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„Thumbs Down - A molecular- morphogenetic approach to avian digit identity“

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Abstract

The problem of avian fore limb digit homology remains one of the standards in EvoDevo research. Various hypotheses have been presented in recent years to resolve the apparent contradiction between embryological and paleontological evidence. The theories have ranged from excluding birds from the dinosaur clade to assuming a hexadactyl tetrapod limb ground state. At the moment there are two predominant approaches: 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 remodeling of digit phenotypes. Here a new model is presented that integrates elements from both hypotheses with the existing experimental data. We trace the main features of both major hypotheses back to a common ontogenetic origin: the reduction of the anterior-most digit. A concerted mechanism of molecular expression and developmental mechanics is proposed that is capable of shifting the boundaries of *hoxD* expression as well as changing the phenotype of digits. The core of this mechanism is directional cell proliferation of digit II to the anterior side, once digit I development ceases. This results in an altered position of digit II cells relative to the zone of polarizing activity (ZPA) and hence a decreased level of Sonic Hedgehog protein. Since Shh is thought to be important in digit specification, this can easily affect digit morphology. Sonic Hedgehog also controls the expression of posterior *hoxD* genes in the limb bud. Therefore we assume that the *hoxD* expression pattern that has been shown in bird fore limbs is caused by this very mechanism as well. We introduce an alternative digit-reduction scheme that reconciles the current fossil evidence with the presented molecular-morphogenetic model. In this work three experiments were carried out: one that tried to reverse the digit modifications in the fore limb, and two, to deliberately cause them in the hind limb. Bead implantations with FGF-8 protein were used to rescue the digit I vestige in the fore limb bud. Mitosis inhibitor injections and invasive manipulations were applied to ablate hind limb digit I. 3D microCT imaging and alcian blue staining of the manipulated limbs revealed that it is possible that one digit takes the place and the morphological phenotype of another, if the development of the latter is

halted. In situ hybridizations detecting *hoxD12* mRNA showed that the expression of *hox* genes also is affected if digits are lost. The integrated morphological and genetic evidence supports the proposed hypothesis, since it shows that the predicted effects the core mechanism are really inducible in embryonic systems. Furthermore the hypothesis also is consistent with currently available developmental and paleontological data.

Zusammenfassung

In dieser Arbeit wird eines der klassischen Probleme der evolutionären Entwicklungsbiologie behandelt: die Homologie der Finger des Vogelflügels. In den vergangenen Jahren versuchten verschiedene Hypothesen, den scheinbaren Widerspruch zwischen embryologischen und paläontologischen Analysen aufzulösen, wobei sowohl in Erwägung gezogen wurde, die Vögel aus der phylogenetischen Gruppe der Dinosaurier herauszunehmen, als auch, dass die Gliedmaßen der Tetrapoda ursprünglich sechs- oder mehrfingrig waren.

Zur Zeit sind zwei große Theorien vorherrschend: Die *Frame Shift* Hypothese und die *Pyramid Reduction* Hypothese. Während erstere eine homeotische Verschiebung der Fingeridentitäten Richtung *anterior* annimmt, geht letztere davon aus, dass die drei zentralen Finger durch eine graduelle, morphologische Umwandlung den Phänotyp der drei *anterioren* angenommen haben. In der vorliegenden Arbeit wird eine neue Herangehensweise vorgestellt, die Aspekte beider Hypothesen sowie aktuelle, experimentelle Ergebnisse integriert. Sowohl der morphologische Umbau als auch die veränderten Expressionsmuster *posteriorer hoxD*-Gene sind dieser neuen Theorie nach auf einen gemeinsamen Grund zurückzuführen: die Reduktion des *anteriorsten* Fingers. Es wird ein entwicklungsbiologisches Szenario vorgestellt, das molekulare Expression und biomechanische Aspekte verbindet. Der Kernmechanismus wäre eine ungleiche Zellproliferation, die eine Verschiebung von Finger II Richtung *anterior* bewirkt. Dadurch verändert dieser Finger seine Position relativ zur *zone of polarizing activity* (ZPA) und wird damit auch einem geringeren Niveau des Sonic Hedgehog (Shh)-Proteins ausgesetzt. Beim gegenwärtigen Stand der Forschung geht man davon aus, dass Shh eine tragende Rolle bei der Spezifizierung der Fingeridentitäten spielt, was nahe legt, dass es die Morphologie der einzelnen Elemente stark beeinflussen kann. Darüber hinaus ist die Expression *posteriorer hoxD*-Gene in der Extremitätenknospe von Shh abhängig; aus diesem Grund nehmen wir hier an, dass auch das Expressionsmuster des Vogelflügels durch den erwähnten Mechanismus zu Stande kommt. Des Weiteren wird ein evolutionäres Szenario vorgestellt, das den Fossilienbefund mit dem

vorgestellten Modell verbindet. Im Rahmen dieser Arbeit wurden Experimente durchgeführt, die entweder den hypothetischen Urzustand im Flügel wiederherstellen oder die morphologisch-genetischen Veränderungen absichtlich im Bein herbeiführen sollten. Dazu wurden entweder Kügelchen, die mit FGF-8-Protein getränkt wurden, in die Extremitätenknospe eingebracht, um den Überrest des ersten Fingers im Flügel fertig zu entwickeln, oder die Entwicklung der ersten Zehe wurde durch invasive Manipulation oder Injektion von Mitose Inhibitoren verhindert. 3D-Darstellungen mikrotomographischer Scans und Alcian Blue-Färbungen belegten, dass es tatsächlich möglich ist, dass ein Finger den Platz und den morphologischen Phänotyp eines anderen einnimmt, wenn dessen Entwicklung gebremst wird. In situ Hybridisierungen, die *hoxD12*-mRNA nachweisen, ergaben außerdem, dass die Expression von *hox*-Genen dadurch ebenso beeinflusst werden kann. Gemeinsam unterstützen die morphologischen und genetischen Ergebnisse die eingeführte Hypothese, da sie eindeutig zeigen, dass die vorhergesagten Effekte des Kernmechanismus der Theorie in Embryonen tatsächlich induzierbar sind. Ferner wird gezeigt, dass die vorgestellte Hypothese mit derzeitigen entwicklungsbiologischen und paläontologischen Ergebnissen vereinbar ist.

Introduction

Assigning homologies to the digits of the avian fore limb has developed into a classic of EvoDevo research (for reviews see [1-4]). The problem has its origins in the question of the phylogenetic origin and the evolution of birds and has persisted for more than 150 years. Because of impressive skeletal similarities, the major theory is that birds are descendants of bipedal theropod dinosaurs (dromaeosaurs). This theory was proposed - or rather rediscovered - by John H. Ostrom[5,6] and gained further support by many researchers such as Paul C. Sereno[7] and Jaques A. Gauthier[8]. The major support came from the fossil of *Archaeopteryx lithographica*, which combines features of birds and dinosaurs[5,6,9].

Contradicting evidence

The major problem with the dinosaurian descent theory is the contradictory evidence different disciplines provide for the identity of the three digits in the bird wing[10-12]. The fossil evidence suggests that the digits of the dromaeosaurian hand, which closely resembles that of Archaeopteryx[7,8], are the anterior ones (thumb, index finger, and middle finger or DI, DII, and DIII). The main lines of evidence here are the resemblance of the first metacarpal of theropods to that of basal, pentadactyl dinosaurs[3,8], and the fact that dinosaurs like *Herrerasaurus*[13] and *Eoraptor*[14], which are considered to represent basal theropods, have two reduced or vestigial digits posterior to the three fully developing ones. This identification is further supported by the morphology of avian digits[7,15]. Also, the expression patterns of genes of the 5' *hoxD* cluster in the bird wing resemble the ones of DI, DII, and DIII in alligators and mice[16-18].

The morphogenetic development of extant birds, however, suggests identification of the digits as DII, DIII, and DIV. The first line of evidence is the so-called primary axis[19], by which the first skeletal elements that form during limb outgrowth are described. The first pre-chondrogenic condensation that is formed in the avian autopod is the one of digit DIV, followed by two more on the anterior

side[15,20,21]. The last digit that is formed is the most anterior one, and therefore a likely candidate to be lost first[15]. In fact this has already been proven experimentally by injecting a mitosis inhibitor into alligator eggs[22].

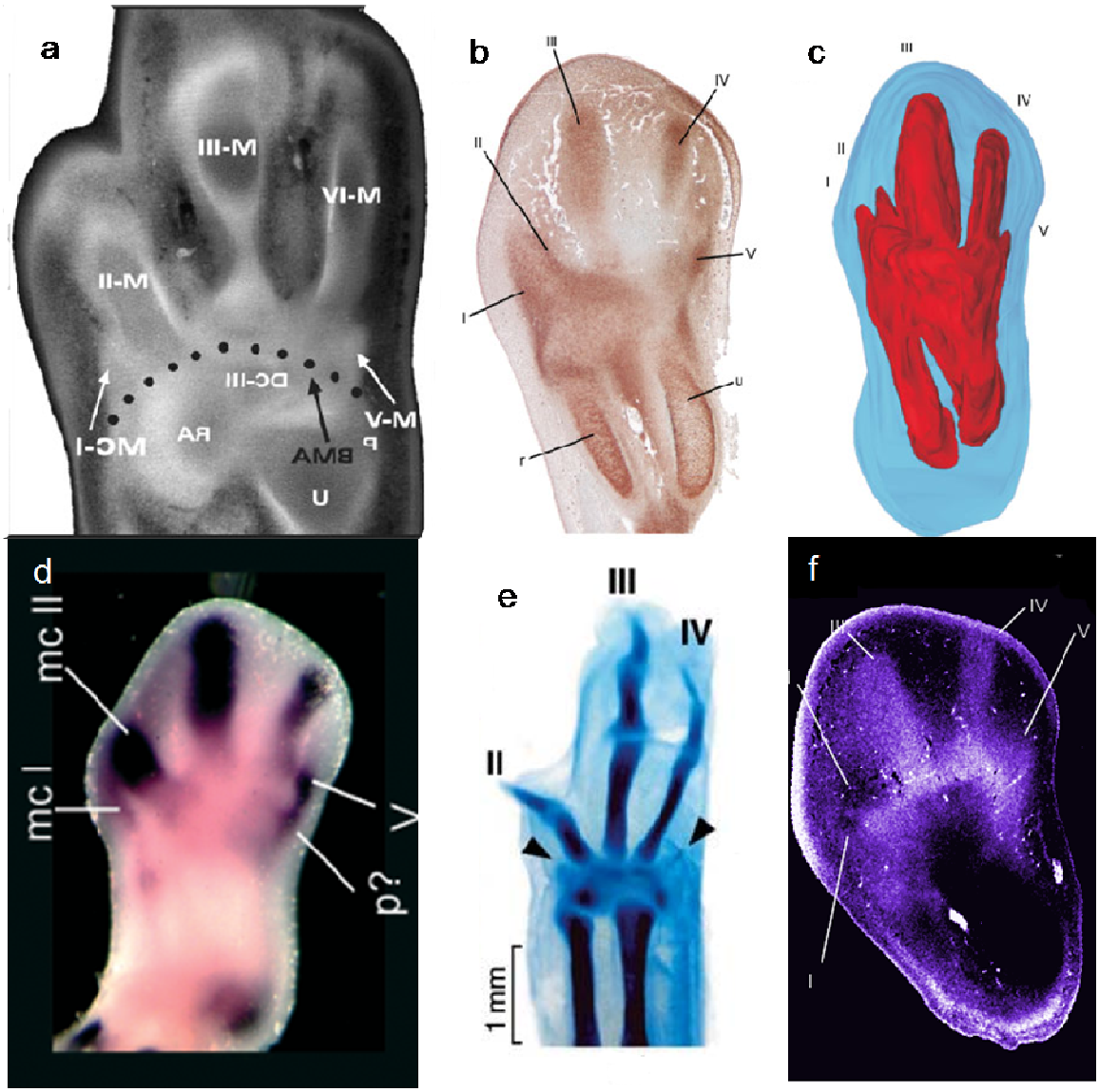

Figure 1: Different approaches to show the vestigial digit on the anterior side of the hand, shown are right fore limbs (a is projected) from dorsal. Roman numerals mark digit position; r = radius, u = ulna, mc = metacarpal, p = pisiform. India Ink injections show avascular zones anterior and posterior of the fully developing digits (a)[23]. b shows sections stained with peanut agglutinin, c a reconstruction of serial sections[24]. Whole mount *in situ* hybridizations were able to detect *sox9* expressing zones in the area of digit I and digit V plus a second posterior structure that could be a pisiform (d)[25]. In ostriches (e) the condensations could be visualized with alcian blue staining[26]. f: X-ray micromorphological approaches of samples stained with phosphotungstic acid also showed the vestige on the anterior margin of the fore limb (Image by Čapek, Bischof, Pokorny, Metscher).

Major support for the DII, DIII, DIV identification came, when a transitory condensation anterior of the three developing digits was discovered in bird embryos. A condensation on the posterior margin that develops until the cartilage stage had already been known[27,28]. The first approach that was successful in finding this anterior condensation, was to inject india ink to the limb bud of chick embryos[23] to visualize the avascular zones that arise with early cartilage formation (Fig. 1a). Shortly that peanut agglutinin staining of histological sections of chicken embryonic limbs[24] (Fig. 1b) and alcian blue staining in ostrich embryos[26] (Fig. 1c) were also able to visualize the digit I vestige. Finally, whole mount *in situ* hybridizations with *sox9* probes provided molecular evidence for the anlage of the anterior-most digit[25] (Fig. 1d). Discoveries of non-avian maniraptorans with feathers like *Protarchaeopteryx robusta* and *Caudipteryx zoui*[29-31] and recently also of the large basal tyrrannosauroid *Yutyrannus huali*[32], support the theropod descent strongly. Although the dinosaurian descent of birds is no longer really in doubt, the problem of the identity of the avian wing digits remains.

Martín Ramírez used this specific problem as an example of ambiguous homology[33] (Fig. 2). He introduces the difference between positional and compositional characteristics of any given structure in an organism. The first marks the location in which the structure is formed - typically relative to other structures - the latter describes the features of the structure itself, like morphological phenotype or the transcriptome of the cells that contribute to the structure. In most cases of homology, corresponding structures can be identified in related organisms by these two types of characteristics, even if one is more or less derived. In the case of avian digits however positional features identify the digits quite clearly as II, III, and IV, whereas compositional information gives strong evidence for them being 1, 2, and 3. In order to be able to distinguish between these two sets of evidence, identification of digits by means of positional information will be referred to with Roman numerals, while Arabic ones will be used for compositional nomenclature.

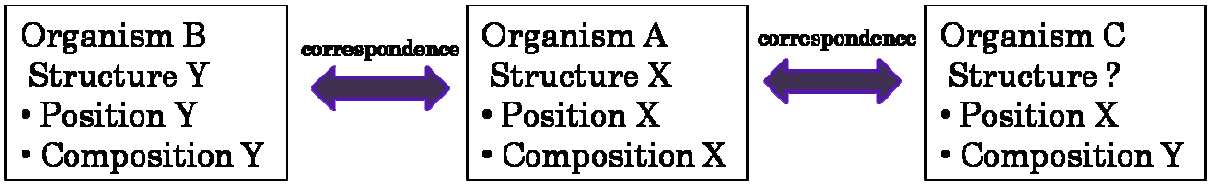

Figure 2 modified after [33]: Assigning homologies to structures in different animals. Typically a structure has specific positional and compositional features, which together give identity to it, and allow to compare it to homologous structures in other animals. Structure Y in animal B can be considered homologous to structure X in Organism 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. Organism C has a structure that shares compositional features with structure Y and positional ones with structure X. Therefore an unambiguous homology to either structure cannot be assigned.

Different approaches to the problem

The aim of Evo-Devo research to integrate data from different fields[3] makes this case especially interesting, because exactly this combination of two data sets (paleontology and embryology) seems to be impossible in the present case. Since this problem does not offer a simple solution, a broad variety of hypotheses have been established in the past. This has even increased in the last two decades, since more techniques have become available.

Birds have a different descent than dinosaurs

This oldest theory had its main renaissance with Alan Feduccia's book *The Origin and Evolution of Birds*[11]. The incompatibility of developmental and paleontological data concerning digit identification is among his prime arguments for excluding birds from the dinosaur clade. Instead he suggests a descent from more basal archosaurs[11]. At that time the identification of the avian digits was mainly based on the argument of the primary axis[15,20,21], because an unambiguous identification of digits in a tetradactyl limb is not possible. Therefore the discovery of a digit I vestige[23-26] was very much in favor of this hypothesis. The major flaw of this approach was from the beginning its lack of parsimony. It solved the problem of digit homology, but at the same time had to explain the entire mosaic form of *Archaeopteryx lithographica* as convergent evolution. With the finding of feathered dinosaurs[29,31,32] however most

researchers do not consider a different origin of birds and dinosaurs any longer, although attempts were made to explain the fossil feather traces with other features than feathers[34].

The Axis Shift Hypothesis

This hypothesis was the first approach that explained the embryological data, while also considering the broad set of synapomorphies, which link birds to dromaeosaurid theropods[8,35,36]. It was based on the fact that the main evidence for identifying bird digits was the primary axis, and that only digits anterior to this axis, that usually represents digit IV are formed. Therefore if this axis would have shifted in avian evolution and would project through digit III in present day birds, this would identify the wing digits as I, II, and III[35,37]. This was somewhat supported by salamander development, in which digit II is the first to be formed and not digit IV[38], but no such case is known in sauropsids. Furthermore *Tyrannosaurus* had a reduced digit IV, leaving only two fingers[35]. As another piece of evidence for the perturbation in the formation of the avian primary axis, Chatterjee[35] mentioned that the *ulnare* usually is a part of the primary axis, but is lost in birds[28]. Finally Garner and Thomas[39] pointed out that with the theropod hand having reduced digit IV, the only possible digit for the bird primary axis is digit III. Therefore an identification as DIV would only be possible by excluding birds from theropods. Thus using the primary axis as support for a non-dinosaurian hypothesis[10] would be tautological and lack a clear separation between hypothesis, evidence, and conclusion[39]. Although this hypothesis offered a plausible and parsimonious solution to the problem, it is not considered longer since the discovery of the digit I vestige[23-26], which identified the bird wing digits clearly as positions II, III, and IV. However, Wagner[3] has pointed out, that it is possible, although not very likely, that the digit I element is some kind of pre-pollex, and the I, II, III assignment could still be correct.

The Frame Shift Hypothesis

The Frame Shift Hypothesis (FSH) was proposed after the ASH but before it was ruled out due to DI discovery in avian embryos[23-26]. Its core assumption is a possible dissociation between the formation of a generic structure and its identity[15]. Examples where organ formation does not directly determine organ identity[40], were the base for this idea[3]. Whereas previous hypotheses have tried to falsify one set of evidence (fossil or developmental) in favor of the other one, the FSH assumes that both identifications are correct (1, 2, 3 = II, III, IV). It argues that dinosaurs faced a selective pressure to reduce posterior digits, but developmental constrains only allowed those digit anlagen that form last to be reduced first[15], i.e. DI followed by DV[38]. The frame shift was originally thought to have taken place after the reduction from 4 to 3 fingers after *Coelophysis*[41] and *Torvosaurus*[42], but before *Allosaurus*[43] branched off[15,44]. After the finding of the ceratosaur *Limusaurus inextricabilis*[45], which displays a tetradactyl fore limb, with digit I reduced further than digit IV, the hypothetical frame shift had to be moved to a time before the loss of the fourth finger[44]. To be more precise, it argues that digit 5 at position V was lost first, then the frame shift occurred, leading to digits 1-4 to be formed at positions II-V with position I being lost; then position V would have been reduced again, thereby losing digit 4, leaving a tridactyl hand with digits 1, 2, and 3 at positions II, III, and IV[44]. The major advantage of the frame-shift hypothesis is that is does not exclude any kind of evidence (paleontological, developmental or morphological). The biggest flaw on the other hand is that it has to assume the possibility that a structure can be removed from its location, to be formed at another one without losing its identity. In support of this it has been suggested that embryological characters, such as the digit position should be weighed less than adult morphological ones or gene expression patterns, because they reflect the identity of structure to a lesser extent[46]. Another point of critique has been the plausibility of such an evolutionary model and the selective pressure that could cause the loss of one digit, and at the same time replacing it by another one[47].

The frame shift faction got molecular support by studies of expression patterns of *hoxD12* and *hoxD13*[16-18]. These were able to show that in the bird hind limb and in pentadactyl limbs of mice and alligators the anterior-most digit is the only one negative for *hoxD12* expression. Also in the bird fore limb the anterior-most digit has no *hoxD12*, but unlike the other examples, it originates from position II and not from position I. Following this approach the transcriptome of digit cells has been analyzed[48]. This also showed a linkage between FL D II (anterior-most) and HL DI (also anterior-most). However it was not possible to link the other 2 wing digits with hind limb digits unambiguously. Furthermore cell labeling showed that DIV in the chicken foot is made from cells of the Zone of Polarizing Activity (ZPA), whereas the cells of fore limb digit IV segregate early from the ZPA and seem to migrate towards the anterior[49].

The Pyramid Reduction Hypothesis

With the discovery of avascular zones at the position of digit I in chicken and ostriches[23], the II, III, IV identification was heavily supported, and hence the question arose, as to whether it was possible that the theropod ancestors also had a bilateral reduced manus and therefore II, III, IV digits[23,47]. Since the reduction of 4 to 3 fingers could have happened by losing V digit and not digit IV, this would be possible. Although this would make things a lot easier, the problem is that the fossils of *Eoraptor* and *Herrerasaurus*, which are considered basal theropods, show reduction of both posterior digits[7,8,50]. Supporters of the Pyramid Reduction (PRH) argue that the attribution of these taxa to the theropods is not unambiguous[47]. And even if they are included in the theropod taxon, their position within it is not clear[47,51]. In the initial publication Kundrát et al.[23] also suggested a mechanism that would be able to derive the *Archaeopteryx* phalangeal formula from the archosaur one. While the archosaur groundstate 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). It has been shown that modulating

interdigital *bmp* signaling[52] or blocking *bmp* with a dominant negative receptor[53] is able to reduce one phalanx from each digit, and therefore a mechanism like that could have caused the archosaur central digits to resemble the *Archaeopteryx* ones. Supporters of the Frame Shift also admitted that the phylogenetic position of *Eoraptor* is problematic, because most apomorphies that link this fossil to the theropods are hand characteristics and therefore not applicable in this case[3]. Taken this into account, it leaves only one fossil (*Herrerasaurus*) that is in conflict with the Pyramid Reduction Hypothesis, therefore the PRH is the most parsimonious approach at the moment.

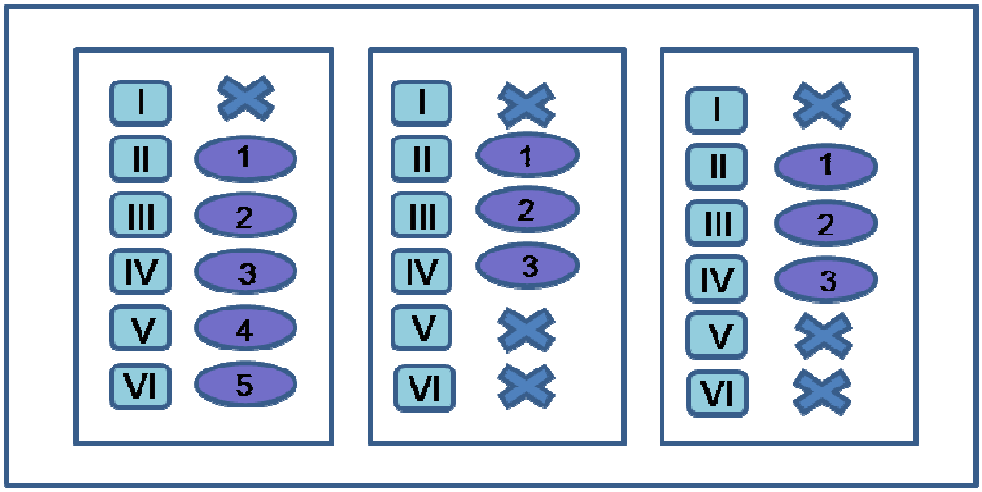

Figure 3 (redrawn after Welten et al.[25]): the polydactylous hypothesis. Squares mark positional, circles compositional features of digits, crosses designate reduced digits. The left column shows the archosaur ancestral condition with position I reduced and the other digits developing normally. The central column shows the theropod trend, where first digit 5 at position VI and then digit 4 at position V are lost (e.g., *Herrerasaurus*, *Eoraptor*, *Coelophysis*). The right column shows the final dromaeosaur condition (e.g. *Deinonychus*, *Gallus*).

The Polydactylous Hypothesis

The most recent hypothesis suggests that the tetrapod ancestor had a six (or more) digit limb[25]. In this case the vestigial digits of *Herrerasaurus* would really be DV and DVI and not DIV and DV (Fig. 3). 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 also have been interpreted as a

vestigial DVI, the same has been suggested for the mammalian pisiform[54,55]. This would also make the digit reduction in dinosaurs bilateral again, as would be expected[20]. The authors themselves however pointed out that there is no evidence for six or more fingered archosaurs and that also the *sox9* expression does not give a hint for a more posterior anlage then digit V[25].

A new approach: the Thumbs Down Hypothesis

None of the current hypotheses is able to explain all of the evidence completely. The axis shift hypothesis is the only one that can be ruled out with some certainty due to embryological evidence, and excluding birds from dinosaurs is very un-parsimonious. Since there is no evidence for hexadactyl tetrapods, the prevalent hypotheses at the moment are the Pyramid Reduction and the Frame Shift. The first question I asked was, whether one or both can be tested by experimental means. This however seems hardly possible, since dinosaur embryos are not available and also digits do not tell us their identity voluntarily. The next logical point to ask is, what the hand of a bird with 4 or 5 digits would look like, and this leads to a new approach to the digit identity question.

The hypothesis presented here argues that the molecular and the morphological changes in the avian fore limb are direct consequences of the loss of the anterior-most digit at position I. Because this effect is thought to be triggered once DI is lost, this model is called the Thumbs Down Hypothesis (TDH).

Morphogenetic aspects

The hypothetical key player for this transformation of molecular and morphological features is differential cell proliferation and, to a certain extent cell migration. At the point in development where the growth of the pre-chondrogenic condensation at position I ceases or falls behind the others, cells that will eventually form the digits II, III, and IV move towards the anterior, to a

region where they form digit phenotypes 1, 2, and 3 instead. This hypothetically happens for biomechanical conditions: if cell populations in the anterior limb region proliferate more slowly or stop completely they make way for faster proliferating populations that will then invade these regions. This results in an altered distance to the ZPA, which causes a change in the cells' fate (D1 instead of D2 phenotype: Fig 4). It has to be pointed out that this mechanism is hypothetical so far, and a reduced proliferation rate in the anterior limb region has not been shown yet.

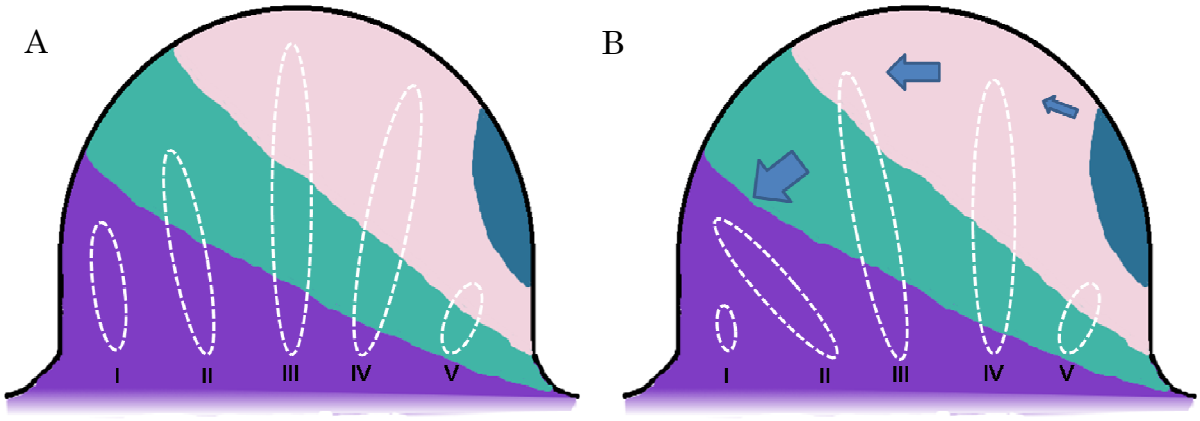

Figure 4: A shows a hypothetical right fore limb bud of a bird or a theropod with digit I developing normally; B shows an actual bird right fore limb bud, where the development of digit I ceases at pre-cartilage stage. Posterior is on the right. The blue region is the ZPA, in the purple region only *hoxd13* is expressed, in the turquoise *hoxd12* and *13* and in the pink region, *hoxD11*, *12*, and *13*. Broken white outlines mark projected digits. Blue arrows mark the proliferation shift in the growth of the respective digits, their size reflects the strength of the effect. The figure shows that the digit forming from position II leaves the zone in which it could express *hoxD12*.

Genetic mechanism

Since digit 1 develops outside of Sonic Hedgehog (Shh) reach, and D2 within a Shh gradient[56], a move of DII towards anterior would cause it to behave as if it was D1, due to a lack of Shh signaling. This would affect the transcriptome and as a consequence the phenotype (e.g. the phalangeal formula). Furthermore digit 2 and partially digit 3 rely on a Shh gradient[56], therefore their cellular behavior would not be altered to the same extent as it would be for D1, which relies on the absence of Shh protein. For the transcriptomes of digit cells to be altered in a way that they resemble another digit, it would be necessary that the Shh gradient

targets a gene that lies quite far upstream. Possible candidates for this could be the genes of the 5' hoxD cluster[57,58].

Sonic Hedgehog and Hox

In early limb development *shh* expression requires HoxA or HoxD signaling[59]. For the early limb bud Tarchini and colleagues[60] have shown that *shh* expression in the ZPA is activated by the 5' genes of the *hoxD* cluster - specificaly *hoxD10* and even more important *hoxD13*, which at that point are expressed only at the posterior end of the limb bud, probably due to anterior repression by Gli3[61]. After the establishment of the ZPA however, under the influence of Shh a new enhancer for the *hoxD* cluster, the general control region (GCR), is activated[57,58]. This enhancer region affects the 5' most gene strongest, hence *hoxD13* is expressed throughout the limb bud, while *hoxD12* leaves out digit I, and *hoxD11* is even more restricted (for a review of hoxD expression in the developing limb see [62]). Therefore the *hox* expression pattern of the chicken fore limb[16] - DII lacks *hoxd12* - can be explained by a larger distance from the ZPA.

Very recently Delpretti et al.[63] showed that genes of the posterior *hoxD* cluster have a direct influence on the length of digits and limb segments in general. In mutant mice with deletions in hoxD12-10 the length of metacarpals and phalanges was reduced to under 90% of the WT length.

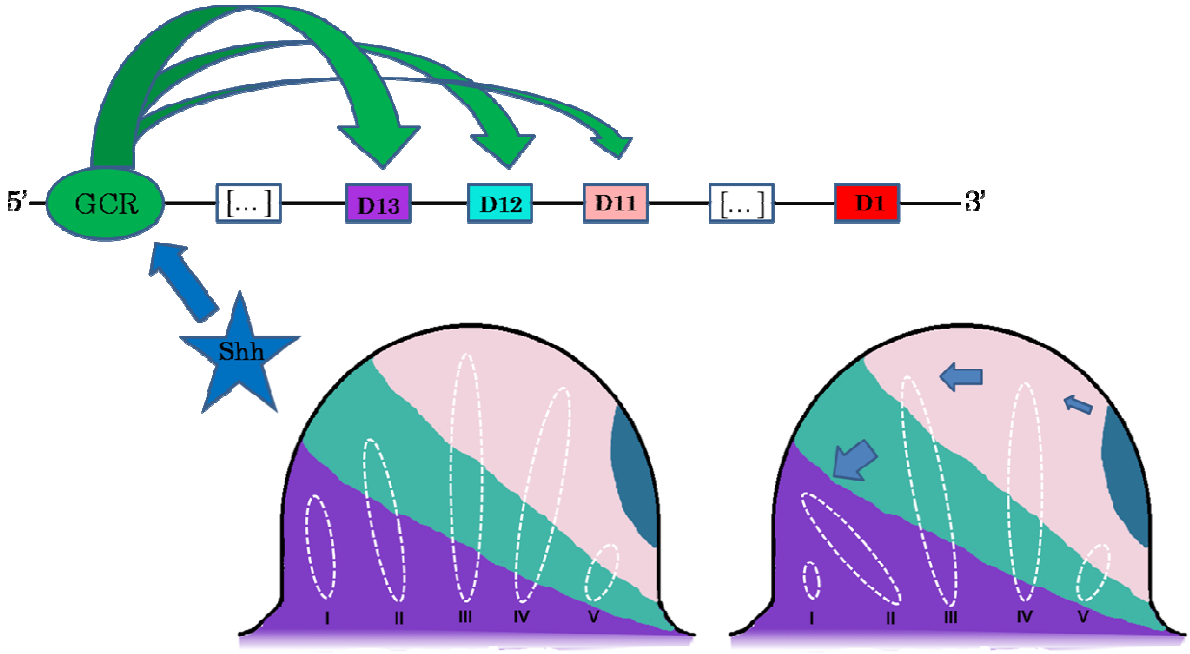

Figure 5 modified after [57]: After the establishment of the ZPA the general control region (GCR) is activated under Shh influence. It affects the closest genes strongest, which is indicated by the green arrows. Therefore *hoxd13* is expressed throughout the limb bud (purple), whereas *hoxD12* (turquoise) and *hoxD11*(pink) are far more limited, *hoxD* genes that lie even further 3' are no longer expressed in the limb bud.

Apical Ectodermal Ridge and Zone of Polarizing Activity

It is plausible that the number of phalanges changes when the transcriptome of the cells forming the digit changes. But also a model in which the number of phalanges is directly depending on the ZPA is conceivable. The proximodistal outgrowth is regulated by FGF-8 signaling from the apical ectodermal ridge (AER) (for a review of vertebrate limb pattering see [64]). Since Shh signaling from the ZPA is required to maintain the AER[56,60,65,66], the number of phalanges is likely to change, if the position of its progenitor cells shift. The maintenance of FGF signaling from the AER works via feedback between *fgf*, *shh*, *bmp* and *gremlin* (*grem*)[64,67]. This loop has the ability to terminate itself once the distance between *grem1* and *shh* expressing cells, the descendants of which cannot express *gremlin* themselves, becomes too large[68]. Thus limb bud outgrowth and patterning is terminated due to a down regulation of *gremlin* at a certain distance from the ZPA[69].

This could also be responsible for lower phalanx numbers in digits situated more anteriorly.

Compatibility with available data

So far it has been shown that the TDH is consistent with the embryological data and to some extend with the molecular results that have been presented in recent years (e.g. the *hox* expression patterns[16,18]). However, the TDH must also be in line with additional data in order to be a viable hypothesis.

Based on previous results[70] Vargas and Wagner[71] have treated chicken limbs with cyclopamine in order to obtain phenotypes with altered digit patterns. Cyclopamine is a steroid alkaloid that inhibits the hedgehog pathway by directly binding to the Smoothened receptor [72]. When they applied cyclopamine between Hamburger & Hamilton[73] stages 18-21, they achieved anterior digit phenotypes at posterior positions: 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 (not the actual protein level) and therefore shifts the boundaries of *hoxD11* (Fig. 4, pink) and *hoxD12* (Fig. 4, turquoise) towards the posterior[71]. Thus the digits arising from this region encounter the *hox* and *shh* environment of their anterior neighbor and consequently adopt its fate.

Another study that supports the TDH is the cell labeling and grafting approach by Tamura et al.[49]. They were able to show that in the chicken hind limb and in mouse fore and hind limbs DIV is formed by cells that originate from the ZPA, while the DIV (D3 phenotype) cells in the chicken fore limb segregate early from the ZPA and migrate towards a region in which digit III usually is formed. Since this is exactly that kind of cellular anteriorization the TDH is based on, this is strong evidence for it.

Recently also the transcriptome of the digit cells was analyzed[48]. It showed a strong linkage between fore limb digit II and hind limb digit I (both are the anteriormost fully ossifying in the respective limb). However for both other fore limb digits the results were not clearly resolvable. FL DIII could not unambiguously be linked with either HL DII or HL DIII. The same was true for the most posterior fore limb digit. Since the TDH predicts that digit II will be 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. 4, blue arrows), this is exactly the result that would be expected. If a homeotic shift would have occurred, which would have moved the entire digit frame towards anterior phenotypes, all digits should be affected in the same way.

Compatibility with the fossil evidence

Beside various molecular results, the fossil evidence has to be taken into account. Intriguingly the most recent developmental evidence was interpreted in favor of the frame shift[48,49,74], whereas the newest fossil findings[45] support the pyramid reduction[44]. The main line of argument for the PRH was that the first reduced digit was digit I followed by the posterior reduction from four to three fingers[47] (Fig 6, upper row). The FSH in contrast assumed that two digits were lost on the posterior side, and then the remaining three digits where shifted in such a way that had them formed from more posterior condensations (Fig. 6, lower row).

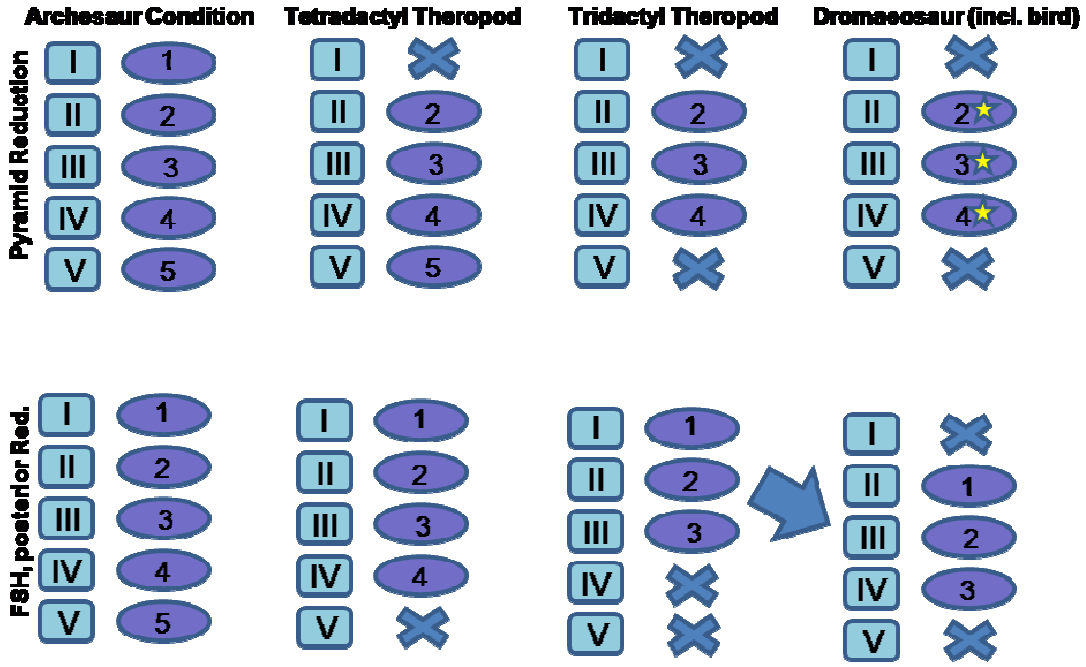

Figure 6: Schematic overview over Pyramid Reduction and original Frame Shift Hypothesis concerning the fossil evidence. Squares with Roman numerals mark digit positions, while circles with Arabic numerals mark compositional features of the digit. Crosses mark lost digits, asterisks mark strongly altered phenotypes. The blue arrow indicates the hypothetical frame shift. The PRH argues that initially digit I was lost in the theropod linage, leaving the four posterior digits (e.g., *Coelophysis*), afterwards DV is reduced, and the three central digits remain. The FSH assumes the loss of both posterior digits, followed by a shift that caused the anterior digits to be formed from the central positions. This FSH reduction scheme is incompatible with *Limusaurus*, since it has a more strongly reduced digit I than IV.

With the discovery of *Limusaurus inextricabilis*[45], however, things got complicated. This ceratosaur had two well developed digits, likely at positions II and III, a partially reduced DIV (only metacarpal and one phalanx) and a vestigial DI metacarpal. This supported a gradual morphological remodeling, and according to the FSH, digit 1 probably was never lost but moved to position II[44]. Hence the FSH had to be adapted so that the shift occurred while still 4 digits were present (Fig. 7, upper row).

The question is how the TDH can accommodate all the fossil evidence. The most parsimonious approach would be to accept a reduction of DV first, in order to include *Herrerasaurus* and *Eoraptor*, and then also assume a partial reduction of digit IV, in a way that leaves the metacarpal and one phalanx (e.g., *Coelophysis*). Then, however, the developmental constraints would prohibit the primary axis

from being reduced further, and digit I starts to overtake digit IV, becoming reduced until it is finally lost (the situation in *Limusaurus*). As soon as the DI development is lost, the biomechanical aspects comes into play, causing the remaining digits to grow further away from the ZPA and therefore adopt phenotypes of more anterior digits (*Deinonychus*, *Archaeopteryx*). Thus this reduction scheme is very favorable, because it does not need to exclude fossils from the phylogenetic tree in order to work.

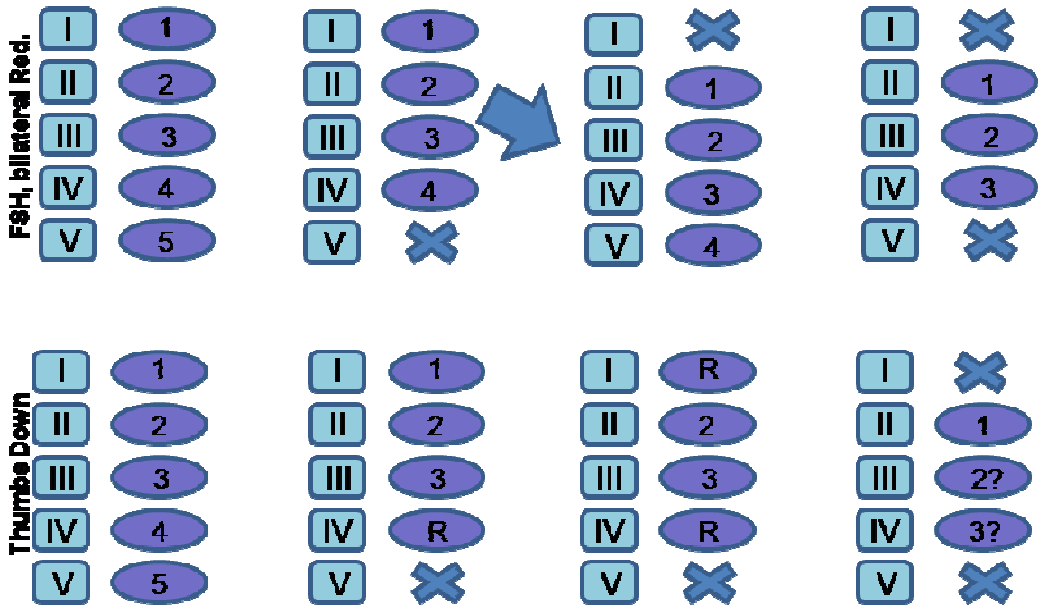

Figure 7: Schematic overview of the updated Frame Shift Hypothesis and the Thumbs Down Hypothesis concerning the fossil evidence. Squares with Roman numerals mark digit positions, while circles with Arabic numerals mark compositional features of the digit. Crosses mark lost digits, asterisks mark strongly altered phenotypes. The blue arrow indicates the hypothetical frame shift. Upper row: After the discovery of *Limusaurus* with its strong reduced digit I, the Frame Shift had to be predated to the 4 digit state. After the FS position V was lost again, this time loosing D4. Lower row: The thumbs down hypothesis accepts the initial loss of DV and partial reduction of DIV (e.g., *Herrerasaurus*, followed by *Coelophysis*). But then digit I is reduced much faster than digit IV. At the point where digit I is lost completely the morphogenetic effect causes the remaining digits to grow more anterior and therefore adopt different fates.

Difference from the Frame Shift Hypothesis

The FSH postulates an evolutionary event in which a dissociation between the developmental formation of a repeated element (in this case the digits) and the subsequent individualization (digit identity) occurs[15]. This can be imagined in a

similar fashion as homeotic changes in the segment identity of *Drosophila* mutants[75]. Thus digits 1,2, and 3 are formed instead of II, III, and IV. According to the TDH no change of identity in a homeotic way happens, but only the phenotypic realization of the developmental process is altered. The digit identity stays the same. Also the TDH assumes that the patterning of the limb bud, by which the digits are laid down, and their morphological realization, are different developmental modules in the first place. The latter uses *shh* signaling and differential *hox* expression, whereas the first essentially is a matter of growth and activator-inhibitor interactions[76]. In our model this effect is a direct consequence of the increased distance of DII cells from the ZPA, which in turn is directly caused by the loss of digit I.

Experiments

As stated before, the problem with many of the established hypotheses is that they are not really testable experimentally. Therefore it is important to think of experiments that can give support to or falsify the TDH. Generally this is not an easy task in developmental biology, since heterochrony and epigenetic mechanisms play a major role in animal development. However there are some predictions of the TDH which can be tested:

1) If birds would have retained their DI, their DII should not display a D1 phenotype, *hox* pattern, and transcriptome. Therefore by reversing DI loss, the phenotypic and transcriptomic effects should be reversible.

2) If a tetrapod reduces its DI until complete loss, its DII should adopt a D1 phenotype, *hox* pattern, and transcriptome. Therefore by inducing DI loss, the phenotypic and transcriptomic effects should be inducible.

Bead implantations

This experiment tries to test prediction 1, that no *hox* shift would occur if the vestigial digit I would develop fully. To rescue digit I, beads soaked with FGF protein are implanted into the anterior AER. It has been shown that FGF-8 signaling from the AER is required to maintain proximo-distal outgrowth[77]. Also it was shown that if the AER is removed, FGFs can replace it and a normal limb is built[78]. In this case, beads soaked with FGF-8 are implanted into stage 27 and stage 29 fore limbs. The tetradactyl fore limbs thus obtained are analyzed with whole mount *in situ* hybridizations for *hoxD12* and *hoxD11*. The working hypothesis is that the tetradactyl limbs have a DI negative for *hoxD12* and a DII positive for *hoxD12*. *HoxD11* served as a control since it should in both cases have a more limited zone of expression.

Cauterization/Injuring of the AER

To test the second assumption the first toe of the chicken hind limb was ablated. This experiment also worked as a backup, in case the bead implantations do not lead to a rescued digit I. The loss of hind limb digit I, according to our hypothesis, should make digit II negative for hoxD12. To achieve tridactyl chicken hind limbs, the anterior hind limb AER of stage 22-24 chicken was be interfered with. Two different approaches were tried here: the cauterization of the AER with a hot needle, and injuring with a sharpened tungsten needle.

Mitosis inhibition with Cytosin Arabinoside

Another approach to test the second assumption tries to place the experiment in a more realistic developmental environment. Instead of specifically interfering with the DI anlage, a global approach was tried. It has been shown[22] that treating alligator embryos with the mitosis inhibitor cytosine arabinoside at the correct stage, removes digit I without impairing the development of the other digits. Effects of this drug have also been confirmed in chicken embryos[79]. Recent

results also showed that overexpressing the cyclin-dependent kinase inhibitor $p21^{cip1}$, inhibits growth and causes anterior elements to be lost in chicken fore limbs[80]. Therefore the cytosine arabinoside treatment was applied to chick embryos of 3 and 4 days of age with the aim to simulate a slower growth rate in the limb bud. Depending on the stage and dose this should yield different phenotypes. The analysis then was done with *hoxD11* and *hoxD12* in situ hybridizations as well as with morphological staining procedures and measurements.

Material and Methods

Animals

Embryos of *Gallus gallus domesticus* (LINNAEUS, 1758) were obtained from Schropper Gmbh, Gloggnitz, Austria. They were incubated at 38.5°C and 39.5°C in the incubator of the Department of Theoretical Biology, University of Vienna. The incubation time was chosen to achieve the respective Hamburger-Hamilton[73] stages for each experiment.

Bead Implantations

Preparation of beads

A number of Affi-Gel Blue beads (Bio-Rad 732-6708) was removed from the cartridge with tweezers and put to a 100µL drop of PBS. A 2µL drop of FGF-8 protein (Sigma SRP 4053) was put in the center of a sterile 50mm petri dish, and around it about 20 8µL drops of PBS were applied for humidification. Then single beads of medium size (about 100µm) were taken from the PBS and first put onto the dry petri dish and then transferred to the FGF-8 drop, in order to avoid dilution of the protein. About 20 beads were collected in this manner. The petri dish was closed and sealed with parafilm, and the beads were soaked overnight at 4°C.

Windowing of the eggs and bead application

The eggs were incubated for 5 or 6 days to Hamburger Hamilton stage 27 or 29 respectively. The eggs were windowed according to Korn & Cramer[81]. First they were cleaned with 70% ethanol and candled with a lamp to locate the embryo. Then a piece of tape (about 1x1cm) was placed on the acute end. The shell was

damaged with dissecting scissors and the egg membrane was pierced with a 0.7mm needle, 3-4ml of albumin were removed. A larger piece of tape was applied to the top of the egg (about 3x2cm) and a window of about 1x1cm was cut into the shell.

1ml of Chicken Ringer Solution (see Appendix) with 1% penicillin-streptomycin (Sigma P0781) was added to the egg. The solution was prewarmed to 40°C.
Sterilized tungsten needles were used to open the amniotic membrane and to cause a wound in the anterior side of one fore limb bud. A bead was picked up from the FGF-8 drop and pushed into the wound with forceps or with a tungsten needle. After surgery the chick embryos were further incubated until stage 35. Beads soaked with PBS instead of FGF-8 were used as negative controls.

Cauterization

For the cauterization of the anterior AER the eggs were grown to Hamburger-Hamilton stage 22-24[73]. The eggs were candled and windowed in the same way as for the bead implantations. The amnion was opened with tungsten needles. The anterior part of the AER was damaged with a 0.6mm needle, that was heated with a flame. After surgery the chick embryos were further incubated until stage 35.

Mitosis Inhibition

The eggs were incubated at 39.5 °C for 3 or 4 days to the Hamburger Hamilton[73] stages 16-25. The egg surfaces were cleaned with 70% ethanol. After the embryo had been located by candling, the egg shell was cautiously cut with a metal saw blade. The egg membrane was not hurt, so that the shell could be removed with the subjacent membrane staying intact. A drop of Chicken Ringer Solution (no

antibiotic added) was placed on top of the membrane. A small hole was made at the acute end of the egg, and about 1ml of albumen was removed with a 0.7mm needle. After the embryo sunk down, the egg membrane was opened and the embryo was staged.

Cytosine beta-D-arabinofuranoside (Sigma C1768) in 200µl Chicken Ringer Solution was then injected to the yolk through the hole at the acute end. Both openings were then sealed again with Leukofix. The cytosine arabinoside was diluted to a stock of 50mg/ml, and this was further diluted to between 1:10000 and 1:200. Therefore between 1µg and 50µg of the drug were delivered with a 200µl injection. After surgery the chick embryos were further incubated until stage 35. After the appropriate dose and the approximate time point that yields the required phenotype were roughly determined, a series of injections of the same dose was done. Every hour two eggs were injected, starting at 91 hours of incubation ending at 99 hours. This was done to pinpoint the correct time point of injection more accurate. This was extended later to the period from 100-120 hours. Eventually the incubation temperature in this experiment was dropped to 38.5°C as well in order to stretch the critical phase of digit development a little.

Alcian Blue staining

The Alcian Blue staining procedure was based on a protocol by Yamazaki et al.[82]. For a detailed protocol see Appendix. The chickens were fixed in 3.7% formaldehyde in PBS for 1 hour at room temperature. They were then stored in 70% ethanol over night at 4°C. The samples were stained with Alcian Blue in 70:30 Ethanol:Acetic Acid for 6-16 hours. After a couple of rinses in 70% ethanol they were rehydrated to water and subsequently macerated in 2% KOH for 4 hours. The samples were transferred in a graded series from KOH to Glycerol (4 steps, each 8-24 hours). Eventually they were photographed and stored in Glycerol.

Whole Mount In Situ Hybridization

The *in situ* hybridizations were done according to a protocol from Cepko/Tabin lab, which I received from Christine Hartmann (Institute of Molecular Pathology, Vienna). It was again slightly modified. A detailed protocol can be found in the Appendix.

RNA extraction and Cloning

Chicken embryos of stages 24, 27, 29, and 35 were collected in sterile Eppendorf tubes. The head was removed and the rest was frozen with liquid nitrogen and stored at -80°C. The tissue of all 4 samples was homogenized with an RNAse free plunger and transferred to one tube. 250µl of the blended homogenized tissue were transferred to a fresh Eppendorf tube and 750µl TRIzol-Reagent (Invitrogen 15596-026) were added. The mixture was again homogenized by pipetting. After 5 minutes the liquid was transferred to a Phase Lock Gel Tube (5 prime 2302820), 200µl of chloroform were added and the samples were mixed by shaking. After 3 more minutes they were centrifuged for 15 minutes at 4°C with 12,000 x g. The aqueous phase was transferred to a new tube the rest was discarded. 500µl of Isopropanol were added, after incubation for 10 minutes at room temperature the samples were centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was discarded the remaining RNA pellet washed twice with 75% Ethanol. To do that 1 ml of ethanol was added, the sample was vortexed briefly and then centrifuged for at 7,500 x g 5 minutes at 4°C. After the second wash the pellet was air dried 10 minutes under the fume hood. The RNA was resuspended in RNAse free water and heated to 55°C for 5 minutes. Subsequently it was stored at -80°C.

cDNA was synthesized by reverse transcription with SuperScript III (Invitrogen 18080-044) for 60 minutes at 50°C (for reaction recipe see Appendix). The reaction was terminated by raising the temperature to 75°C for 15 minutes.

Subsequently a PCR with gene-specific primers was performed and the results checked on a 1% Agarose in TAE-buffer Gel.

The PCR products were ligated into the pGEM-T easy II vector (Promega A1380) for 1 hour at room temperature and over night at 4°C (for the detailed recipe see Appendix). The vectors with the ligated insert were transformed into competent *E.coli* bacteria, which were spread out on agar plates containing ampicillin and incubated over night at 37°C (the protocol can be found in the Appendix). Six colonies of each plate were picked and stirred into 5µl of water; before that a small scratch was made on a new agar plate with ampicillin, the colonies and scratches were labeled correspondingly. For each clones 2 PCR reactions were performed using Phusion Flash High Fidelity Master Mix (Finnzymes F-548S) to check the direction of the insert (for the recipe see the Appendix). Both used the same gene specific primer and one that binds to a promoter site (T7 or SP6) for RNA polymerase.

One clone per gene was selected and picked into 5ml of LB medium with ampicillin and incubated over night at 37°C in a shaker. The plasmids were harvested using a Qiagen QIAprep Spin Miniprep kit (Qiagen 27104) and stored at -20°C. Eventually the inserts were sequenced with T7 and SP6 primers and aligned to *hoxD11* and *hoxD12* mRNA sequences (downloaded from NCBI, accession numbers: NM_204620 & NM_205249), using the plasmid editor ApE from University of Utah (http://biologylabs.utah.edu/jorgensen/wayned/ape/).

Probe synthesis

First the insert of the respective plasmid was amplified with PCR using T7 and SP6 primers for *sox9* probes and M13 primers for *hoxD* probes (for the recipe see Appendix). The products were run on a 1% agarose-TAE-gel. The correct bands were cut out and purified using Qiagen Gel Extraction kit (Qiagen 28704). The purified DNA then was used as template for the digoxygenin labeling. For this an in vitro transcription was performed(for the recipe see Appendix) with a DIG

RNA Labeling Mix (Roche) for 6 hours or overnight at 37°C. The product was cleaned up with Qiagen RNeasy MinElute Cleanup kit (Qiagen 74204). Alternatively 0.5µl of TurboDNAse (Invitrogen AM2238) was added and incubated for 15 minutes at 37°C. Thereafter 10µl nuclease-free water and 10µl of 7M Lithium chloride was added and the RNA was precipitated overnight at -20°C. On the next day the probe was centrifuged at 14,000 rpm for 20 minutes at 4°C and washed twice in 75% Ethanol. After air drying the RNA pellet was re-suspended in 50µl of RNAse-free water, and 1µl was run on a agarose gel. The concentration was determined by nanodrop measuring. The probes were diluted to 50ng/µl with hybridization buffer and stored at -20°C.

In situ Hybridisation

Chicken embryos of 5, 6, or 8 days of age were fixed overnight in 3.7% formaldehyde at 4°C. They were then washed in methanol and stored in fresh methanol at -20°C. The embryonic membranes were dissected away in methanol and they were bleached in 3% H_2O_2 in Methanol to inhibit endogenous peroxidase activity. Thereafter the samples were rehydrated in a graded series to PBT (PBS + 0.1%Tween 20). The embryos were digested with Proteinase K in PBT for 15 minutes. The concentration was 20µg/ml for stage 27 samples, 30µg for stage 29 and 60µg for stage 35 chicken. The digest was stopped with glycine and the specimens were refixed in 4% paraformaldehyde. The samples were then transferred to hybe-buffer and incubated between 1 and 4 hours at either 70°C. Thereafter the hybe-buffer was replaced with one that included RNA probes and the samples were incubated overnight or over weekend at 60 or70°C. On the next day the samples were washed thrice in washing solution I at 60 or 70°C and thrice in washing solution three at 55 or 65°C for raising the stringency.

Antibody detection

After that they were washed three times in TBST and then blocked in TBST + 10% heat-inactivated sheep serum. A horse-radish peroxidase labeled anti-

digoxygenin antibody (abcam ab6212) was diluted 1:5000 in TBST + 1% heat-inactivated sheep serum. The samples were blocked between 1 and 6 hours and then incubated with the antibody over night at 4°C on a rocker. They were washed in TBST the next day at room temperature and over night at 4°C. On the next day they were transferred to water and washed a couple of times.

Colour reaction

Since the antibody was conjugated with horse-radish peroxidase instead of alkaline phosphatase, in order to be also able to precipitate silver nitrate[83], diamino-benzidine (DAB) (Zymed 00-2013) was used instead of NBT/BCIP as a staining substrate. The samples were incubated 3-5 minutes in the solution and thereafter washed with water. Eventually they were transferred to glycerol.

Imaging

Photography of Mounted Samples

Samples were transferred to Glycerol and mounted on microscopy slides. They were then analyzed with a Zeiss Imager.A1 microscope with 5x Magnification. Pictures were taken with ProgRes Mac CapturePro software. Image Processing was done using GIMP.

MicroCT Scans

Additionally some samples were scanned with microCT, in order to have 3D images of the limbs. The specimens were stained with 0.3% (w/v) phosphotungstic acid (PTA) in 70% ethanol or 1% (w/v) iodine in 100% methanol (I2M) for 16-24 hours to enhance the contrast, which is otherwise weak in non-mineralized embryonic tissue[84,85]. They were then rinsed with 70% ethanol or 100% methanol respectively and mounted in pipette tips in 0.5% agarose. The samples were

scanned with a Xradia MicroXCT scanner with the source set to 40keV and 100μA. Reconstructions were performed with the Xradia software, 2D image processing of the image stacks with ImageJ. The 3D processing, volume rendering and surface rendering was done with Amira.

Results

Bead Implantations

This approach had the aim to rescue the vestigial digit I of the avian fore limb by implanting a bead soaked with FGF-8 protein. This was given up after some time for two reasons. First the lethality among the embryos was far too high. Not a single one survived until stage 35 and only one until day 7. The majority died in the first night after surgery. The second point is that the injury from the bead implantation was very close to the site of interest, i.e. digit I and II, and therefore the surgery could possibly interfere directly with digit growth.

The one embryo that lived until day 7 was stained with PTA and scanned with microCT. The scan clearly showed an effect of the FGF-8 protein. The treated limb was lacking a condensation in the region where DII usually forms (Fig. 8). Instead a condensation in the interdigital mesenchyme anterior of digit III was visible.

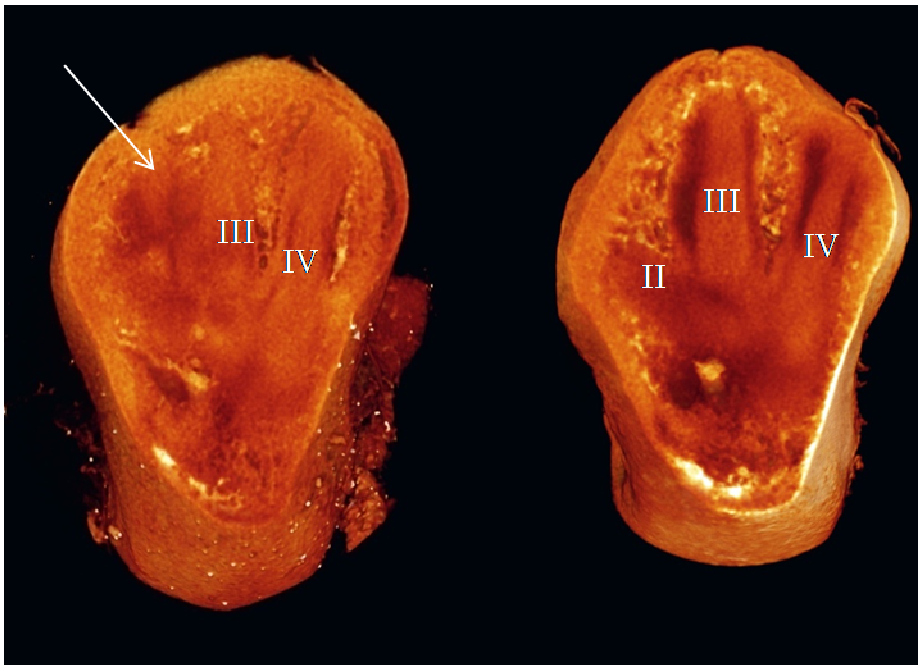

Figure 8: 3D image of a microCT scan of a bead implanted (left) and an unimplanted (right) limb of stage 31 (7 days old). Roman numerals mark the digits. The untreated limb bud shows three digits, whereas the treated one shows only two digits plus a third condensation that is situated more distal than the normal digit II condensation would be.

Ablation of hind limb digit I

The cauterization experiments did not alter the digit phenotype of the embryos. Neither cauterizing the anterior AER with a hot needle, nor injuring it with the tungsten needle were able to ablate digit I. The survival rate in this experiment was about 50%. All specimens were examined, but none showed digit reduction.

Mitosis inhibition experiment

The first step in this experiment was to determine the correct dose and time point for the mitosis inhibitor injections. The first set of embryos (labeled A1-A20) was injected at stages 16-18, when the limb bud was just starting to form. The injection doses were 5, 10, 15, and 20μg of cytosine arabinoside in chicken ringer solution. Except one all embryos died, without any significant post-injection-growth. The one sample that survived did not show any interesting digit phenotype, so it is possible that the injection did not hit the yolk, and therefore had no effect. For the next series (labeled B1-B20) more subtle doses were used: 1, 2.5, 5, and 10 μg, and three samples were injected with Ringer solution only. This time the drug caused an effect. The weight of the treated embryos decreased as the injected dose increased (Fig. 9).

The samples of this injection series did not display reduction of entire digits but only distal phalanges were reduced (Tab. 1). It also was interesting that neither the fore limbs nor hind limb digit I showed any reductions. Reductions were limited to the more-phalanxed digits of the hind limb. The next series was injected at stage 20 and generally showed the same picture. The animals became smaller if the dose was higher, and no digits were reduced. The next series labeled D, was injected at a later time point, when they reached stage 25. Since the embryos are larger at this stage, higher doses were injected: 10, 20, 30, 40, and 50μg. Ringer Injections were used as controls.

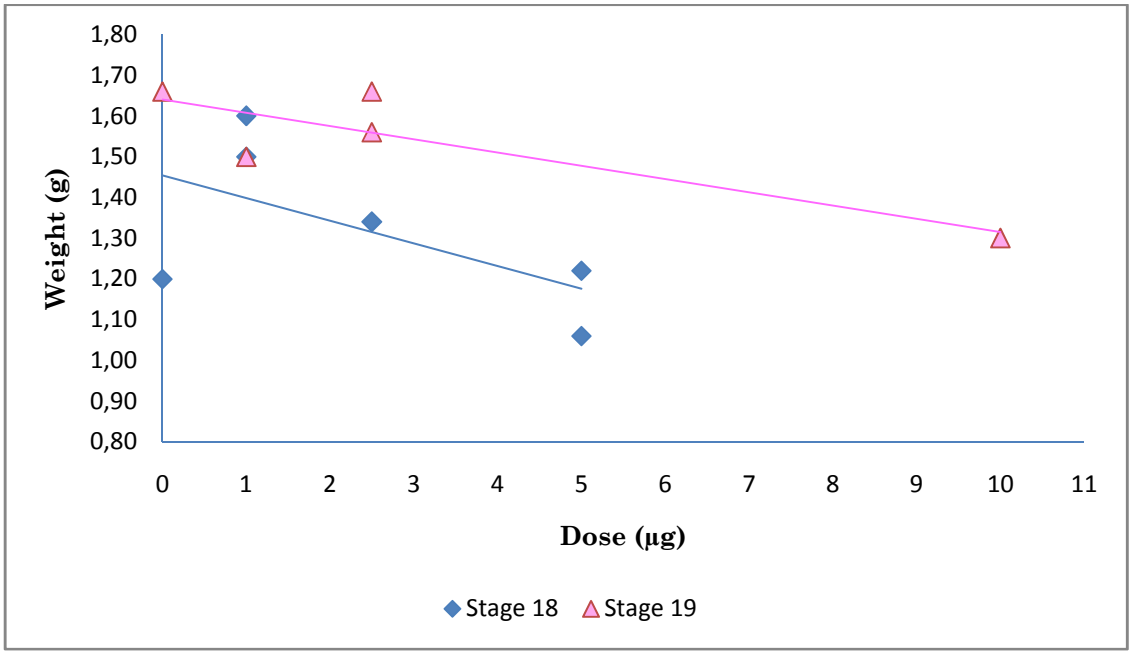

Figure 9: showing the correlation between the weight of the samples and the injected dose of series B. The weight is measured in g, while the injected dose' unit is µg. Although the embryos were incubated for the same time, some were rather stage 18 (blue rhombuses) and some stage 19 (pink triangles). The general trend is that the weight decreases with increasing injection dose, but the sample size is too small to show this clearly. Also the stage at the time of injection has a major influence.

Table 1: Results of the analysis of the B-series. Stage was determined after Hamburger and Hamilton[73]. The unit of the dose is µg, weight is measured in grams. The phalanx formulas go from anterior to posterior.

Sample	Stage	Dose	Weight	Hind Limb	Fore Limb
B2	18	2.5	1.34	2-2-3-4	1-2-1
B3	18	1	1.60	2-3-4-5	1-2-1
B5	18	5	1.22	2-2-3-4	1-2-1
B6	19	1	1.50	2-3-4-5	1-2-1
B8	19	2.5	1.56	2-3-3-4	1-2-1
B9	19	10	1.30	2-2-3-4	1-2-1
B10	18	Ringer	1.20	2-3-3-4	1-2-1
B12	19	Ringer	1.66	2-3-3-4	1-2-1
B13	18	1	1.50	2-3-3-4	1-2-1
B15	19	2.5	1.66	2-3-3-4	1-2-1

Interestingly, this time the mortality was almost 75%, including both Ringer control samples. However among the survivors there were some interesting phenotypes (Fig. 10). The embryos with injections of 40 and 50µg reduced most of the autopod (Fig. 10B), leaving only two metatarsals. Also one of the samples injected with 10µg reduced all phalanges of digit IV, without impairing the development of the other digits visibly (Fig 10C). Another sample injected with the same dose did not alter the phenotype at all.

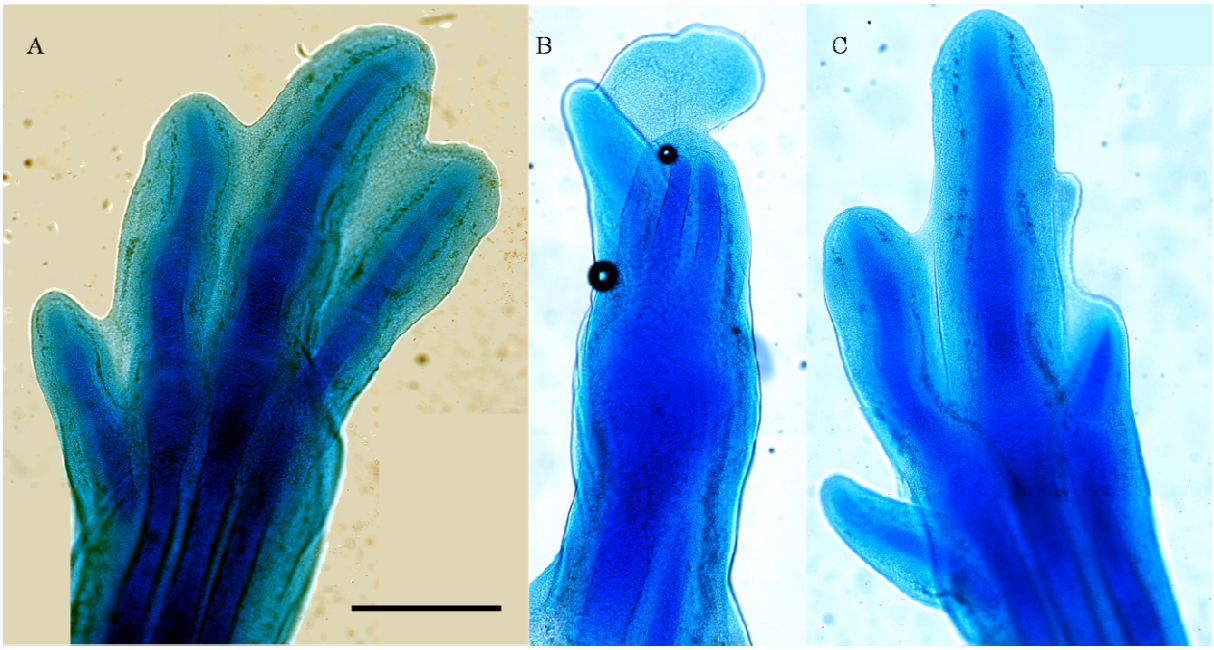

Figure 10: Three phenotypes of the D-series. A shows the regular wild type pattern of a sample injected with 10µg. B shows the typical phenotype of 40 & 50µg samples, most of the autopod is reduced, only two metatarsals can be seen. C also shows an embryos injected with 10µg, D IV is severely reduced. All are right hind limbs from dorsal. Scalebar is 1mm.

Because of the high mortality, this approach was repeated, but the injections were done at stage 24 rather than 25. In this series E two things could be determined: usually an injection of 10µg will not lead to a phenotype at this stage and injections of 30µg or higher lead to strong reduction of the autopod and are therefore not of any use for this experiment. Furthermore the combined data of series D and E showed connections between the injected dose and the weight of the embryo better, than the previous samples did (Fig. 11). Also this series contained another sample with reduced digit IV, but this was one injected with

20µg. After that it was clear that the correct dose for reducing specific digits – if possible at all at that stage – had to be between 10µg and 30µg. Therefore the next 2 series (F and G) were injected 15, 17.5, 20, and 25µg of the drug. F was injected slightly later than G (102hours vs. 96 hours).

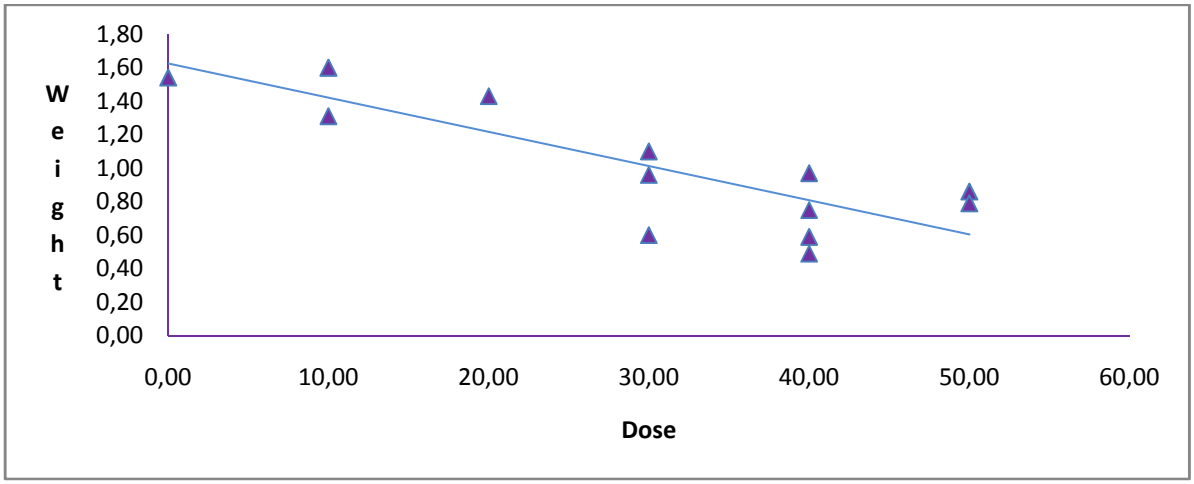

Figure 11: showing the correlation between the weight of the samples and the injected dose of series D and E. The weight is measured in grams, while the injected dose unit is µg. The trend that the bodyweight decreases with increasing cytosine arabinoside dose is here clearer than in sample B (Fig. 9).

In general the samples of the F and G series showed that the injection of 20 and 25µg usually is still too much. Most of the samples had the autopod reduced too strongly. In the F-series a couple of samples again displayed the reduction of digit IV to one phalanx plus the metatarsal. Among the G embryos there were more diverse phenotypes: one embryo had digit III reduced, in such a way that only half of the metacarpal was left (Fig. 12C); in another one the same applied to digit IV, and digit III was bent to the posterior side (Fig. 12D). The most interesting sample had two different reductions: both legs had digit IV reduced in the usual manner, but on the left leg only the metatarsal of digit I was left. The adjacent digit II was bent towards anterior and lacked one phalanx (Fig. 13). This sample was also dehydrated, stained with PTA, and scanned with microCT. However, the treatment seemed to be too harsh after the alcian blue staining procedure, because shrinking and warping made the effect rather difficult to recognize (Fig. 13C.).

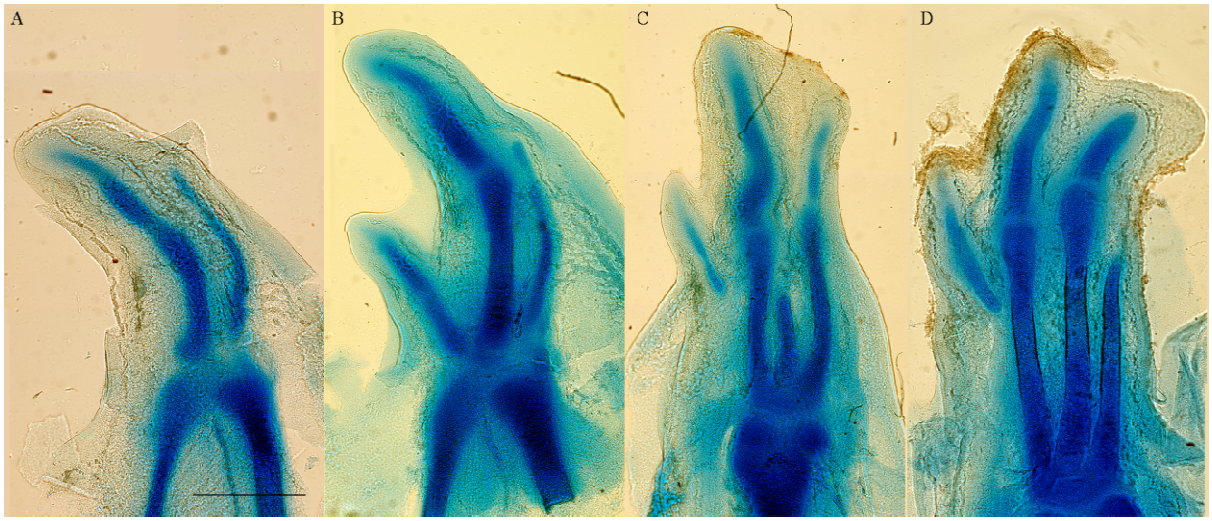

Figure 12: samples G4 (A & C), injected with 15µg, and G6 (B & D), with 17.5µg. A and B show fore, C and D hind limbs. A shows that DI is reduced completely. In C DIII is reduced to the half of the metatarsal only and the phalanges of DII have taken the place of DIII. D shows a strong DIV reduction only the majority of the metatarsal is left, DIII is bent to the left, probably also occupying DIV space. Scalebar is 1mm.

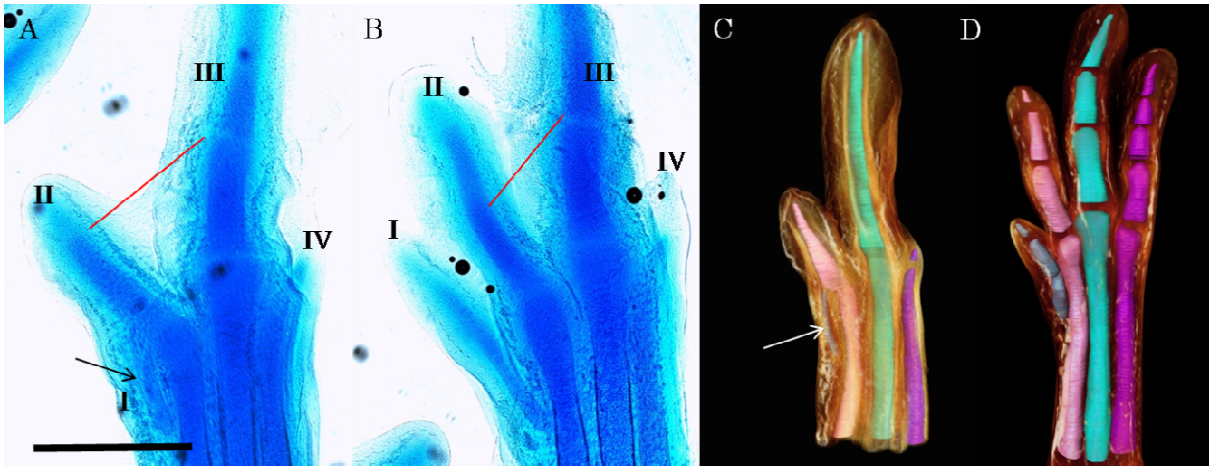

Figure 13: The most exciting phenotype of the G-series showed a reduction of DI and a partial acquisition of D1 phenotype by DII in the left hind limb (A). Only the metatarsal is left and can be seen adjacent to digit II (black arrow). Also digit II is bent further towards anterior and has reduced one phalanx, in comparison to the right leg (B). A is a mirror image of a left hind limb. Roman numerals mark digit positions. Scalebar is 1mm. C shows a 3D image of the same sample. The digits are surface-rendered the general limb form is volume rendered with limited transparence, greyish Blue is DI, pink DII, turquoise DIII and purple DIV. The bent away DII is visible, but not as strong as it is in the microscopic image (A). The vestigial DI is indicated by the white arrow. D shows a normal hind limb, the general limb form is outlined by a semi-transparent volume rendering the digits are surface-rendered.

In most of the cases the fore limbs were rather unaffected, but one sample lost DI entirely (Fig. 12A). In the next two series all samples were injected with 15µg (H) or 17.5µg (I) respectively. The injections were made from 91 to 99 hours of incubation, two embryos per time point. The samples H2 (Fig. 14A) and H6 (14B) looked most interesting, and therefore they were scanned with microCT. H2 showed a fusion between digits I and II, and a mixed phenotype, in that they separate late from the main limb (like DII) but in a rather orthogonal angle (like DI). Additionally this sample has lost the metatarsal of DIII, and the DIV metatarsal is bent towards anterior. Most of DIV however is reduced. H6 shows a reduction of distal DIII elements. The space and phenotype of DIII is then taken by digit DII, and DIV develops rather normal here. It also is remarkable that none of the drug-treated limbs displays the joints in the CT images as clearly as the wild type does.

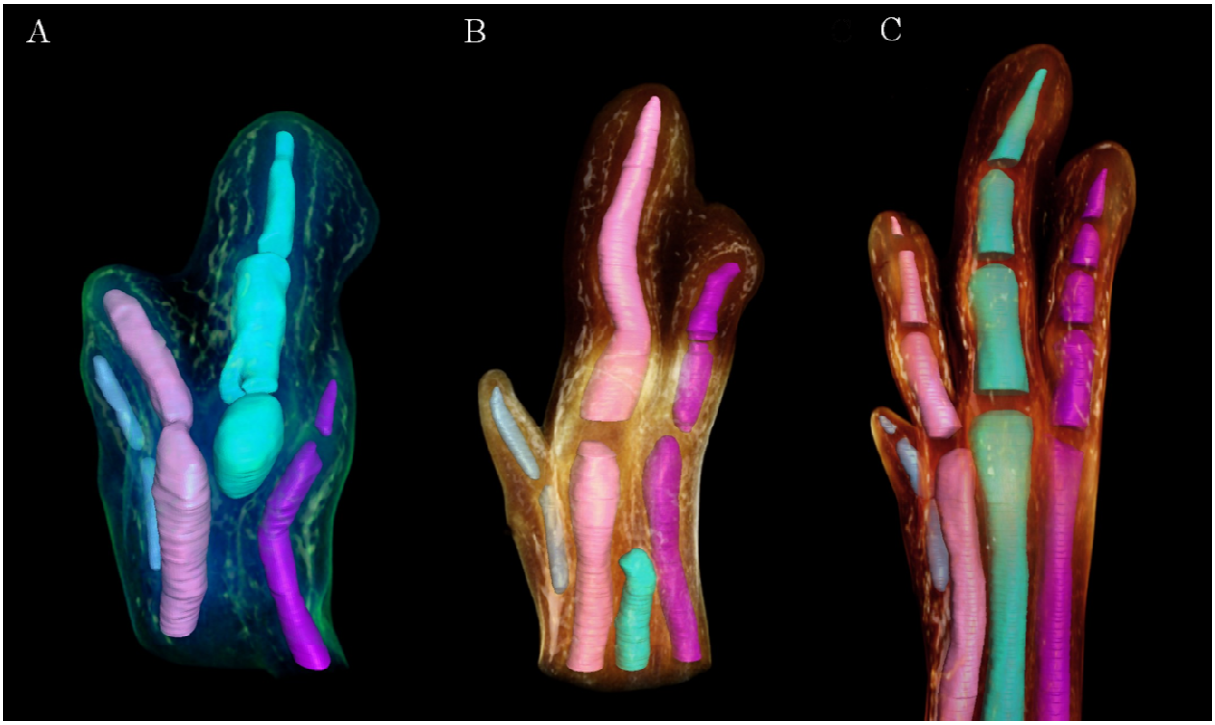

Figure 14: 3D images of chicken hind limbs, stage 35. View is from dorsal, A and B are actual left limbs, but are mirror images, C is a wild type right limb. Shown are semi-transparent volume renderings of the limbs with surface renderings of the digits. Greyish Blue is DI, pink DII, turquoise DIII and purple DIV. A has only the distal half of the metatarsal at position II, metatarsal IV is involuted to its position. Digits I and II are not separated, they branch of the rest of the limb in a digit I like manner, but rather distal. In B only the proximal half of metatarsal III is formed. From the phalanges on digit II occupies the space of digit III, also the phenotype is more digit III like, since it is longer and directly in the PD axis. Note also that none of the cytosine-arabinoside treated limbs displays the joints as clearly as the normal one does.

The following treatments narrowed down the injection dose further: in the majority of cases 15µg will not cause digit loss (e.g. 12 of 17 in the H series) and 20µg will cause all phalanges or even the entire autopod to be reduced. A dose of 17.5µg can cause either result, or really reduce single digits partially or completely. In the I series 11 of 20 samples showed no phenotype, 5 had DIV only reduced, and 3 showed DIV reduction and limited anterior reduction. Generally, the samples that were injected earlier (90-93 hours of incubation) showed no digit phenotype, while the later ones (93-97 hours) showed digit IV reduction.

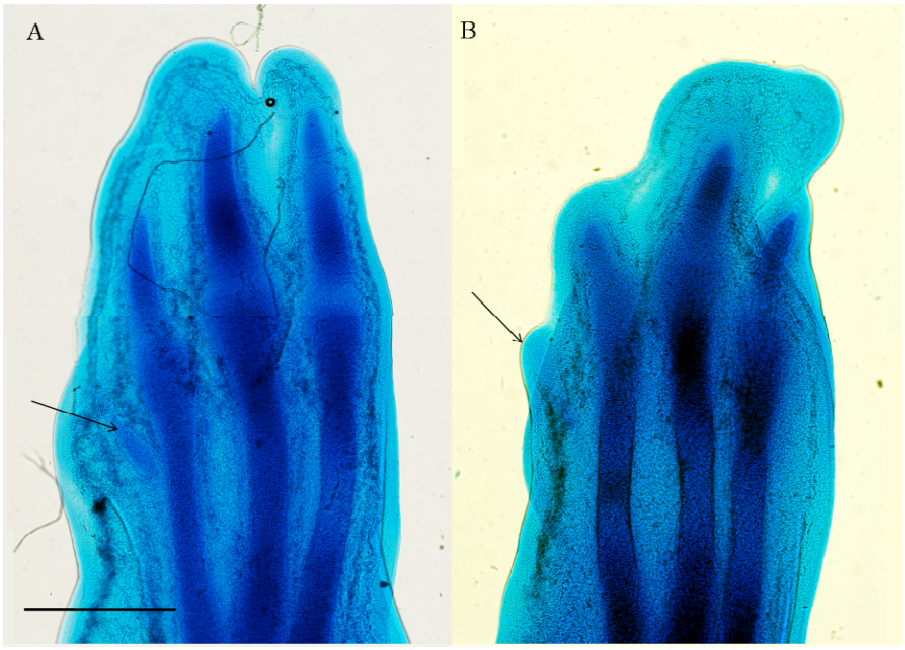

Figure 15: Alcian Blue stained hind limbs of embryos inject with cytosine-arabinoside after 120 (A) and 109 (B) hours of incubation. Arrows indicate the vestige of digit I, the scalebar is 1mm. While the phalanx of DII in the 120 hour sample is straight, the one of the 109 hour sample points somewhat to the anterior.

The chance of getting rid of digit I seemed higher if the animals were injected later. Thus the series M contained embryos injected after 120 hours of incubation and the N series as a whole was injected after 108 to 109 hours of incubation. Some samples really showed DI reduction, but at the same time all phalanges except the first were reduced as well, making it extremely difficult to decide if DII has a D2 or D1 phenotype.

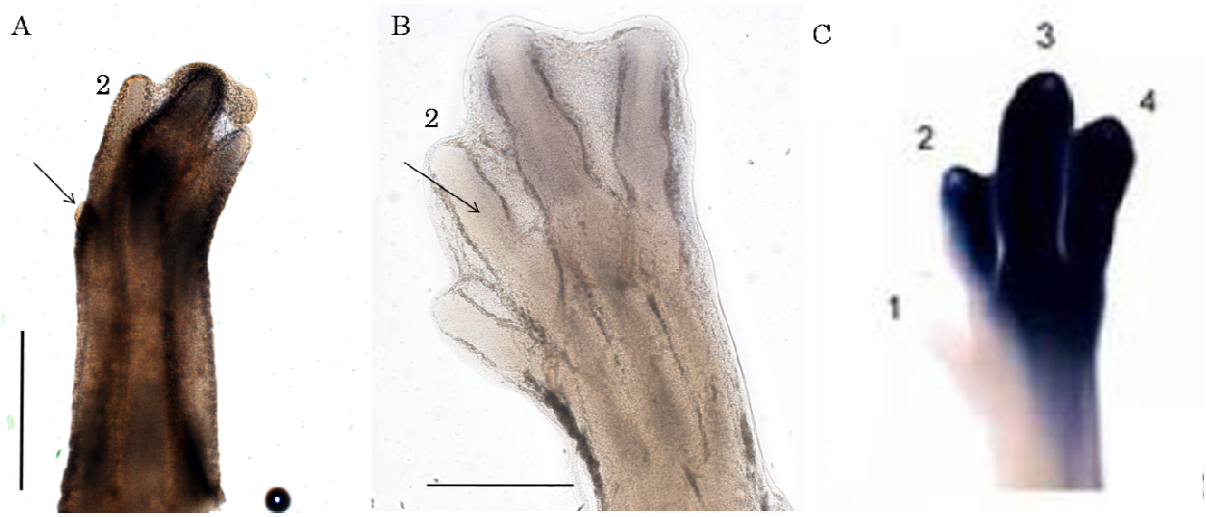

Figure 16: Results of whole mount in situ hybridizations developed with DAB (A & B) or NBT/BCIP (C). C is taken from Vargas & Fallon[16]. Scalebars are 1mm. The Arrow in A indicates the vestige of digit I, the arrow in B marks the border of hoxD12 expression in digit II of a sample that retained digit I. Only the sample with reduced digit I, has lost hoxD12 expression in digit II completely.

However there was a difference between the sample from 120 hours, in which the DII phalanx pointed straightly (Fig. 15A) and the 109 hour samples, in which the phalanx was pointing towards the anterior (Fig. 15B).

In situ hybridization on the other limb of the 109 hour specimen revealed that there is no *hoxD12* expression in digit II (Fig. 16A), whereas in the wild type only the first digit is negative for *hoxD12* (Fig. 16C). Interestingly some of the samples that retained digit I despite drug treatment lacked hoxD12 expression in the anterior portion of digit II (Fig. 16B).

Discussion

The aim of this study was to shed light on the complex changes that occurred in the development of the avian hand during the evolution of birds and their dinosaur ancestors. More specifically the goal was to either rescue the vestige of the anterior most digit in the avian fore limb or to deliberately lose this digit in the hind limb. The results of these experiments were then used to support or falsify the new Thumbs Down Hypothesis.

The experiments however turned out to be not so easy to accomplish. Of three experiments, only one yielded significant results. Both other approaches failed so far. Therefore these two shall be discussed briefly, before I proceed to the mitosis inhibitor injection results.

Bead Implantations

The initial thought behind this approach was that the vestigial digit I can be rescued if the signaling from the apical ectodermal ridge (AER) could be prolonged. This was based on the fact that beads soaked with FGF protein, that were implanted into a developing limb bud, could sustain growth although the AER was removed surgically[78]. Among the proteins of the FGF family, FGF-8 was chosen, because it is the main signal that comes from the AER[77]. The major problem of this approach was the high mortality of the embryos after surgery. The reason for this was very likely the time that was necessary to implant the bead successfully into the limb bud. The other main factor was injury during the surgery. Both of these problems were due to my lacking experience with embryonic surgery, and would have been possible to overcome with a lot of training. However there were two other things that were considered before this approach was stopped.

First the implantation causes a wound that lies directly at the side of interest and therefore could have interfered easily with developing digits at positions I

and II (Gerd Müller, personal communication). Although negative controls – implanting a bead soaked with PBS only – can rule these effects out to a certain extent, it would remain problematic, since the implanted beads will never lie at the exact same position.

The next concern was the FGF-8 protein itself. The main function of FGF-8 in the proximo-distal limb outgrowth is to keep the cells proliferating (reviewed in [64]). Beside maintaining growth, the proliferative state of the limb mesenchyme cells also keeps them from forming pre-chondrogenic condensations[76]. Therefore a higher level of FGF-8 signaling not only causes the limb bud to grow stronger, but also doesn't allow it to form digits. If we take a look at the only sample that survived long enough (Fig. 8), we see that instead of a proper digit II it has a mesenchymal condensation next to digit III.

It is possible that this shows that digit II could not form because the stronger FGF-8 signaling kept the cells from condensing at the proper time and position. The visible condensation would then have formed later (and therefore more distally), probably after all the protein had diffused from the bead.

On the other hand this can also be interpreted as digit II returning to its "correct" phenotype, because of stronger proliferation on the anterior side. If this interpretation would be correct, this would give strong support to the Thumbs Down Hypothesis, since it claims that the adaption of the digit 1 phenotype by digit II is due to the loss of digit I. Therefore elevated cell proliferation on the anterior side, could be enough to keep digit II from moving there and adopt the phenotype.

Ablation of hind limb digit I

This experiment was undertaken because the bead implantations gave reasons for concern. The thought was that if fore limb digit I cannot be rescued, what about deliberately losing digit I in the hind limb. Instead of trying to revert the

fore limb, I tried to repeat the fore limb evolution effect in the hind limb. The main reason for why this approach did not yield any results is probably that it was too random. To injure the AER at the anterior side obviously is not enough to prevent entire the digit I from growing. Also the "tickling of the toes" with a hot needle did not harm the AER seriously. To really see an effect, it would probably have been necessary to remove the anterior portion of the AER entirely. Still it is not clear whether this would have been enough to entirely remove digit I, since signaling from other regions of the AER could still have reached its anlage and caused outgrowth. For these reasons this approach was given up in favor of the mitosis inhibitor injections.

Injections of Cytosine Arabinoside

At a first glance this seems to be an easy experiment. It was already known that the injection of the mitosis inhibitor cytosine arabinoside to the yolk of chicken eggs causes, among other effects, malformations of the limbs[79]. Furthermore it has been shown in alligators that the drug can cause the loss of digit I without removing any other elements of the limb[22]. Therefore it seems plausible that digit I can be removed in the chicken hind limb by this procedures as well, given that the correct time point and drug dose are used. To determine these two factors was the first issue, because they tend to influence each other. A given dose can kill the embryo on day 3 right away but may have no effect on day 4, simply due to the increased size of the embryo on that day. Although there were no usable phenotypes at the beginning, it was clear that the drug was working and also was taken up by the embryo from the yolk, because a clear decrease in the size of the embryos could be seen when the dose was increased (Fig. 9 & Fig. 11). Since the only structures that could be reduced were phalanges and never entire digits, the injections were then performed on four day old embryos. At this stage the correct dose again needed to be determined and was determined to work best at about 17.5µg of cytosine arabinoside (see results section). But again this was only true on average, there were also samples that did not show any phenotype and some

that showed severe reduction. The reason for this is probably the diffusion within the yolk and the amount of the drug that really reached the embryos' limb buds. The digit that was reduced most easily was digit IV, which is surprising since this is opposing Morse's Law[15]. This law is thought to apply to digit reduction by evolutionary means, not caused by embryo manipulations. If digits were reduced in the fore limb, however, digit I was always reduced first. Taking further into account that during the evolution of birds digit V of the hind limb was reduced instead of digit I, it can be questioned whether this law applies to birds at all.

Another interesting aspect is that according to the digit reduction scheme of the TDH (Fig. 7), digit IV of the bird fore limb had a state in which it was only metacarpal and 1 phalanx (e.g. *Dilophosaurus*, *Limusaurus*), before it grew longer again after the loss of digit I. The abundance of embryos that showed this exact phenotype in the hind limb supports the TDH digit reduction scheme to some extent.

The most digit IV reduced embryos were obtained, when the injections were made after 93-97 hours of incubation (at 39.5°C). But the other phenotypes, e.g. loss of digit III (Fig. 14B) or loss of digit I (Fig. 13A & C) were also injected after approximately 96 hours of incubation. This suggests two things: first, the digits in the avian hind limb are determined at an age of about 96 hours (the 93 hours of the experiment plus some time for diffusion of the drug) and second the time frame in which the digit anlagen arise is very narrow.

Among those injected at this approximate time, there was only one embryo that had reduced a digit I. This sample had reduced digit I only on the left limb, and digit IV on both limbs. All other specimens that lacked digit I, also lacked the majority of their phalanges. This suggests that digit I is formed at a time point (approximately 108-120 hours of incubation), in which the second phalanges of the digits usually develop.

Conclusions and implications for the Thumbs Down Hypothesis

The thumbs down hypothesis argues that the digit 1 phenotype in digit II is due to a move of digit II cells towards the anterior of the limb bud, following the reduction of digit I. Therefore the strongest support would have come from an embryo that reduced hind limb digit I, has a digit II with a digit 1 phenotype that is negative for *hoxD12*, and wild type digits III and IV. However, this pattern did not come up. Still there was one sample with reduced digit I and digit 1 phenotype in digit II, digit III was normal, digit IV was partially reduced. The major flaw of this specimen was that it only had one leg, that looked this way. In the other one digit I was in place, and hence digit II looked normal. Although this is strong morphological evidence for the TDH, the availability of only one such leg ruled out the possibility of subsequent in situ hybridization.

Among the samples in which digit I and several phalanges were reduced, there were some in which the first phalanx of digit II pointed straight, and two specimens in which it pointed to the anterior side. In the latter embryos in situ hybridization did not show any *hoxD12* expression in digit II, although wild type digit II has it. Again this is good evidence for the TDH, but since most phalanges are missing it is not as strong as it could be.

Specimens that lost other digits than DI can also be useful in evaluating the TDH. The core assumption of the TDH is that if one digit stops growing, another one can take its position and thereby also – entirely or partially – adopt its phenotype. This is exactly what we see in the embryo with reduced digit III (Fig. 13B). Digit II takes the position of digit III as soon as the metatarsal stops growing and from the phalanges on even looks like digit 3 usually does.

Taken together, the morphological evidence of the mitosis inhibition and the bead implantation experiments (although there is only one sample from the latter) has shown that the core mechanism underlying the TDH is viable. Furthermore it is

plausible that this mechanism is responsible for the difference between positional and compositional information[33] of digit II in the avian fore limb.

The genetic evidence of the *hoxD12* in situ hybridizations further suggests that the expression pattern in the avian wing[16-18] is caused by the same mechanism. However this evidence is not sufficient to decide whether the *hoxD12* expression is directly linked to the digit's morphological phenotype, and if it is, what is upstream. Thus only the core concept of the TDH can be supported so far. The assumption that the effect is caused by lower Shh levels due to increased distance from the ZPA remains hypothetical.

Are there other possible explanations?

When examining the results of the mitosis inhibition experiment, I found it intriguing that hind limb digit IV was so much more often reduced than all the other digits, although it should be the other way round.

The primary axis[19-21,38] is the structure that should be lost least not most often. If we take into account that the primary axis was one of the major hints for the 'two, three, four' interpretation[3,15], the question comes to mind, if it is possible that the digits in the fore limb really are I, II, and III. Given that in the avian hind limb, digit V was lost during evolution instead of digit I, and that now digit IV was lost much more frequently than digit I, it makes me think that the fore limb could have behaved the same way.

This also would agree with the reduction pattern we see in the fossil evidence (reviewed e.g. by Bever et al.[44]) with the exception of *Limusaurus*[45]. Wagner has mentioned the possibility that the structure that is usually identified as fore limb digit I[23,24,26] could also be some kind of pre-pollex[3]. If this was true the axis shift hypothesis[35,36,39] would be back in play and it would also make the polydactylous[25] hypothesis more interesting.

On the other hand, it has to be kept in mind that all experiments are done on extant birds, in which development, and especially limb development is much derived. In particular, reptilian egg development is known to proceed much slower, and hence mitotic inhibition experiments are able to target individual digits much more easily. The sequence of digit loss achieved experimentally in Alligators is in accordance with the TDH (Gerd Müller, personal communication). Since alligators my better represent reptilian development at the time of the evolutionary digit loss, experimental results in extant birds must generally be taken with caution.

Outlook

In this work I presented a new approach to a long-standing problem. The major difference to the traditional hypotheses is that it integrates the morphogenetic and biomechanical aspects of development. The Thumbs Down Hypothesis was formulated in such a way that it is in agreement with currently available results from developmental biology as well as with the currently known fossil evidence. In this thesis I have presented experimental data that give further support to the hypothesis. However, all of the experiments could have yielded better results, therefore they should be refined and repeated until we have more solid evidence to support or to falsify the new approach. For example, the specimens that lost digit III should be analyzed with in situ tools for their *hoxD* expression patterns – not only for *hoxD12*, but also for *hoxD11* and *hoxD13*. If there really exists differential cell proliferation in the avian fore limb this should be detectable with cell proliferation markers such as EdU and BrdU. Once the procedure for obtaining embryos without digit I in their hind limb is established, the transcriptome of their digits needs to be analyzed. And finally we will need to know about the gene regulatory network that controls digit differentiation to know if different levels of Sonic Hedgehog can really cause digits to change their phenotype.

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Appendix

Protocols

Alcian Blue Staining

Fix chicken (st. 35) in 4% Paraformaldehyd in PBS for 1hr @ 4°C

rinse in 70% EtOH

store in 70% EtOH o/n @ 4°C

soak 6-16 hrs in Alcian Blue (10mg Alcian Blue in 70 ml of 95% EtOH and 30ml of 99,7% Acetic Acid)

wash 2-3x in 70% EtOH (10' @ RT each)

rehydrate to Water (50%, 35%, 20%)

soak it 4 hrs in 2% KOH (aqu.) @ RT

clear in 3:1 (0.5% KOH:Glycerin)
1:1 (0.5% KOH:Glycerin)
1:3 (0.5% KOH:Glycerin)
for 8-24 hrs each

store in 100% Glycerol

Transformation of competent bacteria

Let competent cells thaw on ice

Add 3µl of ligation product to the cells, mix gently

Leave on ice for 10 minutes

Heat shock the cells for 45 seconds at 42°C (do not shake)

Put on ice for 2 minutes

Add 250µl of SOC medium (no antibiotic added)

Shake for 30 to 60 minutes at 37°C

Spread the 300µl with glass beads on a agar plate containing ampicillin

incubate at 37°C over night.

In Situ Hybridization

DAY 1 - Hybridization of embryos with riboprobe
(every step is done on the rocker, unless stated otherwise)

1.) Bleach embryos with 3% hydrogen peroxide in MeOH for 1 hour at RT with gentle rocking (Note: embryos must have been previously dehydrated, if you are going to include the bleaching step).

2.) Rehydrate embryos in a graded series of methanol/PBT (75%, 50% and 25%) at RT for 5-10 min.

3.) Wash thrice in PBT for 5 min (use 0,1% Tween 20 for chick).

4.) Proteinase K treatment:

Chick embryos: For younger embryos (<st10), treat with 1 to 3 µg/ml proteinase K in PBT for 15 minutes at RT (this will also work for ectodermal gene expression in older animals). Embryos < st18 are treated with 10µg/ml proteinase K for about 15 min at RT. For stages 18-24 the time can be extended to 20-25 min. For stages 26-29 the treatment can last up to 40 min. Alternatively the time can stay at 15 min, but the concentration can be raised.

stage	**concentration of proteinase K**
26-27	20µg/ml
28-29	30µg/ml
30-31	40 µg/ml
33 and older	50-60µg/ml

5.) Wash 10 min in 2mg/ml glycine in PBT (make fresh). This stops the proteinase K reaction.

6.) Wash twice for 5 min with PBT.

7.) Postfix with 4% paraformaldehyd and 0,2% glutaraldehyde (0,2 ml of 25% stock per 25 ml) in PBT for 20 min at RT.

8.) Wash 2 times for 5 min with PBT.

9.) Wash 10 min in a 1:1 mixture of hybridization solution/PBT.

10.) Wash 10 min in hybridization solution.

11.) Incubate at 70°C in hybridization solution for at least 1 hour (lower the temperature to 60°C for shorter probes).

12.) In a separate tube mix RNA probe into minimal volume of hybridization buffer (500µl - 1ml) (typically 1/50 to 1/10 of a transcription solution)
I did 1ml of hybmix with 2uL of probe.

13.) Replace pre-hyb buffer with buffer containing RNA probe. Hybridize over night at 70°C in shaking water bath (lower the temperature to 60°C for shorter probes).

DAY 2 - Post hybridization washes, blocking and antibody incubation

1.) Preheat solution I at 70°C and soultion III at 65°C (60°C and 55°C for shorter probes).

2.) Wash embryos 3 times in pre-warmed soultion I for 30 min. at 70°C.
Then change shaking water bath temperature to 65°C.

In the meantime prepare blocking solutions and block antibody (see below).
I blocked the antibody for 4 hrs.

3.) Wash embryos 3 times for 30 min. each in 65°C with prewarmed solution III.

4.) Wash 3 times with TBST for 5 min each at RT.

Blocking of anti-DIG antibody

During the above washes prepare blocking reagents for the antibody and the embryos.

For chick embryos:
Blocking solution for the antibody is 1% heat inactivated sheep serum in TBST

(Make enough to dilute antibody to the desired concentration). I did 0.5uL Antibody in 2.5ml Blocking solution (i.e. 1:5000).

Blocking solution for the embryos is 10% heat-inactivated sheep serum in TBST. (Make approx. 1ml per vial)

Keep both blocking reagents on ice or at 4°C until use.

5.) Rock the embryos in the embryo blocking solution for at least 1 hour (I did 2.5) at RT.

6.) Remove blocking solution and add antibody solution and incubate over night at 4 °C with rocking. (Concentration of the antibody should be 1:4000 to 1:10000)

DAY 3 - Washes

1.) Wash embryos 3x for 5 min with TBST at RT (0,1 % Tween).

2.) Wash 5-8 times for 1 to 1,5 hrs. in TBST at room temperature (the more washes the better).

3.) Wash overnight in TBST at 4°C with gentle rocking.

Solutions and Buffers

Chicken Ringer

NaCl	7.2g
$CaCl_2$	0.17g
KCl,	0.37g
$_{dd}H_2O$	1000ml

PBS

NaCl	8g
KCl	0.2g
Na_2PO_4	1.22g
KH_2PO_4	2.4g
$_{dd}H_2O$	1000ml
pH	7.4

Hybridization Solution (50ml)

formamide	25ml
SSC (20X, pH 4.5)	12.5ml
20% SDS	2.5ml
$_{molbio}H_2O$	9.7ml
tRNA (10mg/µl)	250µl
heparin (100mg/ml)	25µl

Washing Solution I

formamide	25ml
SSC (20X, pH 4.5)	12.5ml
20% SDS	2.5ml
$_{molbio}H_2O$	10ml

Washing Solution III

formamide	25ml
SSC (20X, pH 4.5)	5ml
$_{molbio}H_2O$	20ml

TBS

NaCl	8g
KCl	0.2g
Tris.HCl (pH 7.5)	250ml
$_{dd}H_2O$	750ml

TBST

TBS + 0.1% Tween-20

Reaction Recipes

Reverse Transcription

1µl	oligoDT_{17} (Stock 100µM)
2µl	mRNA (1µg/µl)
1µl	dNTP mix (10mM each)
9µl	$_{molbio}H_2O$
13µl	

Heat to 65°C for 5 minutes, than incubate on ice for 1 minute.

Centrifuge briefly and add:

4µl	First Strand Buffer
1µl	DTT (0.1 M)
1µl	RNAseOUT (Invitrogen 10777-019)
1µl	SuperScript III Reverse Transcriptase
20µl	

PCR Amplification of cDNA

25µl	GoTaq Green Master Mix (Promega M7112)
1µl	sense primer (10µM)
1µl	antisense primer (10µM)
2µl	cDNA
21µl	$_{molbio}H_2O$
50µl	

PCR Programme:

82°C	Hot Start	
94°C	2 minutes	
94°C	15 seconds	
55°C	15 seconds	x 33 cycles
72°C	90 seconds	
72°C	8 minutes	

Primers:

hoxD11 forward: TTGCCGGTCAGTGAGGTTGAGC

hoxD11 reverse: CAACGCATTGAAGCCTCCCGGT

hoxD12 forward: CCACAAAACACGCGAGCGCC

hoxD12 reverse: GGCGGCTCTGCCCACCATTT

hoxD13 forward: GGACTCCGGCAATGCGGCTT

hoxD13 reverse: CCCGGGCAGTGCCGTAACTT

Ligation into pGEM-T easy II vector

	hoxD11	*hoxD12*	Pos. control	Neg. control
2X buffer	5µl	5µl	5µl	5µl
Vector	1µl	1µl	1µl	1µl
PCR product	3µl	3µl	-	-
Control DNA	-	-	2µl	-
T4 Ligase	1µl	1µl	1µl	1µl
$_{dd}H_2O$	-	-	1µl	3µl

Colony Check PCR

6µl	$_{dd}H_2O$
10µl	Phusion Flash Master Mix (2X)
1µl	specific primer (*hoxD11*/*hoxD12* reverse)
1µl	promoter-binding primer (SP6/T7)
2µl	bacterial suspension
20µl	

PCR programme:

98°C 10 seconds

98°C 1 second
50°C 5 seconds 30 cycles
72°C 20 seconds

72°C 1 minute

Insert amplification PCR

19.5µl	$_{dd}H_2O$
25µl	Phusion Flash Master Mix (2X)
2.5µl	T7 or M13 forward primer (10µM)
2.5µl	SP6 or M13 reverse primer (10µM)
0.5µl	plasmid (25ng/µl)
50µl	

PCR programme for T7/SP6:

98°C 10 seconds

98°C 1 second
48°C 5 seconds 30 cycles
72°C 20 seconds
72°C 1 minute

PCR programme for M13:

98°C 10 seconds

98°C 1 second
55°C 5 seconds 30 cycles
72°C 20 seconds

72°C 1 minute

In vitro transcription for synthesis of digoxygenin-labeled RNA probes

6.5µl $_{molbio}H_2O$
5µl purified template
2µl DIG RNA labeling mix (10X)
4µl transcription buffer (5X) (Promega P1181)
0.5µl RNAsin (Promega N2511)
2µl T7/SP6 Polymerase (Promega T2075/P1085)
20µl

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Curriculum Vitae

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Mag. phil., Celtic Studies,
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