

# Birds Have Dinosaur Wings: The Molecular Evidence

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**ABSTRACT** Within developmental biology, the digits of the wing of birds are considered on embryological grounds to be digits 2, 3 and 4. In contrast, within paleontology, wing digits are named 1, 2, 3 as a result of phylogenetic analysis of fossil taxa indicating that birds descended from theropod dinosaurs that had lost digits 4 and 5. It has been argued that the development of the wing does not support the conclusion that birds are theropods, and that birds must have descended from ancestors that had lost digits 1 and 5. Here we use highly conserved gene expression patterns in the developing limbs of mouse and chicken, including the chicken *talpid*<sup>2</sup> mutant and polydactylous Silkie breed (Silkie mutant), to aid the assessment of digital identity in the wing. Digit 1 in developing limbs does not express *Hoxd12*, but expresses *Hoxd13*. All other digits express both *Hoxd12* and *Hoxd13*. We found this signature expression pattern identifies the anteriormost digit of the wing as digit 1, in accordance with the hypothesis these digits are 1, 2 and 3, as in theropod dinosaurs. Our evidence contradicts the long-standing argument that the development of the wing does not support the hypothesis that birds are living dinosaurs. *J. Exp. Zool. (Mol. Dev. Evol.)* 304B:86–90, 2005. © 2005 Wiley-Liss, Inc.

## INTRODUCTION

A remarkable difference currently exists between the fields of developmental biology and paleontology regarding the naming of the digits of the tridactyl wing of birds. Within developmental biology, wing digits are named 2, 3 and 4. This identification is supported by the position of the primary axis of cartilage formation, a conserved sequence of cartilage formation and spatial proximity among skeletal elements of developing limbs which runs along the ulna and digit 4 in pentadactyl amniotes (Fig. 1a i-ii; Oster et al., '88; Burke and Feduccia, '97), including crocodiles (Müller and Alberch, '90), the closest living relatives of birds. In the wing, the primary axis is observed to run through the ulna into the most posterior functional digit, suggesting that this digit is the 4th and, by inference, that the digits anterior to it are digits 2 and 3 (Fig. 1a iii; Hinchliffe, '84; Burke and Feduccia, '97; Hinchliffe, 2002). Additionally, small mesenchymal condensations flanking the developing functional digits are present in the embryonic wing that can be interpreted as vestiges of digital condensations 1 and 5 (Fig. 1a iii; Larsson and Wagner, 2002; Kundrát et al. 2002; Nowicki and Feduccia, 2002),

providing further support to the 2, 3, 4 identification of wing digital condensations (Fig. 1a iii).

The embryological nomenclature of the wing digits is in contrast with that used in the field of paleontology. Extensive phylogenetic analyses of fossil taxa have repeatedly indicated that birds are living theropod dinosaurs (Holtz, '98; Sereno, '99; Witmer, 2002). The three-fingered theropod ancestors of birds had lost digits 4 and 5, as indicated by the presence of reduced digits 4 and 5 in early theropods, and the striking morphological resemblance of the three remaining digits to those of *Archaeopteryx* (Fig. 1b i-vi; Ostrom, '77; Sereno, '93; Padian and Chiappe, '98; Wagner and Gauthier, '99). This has led to the alternative naming of the digits of the wing of birds as digits 1, 2 and 3. The apparent discrepancy between paleontological and developmental information has led to fundamentally different interpretations. It has

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This work was supported by grants of CONICYT (Chile) and the Company of Biologists to A.O.V. and an NIH Grant #32551 to J.F.F.

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Received 24 August 2004; Accepted 24 September 2004

Published online 28 October 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21023

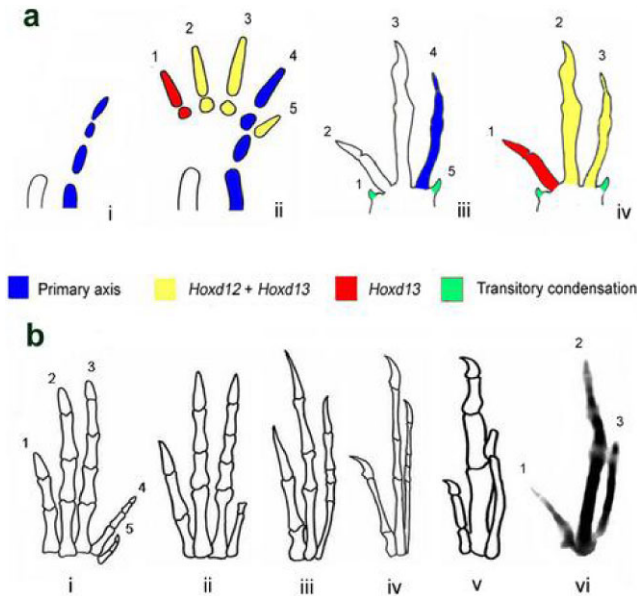


Fig. 1. Digital identity in the bird wing. (a) Schematic representations of developing limbs. (i) early stage (ii) late stage of a pentadactyl limb (iii) embryological naming of digital condensations (iv) digital identity as suggested by the evolutionary origin of birds and the expression of *Hoxd12* and *Hoxd13*. (b) Phylogenetic homology of the wing digits to digits 1, 2 and 3 of dinosaurs. Taxa to the right share a more recent common ancestor with living birds (Neornithes). (i) *Heterodontosaurus* (ii) *Coelophysis* (iii) *Deinonychus* (iv) *Archaeopteryx* (v) *Sinornis* (vi) duck embryo (day 12.5).

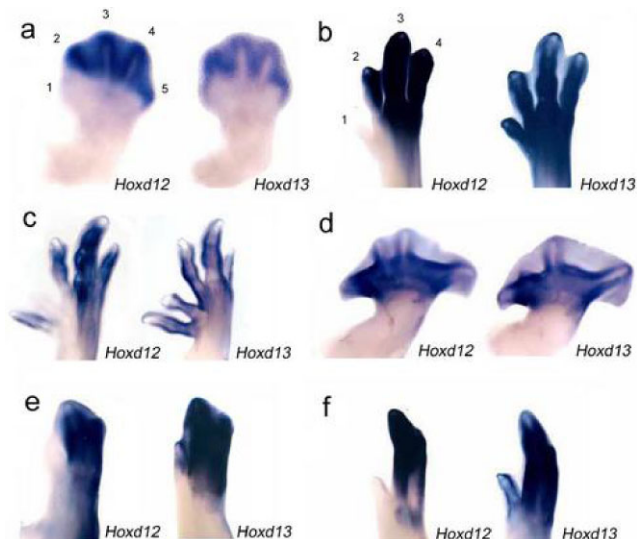


Fig. 2. Expression of *Hoxd12* (left) and *Hoxd13* (right) of developing limbs. (a) Forelimb of wildtype mouse (stage 12.5, n=6 for each gene) (b) Foot of wildtype chicken (day 8, n=8) (c) Foot of the *Silkie* chicken mutant (day 8, n=8) *Hoxd12* is expressed in ectopic digit 2 (d) Foot of the *talpid2* chicken mutant (day 7, n=2). No digit 1 is formed. (e) Wing of the chicken (day 7, n=8) (f) Wing of the chicken (day 8, n=7).

been argued that the embryological condition of the bird wing does not support the proposal that birds are theropod dinosaurs, and that birds must have descended from an ancestor that had already lost digits 1 and 5 (Burke and Feduccia, '97; Feduccia, '99a,b; Galis et al., 2003; Feduccia, 2003). Alternatively, the well supported relations of birds among theropod dinosaurs have led to different proposals of change in the embryological pathway of digits 1, 2 and 3 (Chatterjee, '98; Garner and Thomas, '98; Wagner and Gauthier, '99). One of these hypotheses (Wagner and Gauthier, '99; Larsson and Wagner, 2001) proposes that a homeotic transformation of the identity of digital condensations has occurred, such that digital condensations 2, 3 and 4 actually develop into digits 1, 2 and 3.

Here we introduce molecular evidence to aid the assessment of digital identity in the wing of birds. Recent work has shown that a signature pattern of expression of the *Hoxd12* and *Hoxd13* genes is related to the development of digit 1 (Chiang et al., 2001; Litington et al., 2002; Ros et al., 2003). At late stages of both mouse and chicken embryos (12.5 dpc in mouse, day 5 in chicken and later), *Hoxd12* is not expressed in digit 1, but it is expressed in all other digits (Knezevic et al., '97; Chiang et al., 2001; Ros et al., 2003; Chen et al., 2004). *Hoxd13*, in turn, is expressed in all digits, including digit 1 (Fig. 1a ii; Chiang et al., 2001; Ros et al., 2003; Chen et al., 2004). The use of the expression patterns of *Hoxd12* and *Hoxd13* to identify digit 1 is further supported by experiments in mouse indicating the expression pattern of these genes is causally relevant for normal development of this digit. Forced expression of *Hoxd12* in all digits leads to homeotic transformation of digit 1 to a more posterior identity, as suggested by increased length and the presence of three phalanges (Knezevic et al., '97). The knockout of *Hoxd13* leads to abnormal development of digit 1, which is shorter and presents fusion of both phalanges into a single element (Fromental-Ramain et al., '96; Zákány et al., '97).

We examined and analyzed the expression of *Hoxd12* and *Hoxd13* in late stages of mouse and chicken embryos. To test further whether the expression of these genes can be used to distinguish digit 1, we also examined mutant chickens in which the antero-posterior patterning of the autopod is affected. We have synthesized molecular evidence that identifies the anteriormost functional digit of the wing to be digit 1, in accordance with the 1, 2, 3 identity of the wing

digits as observed in the hand of theropod dinosaurs (Fig. 1a iv).

## MATERIALS AND METHODS

In situ hybridizations were carried out in developing limbs at late stages, when individual digits can be easily identified. Mouse embryos were collected at 12.5 dpc. Embryos of wildtype chicken were collected at 7 and 8 days of incubation. Embryos of the chicken polydactylous Silkie breed were collected on day 7 and day 8. Embryos of the chicken *talpid*<sup>2</sup> mutant were collected on day 7 of incubation. Embryos were then fixed overnight in 4% paraformaldehyde, rinsed in PBS, dehydrated in a sequence of methanol concentrations and preserved in methanol 100% at  $-20^{\circ}\text{C}$ . Antisense probes for chicken and mouse *Hoxd12* and *Hoxd13* labeled with digoxigenin were prepared to visualize the transcripts of these genes in the developing limbs. In situ hybridization was carried out following standard procedures described by Nieto et al. ('96). *Hoxd12* and *Hoxd13* cDNA probes were obtained from C. Tabin (chick) and D. Duboule (mouse). Chick polydactylous Silkie breed and *talpid*<sup>2</sup> mutants were obtained from chicken flocks maintained at the University of Wisconsin-Madison.

## RESULTS AND DISCUSSION

In both forelimbs and hindlimbs of the mouse, and in the hindlimb of the chicken, there is no controversy regarding the identity of digit 1. In all of these limbs, the biphalaingeal digit 1 expresses *Hoxd13* but does not express *Hoxd12*. All other digits express both *Hoxd12* and *Hoxd13* (Fig. 2 a,b). Additionally, the expression pattern related to digit 1 formation is also conserved in mutants presenting an altered antero-posterior patterning of the autopod. In Silkie mutants, an ectopic digit frequently develops anterior to digit 1. This ectopic digit closely resembles digit 2 in being at the same time triphalaingeal, longer than digit 1, and shorter than digit 3 (which has four phalanges in the foot). Despite its anterior position, this ectopic digit expresses *Hoxd12* according to its morphological resemblance to digit 2 (Fig. 2c). Expression of *Hoxd12* in Silkie mutants remains absent in digit 1. In the *talpid*<sup>2</sup> mutant, survivors to late stages form hindlimbs with only posterior digits, and a digit 1 does not form (Litingtung et al., 2002). In the *talpid*<sup>2</sup> mutant, absence of

digit 1 is accompanied by coincident expression of *Hoxd12* and *Hoxd13* along the entire autopod (Fig. 2d). The expression of *Hoxd12* and *Hoxd13* has also been shown to relate to the development of digit 1 in the limbs of mutant mice (Litingtung et al. 2002). In *Gli3*<sup>-/-</sup> or *Shh*<sup>-/-</sup>*Gli3*<sup>-/-</sup> mice, posteriorized limbs where no digit 1 is formed, a coincident expression of *Hoxd12* and *Hoxd13* is reported along the entire digital arch, resembling that found in the chicken mutant *talpid*<sup>2</sup>. In the *Shh*<sup>-/-</sup>*Gli3*<sup>+/-</sup> mouse, additional biphalaingeal digits resembling digit 1 are reported not to express *Hoxd12* but to express *Hoxd13* (Litingtung et al., 2002). It is notable, therefore, that in the wing of the chicken, expression of *Hoxd12* is absent in the anteriormost digit, and expression of *Hoxd13* is present (Fig 2 e,f). Despite the fact that this digit is considered to develop from digital condensation 2, the expression of *Hoxd12* and *Hoxd13* suggests it is digit 1 (Fig. 1a iv). A digit 1 identity for the anteriormost digit of the wing is also suggested by its biphalaingeal morphology. The difference between biphalaingeal digit 1 and triphalaingeal digit 2 is present in most pentadactyl amniotes (Carroll, '88) and is found as far back as the ancestors shared with the Devonian pantetrapod *Tulerpeton* (Wagner and Gauthier, '99).

Published analysis of *Hox* gene expression in chicken limbs at early stages, previous to the morphological definition of individual digital condensations, is not of much help for distinguishing the precursor cells of digit 1 (Nelson et al. '96). The signature expression pattern allowing identification of digit 1 may not be fully established until later stages. Other descriptions of early stages report that expression of *Hoxd13* is absent in the precursor cells of digit 1 (Vargesson et al., '97; Zákány et al., 2004). Cells of digit 1 must therefore acquire the expression of *Hoxd13* at later stages. Strong support for this view is indicated by the aforementioned observation that, in the absence of *Shh* function in both mouse and chick limbs, only a digit 1 forms (Chiang et al., 2001, Ros et al., 2003). In limb bud stages of *Shh*<sup>-/-</sup> and *oligozeudactyly* mutants, *Hoxd13* is initially expressed in limb buds and then becomes undetectable. However, *Hoxd13* is re-expressed as a prelude to the condensation of the single biphalaingeal digit that then forms; *Hoxd12* is not expressed at this time (Chiang et al., 2001; Ros et al., 2003). It is reasonable to conclude that there is a digit 1 specific locus in the *Hoxd13* promoter and its function is *Shh* independent. Despite recent fossil evidence, such as the discovery of feathered

oviraptorid and dromaeosaurid theropods (Ji et al., '98; Xu et al., 2003), doubt on the ancestry of birds has persisted due to the argument that the development of the wing does not support the relationship of birds among theropods (Galis et al., 2003; Feduccia, 2003). In contrast, our molecular evidence on wing development is in accordance with a 1, 2 and 3 identity of the wing digits, as expected for theropods. The question of whether birds are theropods is fairly general and an abundance of fossil taxa are available to answer it (Witmer, 2002). Developmental observations, although apparently compelling, must be regarded with caution when used as substitutes for phylogenetic analysis in identifying the relationships among organisms.

The combined evidence indicating the anteriormost functional digit of the wing is digit 1 opens an interesting question regarding the identity of the anteriormost transitory mesenchymal condensation, which has been proposed to be a vestige of digital condensation 1. According to the hypothesis of a homeotic transformation, the development of digits 1, 2 and 3 was taken over by digital condensations 2, 3 and 4. From this perspective, the anteriormost mesenchymal condensation may correspond to a dormant digital condensation that developed into digit 1 before the occurrence of the homeotic transformation (Larsson and Wagner, 2002). Recent work on the development of digital identity has shown that digital condensations remain undetermined until late stages, allowing for the experimental homeotic transformation of digital identity (Dahn and Fallon, 2000). This suggests that the natural occurrence of homeotic transformation of digits is developmentally possible. The hands of kiwis and tyrannosaurs may represent further cases of natural homeotic transformation of digits (Wagner and Gauthier, '99). An alternative interpretation is that the anterior transitory mesenchymal condensation of the wing is comparable to a prepollex. The prepollex is a small mesenchymal condensation found anterior to digit 1 in the embryos of such distantly related tetrapods as the frog, opossum, and guinea-pig (reviewed in Galis et al., 2001). Generally the prepollex resembles the anteriormost mesenchymal condensation of the bird wing in being small-sized and transitory, without developing into a digit, but in some anuran species it can ossify and even present several phalanx-like elements (Fabrezi, 2001).

The expression of *Hoxd12* and *Hoxd13* in relation to digit 1 formation both in the hand

and foot of the distantly related mouse and chicken suggests this signature pattern will be present in most amniotes. Although within developmental biology the naming of the wing digits as 2, 3 and 4 has become a standard procedure, our molecular evidence in combination with the evolutionary origin of birds suggests these digits are in fact 1, 2 and 3.

## ACKNOWLEDGMENTS

This work was supported by grants of CONICYT (Chile) and the Company of Biologists to A.O.V. and an NIH Grant #32551 to J.F.F. We thank lab members of the Fallon lab and Matt Harris for helpful discussions and technical support. Thanks to G. Wagner for discussion on the prepollex. Special thanks to Jim Bitgood for help with the chicken mutant flocks.

## LITERATURE CITED

- Burke AC, Feduccia A. 1997. Developmental patterns and the identification of homologies in the avian hand. *Science* 278:666–668.
- Chatterjee S. 1998. Counting the fingers of birds and dinosaurs. *Science* 280:355a.
- Carroll R. 1988. *Vertebrate paleontology and evolution*. W.H. Freeman & Co., New York.
- Chen Y, Knezevic V, Ervin V., Hutson R., Ward Y, Mackem S. 2004. Direct interaction with Hoxd proteins reverses Gli3-repressor function to promote digit formation downstream of Shh. *Development* 131:2339–2347.
- Chiang C, Litingtung Y, Harris MP, Simandl BK, Li Y, Beachy PA, Fallon JF. 2001. Manifestation of the Limb Prepattern: Limb Development in the Absence of Sonic Hedgehog Function. *Dev Biol* 236:421–435.
- Dahn R, Fallon J. 2000. Interdigital regulation of digit identity and homeotic transformation by modulated BMP signaling. *Science* 289:438–441.
- Fabrezi M. 2001. A survey of prepollex and prehallux variation in anuran limbs. *Zool J Linnean Soc* 131:227–248.
- Feduccia A. 1999a. 1,2,3=2,3,4: Accommodating the cladogram. *Proc Natl Acad Sci USA* 96:4740–4742.
- Feduccia A. 1999b. *The Origin and Evolution of Birds*. New Haven, CT: Yale Univ. Press.
- Feduccia A. 2003. Bird origins: problem solved, but the debate continues. *Trends Ecol Evol* 18:9–10.
- Fromental-Ramain C, Warot X, Messadecq N, LeMeur M, Dollé P, Chambon P. 1996. Hoxa-13 and Hoxd-13 play a crucial role in the patterning of the limb autopod. *Development* 122:2997–3011.
- Galis F, van Alphen FJJM, Metz JAJ. 2001. Why five fingers? Evolutionary constraints on digit numbers. *Trends Ecol Evol* 16:637–646.
- Galis F, Kundrát M, Sinervo B. 2003. An old controversy solved: bird embryos have five fingers. *Trends Ecol Evol* 18:7–9.
- Garner JP, Thomas ALR. 1998. Counting the fingers of birds and dinosaurs. *Science* 280:355.

- Hinchliffe JR. 2002. Developmental basis of limb evolution. *Int J Dev Biol* 46:835–845.
- Hinchliffe JR. 1984. “One, two, three” or “Two, three, four”: An embryologist’s view of the homologies of the digits and carpus of modern birds. In: Hecht M, Ostrom JH, Viohl G, Wellnhofer P, editors. *The beginnings of birds*. Eichstätt: Freunde des Jura-Museum. p 141–147.
- Holtz TR. 1998. A new phylogeny of the carnivorous dinosaurs. *Gaia* 15:5–61.
- Ji Q, Currie P, Norell MA, Ji S-A. 1998. Two feathered dinosaurs from northeastern China. *Nature* 393:753–761.
- Kundrát M, Seichert V, Russell AP, Smetana K. 2002. Pentadactyl pattern of the avian wing autopodium and pyramid reduction hypothesis. *J Exp Zool B (Mol Dev Evol)* 294:152–159.
- Knezevic V, De Santo R, Schughart K, Huffstadt U, Chiang C, Mahon KA, Mackem S. 1997. Hoxd-12 differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates Sonic hedgehog in a positive feedback loop. *Development* 124:4523–4536.
- Larsson HC, Wagner GP. 2002. Pentadactyl ground state of the avian wing. *J Exp Zool B (Mol Dev Evol)* 294:146–151.
- Larsson HC, Wagner GP. 2003. Old morphologies misinterpreted. *Trends Ecol Evol* 18:9–10.
- Litingtung Y, Dahn RD, Li Y, Fallon JF, Chiang C. 2002. Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. *Nature* 418:979–983.
- Müller G, Alberch P. 1990. Ontogeny of the limb skeleton in *Alligator mississippiensis*: Developmental invariance and change in the evolution of Archosaur limbs. *J Morphol* 203:151–164.
- Nieto MA, Patel K, Wilkinson DG. 1996. In situ analysis of chick embryos in whole mount and tissue sections. In: Bronner-Fraser M, editor. *Methods in Cell Biology*, Vol. 51. New York: Academic Press. p 219–235.
- Nelson CE, Morgan BA, Burke AC, Laufer E, DiMambro E, Murtaugh LC, Gonzales E, Tessarollo L, Parada LF, Tabin C. 1996. Analysis of Hox gene expression in the chick limb bud. *Development* 122:1449–1466.
- Nowicki J, Feduccia A. 2002. The hand of birds revealed by early ostrich embryos. *Naturwissenschaften* 89:391–393.
- Oster GF, Shubin N, Murray JD, Alberch P. 1988. Evolution of the morphogenetic rules: the shape of the vertebrate limb in ontogeny and phylogeny. *Evolution* 42:862–884.
- Ostrom J. 1977. Archaeopteryx and the origin of birds. *Biol J Linn Soc* 8:91–182.
- Padian K, Chiappe L. 1998. The origin and early evolution of birds. *Biol Rev* 73:1–42.
- Ros MA, Dahn RD, Fernandez-Teran M, Rashka K, Caruccio NC, Hasso SM, Bitgood J, Lancman JJ, Fallon JF. 2003. The chick oligozeugodactyly (ozd) mutant lacks sonic hedgehog function in the limb. *Development* 130:527–537.
- Sereno PC. 1993. Shoulder girdle and forelimb of *Herrerasaurus*. *J Vert Paleont* 13:425–450.
- Sereno PC. 1999. The evolution of dinosaurs. *Science* 284:2137–2147.
- Vargesson N, Clarke JDW, Vincent K, Coles C, Wolpert L, Tickle C. 1997. Cell fate in the chick limb bud and relationship to gene expression. *Development* 124:1909–1918.
- Wagner GP, Gauthier JA. 1999. 1,2,3=2,3,4: a solution to the problem of the homology of the digits in the avian hand. *Proc Natl Acad Sci USA* 96:5111–5116.
- Witmer LM. 2002. The debate on avian ancestry: phylogeny, function, and fossils. In: Chiappe LM, Witmer LM, editors. *Mesozoic birds: above the heads of dinosaurs*. Berkeley, University of California Press. p 3–30.
- Xu X, Zhou Z, Wang X, Kuang X, Zhang F, Du X. 2003. Four-winged dinosaurs from China. *Nature* 421:335–340.
- Zákány J, Fromental-Ramain C, Warot X, Duboule D. 1997. Regulation of number and size of digits by posterior *hox* genes: A dose-dependent mechanism with potential evolutionary implications. *Proc Natl Acad Sci USA* 94:13695–13700.
- Zákány J, Kmita M, and Duboule D. 2004. A dual role for *Hox* genes in limb anterior-posterior asymmetry. *Science* 304:1669–1672.