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Heterochrony in Limb Evolution: Developmental Mechanisms and Natural Selection

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ABSTRACT The tetrapod limb provides several examples of *heterochrony*—changes in the timing of developmental events. These include species differences in the sequence of skeletal chondrogenesis, in gene transcription in the developing limbs, and in the relative time at which forelimb and hind limb buds develop. Here, we examine (i) phylogenetic trends in limb heterochrony; (ii) changes in developmental mechanisms that may lead to heterochrony; and (iii) the possible role that heterochrony plays in generating adaptive traits. We analyze the published literature and present preliminary data on turtle (*Emys orbicularis*) and bat (*Rousettus amplexicaudatus*) limb development. Teleosts, marsupials, and some urodeles show extreme timing differences between forelimb (or pectoral fin) and hind limb (or pelvic fin) development; this heterochrony may, in some cases, be adaptive. Published data on limb chondrogenesis reveal sequence elements that are strongly conserved (possibly owing to constraints); and others that vary between higher taxa (for unknown reasons). We find little evidence that chondrogenic sequences are modified by selection for limb functional traits. There are a few examples of developmental mechanisms that may be modified under heterochrony to produce adaptive changes in the limb (e.g. some cases of hyperphalangy or limb reduction). In conclusion, numerous examples of limb heterochrony have been recorded. However, few cases are obviously adaptive. Indeed, current data and methodologies make it difficult to identify the developmental changes, or selective pressures, that may underlie limb heterochrony. More integrative studies, including studies of heterochrony within populations, are needed to assess the role of timing shifts in limb evolution. *J. Exp. Zool. (Mol. Dev. Evol.) 312B:639–664, 2009.* © 2009 Wiley-Liss, Inc.

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The term "heterochrony" refers to timing differences in development. It is most often studied in an evolutionary context, when the timing difference is revealed by comparison of individuals from different species (de Beer, '51; Gould, '77, '82; Raff and Wray, '89; McKinney and McNamara, '91; Gould, '92; Richardson, '95; Klingenberg, '98; Smith, 2001). If a robust phylogeny is available, the polarity of the heterochrony can be inferred, and may be described as timing "change" in evolution. The ancestral

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timing relations represent euchrony or isochrony (Schlosser, 2001).

Heterochrony can also be identified between individuals of the same species, in which case it describes polymorphism or phenotypic plasticity (''individual variation'' sensu Keibel and Abraham, 1900). Such intraspecific heterochrony has been studied in relatively few cases (Mabee and Trendler, '96; Mabee et al., 2000; Irmler et al., 2004). It may even be legitimate to describe heterochrony in a single individual, as when serially homologous structures develop at different stages in one ontogeny.

The parameters of developmental timing used to record heterochrony include chronological age, developmental stage, rate, and the order or sequence in which developmental events occur (Alberch, '85; Richardson, '95; Nunn and Smith, '98; Richardson et al., '98a,b; Smith, 2001; Jeffery et al., 2002; Sánchez-Villagra, 2002; Sánchez-Villagra et al., 2008a,b; Weisbecker et al., 2008). The ''developmental events'' whose time relations are altered in sequence heterochrony are essentially transformations between two developmental character states (Bininda-Emonds et al., 2002). These may be, for example, states of cell behavior, gene transcription, or morphological phenotype. Heterochrony may also affect multi-component or compound events such as birth or hatching (both of which, incidentally, take place at widely differing stages of ontogeny in different taxa). Physiological activities can be included a discussion of heterochrony (Spicer and Burggren, 2003), although we concentrate in this article on developmental patterning and morphology.

One way of thinking about heterochrony is to make a distinction between heterochronic *patterns* (phenotypic expressions of timing change) and *processes* (changes in developmental mechanisms that generate such patterns; reviewed by Raff and Wray, '89; Cubo, 2000). Some care is needed to avoid using the pattern-process distinction too dogmatically. As previously suggested (reviewed by Raff and Wray, '89), the distinction may be arbitrary, even relative. Thus, a heterochronic ''process,'' such as a shift in the timing of developmental gene transcription, might equally be viewed as a heterochronic ''pattern'' when its distribution in an embryo or across a phylogeny is described. Furthermore, the term ''heterochronic process'' (or ''process heterochrony'') is ambiguous: some developmental changes that lead to morphological heterochrony are not, in themselves, heterochronic (reviewed by Cubo, 2000). This creates an awkward category of ''nonheterochronic process heterochronies.''

Here, we shall use the terms in a purely relative sense to distinguish the upstream ''process'' and downstream ''pattern'' in any given heterochronic shift. In developmental terms, ''downstream'' means occurring at a later stage; in molecular terms, ''downstream'' means that an event is distal on a pathway. The causal relationship between process and pattern could, in principle, be proven if it were replicated by suitable experimental manipulations of embryos.

As noted above, ''processes'' may themselves be heterochronic or nonheterochronic (Fig. 1). Transcriptional heterochrony is an example of a timing change in developmental mechanisms that can lead to heterochrony (Mackem and Mahon, '91; Dollé et al., '93; Belting et al., '98). Other developmental alterations leading to pattern heterochrony do not themselves involve a timing change. For example, changes in cell allocation can lead to pattern heterochrony if the resulting skeletal primordium has fewer cells in its growth plate (Cubo, 2000). A further example is provided by nonheterochronic disruption of *Hoxd13* function leading to a pattern heterochrony (delayed limb ossification; Dollé et al., '93). Some nonheterochronic developmental changes, which underlie pattern heterochronies, have been called process heteroposies (reviewed by Cubo, 2000).

In the context of limb development, morphological pattern heterochronies (Figs. 2 and 3) include differences in the stage at which forelimbs and hind limbs develop in different species (Bininda-Emonds et al., 2007), and differences in the sequence in which various skeletal elements develop in the limbs (Rabl, '10; Shubin and Alberch, '86). These heterochronies are expressed in embryonic or larval morphology, and might therefore be open to selection at those developmental stages. However, they do not necessarily lead to changes in adult limb morphology. For example, some salamander species differ in the sequence in which chondrogenic primordia develop in their limbs, yet develop very similar adult limb skeletons (Blanco and Alberch, '92). Furthermore, the early development of the radius is delayed in limbs lacking *Gli3* and a copy of *Alx4*, but later shows catch-up growth to restore normal dimensions (Panman et al., 2005).

In the lizard *Calotes versicolor*, the hind limbs start developing later than the forelimbs, but grow faster, until eventually the forelimb and hind limb reach a comparable level of differentiation (Goel

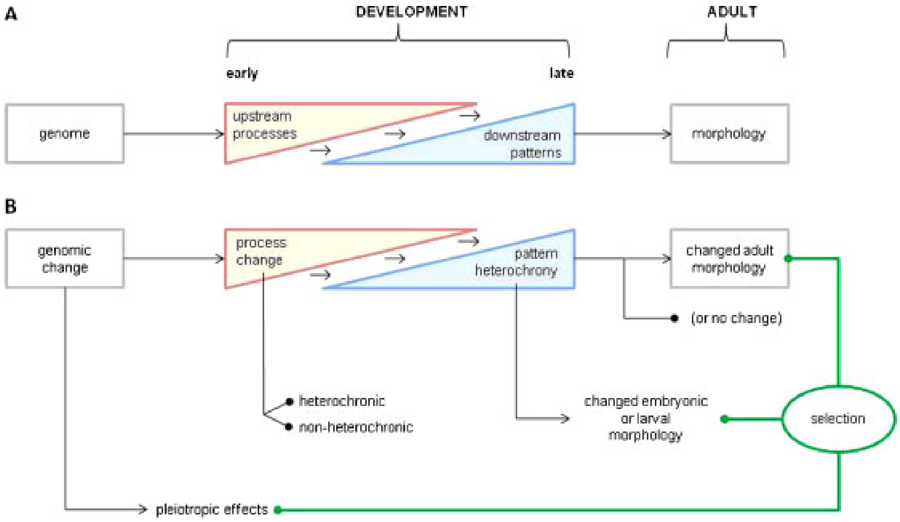

Fig. 1. Schematic diagram showing some of the concepts of limb heterochrony discussed in this article. (**A**) The ancestral state (isochrony or euchrony). Developmental changes associated with heterochrony may be arbitrarily divided into *processes* (upstream changes in developmental mechanism) and *patterns* (the resultant, downstream changes in timing of developmental events). Early developmental events are more likely to be classed as processes, whereas late events are more likely to be seen as patterns, although the distinction is blurred. This blurring is indicated by the sloping faces of the triangles. (**B**) Descendant that shows heterochrony relative to (A). There is a chain of events leading from genomic change, via altered developmental processes, to a downstream (''pattern'') heterochrony. The alterations in developmental processes may themselves be heterochronic or nonheterochronic. Pattern heterochronies are, by definition, expressed during developmental stages, and may be open to selection. They may also lead to changes in adult morphology, or alternatively, have no effect on adult phenotype. As shown at the bottom of (B), genomic change may alter developmental processes in more than one system, leading to pleiotropic effects. These, together with functional changes in the larva or embryo, and possible changes in adult form and function, may be open to selection.

and Mathur, '77). The situation in the *Caiman* appears to be reversed, so that the forelimbs are initiated first, but the hind limbs then outstrip them in their subsequent development (Iungman et al., 2008). These examples suggest that heterochrony present in early stages may be silent with respect to adult morphology; in a sense, its effects are transient and can be overwritten by compensatory changes at later stages.

OBJECTIVES AND APPROACH

We summarize the above arguments in Figure 1, which shows our view of key elements involved in the emergence of heterochrony during evolution. From this starting point, our aim is to ask what role heterochrony plays in limb evolution. Are there any known changes in developmental mechanisms that are necessary and sufficient to account for heterochronic patterns? Can developmental timing shifts lead to adaptive changes, or do they reflect a phylogenetic trend that is unrelated to ecological niche? These questions are far from being resolved. We lack complete, integrative data sets that encompass functional morphology, comparative developmental biology, ecology and genomics, and which are drawn from a phylogenetically informative species sample.

Nonetheless, we can at least survey the available evidence to see whether it can point to some answers, or indicate promising lines for future research. In the section ''Morphological Patterns of Heterochrony in Limb Evolution and Development'' (below) we review what is known about the patterns of morphological heterochrony in limb evolution and their phylogenetic distribution. In the section ''Developmental Mechanisms and Heterochrony in Limb Evolution'', we consider changes in developmental mechanisms that could lead to heterochrony. Finally (the section ''Natural Selection and Heterochrony''), we consider the possible significance of heterochrony in adaptive scenarios of limb evolution. We chose the tetrapod limb as our subject because there is an extensive literature on both pattern and process heterochronies in the limb. We use the terms ''limbs'' and ''paired fins'' in this article to mean the paired appendages in gnathostomes that develop from the somatopleure, and that commonly have locomotor function. We include some discussion of pectoral and pelvic fins in cartilaginous and bony fishes. For this purpose, we shall consider the

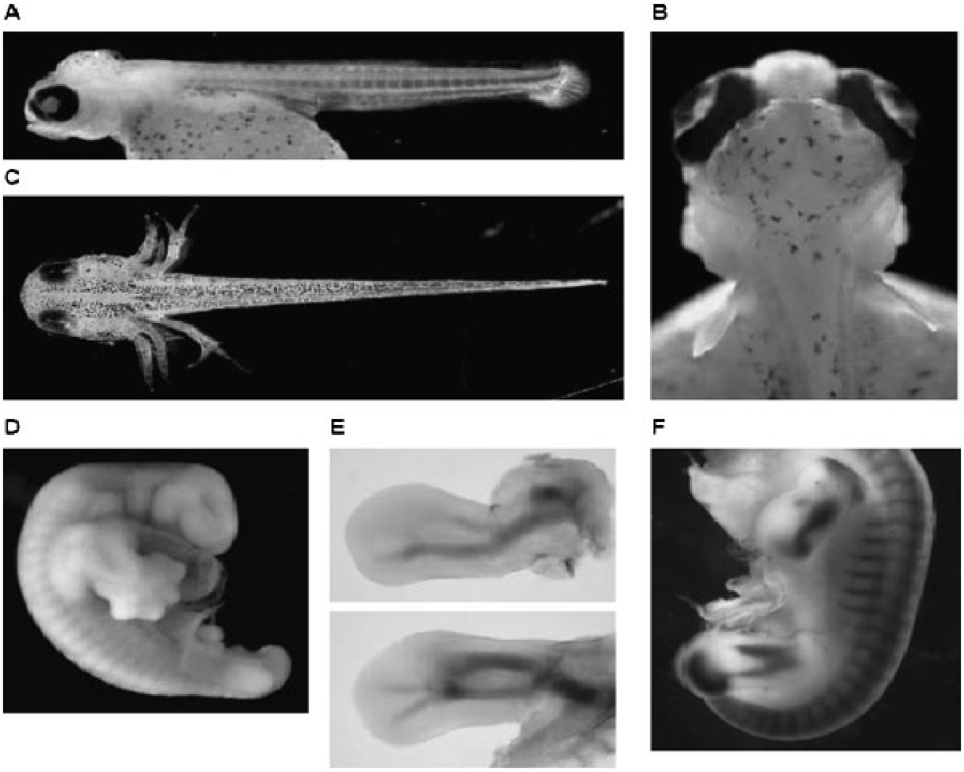

Fig. 2. Examples of heterochrony in the relative time of development of the pectoral and pelvic appendages. (**A**, **B**) The cichlid (teleost) *Haplochromis piceatus* (de Jong et al., unpublished data; embryo C0876). (A) Lateral view, rostral is to the left; no pelvic fin primordium is visible in the region of the cloaca. (B) Detail of same showing the dorsal aspect of the head region. A well-developed pectoral fin is visible. (**C**) This salamander (*Triturus dobrogicus*) larva has well-developed forelimbs, but no signs yet of hind limb primordia (Poot et al., unpublished data). (**D**) Embryo of the brushtail possum *Trichosurus vulpecula* (Hubrecht collection MA194d, 4 mm CR length) showing digital webbing in the relatively large forelimb while the hind limb is still a bud. (**E**) Alcian blue preparation of chick forelimb (top) and hind limb (below) showing primary axis in the forelimb, and a more advanced development of skeletal elements in the hind limb. (**F**) *sox9* in situ hybridization of a chick embryo at stage 25, showing a more advanced development of hind limb skeletal Anlagen, including the more advanced digital arch (image inverted; Richardson and de Bakker, unpublished data). Note that chondrogenic sequences are easily visualized by staining with Alcian blue (for cartilage matrix), in which case, all condensations formed can be seen simultaneously in one specimen (E). By contrast, with transiently expressed markers such as *sox9* mRNA (for precartilage mesenchyme), only condensations undergoing early stages of formation are seen in one specimen (F).

paired fins and limbs to be homologous as paired appendages among tetrapods (although the homologies of individual skeletal elements is debatable; see Mabee, 2000).

MORPHOLOGICAL PATTERNS OF HETEROCHRONY IN LIMB EVOLUTION AND DEVELOPMENT

In this section, we consider patterns of morphological heterochrony in limb development. Several timing relations, which are normal features of limb development, may be modified to produce pattern heterochronies. These include (i) a timing gradient in chondrogenic differentiation along the proximodistal axis of the limb; (ii) one or more timing gradients along the anteroposterior axis of the limb; (iii) a difference in the stage at which the forelimbs and hind limbs develop. Where these timing relations are identical in two species, they may be said to form an isochronic suite (Schlosser, 2001), whereas differences between them constitute heterochrony.

Developmental sequences along the limb axes

If a series of developing limbs is stained for cartilage matrix (Figs. 4 and 5), it can be seen that the various skeletal elements develop in sequence, not simultaneously. The sequence varies considerably between taxa, and may even show minor variations between individuals in one species. It is claimed to be strongly modified in species with

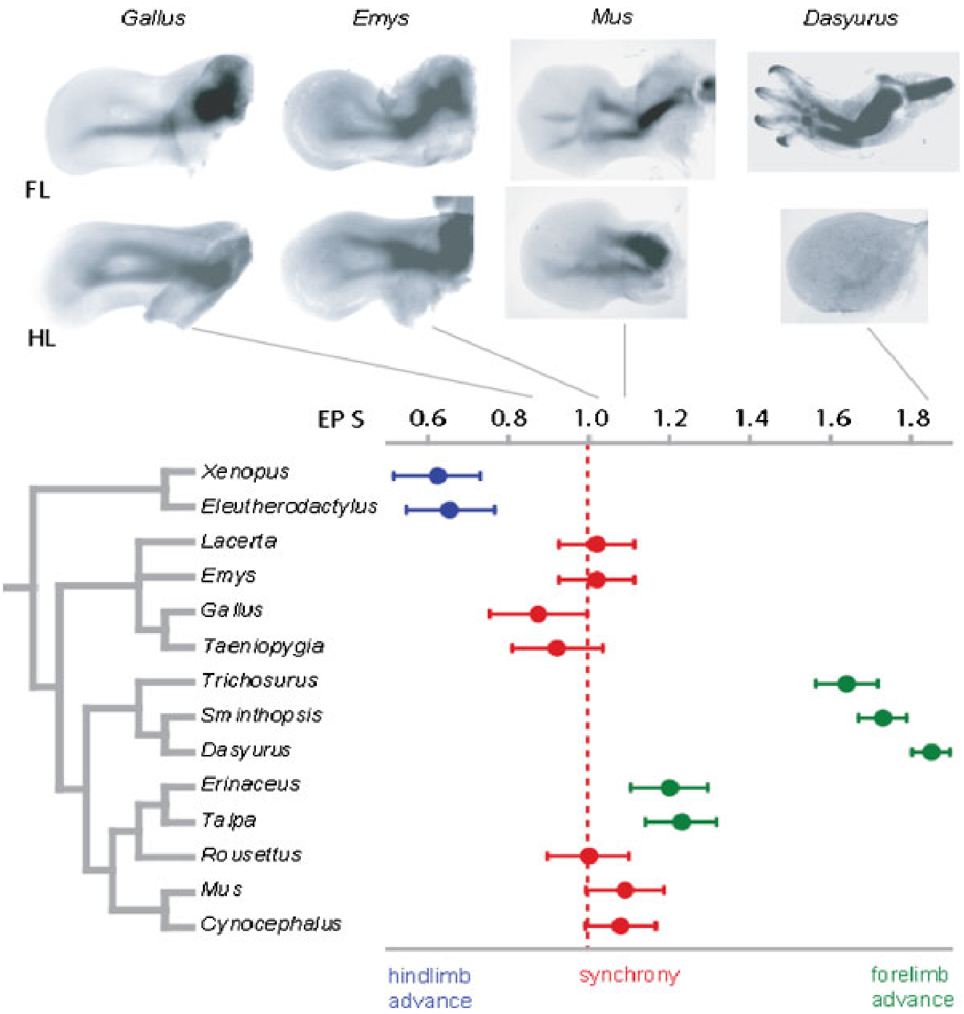

Fig. 3. Degree of timing differential between development of the hind limb and forelimb (*E* value) for several tetrapods. This quantitative measure is derived from a comparison of the sequence of homologous developmental events in the hind limb and forelimb. The hind limbs develop earlier than the forelimbs in the anurans and birds studied; the forelimbs and hind limbs develop synchronously or nearly so in the bat, turtle, and lizard. Forelimbs are moderately accelerated in the hedgehog and mole, and strongly accelerated in the marsupials. Photos in top two rows show limbs from the same individual stained with Alcian blue. FL, forelimb; HL, hind limb. From Bininda-Emonds et al. (2007).

reduced numbers of skeletal elements (e.g. species showing fewer than five digits on the limb; Mehnert, 1897).

For these reasons, it may be simplest to begin our discussion of chondrification sequences in limbs that have five equal or subequal digits, arranged symmetrically with respect to the middle digit. As an example, we consider here the limb chondrification sequence in the European pond terrapin *Emys orbicularis* (Figs. 4–6). The hand of this turtle has five subequal digits with a phalangeal formula of [2-3-3-3-2], whereas the foot has a partially reduced digit V and a phalangeal formula of [2-3-3-3-2]. To analyze the spatial component of the developmental sequence, the limb skeleton can be divided into anatomical subregions along the proximodistal axis (see Fig. 6E). Where these subregions contain multiple elements, they are typically arranged in a transverse row along the anteroposterior axis.

Using this spatial matrix, one can see that the initiation of chondrogenesis in the different regions follows a proximodistal sequence. Thus, the stylopod region (humerus) is the first to show chondrification, followed by the zeugopod, and so on until the last region to show onset of chondrogenesis is that containing the distal phalanges (Figs. 4 and 6). This proximodistal sequence is violated by the centralia, which develop relatively late, and therefore ''out of sequence,'' both in the

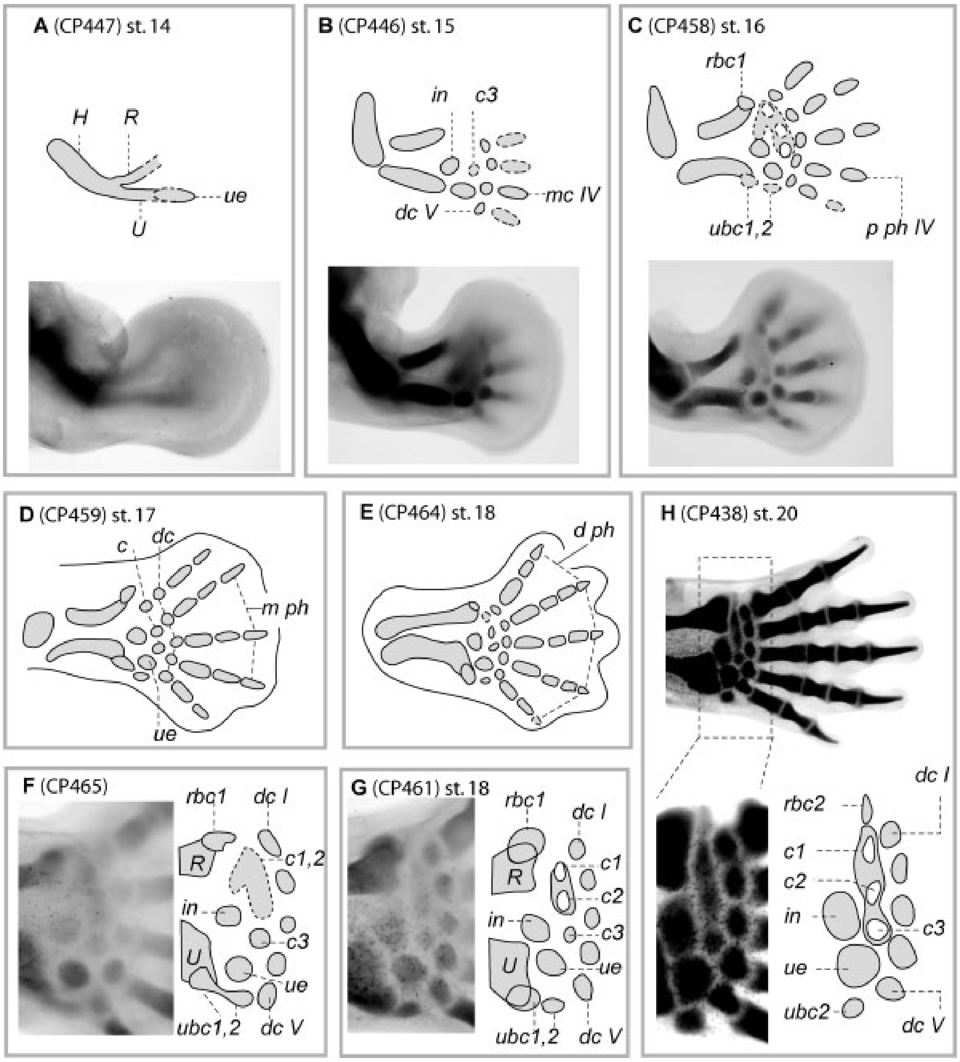

Fig. 4. Chondrification patterns in the distal forelimb of *Emys orbicularis* (not to scale). ''CP'' numbers are the embryo code numbers in Appendix C, where the full data set can be found (with the exception of CP465, which is illustrated here, but not included in the data table because the hind limbs were lost). The schematic figures represent our subjective scoring of chondrogenesis as follows: gray shape outlined with a broken line = faint condensation (scored ''1'' in Supplmentary Table 2); gray shape with continuous outline = distinct condensation (scored ''2'' in Supplmentary Table 2). Abbreviations: *c*, centrale; *dc*, distal carpal; *d ph*, distal phalanx; *H*, humerus; *in*, intermedium; *mc*, metacarpal; *m ph*, middle phalanx; *p ph*, proximal phalanx; *R*, radius; *rbc 1*, *2*, proximal and distal radial border cartilages, respectively; st., Yntema ('68) stage; *U*, ulna; *ubc1*, *2*, proximal and distal ulnar border cartilages, respectively; *ue*, ulnare. The roman numerals refer to the digits.

hand (Fig. 6E) and foot. Within a single region, such as the metacarpals, it can be seen that the component elements do not chondrify synchronously, but according to two sequences, with opposite polarity, along the anteroposterior axis. The first runs from metacarpal IV to metacarpal I; the second from metacarpal IV to metacarpal V. Similar sequences are seen in the hind limb (Fig. 5). In both cases, new cartilage foci tend to form in regions of condensed mesenchyme that are connected to older foci (Shubin, '95, p 59).

In summary, *E. orbicularis* shows a complex spatiotemporal pattern of chondrification that is expressed in at least three superimposed sequences: (i) a proximal-to-distal sequence of initiation of chondrification in contiguous proximodistal regions; (ii) a posterior-to-anterior sequence within each proximodistal region; and (iii) an anterior-to-posterior sequence in that same region. The proximodistal sequence does not apply to individual elements, but to the initiation of chondrogenesis in adjacent regions along the proximodistal

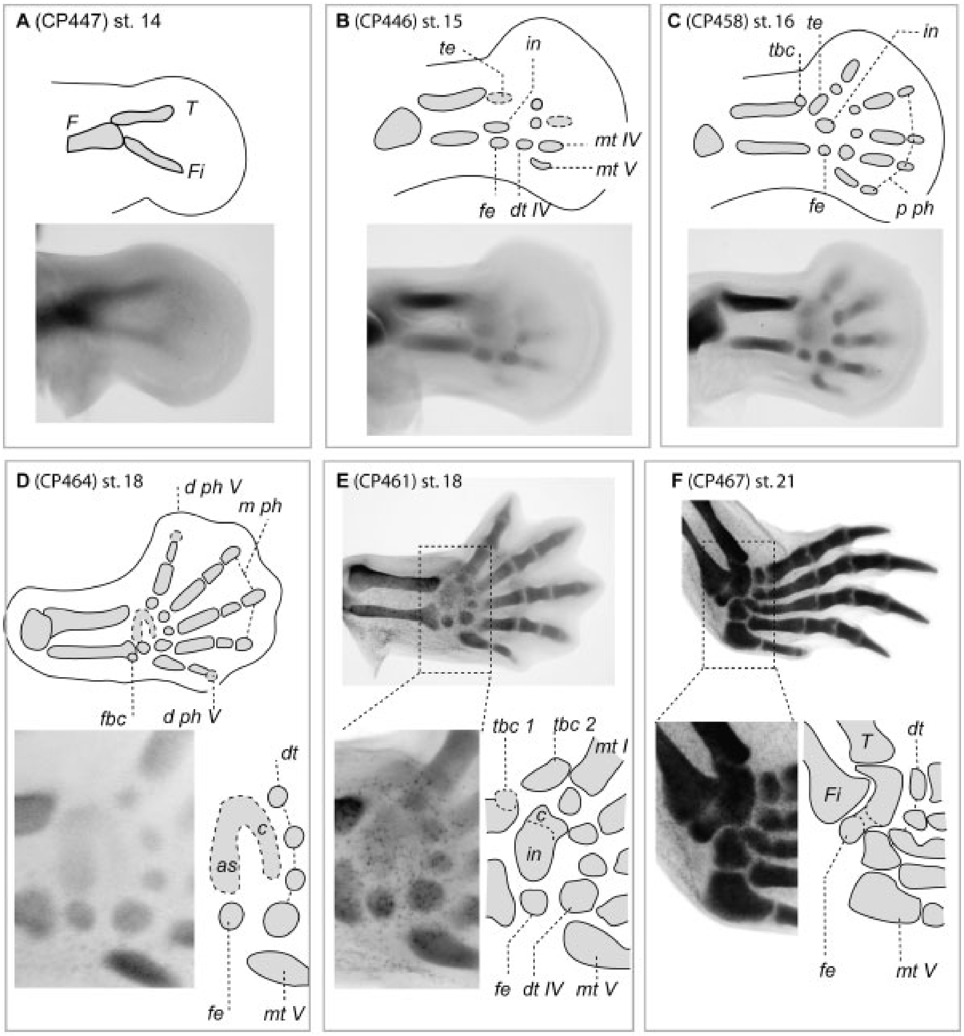

Fig. 5. Chondrification patterns in the distal hind limb of *Emys orbicularis*. Not to scale. For original specimens and data see Appendix C. Numbers refer to Yntema ('68) stages and to the embryo code number in Appendix C. Abbreviations: as, astragalus (intermedium/tibiale); c, centrale; d ph, distal phalanx; dt, distal tarsal; *F*, fibula; *fbc*, fibular border cartilage (pisiform); *fe*, fibulare; *in*, intermedium; *m ph*, middle phalanx; *mt*, metatarsal; *p ph*, proximal phalanx; st., Yntema ('68) stage; *T*, tibia; *tbc1*, *2*, proximal and distal tibia border cartilage, respectively; *te*, tibiale. The roman numerals refer to the digits.

axis. Thus, some elements at a given proximodistal level may still be un-chondrified whereas chondrification has begun at the next level.

One emergent property of the interaction between these sequences is that, at certain stages (Figs. 4B, 5C), two prominent but transient series of chondrified elements are seen in Alcian blue wholemounts (Shubin and Alberch, '86). One, series, the *digital arch* or *carpal arch*, runs transversely, and is composed of the distal carpal or distal tarsal elements (Holmgren, '33). The other ''column of precociously developed elements'' (Shubin and Alberch, '86, p 348) runs from the base to the tip of the limb. It is called the *primary axis* (Burke and Alberch, '85).

Variation in anteroposterior and proximodistal timing gradients

The axial timing gradients described above vary between vertebrate taxa as we shall now discuss.

The primary axis

We summarize the developmental sequence of selected elements along the primary axis in different groups as follows (after Shubin and Alberch, '86 and data here in Figs. 4 and 6 and Appendix A): urodeles: [basal commune, digits (II, I), III, IV] in the forelimb; and [basal commune, digits (II, I), III, IV, V] in the hind limb. Anurans: [ulnare, distal carpal IV, digits III, (IV, II), I] in

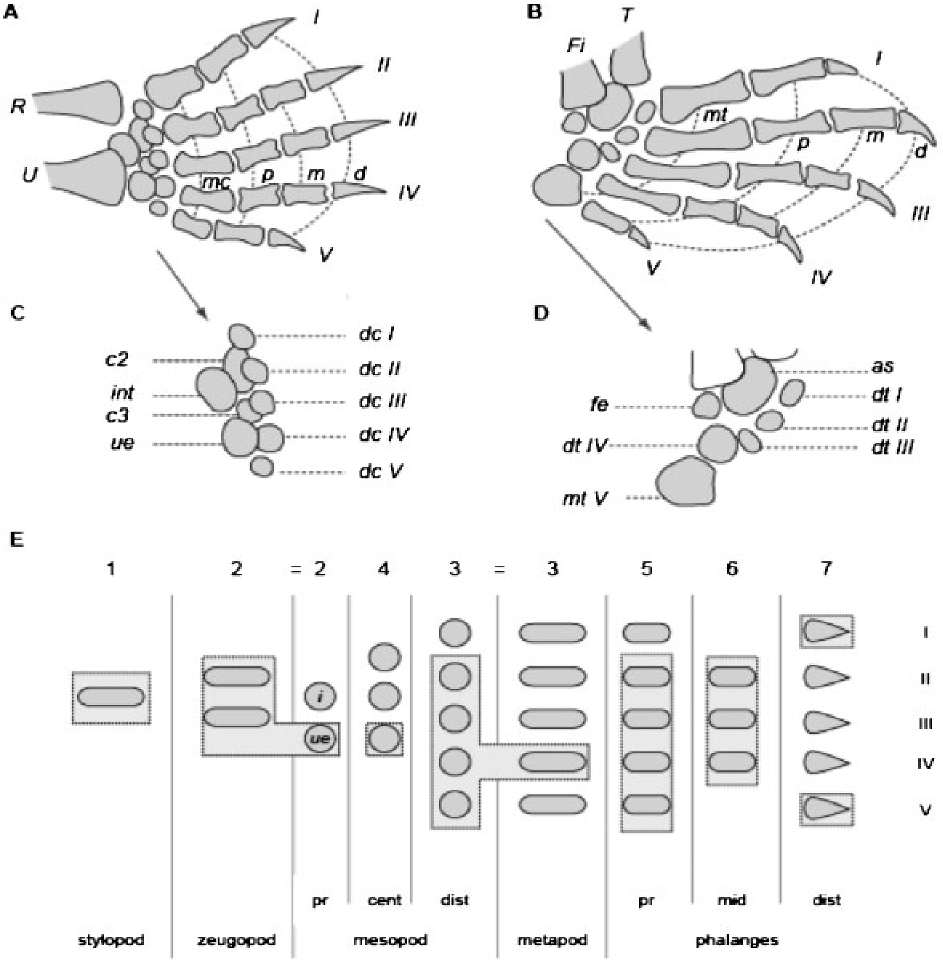

Fig. 6. Schematic diagram based on X-ray of skeletal autopodials of distal forelimb (**A**) and hind limb (**B**) in *Emys orbicularis*. (**C**, **D**) Detail of mesopodial elements from (A) and (B), respectively. Not to scale. For original specimens and data see Supplementary Tables 2 and 3. No pisiform was seen on the X-ray, although a pisiform is reported in this species (Rabl, '10). It is possible, therefore, that a pisiform was present but radiolucent, although we did not determine this. Abbreviations: a, astragalus; c, central; ca, calcaneum; d, distal carpal or distal tarsal; *F*, fibula, *Vhm*: hooked fifth metatarsal, *i*, intermedium; *R*, radius; *T*, tibia; *U*, ulna; *ue*, ulnare. (**E**) Schematic diagram showing proximodistal anatomical regions mapped onto the principal forelimb chondrogenic elements (dark gray) of *Emys orbicularis*. The numbers 1–7, top, indicate the sequence of regions in which the first signs of chondrogenesis appear. (=), synchrony. Roman numerals indicate digit number. Pale gray boxes indicate elements that develop synchronously in a given embryo. Note that the centralia are relatively delayed in appearance, and so develop out of sequence with other elements on the proximodistal axis. Metacarpal IV is relatively accelerated.

the forelimb; and [fibulare, distal tarsal IV, digits IV, V, III, II, I] in the hind limb. Amniotes: [ulnare/fibulare, distal carpal/distal tarsal IV, digits IV, (V, III), II, I].

Note that in urodeles, the [basal commune+ digit I+II] complex is accelerated relative to the other digits, whereas in anurans and amniotes, distal mesopodial IV and digit IV (or digit III in the forelimb of some anurans) are precocious (Goette, 1879; Strasser, 1879; Baur, 1885, 1888; Sewertzoff, '04, '08; Holmgren, '33; Blanco and Alberch, '92; Hinchliffe and Vorobyeva, '99). *Triturus marmoratus* is interesting in showing (i) a distal-to-proximal time sequence extending from the basale commune to the centrale and then the intermedium (the ''central axis''); (ii) a primary axis involving digits I and II; and (iii) an anuran-amniote type primary axis involving the ulnare/fibulare and digit IV (Blanco and Alberch, '92).

In neobatrachian frogs, digit V is part of the primary axis and distal carpal V develops earlier than distal carpal IV (Fabrezi and Barg, 2001). Fröbisch et al. (2007) hypothesized that branchiosaurids, a group of temnospondyle ''amphibians,'' had a pattern of early limb chondrogenesis similar to that of urodeles.

In the chicken, the blastemas of the early wing skeleton are indistinct, and merge into one another (Holmgren, '33); the digital arch forms, but fails to segment in the leg, and is poorly defined in the wing (Hinchliffe, 2002). The carpal arch may not be formed from the distal carpals alone in birds; the radius and ulna appear to expand their distal ends into a common tissue plate, in which the radiale, pisiform, ulnare, and intermedium differentiate, followed later by the three distal carpals (Prein, '14).

Sheil (2008) and Sánchez-Villagra et al. (2008b) pointed out some variations in the sequence of chondrification in the middle and distal row of carpal and tarsal Anlagen in hidden-necked (cryptodire) turtles. According to some reports, the distal carpal (tarsal) III is one of the products of branching of distal carpal IV (the other product being metacarpal IV, part of the primary axis). This implies an "origin" of the digital arch via the distal carpal IV (e.g. Shubin and Alberch, '86, p 346 for the snapping turtle *Chelydra serpentina* and for *Crocodylus*). By contrast, the digital arch in other cases arises from centrale IV, as reported for example by Shubin and Alberch ('86) also for *C. serpentina*.

Another variable is in the commonly seen pattern in which distal carpal (tarsal) V precedes the appearance of metapodial V. In some cases, there are Anlagen of both a distal carpal V and a metacarpal V, but the hind limb usually exhibits a single Anlage there, identified as metatarsal V, with no distal tarsal V (Burke and Alberch, '85). Shubin and Alberch ('86) reported that digit V "forms independently of the digital arch by a de novo condensation" (see also Wagner et al., 2000, p 825). Rabl ('10) reported a [ulna-ulnare-IV] primary axis in turtles, crocodilians, and lacertids, although he found that this axis then shifts so that digit III develops its second phalanx earlier than does digit IV. Fossil evidence suggests that basal ichthyosaurs often retain only an ossified ulnare, intermedium, and distal carpal IV of the mesopodium, and therefore show a primary axis (Motani and You, '98).

The anteroposterior axis in amniote limbs shows an example of deviation from the predictions of a simple anteroposterior timing gradient. Thus, the first elements to form in the digital plate are those of digit IV, and not those of the most posterior digit (i.e. digit V). There exist in effect two anteroposterior timing gradients, sharing a common boundary at digit IV but having opposite polarity.

Delay of wrist and ankle development

Mehnert (1897, p 47) found that turtle and ostrich limbs show a strict proximodistal sequence of elements, whereas in mammals, urodeles, anurans, and carinate birds, the wrist and ankle (mesopodial) elements are delayed and therefore develop out of sequence (see also Sewertzoff, '04). Thus, in the *E. carpus*, he found that the intermedium develops first, then the ulnare and radiale, and then, synchronously, all five distal carpals.[1] In the ostrich foot, which is remarkable among living birds for having only two adult toes, he found that the skeletal elements appear as cell condensations in strict proximodistal sequence1. The tibia and fibula condense synchronously, followed by the tibiale and fibulare.

Delayed mesopodial development, relative to digit development, has been confirmed for *Xenopus laevis* (Alberch and Gale, '83) and several *Triturus* species (Blanco and Alberch, '92). Basal ichthyosaurs had delayed mesopodial ossification, as do many other diapsids, but not advanced ichthyosaurs, which seem to have lost this trait (Motani and You, '98). In the lizard *C. versicolor*, distal tarsal I appears long after the phalanges, and is therefore delayed with respect to its position along the proxiomodistal axis (Mathur and Goel, '76).

Forelimb versus hind limb heterochronies

An analysis by Schlosser (2001) suggested that the primitive tetrapod condition is for the forelimbs to develop early (for example, around the time of pharyngeal cartilage differentiation) and for the hind limbs to develop slightly later. Development of the limbs is retarded[2] in indirect developing frogs, relative to the primitive tetrapod pattern, but accelerated in the direct developing frog *Eleutherodactylus coqui*. Amniotes may also show some acceleration of limb development relative to the presumed ancestral tetrapod condition (Schlosser, 2001).

The stage at which the forelimb buds first develop, relative to the hind limb buds, varies between taxa (we provide a review of the literature

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[1] The only exceptions were those carpal and tarsal elements that are variable, fused, or poorly developed in adults (e.g. the radiale externum, centrals, etc. in *Emys*.). These "torpid" elements develop late, and therefore out of sequence, with respect to the proximodistal axis (Mehnert, 1897).

[2] In this specific context, we use the terms "retardation" and "acceleration" simply to describe the pair wise relationship in the timing of development of forelimb and hind limb, respectively. We are not necessarily making a statement about the polarity of the change in phylogeny.

in Appendix B). In many mammals, urodeles, turtles and tortoises, some squamates, and *Sphenodon punctatus*, the forelimb buds develop earlier than the hind limb buds. In some Ranidae, a few eutherian mammals and the squamate *Lacerta viridis*, forelimb and hind limb buds develop synchronously. In *X. laevis*, the tree frog *E. coqui*, and *Alligator mississippiensis*, the hind limb buds develop earlier than the forelimb buds.

In the literature that we surveyed (Appendix B) there was at least one bird species in each of the three heterochronic categories (i.e. "forelimb acceleration," "hind limb acceleration," and "synchrony"). According to Holmgren ('55), the hind limb in birds (both ratites and carinates) generally develops earlier than the forelimb. But Sieglbauer ('11) claimed that the avian wing and hind limb rudiments develop synchronously at earlier stages, but that the hind limb later becomes accelerated at stages when the digits are appearing. For further details of the complex situation regarding birds, see Appendix B.

In all cartilaginous and bony fishes in our literature survey (Appendix A), the pectoral fin buds are reported to make their first appearance at an earlier stage than the pelvic fin buds. In teleosts, the pelvic fin develops much later than the pectoral fin. In the zebrafish *Danio rerio*, for example, the pelvic fin buds only begin to develop at metamorphosis, and only then do they start to express the pelvic-fin marker *Tbx4*. The pectoral fins develop much earlier and also show appropriately early expression of the pectoral-fin marker *Tbx5* (Tamura et al., '99).

The identification of patterns of forelimb–hind limb heterochrony may be critically sensitive to the characters studied. Thus, Mehnert (1897) found that, in terms of size and shape until the digital plate stage, the forelimbs and hind limbs of *E. orbicularis* develop synchronously; but in terms of chondrogenesis after the digital plate stage, the forelimbs were accelerated. Further, in *Caiman latirostris*, the forelimb bud appears as a mesenchymal condensation earlier (stage 5L) than does the hind limb (stage 6L); but then the hind limb develops its apical ectodermal ridge (AER) at an earlier stage (stage 8L) than does the forelimb (stage 9L); later still, the nail anlagen appear earlier on the hind limb than the forelimb (Iungman et al., 2008). The authors concluded that the forelimb buds are initiated earlier than the hind limbs, but the hind limbs then grow and differentiate more rapidly than the forelimbs (Iungman et al., 2008). These and other observations suggest that different aspects of limb development may show different forelimb–hind limb timing relations.

In an effort to address these problems, and quantify forelimb–hind limb pattern heterochronies, Bininda-Emonds et al. (2007) studied the relative sequence of ten events in the early development of the forelimbs versus the hind limbs in 14 tetrapod species spanning a diverse taxonomic, ecomorphological, and life-history range. Wholemounts and histological sections of 138 embryos were used to code events ranging from the early limb-bud stage to late chondrogenesis. Statistical analysis was used to yield an *E* value, a quantitative measure of the degree of timing difference between the forelimb and hind limb (Fig. 3). The analysis revealed a phylogenetic pattern of change across tetrapods. In the plesiomorphic condition, as seen in Chondrichthyes and Osteichthyes, the pectoral appendage develops earlier than the pelvic. This pattern is either retained or re-evolved in some placental mammals (e.g. shrews, moles) and in marsupials to a more extreme degree, as indicated by *E* value (Fig. 3). No major heterochronic changes in early limb development and chondrogenesis were found within major clades except Lissamphibia. Two anurans examined displayed a significant advance in hind limb development relative to the forelimb (Bininda-Emonds et al., 2007). However, contrary examples, as well as much variation, are recorded within Urodela (see Appendix A). It is only in amniotes that constrained (i.e. clade-specific) patterns were seen.

DEVELOPMENTAL MECHANISMS AND HETEROCHRONY IN LIMB EVOLUTION

In this section, we consider whether changes in developmental mechanism may be necessary and sufficient to account for at least some examples of pattern heterochrony. As noted above, changes in developmental pathways that lead to pattern heterochrony may themselves be heterochronic or nonheterochronic.

Heterochronic change in developmental mechanisms as a cause of pattern heterochrony

Proximodistal axis

Patterning mechanisms based on timing represent an obvious vehicle for heterochronic change. The progress zone is a timing-based model of pattern formation along the proximodistal axis of the limb (Summerbell et al., '73), although the validity of the model has been debated (Tabin and Wolpert, 2007). Cells are suggested to read their proximodistal position by counting the number of cell divisions that they undergo in the progress zone mesenchyme underlying the AER. In principle, a change in the duration of AER activity, and consequent limb outgrowth, may produce an altered time sequence of skeletal element formation, and also a change in adult morphology (with fewer or more elements formed along the proximodistal axis). For convenience, we here consider proximodistal patterning in isolation, although it should be noted that it is linked mechanistically to anteroposterior patterning through reciprocal interactions between the AER and zone of polarizing activity (ZPA or polarizing region; see Laufer et al., '94; Niswander et al., '94).

Some limb defects in mouse mutants may be owing to changes in the length of time over which the AER is active (Johnson '86). There is also some evidence that a prolonged period of limb outgrowth may be associated with hyperphalangy in dolphins; the opposite case—premature termination of limb outgrowth—may explain reduction of the hind limb in these species (Richardson and Oelschläger, 2002). Another example of a possible truncation in outgrowth is provided by the lizard genus *Bachia*; adults show a loss of skeletal elements in distal-to-proximal sequence, and the morphology of the adult resembles embryonic stages of other squamates (Presch, '75).

Models based on truncation of proximodistal outgrowth may be applied to the extinct flightless bird *Apterornis*, in which the manus is truncated distally and resembles an embryonic stage of the chicken wing (Livezey, '94). The plethodontid salamander *Bolitoglossa occidentalis* exhibits small body size, webbed feet, and loss of phalangeal elements. These features may be interpreted as paedomorphosis, or truncation at a small body size (Alberch and Alberch, '81). Furthermore, Sanz-Ezquerro and Tickle (2003) found that implantation of sonic hedgehog beads into the chick interdigital mesenchyme led to hyperphalangy, possibly by prolonging the time of *fgf8* expression in the AER over the digit. In the proximodistally truncated hind limb of the python, Cohn and Tickle ('99) found no AER in the hind limb bud or expression of AER markers; and though the mesoderm was competent to form a ZPA, it lacks signals from the ridge needed to do so.

Further evidence that morphological changes can be induced by changes in AER function come from studies of murine *Msx1*$^{-/-}$; *Msx2*$^{-/-}$ homozygous double mutants (Lallemand et al., 2005). These show delayed and incomplete regression of the AER, leading to persistent interdigital webs, overgrowth of the preaxial mesenchyme, delayed phalangeal development, and other morphological changes.

However, not all cases of morphological change along the proximodistal axis are obviously related to the duration of limb outgrowth. Alberch and Gale ('83) studied experimental digit loss after colchicine treatment in *Xenopus*. They found no evidence that digit loss involves proximodistal truncation, because the loss of a toe was not associated with loss of terminal phalanges. This lack of distal truncation was also noted in the phylogenetic series they examined.

In some skinks too, limb reduction may not always involve a simple truncation of proximodistal outgrowth. Shapiro (2002) studied *Hemiergis* populations and found that, in the species with reduced numbers of digits, the remaining digits were complete, not truncated. There was no delay in ossification either in the reduced manus or pes, and the latter did not correspond to the embryonic condition of condensations in any known reptile. However, digits IV and V in the 5/5 *Hemiergis* phenotypes did have one phalanx fewer than the primitive reptilian pattern, indicating an incipient truncation.

As noted in the section ''Morphological Patterns of Heterochrony in Limb Evolution and Development'', the wrist and ankle bones are delayed with respect to their position on the proximodistal axis in several taxa. It is therefore interesting that the presumptive wrist mesenchyme cells in the chick limb bud proliferate very slowly, relative to cells that give rise to other skeletal elements. This was shown by tritiated thymidine labelling (Lewis, '77), and could be an example of a rate heterochrony that affects patterning.

Transcriptional heterochrony

Transcriptional heterochrony (change in the timing of initiation or silencing of gene expression) is a potential mechanism of body plan evolution, even outside the context of the tetrapod limb. Thus, in three closely related *Drosophila* species, temporal differences in expression of the gene *hairy* were present, even at the earliest stages of development examined (Kim et al., 2000). This led the authors to suggest that there might be a continuous phylogenetic evolution of timing trajectories during microevolution (Kim et al., 2000).

There are examples of transcriptional heterochrony in Hox gene expression that may be associated with changes in morphology of the axial skeleton (Gerard et al., '97; Belting et al., '98). These and other findings have led to the suggestion that temporospatial differences in gene expression may underlie diversification of limb morphology (Mackem and Mahon, '91; Dollé et al., '93; Belting et al., '98).

The onset of *hodx12* expression in the chick wing is delayed relative to its expression in the hind limb, and persists to a later stage (Mackem and Mahon, '91), although it is not clear whether these differences are related to phenotypic differences between the developed limbs. Interestingly, Blanco et al. ('98) found evidence of a heterochronic prolongation in *hoxa11* expression in the *X. laevis* hind limb that might account for differences in carpal and tarsal morphology.

In the Australian skink genus *Hemiergis*, there are species or populations that differ with respect to the number of digits on their limbs. In a comparative study of the genus, it was found that species with fewer digits also showed precocious reduction in sonic hedgehog (SHH) protein levels in the ZPA (Shapiro et al., 2003). Yang et al. ('97) produced an experimental process heterochrony in the chick limb by changing the duration of exposure to SHH; more prolonged exposure ''promoted'' digits to a more posterior identity and in the mouse limb, cells contributing to digit III are exposed to shh for less time than those that form digit V (Harfe et al., 2004). Ahn and Joyner (2004) found that cells responsive to shh signalling from the ZPA in the mouse limb remain in the posterior part of the limb; these could be potential primary axis cells.

The rudimentary hind limb of dolphins develops from a limb bud, which initially has an AER. However, the AER soon loses its columnar shape and disappears at an earlier stage than that of tetrapods with complete hind limbs (Thewissen et al., 2006). FGF8 protein is initially expressed in both the hind limb and forelimb AER of dolphins, but then disappears from the hind limb. The shh protein is expressed in the forelimb ZPA but not in the hind limb, as is *Hand2*, an upstream regulator of *shh*. The authors conclude that the gradual reduction of the hind limb seen in the fossil record of cetaceans is consistent with a progressive temporal delay in *shh* expression (Thewissen et al., 2006).

One situation in which timing shifts in development could lead to morphological change is when pattern formation is limited to a narrow time window. In such cases, a delay in one event could lead to the responsive window being missed. A possible example comes from studies on stickleback populations lacking the pelvic spine (Cole et al., 2003). The authors of that study suggested that the pelvic spines fail to develop because a *Pitx1* expression domain is absent, and the pelvic fin develops too late to gain compensatory *Pitx2* expression. Finally, it should be noted that changes in the timing and level of expression of members of the Bmp, Hox, and Fgf gene families are correlated with evolutionary changes in bat limb morphology (Sears et al., 2006; Sears, 2008).

Timing of cell death

Cell death is seen in the necrotic zones of the chick wing (Hinchliffe, '76). In the *wingless* chicken mutants, the forelimb bud develops transiently, but then regresses. In these mutants, cell death in the anterior necrotic zone of the limb is precociously activated, compared with wild type chickens, and the zone spreads further across the limb bud (Hinchliffe, '76). In chameleon limb development, interdigital cell death is precocious in the cleft region of the prehensile autopodium (Hurle, '87).

Nonheterochronic changes in developmental mechanisms that lead to pattern heterochrony

There are cases where the changes in developmental mechanisms, which lead to pattern heterochrony, are not themselves heterochronic. These include neoteny in urodeles owing to failure of hormone release or reduced tissue sensitivity to hormones (reviewed by Raff and Wray, '89; Cubo, 2000); changes in the spatial domains of gene expression; and loss of gene function, without obvious transcriptional heterochrony. As an example of the latter case, in mutant mice lacking *Gli3* and one copy of *Alx4*, the formation of the radius primordium is delayed (Panman et al., 2005). Furthermore, nonheterochronic disruption of *hoxd13* function can lead to heterochronic change (delay) in limb ossification (Dollé et al., '93).

Another example of nonheterochronic change in developmental mechanisms leading to heterochrony comes from a study of growth rates in the heron hind limb skeleton (Cubo et al., 2000). It was found that heterochrony was related not to differences in mitotic rates but to the number of cells in the growth plate.

NATURAL SELECTION AND HETEROCHRONY

It may be tempting to ascribe adaptive significance to limb heterochrony. In principle, though, factors other than adaptation to particular habitat variables may also be important in explaining the occurrence of limb heterochrony. In this section, we consider whether limb heterochrony is indeed open to natural selection for traits such as locomotor function. This topic is of current interest in fields such as ecological genomics, which are concerned with identifying causal relationships between environment and allelic variation (Fitzpatrick and Shaffer, 2004).

We shall examine the following outcomes of heterochrony: (i) functional changes in adult morphology; (ii) functional changes in the embryonic or larval limb (with or without changes in adult morphology); (iii) changes that are not related to selection for adaptive traits of the limb. The first two categories of change can be analyzed in a functionalist framework, the third includes examples of constraints (Alberch, '90; Richardson and Chipman, 2003). Heterochronic changes in limb development might also produce changes in other systems leading to pleiotropic effects (Fig. 1). A possible example of functional pleiotropy is provided by the HoxD cluster genes, which are important in patterning not only the limb, but also the primary body axis and genitalia (Dollé et al., '91).

In some cases, heterochrony bears little obvious relation to either selection or constraints, appearing, in this sense, to be random. Chipman et al. (2000) studied heterochrony during organogenesis in 12 anuran species, and found that the timing shifts were not correlated either to phylogeny or to various adaptive scenarios. Even closely related species showed some wide differences in timing.

Heterochrony and functional changes in adult morphology

Rabl ('10) noted that digit IV of reptiles was the first to develop. He attributed this precocity to the functional importance of the postaxial part of the limb for standing in water. He saw the same postaxial heterochrony in the bird wing and foot and remarked that flying is simply "swimming in the air." Iungman et al. (2008) found that, although the forelimb bud in the *Caiman* is initiated earlier than the hind limb, the latter soon outpaces the forelimb in its growth and differentiation. The authors Iungman et al. (2008) attributed this heterochrony to a greater functional importance of the hind limb than the forelimb in crocodilians, and pointed to evidence (Colbert and Mook, '51) that ancestral crocodilians had massively developed hind limbs.

Mehnert (1897, p 99) argued that most heterochronies in limb development (including forelimb–hind limb differences and chondrogenic sequence changes) could be linked to changes in mechanical function of the adult limb. He found that bones with major weight-bearing functions in the adult tended to differentiate earlier, and grow faster than other bones. By contrast, bones that showed a phylogenetic trend to functional reduction, such as the marginal digits of the archosaur wing, were delayed in their development.

In the *Emys* forelimb, for example, he claimed that the five subequal digits developed from digital blastemas that develop almost synchronously. Regressive elements, he stated, develop out of sequence, because they differentiate more slowly, may fuse with neighboring elements, or remain cartilaginous in the adult (e.g. the radiale externum) or attain a smaller adult size (e.g. digit V in the *Emys* hind limb).

Mehnert's ideas are interesting as a functionalist explanation of sequence heterochrony, and deserve re-examination. However, it should be noted that our findings (Figs. 4–6) and those of other authors (Burke and Alberch, '85; Sheil, 2003, 2005; Crumly and Sánchez-Villagra, 2004; Sheil and Greenbaum, 2005; Sánchez-Villagra et al., 2007, 2008b), are not entirely consistent with Mehnert's view. Thus, most evidence indicates that the digits in *Emys*, contrary to Mehnert, do not develop synchronously; instead, metapodial IV is accelerated with respect to the others.

A clear example of adaptive evolution in the limb is the greatly enlarged and specialized forelimb of the bat (Adams, 2008). Adapted for flying, the bat forelimb is larger than the hind limb of the same individual, and larger than the forelimb of other eutherian mammals such as the mouse. It, therefore, provides a useful model for testing functionalist approaches to heterochrony. Thus, one might predict that selection for large adult size should result in sequence heterochronies, such as precocious appearance or chondrification of the bat forelimb. This prediction does not appear to hold true, however.

Sears et al. (2006) reported that in the microchiropteran *Carollia perspicillata* and the mouse *Mus musculus* the digits are initially similar in development, and that subsequently those of the bat forelimb lengthen. The developmental elongation of the bat wing digits is achieved relatively late in development via an accelerated rate of proliferation and differentiation of cartilage cells (see also Farnum et al., 2008a,b, for data on another microbat, *Eptesicus fuscus*). The study by Sears et al. (2006) emphasized the similarity in early development of limb structures in the bat and mouse, followed by their later divergence.

Our own studies on limb development in another chiropteran, the fruit bat *Rousettus amplexicaudatus* (Fig. 7), confirms the pattern of early limb growth described by Sears et al. (2006) for a microchiropteran. The fruit bat, with its greatly enlarged forelimbs modified as wings in the adult, shows near-synchrony in the development of the forelimb and hind limb (Giannini et al., 2006; Bininda-Emonds et al., 2007). Figure 4 illustrates that the mouse and the fruit bat have a similar forelimb and hind limb size differential in the earliest limb buds. Perhaps surprisingly, the *E* value (Fig. 3) shows that the *Rousettus* forelimb is actually *less* accelerated than the hind limb, compared with other eutherians. In the fruit bat, faster size increase of forelimb in comparison with hind limb was recorded: differential growth rate is associated with the development of the limb differential.

Although no morphometric data were provided, the figures published by Adams ('92) for *Myotis lucifugus* and Tokita (2006) for *Pipistrellus abramus*, suggest a similar pattern of growth for the forelimb–hind limb differential in these bats to that reported here for *R. amplexicaudatus*. In summary, the dramatic evolutionary changes of the bat forelimb appear to have been achieved mainly through differential growth, and not sequence heterochrony.

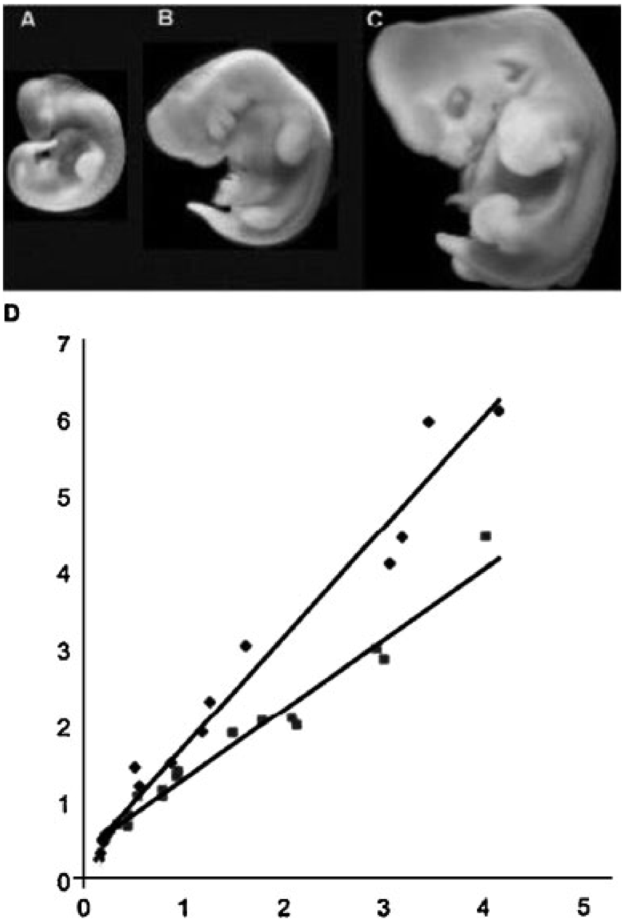

Fig. 7. Embryonic series of the fruit bat *Rousettus amplexicaudatus*, showing the relative development of forelimb and hind limb. Approximately to scale. (**A**) CR (crown-rump length) = 3.76 mm; (**B**) CR approx. 5.3 mm; (**C**) CR approx. 8.2 mm. (**D**) Relationship between forelimb and hind limb size in the mouse *Mus musculus* and the fruit bat *Rousettus amplexicaudatus*. Although the forelimb appears larger in the early bat embryo (A), statistical analysis (Bininda-Emonds et al., 2007) shows that it is not accelerated relative to that of the mouse. Our data in (D) show that the size differential appears at later stages, owing to differential growth in the bat forelimb. See Appendix D for statistics of regression analyses and data.

Heterochrony and adaptive changes in larval limb morphology

Pattern heterochronies appearing in embryonic life do not necessarily affect adult morphology. They may, however, be open to selection for their functional effects on early life stages. Such adaptations come under the heading of caenogenesis (Blanco and Alberch, '92). Keibel (1897) suggested that heterochrony in embryonic stages is driven by functional demands in early life, and therefore reflects the differing times at which organs becomes functional in ontogeny. However, Keibel's hypothesis is not easy to reconcile with the timing of limb development in amniotes. Although the limbs develop relatively early in amniotes, they are not functional until much later because the embryo develops for a long time within the egg, womb, or pouch.

Holmgren ('39, p 32) invoked adaptive explanations for the fact that, in urodeles, the forelimb develops much earlier than the hind limb, and in many anurans, the pattern is reversed. He suggested that the precocity of the anuran hind limb might reflect its locomotory function. In the metamorphosing larva the hind limb is used in swimming, and in the adult it is used in saltatory locomotion (jumping; see Handrigan and Wassersug, 2007). Shubin and Alberch ('86, p 373) supported this view.

Among urodeles, larvae of *Desmognathus ocoee* are partially terrestrial and show more rapid relative development of their functional hind limbs compared with the pond-dwelling larvae of *Hemidactylium scutatum* and *Ambystoma jeffersonianum* (Babcock and Blais, 2001) in which hind limb development is strongly retarded relative to forelimb development. In *A. macrodactylum krausei*, the forelimb already has two digit Anlagen at a stage when the hind limb has only just appeared as a bud; the hind limb only ''catches-up,'' to become fully developed, at metamorphosis (Watson and Russell, 2000). This pattern is similar in other ambystomatid species (Watson and Russell, 2000). In *Notophthalmus viridescens*, the hind limb bud does not appear until the stage at which the forelimb has three or four digits with chondrified elements (Wong and Liversage, 2005). According to Watson and Russell (2000), direct developers, such as desmognathines as well as direct-developing plethodontids, show almost simultaneous appearance of forelimb and hind limb.

These patterns of heterochrony are thought to be strongly affected by larval habitat variables, and may have no effect on adult limb morphology (Blanco and Alberch, '92; Shubin, '95, p 62–63). To summarize the relationship between habitat and forelimb–hind limb heterochrony in urodele larvae: pond-dwellers (e.g. *A. mexicanum* or *T. dobrogicus*), show great acceleration of forelimb development relative to hind limb development (Fig. 2); stream-dwellers (e.g. *D. quadramaculatus* and *Dicamptodon tenebrosus*) and direct-developers (e.g. *D. aeneus*), show a smaller degree of forelimb acceleration (Wake and Shubin, '98; Franssen et al., 2005).

The precocious development of preaxial digits in the urodele limb—in contrast to the digit IV primary axis in some anurans and amniotes—is sometimes seen as adaptive (Blanco and Alberch, '92; Hinchliffe and Vorobyeva, '99). Thus, larvae of some urodele species could use their preaxial forelimb digits for gripping the substratum in flowing water (Hinchliffe and Vorobyeva, '99). Holmgren ('33) however rejected such explanations on the grounds that, though urodeles share a preaxial primary axis, they show different larval environments.

In vertebrates with free-living larvae, trunk muscle fibers may develop earlier than they do in amniote embryos. Thus in *Xenopus* and *Danio*, transcripts of muscle differentiation genes accumulate in the presomitic mesoderm, whereas in the mouse, these transcripts do not appear until after the somites have formed (Coutelle et al., 2001). Although this is not a limb skeletal heterochrony, it may be an illustration of the effect of natural selection on the timing of development of the locomotor apparatus. However, the polarity of this change needs to be established before firm conclusions can be drawn.

In marsupials (Figs. 2 and 3), as in urodeles, the forelimb bud appears at a much earlier stage than the hind limb (Appendix B). Weisbecker et al. (2008) recently used Parsimov (Jeffery et al., 2005) to examine ossification heterochronies between marsupials and eutherian mammals. They concluded that the timing differential in limb development in marsupials is related to hind limb retardation and not forelimb acceleration, as had often been assumed (see also Harrison and Larsson, 2008). They hypothesized that ''the differing ossification patterns in marsupials can be explained with a combination of muscular strain and energy allocation constraints, both resulting from the requirement of active movement of the altricial marsupial neonates towards the teat.'' (Weisbecker et al., 2008, p 1).

Heterochrony and constraints

Shubin and Alberch ('86) proposed a model of limb skeletal patterning in which the spatial and temporal pattern of chondrogenesis was influenced more by developmental constraints than by selection for adult limb functional traits. They noted that though skeletal condensations could form de novo, they more often showed *connectivity*, whereby adjacent elements forming in sequence were linked by a band of tissue possessing high cell density. They suggested that local changes in cell–matrix interactions might trigger the formation of the next element in the connected series. An attractive feature of this model, and others that invoke developmental constraints to account for timing relations in limb development, is that it can explain the persistence in phylogeny of features such as the primary axis.

CONCLUSIONS AND FUTURE PROSPECTS

We have seen that the literature on tetrapod limb evolution and development provides an impressive catalog of timing shifts. The remarkable differential of marsupial and newt forelimb relative to hind limb development is just one striking example (Fig. 2). We have also described cases where changes to developmental mechanisms can persuasively be linked to downstream timing shifts. Finally, there are some cases where limb heterochronies appear to correlate with environmental variables in early life. The timing differences between forelimb and hind limb development among urodeles and marsupials are particularly interesting examples. However, it is unwise to build ambitious adaptive scenarios for heterochrony, based solely on isolated cases where timing shifts appear to correlate with developmental changes or habitat variables.

And so, is heterochrony indeed a major evolutionary route to new adaptive limb phenotypes? On the basis of the evidence and arguments in this article, we suggest that the Scottish verdict of ''not proven'' should be delivered at this time. In order to answer the question with more certainty, we need data on the entire chain of events associated with the emergence of a given heterochronic shift in a population. This chain presumably includes changes in the genome; developmental gene function or expression; embryonic cell properties and behavior; developmental sequences; and limb function. There may be positive or stabilizing natural selection for any of these changes, including selection for traits that are adaptive with respect to habitat variables. To the best of our knowledge, such comprehensive data sets are not available at this time for the limb.

Future studies could examine taxa in which habitat changes are already known to show some correlation with limb heterochrony, such as in urodele larvae. The ratites are also a promising test case because they are amenable to developmental manipulations, and their limbs show a host of variations associated with changed locomotor function. These include flightlessness (reflected in the miniaturized ostrich wing and the one-fingered emu wing); and running (exemplified by the two-toed ostrich foot). It would be interesting to see if these adaptations have led to any changes in developmental timing parameters such as the sequence of chondrification of limb elements. For example, ratites could be studied in order to test hypotheses about a relationship between the embryonic limb primary axis and the functional adult limb axis. Thus, in the ostrich, the functional axis of the adult limb has shifted to digit III, and it would be interesting to know if this is reflected in a shift in the primary axis. Similar questions arise in relation to digit reduction and changes in functional axes in ungulates (the horse, for example, also has a functional axis passing through digit III). Comparative developmental studies along these lines, coupled with ecological data and the increasing availability of genomic sequences and techniques for developmental gene manipulation, may bring us closer to understanding the role of heterochrony in limb evolution.

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APPENDIX A
LITERATURE ON PATTERN HETEROCRHONY IN CHONDROGENIC PATTERNS

Summary of selected pattern heterochronies in limb development in different vertebrates. (*) Prein ('14) was unable to find any evidence of a precocious ulnare in the chick wing. (¶) Digit III, assuming that the digits of the hand are prepollex+ digits I–IV; however, the primary axis passes through digit IV if there are five digits numbered Tables I–V.

Species	Elements included in primary axis	Mesopodium	Reference
Xenopus laevis		Digits develop earlier than carpus	Alberch and Gale ('83)
Rana	Digits III or IV (forelimb)[¶]		Holmgren ('33)
Urodeles	Digits I and II (hind limb)		Alberch and Gale ('83)
Urodeles	Digits I+II basal commune		Hinchliffe and Vorobyeva ('99)
Triturus vulgaris	Digit II	Distal carpals chondrify earlier than proximal	Strasser (1879)
Triturus marmoratus	(i) Preaxial axis: basale commune, digits I and II (forelimb and hind limb); (ii) primary axis (ulnare/fibulare, distal mesopodial IV, digit IV)	Distal mesopodials and digits	Blanco and Alberch ('92)
Triturus	Digits II+III (forelimb)		Glaesner ('25)
Ostrich (*Struthio camelus*)	Digit IV (forelimb and hind limb)	Carpal arch fuses with radial blastema	Holmgren ('55)
Penguin	Digit IV (forelimb and hind limb)		Sieglbauer ('11)
Chick		Metacarpals accelerated relative to carpals	Lansdown ('69), Montagna ('45)
Plotius (a pelaciniform)	Ulnare, digit V (forelimb)		Schestakowa ('27)
Chick (*Gallus gallus*)	Digit IV (wing)[*]		Prein ('14)
Chick		Carpal arch forms in leg but fails to segment; poorly defined in wing	Hinchliffe (2002)
Chrysemys, Agama	Ulnare+digit IV (forelimb)		Holmgren ('33)
Crocodile	Distal carpal IV (forelimb)	Carpal arch	Holmgren ('33)
Calotes versicolor	Distal carpal IV, ulnare (forelimb); distal tarsal IV, fibulare (hind limb)	Carpus delayed relative to digits	Mathur and Goel ('76)
Turtles, crocodilians, lacertids	Ulna, ulnare, digit IV (forelimb)		Rabl ('10)
Chamaeleo chamaeleo	Digit IV		Hurle ('87)
Gecko, *Ascalabotes fascicularis*	Digit IV (forelimb and hind limb)		Severtsov ('50)
Cetacea		Carpals poorly differentiated in adult	Eales ('54)
Hemiergis	Ulnare and centrale (forelimb)		Shapiro (2002)
Alligator mississippiensis, Gallus gallus, Chelydra serpentina	Ulnare[*], metacarpal IV, digit IV (forelimb); fibulare, metatarsal IV, digit IV (hind limb)	Digital arch	Burke and Feduccia ('97)
Alligator mississippiensis	Ulnare, distal carpal IV, metacarpal IV (forelimb); fibulare, distal tarsal IV, metatarsal IV (hind limb)	Distal carpals or tarsals	Müller and Alberch ('90)
Chrysemys picta, Chelydra serpentina	Ulna, ulnare, distal carpal IV, metacarpal IV (forelimb); fibula, fibulare, distal tarsal IV, digit IV (hind limb)	Distal carpals I–III (forelimb); distal tarsals I–III (hind limb).	Burke and Alberch ('85)

APPENDIX B
LITERATURE ON FORELIMB–HIND LIMB HETEROCHRONY

Summary of forelimb–hind limb heterochrony in vertebrates based on the literature. The time of initial bud formation is considered here as the criterion, although there may be later changes that reverse the polarity. For example, Minot and Taylor (1905) found that the rabbit forelimb and hind limb buds appear synchronously but at later stages, the forelimb skeleton ossifies slightly in advance of the hind limb. Quantitative analysis of the timing differential may provide different categorizations to those presented here (cf. Bininda-Emonds et al., 2007). Taxonomy is based on the Entrez Taxonomy database.[3]

In anurans, the picture is confused because the forelimbs in indirect-developers are initially

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[3] http://www.ncbi.nlm.nih.gov/sites/entrez?db = taxonomy

hidden by the operculum; however, the hind limbs do appear to develop in advance of the forelimbs in *X. laevis*, and this differential may be greater in semi-terrestrial forms (reviewed by McDiarmid and Altig, '99). In *X. laevis*, Trueb and Hanken ('92) reported that hind limb elements are formed in cartilage about one stage earlier than those in the forelimb, although the forelimb and hind limb buds appear synchronously. Tarin and Sturdee ('71) also reported that forelimb development lags slightly behind the hind limb in *X. laevis* in terms of AER formation. In *Rana temporaria*, by contrast, the forelimb and hind limb are synchronous at all stages (Kopsch, '52).

Data on birds are also inconsistent, with some authors finding that the forelimb is advanced over the hind limb at the early limb-bud stage. Thus, Grosser and Tandler ('09) found in the lapwing *Vanellus cristatus* that the forelimb appears slightly earlier than the hind and develops its AER slightly earlier. Later, however, the hind limb exceeds the forelimb in size, but not in differentiation. For the chick, Saunders ('77) found that the wing bud appears at stage 16, the leg at stage 17 (stages of Hamburger and Hamilton, '51). Other authors however (see table) found the chick limb buds to appear synchronously. The characters chosen may be important; thus, Hornbruch and Wolpert ('91) assayed polarizing activity in the chick and found that forelimb activity appears earlier than hind limb activity.

Peter ('02) found that the forelimbs develop very slightly earlier than the hind limbs in *Lacerta* and also that the AER appears slightly earlier on the forelimb bud. In birds and mammals, he found that the AER also appears earliest on the forelimb buds.

Scientific name	Common name	Clade	Ref.
A. Forelimb acceleration			
Callorhinchus milii	Ghost shark	Chimaeriformes: Callorhinchidae	Didier et al. ('98)
Squalus acanthias	Spiny dogfish	Squaliformes: Squalidae	Scammon ('11)
Scyliorhinus canicula	Smaller spotted catshark	Carcharhiniformes: Scyliorhinidae	Ballard et al. ('93)
Polypterus senegalus	Gray bichir	Polypteriformes: Polypteridae	Kerr ('07)
Lepisosteus osseus	Longnose gar	Semionotiformes; Lepisosteidae	Long and Ballard (2001)
Polyodon spathula	Mississippi paddlefish	Acipenseriformes: Polyodontidae	Ballard and Needham ('64)
Oncorhynchus mykiss (Salmo gairdneri) & Salvelinus [Salmo] fontinalis	Rainbow trout and brook trout	Salmoniformes: Salmonidae	Ballard ('73)
Hiodon alosoides	Goldeye	Osteoglossiformes: Hiodontidae	Battle and Sprules ('60)
Xiphophorus sp.	Platyfish and swordtail	Cyprinodontiformes: Poeciliidae	Tavolga ('49)
Danio rerio	Zebrafish	Cypriniformes: Cyprinidae	Kimmel et al. ('95), Tamura et al. ('99)
Neoceratodus fosteri	Australian lungfish	Ceratodontiformes: Ceratodontidae	Kemp ('82), Semon ('01)
Lissotriton [Triturus] vulgaris	Smooth or common newt	Caudata: Salamandridae	Glaesner ('25)
Triturus marmoratus	Marbled newt	Caudata: Salamandridae	Blanco and Alberch ('92)
Hynobius nigrescens	Sendai salamander	Caudata: Hynobiidae	Wong and Liversage (2005), Iwasawa and Yamashita ('91)
Ambystoma mexicanum	Axolotl	Caudata: Ambystomatidae	Nye et al. (2003)
Hemidactylium scutatum	Four-toed salamander	Cuadata: Plethodontidae	Babcock and Blais (2001)
Desmognathus ocoee	Ocoee salamander	Caudata: Plethodontidae	Babcock and Blais (2001)
Ambystoma macrodactylum krausei	Western long-toed salamander	Caudata: Ambystomatidae	Watson and Russell (2000)
Notophthalmus viridescens	Eastern newt	Caudata: Salamandridae	Wong and Liversage (2005)
Cryptobranchus alleganiensis	Hellbender	Caudata: Cryptobranchidae	Smith ('12)
Andrias japonicus	Japanese giant salamander	Caudata: Cryptobranchidae	Kudo ('38)
Necturus maculosus	Mudpuppy	Caudata: Proteidae	Eycleshymer and Wilson ('10)
Dermochelys coriacea	Leatherback sea turtle	Testudines: Dermochelyidae	Renous et al. ('89)
Testudo hermanni	Tortoise	Testudines: Testudinidae	Guyot et al. ('94)

Chrysemys picta bellii	Western painted turtle	Testudines: Emydidae	Mahmoud et al. ('73)
Calotes versicolor	Garden lizard	Squamata: Agamidae	Goel and Mathur ('77)
Chamaeleo chamaeleon	Chamaeleon	Squamata: Chamaeleonidae	Hurle ('87)
Sphenodon punctatus	Tuatara	Sphenodontia: Sphenodontidae	Dendy (1899)
Vanellus vanellus	Northern lapwing	Charadriiformes: Charadriidae	Grosser and Tandler ('09)
Gallus gallus	Chicken	Galliformes: Phasianidae	Saunders ('77)
Tachyglossus aculeatus	Australian echidna	Monotremata: Tachyglossidae	Semon (1894)
Didelphis virginiana	North American opossum	Didelphimorphia: Didelphidae	McCrady ('38)
Monodelphis domestica	Gray short-tailed opossum	Didelphimorphia: Didelphidae	Martin and Mackay (2003)
Sminthopsis macroura	Stripe-faced dunnart	Dasyuromorphia: Dasyuridae	Selwood and Woolley ('91)
Dasyurus hallucatus	Northern quoll	Dasyuromorphia: Dasyuridae	Nelson ('92)
Petauroides volans	Greater glider	Diprotodontia: Pseudocheiridae	Bancroft ('73)
Trichosurus vulpecula	Brushtail possum	Diprotodontia: Phalangeridae	Bancroft ('73)
Erinaceus europaeus	Western European hedgehog	Eulipotyphla: Erinaceidae	Jacobfeuerborn ('08)
Tupaia javanica	Javan tree shrew	Scandentia: Tupaiidae	De Lange and Nierstrasz ('32)
Sus scrofa	Pig	Cetartiodactyla: Suidae	Keibel (1897)
Equus caballus	Horse	Perissodactyla; Equidae	Drahn ('27)
Mus musculus	House mouse	Rodentia: Muridae	Martin ('90), Wanek et al. ('89)
Rattus norvegicus	Norway rat	Rodentia: Muridae	Henneberg ('37)
Cricetulus barabensis	Chinese striped hamster	Rodentia: Cricetidae	ten Donkelaar et al. ('79)
Spermophilus citellus	European suslik	Rodentia: Sciuridae	Völker ('22)
Homo sapiens	Human	Primates: Hominidae	Keibel and Elze ('08), O'Rahilly and Gardner ('56), O'Rahilly and Müller ('87)
B. Forelimb–hind limb synchrony			
Rana dalmatina	Agile frog	Anura: Ranidae	Cambar and Marrot ('54)
Rana temporaria	Common frog	Anura: Ranidae	Kopsch ('52)
Lacerta agilis	Sand lizard	Squamata: Lacertidae	Peter ('04)
Melopsittacus undulatus	Budgerigar	Psittaciformes: Psittacidae	Abraham ('01)
Gallus gallus	Chicken	Galliformes: Phasianidae	Hamburger and Hamilton ('51), Keibel and Abraham (1900)
Manis javanica	Malayan pangolin	Pholidota: Manidae	Huisman and De Lange ('37)
Oryctolagus cuniculus	Rabbit	Lagomorpha: Leporidae	Minot and Taylor ('05)
C. Hind limb acceleration			
Xenopus laevis	African clawed frog	Anura: Pipidae	Nieuwkoop and Faber ('94), Tarin and Sturdee ('71)
Eleutherodactylus coqui	Puerto Rican treefrog	Anura: Leptodactylidae	Richardson et al. ([131]'98a,b)
Alligator mississippiensis	American alligator	Crocodilia: Alligatoridae	Ferguson ('85), but cf. Reese ('08)
Struthio camelus	Ostrich	Struthioniformes; Struthionidae	Holmgren ('55)
Anas platyrhynchos	Duck	Anseriformes; Anatidae;	Holmgren ('55), Sieglbauer ('11)

APPENDIX C
E. ORBICULARIS DATA

Materials and methods

We examined the early limb chondrogenesis in the turtle *E. orbicularis*. Terminology follows Burke and Alberch ('85); numbering of centralia is avoided given the uncertain homologies of specific elements in that area of the autopods.

Gravid females were collected under licence from the French Government. Standard injection with oxytocin was used to induce laying. Eggs were placed on a layer of sand in an incubator. Periodically an egg was taken, the embryo was anesthetized, euthanized, and preserved. After fixation embryos were dehydrated in graded ethanols to acid alcohol (70% ethanol+1% concentrated hydrochloric acid) for 1 hr. Staining was done in 0.03% Alcian blue in acid alcohol (4 hr). Specimens were then differentiated in acid alcohol (overnight) and dehydrated in graded ethanols to 100%, and cleared in methyl salicylate.

An ontogenetic series consisting of eight cleared and stained limbs was used to record the chondrification sequence, selected from a total of 35 specimens examined (C.P., Claude Pieau collection). Specimens were staged following Yntema ('68) and Mahmoud et al. ('73), and those recording the most significant changes documented here are in stages 13–19.

X-rays of the limbs of an adult specimen (M.K. Richardson, personal collection) were made to document the adult skeletal anatomy in the species and compare it with published reports on the subject (Baur, 1892; Rosenberg, 1892; Rabl, '10).

Appendix. Table 2. Chondrification in the limbs of *E. orbicularis* based on Alcian blue-stained wholemounts.

Key: I–V, digits I–V; in, intermedium; mt, metatarsal; mc, metacarpal; prox ph, proximal phalanx; mid ph, middle phalanx; dist ph, distal phalanx; dc, distal carpal; dt, distal tarsal; 0, element not visible in Alcian blue preparations; CR, crown-rump; * embryos used in reconstructions in Figures 6 and 7. *Scoring of condensations*: 0, no condensation visible at that stage; 1, indistinct (darker-staining tissue is visible, but its boundaries are poorly defined); 2, condensation with distinct boundaries that demarcate it from other elements (although the boundaries may simply be lighter-staining areas, and not necessarily fully formed joint spaces). Elements scored as "1" and "2" are outlined with broken or continuous lines, respectively, on Figures 6 and 7. Note that the scoring is subjective based on an analysis by one of the authors (M.K.R.) of the original specimens, whereas the independent observations of the same specimens were carried out by S.G.

Embryo code number	CP455	CP444	CP447	CP446	CP458	CP457	CP459	CP463	CP435	CP464	CP461	CP438	CP467	Adult
			*	*	*	*	*	*	*	*	*	*	*	
Yntema ('68) stage	12–13	13–14	14	15	16	17	17	18	18	18	18	20	21	
Forelimb														
Humerus	0	1	2	2	2	2	2	2	2	2	2	2	2	
Radius	0	0	2	2	2	2	2	2	2	2	2	2	2	
Ulna	0	0	2	2	2	2	2	2	2	2	2	2	2	
Ulnare	0	0	2	2	2	2	2	2	2	2	2	2	2	
Intermedium	0	0	0	2	2	2	2	2	2	2	2	2	2	
Centrale 1	0	0	0	0	1	1	2	2	2	1	2	2	2	
Centrale 2	0	0	0	0	1	2	2	2	2	2	2	2	2	
Centrale 3	0	0	0	1	2	2	2	2	2	2	2	2	2	
Distal carpal 1	0	0	0	0	2	2	2	2	2	2	2	2	0	
Distal carpal 2	0	0	0	2	2	2	2	2	2	2	2	2	2	
Distal carpal 3	0	0	0	2	2	2	2	2	2	2	2	2	2	
Distal carpal 4	0	0	0	2	2	2	2	2	2	2	2	2	Fused	
Distal carpal 5	0	0	0	2	2	2	2	2	2	2	2	2	Fused	
mc I	0	0	0	0	2	2	2	2	2	2	2	2	2	
mc II	0	0	0	1	2	2	2	2	2	2	2	2	2	
mc III	0	0	0	1	2	2	2	2	2	2	2	2	2	
mc IV	0	0	0	2	2	2	2	2	2	2	2	2	2	
mc V	0	0	0	1	2	2	2	2	2	2	2	2	2	
prox ph I	0	0	0	0	0	1	2	2	2	2	2	2	2	
prox ph II	0	0	0	0	2	2	2	2	2	2	2	2	2	
prox ph III	0	0	0	0	2	2	2	2	2	2	2	2	2	
prox ph IV	0	0	0	0	2	2	2	2	2	2	2	2	2	
prox ph V	0	0	0	0	1	2	2	2	2	2	2	2	2	
mid ph II	0	0	0	0	0	0	2	2	2	2	2	2	2	
mid ph III	0	0	0	0	0	0	2	2	2	2	2	2	2	
mid ph IV	0	0	0	0	0	0	2	2	2	2	2	2	2	
dist ph I	0	0	0	0	0	0	0	0	2	2	2	2	2	
dist ph II	0	0	0	0	0	0	0	0	0	2	2	2	2	
dist ph III	0	0	0	0	0	0	0	0	0	2	2	2	2	
dist ph IV	0	0	0	0	0	0	0	0	0	2	2	2	2	
dist ph V	0	0	0	0	0	0	0	0	2	1	2	2	2	

Radial sesamoid prox (epiphysis)	0	0	0	0	2	2	2	2	2	2	2	Fused with R	Fused with R
Radial sesamoid distal	0	0	0	0	0	0	0	0	0	0	0	2	2
Ulnar sesamoid prox (ulnar epiphysis)	0	0	0	0	1	2	2	2	2	2	2	Fused with U	Fused with U
Ulnar sesamoid distal	0	0	0	0	1	2	2	2	2	2	2	2	2
Hind limb													
Femur	0	1	2	2	2	2	2	2	2	2	2	2	2
Tibia	0	0	2	2	2	2	2	2	2	2	2	2	2
Fibula	0	0	2	2	2	2	2	2	2	2	2	2	2
Tibiale	0	0	0	1	2	1	1	2	Fusing with in	Fusing with in	Fusing with in	Fused with in	Fused with in
Fibulare	0	0	0	2	2	2	2	2	2	2	2	2	Fusing with in
Intermedium	0	0	0	2	2	1	2	2	2	Fusing with te	Fusing with te	Fused with te	Fused with te
Tibial border cartilage 1	0	0	0	0	2	2	2	2	1	0	2	Fused with T	Fused with T
Tibial border cartilage 2	0	0	0	0	0	0	0	?	0	0	2	0	0
Fibular border cartilage	0	0	0	0	0	0	1	?	1	2	Fused with Fi	Fused with Fi	Fused with Fi
Centrale arch	0	0	0	0	0	0	0	0	1	2	Fused with in	0	0
dt I	0	0	0	0	2	2	2	2	2	2	2	2	2
dt II	0	0	0	2	2	2	2	2	2	2	2	2	Fusing with mt
dt III	0	0	0	2	2	2	2	2	2	2	2	2	Fusing with mt
dt IV	0	0	0	2	2	2	2	2	2	2	2	2	2
mt I	0	0	0	0	2	2	2	2	2	2	2	2	2
mt II	0	0	0	0	2	2	2	2	2	2	2	2	2
mt III	0	0	0	1	2	2	2	2	2	2	2	2	2
mt IV	0	0	0	2	2	2	2	2	2	2	2	2	2
mt V	0	0	0	2	2	2	2	2	2	2	2	2	2
prox ph I	0	0	0	0	0	1	2	2	2	2	2	2	2
prox ph II	0	0	0	0	0	2	2	2	2	2	2	2	2
prox ph III	0	0	0	0	2	2	2	2	2	2	2	2	2
prox ph IV	0	0	0	0	2	2	2	2	2	2	2	2	2
prox ph V	0	0	0	0	2	2	2	2	2	2	2	2	2
mid ph II	0	0	0	0	0	0	0	2	2	2	2	2	2
mid ph III	0	0	0	0	0	0	0	2	2	2	2	2	2
mid ph IV	0	0	0	0	0	0	0	2	2	2	2	2	2
dist ph I	0	0	0	0	0	0	0	0	0	1	2	2	2
dist ph II	0	0	0	0	0	0	0	0	0	0	2	2	2
dist ph III	0	0	0	0	0	0	0	0	0	0	2	2	2
dist ph IV	0	0	0	0	0	0	0	0	0	0	2	2	2
dist ph V	0	0	0	0	0	0	0	0	0	1	2	2	2

APPENDIX D
R. AMPLEXICAUDATUS DATA AND STATISTICAL ANALYSES

Linear regression formulas ($y = ax + b$) and P-values of length and surface area in the mouse and bat. When $P < 0.05$, the tested value is significant. When $P > 0.05$, the tested value is not significant ($P = 0.261$, marked ns).

	A	b	Regression	$y = x$ P-value	$Y = ax$ P-value	$Y = x + b$ P-value
Rousettus						
Length (mm)	1.43	0.23	0.974	0.000	0.009	0.000
Mus						
Length (mm)	0.91	0.33	0.968	0.001	0.000	0.017
Rousettus						
Area (mm^2)	2.38	0.05	0.988	0.000	0.261 ns	0.000
Mus						
Area (mm^2)	0.93	0.24	0.980	0.004	0.000	0.004

Original length data used in the analyses.

	Length hind limb	Length forelimb
Rousettus	0.15	0.21
	0.167	0.28
	0.21	0.43
	0.22	0.49
	0.22	0.53
	0.24	0.54
	0.27	0.56
	0.57	1.14
	0.51	1.39
	0.87	1.45
	1.19	1.85
	1.26	2.26
	1.63	2.98
	3.06	4.04
	3.18	4.38
	3.45	5.9
	4.16	6.03
Mus	0.34	0.65
	0.45	0.63
	0.45	0.76
	0.54	1
	0.8	1.01
	0.79	1.09
	0.94	1.26
	0.96	1.32
	1.5	1.84
	1.8	1.99
	2.1	2.03
	2.14	1.93
	3.01	2.8
	2.94	2.94
	4.03	4.39

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