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Self-organization in the limb: a Turing mechanism for digit development

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The statistician George E. P. Box stated, 'Essentially all models are wrong, but some are useful.' (Box GEP, Draper NR: Empirical Model-Building and Response Surfaces. Wiley; 1987). Modeling biological processes is challenging for many of the reasons classically trained developmental biologists often resist the idea that black and white equations can explain the grayscale subtleties of living things. Although a simplified mathematical model of development will undoubtedly fall short of precision, a good model is exceedingly useful if it raises at least as many testable questions as it answers. Self-organizing Turing models that simulate the pattern of digits in the hand replicate events that have not yet been explained by classical approaches. The union of theory and experimentation has recently identified and validated the minimal components of a Turing network for digit pattern and triggered a cascade of questions that will undoubtedly be well-served by the continued merging of disciplines.

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Introduction

Fingers allow us to grasp objects and to explore and manipulate our three-dimensional environment. Fingers enable me to type this sentence and for you to turn the page or click a mouse. Yet for all of the appreciation that we have for the usefulness of our digits and awe for their intricate construction, there are many remaining questions about how we develop five precisely patterned fingers and toes with a robust and reproducible digit-interdigit periodicity. Our understanding of digit patterning mechanisms has traditionally focused largely on secreted morphogens and their concentration-dependent effects on positional identities across a field of cells. This work has been extensively reviewed elsewhere [1–5] and contributed to a deep understanding of the dynamic processes of pattern formation.

In the background of these classical embryological and developmental genetic studies, theoreticians have long hypothesized that our fingers may have a greater connection to mathematics than our ability to count to ten. Alan Turing's 1952 theory on the chemical basis of self-organization in pattern formation [6•] inspired questions about how such a mechanism, in addition to explaining the stripes and spots of animal coloration, might also explain the formation of structures including the digits of the hand and foot, collectively termed the autopod [7•,8–14,15•,16,17•]. The integration of developmental genetics and computational modeling indicates the ordered array of digits need not depend on a coordinate system for the precise position of each finger. Rather there may be a degree of self-organization whereby the digits establish their own periodicity. Indeed, this work nicely explains the ability of digits to form in re-aggregated limb buds in absence of positional information [18].

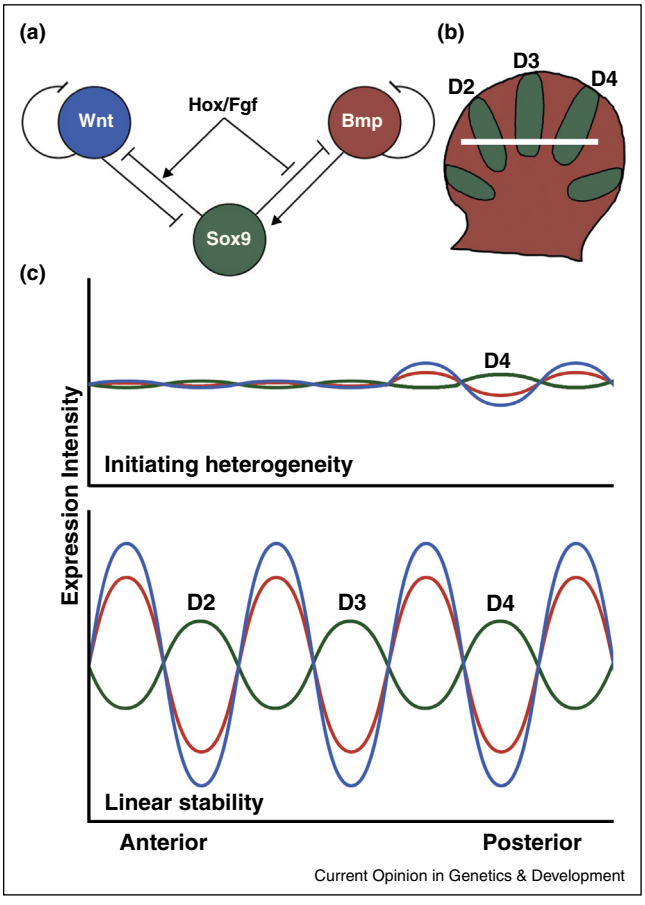
Towards a turing model of digit pattern

Turing's model of the chemical basis of pattern formation centers on the theoretical activities and interactions of diffusible molecules [6•]. Two types of simple molecular networks would conform to the model: an activator and an inhibitor or an activator and the depletion of its substrate [19]. Specifically in the context of digit development, an activator would be predicted to induce digit cartilage differentiation and also to amplify itself by positive feedback. At the same time, the activator either produces an inhibitor of cartilage differentiation or depletes a substrate in the process of amplifying itself. In either case, if all molecules in the network are expressed equivalently with the same reaction rates, the field of tissue would be homogeneous with no cartilage condensations. In order for a pattern of condensations to emerge, an initiating heterogeneity, which can be stochastic, is amplified by differing rates of diffusion of component molecules. The auto-amplification at short range (peak of cartilage) and cross-inhibition at long range (valley between cartilages) will spontaneously form a periodic pattern.

Several Turing models have proposed the identities of activators and inhibitors that are all capable of computationally simulating the ordered array of digits [8,9,14,16]. However, until recently, few had merged theory with experimental validation of model predictions in the intact limb. In the most recent modeling approach

[17•], Raspopovic and colleagues first experimentally pursued a prediction from previous Turing models of digit formation to identify members of key signaling pathways that are expressed in phase or out of phase with *Sox9*, an HMG-box transcription factor that is an early marker and is itself required for digit chondrogenesis [20]. This highlighted both the in phase response to Bmp signaling and the out of phase activation of Wnt signaling and expression of the *Bmp2* ligand. Previous work determined Bmp signaling activates and Wnt signaling inhibits *Sox9* expression. Briefly summarizing extensive supplementary data testing all possible configurations of a model resulting from the three players with these two interactions held constant, a minimal topology was determined to simulate the observed pattern of *Sox9* expression and Bmp activity together out of phase with both the *Bmp2* ligand and Wnt target genes (Figure 1a).

Figure 1



Fundamentals of the BSW network Turing model. **(a)** Network interactions between each of the three key players (Bmp, Sox9, and Wnt) and their modifiers (Hox and Fgf). **(b)** Outline of a mouse forelimb at 12 days post conception (12 dpc) with future digit positions indicated. White line depicts the anatomical location of the simple linear model of Turing pattern. **(c)** Schematic representations of the linear model of initial heterogeneity (top) that is amplified by auto-regulation and cross-regulation to generate a stable pattern (below).

In the Bmp-Sox9-Wnt (BSW) model, it is assumed from experimental data in other developmental systems, that the rate of Bmp diffusion exceeds the rate of Wnt diffusion [17•]. This difference in diffusion amplifies initial heterogeneity by initiating a cascade of events. Bmp induces expression of Sox9 that in turn inhibits the inhibitory effect of Wnt [21] thus producing an autocatalysis of activation (satisfying the requirement for positive feedback in the Turing model). However, according to the best-fit topological model, Sox9 also inhibits the production of *Bmp2* mRNA, which causes a local down-regulation of Bmp signaling, attenuates the production of Sox9, and thus relinquishes the inhibition of Wnt (satisfying the requirement for inhibition in the Turing model). This restricts the amplifying effect to local islands of cells, and thus a pattern takes form where Sox9 (digit) is expressed out of phase with both *Bmp2* ligand and Wnt target genes (interdigit) (Figure 1b,c).

On their own, these three signals form a computational pattern remarkably similar to that formed by chondrogenic nodules in micromass cultures of limb bud mesenchyme in absence of any additional signals [17•]. A theoretical role for Hox and Fgf activities restricts the physical space in which heterogeneity initiates a Turing pattern and organizes the pattern into digit-like stripes rather than meandering lines. The latter is predicted to occur in response to a gradient of Fgf signal from the AER that, together with Hox function, coordinately decreases the inhibition and increases the activation of the Turing network to set the thickness and orientation of stripes. This causative relationship between levels of Hox and Fgf and limb size ensures that as the modeled limb scales larger (higher Hox and Fgf) or smaller (lower Hox and Fgf), digit thickness also scales to predict that five smaller or larger digits will form as a function of limb bud size.

Future challenges for the turing model

It is exciting that developmental genetics has advanced to a point where theoreticians have enough 'knowns' on which to base computational predictions and that those models, in turn, can inspire experimental questions creating an overall iterative approach. Although the current BSW network model simulates the periodicity of digit and interdigit formation in the mouse autopod, it does not yet test hypotheses as to the order of the appearance of digits. Zhu and colleagues proposed an unexpected alternating order to the emergence of mouse digits; digit 4 appears first, followed by 2, then 5, 3, and 1 [22••]. This pattern would invoke a splitting of the interdigit field to allow digit 3 to form in between nascent digits 2 and 4. There is some precedent for this in other proposed models of stripe morphogenesis, namely in the Shh/Fgf model of ruggae formation in the hard palate of the mouth [23]. Growth of the tissue separates ruggae stripes such that a new stripe is laid down in the space where inhibition is released at a distance. Although the hypothesized Turing

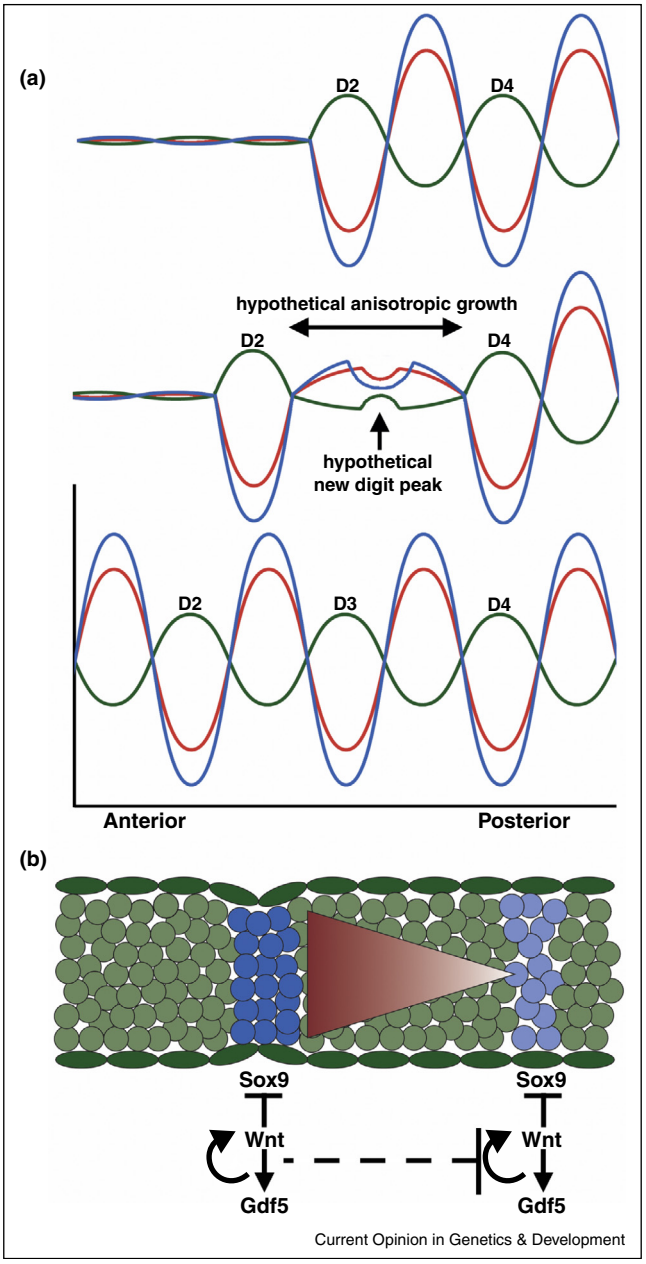
model of ruggae formation did not take a computational approach to mathematically explain this phenomenon, these experimental observations may serve as inspiration to refine the digit model. Pairing computational modeling and experimental approaches can determine whether regional anisotropic growth would allow for the alternating appearance of digits. Separation of the future digits 2 and 4 by growth may expand the out of phase (interdigit) field large enough for the appearance of a new peak (digit) (Figure 2a).

In addition to the sequence of digit-interdigit-digit in the anterior-posterior axis, there is a periodicity to the formation of the phalanx-joint-phalanx pattern in the proximal-distal axis. It is curious that the molecular mechanisms of joint induction closely parallel the BSW network of digit morphogenesis (Figure 2b). Gdf5, a member of the Tgfβ superfamily of proteins that also includes the Bmps, is expressed in the developing joint interzone and is required for normal joint induction in mice and humans [24,25]. Several Wnt ligands are also expressed in joints, and Wnt signaling through β-catenin is both necessary and sufficient for joint induction [26]. Experimental manipulation in chick embryos indicates that Wnt9a is sufficient to induce ectopic joints, and that the presence of a joint inhibits the formation of other joints at close proximity [27••]. Furthermore, interactions between the newly formed phalanx and the next nascent phalanx seem to influence the size, and thus joint positions, along the series of phalanges [28]. Together these observations of a remarkably similar set of molecules communicating as activators and inhibitors across a field of tissue suggest a holistic approach to modeling the autopod may eventually lead to an understanding of coordinated self-organization in two orthogonal axes.

Implications for a Turing mechanism in the evolution of the autopod

To date, Turing models have been closely analyzed for their ability to explain the elaboration of the autopod, the evolutionary origin of digits, and subsequent canalization of pentadactyly [13,15•]. These studies have focused on modeling the observed pattern of endochondral skeletal elements in basal fish and fossilized early tetrapods and, while valuable, are challenging to experimentally validate due to the paucity of extant species. However, while all living tetrapods arose from a pentadactyl ancestor [29], the limb has continued to evolve in remarkable ways including the convergent loss of digits again and again throughout all major clades. Two recent papers investigated the mechanisms of digit loss in a variety of mammalian species and identified distinct motifs of *Ptch1* attenuation during early limb bud pattern, and of expanded cell death during post-patterning chondrogenesis [30••,31]. Although this work identifies important mechanisms of digit loss, it also highlights the complex and multifaceted process of limb evolution and leaves open questions about additional mechanisms that complement and reinforce these motifs and that determine the position of digit condensations — questions that may best be addressed by pairing computational predictions and

Figure 2

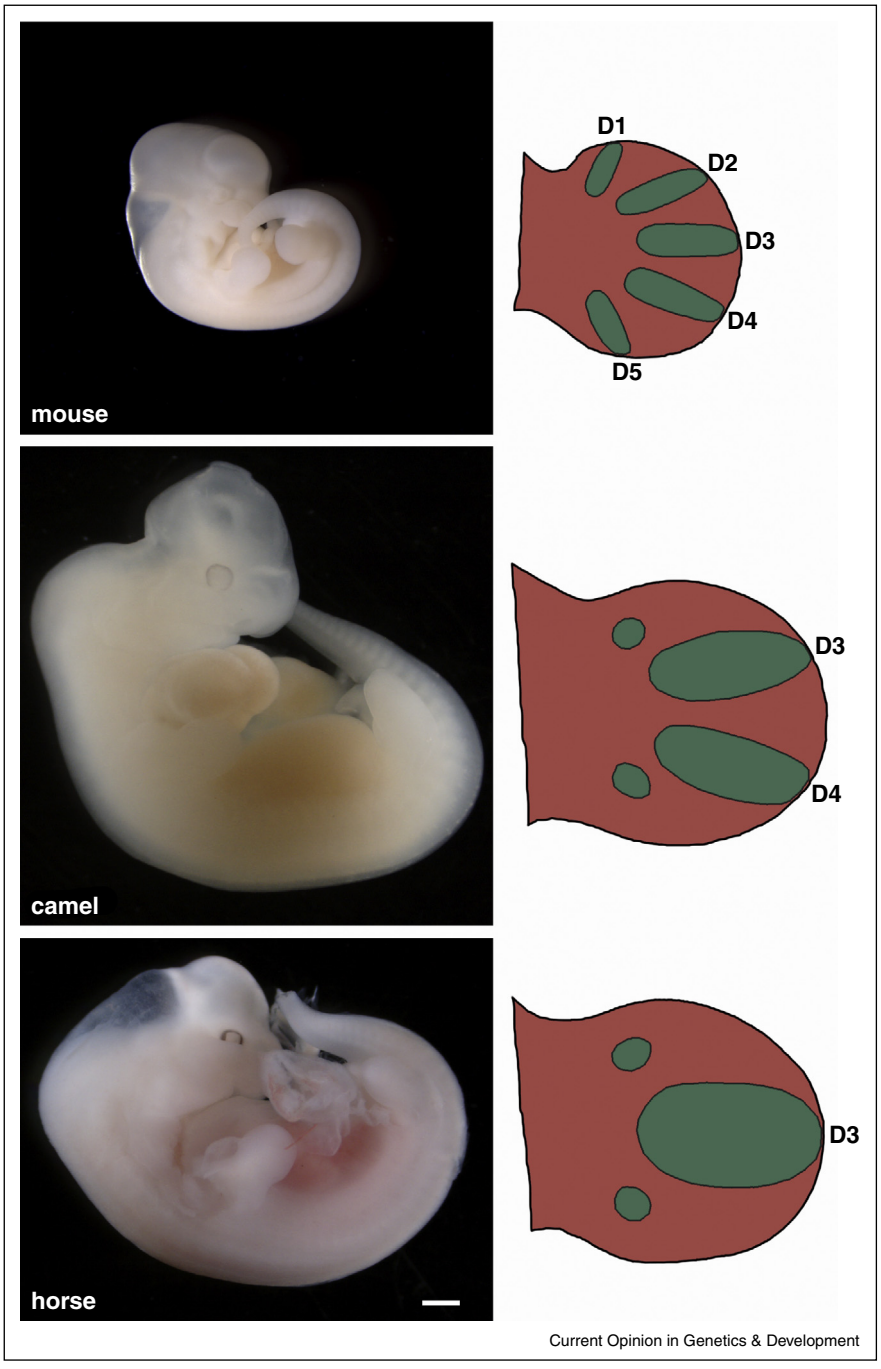


(a) A hypothetical model for the alternating appearance of digits in the limb. The first and second peaks that form may coincide with digits 4 and 2, respectively (top). Subsequent anisotropic growth may create enough space for an additional peak to begin to form between these peaks (middle). This could create the same stable pattern of digits with an alternating order of appearance (bottom). **(b)** Similar molecules function in the periodic pattern of phalanx (green) and joint interzone (blue) development. Wnt induces the joint interzone by suppression of Sox9 and activation of Gdf5. A secreted signal prevents the formation of the next joint within closer range of the prior joint (red).

experimental validation given the scarcity of some embryonic specimens.

In order for pattern to self-organize in a Turing model, the initially low and homogeneous expression of activator and inhibitor must be destabilized, and yet this does not occur randomly in the autopod. Most living pentatactyl species, including the mouse, have a robust and reproducible position of digits with a central axis of symmetry about the middle digit (digit 3). Indeed, when the diffusion

Figure 3



Approximately equivalent developmentally staged mouse (12 dpc), camel (34 dpc), and horse (30 dpc) embryos at the same magnification together with outlines of the limb buds. Limb bud drawings are overlaid with a depiction of the approximate size and position of the future digit condensations as interpreted by the space outlined by *Msx2* expression at stages for each species that are approximately equivalent to 13 dpc mouse (depicted in Figure 4 of Cooper *et al*. [30••]). Scalebar = 1 mm for embryos and 200 μm for limb buds.

parameters of the BSW model are limited to the early posterior-restricted position of *Hoxd13* expression as the point of initial heterogeneity, a very reproducible and stereotyped series of stripes arises that recapitulates the conserved pentadactyl form [17•]. Artiodactyls, in contrast, have shifted the position of digits such that the axis of autopod symmetry centers on the interdigital space between digits 3 and 4 [32]. If the early autopod expression of *Hoxd13* establishes the position of the first digit to form, digit 4, perhaps the distal shift in *Hoxd13* expression observed in the pig and cow functionally relates to the shift in digit position in these species [30••,31]. Later expression of *Hoxd13* in the camel limb bud shows a posterior bias, but earlier specimens should be analyzed to determine if a similar early distalization of *Hoxd13* may be independent of the *Ptch1* attenuation that is present in cow and pig but not in camel.

Surprisingly, while the early limb buds of the horse and camel appear smaller than mouse relative to total body size, the width/length ratio seems greater than one might expect given the fact the horse forms only a single digit flanked by two truncated condensations, and the camel forms only two complete digits with flanking nodules [30••]. Furthermore, while these morphometries have not yet been quantified, each of the digits that form in these species seems larger than expected as a proportion of the autopod field (Figure 3). Though logistically challenging, it will be interesting to collect a series of horse and camel embryonic specimens in order to generate MorphoMovies simulating the growth of each species [17•]. Given the apparently wider than expected autopod, it would be predicted that more digits should form in each of these species than what occurs in nature. Adjusting the model parameters may lead to equations that predict altered levels of signaling output in either or both of the Bmp and/or Wnt pathways that recapitulate the observed digit pattern and size. These models can then be experimentally validated in embryos thus limiting the number of specimens required to address the fundamental and unanswered question of how digit size and number are coordinately modified.

Our understanding of limb development mechanisms has advanced in strides since the earliest embryological manipulations and mathematical theories, aided in part by developmental genetics and increasing computational power. Alan Turing would likely be pleased with the current and future state of the field given the fact we are finally near an explanation not only for the zebra's stripes but also for some of 'the horse part'.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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Alan Turing turned from computer engineering to the mathematics of nature in order to explain the patterns that emerge in organisms. This is the single paper that resulted from that work before his death just 2 years later. His model rests on the chemical properties of morphogens diffusing at different rates through a tissue. Homogeneity is disrupted by a stochastic imbalance leading to auto amplification and cross inhibition that produces a self-organized pattern. Varying each of the reaction and diffusion rates can produce patterns that explain a remarkable diversity of coloration pattern and biological structures.

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Twenty-seven years after Alan Turing's paper on the chemical basis of morphogenesis, Newman and Frisch were the first to apply a mathematical approach to explain the ordered formation of digits in the chick limb. Their paper models a reaction diffusion mechanism to explain the number of elements that form in each of the proximal-distal segments: the humerus, radius/ulna, and digits.

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In the background of Gli3 loss of function, the sequential deletion of distal Hox genes results in more and thinner digits. This result suggested digits do not form in response to a morphogen gradient but rather self-organize

by a Turing mechanism. The mathematical model suggests Hox genes modulate a Turing network to set the thickness of digits while a gradient of Fgf orients the digital rays. The model also established parameters for the subsequent identification of the activator and inhibitor models by Raspopovic *et al*., 2014.

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This work starts with a transcriptome approach to identify candidate pathways that form a Turing network for digit self-organization — Bmp/Sox9/Wnt. The authors then computationally identify a molecular interaction topology that simulates the pattern of digit formation in the mouse autopod. Experimental manipulation of Bmp and/or Wnt signaling replicates phenotypes that are predicted by altering parameters of the computational model. Lastly, the work integrates with a previous paper by Sheth *et al*., 2012 that outlines a role for Hox and Fgf in establishing digit thickness and orientation of the digit rays.

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