

# Thumbs Down: A Molecular-Morphogenetic Approach to Avian Digit Homology

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## ABSTRACT

Avian forelimb digit homology remains one of the standard themes in comparative biology and EvoDevo research. In order to resolve the apparent contradictions between embryological and paleontological evidence a variety of hypotheses have been presented in recent years. The proposals range from excluding birds from the dinosaur clade, to assignments of homology by different criteria, or even assuming a hexadactyl tetrapod limb ground state. At present two approaches prevail: the frame shift hypothesis and the pyramid reduction hypothesis. While the former postulates a homeotic shift of digit identities, the latter argues for a gradual bilateral reduction of phalanges and digits. Here we present a new model that integrates elements from both hypotheses with the existing experimental and fossil evidence. We start from the main feature common to both earlier concepts, the initiating ontogenetic event: reduction and loss of the anterior-most digit. It is proposed that a concerted mechanism of molecular regulation and developmental mechanics is capable of shifting the boundaries of *hoxD* expression in embryonic forelimb buds as well as changing the digit phenotypes. Based on a distinction between positional (topological) and compositional (phenotypic) homology criteria, we argue that the identity of the avian digits is II, III, IV, despite a partially altered phenotype. Finally, we introduce an alternative digit reduction scheme that reconciles the current fossil evidence with the presented molecular-morphogenetic model. Our approach identifies specific experiments that allow to test whether gene expression can be shifted and digit phenotypes can be altered by induced digit loss or digit gain. *J. Exp. Zool. (Mol. Dev. Evol.)* 9999B: 1–12, 2013. © 2013 Wiley Periodicals, Inc.

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The problem of the avian forelimb homologies has engaged interest and debate in many areas of comparative biology and EvoDevo (for reviews, see Chiappe, '95; Galis et al., 2005; Stopper and Wagner, 2005; Wagner, 2005; Bever et al., 2011; Young and Wagner, 2011; Xu and Mackem, 2013). This far-reaching interdisciplinary interest can be attributed to the multi-level nature of the problem: the phylogenetic origin of birds; the origin of flight; the functional and anatomical transformations of forelimbs; homology assignment based on phenotypic characters, developmental processes, or genetic correlations; the molecular and developmental mechanisms underlying the transition; general insights into the relations of developmental patterning with morphological evolution.

The most widely accepted theory of avian origins, owing mainly to numerous skeletal similarities, is that birds are descendants of

theropod dinosaurs. This theory was proposed (or reestablished) by Ostrom ('73, '76) and gained further support from subsequent research including Gauthier ('86), Müller and Streicher ('89), and

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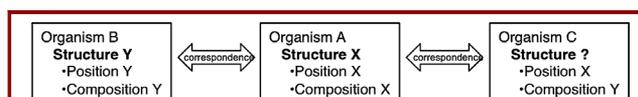
Sereno ('99). The mosaic anatomy of *Archaeopteryx*, which includes features of birds and theropods (Ostrom, '73; Ostrom, '76; Christiansen and Bonde, 2004), as well as other recent discoveries of non-avian maniraptorans with feathers (Ji et al., '98, 2001; Zhou et al., 2003) reinforce the theropod descent hypothesis (Welten et al., 2005). However, not all arguments for the alternative archosaur origin theory have been invalidated (Feduccia, 2012).

The major difficulty with the theropod dinosaur ancestor theory is the persistent discrepancy between anatomical and embryological data regarding the identity of avian forelimb digits. Morphological evidence, especially the adult phalangeal formula, indicates that the three digits of the bird wing are DI, DII, and DIII—thumb, index finger, and middle finger of the pentadactyl hand (Sereno, '99; Wagner and Gauthier, '99). The main lines of evidence are the resemblance of the first metacarpal of theropods to that of basal, pentadactyl dinosaurs (Gauthier, '86; Wagner, 2005), and the fact that dinosaurs such as *Herrerasaurus* (Reig, '63) and *Eoraptor* (Sereno and Arcucci, '94), which are considered to represent basal theropods, have two reduced or vestigial digits posterior to the three fully formed ones. This identification is further supported by the morphology of avian digits (Sereno, '99; Wagner and Gauthier, '99), and, depending on one's point of view, by the expression patterns of genes of the 5' *hoxD* cluster in the bird wing which resemble the ones of DI, DII, and DIII in alligators and mice (Vargas and Fallon, 2005a,b; Vargas et al., 2008). The most recent evidence in support of the anterior identification comes from transcriptomic analyses that indicate molecular commonalities in the anterior most digits of the chicken fore and hind limb buds (Wang et al., 2011).

Contrasting embryological evidence shows that avian digits develop from the anlagen of precartilaginous condensations II, III, and IV (Müller and Alberch, '90; Feduccia, '96; Burke, '97; Hinchliffe, '97; Feduccia and Nowicki, 2002; Kundrát et al., 2002; Larsson and Wagner, 2002; Welten et al., 2005; de Bakker et al., 2013). This finding is consistent with "Morse's Law," a generalization of the phyletic observation that the first digit to be lost from the pentadactyl state in amniotes is usually digit I, followed by digit V. The fact that this sequence of digit loss represents an inversion of the sequence in which the digits form ontogenetically corroborates this observation, and so does the developmental lability of the two digit anlagen which has been experimentally confirmed by mitotic inhibition experiments, showing that DI is the first digit to disappear in treated anuran and amniote embryos, with the exception of salamanders in which DV forms last and disappears first (Müller, '94). Since digit formation in all studied reptilians starts with the digit IV condensation, that is, the primary axis (Burke and Alberch, '85; Shubin and Alberch, '86; Müller and Alberch, '90), followed by III–II–V–I, it is not surprising that morphoclines of limb reduction in extant reptilians, such as skinks (Greer, '87, '90), exhibit the reverse sequence of digit loss: I–V–II–III–IV.

New support for the DII, DIII, DIV identification came from the discovery of a transitory precartilaginous condensation anterior of those three digits in avian forelimb buds. Another condensation on the posterior margin had already been known (Hinchliffe, '77, '85). Injections of India ink into chick limb bud (Kundrát et al., 2002) visualized the condensation by demonstration of an avascular zone that arises with early cartilage formation. Peanut agglutinin staining of histological sections of chick limb buds (Larsson and Wagner, 2002) and alcian blue staining of ostrich embryos (Feduccia and Nowicki, 2002) were also able to visualize the digit I vestige. Whole mount in situ hybridizations with *sax9* probes provided molecular evidence for the anlage of the anterior-most digit (Welten et al., 2005). Furthermore, the discoveries of feathered non-avian maniraptorans, such as *Protarchaeopteryx robusta* and *Caudipteryx zoui* (Ji et al., '98; Ji et al., 2001; Zhou et al., 2003), and recently also of the large basal tyrannosauroid *Yutyrannus huali* (Xu et al., 2012) and the megalosauroid *Sciurumimus albersdoerferi* (Rauhut et al., 2012) strongly support the theropod descent.

Given the accumulating information from both developmental biology and paleontology, the problem of avian digit homology becomes more a problem of homology concepts in general, since positional and compositional homology criteria (Ramírez, 2007) are in conflict (Fig. 1). A positional designation marks the topology in which the structure is formed, typically relative to other structures, while compositional criteria describe the phenotypic features of the structure, such as morphological shape, developmental behavior, or the transcriptome of the cells that contribute to the structure. In the case of birds, the positional features identify the digits quite clearly as II, III, IV, whereas compositional information suggests they could be I, II, III. To distinguish the two approaches, we will use Roman numerals to label digits identified by means of topological position, and Arabic numbers for identifications based on phenotypic composition.



**Figure 1.** Homology assignments to structures in different animals. Typically a structure has specific positional and compositional features, which together give identity to it, and allow it to be compared to homologous structures in other animals. Structure Y in Species B can be considered homologous to structure X in Species A. However, if compositional and positional information are in contradiction with each other no identity can be assigned to the structure and finding homologous structures in other animals is difficult. Species C has a structure that shares compositional features with structure Y and positional ones with structure X. Therefore, an unambiguous homology with either structure cannot be assigned. Modified after Ramírez (2007).

## CONCEPTS OF AVIAN DIGIT HOMOMOLOGY

Over the past two or three decades a significant increase in digit homology concepts took place, primarily induced by new methodologies and discoveries. Departing from different levels of observation, significantly different homology assumptions underlie these concepts.

### The Archosaur Descent Hypothesis

One approach is to exclude birds from the dinosaur clade (Feduccia, '96; Burke, '97; Feduccia and Nowicki, 2002; Feduccia et al., 2005), thus bypassing the theropod stage of forelimb reduction. This classical solution had its main renaissance with Alan Feduccia's book *The Origin and Evolution of Birds* (Feduccia, '96). The incompatibility of developmental and paleontological data concerning digit identification is among the prime arguments for excluding birds from the dinosaur clade. Instead a descent from more basal archosaurs was suggested. At that time the identification of the avian digits hinged much on the recognition of the primary axis in developing limbs (Alberch and Gale, '83; Burke and Alberch, '85; Müller and Alberch, '90), because an unambiguous identification of the adult digits was not possible. Therefore, the discovery of a digit I vestige (Feduccia and Nowicki, 2002; Kundrát et al., 2002; Larsson and Wagner, 2002; Welten et al., 2005) much favored the hypothesis.

A major flaw of the archosaur descent hypothesis (ADH) was its (partial) circularity: Digit identification is used in taxonomical assignment, which in turn is used in establishing digit homology. It had a solution for the problem of digit homology, but at the same time the mosaic features of *Archaeopteryx lithographica* had to be explained as convergent evolution. Since the discovery of feathered dinosaurs more basal than *Archaeopteryx* (Ji et al., '98; Zhou et al., 2003; Xu et al., 2012), most researchers no longer consider a different origin of birds and dinosaurs, although others have argued that most of the "feathered dinosaurs," the maniraptorans, actually represent an early radiation of birds, whereas filamentous traces in real dinosaurs may not be feathers (Feduccia et al., 2005).

### The Axis Shift Hypothesis

The axis shift hypothesis (ASH) was the first approach that included the embryological evidence, while also considering a broad set of synapomorphies that link birds to dromaeosaurid theropods (Gauthier, '86; Chatterjee, '97, '98). It argues that if the primary axis (Alberch and Gale, '83; Burke and Alberch, '85; Müller and Alberch, '90) had shifted in avian evolution and instead projected through digit III in present day birds, this would identify the wing digits as I, II, III (Shubin, '94; Chatterjee, '98).

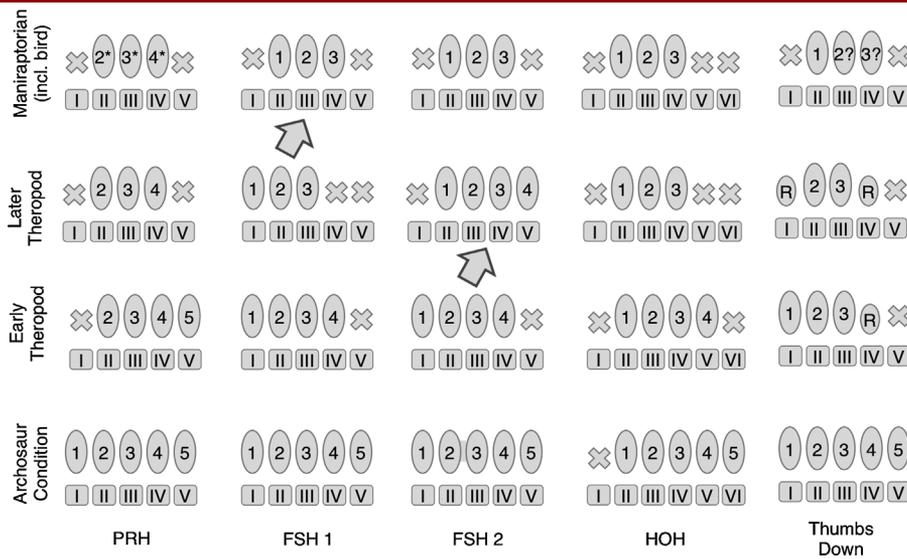
This contention was somewhat supported by salamander development, in which digit II is the first to be formed and not digit IV (Shubin and Alberch, '86)—even though it is not certain that this is true for the condensation stage—but no such case is known in sauropsids. Support for the axis perturbation idea came

from *Tyrannosaurus*, which apparently lost digit IV, leaving only two fingers, and from the fact that the ulnare, which usually forms part of the primary axis, is lost in birds (Hinchliffe, '85). Garner and Thomas ('98) pointed out that if the theropod hand lost digit IV, the only possible digit for the avian primary axis would be digit III. Again, an identification as DIV in birds only would be possible by excluding them from the theropods. Using the primary axis as support for a non-dinosaurian hypothesis, again, would be tautological and lack a clear separation between hypothesis, evidence, and conclusion (Garner and Thomas, '98). Although this ASH offered a plausible and parsimonious solution to the problem, it is not considered any longer since the discovery of the avian digit I vestige, which identified the bird wing digits clearly as positions II, III, IV. However, Wagner (2005) has pointed out that it is possible, although not very likely, that this condensation represents a kind of pre-pollex instead of digit I, and then the I, II, III assignment could still be correct; data from alligator show no condensations anterior to digit I, hence the pre-pollex identification is unlikely (Larsson et al., 2010).

### The Frame Shift Hypothesis

The frame shift hypothesis (FSH) of Wagner and Gauthier ('99) offers yet another kind of solution, in which a homeotic shift causes digits I, II, and III to form instead of II, III, and IV. The FSH was proposed after the ASH, but before the latter was ruled out. Its core assumption is a possible dissociation between the formation of a generic structure and its phenotypic identity (Wagner and Gauthier, '99). Examples in which organ formation does not directly determine organ identity (Hall, '94) were the basis for this idea (Wagner, 2005). Whereas previous hypotheses attempted to reject the inferences from one set of evidence (fossil or developmental) in favor of the other, the FSH assumes that both identifications are correct (1, 2, 3 = II, III, IV; Fig. 2). It argues that dinosaurs faced a selective pressure to reduce posterior digits, but developmental constraints only allowed the digit anlage that formed last to be reduced first, that is, DI followed by DV. Thus the embryonic digit primordia were reduced bilaterally, while the adult ones were lost on the posterior side.

The frame shift was originally thought to have taken place after the reduction from four to three fingers, that is, after *Coelophysis* and *Torvosaurus* (Galton and Jensen, '79) but before *Allosaurus* branched off (Wagner and Gauthier, '99; Bever et al., 2011). After the finding of the ceratosaur *Limusaurus inextricabilis* (Xu et al., 2009), which displays a tetradactyl forelimb with digit I reduced further than digit IV, the hypothetical frame shift had to be moved to a time before the loss of digit IV (Bever et al., 2011). The FSH now argues that digit V at position V was lost first, then the frame shift would have occurred, leading to the formation of digits I–IV at positions II–V with position I being lost; then position V would have been reduced again, thereby losing digit IV, leaving a tridactyl hand with digits I–III at positions II–IV (Bever et al., 2011, Fig. 2).



**Figure 2.** Schematic overview of the major digit reduction hypotheses for the avian lineage. Squares mark positional features (topological identity), and circles compositional features (phenotypic appearance) of digits. Crosses designate reduced digits, asterisks mark strongly derived phenotypes, diagonal arrows indicate hypothetical frame shifts. The bottom row shows the ancestral archosaur condition. The middle rows show the theropod trend, (e.g., *Herrerasaurus*, *Eoraptor*, *Coelophysis*). The top row shows the final tetanuran condition, which includes birds (e.g., *Deinonychus*, *Gallus*). The pyramid reduction hypothesis (PRH) argues that initially digit I was lost in the theropod lineage, leaving the four posterior digits (e.g., *Coelophysis*), then digit V is reduced, and the three central digits remain. The frame shift hypothesis (FSH1) assumes the loss of both posterior digits, followed by a shift that caused the anterior digits to be formed from the central positions. After the discovery of *Limusaurus* with its strong reduced digit I, the shift was predated to the four digit state (FSH2). After the shift, position V would be lost again, this time actually deleting digit IV. The hexadactyl origin hypothesis (HOH) argues that originally a first digit existed that already was a vestige in the archosaur ground state, then the posterior-most digit is lost (digit V from position VI) followed by the adjacent one (digit IV from position V). Modified after Welten et al. (2005).

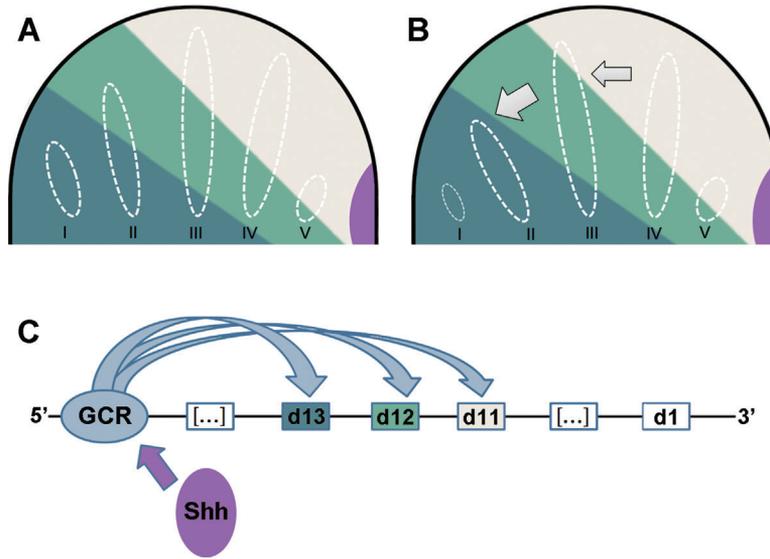
A clear advantage of the FSH is that it does not exclude any kind of evidence (paleontological, developmental, or morphological). Its main drawback is that it has to assume the possibility that a structure can be removed from its location to be formed elsewhere without losing its identity. In support of this idea it has been suggested that embryological characters such as digit position should be weighted less than adult morphological characters or gene expression patterns, because they reflect the identity of structure to a lesser extent (Young and Wagner, 2011). Thus the FSH favors a homology concept that is based on compositional features and not on positional ones. Another point of critique has been the plausibility of such a model and the kind of selective pressure that could cause the loss of one digit, while at the same time replacing it by another one (Galis et al., 2003).

The frame shift view has gained molecular support from studies of *hoxD11*, *hoxD12*, and *hoxD13* expression patterns (Vargas and Fallon, 2005a,b; Vargas et al., 2008). These works showed that in the avian hind limb buds and in the pentadactyl limb buds of mice and alligators the anterior-most digit is the only one negative for

*hoxD12* expression. Also, in the avian forelimb bud the anterior-most digit has no *hoxD12*, but, unlike in the other examples, it originates from position II and not from position I. Following this approach, Wang et al. (2011) analyzed the transcriptome of digit precursor cells. Their study shows a linkage between the anlagen of forelimb DII (anterior-most) and hind limb DI (also anterior-most), but it was not possible to link the other two wing digits with any hind limb digits unambiguously.

#### The Pyramid Reduction Hypothesis

With the discovery of precartilag condensation zones at the position of digit I in chicken and ostrich (Feduccia and Nowicki, 2002; Kundrát et al., 2002), the II, III, IV identification was strongly favored, and hence the question arose as to whether it was possible that the theropod ancestors also had a bilaterally reduced manus with digits II, III, IV (Kundrát et al., 2002; Galis et al., 2003; Fig. 3). Since the reduction from five to four fingers could have happened by losing digit I and not digit V, this would be possible. This would make things a lot easier, but the problem is



**Figure 3.** (A) Schematic of a hypothetical right fore limb autopod of an avian or theropod embryo, with digit I developing normally. (B) Right fore limb autopod of an extant bird (chick), in which the development of digit I ceases at pre-cartilage stage. Anterior is to the left, posterior to the right. The purple region is the zone of polarizing activity (ZPA), and the shaded regions indicate the nested expression of *hoxD13*, *12*, and *11*. Broken white outlines mark prospective digit condensation areas. Arrows mark the proliferation shift in the growth of the respective digits; arrow size reflects the strength of the effect. The presumptive digit forming from position II leaves the zone in which it could express *hoxD12*. C: After the establishment of the ZPA, the general control region (GCR) is activated under Shh influence (after Deschamps, 2004). It affects the closest genes most strongly, as indicated by the arrows. Thus *hoxD13* is expressed throughout the limb bud, whereas *hoxD12* and *hoxD11* are far more restricted, and *hoxD* genes that lie even further toward 3' are no longer expressed in the limb bud.

that the fossils of *Eoraptor* and *Herrerasaurus*, which are considered basal theropods, show reduction of both posterior digits (Gauthier, '86; Padian, '92; Sereno, '99). The pyramid reduction hypothesis (PRH) argues that the attribution of these taxa to the theropods is not unambiguous (Galis et al., 2003), and even if they are included in the theropod clade, their position within it relative to the birds, is not clear (Sereno, '94; Galis et al., 2003). According to the PRH, digit loss proceeds symmetrically from the anterior and posterior rims of the limb, that is, digit I and digit V are lost and the remaining digits in the birds are II, III, IV.

In the initial presentation of the concept, Kundrát et al. (2002) also suggested a mechanism that would be able to derive the *Archaeopteryx* phalangeal formula from the archosaur one. While the archosaur ground state is thought to be DI(2)–DII(3)–DIII(4)–DIV(5)–DV(3), *Archaeopteryx* could have DI(2)–DII(3)–DIII(4)–DV(0)–DV(0), but could also be interpreted as DI(0)–DII(2)–DIII(3)–DIV(4)–DV(0). Experiments with molecular signaling pathways in early limb development have shown that modulating interdigital *bmp* signaling (Dahn and Fallon, 2000) or blocking *bmp* with a dominant negative receptor (Zou and Niswander, '96) is able to remove one phalanx from each digit, and therefore a mechanism like that could have caused the

archosaur central digits to resemble the *Archaeopteryx* ones with regard to their phalangeal numbers.

Further developmental support for the PRH is provided by experimental evidence from studies reducing cell numbers in limb buds locally (Alberch and Gale, '83) or systemically in entire embryos (Müller unpublished, briefly described in Müller, '94). The results show that digits I and V of pentadactyl limbs are the most labile to perturbation and can easily be caused to disappear without much pathological effect on other parts of the autopod skeleton. Furthermore, comparative studies of digit reduction sequences in extant reptilians, such as the skinks (Greer, '87, '90) also attest to the repeated and independent realization of the PRH pattern of reduction. As pointed out above, the sequence of phylogenetic digit loss in these reptilian cases usually represents a reversal of the sequence of their appearance in embryonic development.

The question remains, how much weight should be placed on the fossil evidence? Even Frame Shift supporters admit that the phylogenetic position of *Eoraptor* is problematic, because most apomorphies that link this fossil to the theropods are hand characteristics and, therefore, are not applicable in this case (Wagner, 2005). Taking this into account leaves only one fossil

(*Herrerasaurus*), with its unresolved precise taxonomical position, in conflict with the PRH. This makes the PRH the most parsimonious approach to date.

#### The Hexadactyl Origin Hypothesis

A last alternative, included here for completeness, considers the possibility that the tetrapod ancestor had six (or more) digits (Welten et al., 2005). In this case the vestigial digits of *Herrerasaurus* would really be DV and DVI and not DIV and DV (Fig. 2). The vestigial state of DI would therefore have been the archosaur ancestral condition. Furthermore Welten et al. argue that the pisiform or the Element X of birds could be interpreted as a vestigial digit VI, and the same has been suggested for the mammalian pisiform (Bardeleben, 1889; Holmgren, '52). This would also make digit reduction in dinosaurs bilateral again, but there is no evidence for six or more fingered archosaurs. It also counts against the hexadactyl origin hypothesis (HOH) that the pattern of *sor9* expression or any other condensation marker used so far gives an indication of a digit anlage posterior of digit V (Welten et al., 2005).

None of the current hypotheses on avian digit homology is able to explain all of the evidence completely. Only the ASH and the HOH can be ruled out with some certainty due to embryological and fossil evidence. The ADH boils down to the PRH. At present, this leaves the PRH and the FSH as the most plausible models for avian digit homology. Both have their flaws. Neither can be tested directly, since living dinosaur embryos are not available and digits do not tell their identities voluntarily. In the absence of embryos that demonstrate ancestral forelimb development, it is necessary to stick to bird development and see if it can provide new approaches to the digit identity question.

#### An Integrative Approach: The Thumbs Down Hypothesis

The new approach presented here traces the origins of both the FSH and the PRH to a common source: the loss of digit I. We propose a morphogenetic-molecular model in which the changing transcriptome as well as the altered phalanx number in birds are direct consequences of the reduction of the anterior-most digit. Because this effect is thought to be triggered once digit I is lost, we have dubbed it the thumbs down hypothesis (TDH).

#### Morphogenetic and Biomechanical Aspects

The ontogenetic basis for altered digit morphologies might be found in changes to the spatial orientation of cell proliferation or cell displacement triggered by the absence of the digit I anlage. As soon as the development of the primordium for digit I ceases or merely lags behind the one of the other digits, cells that would usually contribute to the formation of digits II–IV, expand into a more anterior region where they partially behave as if they were digits I–III. The reason for this relocation is likely to be morphogenetic or biomechanical, rather than following from gene expression. If cells in the anterior region proliferate more

slowly or stop completely, they will make way for the more rapidly proliferating cell masses of prospective condensations II, III, IV. The subsequent change in the cell's fate (D1-phenotype instead of D2-phenotype; Fig. 3A and B) would be caused by the increased distance from the zone of polarizing activity (ZPA). It is emphasized that this mechanism is hypothetical, and a reduced cell proliferation rate in the anterior limb bud region has not been shown yet. The positional identities of digits do not change during this process; only the cellular environment, cell behavior, and the morphology of the digits (compositional features) are altered to some extent. The time frame during which this can take place is limited by the condensation of autopod elements, since already condensed cells can no longer proliferate (Newman and Bhat, 2007). Based on *sor9* expression (Welten et al., 2005) this must take place before stage 26.

#### Molecular Mechanisms

Since digit I usually develops outside of the Sonic Hedgehog (Shh) expression domain of the early limb bud, and digit II within a Shh gradient (Harfe et al., 2004), a move of digit II toward anterior would cause it to behave as if it were digit I, due to a lack of Shh signaling (Fig. 3A and B). This would affect the transcriptome and as a consequence the phenotype (e.g., the phalangeal formula). Furthermore, digit II and partially digit III rely on a Shh gradient (Harfe et al., 2004), so their cellular behavior would not be altered to the same extent as it would be for digit I, which relies on the absence of Shh protein. The observation that Shh is not detectable after stage 26 (Harfe et al., 2004, Dunn et al., 2011) supports a terminus ante quem of stage 26/27. For the transcriptomes of digit cells to be altered in such a way that they resemble another digit, it would be necessary that the Shh gradient targets one or more genes that lie quite far upstream. Possible candidates for this could be the genes of the 5' *hoxD* cluster (Deschamps, 2004; Zakany, 2004).

In early limb development *shh* expression requires HoxA or HoxD signaling (Kmita et al., 2005). For the early limb bud Tarchini et al. (2006) have shown that *shh* expression in the ZPA is activated by the 5' genes of the *hoxD* cluster—specifically *hoxD10* and, more important, *hoxD13*—which at that time are expressed only at the posterior end of the limb bud, probably due to anterior repression by Gli3 (Zúñiga and Zeller, '99). However, after the establishment of the ZPA, under the influence of Shh, a new enhancer for the *hoxD* cluster is activated, the general control region or global center region (GCR; Fig. 3C; Deschamps, 2004; Zakany, 2004). This enhancer region affects the 5' most gene most strongly, hence *hoxD13* is expressed throughout the limb bud, while the *hoxD12* domain excludes digit I, and *hoxD11* is even more restricted (for a review of *hoxD* expression in the developing limb, see Wagner and Vargas, 2008). Thus the *hox* expression pattern of the chicken fore limb (Vargas and Fallon, 2005a)—digit II lacks *hoxD12*—can be explained by a greater distance from the ZPA.

Recently Delpretti et al. (2012) showed that genes of the posterior *hoxD* cluster have a direct influence on the lengths of digits and limb segments in general. In mutant mice with deletions in *hoxD12-10* the lengths of metacarpals and phalanges were reduced to less than 90% of the wild-type lengths.

It is plausible that the number of phalanges changes when the transcriptome of the cells forming the digit changes. But a model in which the number of phalanges depends directly on the ZPA is also conceivable. Proximodistal outgrowth is regulated by FGF signaling from the apical ectodermal ridge (AER; for a review of vertebrate limb patterning, see Benazet and Zeller, 2009). Since Shh signaling from the ZPA is required to maintain the AER (Chiang, 2001; Kraus et al., 2001; Harfe et al., 2004; Tarchini et al., 2006), the number of phalanges is likely to change if their progenitor cells receive a lower dose of FGF due to altered positioning. The maintenance of FGF signaling from the AER works via feedback between *fgf*, *shh*, *bmp*, and *grem1* (Benazet et al., 2009; Benazet and Zeller, 2009). This loop has the ability to terminate itself once the distance between *grem1* and *shh* expressing cells, whose descendants cannot express *grem1* themselves, becomes too large (Scherz, 2004). Thus limb bud outgrowth and patterning is terminated due to a down regulation of *grem1* at a certain distance from the ZPA (Verheyden and Sun, 2008). This could also be responsible for lower phalanx numbers in digits situated more anteriorly. The altered transcriptome of digit II cells, however, is not explainable by this mechanism alone.

It has been shown that the digit phenotype can be altered at late stages (34–36) by modulating cell signaling in the inter-digital mesenchyme (Dahn and Fallon, 2000; Suzuki et al., 2008). This suggested that limb bud cells acquire a memory of positional identity from prior Shh signaling, which later determines phalangeal shape (Harfe et al., 2004; Suzuki, 2013). This fits well with the TDH since it argues that encountering a different cellular environment alters the digit phenotype and transcriptome.

#### Models of Vertebrate Limb Patterning

The bony elements in the bird limb are formed in proximo-distal progression: the stylopod forms first, followed by the zeugopod, and the autopod, the most relevant region for our purpose, forms last. Previous models for limb skeleton formation (reviewed in Towers and Tickle, 2009) have suggested that the time a cell spends in the progress zone decides which structure it forms (Summerbell et al., '73; Summerbell and Lewis, '75) or that cell fate is decided very early in this process (Dudley et al., 2002). The most recent model (Newman and Bhat, 2007; Zhu et al., 2010) suggests a reaction diffusion system that patterns the vertebrate limb bud. In this model, three zones are distinguished in the limb bud according to their position relative to the AER: the apical zone, the active zone, and the frozen zone. In the active zone the cells express FGF receptor 1 (FGFR1) that keeps the cells in a proliferative state and suppresses cell condensation in this area. When the cells reach the active zone, FGFR1 is down regulated and instead a second FGF

receptor (FGFR2) is expressed. Those cells start expressing *tgf- $\beta$* , which is positively autoregulatory and also enhances the expression of fibronectin and its own inhibitor. The enrichment of glycoproteins, such as fibronectin in the extracellular matrix favors the forming of cell condensation that will form cartilage later. The simultaneous expression of an inhibitor of TGF- $\beta$  restricts the condensations; therefore, they are limited to the vicinity of the TGF- $\beta$  maxima.

It has also been suggested that an additional lateral inhibition system that uses the *delta/notch* pathway could be involved. This combined local autoactivation–lateral inhibition (LALI; Meinhardt and Gierer, 2000) system is capable of forming repetitive pre-chondrogenic structures that are equally spaced from each other. The number of those elements depends on the size of the LALI-zone. Since available space is a key determinant for size and number of bony structures (e.g., digits), it is well conceivable that its neighbor suppresses a lateral element, if its growth rate is substantially lower. Once the core condensation pattern is laid down, the individual elements grow and differentiate with respect to their morphology and gene expression. Hence single digits can be exposed to variable morphogen gradients after they are originally formed, giving them plenty of opportunities to change their adult phenotype compared to homologous structures in other vertebrates or in the same animal. The reaction-diffusion model (Newman and Bhat, 2007) is in accordance with the TDH and even supports it, since it allows for the number of digits in a vertebrate limb being altered during development. Furthermore it sees the initial formation of digit primordia and the later differentiation as two autonomous modules, which is in favor of the TDH as well.

#### Compatibility With Experimental Results

Based on earlier findings (Scherz et al., 2007), Vargas and Wagner (2009) treated chick limbs with cyclopamine (a hedgehog pathway inhibitor; Chen et al., 2002) in order to generate phenotypes with altered digit patterns. When they applied cyclopamine between stages 18 and 21 (Hamburger and Hamilton, '92), they obtained anterior digit phenotypes at posterior locations: positions III and IV formed digits with D1 and D2 phenotypes (normally forming at positions II and III). These results are consistent with the TDH, and even support it strongly, because cyclopamine reduces the activity of the hedgehog pathway and therefore shifts the boundaries of *hoxD11* (Fig. 3, pink) and *hoxD12* (Fig. 3, turquoise) toward the posterior (Vargas and Wagner, 2009). Thus the digits arising from this region encounter the *hox* and *shh* environment of their anterior neighbor and consequently adopt its fate.

The TDH draws further support from the cell labeling and grafting approach of Tamura et al. (2011). These authors showed that in the chicken hind limb and in mouse fore and hind limbs digit IV is formed by cells that originate from the ZPA, while in the chicken fore limb the digit IV (D3 phenotype) cells segregate early from the ZPA and migrate toward a region in which digit III

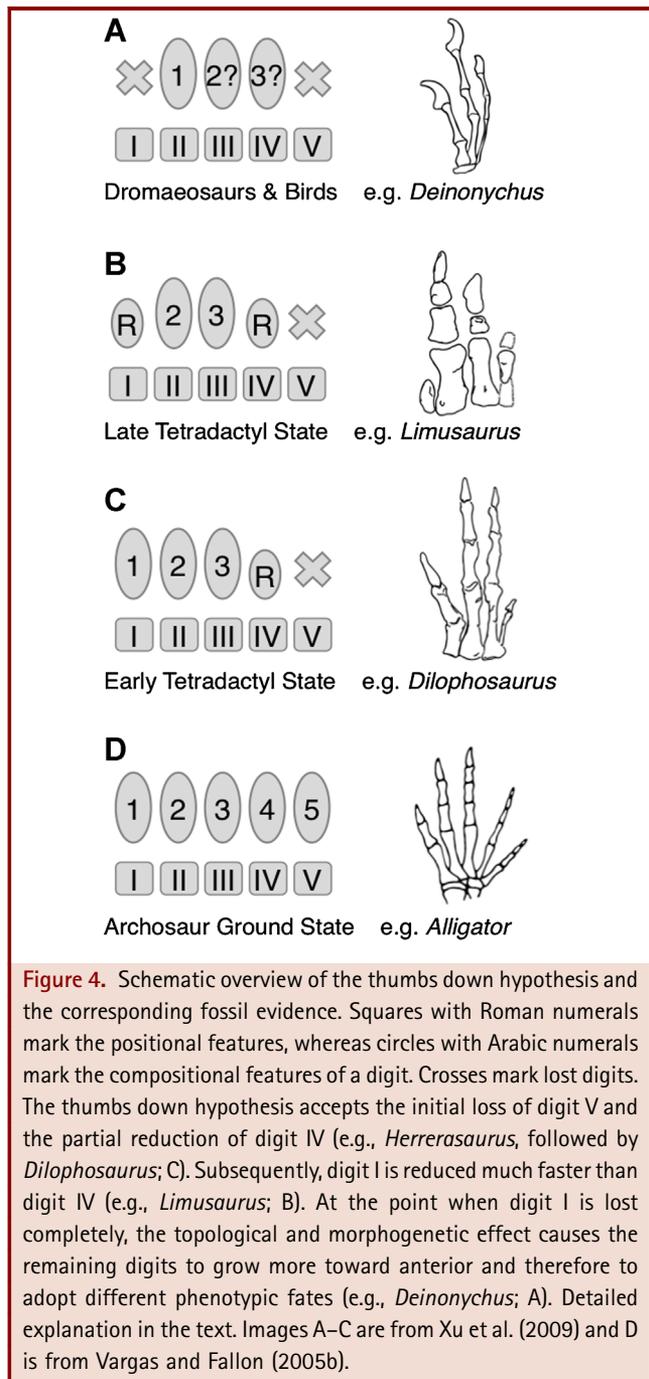
usually is formed. This corresponds precisely to the kind of reoriented growth central to the TDH.

The transcriptomic analysis of digit precursor cells by Wang et al. (2011) showed a strong linkage between fore limb digit II and hind limb digit I (each is the anterior-most fully ossifying digit in its respective limb). However, for both of the other fore limb digits the results were not clearly resolvable: forelimb digit III could not unambiguously be linked with either hind limb digit II or III. The same was true for the most posterior fore limb digit. Again this result would be expected: the TDH predicts that digit II will be the one affected most by the loss of digit I, due to its directly adjacent position, and that the effect has to be smaller for digits further away (Fig. 3B, blue arrows). If a homeotic shift had occurred—moving the entire digit frame toward anterior—all digits should be affected.

#### Compatibility With the Fossil Record

Whereas the most recent developmental evidence was interpreted in favor of the Frame Shift (Tamura et al., 2011; Wang et al., 2011; Welten et al., 2011), the newest fossil findings (Xu et al., 2009) support the PRH (Bever et al., 2011). The main line of argument for the PRH was that the first digit reduced was digit I, followed by the posterior reduction from four to three fingers (Galís et al., 2003; Fig. 2, upper row). The FSH, in contrast, assumed that two digits were lost on the posterior side, and then the remaining three digits were shifted in such a way that they formed from more posterior condensations (Fig. 2, middle rows). The discovery of *Limusaurus* *inextricabilis* (Xu et al., 2009), however, complicated things: this ceratosaur had two well-developed digits, likely at positions II and III, a partially reduced DIV (only a metacarpal plus one phalanx) and a vestigial DI metacarpal.

How does the TDH accommodate the fossil evidence? A new phylogenetic digit reduction scheme for the avian lineage follows from the TDH (Fig. 4). The most parsimonious approach is to accept a reduction of digit V to occur first, in order to accommodate *Herrerasaurus* and *Eoraptor*, and then assume a partial reduction of digit IV, in a way that leaves the metacarpal and one phalanx (e.g., *Coelophysis* or *Dilophosaurus*, Fig. 4). Developmental constraints would prohibit the primary axis from being further reduced, and digit I reduction begins to overtake digit IV until digit I is finally lost (a situation represented by *Limusaurus*; Fig. 4). As soon as DI development regresses, the topological and biomechanical factors come into play, causing the remaining digits to grow further away from the ZPA, therefore, adopting phenotypes of more anterior digits (as represented by *Deinonychus* and *Archaeopteryx*, Fig. 4). This reduction scheme is attractive, because it does not require the exclusion of any fossils from the phylogenetic tree in order to work. Very recently Xu and Mackem (2013) introduced a digit reduction scheme called the lateral shift hypothesis (LSH). Like the TDH, it follows the II, III, IV identification of tetanuran digits, and values the positional criteria over the compositional ones. Unlike the TDH, however, it argues



**Figure 4.** Schematic overview of the thumbs down hypothesis and the corresponding fossil evidence. Squares with Roman numerals mark the positional features, whereas circles with Arabic numerals mark the compositional features of a digit. Crosses mark lost digits. The thumbs down hypothesis accepts the initial loss of digit V and the partial reduction of digit IV (e.g., *Herrerasaurus*, followed by *Dilophosaurus*; C). Subsequently, digit I is reduced much faster than digit IV (e.g., *Limusaurus*; B). At the point when digit I is lost completely, the topological and morphogenetic effect causes the remaining digits to grow more toward anterior and therefore to adopt different phenotypic fates (e.g., *Deinonychus*; A). Detailed explanation in the text. Images A–C are from Xu et al. (2009) and D is from Vargas and Fallon (2005b).

that a (partial) homeotic anterior shift took place and that digit IV was completely re-evolved, following in both points the FSH. Despite these differences, the LSH is quite similar to the digit reduction scheme of the TDH and is almost fully compatible with the TDH's core mechanism.

## CONCLUSIONS

The TDH solves a number of open problems concerning avian forelimb homologies. It also leads to new experimental approaches and has some general consequences for theoretical homology. We begin with a condensed summary of the TDH and its differences from FSH and PRH, respectively.

### Summary of "Thumbs Down"

We argue that limb bud cells that would normally form the digit II condensation proliferate toward a more anterior direction, into the space made physically available by the loss of digit I. This causes the presumptive digit II to leave the Shh activity zone. At the same time its cells do not express *hoxD12* (and other posterior digit markers) any more, and therefore its transcriptome becomes characteristic of digit I. Its phalangeal number is reduced—whether due to weaker anterior FGF8 signaling, caused by lower Shh levels that would otherwise stabilize the expression, or because of different *hox* and downstream gene expression. Thus the change of the phalangeal formula (as in the PRH) is actually caused by the change of the transcriptome (as in the FSH), which in turn is directly caused by the loss of digit I (probably *shh* and *hoxD* mediated). Since the phenotypic changes primarily affect the anterior-most digit (from position II), we rather follow positional criteria for assessing digit identity, since they are stringent for the complete manus. The TDH resolves the major conflicts among the earlier models and accounts for the molecular, developmental, and fossil evidence.

### Differences to the Frame Shift and Pyramid Reduction Hypotheses

The FSH postulates an evolutionary event in which a dissociation occurs between the developmental formation of repeated elements (digits) and their subsequent individualization (identity; Wagner and Gauthier, '99). This can be understood in a similar fashion to homeotic changes of segment identity in *Drosophila* mutants (Lewis, '78): digits I, II, III are formed instead of II, III, IV. According to the TDH no change of identity of a homeotic nature occurs, but only the phenotypic realization of the developmental process is altered due to redirected growth induced by altered tissue topology. Digit identity stays the same. Also the TDH assumes that the patterning of the limb bud, by which the digit primordia are laid down, and their developmental realization, are different developmental modules in the first place. The latter uses *shh* signaling and differential *hox* expression, whereas the former is essentially a matter of growth and activator–inhibitor interactions (Newman and Bhat, 2007). In our model this effect is a consequence of the increased distance of DII cells from the ZPA, which in turn is directly caused by the loss of digit I. Finally, the frame shift argues that D1 was never lost but moved to position II (while retaining its identity), and therefore has difficulty to explain fossils with a reduced digit I, such as *Limusaurus*, whereas the TDH expects this reduction at the anterior-most position.

The PRH, on the other hand, argues that birds have regular digits II, III, and IV, and that digits I and V were lost in the avian dinosaur ancestors. This causes problems with fossils that display a posterior reduction of two digits. While the PRH solves this contradiction by excluding the relevant fossils, the TDH argues that the pressure to reduce digit I was stronger than the one to reduce digit IV. Therefore, what started as a trend toward losing two digits posteriorly, became a bilateral reduction. Genetically, the PRH has difficulties to deal with the *hox* and transcriptomic evidence, which suggests I, II, III interpretation of the bird digits. By contrast, the TDH predicts exactly that. Moreover it actually links the evolutionary event of losing digit I with the altered developmental genetics of the remaining digits.

### Potential Experiments

Future research could include experiments to rescue digit I and examine the resulting change in *hox* expression. The Thumbs Down model also predicts a change in digit II phenotype in the hind limb, if digit I was ablated by mechanical or molecular means. Since a digit reduction similar to the one in birds is known in skinks with reduced limbs (Wagner, 2005), closely related skink species with and without digit I could be compared concerning cell proliferation rates, gene expression domains, as well as transcriptomes of their anterior-most digit. Also needed are experiments to distinguish between cell migration and cell proliferation as factor in the development of digit I phenotype from condensation II, and molecular studies to clarify the interactions between *fgf*, *shh*, and *hoxD* genes in four- and three-digitated limbs.

It is also interesting that although the changes in the transcriptome and phenotype of the digits seem to be caused by the loss of digit I, the loss of digit V appears to have no such effect in either the forelimb or the hind limb. It would be worth investigating the possibility of different mechanisms of digit loss for the forelimbs and hind limbs, or for digits I and V. A ZPA-dependent mechanism that can explain the difference between anterior and posterior digit loss (de Bakker et al., 2013) was suggested recently.

### Notes on Homology

The TDH is an exemplification of the Organizational Homology Concept (Müller, 2003), which stated that "homologues are autonomized elements of the morphological phenotype that are maintained in evolution due to their organizational roles in heritable, genetic, developmental, and structural assemblies." Here a multilevel organizing relation stabilizes building elements of the final phenotype, but in turn, the phenotypic integratedness exerts (via natural selection) an organizing effect on the generative processes. In the case of the avian forelimb, we have argued that gene regulatory and molecular properties of pattern forming limb bud cells are elicited by topological conditions that

organize and maintain structural building elements (digits composed of phalanges), despite their shifted proliferation rates and modified molecular environments, which modulate their phenotypic appearance. In this view, the morphogenetic process itself maintains homology.

The avian hand problem illustrates several pitfalls of biological homology concepts identified previously (Müller, 2003). In particular, a problem occurs when statements about structural sameness (identity as the same element in a body plan) are mixed with statements on developmental individualization (identity as a unique differentiation state). The discussion of Ramirez (2007) only addresses part of the problem, because structural homology is not only about position, and the implied distinction between structural and compositional features is akin to the problem of cross level justification pointed out earlier (Müller, 2003), that is, when homology of structure is justified by shared genetic regulation, cellular origin, or developmental process. In general, though, it had looked like the problem was overcome by a rather widely held consensus, indicating that (morphological) homology represents a manifestation of maintained building elements, precisely despite evolutionary variation in their molecular, developmental, and genetic makeup (True and Haag, 2001).

To say, as was argued in the current debate on digit homology, that a character is not homologous because its derived differentiation state is different from the primitive state, throws the homology discussion back to field one: is homology assignment a statement about structural identity in body plan assemblies or about compositional features of developmental, genetic, or other differentiation processes? If the answer that had been reached, as cited above, is maintained, then homology assignments cannot be altered by process based properties of differentiation state. Hence, even if one considers the FSH to be mechanistically realistic, the homology status of the avian digits would still be II, III, IV. The TDH overcomes these problems through its distinction between identity and phenotypic appearance.

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