UCLA
UCLA Previously Published Works

Title

Evidence of five digits in embryonic horses and developmental stabilization of tetrapod digit number.

Permalink

https://escholarship.org/uc/item/0kn3301w

Journal

Proceedings of the Royal Society B, 287(1920)

ISSN

0962-8452

Authors

Kavanagh, Kathryn D
Bailey, C Scott
Sears, Karen E

Publication Date

2020-02-12

DOI

10.1098/rspb.2019.2756

Peer reviewed

Author for correspondence:
Kathryn D. Kavanagh
e-mail: kkavanagh@umassd.edu

THE ROYAL SOCIETY
PUBLISHING

Evidence of five digits in embryonic horses and developmental stabilization of tetrapod digit number

Kathryn D. Kavanagh[1], C. Scott Bailey[2] and Karen E. Sears[3]

[1]Department of Biology, University of Massachusetts Dartmouth, Dartmouth, MA, USA
[2]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607, USA
[3]Department of Ecology and Evolutionary Biology, University of California Los Angeles, Los Angeles, CA, USA

KDK, 0000-0002-1496-990X; KES, 0000-0001-9744-9602

Previous work comparing the developmental mechanisms involved in digit reduction in horses with other mammals reported that horses have only a 'single digit', with two flanking metapodials identified as remnants of digit II and IV. Here we show that early *Equus* embryos go through a stage with five digit condensations, and that the flanking splint metapodials result from fusions of the two anterior digits I and II and the two posterior digits IV and V, in a striking parallel between ontogeny and phylogeny. Given that even this most extreme case of digit reduction exhibits primary pentadactyly, we re-examined the initial stages of digit condensation of all digit-reduced tetrapods where data are available and found that in all cases, five or four digits initiate (four with digit I missing). The persistent pentadactyl initiation in the horse and other digit-reduced modern taxa underscores a durable developmental stability at the initiation of digits. The digit evodevo model may help illuminate the biological circumstances under which organ systems become highly stabilized versus highly plastic.

1. Introduction

Evolution of the modern horse limb, and in particular reduction of the number of toes during the evolution of the horse lineage, is one of the most iconic evolutionary transitional stories documented in the fossil record. Recent phylogenies and fossil analyses concur with older hypotheses that modern horses arose from a pentadactyl ancestor with intermediate descendants that have reduced numbers of digits [1–5] (figure 1*b*). Modern monodactyl horses are called single-toed because they possess an enlarged central digit III with a thick metapodial called the cannon bone and three smaller distal phalanges that complete the main digit in both their forelimb (FL) and hindlimb (HL). Alongside, the proximal end of the large adult metapodial III lay two very small 'splint' metapodials, identified as remnants of metapodials MII and MIV. Cooper *et al.* [7] report that at about 34 dpc (days post coitus), the central digit of the modern horse, *Equus caballus*, is already relatively large and apoptosis is carving away the distal end of the two splint metapodials proximally. The authors recognized that the horse embryos in their comparisons were slightly older than the stage at which digit condensations initially form, likely due to very limited accessibility of early horse embryos for research. Nevertheless, this limited data on horse embryology suggested that horses only ever form three digit remnants during their development and became an important cornerstone of the general view of the evolutionary developmental biology of digits [8–10]. By contrast, a recent palaeontology paper proposed a novel hypothesis, based on bone articulations and ridges in fossil horses and vasculature in late foetal horses, that the identities of all five ancestral digits might be preserved in the metacarpal anatomy of the modern horse adult FL [6], but direct evidence was lacking. This same study also proposed that the horse 'frog' (distal hoof cartilage) is a distal remnant of all five digit condensations, although embryological evidence was again lacking.
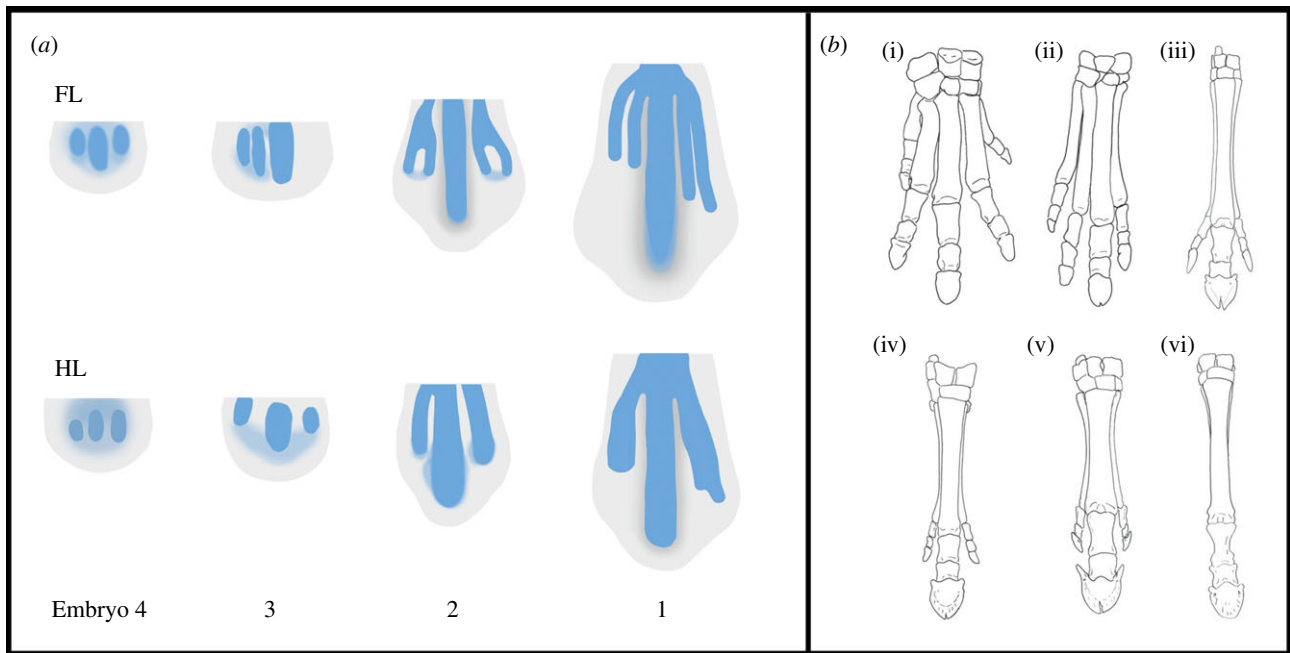
Figure 1. (a) Illustration of arrangement and relative sizes of pre-cartilaginous condensations in developing *Equus* FL and HL digits based on reconstructions of histological sections of 30–35 dpc embryos from this study. (b) Fossil transition series of adult horse FL digits (isometrically scaled) showing the sequence of reduction of anterior and posterior digits and increasing dominance of central digit III. (i) *Phenacodus* (AMNH 4369), (ii) *Hyracotherium* (AMNH 4832), (iii) *Meso-hippus* (AMNH 39480 and AMNH 1477), (iv) *Hypohippus* (AMNH 9407), (v) *Hipparion* (AMNH 109625), (vi) *Dinohippus* (AMNH 17224). Illustration from Solounias *et al*. [6]. (Online version in colour.)

Here, we examine histologically younger horse embryos to directly document the process of digit condensation in horses and thereby test among these conflicting hypotheses.

2. Horse digit genesis

To assess early digit development in the modern horse, several embryonic limbs from horses of estimated ages of 29–35 dpc were paraffin-embedded, serially transverse-sectioned across the distal limb, stained with haematoxylin and eosin (H&E) to high-light condensing cells, and digitally imaged. The sections span the developmental period from the initial flattening of the early autopod tissue prior to digit condensation (Embryo 5; figure 3), through initial condensation (Embryos 3 and 4) to early stages of post-patterning growth and differentiation (Embryos 1 and 2). Both FL and HL were sectioned in Embryos 1–4 and HL only in Embryo 5. Individual rods of condensing cells could be tracked through the proximo-distal direction in sequential sections, extending from the forming carpal–metacarpal joint to approximately 200 µm below the distal tip.

(a) Forelimbs

Starting with the oldest embryos in this series, transverse sections through the FL of an estimated Carnegie Stage 16 (CS16) horse specimens (Embryos 1 and 2) showed a single central digit in distal-most sections (figure 2d,h). In more proximal sections of the same limb, five separate digit condensations were observed in the wider mid-autopod sections (figures 1a and 2b, g). Even more proximally, the two anterior and two posterior digit condensations in these embryos are observed fusing (i.e. the merging of DI with DII, and DIV with DV), clearly showing that cell lineages from all five metacarpals persist in the older horse FL and that DI and DV are not absent or solely carved away as previously thought, but rather they also fuse at this

stage (figure 2a,b,e,f,i,j; electronic supplementary material, figures S1 and S3). This observation is in agreement with the recent palaeontological hypothesis that remnants of all five digits persist in adult *Equus* metacarpals [6]. More proximally, all condensations join at the carpal–metacarpal joint and are hard to distinguish (electronic supplementary material, figures S1 and S3). At the section level at which all five digits are observable, the central DIII is approximately double the diameter of the side digits, which are all of similar diameter (figure 2b,c,g). At this section level, the central digit is also more developmentally advanced than the anterior and posterior digits, having more neatly organized and layered cells with increased extracellular matrix (ECM). Although Embryos 1 and 2 were documented from the supplier as the same age, we scored Embryo 1 as slightly more advanced because the central digit had a more organized cartilage cell structure, the ventral flexor tendon condensation was more developed, and, interestingly, the embryo's limb showed apparent loss of distal portions of DI and DII (figure 2b,c compared with g; electronic supplementary material, figures S1 and S3), as might happen if distal apoptosis had begun (as in [7]).

An examination of younger horse FL (Embryos 3 and 4) shows that the digits initiate in the posterior-to-anterior sequence common to all amniotes (figure 2n shows IV, III). At initiation of the digits, the condensations begin at similar sizes, although the dynamic nature of patterning at this stage begs further detail from additional specimens. Clearly, the central toe only emerges as dominant as a consequence of accelerated growth, development and later apoptosis relative to the other digits *after* the initiation of the five digit condensations (figure 2n,j,g,c).

(b) Hind limbs

HLs were examined from these same embryos. Compared with FL development in the same embryo, HL-DIII was
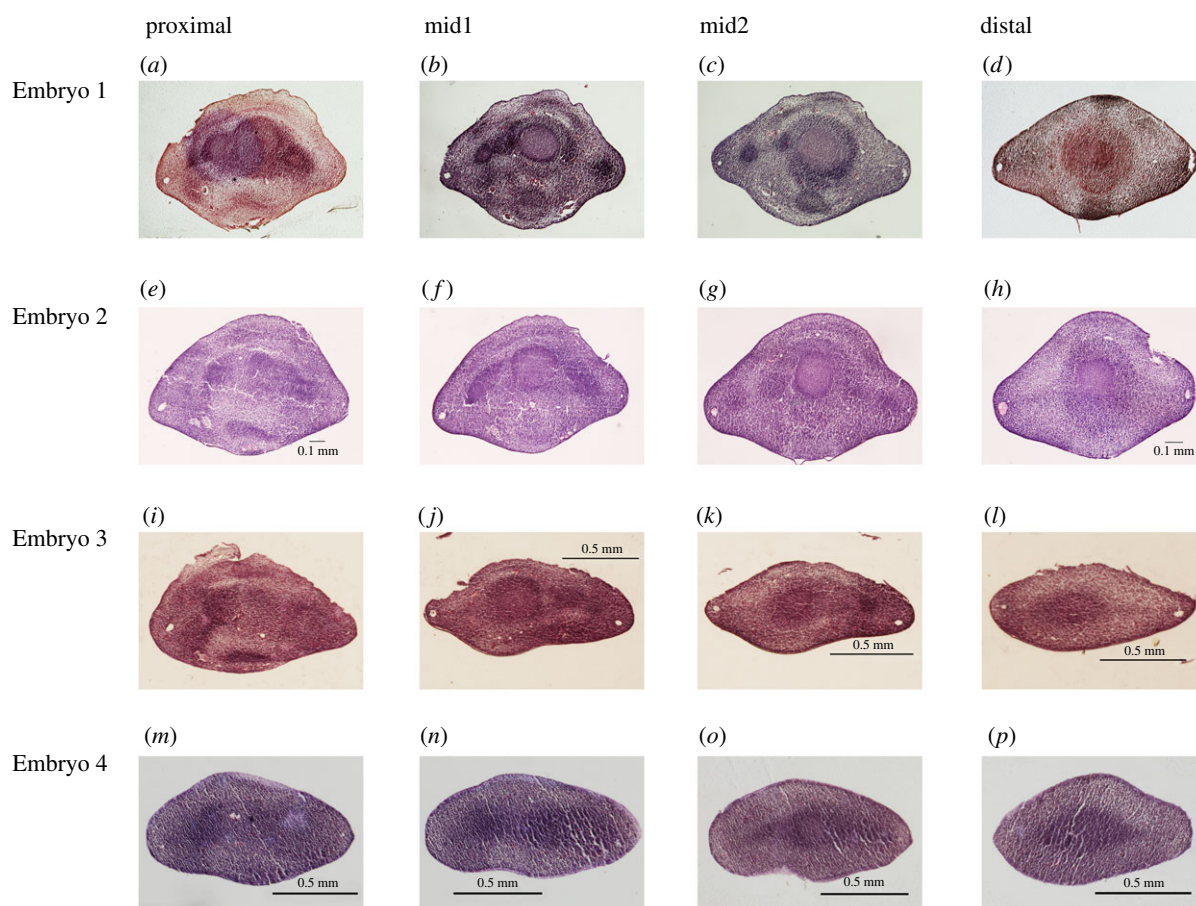
Figure 2. Transverse sections of embryonic horse FL autopods of approximate ages dpc 35 (Embryos 1 and 2), dpc 31–34 (Embryos 3 and 4). Proximal sections are taken from the carpo-metacarpal joint. Mid1 and mid2 sections are taken where the most condensations are most apparent to illustrate separate digits or obvious fusions. Distal sections are taken where the enlarged digit III, if present, is solely visible in the section. Posterior is to the left in each section and dorsal is top. All sections are stained with H&E. (Online version in colour.)

relatively smaller and relatively developmentally advanced at the same stage (figure 3*b,c*). In the oldest embryos, the anterior and posterior digits of the HL were not fully separated distally as seen in the FL but rather had already fused into a more A-P elongated condensation that still appeared to maintain two distinct cell populations (figure 3*b,c*). In the HL, the condensations for digits IV and III were the first to appear, followed by those for II, V and I (figure 3*b,c,k,o*).

No evidence of any mesenchymal anomalies or distal remnant condensations was observed in the FL or HL that might account for digit cartilages contributing to the hoof elements (the frog), as hypothesized in Solounias *et al.* [6] (electronic supplementary material, figures S1–S9).

3. Ontogeny and phylogeny of horse digits

We found that embryonic horse limbs develop five digit condensations that persist into later developmental stages through fusion of cell populations within days after initiation. Thus, even the highly modified monodactyl *Equus* FL maintain an ontogenetic reflection of their pentadactyl ancestors and follow a sequence of development similar to the iconic evolutionary transitions in the horse lineage. For example, adult FL skeletons in the fossil horse lineage include the most ancestral pentadactyl *Phenacodus* and tetradactyl *Protorohippus* (*Hyracotherium*) as well as more derived tridactyl *Mesohippus* and *Hypohippus* with more reduced, closely articulated and/or fused lateral digits, and the most recent, most extreme modern

form, the 'monodactyl' *Equus* with two splint metacarpals [2,3,6] (figure 1*b*). Similarly, the ontogenetic series of *Equus* FL digits also reflects these transitional sequences from pentadactyly to tridactyly with a dominant central digit, here occurring over a short period of approximately 4–5 days of development. Shortly after the appearance of the flattened early autopod paddle, five digit condensations form that are initially similar-sized, similar to *Phenacodus* and *Hyracotherion*. Slightly later, the central digit III becomes larger and extends further distally, while digits I and II fuse, and digits IV and V fuse, into the anterior and posterior metapodials, respectively, flanking the dominant central digit III. Slightly later in development, the distal growth of the splint metapodials is arrested while the central toe continues to grow larger, similar to the digit anatomy of e.g. *Protohippus* and *Hipparion* [3,6] (figure 1*a*). Francioli *et al.* [11] showed a cleared-and-stained 60 dpc horse fetus which clearly has an ossifying dominant central digit and highly reduced splint metapodials, likely to be close to the final adult proportions given that the hoofs are already forming, and thus providing a bound for ontogenetic timing from initial condensation to fully adult proportioned in 30 days or fewer.

A further parallel between evolutionary and developmental timing is seen in the comparison of the FL and HL of modern horses and their ancestors. In the fossil horse series, the HL experiences lateral digit fusion and the 'loss' of digits ahead of the FL, e.g. the fossil species *Mesohippus* and *Protorohippus* have four digits or splints in the FL and only three in the HL [3,4]. In one of our *Equus* embryos (Embryo 1), the HL lateral digits are fully fused while the
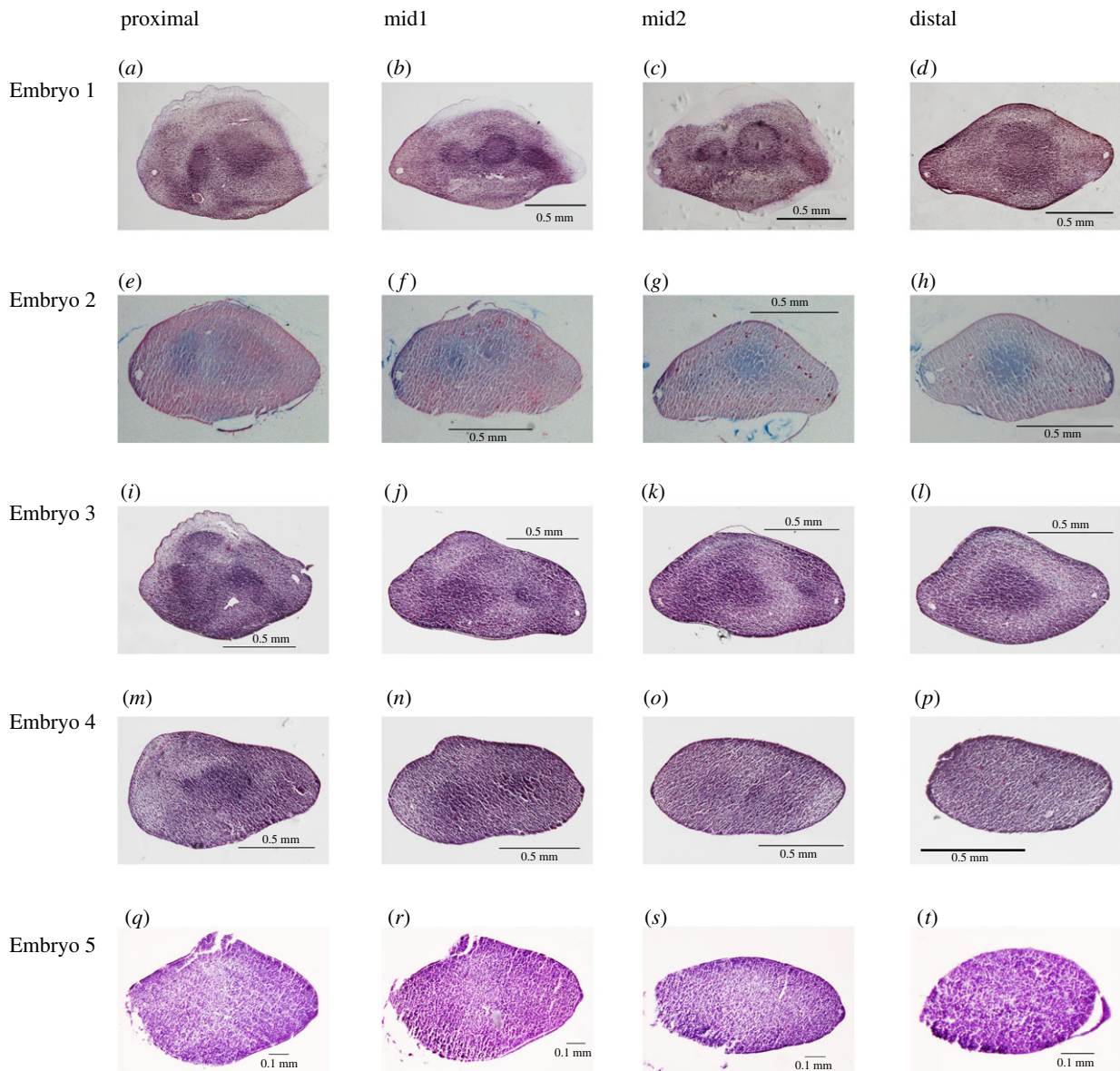
Figure 3. Transverse sections of embryonic horse HL autopods of approximate ages dpc 35 (Embryos 1 and 2), dpc 31–34 (Embryos 3 and 4) and dpc 29.5 (Embryo 5). Proximal sections are taken from the carpo-metacarpal joint. Mid1 and mid2 sections are taken where the most condensations are most apparent to illustrate separate digits or obvious fusions, but are not necessarily spaced equally or matched precisely with other sections. Distal sections are taken where the enlarged digit III, if present, is solely visible in the section. Posterior is to the left in each section and dorsal is top. All sections are stained with H&E, except Embryo 2 sections are stained with eosin and Alcian Blue to show that condensations are pre-cartilaginous. (Online version in colour.)

FL digits remain distally separated (figures 1 and 2*b,f*). Independent evolution of the developmental timing of the FL and HL has been documented in other mammals [7,12–15]. Here it appears to mirror the FL and HL differences in the fossil sequences of horse digit evolution.

The persistence of pentadactyly even in this most extreme and high profile example of digit reduction in modern species led us to seek clarification regarding the developmental status of other digit-reduced taxa where embryonic digit development had been examined.

4. Developmental stabilization of tetrapod digit number

A substantial body of literature in evolutionary developmental biology has focused on the question of what developmental processes are involved in regulating the diversity in the number of digits in tetrapods, with several recent major reviews

on this subject [8–10]. Here, all published observations on early developmental stages in tetrapods with functional digits were re-examined to clarify the number of digits present at the initiation stages of autopod development. One possible source of confusion in the literature is incorrectly equating the term *digit*, which in modern tetrapods refers to only the phalanges, with *digit ray*, which consists of both metapodial elements *and* phalanges. Ancestrally in tetrapod evolution and in some aquatic tetrapods, metapodials are indistinguishable from the graded sequence of phalanges in a given digit; only later in evolution do metapodials typically become a separate variational module [16]. Since no phalanges ever develop in nature without a proximal metapodial, then anatomically, developmentally and evolutionarily the number of metapodials initiating should simply reflect the number of digits initiating. With this perspective, our re-examination of digit-reduced tetrapods found that there are always five or four digits initiated at this patterning stage (table 1). If there are only four digits present, it is always digit I that is missing in amniotes (and, interestingly,

Table 1. Observations of Digits I–V metapodial initiation in embryonic forelimbs and hindlimbs of diverse (mostly) digit-reduced tetrapods. Observation codes: Stage 0 = not observed; Stage 1 = Sox9 expression; Stage 2 = Alcian/cartilage; Stage 3 = Alizarin/bone. 'Number of digits' refers to the commonly reported number of adult digits (usually the large ones with phalanges).

species	'number of digits'	forelimb (I–V)	hindlimb (I–V)	reference
mouse	5/5	3,3,3,3,3	3,3,3,3,3	[7,17]
3-toed jerboa	5/3	3,3,3,3,3	2,3,3,3,2	[7]
5-toed jerboa	5/5	3,3,3,3,3	3,3,3,3,3	[7]
camel	2/2	0,1,3,3,1	0,1,3,3,1	[7]
pig	2/2	0,3,3,3,3	0,3,3,3,3	[7,18]
horse	1/1	2,3,3,3,2	2,3,3,3,2	[7]; this study*
cow	2/2	0,1,3,3,1	0,1,3,3,1	[17]
skink *H. initialis*	5/5	3,3,3,3,3	3,3,3,3,3	[19]
skink *H. peronii*	4/4	0,3,3,3,3	0,3,3,3,3	[19]
skink *H. peronii*	3/3	0,3,3,3,3	0,3,3,3,3	[19]
skink *H quadrilin.*	2/2	0,3,3,3,3	0,3,3,3,3	[19]
crocodile	5/5	3,3,3,3,3	3,3,3,3,2	[20]
Mauremys turtle	5/5	3,3,3,3,3	3,3,3,3,3	[21]
emu	1/3	0,2,3,2,2	2,3,3,3,2	[20]
ostrich	3/2	2,3,3,3,2	2,2,3,3,2	[20]
chicken	3/4	1,3,3,3,2	3,3,3,3,2	[20]
Barbary dove	3/4	0,3,3,3,2	3,3,3,3,2	[20]
zebra finch	3/4	0,3,3,3,2	3,3,3,3,2	[20]
Plethodon salamander	4/5	3,3,3,3,0	3,3,3,3,3	[22]

it is digit V that is first missing in digit-reduced salamanders with 'reversed' sequence of initiation, both in nature [22] and in experiments [23]). In later stages of digit development in amniotes (post-patterning), numerous mechanisms are employed to diversify digit features: growth can be arrested, elements fused or tissue carved away by apoptosis, combined with allometric changes among digits and/or positional changes of proximal elements (e.g. [7–10,17,24–27]. Thus, while digit reduction is a 'repeated theme in tetrapod evolution,' the number of digits at initiation is remarkably conserved and evolutionary changes in later digit development explain most of the diversity of digit patterns [10,28].

The 'patterning-type' digit reduction described in the literature, which refers to changes in the *Shh*-mediated pathway and associated variation in paddle width of the autopod, is thus not likely affecting the initiation of 4 or 5 digit condensations in nature, in contrast with assumptions in current and frequently cited models of this system [29–31] and a large number of experimental manipulations correlating digit number with *Shh*-mediated autopod width [30–32]. The current paradigm of digit number regulation by patterning mechanisms has been interpreted and used to model evolution in the light of experiments in which changes to the width of the limb bud, driven by experimental modification of early autopod developmental networks, leads to reduced numbers of digits (reviews in [9,10,19,30,32]). Certainly developmental regulatory differences in the lab can phenocopy digit-reduced species in nature, e.g. *Gli1* expression in cow and pig [17,18]. Yet, in both of these examples, expression of *Sox9*, an early marker of digit initiation, suggests that four or five digits initiate; *Sox9* marks four digits of the two-toed, three-toed and four-

toed skinks [19], four digits in cow [17] and five digits in the pig [18]. If 'patterning' refers to initiating the positions of digit condensations in the early autopod, we do not see evidence of morphological or genetic markers (such as Sox9) in nature that distinguishes those taxa said to reduce digits by patterning processes versus those that reduce digits by post-patterning processes, with exception of digit I, which is known to form in a slightly different manner from other digits and usually last [27,33]. This conservation at the initiation stages contrasts with the evolutionary diversity in the later-acting developmental mechanisms shaping digit morphology [7,17] and described as 'post-patterning' types of digit reduction [8,10]. The data support the hypothesis of 'construction then deconstruction' as a rule [34], that is, changes in post-patterning, rather than patterning mechanisms are far more universal in the evolution of tetrapod digit number than currently acknowledged.

5. Conclusion

Here we find that in all cases where embryonic anatomy has been evaluated during early autopod formation in amniotes, the initial number of digits that condense is either 5 or 4, and if the number is 4, it is digit I that is lost. In the extreme case of the monodactyl modern horse, we have shown that five digit condensations form in the embryonic *Equus* limb, and their subsequent reduction follows a striking parallel with evolution in the famous fossil transition series in the horse lineage.

In evolutionary discussions of tetrapod digit number, the 'constraint' of five maximum digits in modern taxa has been

long recognized [34]. Notable exceptions to this constraint are the Devonian stem-tetrapods *Ichthyostega* and *Acanthostega* [35]. When the unusual presence of six digits in some modern taxa has been analysed, they are inevitably shown to be formed via convoluted developmental solutions, such as wrist bone modifications, that seem to win out over simpler modification of autopod patterning pathways, e.g. the panda's and mole's extra thumb [36–38]. As such, it appears that in amniote tetrapods, there is both a developmentally favoured maximum (5) and a developmentally favoured minimum number of early digit condensations, which is either five or four with digit I missing. Careful scrutiny is warranted to determine whether digit I initiation is truly missing in species in which only four digit condensations are thus far reported. The critical embryonic stage at which digit initiation occurs is brief but predictable. Probably because of the difficulty in obtaining substantial numbers of staged embryos for diverse digit-reduced taxa, this observation of a 'digit minimum' has been overlooked despite its potential significance as a constraint for the vast array of models of patterning in the limb and digits. Going forward, the conserved nature of tetrapod digit initiation urges further evaluation of the developmental and evolutionary processes acting to stabilize this embryonic stage.

6. Methods

Horse embryos were obtained from mares that were bred via artificial insemination within 48 h before documented ovulation at North Carolina State University. Several embryonic limbs from horses of estimated ages of 29–35 dpc were fixed, dehydrated, paraffin-embedded, serially transverse-sectioned across the distal limb, stained with H&E and digitally imaged. The sections span the developmental period from the initial flattening of the early autopod tissue prior to digit condensation (Embryo 5; figure 2), through initial condensation (Embryos 3 and 4) to early stages of post-patterning growth and differentiation (Embryos 1 and 2). Both FL and HLs were sectioned in Embryos 1–4 (HL only in Embryo 5). In all samples, H&E stain highlighted the condensing cells in the initiating digits by a darker stain of presumably increased cell density and ECM in the centre of the mesenchymal tissue. To confirm that the darker coloured staining in the sections indicated condensing cartilaginous cells, we stained one limb series with Alcian Blue and eosin, and indeed the Alcian Blue stain consistently marked the regions of suspected condensing cells (figure 2m–p).

References

1. Janis CM, Bernor RL. 2019 The evolution of equid monodactyly: a review including a new hypothesis. *Front. Ecol. Evol.* **7**, 119. (doi:10.3389/fevo.2019.00119)

2. McHorse BK, Biewener AA, Pierce SE. 2018 Mechanics of evolutionary digit reduction in fossil horses (Equidae). *Proc. R. Soc. B* **284**, 20171174. (doi:10.1098/rspb.2017.1174)

3. Janis CM. 2007 The horse series. In *Icons of evolution* (ed. B Regal), pp. 257–280. Westport, CT: Greenwood Press.

4. Matthew WD. 1903 The evolution of the horse. American Museum of Natural History, Supplement to the American Museum. *J. Guide Leaflet* **9**, 1–30.

5. Marsh OC. I879 Polydactyl horses, recent and extinct. *Amer. Jour. Sci.* **17**, 499–505.

6. Solounias N, Danowitz M, Stachtiaris E, Khurana A, Araim M, Savegh M, Natale J. 2018 The evolution and anatomy of the horse manus with an emphasis on digit reduction. *R. Soc. open sci.* **5**, 171782. (doi:10.1098/rsos.171782)

7. Cooper KL, Sears KE, Uygur A, Maier J, Baczkowski K, Brosnahan M, Antczak D, Skidmore JA, Tabin CJ. 2014 Patterning and post-patterning modes of evolutionary digit loss in mammals. *Nature* **511**, 41–45. (doi:10.1038/nature13496)

8. Young JJ, Tabin C. 2016 Saunder's framework for understanding limb development as a platform for

investigating limb evolution. *Dev. Biol.* **429**, 401–408. (doi:10.1016/j.ydbio.2016.11.005)

9. Saxena A, Cooper KL. 2017 The origins, scaling, and loss of tetrapod digits. *Proc. Trans. R. Soc. B* **372**, 20150482. (doi:10.1098/rstb.2015.0482)

10. Sears KE, Maier JA, Sadier A, Sorensen D, Urban DJ. 2018 Timing the developmental origins of mammalian limb diversity. *Genesis* **56**, e23079. (doi:10.1002/dvg.23079)

11. Franciolli AL *et al.* 2011 Characteristics of the equine embryo and fetus from days 15 to 107 of pregnancy. *Theriogenology* **76**, 819–832.

12. Beiriger A, Sears KE. 2014 Cellular basis of differential limb growth in postnatal grey short-tailed opossums (*Monodelphis domestica*). *JEZ Part B: Mol. Dev. Evol.* **322**, 221–229. (doi:10.1002/jez.b.22556)

13. Adams RA. 1992 Comparative skeletogenesis of the forearm of the little brown bat (*Myotis lucifugus*) and the Norway rat (*Rattus norvegicus*). *J. Morphol.* **214**, 251–260.

14. Richardson MK, Gobes SMH, Van Leeuwen AC, Polman JAE, Pieau C, Sanchez-Villagra MR. 2009 Heterochrony in limb evolution: developmental mechanisms and natural selection. *J. Exp. Zool. (Mol. Dev. Evol.)* **312B**, 639–664. (doi:10.1002/jez.b.21250)

15. Cooper KL, Oh S, Sung Y, Dasari R, Kirschner MK, Tabin CJ. 2013 Multiple phases of chondrocyte

enlargement underlie differences in skeletal proportions. *Nature* **495**, 375–378. (doi:10.1038/nature11940)

16. Kavanagh KD, Shoval O, Winslow BB, Alon U, Leary BP, Kan A, Tabin CJ. 2013 Developmental bias in the evolution of phalanges. *PNAS* **110**, 18 190–18 195. (doi:10.1073/pnas.1315213110)

17. Lopez-Rios J *et al.* 2014 Attenuated sensing of SHH by Ptch1 underlies evolution of bovine limbs. *Nature* **511**, 46–51. (doi:10.1038/nature13289)

18. Sears KE, Bormet AK, Rockwell A, Powers LE, Cooper LN, Wheeler MB. 2011 Developmental basis of mammalian digit reduction: a case study in pigs. *Evol. Dev.* **13**, 533–541. (doi:10.1111/j.1525-142X.2011.00509.x)

19. Shapiro MD, Hanken J, Rosenthal N. 2003 Developmental basis of evolutionary digit loss in the Australian Lizard *Hemiergis*. *J. Exp. Zool. (Mol. Dev. Evol.)* **297B**, 48–56. (doi:10.1002/jez.b.19)

20. de Bakker MAG, Fowler DA, den Oude K, Dondorp EM, Carmen Garrido Navas M, Horbanczuk JO, Sire J-Y, Szczerbinska D, Richardson MK. 2013 Digit loss in archosaur evolution and the interplay between selection and constraints. *Nature* **500**, 445–449. (doi:10.1038/nature12336)

21. Montero JA, Lorda-Diez CI, Francisco-Morcillo J, Chimal-Monroy J, Garcia-Porrero JA, Hurle JM. 2017 Sox9 expression in amniotes: species-specific

differences in the formation of digits. *Front. Cell Dev. Biol.* **5**, 23. (doi:10.3389.fcell.2017.00023)

22. Kerney RR, Hanken J, Blackburn DC. 2018 Early limb patterning in the direct-developing salamander Plethodon cinereus revealed by sox9 and col2a1. *Evol. Dev.* **2018**, 1–8.

23. Stopper GF, Wagner GP. 2007 Inhibition of Sonic Hedgehog signaling leads to posterior digit loss in *Ambystoma mexicanus*: Parallels to natural digit reduction in urodeles. *Dev. Dyn.* **236**, 321–331. (doi:10.1002/dvdy.21025)

24. Adams RA. 1992 Comparative skeletogenesis of the forearm of the little brown bat (*Myotis lucifugus*) and the Norway rat (*Rattus norvegicus*). *J. Morphol.* **214**, 251–260. (doi:10.1002/jmor.1052140302)

25. Rolian C. 2008 Developmental basis of limb length in rodents: evidence for multiple divisions of labor in mechanisms of endochondral bone growth. *Evol. Dev.* **10**, 15–28. (doi:10.1111/j.1525-142X.2008.00211.x)

26. Sears KE, Behringer RR, Rasweiler JJ, Niswander LA. 2007 The evolutionary and developmental basis of parallel reduction in mammalian zeugopod elements. *Am. Nat.* **169**, 105–117. (doi:10.1086/510259)

27. Stewart TA, Liang C, Cotney JL, Noonan JP, Sanger TJ, Wagner GP. 2019 Evidence against tetrapod-wide digit identities and for a limited frame shift in birds. *Nat. Commun.* **10**, 3244. (doi:10.1038/s41467-019-11215-8)

28. Galis F, van Alphen JM, Metz JAJ. 2002 Digit reduction: via repatterning or developmental arrest? *Evol. Dev.* **4**, 249–251. (doi:10.1046/j.1525-142X.2002.02013.x)

29. Stewart TA, Bhat R, Newman SA. 2017 The evolutionary origin of digit patterning. *EvoDevo* **8**, 21. (doi:10.1186/s13227-017-0084-8)

30. Sheth R, Marcon L, Bastida MF, Junco M, Quintana L, Dahn R, Kmita M, Sharpe J, Ros MA. 2012 Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. *Science* **338**, 1476–1480. (doi:10.1126/science.1226804)

31. Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. 2004 Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* **118**, 517–528. (doi:10.1016/j.cell.2004.07.024)

32. Alberch P, Gale EA. 1983 Size dependence during the development of the amphibian foot: colchicine-induced digital loss and reduction. *J. Embryol. Exp. Morph.* **76**, 177–197.

33. Oberg KC. 2014 Review of the molecular development of the thumb: digit primera. *Clin. Orthop. Relat. Res.* **472**, 1101–1105. (doi:10.1007/s11999-013-3008-5)

34. Galis F, van Alphen JM, Metz JAJ. 2001 Why five fingers? Evolutionary constraints on digit numbers. *TREE* **16**, 637–646. (doi:10.1016/s0169-5347(01)02289-3)

35. Coates MI, Clack JA. 1990 Polydactyly in the earliest known tetrapod limbs. *Nature* **347**, 66–69. (doi:10.1038/347066a0)

36. Mitgusch C *et al.* 2011 Circumventing the polydactyly 'constraint': the mole's thumb. *Biol. Lett.* **8**, 74–77. (doi:10.1098/rsbl.2011.0494)

37. Salesa MJ, Anton M, Peigne S, Morales J. 2006 Evidence of a false thumb in a fossil carnivore clarifies the evolution of pandas. *Proc. Natl Acad. Sci. USA* **103**, 379–382. (doi:10.1073/pnas.0504899102)

38. Davis DD. 1964 *The giant panda, a morphological study of evolutionary mechanisms*. Fieldiana Zool Memoirs 3. Chicago Natural History Museum.