A PRIMER ON THE MORPHOLOGICAL PATTERNING AND
MATHEMATICAL MODELING OF THE DEVELOPING CHICK FORELIMB

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ABSTRACT

The understanding of both the biological processes and current mathematical models which describe the temporospatial patterning of the vertebrate limb require a unique set of interdisciplinary knowledge. In an effort to acclimate researchers who are new to this topic, we provide within a description of the developmental biology of the chick embryo, with an emphasis on the signal transduction of the developing forelimb. In our description of these systems, we include both the classical and molecular biology, along with the relevant variables, equations, and computational methods used to describe them. In addition to the positional axes commonly provided, we interrelate Hamburger-Hamilton stages of development, elapsed time, cell count, and observed measure as potentially useful reference points during navigation of literature on the subject. Particular attention is paid to the role that morphogen diffusion plays, and the effect it has on the differentiation of cells as they divide and migrate throughout the growing limb. Cell and tissue mechanics are not discussed in this revision. A glossary of terminology, as well as a list of relevant literature, cross-referenced by morphogen and region, are provided.

INTRODUCTION

Pattern formation is the process by which cells differentiate and assemble to form various tissue types in a developing organism. The patterning of the vertebrate limb is a complex process in developmental biology, into which much experimentation and data collection has been done. This experimentation has yielded a theoretical model for the changes in cell types and their positional information in the limb. The coordination of these cell movements, changes, and assembly into tissues over the course of development is guided by a network of chemical signals diffused from various areas throughout the developing limb. These chemical signals are received, or bound, by other cells, which have the appropriate receptors for that particular signal. The end result in the ensuing network of reactions within the cell can be motile behavior, as well as further differentiation into its final phenotype. Morphogen is the general term for all chemical signals which result in morphological changes in the cell. The variation of concentration of these morphogens from their diffusion sources through space and time give rise, at least partially, to the locations of various tissue phenotypes observed. Other mechanisms, including the mechanical properties of the varying cell and tissue types, the interactions between cells, and the interaction between the cells and the extracellular matrix, also contribute to the pattern formation.
The processes involved in both the creation and execution of this system are crucial to understanding the modeling methodology, an vice versa.

**EVOLUTIONARY PERSPECTIVE**

While there are many obvious differences between the limbs of the various vertebrates, the similarities on both a genetic and morphological level are numerous as well. This is attributed to the fact that they diverged from all other life on earth into their own unique evolutionary path, or phylogeny, about 500 million years ago. Morphological features of vertebrates, such as the notochord, somites, and the bilateral symmetry of the body plan are common in all members of this subphylum, including zebra fish, salamanders, chickens and humans (Figure 1). Between 400-360 million years ago, the evolution of the tetrapod condition gave rise to terrestrial vertebrates. While their four limbs are identifiably similar in form and function, many of the patterning mechanisms involved in their development are shared with the fins and tails of their water-bound relatives. Evolution continues to specialize the limbs of the vertebrates into the various forms we observe today. The remaining symmetries, however, as well as their observed differences, give us a unique tool for testing our understanding of the patterning involved in forming them.

**APPLICATIONS OF PATTERNING**

The complete sequencing of both the human and chicken genome provides us with a way to compare these two vertebrates. When analyzed, it was found that only 2.5 percent of the human DNA can be matched to chicken DNA. This small amount, however, contains genes that have been preserved over the 310 million years since humans and birds shared a common ancestor. These 70 million nucleotides contain the instructions that are shared between the developing chick limb and our own. Indeed, as evidence of the role specific genes play in the patterning of the limb are discovered, their homologues in the human genome often contribute to development similarly. 1:1000 infants are born with limb malformations [16]. 1:300 have polydactyly. 1:100000 have severe abnormalities [17]. Understanding the developmental processes allows us to detect and prevent these from occurring. In addition, understanding the underlying models involved in limb patterning can lead to methods for understanding patterning in other areas of development. These models can provide a powerful tool for understanding biology.

**SIGNAL TRANSDUCTION AND GENE EXPRESSION**

* DNA and Genes Overview
* Transcription and proteins
* Morphogens and Signal Transduction
* Upregulation / Downregulation
* Feedback Systems and Complexes (Different absorption vs. excretion rate by area)
The understanding we currently have of the patterning of the chick limb is largely due to the experimentation on, and observation of changes in, the developing embryo. In addition, evidence from mouse (Mus musculus) embryo manipulation is used to test new hypotheses on pattern formation.

1. Mechanical cell / tissue manipulation As we will discuss in detail, various regions of the developing limb are responsible for pattern formation. It is often necessary to understand the role of a given region, or to understand whether the cells in it have differentiated, and their cell fates determined. Removal of these regions through in vivo surgical procedures allows for determination of pattern formation without the region excised expressing its pattern. Implantation of this region at various locations of the developing limb allows for testing of axis determination, totipotency, and signal transduction network up / down regulation.

2. Bead implantation / morphogen application Many of the morphogens involved in the pattern formation of the developing limb have been identified. The use of plastic beads soaked in these chemicals allows for testing of accurate deduction of the morphogen’s role in the patterning process. Beads are also used to determine the path which specific regions travel along the developing limb axis.

3. Genetic knockouts and mutants and transcription factor blocking Many of the patterns observed in the limb are caused by expression of specific genes in certain locations. Mutant (naturally occurring and collected) chicks who are missing certain features can be compared with the normal chick gene sequence to observe which genes are likely the cause of the change in pattern formation. Genetic modification of chick top exclude these genes, or to block their transcription through RNA manipulation, can allow for testing of these genes roles.

4. Cell fate maps Through the use of radioactive precursor stains and lipophillic dyes, cells in specific locations in the developing limb can be marked. As these cells divide, migrate, and differentiate throughout the limb, their final location and phenotype can be observed by following the cells with the mark in them. Cell fates maps provide data about which cells will become which tissue types, as well as a temporospatial map of these cells as the limb develops. Fate maps by Tickle, as well as

5. Simulation The understanding of the positional information of the regions of morphogen diffusion, as well as a model for the up/down regulation
of one morphogen on another, as well as one morphogen on gene expression, allows us to create models for how the gradients develop in the limb through time. These models can answer questions, as well as suggest to the location and effect of potentially undiscovered processes.

EMBRYONIC DEVELOPMENT STAGES

As time progresses during development, various morphological events have been used to mark developmental stages. One method (and the most prevalent) of staging chick embryos is the Hamburger-Hamilton (HH) stages of chick development [10]. Their original paper appeared in 1951, and has remained the standard reference point on the developmental time line and its various landmarks. See Figure XX for HH stages relevant to limb development and their correlation to time and developmental landmarks. The entire developmental process, from fertilization to hatching takes between 20 and 21 days to complete, is divided into 46 stages. Additional staging schemes and morphological event cross-reference can be found in various atlas’s of chick development [11]. Each HH stage below 23 is approximately 4 hours long, and stages greater than 23 can be estimated at 6 hours in length [5]. These stages do not begin until the egg is laid, and initial cleavage and shell formation are not included in these stages. Our table uses fertilization as the start time, and the HH stages are adjusted accordingly by starting stage 1 at t=3600 (24 hours). Only stages relevant to forelimb development patterning are listed.

CELL DIFFERENTIATION AND THE FRENCH FLAG MODEL

As the embryo of chick divides from a single cell, and patterns itself into the varying cell phenotypes and tissues we observe in the stage 46 embryo, the individual cells go through a progressive narrowing of possible developmental fates. Totipotency refers to the ability for early stage embryonic cells to become any cell phenotype. As development continues, the degree of totipotency lowers, and they become more specialized (e.g. differentiated). This differentiation is guided by varying levels of morphogen concentration gradients they are exposed to during the course of their lifetime, however the combination of concentration and time that determines each tissue type is still unknown. One of the initial categorical divisions of cell fates occurs during the formation of the germ layers of the blastodisc between stage 1-2. The cells in these three distinct layers give rise to very specific tissue types during development, as is illustrated in Figure XX.

<table>
<thead>
<tr>
<th>Ectoderm</th>
<th>Mesoderm</th>
<th>Endoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Cardiac muscle</td>
<td>Lung</td>
</tr>
<tr>
<td>Neurons</td>
<td>Skeletal Muscle</td>
<td>Thyroid</td>
</tr>
<tr>
<td>Pigment</td>
<td>Bone</td>
<td>Pancreatic</td>
</tr>
<tr>
<td></td>
<td>Kidney (mesonephros)</td>
<td></td>
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<tr>
<td></td>
<td>Blood</td>
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<tr>
<td></td>
<td>Gut (smooth) muscle</td>
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</tbody>
</table>
One model for determining cell fate and controlling differentiation is termed
the French flag model. Proposed by Louis Wolpert, it describes the ability of
a cell to differentiate into several types, determined by the concentration of a
given morphogen that the cell is exposed to. Cell phenotypes exist within the
boundaries of concentration thresholds [12]. The effect of several morphogens
in varying concentration gradients which change over the course of time in de-
velopment suggests are more complex version of this model.

### ORIENTATION OF AXIS IN EMBRYO AND LIMB DEVELOPMENT

Stand up and hold out your arms at shoulder height with your thumbs
up. In front of you, including the way your chest and palms are facing is
ventral, and your back and back of hands are dorsal. From you head to your
 toe, as well as from your thumb to your pinky is anteroposterior. From your
shoulder to your fingertips is proximodistal. These axes are formed during the
course of development through a combination of the direction of cell division
and movement of morphogen diffusion centers over time. The x-axis in the
developing limb is referred to as the DV axis, the y-axis is the AP axis, and the
z-axis is the PD axis. Time should also be considered an axis as well, as the
problems of modeling of limb development require the understanding of changes
through time.

### EARLY EMBRYONIC DEVELOPMENT

We begin with a review of the early stages of development, starting with fer-
tilization. As our concern is with the patterning of the limb and the models used
to represent it, this section can be skipped by those familiar with the biological
processes leading up to the initiation of the limb bud. These initial develop-
mental stages are important however, as they give us necessary framework and
clues to the patterns of development which we observe in the limb.

1. **Fertilization**
   
   The oocyte, or unfertilized egg, cell begins to develop on to
   side of the single functional ovary. The oocyte in the chicken (and all birds
   and reptiles) is relatively large, when compared to other vertebrates. This
   size is due to the protein-rich yellow yolk. This single cell, which makes
   up 33 percent of the liquid weight of the new embryo, provides the rest
   of the embryonic cells with their nutrition as growth continues (1). Once
   ovulation has occurred in an adult female, the single-celled ovum begins
to travel down the oviduct, with the thick albumin holding the yolk in the
center of the oocyte. The unfertilized cell contains only the females DNA,
   divided amongst 39 chromosomes, all contained in the nucleus (haploid)

   (2). The sperm of the male has been stored after mating, and is released
   from the cloaca, a sperm storage organ, between 15 and 45 minutes after
   ovulation occurs. Once the fusion of a males chromosomal counterparts
   occurs during fertilization, the zygote is formed, and is now a diploid cell,
containing sets of both the DNA from the male and the female. As the egg travels down the oviduct, new layers of albumin form, including the white and the shell.

2. Cleavage and the blastodisc Shortly after fertilization, the new diploid cell, which is located in the small cavity of cytoplasm at the top of the yolk, begins to divide via mitosis. Mitotic division, referred to as cleavage, creates duplicate copies of the dividing cell, differing only in the content of their cytoplasm. (Figure 1). The dividing cell mass, and the yolk below, provide our first polarization during development, with the animal pole being the dividing cell mass, and the vegetal pole the yolk. The cell located at the animal end of the embryo continues to divide vertically, from top to yolk. Continued division in this fashion forms a disc of cells afloat atop the yolk, called the blastodisc, or blastoderm. This disc is one to a few cells thick in center of the blastodisc, and becomes thicker, and visibly darker towards the edges. The cells in the center of the blastodisc excrete a fluid between themselves and the yolk, and create the subgerminal space, which differs in pH and charge from the yolk, and polarizes the cells. This polarization is our second, and forms the Dorsoventral axis of the embryo, with the ventral side facing the yolk. The embryo continues down the oviduct, and continues dividing. When laid, the blastodisc is approximately 60,000 cells in total, which is just more than 16 divisions from the original 1 cell. (INSERT Fig 5.30 from Gilbert p189)

3. Primitive streak Our embryos anteroposterior axis is also formed at this point as well. As more cells congregate to one side of the blastodisc during the embryos travel down the oviduct, this denser area is the posterior, of our anteroposterior axis. Our next major milestone is the formation of the hypoblast, epiblast, and ultimately, the primitive streak. This major cell migration and layering of cells, with the epiblast on top, and the hypoblast on bottom, creates a cavity in the center (the chick equivalent of the blastocoel) and gives rise to a thicker, wedge-shaped opening called the primitive streak. As cells continue to migrate toward the primitive streak, they enter the cavity and begin to fill up this space. The first cells to enter it become the endoderm, with the later cells becoming the mesoderm. (T=?)

4. Hensen’s node and the somites The streak continues to elongate as development continues, and then retracts toward the posterior end of the embryo. The end of the streak, referred to as Hensen’s node, excretes various morphogens, and plays a critical role in pattern formation. Cells continue to migrate through it, and the location at which they move through the streak determines their ultimate role in the developed embryo. While the act of cell migration through the streak is not significant, the distance of the cells final destination to the node at the top of the streak is. As the streak begins to retract, cells migrating in become the increasingly poste-
rior components of the chick. e.g. the cells that migrate through first become the head and brain, followed by the somites, the organs of the body, including the heart, kidneys, and gonads, and finally the blood. After the cell migration through the primitive streak, and their placement, they are no longer totipotent, meaning that their fate is now predetermined, which has been proven experimentally. The formation of the somites provides an important indicator for development of the chick forelimb. Numbering schemes for somites are done from anterior to posterior direction. (Insert fig. 18.6 in Gilbert p 705) The forelimb forms on both the right and left side of somites 15-20. The interlimb region, where FGF8 is expressed from, is located between somites 21-25. The hindlimb is formed between somites 26-31.

LIMB FORMATION

While the organs of the body and neural structures continue to develop, we will now shift our focus to the initiation of the limb. Think for a moment of a maple leaf. As it grows out from the bud of a tree, at some point it branches. The location of the developing limbs is much like these branches, growing away from the initial location of growth, and towards the newest tissue being generated in the progress zone of the Hensen's node. After this branching, new division and growth also occurs in the distal tips of the forelimb buds. Another branching occurs in the hindlimb, resulting in a total number of growth centers of 5 in the developing vertebrate. (tail = retinoic acid, limb buds = FGF8). It is quite likely that retinoic acid often provides similar function to Fgf8, Shh, and other morphogens of the limb when provided as an implanted bead, due to it being the original morphogen in the original growth center (Hensen’s node). (See Question 13, 14)

1. Limb field The area that gives rise to the limb is located below the somites (ventrally) on the lateral flank. These cells are not necessarily predetermined as limb cells, as they can be removed, and cells surrounded the excised area form the limb, but rather this region is determined by a morphogen gradient, and is known as the limb field. If the limb field is split in two vertically, two limbs form. This points to the gradient for the limb field being formed in an anteroposterior direction (in reference to the entire embryo). The limb field is located laterally along AP axis parallel to somites 13-18, then 16-19, then 15-20. This location is however directly linked to the level of Hoxc-6 gene expression as we measure this anteroposteriorally. The most anterior region that expresses Hoxc-6, which is also the location of the first thoracic vertebra, is the future location of the limb bud. This location also induces migration of stem cells, called myoblasts from the somites, into the newly forming limb bud. These specializes mesenchyme cells located in the somatic field and the somites migrate to just below the epidermal layer of the lateral plate mesoderm. This forms a bulge at approximately Stage 17, and this is the new limb bud. Hensen's
node is a circular, more dense region in ectoderm at the posterior end of
primitive streak (it defines the posterior end). It descends posteriorally
along the back of the embryo, just below the forming notochord. It ex-
cretes several morphogens, including shh, fgf8, and retinoic acid. It forms
the somites and plays a role in guiding the migration of the myoblasts to
the presumptive limb bud. This is managed by regulation of hoxc-6 gene
expression in a AP gradient along the lateral plate mesoderm, parallel to
the somites. This forms in a circular region, developing laterally along the
AP axis. Limb bud formation sites from reptiles and mammals, grafted on
to chick, will properly control the formation of the limb (Hincliffe 1991).
This points to the fact that the totipotency of cells in the unformed limb
bud region are no different in any of these three, and the rules are the
same. Further development however further determines the cell fate, and
as development progresses, these cells are not interchangeable. At what
point does this occur?

2. Mesonephros, FGF8, and the Interlimb region
The mesonephros, which is
the kidney in chick, is located on either side of the developing embryo.
It’s location between somites 21-25, and it’s diffusion of FGF8 causes the
migration of mesenchyme from the somites and the somatic layer of the
lateral plate mesoderm to the layer under the epiderm of the limb bud site.
This area becomes swollen and these mesenchyme cells divide. As FGF8
is expressed from this one location in the mesonephros, it is possible that
the far ends of the FGF8 gradient (i.e. the lowest concentration areas)

determine the two locations of the limb buds. Limbless mutants do not
form an AER, or ZPA, and the limb bud swells, but then retracts. No
FGF8, FGF4, or Shh are expressed in the swollen bud, as the AER and
ZPA are missing. This shows that the limb buds are preprogrammed to
form, and that the AER, and ZPA only continue the process once formed.

3. Dorsoventral axis features
The dorsoventral axis of the embryo is set al-
most immediately. In the limb, the morphogen responsible for this is
Wnt7a, excreted from the cells int eh dorsal ectoderm. Experiments
removing the dorsal ectoderm result in missing digits and DV features.
Wnt7a induces expression of Lmx1 genes in the dorsal mesenchyme, and
these express feathers, nails, and other dorsal features. Wnt7a plays a
crucial role, in conjunction with FGF4 in upregulating Shh.

4. AER Formation
Proximodistal axis formation in the limb occurs now.
Outgrowth of limb occurs, and interaction of PD proteins which induce
escodermal proliferation, with those of the AP axis and DV axis (and
those changes over time, T-axis) form the patterns of the limb. The AER
forms anteroposteriorally along the border of the DV axis boundary. In
mutants, where the limb is dorsalized (no ventral En1 expression?), no
AER forms. The border from DV morphogen expression must exist. At
stage 15, the cells in the unformed bud located on the dorsal side of the DV
border excrete Radical Fringe. Radical fringe expression is restricted to the ectoderm cells. By stage 17, these cells begin to excrete fgf8 from the ectodermal cells. Radical fringe seems to form the AER, where fgf8 seems to cause the proliferation of the mesenchymal cells underneath the AER. Interestingly, it seems that the fates of mesodermal cells which migrate to the bud are predetermined. If mesenchymal cells from the hindlimb, from either the somites (muscle) or somatic layer (skeletal), are placed directly underneath the AER at stage 17 or so, these cells will form into hindlimb structures at the distal tips of the forelimb. It is unknown whether the mesonephros or the radical fringe cells cause the initiation of FGF8 expression prelimb bud area. The AP Axis is formed in the limb at Stage 16. The AER, and therefore expression of FGF8, FGF4, and possibly FGF2, do two specific things. Keep the mesenchyme cells under the ectoderm dividing mitotically, and restrict the chondrogenesis of these cells until the point when digit formation should occur.

5. Progress Zone The progress zone is located directly underneath the AER in the mesenchyme, and is about 200 um wide proximodistally. When the progress zone from older limbs which have already formed their distal structures are implanted into new limbs, no more distal growth occurs. When PZ tissue from new limb buds is implanted into distally developed AER, extra distal structures develop. The expression of HOX9-13 is thought to be responsible for developing regions of the forelimb. (Insert figure 18.15D on p 710 of Gilbert) The AER keeps these cells in the PZ dividing through excretion of FGF2, FGF4, FGF8.

6. ZPA formation and Shh expression At stage 17, Shh begins to be expressed in the posterior / distal region of the bud. This is induced by exposure of these cells to FGF8 expression. (Why? See experimental questions) By stage 18, Shh expression then induces Fgf4 expression in the posterior portion of the limb bud ectoderm. Fgf4 in turn then regulates more Shh expression. Fgf2 is also formed at this point. It should be noted that two areas are described as expressing FGF8 at this point; both the mesonephros in the interlimb region, and the AER ectoderm cells. Shh specifies the AP axis of the developing limb and is responsible for differences along it. These include the specification of the zeugopod, stylopod, and autopod, and will and specifying digit identity. The true mechanism for how the ZPA and Shh send the positional information and encode this pattern is still unproven, however, three theories exist.

7. AP axis features and digit specification Many of the current questions in limb development center around the signal transduction model The role played by the ZPA, and the Shh diffused from it is a complex one. Short range diffusion and cell migration is our first model to consider. It suggests that Shh is a short-range diffusive, only able to effectively signal near the ZPA. As cells that are near the ZPA are exposed to this
signal and differentiate, they then migrate via chemotaxis to their final location along the AP axis. The second model suggests again that Shh is a short range diffusive, but as cells near the ZPA are exposed to Shh, they then in turn begin secreting another signaling protein. This cascade effect provides the necessary signaling along the AP axis to specify the AP components of the limb [8]. The third model designates Shh as a long range diffusive, and suggests that a gradient of Shh along the AP axis determines digit identity, using a French flag model for digit identity [9]. The cascade model is currently the most likely, due to Shh’s inability to diffuse over long distances. In addition to how Shh diffuses, the expression that it causes is also of major importance. Shh has the ability to express, either directly or by cascade of BMP’s, Hoxd9-Hoxd11. However, retinoic acid was also required for the expression of Hoxd-12 and Hoxd-13. This suggests that retinoic acid gradients are also causing expression of FGF4 in the PZ, along with the BMP expression caused by Shh. This complex combination of cyclic regulation and co-expression successfully forms the digits. Interestingly, retinoic acid can induce BMP2 production, and BMP2 in turn will induce FGF4, as well as express Hoxd11 and hoxd13 in the PZ. [7, p.721] The reason the ZPA forms on the posterior side of the developing limb bud is also a mystery. It is likely that the expression of Hoxb-8, which is only produced in the posterior limb bud, is the cause. What causes expression of Hoxb-8 on the posterior only side of the developing limb is still unknown. Possibly the retinoic acid signals from the fully descended Hensen’s node in combination with the FGF signaling from the mesonephros.

8. Apoptosis Apoptosis also plays an important role in the specification of the AP features. Cells within the space between the radius and ulna in the more distal portion of the forelimb die during developmental via programmed cell death. Cells within the spaces between the digits do as well. Examples of the importance of apoptosis are webbed feet and joints. When a cell’s fate is determined to the death, DNA and RNA activity, and therefore protein synthesis rates drop dramatically. BMP4 is thought to be the signaling mechanism for initiating apoptosis.

MATHEMATICAL MODEL FORMULATION

Much is still unknown about the patterning of the chick limb. The derivation of a mathematical model, and it’s corresponding equations allow us to answer questions that are not able to be quantified experimentally. The formulation of this model is done by breaking it into three primary systems:

1. Production and diffusion of morphogens within the limb is modeled using reaction-diffusion equations. Each one of these equations allow us to model the rate of production \( k \) of a specific morphogen from within a known volume of cells, and determine the concentration \( c \) of this morphogen.
at any point \((x, y, z)\) in the limb as development continues and time \(t\) increases. To accurately model the system, the function not only involves production, but also includes reaction functions which simulate the rate of receptor binding and chemical degradation as these morphogens diffuse through the limb. These accurate modeling of these equations will yield a complete system of equations whose concentration density and relative time match the observed biological data.

2. The tissues of the limb are modeled using the known material properties of a viscoelastic fluid. As concentrations of specific morphogens \((F_1 M, F_8, F_4, Bmp)\) change within the material, expansion and contraction occur to simulate cell growth and death. This deformation is modeled by creating an interconnected grid of points in \(x, y, z\) in the shape of the limb bud at stage 16. This mesh is connected into either tetrahedrons or hectagons. Each of these is referred to as an element, and this element is used to calculate properties of deformation.

3. We propose here, as the third component of the model, a way to differentiate tissue types. Final cell types and positions have been determined to be functions of both exposure to a specific concentration level of a morphogen (threshold) at a specific time, as well as a sum of it’s total exposure over time. Specific morphogens are linked with varying axial positional information and patterning. The resulting gene expression of some morphogens also lends to their association with observed cell type differentiation. One, or several thresholds are assigned to each morphogens production range over the entire developmental process, and a value is assigned to each threshold’s range ordered \(0, 1, 2, \ldots, n\), where \(n\) is the number of thresholds for the given morphogen. The permutation of all of the resulting combinations of threshold values allows us to assign a tissue type. This model is notably an expansion of the french flag model beyond a single morphogen. First, we calculate the sum of the concentration of each morphogen at each element over the simulation time. Next we calculate the cell type at each element. A finer mesh yields more accurately determine the more detailed cell differentiation observed in the actual limb. An important component of this model is the correct timing of the reactions which set off the system of equations. Recent data from Tabin, et. al. has shown that a ‘priming’ period is required for proper concentration threshold gradients to be setup before cell fate is determined. Proper order for establishment of each gradient is determined to be 10 hours, and full expression of that gradient level is maintained for the remainder of the simulation. See Table XX for the order of events of morphogen expression, and their associated embryonic stages, regions, and diffusion rates. The results are plotted using varying colors for each of the discrete tissue types.

Enzyme:
Wnt3a:

Wnt7a: A morphogen which is diffused by the dorsal ectoderm, and is responsible for DV patterning on the dorsal side. As well as some regulation of other morphogens. The gene for the transcription of this morphogen is at 3p25 encoding a 349aa secreted glycoprotein. Wnt7a is secreted protein and binds to extracellular matrix. The mechanism of Wnt7a distribution and its possible cell receptors are still being determined.

BMP2: Expressed in the AP direction of the developing limb mesenchyme. Regulated by Shh, FGF, Wnt7a

BMP4: Expressed in the interdigital spaces, interior, anterior, and posterior necrotic zones, and is the likely signal which causes apoptosis to occur.

dHand:

En1: A morphogen which is diffused from the ventral ectoderm in the developing limb, and contributes to DV patterning on the ventral side.

Fgf2: Expressed by the cells in the AER. Beads with 24 hours worth of FGF2 implanted in PZ of stage 20 chick after AER removal lead to successful limb and digit development.

Fgf8: Expressed by cells in the mesenephros and the AER. Control proliferation of mesenchyme cells in the distal tip of the forming bud. Possibly induced by retinoic acid from the Hensen's node.

Fgf4: Expressed by cells in the ZPA. These control AP axis differentiation in forming bud. Induced by FGF8 initially, and maintained by FGF4 + FGF8 from the AER and possibly FGF8 from the mesenephros.

Fgf10:

Gli1:

Gli2:

Gli3: Enzyme which acts as a transcriptional repressor (and possibly promoter) of dHAND and Gremlin. Regulates Shh expression via concentration gradients.

Gremlin:

hox genes:
Pattern formation:

Protein:

Receptor:

Transcription: Within the nucleus of every cell are the genetic instructions to

Radical-Fringe: Expressed by the ectodermal cells on the dorsal side of the DV border of the stage 16-18 limb bud. (Do they stop expressing after this?) Possibly induces expression of FGF8 from the AER, giving the developing limb two FGF8 sources.

Shh: Expressed in ZPA. Works in combination with the AER to differentially express Hox genes, along the AP axis. Several theories exist for how Shh from the ZPA specifies digit identity, including the Short range diffusion and cell migration, Cascading short range signaling and Long range diffusion specifies digits spatially based on concentration of Shh in gradient. Most likely a short range diffusive, which activates expression of BMP2 and other BMP’s. These BMP’s may be the cascade signals from model 2. Shh is regulated by the combination FGF4 from the AER and Wnt7a from the dorsal ectoderm. Shh is also regulated by the combination of Retinoic acid from the ZPA and FGF4 in the AER. Shh also regulates FGF4 in the AER (cyclical).

Myoblast: A type of stem cells that exist in muscles. Skeletal muscle cells are called muscle fibers and are made when myoblasts fuse together; muscle fibers therefore have multiple nuclei. Myoblasts that do not form muscle fibers differentiate into satellite cells. These satellite cells remain adjacent to a muscle fiber, separated only by its cell membrane and by the endomycium (the connective tissue of collagen surrounding the muscle fiber. http://en.wikipedia.org/wiki/Myoblast

During the course of research on the subject, many questions have been raised. Many potential answers and tests have also been considered. Those that were answered were taken from the list, and those unanswered remain. The reader is cautioned that some of these questions are hypothetical in nature, and are by no means meant as posed hypothesis for further discussion.

1. Do new expression regions and tissue types arise in directional vectors which are the cross product of each morphogen gradients perpendicular vectors? Does the density of one morphogen expressed in a region, when compared with the density of another determine the magnitude of each perpendicular vector? Final observed tissue types can be explained with the French flag model adapted to be the

2. Do cells which exist in overlapping morphogen gradients determine their
fate by reaching the steady state of a non-linear set of feedback loop equations? For example, if we are discussing the fate of a cell that exists in the mesoderm in a stage 18 limb bud, and a dorsal-to-ventral Wnt3a gradient is expressed, as well as a ventral-to-dorsal En1 gradient is expressed, the cells that are located within the overlapping gradients will express both of the proteins which the receptor on that cells which become active code for. If we use a binary French flag model, in which a cell can only become one type or another, based on the value of the steady state system of multiple morphogen expression feedback loops. i.e. Eventually one morphogen will win and express that amount for the remainder of the development. If a cell can either express or not express a given morphogen, this is $2^n$, where $n$ is the number of morphogens overlapping their gradients around this cell. If $n=8$, then we have 256 possible combinations of expression patterns in cells. However, it is possible that a steady state for what is expressed by a cell is a combination of morphogen, and only experimentation will determine this. How many steady states exist for each morphogen gradient combination is the number of thresholds in the French flag model. What are stage 35 cells in the AER expressing? What about the cells that become blood?. Do these still express morphogens, or does their steady stop stop expression and dedicate itself to differentiation via protein expression? If tissue types are the ultimate destination of a differentiating cell, then a fully differentiated cell should be expressing a constant amount of a predictable morphogen.

3. Instead of a 2D French flag model, a n-dimensional French flag, caused by overlapping gradients, with each dimension as a morphogen concentration. Combinations of various thresholds in each dimension give is the resulting tissue phenotypes.

4. Results from differential expression of morphogen diffusion coefficients. How does the existing region sizing work? What portion of our equations is this and how is it scaled with time? Linear?

5. Progress Zone vs. Cell History / Memory Model

6. Revisit Recent Articles from Past 4 years for model changes

7. What role do temperature gradients play on development? 37 C is brooding temp for development, and much variation from this will result in failure of development. How much, and what if a gradient of the maximum and minimum is applied to the whole embryo? What about changes to specific locations? Do temp fluctuations effect various morphogen expression differently?

8. Is it possible that all of the morphogens are expressed in the new limb bud in a single group of cells, and that as the cytoplasm of these cells divides, and the cells migrate, they are only able to express specific morphogens as time goes on? Maybe a combination of morphogen expression capacity
and chemotaxis affinity is the answer to how cells that express specific morphogens end up in specific locations

9. ZPA Expression site: Is this then expressed here because of either the combination of more retinoic acid + the FGF8? Does radical fringe boundary have anything to do with this?

10. Is the expression of FGF8 from two sources responsible for the delay of digit formation until the AER is far enough away from the mesenephros that the gradient is no longer applicable. Digits then form? Does the mesenephros and eventual kidney always produce FGF8, or does this stop at some stage?

11. Check model where each morphogen causes a gene expression to begin, down-regulates one, or up-regulates one. Create equations for unknown morphogens which are expressed in return, assuming all morphogens which bind to a cell cause either an increase in cell division, decrease in cell division, a differentiation if exposed for t seconds, as well. For non-existent (undocumented) morphogen equations, use variable names (F)

12. Discretized cell count and axis formation hypothesis: A zygote begins as a single cell, diffusing an unknown morphogen. It divides, and the result is two cells whose two primary differences are the contents of the cytoplasmic mixture they were given in division, and the ratio of existing cell wall they were given during cytokinesis. The set of receptors is identical in both cells once the cell wall heals, however the one of the two cells will begin producing the morphogen they are programmed to produce earlier than the other. This morphogen is guaranteed to have a receptor on the slower cell, as long as it has a receptor on itself. Are the receptors the same after division? How many receptors are on the stage 1 zygote? What about the 2 cells after initial division? Are they symmetrical in receptor potential? Differentially (with time) expressed genes in the almost identical cells begin producing the proteins they are coded for, up-regulated from 0 by the absorbed morphogen. These proteins are then excreted from the cell as down regulating morphogens of the other cell. Division continues to occur. At 4 divisions, the 4 part rhythm of division is established, with symmetry between the first and second pairs, offset by the time between the initial division. Each pair is then offset by a slight time step. With each division the symmetry is preserved between each of the two pairs resulting from it. If this division is possible only in the surface of the epiblast, then the direction of division is likely caused by the easiest path in one direction. The easiest direction is likely the location on the primitive streak of Hensen’s node, and therefore the posterior axis, on the ventral side. If we can accurately measure and stochastically simulate this rhythm based on observed data on the division rates, we can then predict cell count at various stages by making a time series generating function. The division travels as a wave down the AP axis, along what is referred
to as the primary body axis, soon to become the AP axis. If the x-axis is the DV axis, with the epiblast as the dorsal side (+), then the y-axis is the AP axis, with the posterior (-) side. What landmarks define the +, - direction of each axis?

13. Does the general increase in size of the PZ of the Hensen’s node reach some sort of a physical maximum at the neck / shoulder boundary? Is it likely that the branching into the arms occurs because of this and migration of specific mesenchymal cells get dragged along with this division?

14. What is the chemical makeup and similarity between retinoic acid and FGF8? Why the change for growth center morphogen once limb budding occurs?

15. Does the nucleus dissolve, merge chromosomes, and then reform as a new cell at the top of the yolk, post-fertilization?

16. What happens developmentally to chick without mesenephros? Do limbs form? Do retinoic acid bead implants in place provide formation? Does fgf8 work?

17. Do reaction / diffusion equations take into account degradation of chemical and reduced production over time in the limb? i.e. Plots show flooding of areas with all morphogens over time, but eventually they stop producing and all molecules are absorbed or degraded. Is there a degradation component to the reaction diffusion equation?

5. Cellular mechanisms of pattern formation in the developing limb, Maini and Solursh.
6. (Rudnick and Chaube)
8. (Martinez, et. Al, 1995)
The Mech3d code was created initially to model tumor growth. It’s ability to accurately simulate the coupling of the kinetics and mechanics of growth will allow us to answer the questions we are interested in. Experiments are designed to demonstrate the ability of the sim-limb to grow and or differentiate based on exposure to morphogen gradients as is observed in current in vivo data. Visualizations will be generated for each solution at varying time steps during the simulation. Next, combinations of cells exposed to various morphogen concentration gradients will be studied, and the overlapping regions of morphogen concentration during diffusion will be analyzed through visualization. This will give us insight into evolving gradients. Then, running them in all possible and relevant combinations and observing gradients, especially areas of overlap. Are there distinct concentrations of overlapping morphogens that correspond with specific tissue differentiation patterns? What are the observed thresholds for given morphogens experimentally.

16 additional variables need to be added to the solution vector, each corresponding to the sum of the concentration of the given morphogen over the whole simulation at that given point. These are stored as c(17) - c(32) at each element. This information allows us to relate specific observed exposure data to changes in kinetics and mechanics components.

Experimental Benchmarking
Experiment 1 - Turn off all morphogens other than $F_I M$, which simulates diffusion of retinoic acid from the mesonephros starting at Stage 8 and proceeding until stage 16 when limb bud forms. Observe growth in the distal direction as is shown in /refGeduspan:1990:PIL.pdf.

Experiment 2 - Enable $F_I M$, $W$, $F_8$, and observe expansion of AER along AP axis increased between HH7 and HH14. Wnt is enabled because it has been shown that it is required for AER formation.

Experiment 3 - Enable $F_I M$, $F_8$, $S$ and observe establishment of Shh gradient in AP direction. Shh gradient takes 10 hours to reach its effective, steady state, as shown in /refHarfe:2004:EEB.pdf. Continued maintenance of this gradient for 16 hours allows for normal morphogenesis of digits.
Experiment 4 - Modify boundary conditions for $S$ to accurately reflect findings that former Shh expressing ZPA cells which have migrated away from the ZPA create a barrier which terminates the Shh-Fgf feedback loop. The location of the boundary can be determined by finding the surface that describes elements whose Shh sum value is greater than the sum of the steady state of Shh for t+10 hours. The dynamic change of this boundary help to elucidate changes in downstream expression patterns in Gli3, Bmp, Ptc, Smo, Gre, and other unidentified expression domains. Does this result match findings in Dillon:2003:SLR in regard to long-range signaling of Shh?

Experiment 5 - Repeat Experiment 4 and enable $F_4$. This change should result in regulation of Shh expression and an increase in growth in the PD direction due to increased fgf8, fgf10, fgf 9, fgf17 expression. adi3d limb simulations do not currently reflect the more posterior nature of this expression in posterior and distal region. Is the Fgf4 equation properly coupled with the Shh and Fgf8 equation?

Equations

We assume that Sonic hedgehog (Shh) is produced by cells in the Zone of Polarizing Activity (ZPA) and in the implant, it diffuses into the neighboring cells, binds to Patched (Ptc) receptors on the cell surface, and is also degraded. The Shh production rate is known to be influenced by Fgf4 and Fgf8 activity, and we model these as saturating functions of Fgf concentrations. Shh is not expressed in the ZPA in Wnt7a knockout mutants, and we model this dependence by requiring that both Wnt7a and one Fgf be present in order to have a non-zero Shh production rate in the ZPA. Shh action is assumed to be mediated through the Ptc-Shh complex, and results in the conversion of Smo from its inactive to active form. We model the Patched production rate as being a function of the activated Smo concentration, as in our previous paper.

Similarly, the reaction converting Smo to its inactive form is governed by the concentration of unbound Ptc. We assume that Gli3 is produced in its active form, and converted to the inactive form at a rate that is inversely proportional to the Shh signaling activity as represented by the active Smo concentration. All molecules are assumed to degrade at a rate proportional to their respective concentrations.

Let $S$ represent Shh, which bind reversibly to free Ptc ($P$) to form the complex $SP$. The rate of Ptc production is assumed to be a saturating function of the $SP$ concentration, and $k_P$ and $K_P$ are the associated maximal rate and concentration corresponding to half-max rate respectively. $k_0$ is the basal rate of Ptc production. Let $W$, $F_8$, $F_{10}$, $F_4$ represent the species Wnt7a, Fgf8, Fgf10 and Fgf4 respectively.

The expression of a certain factor in the IM at a location corresponding to the limb fields has been proposed to initiate the limb development process. There is some evidence to suggest that this might in fact be Fgf8, but we refer to this as the IM factor or $F_{IM}$. Let $F_{IM}$, $Gre$, $G3a$, $G3r$, and $Bmp$ denote Gremlin, the activator and represser forms of Gli3, and BMP respectively. Uppercase letters represent the corresponding dimensional concentrations of species. $k^{sup}_{sub}$ represent the rate constants for the reactions shown in Fig. 1, where $sub$ denotes
the complex formed and degraded as a result of the association (sup = +) or
dissociation (sup = -) of the constituents. Absence of a superscript denotes a
first-order rate constant for irreversible conversion or degradation.

Let \( D_i \) represent the diffusivities of species \( i \). Let \( V_e \) and \( V_c \) be the
extracellular and cell volume (total volume = \( V_e + V_c \)), \( a_s \) the cell surface area
per unit total tissue volume, and \( \theta_f \) the ratio of the extracellular volume to
the total volume. \( \chi_Z, \chi_A, \chi_{FIM} \) are used to denote the fraction of the domain
occupied by the ZPA, AER and site of \( F_{IM} \) production, respectively. \( f_Z, f_A, f_{FIM} \)
represent the rate of Shh production in the ZPA, Fgf4 (Fgf8) production in the AER and \( F_{IM} \) production at the IM, respectively. \( \chi_{region} \) is
a binary variable that takes the value of one at spatial coordinates included in
the corresponding region, and zero elsewhere.

The \( F_{IM} \) is produced at a constant rate in a localized zone. \( F_{10} \) is pro-
duced throughout the mesenchyme at a rate which is a function of the local
\( F_{IM} \) concentration. \( F_8 \) is produced in the AER as a function of the local \( F_{10} \)
concentration. Shh is produced in the ZPA. Its production rate depends on a
weighted local Fgf concentration multiplied by the local Wnt7a concentration.
In the absence of Shh, Gli3a gets converted to Gli3r through a first-order reac-
tion. Shh decreases the rate constant of this conversion reaction. In a similar
fashion, Gli3R decreases the rate of formation of Gre, which decreases the for-
mation rate of Bmps which in turn decreases the rate of fgf4 formation in the
AER. The concentration of Wnt7a is assumed to be a constant at the dorsal
ectoderm. Wnt7a, all Fgfs, Shh, \( F_{IM} \), Gre and Bmp diffuse throughout the
limb and degrade with first order kinetics. Gli3 is a transcription factor and
remains inside the cell. Ptc and the Shh-Ptc complex are present on the cell
surface and do not diffuse in the extracellular space.

A mass balance on each species results in the following set of equations:

Equations

\[
\frac{\partial F_{IM} V_e}{\partial t} = D_{FIM} \nabla^2 (F_{IM} V_e) - V_e k_{FIM} F_{IM} + F_{FIM} V_e \chi_{IM} \tag{1}
\]

\[
\frac{\partial F_{10} V_e}{\partial t} = D_{F10} \nabla^2 (F_{10} V_e) + V_e \frac{k^*_{F10} F_{IM}}{K^*_{F10} + F_{IM}} - V_e k_{F10} F_{10} \tag{2}
\]

\[
\frac{\partial F_8 V_e}{\partial t} = D_{F8} \nabla^2 (F_8 V_e) - V_e k_{F8} F_8 + F_{F8} V_e \chi_{AER} \tag{3}
\]

\[
\frac{\partial S V_e}{\partial t} = D_S \nabla^2 (S V_e) + a_s V (-k^+_s S \cdot P + k^-_s S P) - V_e k_s S + F_Z V_e \chi_{Z} \tag{4}
\]

\[
\frac{\partial W V_e}{\partial t} = D_W \nabla^2 (W V_e) - V_e k_W W + F_{Wnt} V_e \chi_{ectod} \tag{5}
\]

\[
\frac{\partial P a V}{\partial t} = a_s V (-k^+_s P \cdot S + k^-_s S P - k^+_m M^a \cdot P + (k^-_m + k_{mp}) M \overline{\nabla P}) + k^*_p + k^-_p M^a - k_P P \nonumber
\]
\[
\frac{\partial S P a_{s}V}{\partial t} = a_s V(k_{sp}^+ P - (k_{sp}^+ + k_{sp})S P - k_{spm}^- M^i \cdot S P + (k_{spm}^- + k_{spm})S P M)
\]

(6)

\[
\frac{\partial M^a a_{s}V}{\partial t} = a_s V(-k_{mp}^+ M^a \cdot P + k_{mp}^- M^a P + k_{spm}^- S P M)
\]

(7)

\[
\frac{\partial M^i a_{s}V}{\partial t} = a_s V(-k_{mp}^+ M^i \cdot S P - (k_{mp}^- + k_{mp})M P)
\]

(8)

\[
\frac{\partial M a_{s}V}{\partial t} = a_s V(k_{mp}^+ M a \cdot P - (k_{mp}^- + k_{mp})M P)
\]

(9)

\[
\frac{\partial S P M a_{s}V}{\partial t} = a_s V(k_{spm}^+ M^i \cdot S P - (k_{spm}^- + k_{spm})S P M)
\]

(10)

\[
\frac{\partial F a_{s}V}{\partial t} = D_{F a} \nabla^2 (F a_{s}V) - V e k_{F a} F a + F F a (1 - \theta_f) V x A E R
\]

(11)

\[
\frac{\partial G a_{s}V}{\partial t} = V c k_{G a}^* G a + K_{G a}^* G a - V e k_{G a} G a
\]

(12)

\[
\frac{\partial G r a_{s}V}{\partial t} = V c k_{G r a}^* G r a + K_{G r a}^* G r a - V e k_{G r a} G r a
\]

(13)

\[
\frac{\partial B m p a_{s}V}{\partial t} = D_{B m p} \nabla^2 (B m p a_{s}V) + V c k_{B m p}^* B m p - V e k_{B m p} B m p
\]

(14)

\[
\frac{\partial B m p a_{s}V}{\partial t} = D_{B m p} \nabla^2 (B m p a_{s}V) + V c k_{B m p}^* B m p - V e k_{B m p} B m p
\]

(15)

The specific rates of production in the ZPA, AER and $F_{1M}$ production region are given by

\[
F_{F_{1M}} = k_{F_{1M}}^*
\]

(16)

\[
F_{ZPA} = \frac{k_{F_{4}}^*(F_{4} + \alpha_{1} F_{8}) W}{K_{S}^* + (F_{4} + \alpha_{1} F_{8}) W}
\]

(17)

\[
F_{F_{4}} = \frac{k_{F_{4}}^*}{1 + K_{F_{4}} B m p}
\]

(18)
\[ F_{F_8} = \frac{k_{F_8}^* F_{10}}{K_{F_8}^* + F_{10}} \]  

\[ F_{W_{nt}} = k_{W_{nt}}^* \]  

We use characteristic length \((L)\), time \((T)\), surface concentration \((C^{II})\) and concentration per unit volume \((C^{I})\) to scale these equations. The scaled version of the equations is given by the following equations, where all concentrations per unit volume are scaled by \(C^{III}\), all surface concentrations are scaled by \(C^{II}\), time is scaled by \(T\) and partial derivatives in rectilinear coordinates included in the operator \(\nabla^2\) are scaled by \(L\). The diffusion and rate parameters are defined in Table 1. Note that although the same symbols are used, the variables and parameters in the following set of equations are distinct from the previous set of equations.

\[ \frac{\partial F_{1M}}{\partial t} = \delta_{F_{1M}} \nabla^2 F_{1M} - k_{F_{1M}} F_{1M} + F_{F_{1M}} \frac{1 - \theta_f}{\theta_f} \chi_{1M} \]  

\[ \frac{\partial F_{10}}{\partial t} = \delta_{F_{10}} \nabla^2 F_{10} + k_{F_{10}}^* F_{1M} \frac{1 - \theta_f}{\theta_f} \chi_{1M} - k_{F_{10}} F_{10} \]  

\[ \frac{\partial F_8}{\partial t} = \delta_{F_8} \nabla^2 F_8 - k_{F_8} F_8 + F_{F_8} \frac{1 - \theta_f}{\theta_f} \chi_{AER} \]  

\[ \frac{\partial S}{\partial t} = \delta_S \nabla^2 S + \gamma (-k_s^+ S \cdot P + k_{sp}^+ S P) - k_S S + F_Z \]  

\[ \frac{\partial W}{\partial t} = \delta_W \nabla^2 (W) - k_W W + F_{W_{nt}} \frac{1 - \theta_f}{\theta_f} \chi_{ectod} \]  

\[ \frac{\partial P}{\partial t} = -k_s^+ S \cdot P + k_{sp}^+ S P - k_{mp} M^a \cdot P + (k_{mp}^+ + k_{mp}) M^P + k_0^+ + k_{sp}^+ M^a - k_P P \]  

\[ \frac{\partial S P}{\partial t} = k_{sp}^+ S \cdot P - (k_s^+ + k_{sp}) S P - k_{spm} M^i \cdot S P + (k_{spm}^+ + k_{spm}) SPM \]  

\[ \frac{\partial M^a}{\partial t} = -k_{mp}^+ M^a \cdot P + k_{mp} M^P + k_{spm} SPM \]  

\[ \frac{\partial M^i}{\partial t} = -k_{spm}^+ M^i \cdot S P + k_{spm} SPM + k_{mp} M^P \]  

\[ \frac{\partial M^P}{\partial t} = k_{mp}^+ M^a \cdot P - (k_{mp}^+ + k_{mp}) M^P \]  

\[ \frac{\partial SPM}{\partial t} = k_{spm}^+ M^i \cdot S P - (k_{spm}^+ + k_{spm}) SPM \]  

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\[ \frac{\partial F_4}{\partial t} = \delta_{F_4} \nabla^2 F_4 - k_{F_4} F_4 + F_{F_4} \frac{1 - \theta_f}{\theta_f - \chi_{AER}} \] (31)

\[ \frac{\partial G_3 r}{\partial t} = \frac{k_{G_3 r}^* G_3 a}{1 + K_{G_3 r}^* M^a} - k_{G3r} G_3 r \] (32)

\[ \frac{\partial G_3 a}{\partial t} = \frac{k_{G_3 a}^* G_3 a}{1 + K_{G_3 a}^* M^a} - k_{G3a} G_3 a \] (33)

\[ \frac{\partial G_{re}}{\partial t} = \delta_{G_{re}} \nabla^2 G_{re} + \frac{1 - \theta_f}{\theta_f} \frac{k_{G_{re}}^*}{1 + K_{G_{re}}^* G_{3r}} - k_{G_{re}} G_{re} \] (34)

\[ \frac{\partial B_{mp}}{\partial t} = \delta_{B_{mp}} \nabla^2 B_{mp} + \frac{1 - \theta_f}{\theta_f} \frac{k_{B_{mp}}^*}{1 + K_{B_{mp}}^* G_{re}} - k_{B_{mp}} B_{mp} \] (35)

The specific rates of production in the ZPA, AER and \( F_{1M} \) production region are given by

\[ F_{F_{1M}}^* = k_{F_{1M}}^* \] (36)

\[ F_{ZPA}^* = \frac{k_S^* (F_4 + \alpha_1 F_8) W}{K_S^* + (F_4 + \alpha_1 F_8) W} \] (37)

\[ F_{F_4} = \frac{k_{F_4}^*}{1 + K_{F_4}^* B_{mp}} \] (38)

\[ F_{F_8} = \frac{k_{F_8}^* F_{10}}{K_{F_8}^* + F_{10}} \] (39)

\[ F_{Wnt}^* = k_{Wnt}^* \] (40)