

Developmental Basis of Limb Evolution

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ABSTRACT Can developmental processes account for vertebrate limb homology, the overall similarity of definitive limb structure despite differences in different taxa which often relate to evolutionary adaptations? Relevant evidence is from molecular studies, from 'cut & paste' experimental embryology and from classical descriptive accounts of embryology and structure. There is striking evidence of a similar pattern of homologous regulatory gene expression (eg *Shh*, and *Hox A & D* genes) in tetrapod limb buds, and both similarity and differences when these are compared with expression patterns in a teleost fish paired fin bud. But these findings are as yet from too few tetrapod species (chick and mouse) to permit a '*molecular bauplan*' for the limb to be proposed with any certainty. Further, the identification of similar networks of regulatory genes common to non-homologous developmental systems limits possibilities for finding a basis for classical structural homology in terms of expression of system-specific genes or gene networks. An integrated approach is needed, combining evidence from the fin-limb transition, and from study of the patterns and processes of amphibian and avian limb embryology, and this points towards a conserved developmental *bauplan* for the pentadactyl skeleton of the type earlier proposed by Alberch. Key features include the digital arch, restriction of digit number to a maximum of 5 and stereotyped connections between prechondrogenic condensations. But this is a dynamic and not rigidly fixed *bauplan*. It has no single set of skeletal elements (except proximally), since the position of joint formation in the prechondrogenic condensations is not stereotyped. Urodele amphibians in particular demonstrate heterochronic differences in the timing of events. Heterochrony may underlie some of the important changes in the pentadactyl pattern during evolution.

KEY WORDS: *anurans, chondroitin-6-SO4, condensation, developmental bauplan, heterochrony, hynobiid*

Introduction

The evolution of the tetrapod limb raises many profound questions in biology. Recognition of the essential similarity of its structure regardless of its functional adaptation – whether for flight, paddling or running – gave rise to the concept of homology. Since definitive structures arise from embryonic developmental processes, how do these account for both the similarity and the differences between the limbs of different tetrapods? And since the limb arose in evolution from a fish fin, can we propose a convincing developmental transformation?

Establishing a basis in embryology and genetics to homology has proved disconcertingly difficult. This is recognised by de Beer (1973) in his paper entitled 'Homology, an unsolved problem'. Similarity in structure is itself sometimes misleading since this may be due to convergence from different starting points rather than descent from a common ancestor. The assumption has often been

made that homologous structure must be the end point of similar embryology. But as de Beer demonstrates, the same structure (eg the lens of the eye) in different taxa may be the consequence of different developmental processes such as inductive ones or it may originate from different germ layers.

While early developmental stages are sometimes unreliable as indicators of homology, later when organ rudiments – such as at the branchial arch or 'phylotypic stage' – are appearing, conserved development may provide such evidence and thus be taken as the basis for definitive structural similarity. An example of this is provided for the limb by the interpretation by Shubin & Alberch (1986) of a generalised early skeletal pattern or developmental *bauplan*** for the skeleton, as discussed later. Analysis of such early pattern gives information on regressions or fusions of elements which cannot be known simply by observing the definitive skeleton.

From whatever taxa have been analysed, the limb bud itself may represent such a conserved developmental stage. It is composed

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** Note: Developmental *bauplan* in this context is the concept of conserved, common developmental processes initiating or acting within vertebrate limb buds and which are assumed to underlie the structural homology of the definitive limb, especially at the skeletal level. See in addition to Shubin & Alberch (1986), the discussion of 'bauplan' in Hall (1999), chapter 6.

of the same tissues of ectoderm with generally a distal thickening, the apical ectodermal ridge (AER) and an inner mesodermal core (part flank and part somitic derived, the latter contributing the musculature) which undergoes at least proximally the same branching patterning and condensation initiated skeletogenesis (Figs. 1,3E). The interactive developmental processes may be interpreted as conserved in the different tetrapod classes. The AER controls mesoderm outgrowth, and is itself dependent for its maintenance on the underlying limb mesoderm – the Saunders-Zwilling hypothesis. In this way the mesoderm generated distally under the AER takes part subsequently in formation of the endoskeleton more proximally. A part of the posterior distal mesoderm (the ZPA – zone of polarising activity) controls the antero-posterior axis. Branching skeletogenesis appears a fundamental property of the chick limb bud mesenchyme even when positional cues such as the ZPA are removed (Pautou 1977). These conclusions were based on the classical analysis by tissue exchanges and ectopic grafting (Saunders 1972). Such experiments were extended to inter-specific exchanges: eg mouse ZPA transplantation preaxially to the chick wing bud (digit duplication resulted). Essentially these inter-specific experiments (for example in mammalian-avian and reptile-avian embryo chimeras) when taken together showed that the same interactive developmental processes were conserved in all the tetrapod classes (reviewed: Hinchliffe 1991).

From an evolutionary view it is interesting to compare and contrast these developmental structures and processes with those of the paired fins of fish – in this case the Zebra fish (Géraudie & François 1973). Unfortunately the value of the comparison is limited as the teleost group to which the Zebra fish belongs is very remote from the fish/tetrapod evolutionary node and in fact knowledge of the fins of extant lobe finned fish such as *Neoceratodus* would be more useful as these are more closely related. But such knowledge is sparse. However the Zebra fish fin bud has both similarities and differences to the limb bud (Hinchliffe *et al.*, 2001). The fin bud must generate both proximal endoskeletal radials and distal exoskeletal or dermal finrays. First a AER-like ridge briefly generates mesoderm from which the endoskeletal radials form. The definitive radials superficially resemble tetrapod elements and both are formed as cartilage-replacement bone, though the cell processes at the condensation stage are fundamentally different (Hinchliffe *et al.*, 2001). Fin rays have no homologue in the limb and are generated by a second process in which a distal finfold is invaded by mesenchyme like cells which may be a second population of neural crest origin (Fig. 4, Thorogood 1991). This second process is not found in the tetrapod limb bud where there is no generation of exoskeleton in the limb. Further discussion of the fin/limb transformation is held back until more of the structural aspects are considered later.

New Molecular Evidence relating to the Development and Evolution of Limb and Fin

The last decade has seen a large growth in the body of evidence of genetic control of limb development, mainly drawn from work on chick and mouse embryos (reviewed; Capdevila & Belmonte 2001, Cohn & Bright 1999). The earlier phase of analysis based mainly on experimental embryology together with some evidence from mutant embryos had led to the identification of a common set of processes at the limb bud stage of tetrapods, as just described.

Initially it was anticipated that this would be underpinned by a common pattern of homologous regulatory gene expression specific to the limb. Early results – e.g. of *Shh*, *Hox A* and *Hox D13* appeared to support this and to suggest that features such as AER and ZPA could be given molecular descriptions. But as further results were obtained eg regarding gene networks it became clear that molecular control was more complex than initially assumed and further that some regulatory genes and gene networks were not specific for the limb bud but common to other non-homologous developmental systems. Thus we have the new concept of a conserved ‘genetic toolbox’ including families of such genes as *Hedgehog*, *Tgf- β* , *Bmp* and *Fgf* which are used at different times and places by the embryo to form not just limbs but other organs. Indeed there may be a set of such genes which is appendage forming but in a general rather than specific way (Shubin *et al.*, 1997, Kondo *et al.*, 1997). One result is to blur the classical distinction in evolutionary theory between homologous and analogous structures since analogous organs such as limbs of insects and tetrapods may have very similar regulatory gene expression patterns. For example Wray writes (1999): ‘There is increasing evidence that several kinds of evolutionary dissociations can evolve between genotype & phenotype, some of which are quite unexpected...these dissociations limit the degree to which it is possible to make inferences about the homology of structures based solely on the expression of homologous genes.’ Similarly, Abouheif (1999) discusses homologous regulatory gene networks underlying classically analogous limbs of insects & birds. The consequence of this is that rather than molecular developmental biology underpinning classical homology it becomes instead a new and separate field of homology.

Turning from such general aspects to the detail of regulatory gene expression and networks in limb buds it should be emphasised that what we know is based largely on chick and mouse, chosen because much is known about mouse genetics and its mutants and from chick mutants also, together with experimental manipulation of chick limb buds including the alteration of normal patterns of gene expression.

It is not the intention here to analyse in any detail the by now large field of knowledge of the molecular control of the limb buds of these two species (Capdevila & Belmonte 2001). Rather a few key points (e.g. Figs. 1,2) will be selected from the viewpoint of illuminating evolutionary developmental biology of the limb, the subject of much current interest (eg Zakany & Duboule 1999, Capdevila & Belmonte 2000).

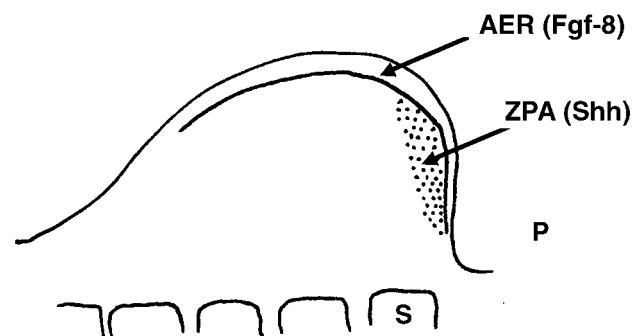


Fig. 1. Schematic diagram of tetrapod limb bud. AER, apical ectodermal ridge; ZPA, zone of polarising activity. (AER & ZPA as sites of chick and mouse *Fgf-8* and *Shh* gene activity are indicated). P, posterior; S somites.

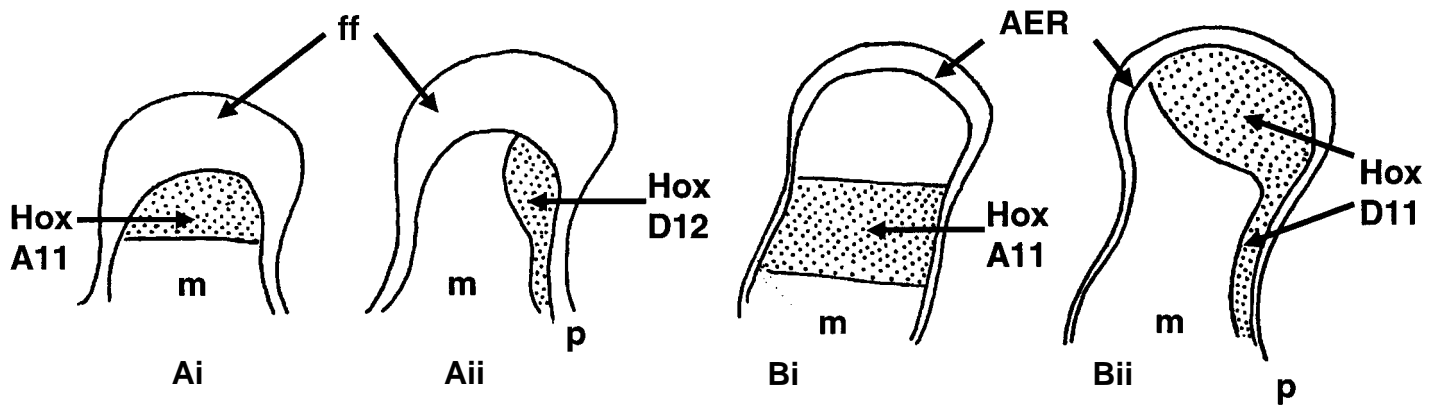


Fig. 2. *Hox A* and *D* expression in Zebra fish paired pectoral fin buds (Ai & ii) and chick & mouse limb buds (Bi & ii). *M*, mesoderm. Note the differences in *Hox D* expression distally, believed to be associated with digit evolution (after Coates 1995).

Hox regulatory genes encoding homeodomain transcription factors are known to regulate position along the antero-posterior axis of both *Drosophila* and mouse and chick embryos. In the latter they also regulate fore and hind limb bud position. Once the limb bud is formed, they continue to have a key role in position control, this time on skeletal development, *Hox A* proximally along the proximo-distal and *Hox D* in the digital plate (Sordino *et al.*, 1996). After limb field establishment, limb budding is mediated by *Fgf-8* from the flank, signalling via *Wnt-2b* in the prospective limb bud itself. Fore and hind limb buds have common molecular mechanisms but the different morphologies are mediated through the T-box family: for the fore, *Tbx5* and the hind, *Tbx4*. Induction by the mesoderm of the AER and its subsequent induction of mesoderm outgrowth are the responsibility of a *Fgf-10* and *Shh* loop which activates *Fgf 8* in the AER thus maintaining the outgrowth of the mesoderm partly through its expression of *Fgf-10*. Various FGFs (2,4,8) are capable of substituting for the AER role if the AER is removed surgically. Dorso-ventral pattern is regulated by the ectoderm: dorsality via *Wnt-7a* expression. The ZPA is known through classic grafting experiments in a number of species to control the antero-posterior axis and this is now shown in the chick and mouse limb buds to have a basis in Sonic hedgehog (*Shh*) expression which also maintains the AER. As far as differentiation within the digital plate is concerned, the mesenchyme cells essentially have a choice between interdigital cell death or chondrogenesis to form the digits. This choice is mediated respectively by BMPs and TGF- β s (Macias *et al.*, 1999). Digit identity according to one theory is specified by BMP levels (Drossopoulou *et al.*, 2000).

An 'out-group' comparison has been made with the fin buds of the teleost Zebra fish. Early in development there are similarities: the positioning of buds of fore and hind fin and limb appears to involve the same *Hox* genes which also (in the case of *Hox A* and *D*) are similarly expressed in early fin and limb buds. Differences in *Hox D* expression arise distally later in fin and limb bud (Fig. 2). *Shh* expression is localised posteriorly in these fin and limb buds. The possible evolutionary significance of this is discussed later (Sordino *et al.*, 1996).

These molecular findings are very striking from an evolutionary viewpoint but essentially they are confined to only 3 species (2 tetrapods and 1 teleost fish) and there should be care not to generalise too broadly - as do some of the molecular reviews - about a 'molecular bauplan' for tetrapod limb development until it is known to apply to a wider range of species.

Molecular theories of tetrapod digit specification have been put forward as in the 'one domain=one digit' of Tabin (1992) hypothesis. This was based on the finding of a nested set of *Hox D* expression domains across the antero-posterior axis of the digital plate in chick and mouse limb buds. All *Hox D 11-13* genes were expressed posteriorly but only *D 11* anteriorly, corresponding with posterior and anterior digits respectively. But later, more evidence was obtained showing that the posterior pattern spread into the anterior plate and such an oversimplified 'one gene domain, one structure' theory became untenable (Goff & Tabin 1997, Zakany *et al.*, 1997) since *Hox D 13* was now expressed uniformly, ahead of digit development. Instead *Hox D 11-13* appears correlate with a general digit formation capacity of the digital plate (Capdevila and Belmonte, 2000). Thus, a theory of one *Hox* domain specifying one structure does not fit well the clearer recognition now that these genes are multipurpose. More recent interpretations involve theories of *Shh* protein from the ZPA acting at a distance to control BMP levels which are specific for particular digits (Drossopoulou *et al.*, 2000). Alternatively Dahn & Fallon (2000) in classical tissue grafting experiments on chick leg buds suggest digit specificity to be under the control of the adjacent posterior interdigit. It is clear we do not yet have a defined molecular coding for digit specificity and in any case this needs explanation also in terms of AER activity and mesenchyme supply, digit ray condensation and joint formation and differential growth of elements, all needing integration as 'down stream' activities (Horder 1993).

Resolving Problems of the Developmental Basis of Limb Evolution

We now address three problems of limb evolution which all have a developmental base. The first concerns the fin/limb transition with its addition of some structures (eg digits), loss of others (fin rays) together with major changes in skeletal patterning. The second is a problem of limb development in the urodele amphibians with its reversal in the normal timing of digit appearance. Thirdly despite it being a favoured experimental subject, the chick wing bud originates a highly specialised form of the pentadactyl limb. It provides important evidence for resolving a key problem of digit identity from embryological evidence.

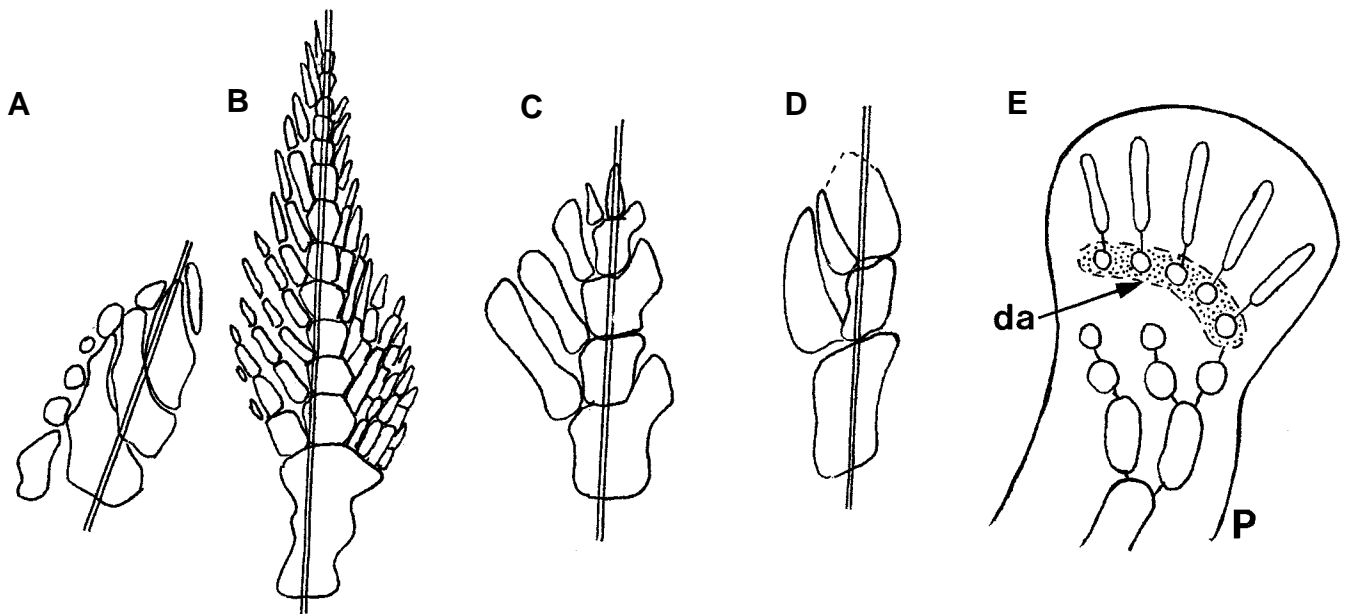


Fig. 3. Definitive paired anterior fin and limb skeleton patterns in (A) the teleost zebra fish (*Danio*) with only endoskeletal radials shown; (B) a dipnoan, *Neoceratodus*; (C) the osteolepiform, *Eusthenopteron*; (D) the Panderichthyid, *Panderichthys* (tetrapod sister group); (E) a generalised pentadactyl limb showing the Shubin-Alberch developmental skeletal bauplan. The hypothetical metapterygial axis is marked for A-D (doubtful for teleosts, A). In (E), it runs postaxially supposedly continuing as the digital arch, da. P, posterior.

Fin to Limb

Lobe-fin or sarcopterygian fish (eg *Eusthenopteron* & *Panderichthys*) rather than teleosts are regarded as the sister group of the early tetrapods (Vorobyeva & Schultze 1991) and as far as the fin to limb evolutionary transition (Fig. 3) is concerned proximal parts (Humerus/femur, radius/tibia, ulna/fibula) are seen as homologous (review in Vorobyeva & Hinchliffe 1996, Coates 1995). Within the lobe fins, the Osteolepiforms are the closest to early tetrapods and they show considerable variety distally in the degree of branching of the fin endoskeleton (Fig. 3, Vorobyeva 1992). Digit origin is more difficult to explain, but the consensus now is that these are newly evolved in the tetrapods. By contrast, the dermal fin rays which fringe the lobe fin are lost in the fin/limb transition.

Since few observations have been made on lobe fin bud development, the Zebra fish paired fin bud has been used as a model for comparison in order to understand the likely developmental basis of the transition. Lobe fin buds (eg *Neoceratodus*) appear to resemble the Zebra fish in having the same two outgrowth phases as described above. First there is a longer lived AER-like ridge which stimulates mesoderm outgrowth relating to the relatively large proximal endoskeleton and then a fin fold invaded by mesenchymal cells which form the fin rays (Fig. 4) (Vorobyeva & Hinchliffe 1996). Thorogood (1991) has proposed a heterochronic theory of fin to limb evolutionary transformation in which the first AER process is prolonged thus generating sufficient mesoderm to form the substantial tetrapod limb skeleton while the final finfold exoskeleton generating phase, reduced in lobe fin buds, is completely eliminated in tetrapods.

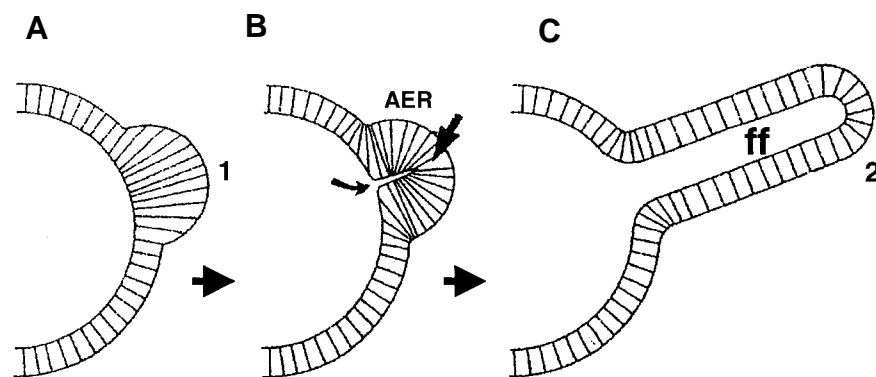


Fig. 4. The ridge (AER) to fin fold (ff) transition in Zebrafish (lobe fins develop similarly). The short-lived AER-like phase 1 generates outgrowth ahead of endoskeletal radial formation. The later fin fold phase 2 is concerned with generating the dermal fin rays. After Thorogood (1991).

Earlier, analysis of the Devonian fossils of *Ichthyostega* (one of the earliest tetrapods), interpreted the tetrapod limb as pentadactyl from the time of its first appearance (Jarvik 1980, 1996). New *Acanthostega* & additional *Ichthyostega* finds together with the Russian Devonian tetrapod *Tulerpeton* have made clear the polydactylous nature of this first limb form and have led to the hypothesis of an aquatic origin of the limb (Coates & Clack 1990, Vorobyeva 1992). Devonian tetrapods have 6-8 digits. Thus digit number has been reduced and stabilised at a maximum of five in the subsequent evolution of the limb**. There is disagreement as to whether pentadactyly arose only once (Laurin 1998) or as Coates (1991, 1996) argues, twice, once on the amniote line and once on the lissamphibian line.

It is best to regard the first forms of the limb in the Devonian as experimental since not merely is the digit number not fixed but unlike those in more recent tetrapods the elements of carpus/tarsus are patterned unusually, while metacarpals/tarsals and phalanges are morphologically poorly differentiated from each other (fig. 37 Coates 1996). Thus it appears that for both the Osteolepiforms and early tetrapods only the proximal parts are stable while distally there is considerable variation (Fig. 3) (Vorobyeva & Hinchliffe 1996). Only in post-Devonian times is the limb development canalised to a maximum digit number and to carpus/tarsus and metacarpals/tarsals and digit phalange morphology (Fig. 3E).

Developmental molecular evidence on the fin-limb transition must remain speculative since comparison is currently between only three species and these very distant from the evolutionary node. This evidence is from a teleost and from two amniote tetrapods, the mouse and chick. There are intriguing initial similarities with *Hox A* and *Hox D* but not in later *Hox A* and *D* expression in Zebra fish fin buds and the limb buds of the two amniotes (Fig. 2) (Sordino *et al.*, 1995). These similarities suggest that proximally Zebra fish fin buds are controlled at least in their early phase by a similar *Hox* (and *Shh*) expression pattern to that in proximal parts of tetrapod limb buds. Teleost paired fish fins have a proximal endoskeleton (the radials) but their definitive structure is quite different from that of tetrapods (Hinchliffe *et al.*, 2001) though skeletal homology in terms of a metapterygial axis (MTA) at the proximal level has been hypothesised (Sordino *et al.*, 1995). The metapterygial axis (Fig. 3) is the main axis of osteolepiforms which is considered to be retained in tetrapods where it passes via ulna/fibula and distally through the distal carpals/tarsals as the 'digital arch' (Coates 1995, Shubin & Alberch 1986 fig.19, but see their discussion of other interpretations of MTA position). The presence of a MTA in teleost fins must be doubted. In fact despite their apparent similarity in molecular control in this particular teleost neither definitive fin endoskeleton nor the underlying skeletogenic processes are at all comparable with those of limbs (Fig. 5 from Hinchliffe *et al.*, 2001, Géraudie 1995). Similar early molecular control may involve early positional cues rather than specification of skeletogenic processes. However, analysing another aspect, Sordino *et al.*, (1995) use the *Hox D11-13* expression pattern of the two tetrapods (found in anterior/distal part of chick and mouse limb buds but not of fish fin buds) to support a theory of a neomorphic origin of digits. They speculate that the new expression pattern is correlated with the digits newly evolved in tetrapods, thus elegantly linking palaeontological discoveries on digit evolution with molecular discoveries.

Digit evolution cannot be separated from the metapterygial axis and its supposed anterior-distal continuation, the 'digital arch'. A single general developmental 'bauplan' for the tetrapod limb skeleton has been proposed in the influential scheme of Shubin & Alberch (1986) (Figs. 3,6,8). This sees the osteolepiform radial branching and metapterygial axis retained in tetrapod evolution respectively as i) radius plus proximal carpals/tarsals and ii) ulna plus digital arch. Newly evolved digits appear to branch in a posterior to anterior sequence from the underlying digital arch (ie on the postaxial side of the MTA) during limb development (Figs. 3,6). The digital arch appears to form initially usually as a single continuous condensation which segments into a series of distal carpals or tarsals each proximal to the meta-element of each digit.

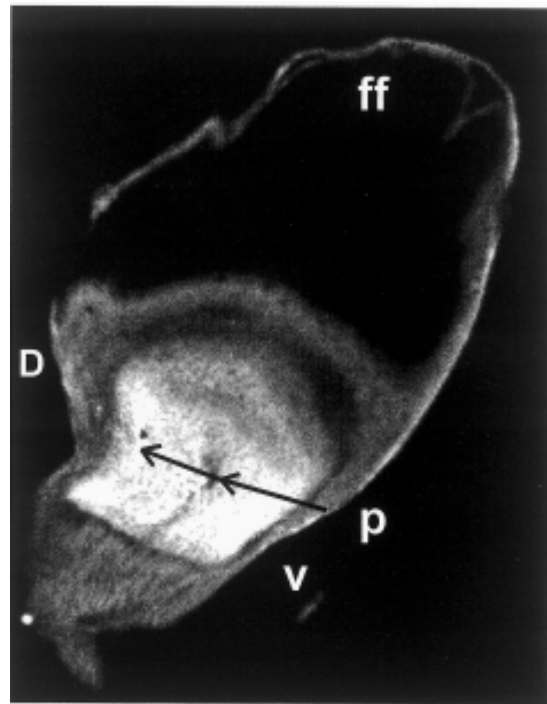


Fig. 5. Pectoral fin development in the Zebrafish. The single prechondrogenic plate (*p*) forms the endoskeletal radials by two subdivisions (the first is beginning). Immunofluorescence using 3B3 for chondroitin-6-SO₄, a condensation marker. (*D*, dorsal; *ff*, fin fold; *V*, ventral).

Digit 4 is usually first, except in urodeles where anterior digits nos 1 & 2 are first (Figs. 7,8). As usual, there are exceptions to any uniform pattern: for example the digital arch forms but fails to segment in the bird leg bud while in the bud of the highly specialised wing, the digital arch is poorly defined. Also the digital arch segments antero-posteriorly in urodeles (see later). While the ulna/fibula lies at the base of the digital branching, pre-axially the radius/ulna only segments off one or two further elements (eg radiale).

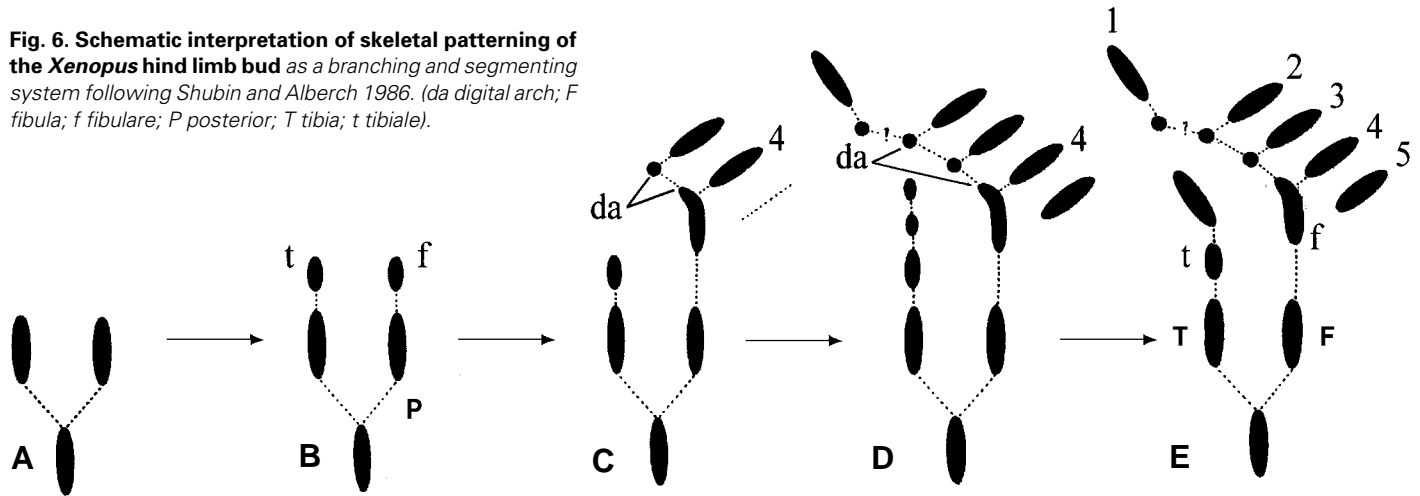
The Shubin and Alberch 'bauplan' scheme attempts also to provide a physico-chemical theory of pre-chondrogenic condensation patterning through branching and segmentation mediated via the extracellular matrix. Here, at the process level, it is on less secure ground (Cohn *et al.*, 2002). The MTA and its supposed extension into the 'digital axis' remain hypotheses rather than morphological facts. But the scheme remains a useful systematisation of many descriptive accounts of patterns of limb development across the vertebrate classes.

Urodele Limb Development: Importance of the Timing of Processes

Urodele amphibians (tailed amphibia: newts and salamanders) are unique among the tetrapods in their unusual sequence of the development of digits which generally form anteriorly to posterior with digits 1 & 2 (plus their associated 'basal commune' at their base) the first to appear. Usually the species used are *Triturus* and the Axolotl (Blanco & Alberch 1992, Shubin & Alberch 1986). The sequence described is distinctly different from that in other

** Footnote. Extant amphibia have a maximum of 4 digits in the forelimb.

Fig. 6. Schematic interpretation of skeletal patterning of the *Xenopus* hind limb bud as a branching and segmenting system following Shubin and Alberch 1986. (da digital arch; F fibula; f fibulare; P posterior; T tibia; t tibiale).



tetrapods where digits develop within the digital plate in posterior to anterior sequence usually beginning with digit 4. Other features of these urodele limbs are the distal to proximal development of carpus and tarsus and the late appearance of the digital arch at digit levels 3-5. These distinctive features appear to represent a major difference from the skeletal developmental bauplan and have led to a theory of the diphyletic origin of tetrapods (Holmgren 1933).

Alberch & his co-workers investigated the properties of the urodele 'bauplan' (and for comparison anuran amphibia) in the Axolotl by experimentally reducing the number of mesenchyme cells in the limb buds (Alberch & Gale 1985). This resulted in stereotyped skeletal losses on the principle of 'last in, first out' – the first forming elements were most likely to survive the treatment. Thus in urodeles digits 4 & 5 were the first to be lost (in anurans it was digit 1, the last to develop) while digits 1 & 2 were the last to be lost. In some species such as *Proteus* the limb buds are naturally miniaturised and here again the surviving hind limb digits may be identified as 1 & 2, while 3,4 & 5 and their associated distal tarsals are missing (Shubin & Alberch 1986). Thus *Proteus* mirrors digit reduction obtained experimentally. It is a paedomorphic (early features retained into the definitive morphology) species and Shubin & Alberch (1986) ascribe its perturbed skeletal pattern not to a truncation of development as a whole but to a truncation of individual branching and segmentation events. These studies provide an insight into the way by which evolutionary changes may occur: in this case through alterations to the timing of developmental processes (Alberch & Blanco 1996). But the variation in pattern is developmentally constrained and particular variants recur both in experimentation and evolution (Alberch & Blanco 1993). Studies on a wide range of urodele species, regardless of phylogenetic relation demonstrate the same limited repertoire of variants, interspecifically and intraspecifically, especially of the carpus/tarsus, suggesting that urodeles have a phylogenetically conserved generative mechanism (Shubin *et al.*, 1995, Schmalhausen 1917, Hanken 1983, Vorobyeva & Hinchliffe 1998).

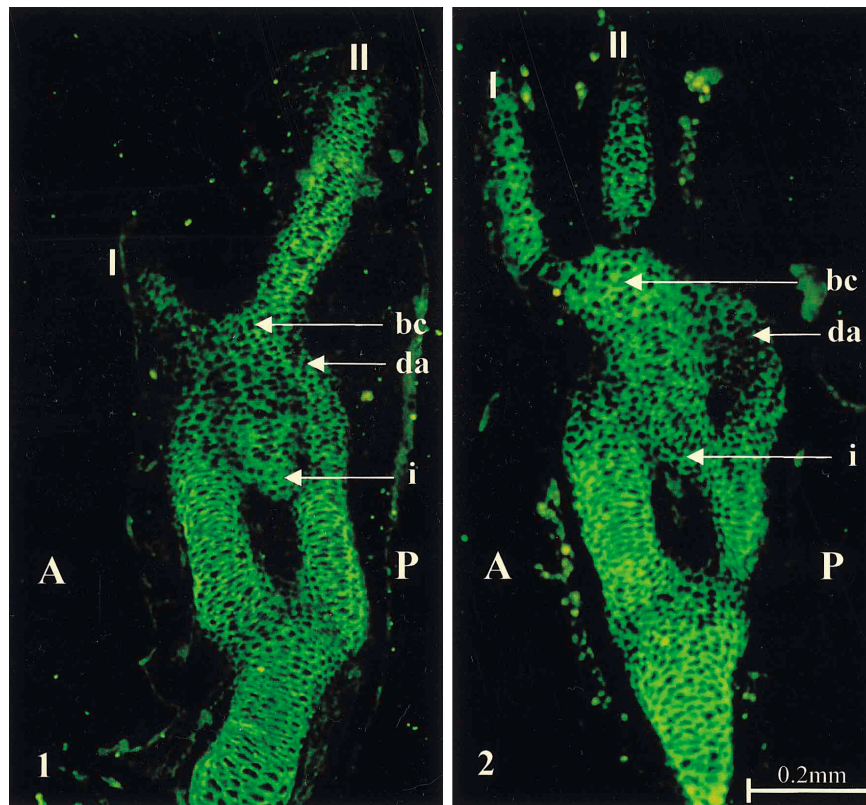


Fig. 7. Skeletal development in the forelimb of two urodeles, the basal hynobiid Salamandrella (1) and the Axolotl (2). In (1), carpus differentiation is proximo-distal with the intermedium (i) in advance of 'bc' and with a relatively early digital arch, da. Apart from early development of digits I and II, this pattern resembles that of other tetrapods (Fig. 8). Immunofluorescence using 3B3 for chondroitin-6-SO4. See Hinchliffe and Vorobyeva, 1999.

While the urodele skeletal 'bauplan' appears strikingly different from that of other tetrapods these differences may be less than has been assumed. The differences are greatest in the more evolutionary advanced urodeles such as *Triturus* and the Axolotl and it these which have

been taken to be generally typical of urodeles (Holmgren 1933). But if more basal urodeles such as the hynobiid family (eg *Salamandrella*, the Siberian Newt) are examined the differences are less marked (Figs. 7,8 – Vorobyeva & Hinchliffe 1996, Hinchliffe & Vorobyeva 1999, Hinchliffe *et al.*, 2001). *Salamandrella* deserves attention as probably the most primitive extant tetrapod which does not have limb reduction. Here - as in other urodeles - digits 1&2 develop first, but the carpus (tarsus) develops more proximo-distally with the proximal intermedium in advance of the more distal 'bc' while the digital arch appears relatively early.

Direct developing urodeles (which omit the free living larval stage) demonstrate some similarities with the hynobiid pattern (Wake & Hanken 1996). The advanced pattern with its early appearance of digits 1&2 and the 'bc' may well represent an adaptive heterochronic variation of the general tetrapod pattern. Uniquely amongst tetrapods many urodele larvae use their limbs while they are developing and contacting the substratum through early development of digits 1&2 and the 'bc' may well give survival value (Hinchliffe & Vorobyeva 1999, Blanco & Alberch 1992). Such selective pressure for acceleration of digit 1 & 2 formation may well be relaxed in direct developers thus revealing the general tetrapod 'bauplan' (Shubin & Wake 1991, Wake & Hanken 1996). Overall these studies show the urodeles have a range of limb skeletal patterning in which the basal hynobiids and direct developers are closer than is generally recognised to the other tetrapods (Fig. 8).

A Theory of Digit Identity Transformation based on Hox Expression

While this account raises no problems for identifying urodele digits as 1-5 as in other tetrapods, the differences in sequence of their formation in the advanced forms such as *Triturus* has been sufficiently puzzling to provoke a theory of digit identity transformation via Hox gene expression by way of explanation. Wagner (1999) notes that in other tetrapods such as mouse, chick and *Xenopus* the domain for *Hox A11* marks out the future zeugopod (eg radius/ulna) region, but the gene is not expressed in the digital plate. But in the Axolotl, a urodele, *Hox A 11* is expressed in the developing digit 3 (and later 4 & 5) which is forming posteriorly and proximally and distinctly later than digits 1 & 2. The gene is not expressed in urodele digits 1 & 2 and not in any digits in non-urodele tetrapods. Wagner's theory is that the first forming urodele digits, conventionally identified as 1&2, in fact represent the original digits 3 & 4 of the pentadactyl pattern, that digit reduction (fairly frequent in urodele evolution eg in *Proteus* – Shubin & Alberch, 1986) has occurred in the evolutionary past when original digits 1 & 2 have been lost, but then at a later date new digits (conventionally 3+4 and in the hind limb, also 5) have evolved. Thus according to the theory, the 'new' digits (3-5) of urodeles are not homologous with specific digits 3-5 in other tetrapods.

One reason for scepticism about this theory is the presence in Axolotls of a well differentiated digital arch, a good general tetrapod marker for digit position (Hinchliffe *et al.*, 2001). Even though the digit formation sequence usual for other tetrapods is reversed in urodeles, their digits 3-5 still form in their normal tetrapod position. Moreover, other molecular evidence does not support Wagner's interpretation. In the axolotl future digit 1 cells do not express *Hox D 11* but the remaining digit forming areas do express it (Torok *et al.*, 1998). In this case the authors argue that the *Hox D 11* anterior border is conserved in all tetrapods between prospective digit 1 & 2. Thus this evidence

from these Hox domains supports the conventional interpretation of urodele digit identities as homologous to those of other tetrapods. It is also the simpler explanation.

Bird Wing Digits, their Development, Identity and the Theory of Dinosaur Origin of Birds

Owing to its accessibility, for at least fifty years the chick wing bud has been favoured for experimental analysis (eg Saunders 1972) and subsequently for molecular study (Capdevila & Belmonte 2001). But the wing bud generates a definitive wing skeleton which is such a highly specialised form of the pentadactyl limb (Fig. 9) that even the identity of its digits is unclear. Study of the digit patterning (including its molecular control) within the limb bud is therefore relevant. On this issue hangs an important and controversial evolutionary question – the possible origin of birds from dinosaurs. But in discussing this we find we are also addressing the developmental 'bauplan' question.

In birds the definitive wing skeleton has 3 digits while the chick wing bud also has posteriorly a small rudiment of a 4th. But which one of the original complement of pentadactyl digits has been lost during the evolutionary digit reduction? This question is not answered by the famous Jurassic fossil of the 'first bird', *Archaeopteryx* since it has a wing already reduced to 3. Palaeontologists on the whole are convinced these represent the 3 theropod dinosaur digits, identified as 1-2-3 on the grounds that primitive forms such as *Herrerasaurus* have three main digits and two posterior digit rudiments (Sereno 1993). Supporting evidence cited is the similarity in the detail of digit phalangeal and carpus structure in birds and in some theropods, such as *Deinonychus* (Ostrom 1977). Cladistic

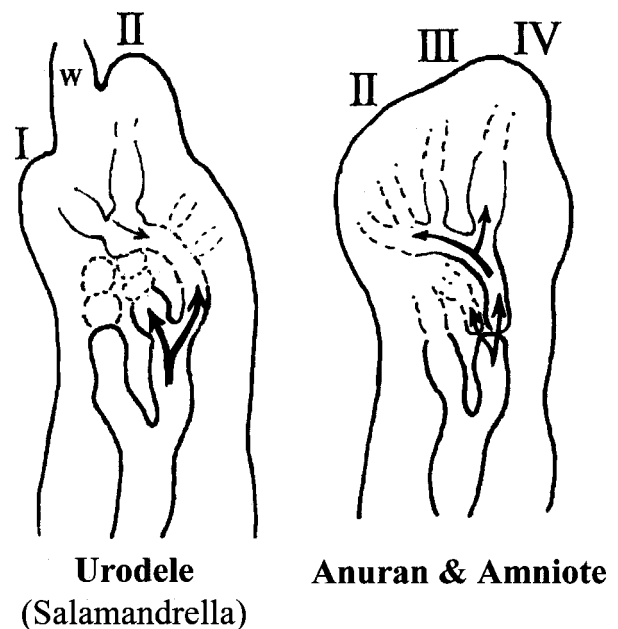


Fig. 8. A single developmental bauplan for the tetrapod limb? Comparison of the basal urodele *Salamandrella* skeletogenic pattern with the anuran and amniote pattern suggests few differences apart from the digit sequence. Earlier accounts (Holmgren, 1933) described an advanced urodele, *Triturus*, with a greater degree of difference. Arrows indicate the sequence of chondrogenic patterning. (w, web, a larval adaptation of *Salamandrella*).

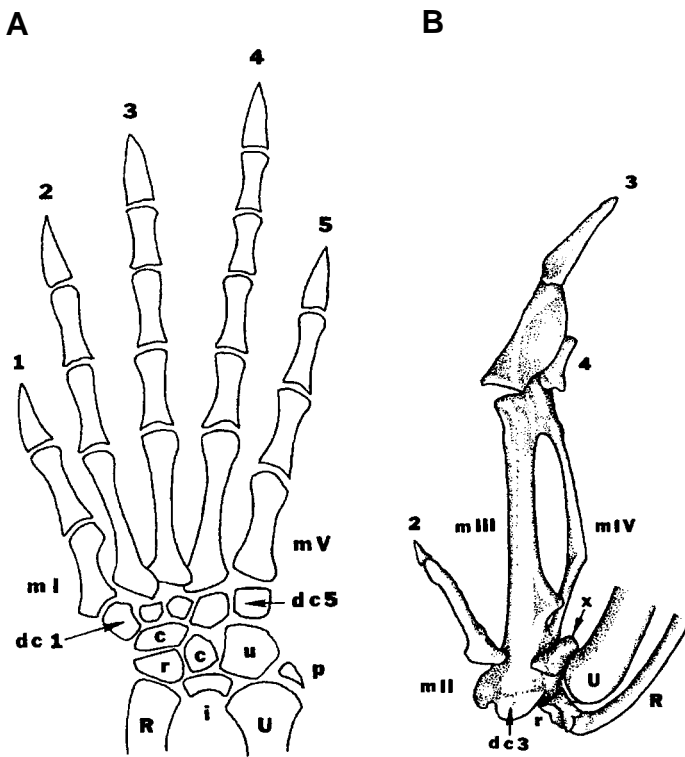


Fig. 9. (A) Reptilian ancestral hand and (B) bird (chicken) wing skeleton. Abbreviations (also for Fig. 10): *c*, central; *dc*, distal carpal; *i*, intermedium; *m*, metacarpal; *p*, pisiform; *R*, radius; *r*, radiale; *U*, ulna; *u*, ulnare; *x*, element *x*.

interpretation assigns significance to the similarity of phalangeal formula in the three theropod and *Archaeopteryx* forelimb digits. There are impressive similarities in many features of the non-limb skeleton. The theropod dinosaur origin (Padian & Chiappe 1998) has become the orthodox theory cited in vertebrate textbooks and museum presentations.

Embryological evidence from the chick wing bud is one of the principal barriers to acceptance of this orthodoxy (Hinchliffe 1997). The convention that developmental biologists adopt for wing digit identity is 2-3-4 (Hinchliffe & Hecht 1984) based on the classic principle of position and connection in a comparison with the bird leg bud and with reptile limb buds. Most studies on 'birds' are those on chick and duck limb buds. Burke and Feduccia (1997) made a comparison between the buds of bird wing and leg (in which all 5 digits are present) and those of crocodiles (the birds' closest living relative) and turtles. Apart from the wing bud all these retained the

5 digits and had a stereotyped pattern of digit position during development. Digit 4 was first to develop and a digital arch identified. Also in experiments rather similar to those of Alberch's on urodeles, in reptile (lizard) limb buds when the mesoderm is reduced, digit 4 is the last survivor where other digits are reduced or lost (Raynaud 1990, Greer 1990). Thus, on similarity of position/connections and timing (4 as the first to form) in development (Fig. 10), wing bud digits were identified as 2-3-4, providing important evidence against the dinosaur origin theory.

A recent observation of Feduccia (personal communication) on the developing wing skeleton in the ostrich supports the 2-3-4 theory. A small temporary additional digit ('1') with 2 elements, anterior to digit 2, was discovered. Ostriches are usually classified as primitive birds and on this evidence during one phase of development their wings briefly may be pentadactyl, clarifying digit identity.

This is a simplified account of a complex and contentious issue which draws in evidence from beyond the limb. The controversies can only briefly be mentioned here (Padian & Chiappe 1998, Feduccia 1999). Dinosaur origin is supported by many skeletal resemblances (Gauthier 1986) – but those of the limb may be a result of convergence. The most bird-like dinosaurs post date *Archaeopteryx* by tens of millions of years. Dinosaur 'protofeathers' are unconvincing while much the most convincing fossil feathers are found in a lizard-like thecodont, *Longisquama* and these predate *Archaeopteryx*, thus raising the possibility of thecodont ancestry. But it is possible that in forming such a specialised limb (including loss of two main digits) as the wing, its bud may have been reorganised at the skeletal bauplan stage (Fig. 10): eg the form of the digital arch is unusual (Figs. 1,2, Hinchliffe 1977) making the distal carpal digit reference points difficult to read (Garner & Thomas 1998). The 2-3-4 digit identity therefore remains a hypothesis, though probably the more likely one.

Molecular evidence is difficult to use to resolve this problem, but Wagner & Gauthier (1999) propose a hypothesis, in line with a dinosaur origin of birds, of transformation of digit identity possibly through the agency of Hox genes. As in Wagner's discussion of urodele digit identity it is a 'frame shift' theory. The authors propose that, based on position, the 3 main wing digit condensations are 2-3-4 in terms of their origin but that they take on the digit 1-2-3 identities of theropod dinosaurs (thus condensation 2 becomes morphologically digit 1, 3 becomes 2 etc.). According to Galis (2001) a possible model is provided by the recent discovery that the boundary between cervical and thoracic vertebrae is controlled by Hox genes which may be manipulated to switch the boundary. Work by Drossopoulou *et al.*, 2000 demonstrated the dissociation of digit number from identity. The gene *Shh* plays a role in the first and *Bmp* level in the second. But as yet there is no molecular

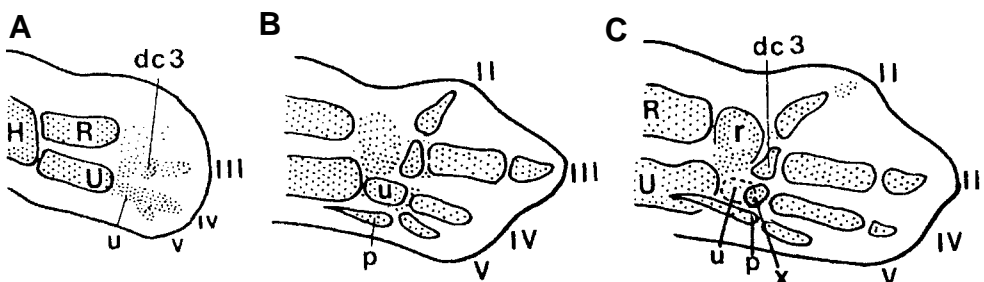


Fig. 10. The pattern of condensations and cartilage elements in the chick wing bud, based on SO4 autoradiographs (Hinchliffe and Hecht 1985). (A) stage 27, (B) stage 28; (C) stage 30. Ulnare is regressing at stage 30. For captions, see Fig. 9.

'signature' known for specific digits and thus the theory remains highly speculative especially as the key embryos are unavailable and other problems with the 'dinosaur origin' hypothesis remain.

The evolution of the bird wing including its ancestry remains difficult to resolve from the available morphological, embryological and molecular evidence. The data needs interpretation and must be assessed in conjunction with other evidence. But the identification of wing digits as 2-3-4 appears to present fewer problems and to involve fewer assumptions.

A Conserved Developmental 'Bauplan' of the Pentadactyl Skeleton?

Even when such deviant limbs as those of urodeles and bird wings are considered there appears to be a general developmental 'bauplan' for the skeleton of tetrapods (Figs. 3,8), including the following features:

- 1 Proximo-distal development
- 2 A preaxial axis of segmentation into radius (tibia) and radiale (tibiale)
- 3 A postaxial axis of a single zeugopod element (ulna/fibula) running via ulnare (fibulare) into the digital arch which forms distal carpal (tarsal) elements which are the base for the apparent branching** of the digits.
- 4 Digit 4 begins the posterior to anterior sequence of digit formation (except for urodeles: see section 3b).
- 5 A maximum of 5 digit rays.
- 6 Connections of the prechondrogenic condensations are stereotyped.

But this is a dynamic and not rigidly fixed stereotypic bauplan. There is no common single set of skeletal elements (except proximally) nor is there a single pattern of segmentation positions (eg chick in Hinchliffe 1977). The latter applies particularly to the digital arch elements and to segmentation of the digital rays into a phalangeal number which is very varied between different taxa. There are some differences in the timing of some of the events, for example in urodeles, and such heterochronic differences may underlie at least some of the important evolutionary changes to the pentadactyl pattern (Müller 1991). Others follow from differential growth of chondrogenic elements such as the fast growing tibia and slow growing fibula in the bird. Reduction in digit number is fairly common throughout tetrapods. The bauplan term is not of course used as signifying a 'developmental cause', but to describe a set of conserved and integrated processes at different hierarchical levels: regulatory gene activity, localised cell surface and migration properties, differential cell division and matrix synthesis, inductive interactions, in fact all the many processes which build the limb.

One interesting test of the conserved nature of the bauplan is whether since the first polydactylous tetrapods the 'five digit maximum' rule can be exceeded. A number of extant tetrapods appear to be polydactylous developing additional 'digits' sometimes fulfilling a clear adaptive role. In the definitive mole and panda limb skeleton there are anteriorly positioned 'extradigits' which

serve respectively digging and bamboo leaf stripping roles (Gould 1983). But these panda extradigits are unlike normal digits and comprise single unjointed rods (the mole structure is curved and at carpus level). Their development is virtually unknown (Milaire 1963 describes a cartilaginous precursor in the mole) but they are usually described as sesamoid implying they form by direct ossification in fibrous or tendon forming tissue. Thus mole and panda keep to the 5-digit rule

In addition, anuran amphibia in almost all cases have a prepollex anterior to the thumb and a pre-hallux anterior to the toe. As described by Fabrezi (2001) these usually have two elements, a proximal carpus like part and a more variable distal part which is sometimes elongated and in burrowing species expanded. Developmentally they differ from digits in their time of appearance and in species where digit 1 is lost, pre hallux and pre pollex are unaffected. Fabrezi (2001) argues they are not homologous with digits.

Among tetrapods only ichthyosaurs (Carroll 1985) appear to evade the constraint of the 5-digit bauplan – their fossils have up to 10 digital rays, often branched. But their limbs are highly unusual as carpus/tarsus and digit distinctions break down as do specific morphological differences between elements such as metacarpals and phalanges.

The difficulty which tetrapods have in evolution in escaping the 5-digit bauplan is a remarkable paradox. Mechanisms have been discovered in experiment and in classical genetics which appear perfectly capable of generating polydactyly in natural species. Extradigits can be provoked in numerous experiments, for example anteriorly following preaxial ZPA grafts or in the interdigit in the chick leg bud by means of incision (Hinchliffe & Horder 1993) or removal of AER or distal ectoderm (Macias *et al.*, 1996). Polydactylous mutants are too numerous to describe. Natural populations of a species frequently demonstrate a remarkable variety of limb skeletal patterning (Hanken 1983, Vorobyeva & Hinchliffe 1998) though the different morphologies provide evidence of design constraints (Shubin *et al.*, 1995). But in nature, the limb remains stubbornly pentadactyl.

Prospects

Explaining at the developmental level the similarity and differences in limb structure during its evolution continues to present a number of challenges. Criteria for classical homology and the homology of the regulatory gene networks which play a role in development of the limb and of other structures, are now recognised as different, making problematic the identification of specific 'genes for limbs' since many have multipurpose roles. Nonetheless, though very few species have been analysed, a common pattern of gene expression found in both early fin buds and limb buds of a teleost fish species and two amniote tetrapods respectively has been identified, together with later differences which may relate to digit evolution at the time of the fin to limb transformation. Three specific problems of evolution are examined in terms of limb development in more detail: the fin to limb transition, and urodele amphibian and bird digit identity & evolution. The theory due to Shubin & Alberch of a developmental 'bauplan' for the limb skeleton is examined and largely supported. Timing differences affecting the same set of developmental processes in different taxa appear responsible for many of the variants in limb structure. The pentadactyl developmental bauplan appears canalised, suggest-

** Footnote Branching has been criticised as a description (Cohn *et al.*, 2002) since it is not strictly proximo-distal. The digit metacarpal/tarsal condensation may precede the related distal carpal (tarsal) condensation. But the resulting pattern appears branch-like and *in vitro* limb mesenchyme has branching properties.

ing it has design constraints, which regulatory genes may affect rather indirectly. Much will be learned from an integrated approach which combines molecular and palaeontological finds with analysis of the processes (including their timing) that build the limb: cell surface changes and migration, local differences in cell division and matrix synthesis, tissue interactions through induction and chondrogenic patterning across the whole range of tetrapod taxa.

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