence indicates that growth plate fusion does not cause cessation of growth, but fusion follows cessation of growth (Nilsson & Baron, 2004). Moreover, fusion is not simply the result of continued cartilage replacement, but a different process (Parfitt, 2002). Empirical evidence for a decoupling of cessation of growth and growth plate closure was moreover found by Roach *et al.* (2003). Therefore, growth plate fusion does not necessarily reflect skeletal maturity, and any adaptational conclusions about growth heterochrony that might arise from discovery of patterns of fusion are insubstantial.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Complete set of heterochronic changes in growth plate fusion that was computed using Parsimov-based Genetic Inference (PGI) (Harrison & Larsson, 2008).

**Table S1** Species per genus (Wilson & Reeder, 2005) examined in this study.

**Table S2** Sequences of growth plate closure

**Table S3** Sequences of growth plate closure.

**Table S4** Summary of literature data about growth plate closure sequences of different taxa.

**Table S5** Growth plate closure sequences of literature data.

**Table S6** Marsupial species and genera investigated in this study and growth plates that show lapsed (never completed) union (lu).

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