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REVIEW

Development and embryonic staging in non-model organisms: the case of an afrotherian mammal

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Abstract

Studies of evolutionary developmental biology commonly use 'model organisms' such as fruit flies or mice, and questions are often functional or epigenetic. Phylogenetic investigations, in contrast, typically use species that are less common and mostly deal with broad scale analyses in the tree of life. However, important evolutionary transformations have taken place at all taxonomic levels, resulting in such diverse forms as elephants and shrews. To understand the mechanisms underlying morphological diversification, broader sampling and comparative approaches are paramount. Using a simple, standardized protocol, we describe for the first time the development of soft tissues and some parts of the skeleton, using μCT-imaging of developmental series of *Echinops telfairi* and *Tenrec ecaudatus,* two tenrecid afrotherian mammals. The developmental timing of soft tissue and skeletal characters described for the tenrecids is briefly compared with that of other mammals, including mouse, echidna, and the opossum. We found relatively few heterochronic differences in development in the armadillo vs. tenrec, consistent with a close relationship of Xenarthra and Afrotheria. Ossification in *T. ecaudatus* continues well into the second half of overall gestation, resembling the pattern seen in other small mammals and differing markedly from the advanced state of ossification evident early in the gestation of elephants, sheep, and humans.
Key words: Afrotheria, embryogenesis, *Mus*, ontogeny, skeleton, staging.

Introduction

The use of 'model organisms' is very important in unravelling some of the mechanisms behind evolutionary novelties, as in these a sophisticated analytical toolkit can be used (Sommer, 2009). However, hypotheses about evolution based solely on model organisms are very limited if we wish to understand the variation of anatomical forms not only across, but also within, a 'body plan' (Milinkovitch & Tzika, 2007). For example, the ecomorphological diversity of mammals is a rich subject of investigation (Sears, 2011), with additional phenotypic diversity available when considering the rich fossil record (Sánchez-Villagra, 2010).

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Knowledge of developmental genetics in the mouse can inspire and guide comparisons of other species, as when examining limb reduction in whales (Thewissen et al. 2006), digit elongation (Sears et al. 2006), interdigital webbing in the forelimbs of bats (Weatherbee et al. 2006), early limb development in opossums (Keyte & Smith, 2010), and autopodial specializations in talpid moles (Mitgutsch et al. 2012). But a molecular genetic approach is not always necessary to unravel a significant change in developmental patterning. For example, Hautier et al. (2010) discovered the basis of the divergence of sloths in the otherwise widespread presence of seven neck vertebrae of mammals after a comparative study of μCT data from several embryos and fetuses. In fact, late embryonic or postnatal studies of development are bound to provide major insights into the morphological diversification of mammals (Maier, 1999), whose molecular determinism can then be investigated. For example, rostral elongation in carnivoran mammals is coupled with protein-coding changes in the transcription factor *Runx2* (Sears et al. 2007), and sutural closure patterns in the skull are very much linked with growth factors

and bone morphogenetic proteins (Morriss-Kay & Wilkie, 2005).

Unravelling mechanisms behind mammalian diversity is possible only with access to embryos of diverse species. New breeding programmes of unconventional model species (Tzika & Milinkovitch, 2008) and museum collections are often the only source of animals no longer available for study from natural populations because of legal restrictions and/or conservation concerns. Thanks to new imaging techniques, it is possible to document the morphology of rare embryos non-invasively (Hautier et al. 2010, 2011; Kim et al. 2011).

Recent conceptual and methodological approaches also promise to open new possibilities of comparison and quantification of developmental changes. These approaches concern staging systems, heterochrony, and phylogeny. They are starting to capitalize on a large embryological tradition that, with analytical and quantitative tools, promises to address some of the most fundamental questions about the evolution of development (Bininda-Emonds et al. 2003a,b).

In this manuscript we describe and categorize the development of two tenrec species, helping to cover the gap in information on afrotherian mammals and addressing specific issues for placental mammal evolution as well as methodological ones related to staging, heterochrony, phylogeny, organogenesis, and life history.

Staging systems

In recent years more and more descriptive work on development and 'staging' of different species has been presented, allowing studies of evolutionary development to benefit from broader taxonomic sampling (e.g. Cretekos et al. 2006; Sanger et al. 2008; Kerney, 2011). The staging makes reference to previous standard studies (e.g., Yntema, 1968 for turtles). Often, similar character descriptions were used, and the embryos of newly described species were assigned to the stages once created for the reference species. However, the stages were subjectively created based on an individual understanding of similarity among individual embryos of the reference species. Similarly, the 'normal plates and tables' (Hopwood, 2007) were handled in a typological sense (Richardson et al. 1999), as variation in developmental sequences (de Jong et al. 2009) was largely ignored and an ideal sequential series was presented. Werneburg (2009) developed a system to study vertebrate embryos and presented a set of more than 100 soft-tissue characters that can be coded for any vertebrate embryo (e.g. Werneburg et al. 2009; see Table 2). Contrary to traditional staging criteria, these 'atomize' the body into clearly defined homologous structures, or 'standard event system' (SES) characters, which serve to describe the progress of individual development. The developmental timing of these SES characters can be compared analytically in a phylogenetic framework (Werneburg et al. 2009; Werneburg & Sánchez-Villagra, 2009, 2011). SES stages are created from a developmental series. A new SES stage is defined when particular specimens show new SES characters. As such, each SES stage represents a distinct composition of clearly defined characters. Characters which appear in earlier stages are not redescribed in a new SES stage description. Thus, for stage description, the variability in the timing of characters' development is fixed to the first appearance of those characters in a developmental series.

Heterochrony

Phylogenetic comparisons can uncover apomorphic features of a species' developmental sequence (Maxwell et al. 2010). Methods dealing with this approach are event-pairing (Mabee & Trendler, 1996; Smith, 1997; Velhagen, 1997), event-pair-cracking (Jeffery et al. 2002a), Parsimov (Jeffery et al. 2005), and PGi (Maxwell & Harrison, 2009). These concern non-independent characters and the results can be hard to interpret in a biological sense (see Schoch 2006; Maxwell & Larsson, 2009; Werneburg & Sánchez-Villagra, 2009, 2011). The squared-change parsimony approach used by Germain & Laurin (2009) also seems to be a promising kind of analysis of developmental sequence evolution. Along with that work, Laurin & Germain (2011) presented a new method to reconstruct a phylogeny based on developmental data that avoids some of the problems of event-pairing data for this task (Sánchez-Villagra, 2002; Ziermann 2008; Werneburg & Sánchez-Villagra, 2009).

In addition, and as reviewed by Maxwell & Harrison (2009), several statistical methods exist to study developmental sequences besides those with a phylogenetic emphasis described above. These include, for example, non-parametric methods to test for the overall divergence of ranked data (Nunn & Smith, 1998), for the evolutionary lability or amount of change in sequences (Poe & Wake, 2004) or for the relative timing of development between serially homologous structures (Bininda-Emonds et al. 2003a,b).

Mammal development

Developmental sequences in mammals have been examined quantitatively concerning suture formation (Wilson & Sánchez-Villagra, 2009; Bärmann & Sánchez-Villagra, 2012), skeletogenesis (Sánchez-Villagra, 2002; Sánchez-Villagra et al. 2008; Weisbecker et al. 2008; Wilson et al. 2010; Hautier et al. 2010, 2011; Weisbecker, 2011), and craniofacial tissues (Smith, 2001). Relatively less work has been done on the timing of organogenesis of Mammalia with a phylogenetic perspective (Jeffery et al. 2002b; Bininda-Emonds et al. 2003a,b; Werneburg & Sánchez-Villagra, 2011). For many mammal species it is a challenge to establish a breeding colony. Several species show a k-strategy in their reproduction, meaning few offspring, and embryos are often only accessible through maternal or fetal mortality. Over the past centuries, a few such specimens have become part of

museum collections. Although arrested development in late-stage embryos yield either expulsion of the fetus or mortality of an obviously pregnant mother (from which the embryo can then be sampled), aborted small embryos tend to be resorbed by the mother, leading to a lack of young specimens in zoological collections. As such, comprehensive embryonic series are rare for most mammalian species and often only perinatal specimens are available (Fig. 1), while earlier stages are the exception (Fig. 1F).

The mammalian phylogenetic framework

In recent years, a new and well supported framework of placental phylogeny has emerged (Springer & Murphy, 2007; Asher et al. 2009; Meredith et al. 2011). A fundamental discovery has been that of Afrotheria, a morphologically diverse clade including Tenrecidae (tenrecids), Chrysochloridae (golden moles), Macroscelididae (elephant shrews), Tubulidentata (aardvarks), Proboscidea (elephants), Hyracoidea (damans or hyraxes), and Sirenia (dugongs and manatees).

To date, relatively few adult morphological features have been identified that support the Afrotheria (Seiffert, 2007; Tabuce et al. 2008). The full spectrum of developmental data has not been examined in this regard, although progress is being made in areas including the dentition (Asher & Lehmann 2008), vertebral column (Sánchez-Villagra et al. 2007; Asher et al. 2009, 2011), and placentation (Mess & Carter, 2006).

Fig. 1 Afrotherian (A,C–E,G,J,K) and xenarthran (B,H) embryos. (A) African Elephant, *Loxodonta africana* (UMZC), (B) Southern Tamandua, *Tamandua tetradactyla* (ZMB 40639; anteater with placenta), (C) North African Elephant Shrew, *Elephantulus rozeti* (ZMB: Macroscelides 34a), (D) Rock Hyrax, *Procavia capensis* (UP: Hyrax), (E) Aardvark, *Orycteropus afer* (UP), (F) Giant Otter Shrew, *Potamogale velox* (ZMB: IN. 38), (G) West Indian Manatee, *Trichechus manatus* (BMNH 65.4.28.9), (H) Three-Toed Sloth, *Bradypus* spec. (ZMB 18835, *Bradypus*), (J) Cape Golden Mole, *Chrysochloris asiatica* (ZMB: N.N.), (K) Giant Otter Shrew, *Potamogale velox* (ZMB: IN. 44). Scale bars: (A,B,D,E,H–K) 1 cm, (C,F) 0.25 cm, (G) 10 cm. UP, University of Pretoria Collection; BMNH, British Museum of Natural History; ZMB, Zoologisches Museum Berlin.

Materials and methods

Specimens

A breeding colony of *Echinops telfairi,* the lesser hedgehog tenrec, was established in the laboratory of MCM and 48 embryos were collected in 2008, 2009, and 2010 (Table 1, Fig. 2, Appendix S1). The youngest and oldest embryos were collected at 39 and 48 days post-copulation, respectively. The full gestation length of this species varies considerably (from 57 to 75 days). In addition, we examined 11 advanced embryos of *Tenrec ecaudatus* from different embryological collections (Table 1, Fig. 3, Appendix S2), the absolute ages of which were unknown.

μCT-scans

Skeletons were imaged using high-resolution X-ray microtomography (μCT) at the Helmholtz Zentrum in Berlin, at the University of Cambridge Department of Engineering, at the Anthropological Institute of the University of Zürich and at the Institut des Sciences de l'Evolution de Montpellier 2. Threshold density values between ossified parts and soft tissues were substantial and easily allowed osteological reconstructions. 3-D rendering and visualization were performed using DRISHTI v.1.0 (Drishti Paint and Render; Limaye, 2006) (Fig. 4, Appendices S1 and S2).

Clearing and double staining

Selected specimens of *T. ecaudatus* and *E. telfairi* were cleared and double-stained (Table 1) following standard protocols (Dingerkus & Uhler, 1977).

Description of soft-tissue events

For the description and illustration of soft-tissue and external characters, we follow the protocol of Werneburg (2009) in which the first occurrence of a clearly defined developmental character is recorded.

A detailed SES formula for each tenrec species with depictions can be found in Appendices S1 and S2. All specimens were assigned SES stages based on SES characters. If no new SES character could be detected (e.g. *E. telfairi* SES stage 7), and only an obvious progress of development was observed, such as hair density or a change in the proportions, this was used to define a new SES stage. Changes that cannot be described with the original SES characters (Werneburg, 2009) and information on age are written in italics. All tenrec embryos observed are prenatal specimens. For each species we created an additional SES stage for 'birth' as information on this is available in the literature.

Comparisons among mammals

The tenrecids described herein are compared to four other mammalian species. SES coding of *Tachyglossus aculeatus* (Monotremata) is that of Werneburg & Sánchez-Villagra (2011). Codings of *Didelphis virginiana* (Marsupialia) and *Dasypus hybridus* (Placentalia: Xenarthra) are expanded after Werneburg & Sánchez-Villagra (2009). In addition, we present SES coding of *Mus musculus* derived from the staging system of Theiler (1989) (see Table 2).

The developmental series of those species were scaled from copulation/fertilization until the last SES stage (Fig. 5). In each species, the SES stages were equally distributed as ranks because absolute time data were only available for the mouse (Theiler, 1989) and only for some specimens of the other species analysed. The last SES stage does not correspond to a particular homologous SES character, as such information was not available. However, in most cases the oldest specimens were near birth and show a very advanced development with at least a few hairs, making them more or less comparable. All corresponding SES characters between species depicted next to each other (Fig. 5) were connected with lines starting at the respective SES stage of each species. The scheme derived from this comparison serves to estimate graphically differences in the relative timing of SES characters (see Schlosser, 2001, for another graphic approach to study sequences). If two characters of SES stage A in species X correspond to the same SES characters of SES stage B in species Y at the same time, a thicker line was drawn. And if several characters correspond like this, the number of those characters is indicated on the lines. Those numbers were considered when counting the intercrossing lines.

Results

The stage descriptions for *E. telfairi* and *T. ecaudatus* below follow Werneburg (2009) and are based on the possession of a concrete set of anatomical features, as summarized in Table 2 and illustrated in Appendices S1 and S2.

Staging system of *E. telfairi*

E. telfairi 1

The external nares, otic vesicle, and lens vesicle are present (Fig. 2, Appendix S1). The lens already has a sharp contour. The first three pharyngeal arches and the first two pharyngeal slits are visible. The mandibular bud reached the posterior end of the eye and its bud-shaped maxillary process reached the anterior border of the lens. The neck shows a cervical flexure. The ventricle bulbus is visible and contains an S-shaped heart. About 42 somites are present and its trunk is coiled. The forelimb bud has an apical epidermal ridge (AER) at its distal end and the hind limb appears as an elongated ridge. Between the hind limbs, a urogenital bud is visible.

E. telfairi 2

The maxillary process has reached the anterior border of the eye. There are about 44 somites visible. The forelimb is elongated and the hind limb bud is present.

E. telfairi 3

The mandibular process has reached the midline of the eye, in which an optic fissure is visible and the pupil is forming. In this stage, approximately 47 somites are present. The forelimb and the hind limb are paddle-shaped and a digital plate has begun to form. The hind limb shows an AER at its distal end.

Table 1. Specimens used in this study for SES staging and skeletal analysis. The collection numbers of *Echinops telfairi* from the collection of M.C.M. refer to the litter (numbers) and specimen (letters).

SES stage	Collection	Collection numbers	Skeletal ranks (Table 3)
Echinops telfairi			
1	Collection of M.C.M.	3a; 3b; 3c	x
1	Collection of M.C.M.	4a; 4d	x
3	Collection of M.C.M.	4b; 4c; 4e; 4f	x
4	Collection of M.C.M.	2a, 5a; 5b; 5c; 5d; 6a; 6b; 7d	x
5	Collection of M.C.M.	7a; 7b; 7c; 9a; 10a; 10b; 10c; 10d; 11a; 11b; 12a	x
6	Collection of M.C.M.	13a; 14a; 14a; 14b; 14c; 14d;	x
7	Collection of M.C.M.	8a; 8b; 14e; 14f	x
8	Collection of M.C.M.	15a* (CRL = 19 mm); 16a; 16b; 16c; 16d	1
9	Collection of M.C.M.	17a* (CRL = 31 mm); 17b; 18a; 18b; 18c	2
Unstaged	Laboratory of Paläontologisches Institut und Museum Zürich (PIMUZ lab#)	ET2095M°	3
		ET2095K°	4
		ET2103°	5
Tenrec ecaudatus			
1	Naturkundemuseum Berlin (Zoologisches Museum Berlin) (ZMB)	Centetes 44580/1	x
2	Laboratory of Paläontologisches Institut und Museum Zürich (PIMUZ lab#)	2012.IW24 (=MSV-TEC-11)* (CRL = 24 mm)	1
	Embryological Collection Berlin	Tenrec Hubrecht 2, several specimens	x
	American Museum of Natural History (AMNH)	Tenrec 1570 (length: 21 mm)	x
	American Museum of Natural History (AMNH)	Centetes 1063 (length: 23 mm)	x
3	Embryological Collection Berlin	Tenrec Hubrecht 3 (several specimens), specimen 3A* (CRL = 35 mm)	3
	Museum d'Histoire Naturelle de Paris (MNHN)	Tenrec 1890–2750*	6
4	American Museum of Natural History (AMNH)	Centetes 1139* (CRL = 42 mm)	3
	Embryological Collection Berlin	Tenrec Hubrecht 4 (several specimens), specimen 4A* (CRL = 22 mm)	4
5	Naturkundemuseum Berlin (Zoologisches Museum Berlin)	Centetes_44579* (CRL = 53.5 mm)	5
	American Museum of Natural History (AMNH)	Tenrek 1139 (length: 62mm)*, Tenrek 1139 (length: 53mm)*, Tenrek 1139 (length: 49mm)*	5
6	University Museum of Zoology Cambridge (UMZC)	Tenrec 6* (CRL = 46 mm)	5
	Naturkundemuseum Berlin (Zoologisches Museum Berlin) (ZMB)	Tenrec 1880a* (CRL = 37.5 mm), b* (CRL = 64.5 mm), c* (CRL = 51.5 mm), d* (CRL = 68 mm), e* (CRL = 72 mm), f* (CRL = 69 mm)	6
		Hildebrandt 16.XII.1880a, b, f	x
Unstaged	Laboratory of Paläontologisches Institut und Museum Zürich (PIMUZ lab#)	MSV Tek 2 ak°, 13°, 2008.14°	2
		2008.126°	3
		2008.110°	5

CRL, crown rump length (measured for selected specimens).
Specimens labelled with asterisks (*) were μCT-scanned (see Appendices S1 and S2), specimens labelled with a circle (°) were cleared and double-stained. SES stages correspond to the ranks of SES characters (Table 2). For ranks representing the progress of skeletogenesis, see Table 3.

E. telfairi 4

The maxillary process is fused with the frontonasal process. The mandibular process reached the level of the frontonasal region. All pharyngeal slits are closed. A lower eyelid has formed as well as the pinna fold. As such, the otic capsule is also inconspicuous. The number of somites is unclear, but

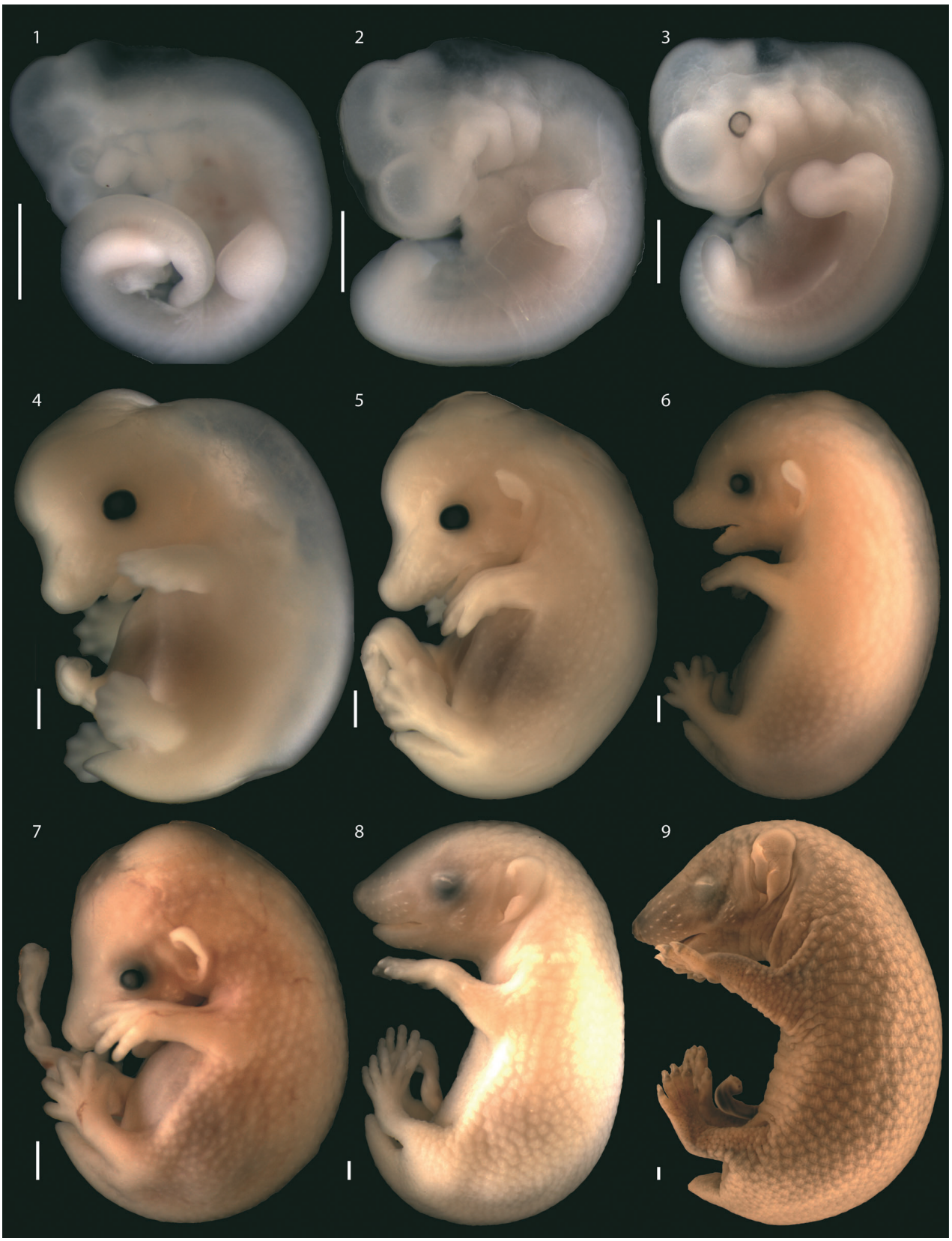

Fig. 2 Embryonic series of *Echinops telfairi*. Numbers refer to SES stages. Specimens shown are 1: MCM 3a, 2: MCM 4a, 3: MCM 4f, 4: MCM 5b, 5: MCM 7a, 6: MCM 14a, 7: MCM 8a, 8: MCM 16c, 9: MCM 17a. Specimens mirrored: 1, 6–9. Scale bar: 1 mm. For the SES formula see Appendix S1.

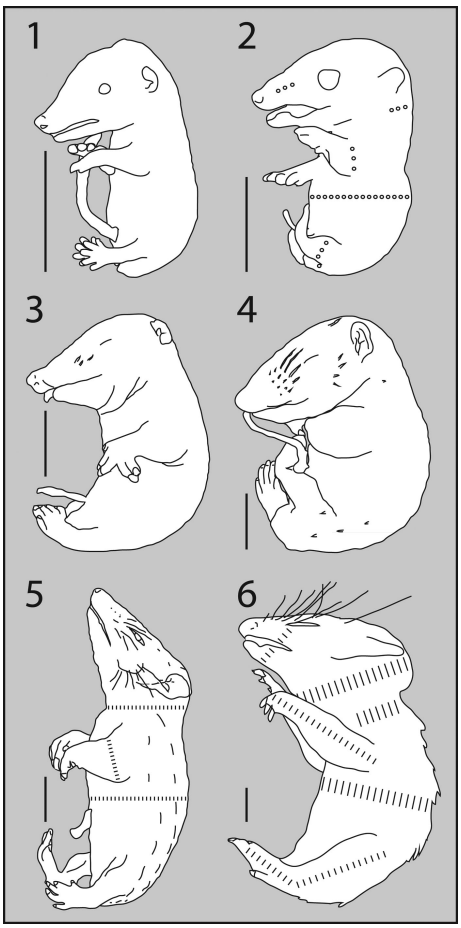

Fig. 3 Embryonic series of *Tenrec ecaudatus*. Simplified schemes concentrating on SES characters, derived from photographs. Hair follicles (circles) and hairs (dotted lines) are indicated only in the regions relevant for SES coding. For a full SES formula and photographs, see Appendix S2. Numbers refer to SES stages. 1: ZMB 44580/1; 2: Hubrecht 2; 3: Hubrecht 3; 4: Hubrecht 4; 5: ZMB 44579; 6: Hildebrandt 16.XII.1880a. Embryos mirrored except for (A) and (E). Scale bar: 1 cm.

the coiling of the trunk has disappeared. Both forelimb and hind limb show digital grooves and serrations. The mammary glands are visible. The specimens are about 39 days post-copulation.

E. telfairi 5
Fingers are present, the thoracal protrusion has disappeared and the urogenital papilla is inconspicuous. Forming hair and spine follicles or hair and spine placodes are loosely spread over snout, head, neck, belly, and back.

E. telfairi 6
The cervical flexure has disappeared. Toes are present. Hair follicles are visible on the limbs and the belly and the follicle density of the whole body is increased.

E. telfairi 7
No SES characters detected. The snout region has elongated. Fingers and toes show joints. The pinna fold is enlarged dorsally. More snout and cheek hairs are visible. The specimens are around 41 days post-conception.

E. telfairi 8
The mandibular process has reached the occlusion point with the upper jaw. The lower eyelid covers more than half of the eye and hairs are visible on the snout. The forelimb has an elbow and the hind limb shows a knee. In contrast to the original character description (Werneburg, 2009), we define the first appearance of a clear joint in the limb as elbow and knee. All limbs have claws on fingers and toes. Spine anlagen are visible in the spine follicles. The specimens are about 48 days post-conception.

E. telfairi 9
Hairs are visible on the forelimb and the neck, whereas spines start to appear on the dorsal side The specimens are about 48 days old.

E. telfairi 10
Hayssen et al. (1993) documented that the animals are born after 49 days post-conception. However, in our breeding colony, depending on the conditions in which the animals are kept, the gestation varies between 57 and 75 days.

Staging system of *T. ecaudatus*

T. ecaudatus 1
The maxillary process is fused with the frontonasal process and the mandibular process reached the occlusion point with the upper jaw (Fig. 3, Appendix S2). All pharyngeal slits are closed and the otic capsule is inconspicuous. A pinna fold is present. The cervical flexure and the trunk coiling has disappeared. Somites are not yet discrete and therefore are difficult to count. The thoracal protrusion is absent and the urogenital papillae are inconspicuous. Fingers and toes are present and the forelimb has an elbow. The nostrils are distinct. The eyes are not covered by the lower eyelid and due to the long preservation, no details of the eye are visible.

T. ecaudatus 2
The posterodorsal head projection has disappeared and the hind limb has a knee. Hair follicles are present on snout, back and belly.

T. ecaudatus 3
The lower eyelid covers more than half of the eye and hairs are present on the snout. The first claws are present both on the forelimb and the hind limb.

T. ecaudatus 4
Hairs are present on the neck, the top of the head and on the back of the body. Snout hairs elongated.

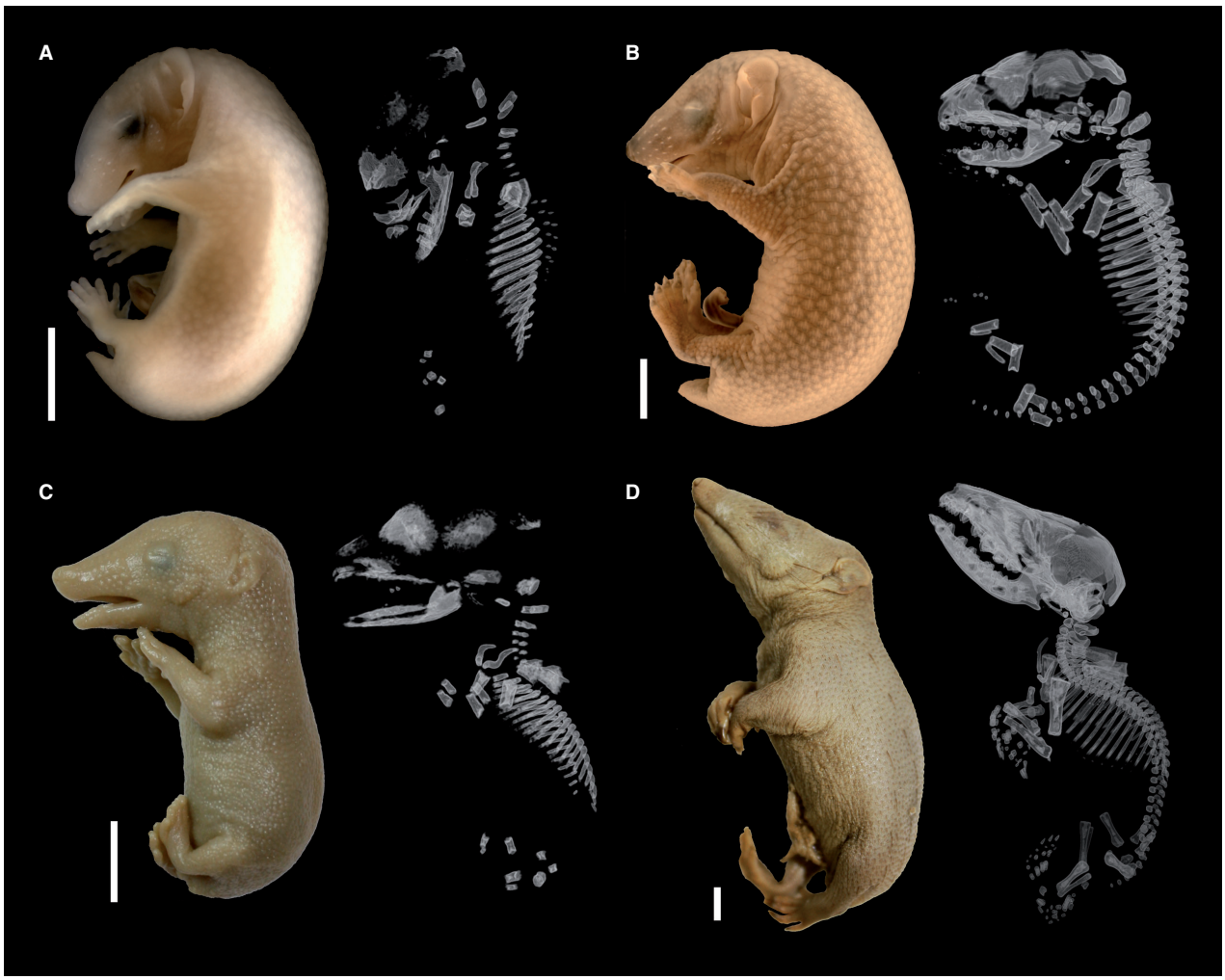

Fig. 4 Selected ontogenetic stages of *Echinops telfairi* and *Tenrec ecaudatus*. Lateral, external view of the specimens (left) and three-dimensional reconstruction of the skeleton using μCT-scans (right). (A) *E. telfairi* (SES stage 8, 48 day-old specimen, MCM 15-a), CRL = 19 mm; (B) *E. telfairi* (SES stage 9, 48 day-old specimen, MCM 17-a); (C) *T. ecaudatus* (SES stage 2, PIMUZ MSV-Tec-11); (D) *T. ecaudatus* (SES stage 5, ZMB 44579). Scale bars: 5 mm.

T. ecaudatus 5
Hairs are present on the throat, the whole belly, the tail and on the forelimb. Fingers and toes show joints. Snout hairs are further elongated.

T. ecaudatus 6
Hairs are present on the entire fore- and hindlimbs. The hair length has increased and forms a dense fur.

T. ecaudatus 7
The animals are born 53 days post-conception (Hayssen et al. 1993).

Heterochronic changes in soft-tissue development

The crossing points of the SES connections are interpreted as heterochronic changes between species (see also Fig. 5: top). Among the soft-tissue events defined here (Figs 2–3, Table 2, Appendices S1 and S2), we found 94 heterochronic changes between *T. aculeatus* and *Di. virginiana*, 128 between *Di. virginiana* and *M. musculus*, 141 between *M. musculus* and *Da. hybridus*, 44 between *Da. hybridus* and *E. telfairi*, and 0 between *E. telfairi* and *T. ecaudatus*.

The crossing points – hypotheses of heterochronies requiring further testing – appear in most cases with rank changes ranging between 1 and 3. No clear clusters of features exhibit heterochronic changes. Some of the recorded major shifts (more than three rank changes) in the timing of particular structures are listed below:

1 The hind limb paddle is relatively delayed in *Di. virginiana* when compared to *T. aculeatus*.

2 The closure of the anterior and posterior neuropore, the formation of the otic pit, and the appearance of the contour of the lens/iris are all relatively delayed in

Table 2 Matrix of SES coding for the mammals compared herein (for non-tenrecid species, SES coding is expanded after Werneburg & Sánchez-Villagra, 2009, 2011). Characters as described by Werneburg (2009) with expansions of Werneburg & Sánchez-Villagra (2011).

Standard code for character	Character	*Tachyglossus aculeatus*	*Didelphis virginiana*	*Mus musculus*	*Dasypus hybridus*	*Echinops telfairi*	*Tenrec ecaudatus*
V01a	Egg-laying	12	?	?	?	?	?
V02a	Blastoporus	5	16	9	?	?	?
V03a	Primitive streak	6	17	9	12	?	?
V03b	Neural folds closure	?	25	12	16	?	?
V03c	Anterior neuropore closed	?	24	14	20	?	?
V03d	Posterior neuropore closed	?	25	17	20	?	?
V04a	Somites hard count	19	31	25	29	4	1
V04b	1–5 somite pairs	7	23	12	18	?	?
V04c	6–10 somite pairs	9	?	12	19	?	?
V04d	11–15 somite pairs	10	25	13	20	?	?
V04e	16–20 somite pairs	12	26	14	?	?	?
V04f	21–25 somite pairs	13	?	15	24	?	?
V04g	26–30 somite pairs	15	?	15	?	?	?
V04h	31–35 somite pairs	?	?	16	26	?	?
V04i	36–40 somite pairs	17	?	17	?	?	?
V04j	41–45 somite pairs	?	?	18	28	1	?
V04k	46–50 somite pairs	?	?	19	?	3	?
V04l	51–55 somite pairs	?	?	21	?	?	?
V04m	56–60 somite pairs	?	?	22	?	?	?
V05a	Head bulbus	?	24	11	19	?	?
V05b	Anterior cephalic projection	7	26	12	20	?	?
V05c	Head projection disappeared	23	34	24	35	?	2
V06a	Olfactory pit	15	?	18	25	?	?
V06b	External nares	17	28	19	28	1	?
V07a	Otic pit	13	24	14	19	?	?
V07b	Otic vesicle	14	28	15	20	1	?
V07c	Otic capsule inconspicuous	24	?	20	?	4	1
V07d	Pinna fold	?	?	21	29	4	1
V08a	Optic vesicle	15	24	12	19	?	?
V08b	Lens vesicle	18	?	16	25	1	?
V08c	Optic fissure	15	?	?	30	3	?
V08d	Contour lens/iris	22	33	16	31	1	?
V08e	Pupil forms	?	?	?	30	3	?
V10a	Ventricle bulbus	15	26	12	20	1	?
V10b	Thoracal bulbus disappeared	22	33	21	35	5	1
V10c	Ventricle S-shaped	?	?	14	?	1	?
V11a	Tail bud	15	27	16	23	?	?
V12a	Forelimb ridge	14	26	14	25	?	?
V12o	Hind limb ridge	15	27	16	24	1	?
V12b	Forelimb bud	15	?	15	27	1	?
V12s	Hind limb bud	17	?	17	27	2	?
V12c	Forelimb elongated	17	28	16	28	2	?
V12p	Hind limb elongated	19	28	18	28	?	?
V12d	Forelimb AER	?	28	17	?	1	?
V12e	Hindlimb AER	?	28	19	?	3	?
V12f	Forelimb elbow	?	32	23	33	8	1
V12t	Hind limb knee	20	33	25	34	8	2
V12g	Forelimb paddle	17	29	19	28	3	?

Table 2 (Continued)

Standard code for character	Character	*Tachyglossus aculeatus*	*Didelphis virginiana*	*Mus musculus*	*Dasypus hybridus*	*Echinops telfairi*	*Tenrec ecaudatus*
V12h	Hindlimb paddle	17	32	19	28	3	?
V12i	Forelimb digital plate	17	30	21	29	3	?
V12j	Hindlimb digital plate	20	32	21	29	3	?
V12k	Digital grooves (forelimb)	20	30-31	21	29	4	?
V12u	Digital grooves (hind limb)	22	33	21	31	4	?
V12l	Digital serration (forelimb)	20	31	21	30	4	?
V12v	Digital serration (hind limb)	22	33	21	31	4	?
V12m	Finger	22	33	23	33	5	1
V12w	Toe	25	35	23	34	6	1
V12n	First claw (forelimb)	28	34	27	35	8	3
V12x	First claw (hind limb)	29	?	27	35	8	3
V12q	Hind limb sporn	28	?	?	?	?	?
V12r	Hind limb sporn pouch	26	?	?	?	?	?
V14a	Hatch	24	?	?	?	?	?
V14b	Birth	?	36	27	36	10	7
G01a	Max. bud	15	27	17	24	1	?
G01b	Max posterior eye	15	27-28		24	?	?
G01c	Max. midline eye	?	28	18	25	?	?
G01d	Max. anterior lens	16	29	?	26	1	?
G01e	Max. anterior eye	17	30	?	27	2	?
G01f	Max. frontonasal fuse	22	31	22	33	4	1
G02a	Mand. arch bud	8	27	12	23	?	?
G02b	Mand. posterior eye	18	28	13	?	1	?
G02d	Mand. midline eye	19	29	19	24	3	?
G02e	Mand. anterior lens	20	?	?	25	?	?
G02f	Mand. anterior eye	21	29-30	21	26	?	?
G02g	Mand. level frontonasal	22	32	22	27	4	?
G02h	Mand. occlusion point	25	34	24	34-35	8	1
G03a	2nd arch	9	27	13	24	1	?
G03b	3rd arch	12	27	14	24	1	?
G03c	4th arch	15	27	16	25	?	?
G04a	1st slit	11	27	14	?	1	?
G04b	2nd slit	12	27	18	?	1	?
G04c	3rd slit	15	28	?	?	?	?
G04d	4th slit	15	?	?	?	?	?
G04e	Slits closed	21	30	21	?	4	1
G05a	Urogenital papilla bud	22	29	17	?	1	?
G05b	Urogenital papilla inconspicuous	28	35	?	?	5	1
T01a	Cervical flexure 90°	?	28	14	25	1	?
T01b	Cervical flexure disappeared	?	34	23	30	6	1
T01c	Wrinkles on neck	?	35	25	?	?	?
A01a	Lower lid	21	31	24	31	4	?
A01b	Eyelid begun overgrow	22	?	25	33	?	?
A01c	Eyelid at scleral papillae	?	?	?	33	?	?
A01d	Eyelid ventral lens	23	33	?	34	?	?
A01e	Eyelid half eye	24	34	?	35	8	3
A01f	Membrana nictitans	?	?	23	?	?	?
A02a	Caruncle	22	?	?	?	?	?
M01a	Back hairs	30	?	?	?	?	4
M01b	Belly hairs	31	?	?	?	?	5
M01c	Whole belly hairs	33	?	?	?	?	5
M01d	Forelimb hairs	30	?	?	?	9	5
M01e	Whole forlimb hairs	30	?	?	?	?	6

Table 2 (Continued)

Standard code for character	Character	*Tachyglossus aculeatus*	*Didelphis virginiana*	*Mus musculus*	*Dasypus hybridus*	*Echinops telfairi*	*Tenrec ecaudatus*
M01f	Tail hairs	30	?	?	?	?	5
M01g	Hind limb hairs	32	?	?	?	?	6
M01h	Whole hind limb hairs	33	?	?	?	?	6
M01i	Neck hairs	30	?	?	?	9	4
M01j	Head hairs	30	?	?	?	?	4
M01k	Throat hairs	33	?	?	?	?	5
M01l	Snout hairs	33	?	21	?	8	3
M02a	Trunk coiling	16	28	13	20	1	?
M02b	Trunk coiling dissappeared	18	29	14	?	4	1
M03a	Marsupium Anlage	25	?	?	?	?	?
M03b	Marsupium dent	28	?	?	?	?	?
MO01a	Monotreme beak	29	?	?	?	?	?

SES characters that are not listed were not scored for any mammal compared here.

Di. virginiana when compared to these events in *M. musculus*.

3 When comparing *M. musculus* and *Da. hybridus*, the closure of the posterior neuropore and the time when somites become hard to count is delayed in *M. musculus*; the timing of the mandibular process reaching the level of the frontonasal groove is advanced in *Da. hybridus*, whereas the disappearance of the thoracal bulbus is delayed in *Da. hybridus*.

Comparison of skeletons

Table 3 presents the onset of ossification in cranial and postcranial bones of *T. ecaudatus* and *E. telfairi*, based on examination of CT-scan images (Fig. 4). Apart from a few differences that are due to artefacts of sampling in the earliest stages, *E. telfairi* and *T. ecaudatus* display very similar ossification sequences for postcranial and cranial elements (Table 3). In terms of timing of ossification, the first centres of ossification were detected in stage 8 of *E. telfairi*, so the skeleton should start ossifying slightly before day 48 of gestation (Fig. 4). Despite the variable length of gestation of *E. telfairi* (from 57 to 75 days), we can estimate that most of the ossification centres appear during the second half of gestation.

Discussion

The comparison of SES characters between species depicted next to each other (Fig. 5) illustrates differences in the sequence of characters. The crossing points of the character-lines are hypothesized as potential heterochronic differences between two neighbouring species. The timing differences are detected from a direct comparison of two species and not from a comprehensive phylogenetic examination. The number of crossing points is likely correlated with the number of sampled characters connecting two species. As such, the low numbers between *Da. hybridus*, *E. telfairi*, and *T. ecaudatus* are obviously dependent on the lack of early embryonic stages of the latter two species (see below). However, in the remaining 19 characters no heterochronic SES characters can be recognized between the tenrecids. This is likely to reflect the sister-group relationship of the two species (which have a similar SES sequence in their development) as one might assume a phylogenetic relevance of the number of heterochronic changes.

For comparison, we chose mammalian species of a more or less similar overall adult shape for which specimens were available (Werneburg & Sánchez-Villagra, 2009, 2011). This prevented a large number of heterochronic differences that one would find when comparing a whale with a mouse or a bat with a kangaroo. As such, the number of heterochronic events is reduced to a minimum. Although we may be able to estimate some phylogenetic value in the numbers of heterochronic characters, these numbers differ significantly among the distantly related mammalian clades. The comparison among clades should be performed in regard to the phylogenetic position of the armadillo (Xenarthra, *Da. hybridus*). The uncertainty of early stage resolution aside (see below), the distribution of characters best optimizes on a sister-group relationship with tenrecids (Afrotheria) and not with the mouse (Euarchontoglires/Laurasiatheria-clade, Rodentia) (Fig. 5). Indeed, following the principle of parsimony, one may assume that sister clades exhibit fewer heterochronic character changes, as discussed for the tenrecid species above. Hence, the armadillo shows fewer heterochronic differences relative to the tenrecids than to the mouse. This could indicate either a Xenarthra/Afrotheria-clade opposing the Euarchontoglires/Laurasiatheria-clade, or simply shared plesiomorphic features.

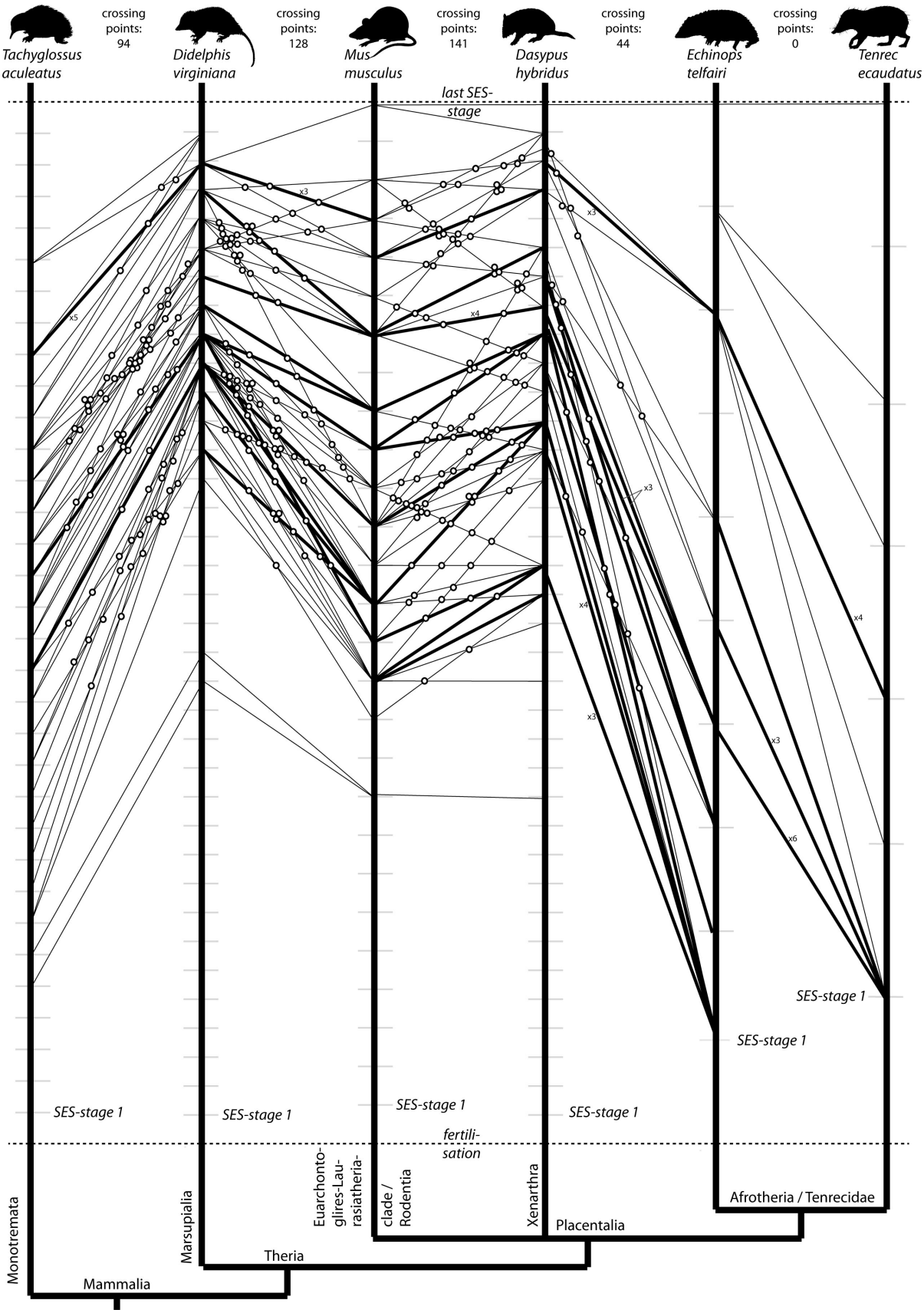

Fig. 5 Comparison of SES characters among six mammal species. SES stage 1 of each species is labelled. The horizontal dotted line at bottom indicates fertilization ('SES 0') and that depicted at the top indicates birth, i.e. the most advanced SES stage (33 in *Tachyglossus aculeatus*, 36 in *Didelphis virginiana*, 27 in *Mus musculus*, 36 in *Dasypus hybridus*, 10 in *Echinops telfairi*, and 7 in *Tenrec ecaudatus*). SES characters, which are present in two neighbouring species, are connected via a line (for character names compare with Table 2). In cases of more than one SES character connecting the same SES stages, thicker lines are used. If there are more than two SES characters, the count is added to the line.

When the timing of SES characters is compared between mouse and *E. telfairi* only (Appendix S3) a higher number (51) of heterochronic differences is detectable than between armadillo and *E. telfairi* (44) (Fig. 5). This may illustrate a higher affinity of tenrec and armadillo. To further test this possibility, additional developmental series of other species must be analysed.

Methodological and sampling issues

The lines that connect the same characters of different species in Fig. 5 serve to compare the relative timing of events between neighbouring species as depicted in the tree. Their uneven distribution has different causes, for which methodological and sampling aspects are relevant:

1 The species examined do not possess the same number of SES stages. This results in a fan-shaped distribution of connecting lines from one species to the next. Absolute time data, which are not available for most specimens, would provide more details about interspecific character alignment. The illustration shows that any comparison of ranked data, as often done for ossification data, carries a large amount of variance. However, the number of heterochronic changes between species would not change if absolute time data were used, as the composition of SES characters in one stage would stay the same. This approach, however, would raise the issue of standardizing gestation length in animals with very different reproductive strategies (e.g. marsupial vs. placental).

2 All non-tenricid mammals compared in this study are known by a comprehensive set of SES characters, which are distributed from the earliest phase of organogenesis until the perinatal period. However, both tenrec species are only known from late embryogenesis. This results in

Table 3 Onset of ossification in the cranial and postcranial skeleton of *Tenrec ecaudatus* and *Echinops telfairi* derived from μCT-data and cleared and double-stained specimens (Table 1). In both species, the periotic is ossified in adults and the epipubic is not present in tenrecids. For specimens corresponding to the ranks see Table 1.

Cranial elements	*E. telfairi*	*T. ecaudatus*	Postcranial elements	*E. telfairi*	*T. ecaudatus*
Premaxilla	1	1	Clavicle	1	1
Maxilla	1	1	Humerus	1	1
Palatine	1	1	Ribs	1	1
Dentary	1	1	Femur	1	1
Frontal	1	1	Radius	1	1
Parietal	1	1	Ulna	1	1
Squamosal	2	1	Scapula	1	1
Basioccipital	1	1	Cervical vertebrae	1	1
Nasal	2	1	Thoracic vertebrae	1	2
Pterygoid	1	1	Tibia	1	1
Exoccipital	1	1	Fibula	1	1
Basisphenoid	2	2	Lumbar	2	3
Jugal	?	?	Sacral	2	3
Lacrimal	2	2	Caudal	2	3
Alisphenoid	2	2	Ilium	2	1
Orbitosphenoid	2	2	Manual phalanges	2	3
Periotic	?	?	Pedal phalanges	3	3
			Ischium	3	3
			Pubis	3	4
			Metacarpals	2	3
			Metatarsals	2	3
			Tarsals	4	5
			Carpals	5	5
			Sternum	2	3

a decline of the 'character curve' in those species. In this sense, the number of SES stages (Werneburg & Sánchez-Villagra, 2009) influences the slopes of the connecting lines (Fig. 5). The resolution of the tenrec sequences is low compared with that in the mouse and the armadillo. However, these few tenrec stages include most of the SES characters, which were also compared between mouse and armadillo in the respective stages (starting at mouse SES stage 12 and armadillo SES stage 20) and only two crossing points appear in earlier development between mouse and armadillo (Fig. 5). Hence, the stage-resolution of our study may not significantly influence the count of heterochronic differences in comparable stages. A lower SES stage-resolution could result in a higher as well as in a lower count of intercrossing points depending on the width of the developmental window observed (e.g. early to late or just late embryogenesis). Nevertheless, the total number of documented SES characters differs between species; mouse and armadillo have 62 in common (seven of which appear before the comparable SES stage 1 of the tenrecids), armadillo and *E. telfairi* have 44 (Fig. 5), and mouse and *E. telfairi* have 32 characters (Appendix S3) in common. This count certainly influences the number of heterochronic differences. Most SES characters themselves appear for some time before they are 'replaced' by other SES characters. Several of those characters are first recorded for the tenrecids at SES stage 1 but may have already been present much earlier in development (pseudo-shifts *sensu* Werneburg & Sánchez-Villagra, 2011). Hence, a higher resolution of early organogenesis would eventually reduce the number of heterochronies, as the first occurrence of SES characters is usually recorded.

Werneburg & Sánchez-Villagra (2009, 2011) used the Parsimov-approach (Jeffery et al. 2005) to test the phylogenetic relevance of heterochronic changes during development. They, as previous authors (e.g. Maxwell & Harrison, 2009), also criticized the problem of non-independent characters involved in the analysis and the problem of pseudo-shifts related to 'missing' embryonic stages. The issue of pseudo-shifts can potentially influence the analysis performed here. A pairwise comparison (event-pairs) of individual developmental characters helps to detect heterochronic shifts but these are difficult to interpret in a biological sense. The approach presented here (Fig. 5) may be an alternative way to visualize changes in developmental timing. The actual composition of developmental characters within one stage may be more important than a relative shift of one character against half of the characters during the whole developmental period. The event-pairing approach, although providing a clear analytical framework to examine heterochrony, can result in an abundance of comparisons that are difficult to trace.

A methodological issue is the scaling of ranked developmental stages. The approach of Germain & Laurin (2009) and Laurin & Germain (2011) treat the ranked sequence as a unit (Schulmeister & Wheeler 2004; Maxwell & Harrison, 2009) and may then help to reconstruct the ground pattern of a developmental sequence. Herein, non-independent characters are used and, with an underlying geological time scale, the appearance of developmental characters within an embryological time scale is optimized parsimoniously onto a topology.

Ossification timing and life history

Hautier et al. (2011) found that xenarthrans differ from other placentals in the late ossification of the sternum and in the early ossification of the phalanges and of the pubis. On the one hand, elephants clearly depart from what is observed for xenarthrans, notably in showing a late ossification of the phalanges (Hautier et al. 2012). On the other hand, the timing of these events in tenrecids is the general one of boreoeutherians (Weisbecker et al. 2008; Sánchez-Villagra et al. 2008). Hence, the examination of ossification timing in the developmental series of the two afrotherian mammals studied so far revealed neither a major deviation from the placental pattern nor any similarity with that reportedly derived for xenarthran mammals.

Hautier et al. (2012) showed that the ossification of the skeleton in elephants starts very early, as in human and cow, i.e. before the end of the first third of their gestation period. This clearly departs from the pattern observed in rodents, which start ossifying their skeleton by the end of the entire gestation period. For small mammals, tenrecids are not characterized by a short gestation period as in most rodents, but nevertheless show a rodent-like, late onset of ossification, towards the end of intrauterine pregnancy.

Hautier et al. (2010, 2011) discovered differences in ossification sequences in xenarthrans, particularly sloths, compared to other mammals. Data collected here enable us to compare vertebral ossification in two tenrecid species with that of xenarthrans. With the exception of two specimens (*E. telfairi*: MCM17a; *T. ecaudatus*: PIMUZ lab#2012.IW24), all the specimens display well ossified centra and neural arches in the cervical and thoracic vertebrae (Appendices S1 and S2). *Echinops telfairi* (MCM 15-a, SES stage 8) displayed ossified neural arches in the upper cervical and upper thoracic areas (the lower neck region is not yet ossified), and showed ossification of the vertebral centra in the upper thoracics (Appendix S1). *Tenrec ecaudatus* (PIMUZ lab#2012.IW24, SES stage 2) displayed well ossified neural arches in the cranial cervical region and barely detectable ossification in the lower cervical and upper thoracic regions; all centra were still unossified (Appendix S2: Fig. F2.2). The timing of ossification in the tenrecid vertebral column is therefore very similar to the pattern observed in *Dasypus novemcinctus* (Hautier et al. 2010).

Future investigations will clearly benefit from considering non-model organisms like tenrecids in order to estimate which life-history traits are linked to the timing of ossification and the events of soft tissue development in placental mammals. Ultimately, this enables a comprehensive understanding of evolutionary change via developmental change.

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Authors' contributions

This study was conceived by all authors. Data acquisition on soft-tissue development was performed by I.W. and A.C.T., on ossification by L.H. and R.J.A., and breeding methods and embryo staging by ultrasounds were developed by A.C.T. and M.C.M. Drafting of manuscript was performed by M.R.S.-V and I.W., and all authors critically revised it.

References

Asher RJ, Bennett N, Lehmann T (2009) The new framework for understanding placental mammal evolution. *BioEssays* **31**, 853–864.

Asher RJ, Lehmann T (2008) Dental eruption in afrotherian mammals. *BMC Biol* **6**, 14.

Asher RJ, Lin KH, Kardjilov N, et al. (2011) Variability and constraint in the mammalian vertebral column. *J Evol Biol* **24**, 1080–1090.

Bärmann EV, Sánchez-Villagra MR (2012) A phylogenetic study of late growth events in a mammalian evolutionary radiation. The cranial sutures of terrestrial cetartiodactyl mammals. *J Mamm Evol* **19**, 43–56.

Bininda-Emonds ORP, Jeffery JE, Richardson MK (2003a) Inverting the hourglass: quantitative evidence against the phylotypic stage in vertebrate development. *Proc Biol Sci* **270**, 341–346.

Bininda-Emonds ORP, Jeffery JE, Richardson MK (2003b) Is sequence heterochrony an important evolutionary mechanism in mammals? *J Mamm Evol* **10**, 335–361.

Cretekos CJ, Weatherbee SD, Chen C-H, et al. (2005) Embryonic staging system for the short-tailed fruit bat, *Carollia perspicillata*, a model organism for the mammalian order Chiroptera, based upon timed pregnancies in captivebred animals. *Dev Dyn* **233**, 721–738.

Dingerkus G, Uhler LD (1977) Enzyme clearing of Alcian Blue stained whole small vertebrates for demonstration of cartilage. *Stain Technol* **52**, 229–232.

Germain D, Laurin M (2009) Evolution of ossification sequences in salamanders and urodele origins assessed through event-pairing and new methods. *Evol Dev* **11**, 170–190.

Hautier L, Weisbecker V, Sánchez-Villagra MR, et al. (2010) Skeletal development in sloths and the evolution of mammalian vertebral patterning. *Proc Natl Acad Sci U S A* **107**, 18903–18908.

Hautier L, Weisbecker V, Goswami A, et al. (2011) Skeletal ossification and sequence heterochrony in xenarthran evolution. *Evol Dev* **13**, 460–476.

Hautier L, Stansfield FJ, Allen WRT, et al. (2012) Skeletal development in the African elephant and ossification timing in placental mammals. *Proc Biol Sci*, doi: 10.1098/rspb.2011.2481.

Hayssen V, van Tienhoven A, van Tienhoven A (1993) *Asdell's Patterns of Mammalian Reproduction: A Compendium of Species-Specific Data*. Ithaca, NY: Cornell University Press.

Hopwood N (2007) A history of normal plates, tables and stages in vertebrate embryology. *Int J Dev Biol* **51**, 1–26.

Jeffery JE, Bininda-Emonds ORP, Coates MI, et al. (2002a) Analyzing evolutionary patterns in amniote embryonic development. *Evol Dev* **4**, 292–302.

Jeffery JE, Richardson MK, Coates MI, et al. (2002b) Analyzing developmental sequences within a phylogenetic framework. *Syst Biol* **51**, 478–491.

Jeffery JE, Bininda-Emonds ORP, Coates MI, et al. (2005) A new technique for identifying sequence heterochrony. *Syst Biol* **54**, 230–240.

de Jong IML, Colbert MW, Witte F, et al. (2009) Polymorphism in developmental timing: intraspecific heterochrony in a Lake Victoria cichlid. *Evol Dev* **11**, 625–635.

Kerney R (2011) Embryonic staging table for a direct-developing salamander, *Plethodon cinereus* (Plethodontidae). *Anat Rec* **294**, 1796–1808.

Keyte AL, Smith KK (2010) Developmental origins of precocial forelimbs in marsupial neonates. *Development* **137**, 4283–4294.

Kim JS, Recknagel AK, Riccio M, et al. (2011) Quantitative three-dimensional analysis of embryonic chick morphogenesis via microcomputed tomography. *Anat Rec* **294**, 1–10.

Laurin M, Germain D (2011) Developmental characters in phylogenetic inference, and their absolute timing information. *Syst Biol* **60**, 630–644.

Limaye A (2006) Drishti - Volume Exploration and Presentation Tool, Poster presentation, VisBaltimore.

Mabee PM, Trendler TA (1996) Development of the cranium and paired fins in *Betta splendens* (Teleostei: Percomorpha): intraspecific variation and interspecific comparisons. *J Morphol* **227**, 249–287.

Maier W (1999) On the evolutionary biology of early mammals – with methodological remarks on the interaction between ontogenetic adaptation and phylogenetic transformation. *Zool Anz* **238**, 55–74.

Maxwell EE, Harrison LB (2009) Methods for the analysis of developmental sequence data. *Evol Dev* **11**, 109–119.

Maxwell EE, Larsson HCE (2009) Comparative ossification sequence and skeletal development of the postcranium of palaeognathous birds (Aves: Palaeognathae). *Zool J Linn Soc* **157**, 169–196.

Maxwell EE, Harrison LB, Larsson HCE (2010) Assessing the phylogenetic utility of sequence heterochrony: evolution of avian ossification sequences as a case study. *Zoology* **113**, 57–66.

Meredith RW, Janečka JE, Gatesy J, et al. (2011) Impacts of the cretaceous terrestrial revolution and KPg extinction on mammal diversification. *Science* **334**, 521–524.

Mess A, Carter AM (2006) Evolutionary transformations of fetal membrane characters in Eutheria with special reference to Afrotheria. *J Exp Zool B Mol Dev Evol* **306(2)**, 140–163.

Milinkovitch MC, Tzika AC (2007) Escaping the mouse trap; the selection of new evo-devo model species. *J Exp Zool B Mol Dev Evol* **308(2)**, 337–346.

Mitgutsch C, Richardson MK, Jiménez R, et al. (2012) Circumventing the pentadactyly 'constraint' – autopodial recruitment of pre-axial structures in true moles. *Biol Lett* **8**, 74–77.

Morriss-Kay GM, Wilkie AOM (2005) Growth of the normal skull vault and its alteration in craniosynostosis: insights from human genetics and experimental studies. *J Anat* **207**, 637–653.

Nunn CL, Smith KK (1998) Statistical analyses of developmental sequences: the craniofacial region in marsupial and placental mammals. *Am Nat* **152**, 82–101.

Poe S, Wake MH (2004) Quantitative tests of general models for the evolution of development. *Am Nat* **164**, 415–422.

Richardson MK, Minelli A, Coates MI (1999) Some problems with typological thinking in evolution and development. *Evol Dev* **1**, 5–7.

Sánchez-Villagra MR (2002) Comparative patterns of postcranial ontogeny in therian mammals: an analysis of relative timing of ossification events. *J Exp Zool B Mol Dev Evol* **294**, 264–273.

Sánchez-Villagra MR (2010) Developmental palaeontology in synapsids: the fossil record of ontogeny in mammals and their closest relatives. *Proc Biol Sci* **277**, 1139–1147.

Sánchez-Villagra MR, Goswami A, Weisbecker V, et al. (2008) Conserved relative timing of cranial ossification patterns in early mammalian evolution. *Evol Dev* **10**, 519–530.

Sánchez-Villagra MR, Narita Y, Kuratani S (2007) Thoracolumbar vertebral number: the first skeletal synapomorphy for afrotherian mammals. *Syst Biodivers* **5**, 1–7.

Sanger TJ, Losos JB, Gibson-Brown JJ (2008) A developmental staging series for the lizard genus *Anolis*: a new system for the integration of evolution, development, and ecology. *J Morphol* **269**, 129–137.

Schlosser G (2001) Using heterochrony plots to detect the dissociated coevolution of characters. *J Exp Zool B Mol Dev Evol* **291**, 282–304.

Schoch RR (2006) Skull ontogeny: developmental patterns of fishes conserved in across major developmental stages. *Evol Dev* **8**, 524–536.

Schulmeister D, Wheeler WC (2004) Phylogenetic analysis of developmental sequence heterochrony: an application of search-based character optimization. *Evol Dev* **6**, 50–57.

Sears KE (2011) Novel insights into the regulation of limb development from 'natural mammalian mutants'. *BioEssays* **33**, 327–331.

Sears KE, Behringer RR, Rasweiler JJ, et al. (2006) The development of bat flight: morphologic and molecular evolution of bat forelimb digit. *Proc Natl Acad Sci U S A* **103**, 6581–6586.

Sears KE, Goswami A, Flynn JJ, et al. (2007) The correlated evolution of Runx2 tandem repeats, transcriptional activity, and facial length in Carnivora. *Evol Dev* **9**, 555–565.

Seiffert ER (2007) A new estimate of afrotherian phylogeny based on simultaneous analysis of genomic, morphological, and fossil evidence. *BMC Evol Biol* **7**, 224.

Smith KK (2001) Heterochrony revisited: the evolution of developmental sequences. *Biol J Linn Soc* **73**, 169–186.

Smith KK (1997) Comparative patterns of craniofacial development in eutherian and metatherian mammals. *Evolution* **51**, 1663–1678.

Sommer RJ (2009) The future of evo-devo: model systems and evolutionary theory. *Nat Rev Genet* **10**, 416–422.

Springer MS, Murphy WJ (2007) Mammalian evolution and biomedicine: new views from phylogeny. *Biol Rev* **82**, 375–392.

Tabuce R, Asher RJ, Lehmann T (2008) Afrotherian mammals: a review of current data. *Mammalia* **72**, 2–14.

Theiler K (1989) *The House Mouse: Atlas of Embryonic Development*. New York: Spinger Verlag.

Thewissen JG, Cohn MJ, Stevens LS, et al. (2006) Developmental basis for hind limb loss in dolphins and origin of the cetacean body plan. *Proc Natl Acad Sci U S A* **103**, 8414–8418.

Tzika A, Milinkovitch MC (2008) A pragmatic approach for selecting evo-devo model species in amniotes. In *Evolving Pathways. Key Themes in Evolutionary Developmental Biology* (eds Minelli A, Fusco G), pp. 123–143. Cambridge: Cambridge University Press.

Velhagen WA (1997) Analyzing developmental sequences using sequence units. *Syst Biol* **46**, 204–210.

Weatherbee SD, Behringer RR, Rasweiler JJ IV, et al. (2006) Interdigital webbing retention in bat wings illustrates genetic changes underlying amniote limb diversification. *Proc Natl Acad Sci U S A* **103**, 15103–15107.

Weisbecker V (2011) Monotreme ossification sequences and the riddle of mammalian skeletal development. *Evolution* **65**, 1323–1335.

Weisbecker V, Goswami A, Wroe S, et al. (2008) Ossification heterochrony in the mammalian postcranial skeleton and the marsupial-placental dichotomy. *Evolution* **62**, 2027–2041.

Werneburg I (2009) A standard system to study vertebrate embryos. *PLoS ONE* **4**, e5887. Doi: 10.1371/journal.pone.0005887.

Werneburg I, Hugi J, Müller J, et al. (2009) Embryogenesis and ossification of *Emydura subglobosa* (Testudines, Pleurodira, Chelidae) and patterns of turtle development. *Dev Dyn* **238**, 2770–2786.

Werneburg I, Sánchez-Villagra MR (2009) Timing of organogenesis support basal position of turtles in the amniote tree of life. *BMC Evol Biol* **9**, 82. Doi: 10.1186/1471-2148-9-82.

Werneburg I, Sánchez-Villagra MR (2011) The early development of the echidna, *Tachyglossus aculeatus* (Mammalia: Monotremata) and patterns of mammalian development. *Acta Zool* **82**, 75–88.

Wilson LAB, Sánchez-Villagra MR (2009) Heterochrony and patterns of cranial suture closure in hystricognath rodents. *J Anat* **214**, 339–354.

Wilson LAB, Schradin C, Mitgutsch C, et al. (2010) Skeletogenesis and sequence heterochrony in rodent evolution, with particular emphasis on the African striped mouse, *Rhabdomys pumilio* (Mammalia). *Org Divers Evol* **10**, 243–258.

Yntema CL (1968) A series of stages in the embryonic development of *Chelydra serpentina*. *J Morphol* **125**, 219–251.

Ziermann JM (2008) Evolutionäre Entwicklung larvaler Cranialmuskulatur der Anura und der Einfluss von Sequenzheterochronien. In *Biologisch-Pharmazeutische Fakultät*, p. 347. Jena: Friedrich-Schiller-Universität.

Supporting Information

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

Appendix S1. SES formula of *Echinops telfairi* with images of the embryos and μCT-scans.

Appendix S2. SES formula of *Tenrec ecaudatus* with images of the embryos and μCT-scans.

Appendix S3. Comparison of SES stages and SES characters between *Mus musculus* and *Echinops telfairi*. For further information compare to the description of Fig. 5.

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