

RESEARCH HIGHLIGHT

# 1, 2, 3: Counting the fingers on a chicken wing

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## Abstract

RNA-seq transcriptomics of digit primordia in the developing chick wing and leg has clarified a long-standing dispute between paleontologists and embryologists about evolutionary homology.

**Keywords** Chick digit identity, digit loss, RNA-seq, theropod evolution.

The forelimbs and hindlimbs of vertebrates are serially homologous structures. Bird limbs differ from many other vertebrates in that they have only three digits in the forelimb (wing), but four digits in the hindlimb (leg). This pattern has emerged through a process of digit loss during evolution. While the morphological digit identities of the hindlimb are widely accepted as digits I to IV, corresponding to digits I to IV of the ancestral pentadactyl (five digit) limb (Figure 1), such definitive identities have yet to be designated to the digits of the wing. The precise identities of these digits have been the focus of a long-standing and ardent debate amongst paleontologists and embryologists, since they underpin the mechanism by which the bird wing has evolved from the theropod dinosaur lineage; however, a recent article by Wagner *et al.* [1] has shed light on this conundrum.

Paleontological evidence demonstrates the progressive loss of the two most posterior digits, V and IV, in theropods, implying digits I to III from the ancestral hand remain in the avian wing. However, embryological evidence identifies the digits as II to IV, since the most posterior digit of the wing is the first to condense (the first visible digit) and forms in alignment with the primary axis of cartilage condensation [2]; this digit is consistent with digit IV in the ancestral hand, which is a pattern retained in the mouse.

In order to settle this debate, Wagner *et al.* [1] utilized contemporary RNA-seq techniques to uncover a gene expression 'signature' of digit identity taking the rationale

that morphological digit identity is ultimately determined by gene expression profiles, which are unique to each digit.

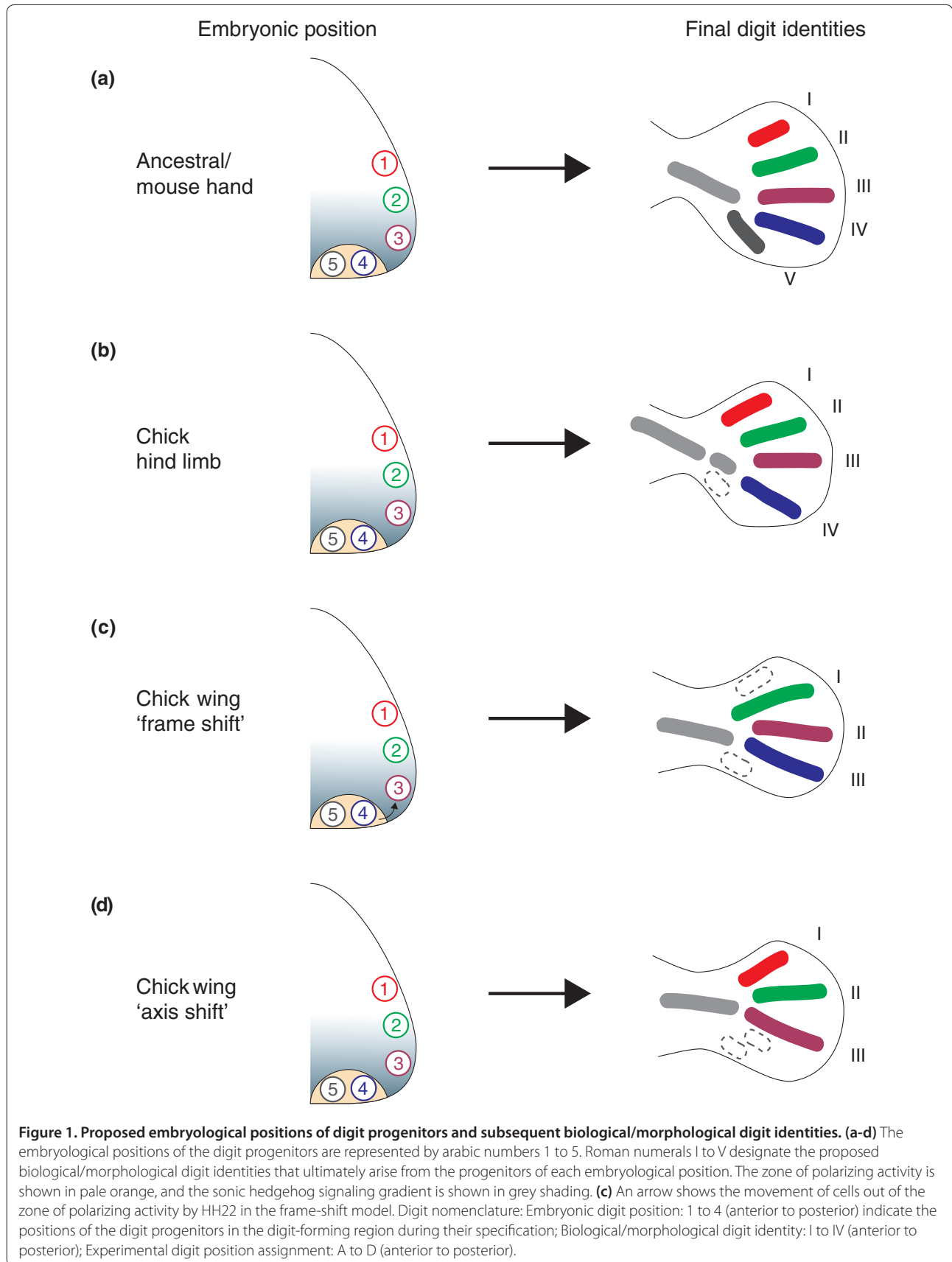
## Testing, testing, one, two, three

Specifically, the group sequenced the transcriptomes of the digit primordia of the forming wing and leg dissected from chicks at two different stages (HH28/29 and HH31). To establish a neutral digit annotation, the anterior-most digit was designated digit A and, in sequence, the more posterior digits were designated B and C in the wing, and B, C and D in the leg. Multidimensional scaling analysis, a method of graphically mapping populations based on similarities in gene expression patterns, was used in conjunction with Pearson's correlation coefficient heat map analysis. This analysis individually compares all populations one-to-one to distinguish correlations between any two populations, in order to investigate similarities in gene expression profiles between digits of the forelimbs and digits of the hindlimbs.

A key observation from this study is that digit A (anterior most) of the forelimb exhibits strong correlation, at both developmental stages, to digit A of the hindlimb, which is widely perceived to be corresponding to the ancestral digit I. Furthermore, four genetic markers were detected for both digits, suggesting a robust homology at the level of gene expression. While *HOXD12* and *HAND2* were shown to be significantly downregulated in both digit As, two markers, *ZIC3* and *LHX9*, were shown to be highly expressed exclusively in the most anterior of both limbs. The results of the transcriptome analyses were supported by classical whole mount *in situ* hybridization staining that confirmed the regional restriction and enrichment of mRNA transcripts.

No such homology was recorded between digit A of the forelimb and digit B of the hindlimb, implying that digit A of the forelimb is thus a true homolog of the ancestral digit I. The same group has proposed, in a previous study, that a so-called 'frame shift' may have occurred in the avian wing whereby digits of the biological identity I to III have been shifted to the embryological positions of 2 to 4 (Figure 1), thus resolving the conflict concerning the position of the posterior-most digit to the primary axis [3]. These data support such a frame-shift model and, moreover, are consistent with conclusions drawn from two other recent fate-mapping studies [4,5].

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## Shifting models

These two independent fate-mapping experiments in the chick have recently revealed the origin of digit progenitor cells in both the forelimb and the hindlimb [4,5]. The posterior-most digit in the chick wing is formed from progenitors that originate adjacent to the zone of polarizing activity (a group of cells found at the posterior margin of the limb bud; the cells express the morphogen sonic hedgehog, which is critical for anterior-posterior patterning of the digits). Cells in the zone of polarizing activity never contribute to the skeletal elements of the third digit (C) in the chick wing. Conversely, it was found that progenitors originating in the zone of polarizing activity of the hindlimb always contribute to digit IV (D, posterior most), but never to the skeletal structure of digit III. The results suggest that the posterior-most digit of the chick wing corresponds to the third digit (C) of the chick hindlimb and mouse hand. Consequently, the digits of the avian wing are assigned the identity I to III, consistent with the findings of Wagner *et al.*

Tamura *et al.* [4] go further to propose how the frame shift may occur during development. They suggest that, at HH20 in the wing, digit III progenitors reside in the zone of polarizing activity, but move out by HH22 where they become exposed to paracrine sonic hedgehog signaling that determines their digit III identity. This shift of cells from embryonic position 4 to 3 in the early limb bud could account for the hypothesized 'frame shift' and how the primary axis becomes aligned with digit III. Correspondingly, the use of the primary axis to designate digit identity or, indeed, embryological position is considered outmoded. However, Towers *et al.* [5] have contested the need for a frame-shift model, alternatively suggesting an axis shift that stipulates the primary axis of cartilage condensation has shifted to align with the embryonic position 3 instead of position 4 (Figure 1). Despite the discrepancies in mechanisms, all models are consistent in designating avian wing digits as I to III.

## Posterior digit identities

The RNA-seq transcriptome analysis of chick digits was unable to identify similar gene expression profiles between forelimb digits B to C, and hindlimb digits B to C; this would lend further support to a model in which forelimb digits correspond to digits I to III of the ancestral hand.

However, it was observed that the forelimb digit B profile, at HH28/29, did show correlation to the profile of digit B in the hindlimb, staged HH31. The difference in stage could be a reflection of the heterochrony in the

development of the forelimb and hindlimb. The forelimb emerges before the hindlimb in chick and mouse. Notably, results did not show consistent homology between forelimb digits B to C and hindlimb digits C to D. Taken as a whole, this transcriptome analysis suggests that the digits of the forelimb show no definite correspondence to any of the digits of the hindlimb, and they were further shown to exhibit a higher degree of differentiation than their hindlimb counterparts. Interestingly, these data also offer a glimpse of the unique gene expression profiles underlying differences in limb-type morphologies.

Although many questions remain, using RNA-seq to profile the transcriptome of digit progenitors has added to a growing body of evidence supporting the hypothesis that avian wing digits are homologous to digits I, II and III of the mouse limb. Whether this is achieved through an axis shift or frame shift remains unresolved. These findings provide an example of how modern sequencing techniques can complement studies using more classical methods to provide insights into the genetic mechanisms that underlie evolution and development. RNA-seq and the establishment of a growing resource of limb transcriptomics [6] will undoubtedly prove a powerful tool for the future.

## Competing interests

The authors declare that they have no competing interests.

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