Proximodistal patterning of the limb: insights from evolutionary morphology

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SUMMARY There is an active debate about how skeletal elements are encoded along the proximodistal (PD) axis of the developing limb. Our aim here is to see whether consideration of the evolutionary morphology of the limb can contribute to our understanding of patterning mechanisms. Of special interest in this context are animals showing reiterated skeletal elements along the PD axis (e.g., dolphins and plesiosaurs with hyperphalangy). We build on previous hypotheses to propose a two-step model of PD patterning in which specification of broad domains in the early limb bud is distinct from subsequent processes that divides an initial anlage into a segmental pattern to yield individual skeletal elements. This model overcomes a major evolutionary problem with the progress zone model, which has not previously been noted: pleiotropy. Parallels with other developmental systems are briefly discussed.

INTRODUCTION

Classical morphological and histological studies showed that the developing tetrapod limb skeleton differentiates in a time sequence from proximal to distal (the carpal and tarsal regions do not follow this sequence, being relatively delayed; Lewis 1975). Surgical ablation and irradiation experiments suggest that the sequential laying down of parts is somehow dependent on the activity of the apical ectodermal ridge (AER) and the underlying mesenchymal cells of the progress zone (PZ). Interactions between the AER and PZ are, at least in part, mediated by the products of the fibroblast growth factor genes.

Although much is known about the cellular and molecular mechanisms involved in proximodistal (PD) patterning (reviewed by Tickle 2003), at least two things are unclear: the importance of timing as a mechanism for encoding position and the mechanism that leads to regionalization of patterning along the limb (by which we mean that the humerus, for instance, is a different size and shape from the carpal bones).

The PZ model

In the embryo, limb cells undergo proliferation at a constant rate throughout the limb bud but remain undifferentiated in a strip of tissue at the tip of the limb, called the PZ, and then drop out of the PZ to differentiate into a series of bones along the PD axis (Saunders 1948; Summerbell et al. 1973). According to the original PZ model, many aspects of the skeletal pattern along the PD axis, including the number, type, and positions of bones, are determined by the length of time that cells spend in the PZ (Summerbell et al. 1973). Thus, early drop-outs will make humerus, and late ones will make finger-tip bones (phalanges).

New findings: early specification

Recent experimental studies challenge the PZ model and suggest that the surgical experiments on which it was based are open to reinterpretation (Dudley et al. 2002; Sun et al. 2002). It is likely that some components of the skeletal pattern are specified much earlier than assumed by the PZ model and may be independent of drop-out time (Dudley et al. 2002). Specifically, the new studies suggest that the limb is divided at an early stage into broad domains with distinct positional value that may function as developmental compartments, such that there is little or no cell mixing between them. These compartments are marked by genes activated, not by time spent in the PZ but by proximity to proximal or distal signaling centers (e.g., activation of *Meis* expression by proximity to retinoids produced by the flank; Mercader et al. 2000). Subsequent stages of limb outgrowth are concerned with the sequential differentiation and expansion of the prepattern and not with the sequential encoding of the pattern (Dudley et al. 2002). The interpretation of these

experiments are controversial, and as a result ideas about PD patterning are currently in a state of flux (Saunders 2002; Wolpert 2002).

LIMB EVOLUTIONARY MORPHOLOGY

Can considerations of evolutionary morphology give insights into this problem? There are various patterns in limb evolution that may have involved changes in PD patterning mechanisms. The most obvious is the evolution of hyperphalangy—an increase in the number of phalanges (as seen in cetaceans, ichthyosaurs, and plesiosaurs; Richardson and Chipman 2003). Lungfishes (the closest living relatives of tetrapods) also show reiteration of elements along the PD axis of their pectoral fins (Fig. 1).

What does hyperphalangy show that might be relevant to our discussion? Two interesting features are seen in limbs with hyperphalangy (Fig. 1). First, the "extra" or reiterated elements all have the appearance of distal elements; proximal elements are never duplicated along the PD axis. Second, there is no obvious regionalization within the series of extra elements. Thus, apart from a gradient in size they look similar. A comparable result can be obtained experimentally when a bead soaked in Shh protein is placed at the growing tip of a chick digit at the stage when endogenous Shh normally disappears. Continued *Fgf* expression is induced in the overlying AER, resulting in continued outgrowth and the formation of an extra distal phalanx, similar in morphology but tapered relative to the more proximal phalanges (Dahn and Fallon 2000). These observations need to be explained by any model of limb patterning.

INTEGRATING MECHANISM AND MORPHOLOGY

At first sight, the PZ model appears to be consistent with findings in cetaceans, such as dolphins, that show hyperphalangy (numerous finger bones) on their forelimbs (flippers). Thus, in the spotted dolphin, morphological studies reveal a persistent bud of tissue at the tip of the developing flipper that continues to lay down phalanges into relatively advanced stages of development (Richardson and Oelschläger 2002). Similarly, in the experimental model, extended FGF exposure results in prolonged maintenance of the AER and, hence, the underlying PZ.

However, if positional values are encoded by the length of time spent in the PZ, then it should be possible for animals with hyperphalangy and prolonged outgrowth to show a greater diversity of bone types. However, what we see is either no increase in diversity of bone types in hyperphalangy or even a loss of diversity (in plesiosaurs, the phalanges closely resemble the more proximal elements). Thus, other than the number of elements, there is no evidence for an increase in number of positional identities.

So it seems that the number of different types of bones along the PD axis is intrinsically limited and is not a simple function of the number of cell cycles spent in the PZ. The early specification model is consistent with these findings because the fixed number of domains specified in early development will not be changed simply because of prolonged outgrowth, although the increase in numbers of elements needs to be accounted for.

A major objection to the PZ model, not previously been noted, is pleiotropy. In its strict version, the PZ model places a large burden of phenotypic outcomes on a single mechanism (timing). Thus, the length of time spent in the PZ determines numerous aspects of the skeletal pattern, including size of the initial condensation, branching, joint spacing, and degree of subsequent growth. This is a problem because systems with high pleiotropy are generally considered to be evolutionarily inflexible (Galis et al. 2001).

For its part, the early specification model, with its fixed number of PD positioned units, has difficulty with the large number of bones per se. If a pilot whale has around 20 bones in series along the PD axis of its flipper (Kükenthal 1889) and a plesiosaur up to 30, it becomes difficult to see how such complexity could be encoded as a prepattern within the limited dimensions of an early limb bud. Furthermore, if outgrowth is a matter of expanding an existing prepattern, then the prolonged outgrowth seen in the normal dolphin flipper, or experimentally induced with Shh and Fgf in chick limbs, ought to lead to bigger phalanges, not more numerous ones. In fact this is not observed (Richardson and Oelschläger 2002).

PROPOSAL: REGIONALIZATION IS UNCOUPLED FROM SEGMENTATION

We propose a new model to accommodate the data outlined above (Fig. 2). It is based on a two-step process in which the PD axis is first subdivided at early stages into a limited number of broad spatial domains, corresponding to different PD limb regions (Dudley et al. 2002). These could arise all at once or by a series of sequential subdivisions during early limb bud growth. Each of these broad domains is characterized by a set of cellular parameters that modulate the subsequent processes of chondrogenesis. This would include cellular properties affecting the number condensations forming along the anteroposterior axis, with discontinuities reflected as the pattern of branching of the forming condensation. In addition, the different PD domains have distinct programs of segmentation.

In lungfish (the closest living relatives of tetrapods) the distal skeleton of the pectoral fin is comprised of a long metameric axis with approximately 40 segments proximodis-
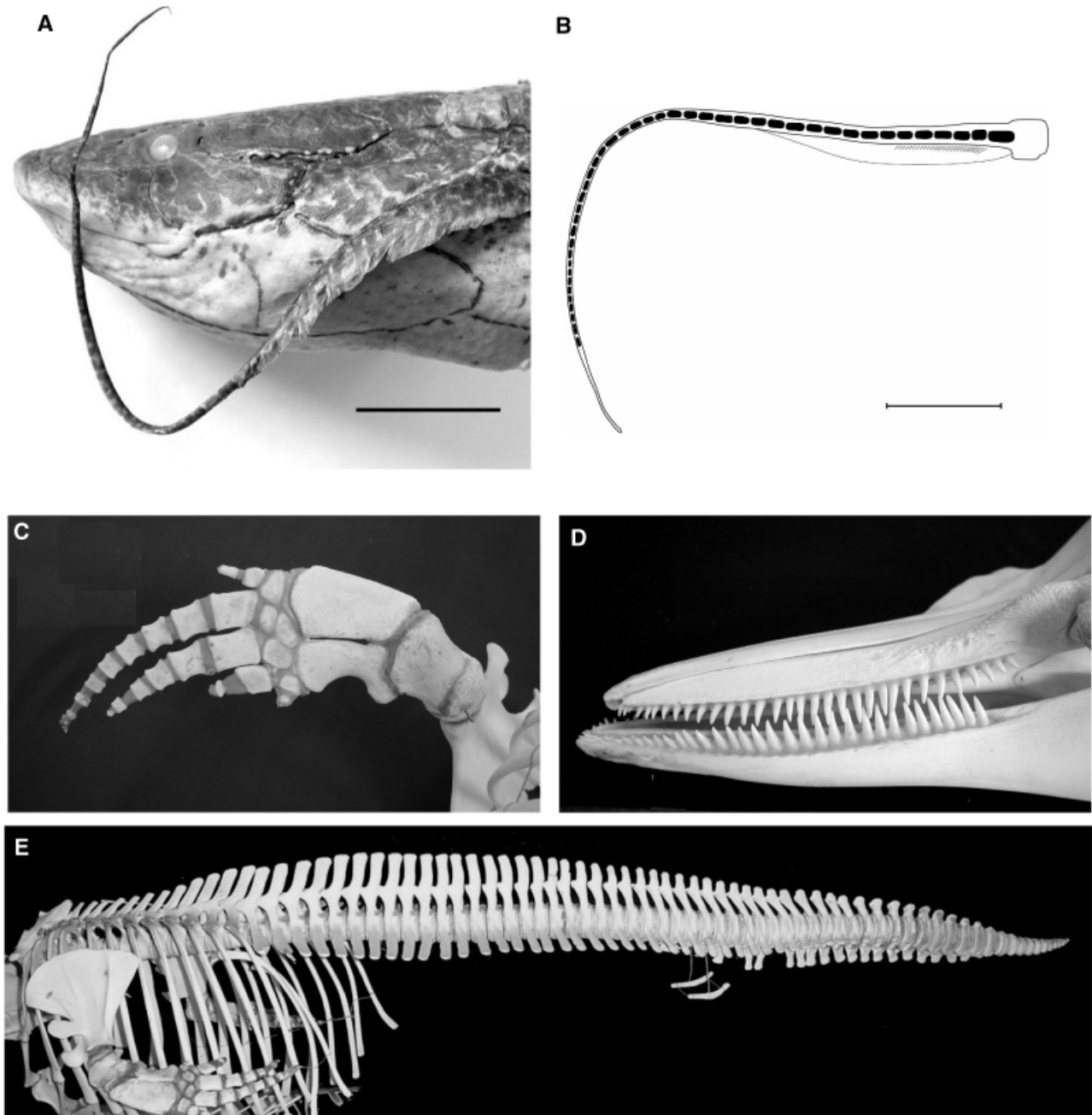
Fig. 1. (A) Head of African lungfish, *Protopterus aethiopicus aethiopicus* (Heckel, 1851). Note the highly attenuated and elongated pectoral fin (arrows). Adult, gender not determined. Collected in the Mwanza Gulf, Lake Victoria by the Haplochromis Ecology Survey Team. Courtesy of Dr. Frans Witte. Right side, image inverted. Scale bar, 3 cm. (B) Left fin from A, redrawn schematically after clearing in methyl salicylate. Note large number of reiterated cartilages along the axis of the fin. (The fin has a different curvature from the one shown in A.) Scale bar, 3 cm. (C–E). Dolphins and porpoises often show hyperphalangy (C) (Richardson and Chipman 2003), a large number of teeth (D), and a high vertebral number (E) compared with typical terrestrial mammals. In the genus *Phocenoides*, for example, the vertebral count approaches 100 (Anderson and Jones 1984). Whether these are independent adaptations to piscivory and swimming mode or are a reflection of developmental constraints is not known. The species shown here is *Lagenorhynchus acutus* (Gray, 1828), the white-sided dolphin; female juvenile, reg. no. 19535, Nationaal Natuurhistorisch Museum, Leiden (Naturalis). C shows the ventral aspect of the right flipper; D are E are left lateral views.
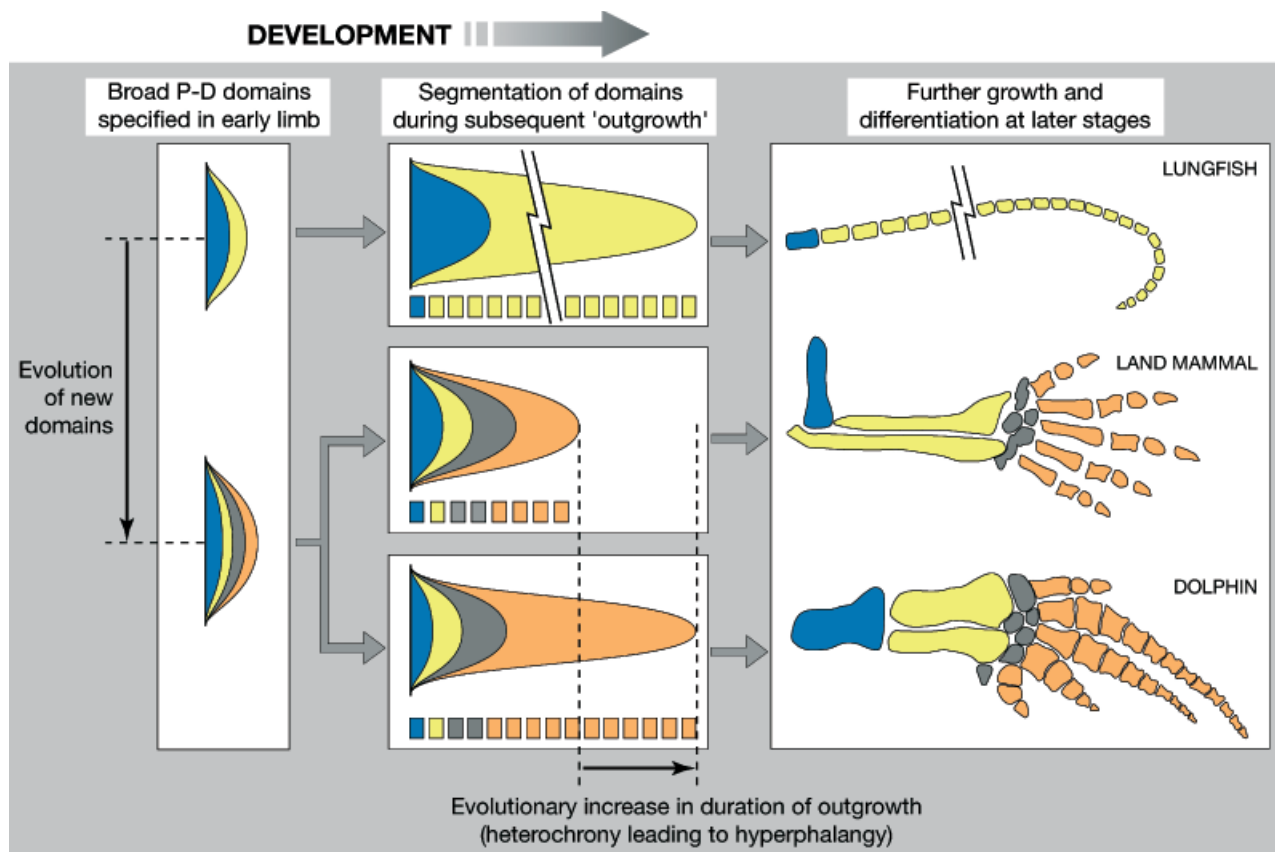
Fig. 2. Summary of our model to account for aspects of limb pattern formation and evolution. It is possible that an early stage of limb development involves the specification of broad domains of positional identity (represented in the far left-hand column by different colored vertical stripes). In this diagram, we assume that tetrapods have evolved a greater number of early domains than lungfish. The domains, as depicted here, are purely arbitrary, as is their mapping to the skeletal domains seen in the far right of the figure. In a later stage of limb development, during the outgrowth phase, we envisage segmentation of the domains into cartilage and joint regions through a temporal AER-dependent mechanism. In the dolphin, we assume that outgrowth is prolonged to give more elements with the same distal (pink) character. A possible outcome in the formed limb is shown schematically on the far right: Limb regions, corresponding to the early domains, are subdivided into skeletal elements. Further work is required to examine this hypothesis.

tally (Fig. 1, A and B). There is no obvious regionalization of the distal skeleton because, we suggest, it comprises a single distal developmental domain. Within this domain, a specified periodicity for segmentation would mean that as the distal domain continues to grow, the condensations segment into metameric elements of defined size and shape. Thus, the repeating segments are not directly specified but produced by continued outgrowth of a fin with a defined program of reiterative segmentation, not via the evolution of a large number of uniquely defined fin bones.

Tetrapod limbs have fewer PD segments than lungfish fins (typically 8–10) but much clearer regionalization along the PD axis. This suggests a greater number of unique PD domains specified in tetrapod limb buds. For example, it has been known for some time that the distal region (the wrist and hand, or "autopod") follows a morphogenetic program distinct from the rest of the limb. This includes a distinct

pattern of *Hox* gene expression not seen in non-tetrapods (Sordino et al. 1995; Nelson et al. 1996). The autopod domain may have evolved via the recruitment of a new distal limb *Hox* gene enhancer (Herault et al. 1999).

Within the tetrapods, prolonged outgrowth resulting in an increased number of PD segments (approximately 15) has been observed in dolphin flippers (Richardson and Oelschläger 2002). These highly derived fin-like limbs retain the primitive tetrapod morphology proximally (humerus, ulna, radius) but show hyperphalangy in the digits. As with lungfish, we believe that the increased number of elements is the result of the prolongation of a cyclic program of segmentation within the distal domain (in this case the autopod). An attractive feature of our model is that the uncoupling of the establishment of PD domains from the determination of the number of segmented skeletal elements allows greater scope for independent evolutionary changes.

COMPARISON WITH OTHER DEVELOPMENTAL SYSTEMS

In summary, our model states that the specification of joints between the bones occurs in the context of the parameters specified for each limb segment, but the process of continued outgrowth and reiterative formation of skeletal elements is distinct from the earlier patterning events that set up the broad limb domains. This uncoupling of mechanisms is also seen in wing feather patterning in the quail. The feather buds are laid down by a spacing mechanism—quite distinct from the positional signals from the polarizing region that give different feathers their characteristic growth properties (Richardson et al. 1990).

In the vertebral column too there are separate mechanisms for regionalization and segmentation. Like the tetrapod autopod, the somites form in a reiterative pattern, as long as the program is maintained, in this case by the caudal growth of the presegmental mesoderm (Pourquie 2003). Although this process is coupled to the expression of *Hox* genes that define morphological differences between vertebral regions (Dubrulle et al. 2001; Zakany et al. 2001), the mechanism by which *Hox* genes are initially turned on in particular domains is distinct from the segmentation mechanism. Thus, the number of vertebrae within each region (cervical, thoracic, lumbar, sacral) can be varied in evolution (Burke et al. 1995). Similarly, according to our proposal, there is a decoupling of specification and segmentation in the distal limb. In principle, this decoupling allows great evolutionary flexibility because it means that different properties of the axial skeleton can be modified independently by natural selection (Burke et al. 1995; Richardson et al. 1998).

A final tantalizing observation is that in some dolphins not only are the phalanges multiplied, but the teeth and vertebrae have also increased in number over the primitive mammalian pattern (Fig. 1). Whether these multiplications represent independent adaptations or reflect some deeper link between the control of segment number in different systems is not yet clear. However, it is interesting to note that FGF signaling may be a common component in outgrowth of both the limb and the tail bud (reviewed by Pourquie 2003).

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