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Documenting the Spatial and Temporal Dynamics of Pair-Rule Genes in the Early Tribolium Embryo

Hasan Mahmud
The University of Texas Rio Grande Valley

DOCUMENTING THE SPATIAL AND TEMPORAL DYNAMICS OF PAIR-RULE GENES IN THE EARLY TRIBOLIUM EMBRYO

A Thesis

by

HASAN MAHMUD

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COMMITTEE MEMBERS

Dr. Ezzat El-Sherif
Chair of Committee

Dr Yonghong Zhang
Committee Member

Dr Robert Gilkerson
Committee Member

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ABSTRACT

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Although Insects and vertebrates have different anatomical structures, they possess similar mechanisms for segmenting during embryogenesis. Clock and wavefront model exhibit a similar pattern of division as observed in Drosophila, like vertebrates, which emphasizes fundamental principles in the field of developmental biology. In contrast to Drosophila, the insect Tribolium castaneum exhibits posterior segmentation originating from the growth zone. The pair-rule genes' expression is characterized by oscillatory patterns, which are controlled by a segmentation clock. Researchers found that the speed regulation model combines oscillatory and sequential gene activities to shape both elongating and non-elongating embryonic tissues. The adaptability of this model is responsible for the evolutionary shift from short-germ to long-germ segmentation. The model demonstrates hierarchical gene activation and periodic expressions of pair-rule genes in Tribolium and the segmentation clock mechanism. The extensive research on segmentation processes among different species highlights the significance of principles in developmental biology.

DEDICATION

I want to dedicate my Master's thesis to my family and friends, whose constant support and guidance have played a critical role in helping me reach this significant milestone. Their unwavering love and encouragement have been instrumental in my academic success and have inspired me to push through the challenges I faced. This thesis is a testament to the values and principles they have instilled in me, and I am deeply grateful for all their sacrifices and efforts that have led me to this point.

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CHAPTER I

INTRODUCTION

Pattern formation in Drosophila melanogaster

The Drosophila melanogaster is an important model organism for genetics research. Their body consists of numerous distinct body segments. As it has a comparatively short reproductive cycle and a prolific birth rate, the fruit fly has emerged as a prime specimen for scientists studying insect evolutionary patterns. Around twenty-four hours after fertilization, their larva becomes visible quickly with clearly defined segments. Throughout the development of the embryo, the sequential activation of various gene types helps to form different segments. The genes produce the transcription factors, and they are involved in each step to regulate the synthesis of additional transcription factors that act on the subsequent set of genes. The mother's nurse cells deposit two distinct types of mRNA into the egg. The mRNAs are called Bicoid and Nanos. The mRNA encoding the Bicoid protein is localized at the egg's anterior pole, whereas the mRNA encoding the Nanos protein is localized at the posterior pole of the egg. The maternal genes Bicoid and Nanos determine the anterior and posterior ends of the embryo because their concentration gradients are opposite to each other. The genes responsible for the maternal effect are the initial genes in a sequence that will control the development of early patterns in the Drosophila embryo.

The Anterior-posterior fates in almost all insects are differentiated in two distinct phases (Davis & Patel, 2002). The developmental stages can be categorized into two phases: (i) the blastoderm, when the

anteroposterior (AP) axis remains constant in length, and (ii) the germband, when the AP axis elongates. Fates in short-germ insects can be determined during the development of the germband. On the other hand, in long-germ insects, for example, the fruit fly Drosophila melanogaster (Schroeder et al., 2011), Most of the developmental stages can be determined during the early stage of embryonic development, which is known as the blastoderm. Intermediate-germ insects exist between these two scenarios. The intermediate-germ insect Tribolium castaneum's germband is responsible for determining the abdominal fates (Patel et al., 1994).

Gap genes are responsible for regulating pair-rule genes. Inside the embryo, researchers found different types of expression patterns of pair-rule genes that divide the embryo into units composed of two body segments each. In contrast to the developmental process of the red flour beetle Tribolium castaneum, the patterning in the Drosophila blastoderm occurs concurrently. A substantial proportion of these genes encode transcription factors that control the expression of another group of genes called segment polarity genes. When the segment polarity genes are activated, they lead to the formation of a complex striped pattern. Ultimately, this acts as a pioneer to the segmented body of the adult fly's structure. Finally, in the cascade, the Hox genes has the accountability for specifying the arrangement and functions of each segment. The Hox genes encode a group of transcription factors, which are expressed in different combinations throughout the embryo. These Hox genes are involved in determining the destiny of cells within each segment.

Similarities between vertebrates and insects

All animals undergo embryogenesis during their development. In all animal species, Segmentation occurs along the anterior-posterior axis. Several distinct genes regulate this process. Both segmentation genes and homeotic genes play essential roles in the sequential formation of each segment. The embryonic development of insects and vertebrates exhibits significant

similarities despite their distinct anatomical differences. Somitogenesis is referred to as vertebrate segmentation. An elongating embryo carries out the somitogenesis process in a sequential manner. The researchers (Palmeirim et al., 1997) found that the presomitic mesoderm is responsible for the manifestation of periodic waves of genes. According to the clock and wavefront model (Cooke & Zeeman, 1976), the levels at which genes are expressed and move forward in the body become fixed in stationary patterns. After that, the patterns are defined as somites. The clock and wavefront model is essential for vertebrate segmentation. The model can be represented as a clock (indicating the oscillatory waves of genes) and a wavefront (illustrating the distinct stripes of gene expression). This procedure is analogous to the segmentation that takes place in insects.

Segmentation in Tribolium Castaneum

The research on short germ insect Tribolium castaneum is necessary since the development of Drosophila needs to represent the development of all insects accurately. There are several differences in the patterning of long and short-germ insects. An essential difference between short germs and long-germ insects exists in the anterior-posterior axis formation and the expression of gap genes and pair-rule genes. Long-germ insects establish the anterior-posterior axis during the blastoderm stage. The short germ insects specify the anterior-posterior axis during the germband stage. At the blastoderm stage, the initial patterns of gene expression in long-germ insects arise simultaneously in seven distinct stripes. In contrast, in short-germ insects, the gap gene and pair-rule gene patterns are observed in a series of waves that occur at consistent intervals within the posterior growth zone. Most other arthropods have a cellularized environment where the “growth zone” cells specify the posterior segments sequentially (Davis & Patel, 2002).

The morphological structures of Drosophila and other insects exhibit apparent differences. While the researchers found distinct types of maternal genes of Drosophila, Tribolium exhibits

posterior Nanos and caudal expression but lacks the bicoid gene. The researchers (El-Sherif et al., 2014) found that the expression of the caudal gene is moved towards the anterior part of the body rather than the presence of the bicoid gene. The caudal region in the tribolium not only contributes to posterior patterning but is also responsible for activating gap genes and pair-rule genes. In short germ insects, thresholds of morphogene gradients ultimately determine the various gene expressions. The pair-rule genes have responsibility for determining the segments. The even-skipped (eve), runt (run), and odd-skipped genes oscillate to facilitate the emergence of a striped pattern. This subsequently develops into a parasegment. After the process of invagination, the blastoderm changes and becomes an elongated embryo which is called the germband. The posterior end, which is called the growth zone, undergoes wave-like expansion. The germband follows the formation of the clock and wavefront model.

Both the pair-rule genes and the gap genes play an undoubtedly significant role in the development of parasegments in Tribolium. The pair-rule genes exhibit striped expression. These striped expression patterns indicate the embryo's segmentation. They receive further positional information from maternal and gap gene gradients (El-Sherif et al., 2014) . The gap genes are responsible for specifying the anterior and posterior developmental fates. The researchers state (Sarrazin et al., 2012) that the growth zone extends, which leads to the creation of a new segment and an increase in the length of the embryo.

The French Flag Model

The French Flag Model is essential in the development biology research. This model serves as a visual representation of positional information. Through the concentration-specific response of target genes, the signaling gradient is distributed throughout the area into a pattern of gene expression domains (Sharpe, 2019). One of Two important models for explaining this pattern formation is the French flag model. Lewis Wolpert devised this vital model in 1969 (Wolpert,

1969). Morphogens are utilized in the model. Turing was the first researcher to propose the concept of a morphogen. These morphogens are chemical substances responsible for morphogenesis (Vadde et al., 2020). Morphogen is defined as a signal that triggers specific responses in cells and determines various cell fates. They spread throughout the tissue, create a gradient of concentration, and provide positional information (Green & Sharpe, 2015). Morphogen gradients form within a tissue when they are released from an induction site and diffuse throughout the tissue (Dalessi et al., 2012). The researchers (Rivera-Pomar & Jäckle, 1996) found that maternal factors are responsible for initiating long-range morphogen gradients in Drosophila. Researcher Wolpert introduced the French flag problem, which explores the origin of complex patterns, like the blue, white, and red stripes on the French flag. One of his proposed solutions involved transmitting positional information through the concentration gradient of a morphogen. This morphogen or signaling molecule would be produced at a specific location called an organizing center and then spread throughout the tissue through a diffusion mechanism. In the French flag model, cells positioned below the gradient are determined by the threshold of morphogen gradients and finally acquire distinct fates (Vadde et al., 2020; Wolpert, 1969). This model shows that for the division of developing tissues, downstream genes interpret the threshold concentrations of morphogen gradients (Rogers & Schier, 2011; Wolpert, 1969). The concept has successfully helped researchers understand several critical developmental processes in embryos, including the anterior-posterior polarity's establishment in the Drosophila egg by the difference in the concentration gradient of bicoid protein (Driever & Nüsslein-Volhard, 1988b). The maternal bicoid gradient is widely recognized, extensively studied, and modeled in Drosophila by various researchers (Xie & Hu, 2016).

Pattern formation is a complex process. Two homeodomain proteins, Caudal (Cad) and Bicoid (Bcd), are involved in this process. The Bcd proteins facilitate the diffusion of mRNA translation products, and in this way, a long-range anterior-posterior (AP) gradient is formed finally. Thus, Bicoid activates the target genes, which induces the establishment of boundaries at predetermined positions along the AP axis. This situation ultimately results in the development of a segmented body plan (Driever & Nüsslein-Volhard, 1988a; Dubnau et al., 1997; Little et al., 2011; Niessing et al., 2002).

The neural tube in vertebrates is another example by which researchers can illustrate pattern formation mechanisms by morphogens. Though there are distinctions, pattern formation in both cases shows essential similarities. Both systems respond to the presence or absence of the morphogen by undergoing the loss of specific cell types and substantial changes and expansions in the identities of the cells that continue to be generated. The French Flag model is applicable to both systems. Because the model can operate on the fundamental assumption that there are certain concentration levels of the patterning signal that can be converted into positional information. The researchers found that there is a correlation between the concentrations of morphogens and the differential expressions of genes in both systems. Based on their research, researchers have proposed that morphogen gradients play an essential role in establishing the initial conditions for pattern formation in these tissues (Briscoe et al., 2000; Briscoe & Small, 2015; Vallstedt et al., 2005).

The Speed Regulation Model

The patterning of elongating tissues depends on the activation of genes in an oscillatory and sequential manner. This situation is very similar to the clock-and-wavefront mechanism. However, oscillatory and sequential gene expressions exist in the formation of different types of embryonic formation—these specific structures exhibit elongation, such as the AP axis of vertebrates and short-germ insects. The speed regulation model employs oscillatory and sequential gene activity

to pattern elongating and non-elongating embryonic structures (Zhu et al., 2017). In this model, the rate of sequential gene activation is controlled by the concentration of molecular factors. The scientists found that the gap genes are activated in a sequential manner in both the blastoderm and the germband of the beetle *Tribolium castaneum* (Zhu et al., 2017).

Next, the researchers found that arthropods can utilize two different methods of segmentation: one like Drosophila in the blastoderm and another clock-based segmentation in the germband. This report presents that the beetle Tribolium castaneum follows clock-based mechanism for segmentation during both the blastoderm and germband stages (Davis & Patel, 2002). They also found from experimental evidence that pair-rule genes in short germs and intermediate germs are interconnected in a segmentation clock. The rhythmic movements of this segmentation clock are arranged in spatial waves. The researchers found that the segmentation clock functions in both the germband (Sarrazin et al., 2012) and the blastoderm (El-Sherif et al., 2012) of the short-germ insect Tribolium castaneum. They also found that the even-skipped gene in the red flour beetle Tribolium castaneum, is expressed like gene expression waves from the posterior to the anterior part of the beetle's body. Eventually, It stops the formation of different segmented stripes. The researchers found that the presence of the segmentation clock was observed as stripe formation in the Tribolium embryo (El-Sherif et al., 2012, 2014).

Pattern formation in short-, intermediate and long-germ insects

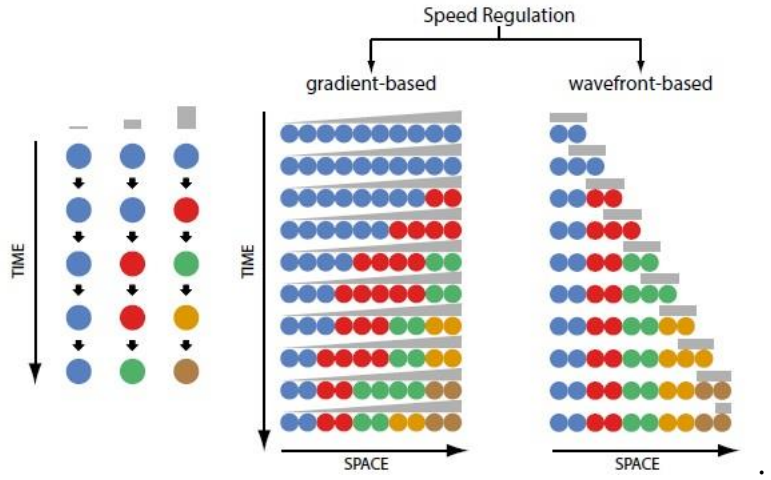

Figure 1: Pattern formation in insects: The picture displays different states with different colors, indicating the single gene expression and the simultaneous expression of multiple genes. The speed regulator regulates the rate of transition between states. Cells transit through progressing states at varying speeds, depending on the value of the speed regulator. This happens simultaneously (El-Sherif et al., 2014).

During the pattern formation, the cells are exposed to a constant gradient of the speed regulator. Gene expression waves propagate from higher regions to low-speed gradients, and the cells transition through successive states. However, they transit through lower when the speed gradient is lower. The gradient-based speed regulation leads to a clock-and-wavefront model when the gradient is very steep and moves back towards higher gradient levels. All cells initially start with the blue state. Cells migrate from the high peak value of the wavefront to the neutral value during the wavefront progression. Cells that are under the influence of the wavefront will transition to the next state. On the other hand, other remaining cells persist in the blue state. This procedure goes through until all the cells along the spatial axis go to distinct states. The researchers mentioned this mode of speed regulation "wavefront-based." When the researchers used oscillators for sequential processes, they used the same model to produce a recurring pattern, referred to as segments (Zhu et al., 2017). These Segments are generated sequentially over an extended period, going through cellular division and reconfiguration (Brown et al., 1994) .

In insects, after a sequence of divisions, the nuclei migrate toward the outer edge, where they ultimately create blastoderm. The initial stage of the embryo starts to elongate, which leads to the development of a germband. The researchers classified the three main classes of embryogenesis based on the number of fates (Sarrazin et al., 2012). The scientists found that in long-germ insects, all segments are specified simultaneously within the blastoderm (Davis & Patel, 2002). The fate map of blastoderm shows the complete blueprint of the future body segments (Hartenstein & Campos-Ortega, 1985). However, at the blastoderm stage of short-germ and intermediate embryonic development, only the insect's anterior segments are initially determined. During the secondary growth phase, The researchers found that the posterior region of the germband undergoes significant elongation (Liu & Kaufman, 2005). This leads to the development of posterior segments and their final elongation.

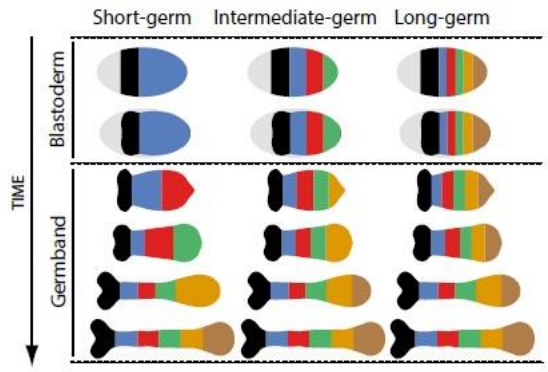

Figure 2: The early stages of insect development: The early stages of development involve two distinct phases: blastoderm and germband. These stages are responsible for determining the anterior and posterior fates (El-Sherif et al., 2014).

In short-germ insects, most cell fates are determined during the germband stage (Figure 2). On the other hand, in long-germ insects, cell fates are determined during the blastoderm stage. The insects classified as intermediate germs are defined between the two extreme cases depicted in Figure 2. Through the speed regulation model, both gradient-based (Fig. 1, Left) and wavefront-

based (Fig. 1B, Right) are patterned in insects. In this way, this model allows for the transformation of short germs into long germs during the evolution process. In the short-germ insect, the determination of AP fates occurs in a wavefront-based manner during the germband stage. Thus, the initial few cell fates spread throughout the blastoderm in a gradient-based manner. After the blastoderm stage, the germband occurs in a wavefront-based manner.

In situ HCR

HCR, or hybridization chain reaction, involves the initiation of a hybridization, which leads to the polymerization of oligonucleotides into long double-stranded DNA molecules. This is an amplification system that uses nucleic acids to identify specific sequences of RNA or DNA. The amplifiers are called DNA hairpin molecules, which are polymerized, and they are dependent on the existence of particular DNA sequences, which are called initiators. Before the hybridization with a DNA probe to a target molecule, one or more initiator sequences must be attached to the probe. So, after the incubation with the DNA hairpin amplifiers, polymerization occurs. This results in the buildup of a label at the location where the probe is attached. The amplification and detection reaction's rapidity can be conducted under mild conditions (Zhuang et al., 2020).

The researchers (Ikbal et al., 2015) found that HCR can effectively amplify signals without the need for enzymes while maintaining a constant temperature. The HCR amplifier consists of two distinct DNA hairpins (H1 and H2), and they are kinetically trapped, as shown in Figure 3. They trigger a specific self-assembly process when they face a matching DNA initiator sequence (I1) (H. M. T. Choi et al., 2014; Dirks & Pierce, 2004; Lindstrom et al., 2023). The initiator I1 hybridizes with the hairpin H1's input domain and causes the hairpin to unfold and reveal its output domain. After that, this goes for hybridization with the input region of hairpin H2 and causes the output

region of H2 to get exposed. This establishes the basis, which forms a series of interconnected reactions. This involves the formation of polymers by the alternating steps between H1 and H2 (H. M. T. Choi et al., 2018).

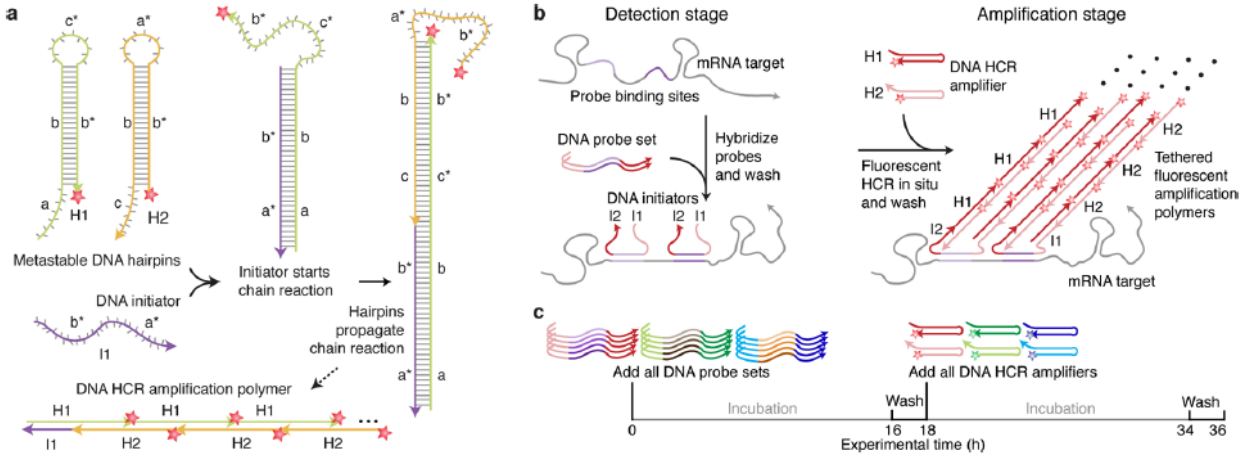

Figure 3: In situ hybridization using HCR in situ amplification (H. M. T. Choi et al., 2014).

In ISH, the initiator I1 establishes a nucleation complex with the hairpin H1. Subsequently, this intricate compound facilitates the process called branch migration, which leads to the hairpin unfolding and transforming into a more complicated configuration referred to as I1- H1. Within this complex, there is a segment labeled "c*-b*" (Figure 3) that consists of a single strand. The hairpin H2 undergoes base pairing with the "c", resulting in the nucleation of this complex. Thus, this binding enables the movement of branches. This causes the hairpin to open and goes for the formation of the complex I1-H1-H2. A constituent of this intricate structure is the unpaired portion "b*-a*." This leads to the re-establishment of the initiator sequence, which forms the basis for a cascade of events involving successive stages of polymerization of H1 and H2 molecules. When the initiator arrives, a cascade of events is initiated. The cascade events involve the sequential formation and unfolding of H1 and H2 hairpins. These events lead to the creation of a lengthy double-stranded polymer with nicks in it, which is amplified (J. Choi et al., 2011). Fluorophores,

represented as red stars, symbolize the in-situ hybridization process. During the detection stage, probe sets are used to hybridize with mRNA targets to form a binding. In the amplification stage, initiators are activated to induce the spontaneous formation of fluorescent amplification polymers. This stage is mentioned as the amplification stage.

HCR signal amplification has been devised for a diverse range of technological applications in different types of laboratory settings (H. M. T. Choi et al., 2014) and used for a wide range of output signals. These signals include fluorescence (J. Choi et al., 2011). HCR can identify different initiator sequences and execute their tasks autonomously (H. M. T. Choi et al., 2014). Although in situ hybridization chain reaction (HCR) possesses several technical advantages, it has yet to be widely adopted as the standard method for mRNA detection. This is because in situ HCR utilizes DNA hairpins that are costly and exceed that of conventional ISH methods.

CHAPTER II

EXPERIMENTAL

Materials and Methods

Preparation of Rearing Flour (RF) for Stock Keeping and Resting Adult Beetles

Composition: 1 kg of whole grain flour was mixed with 50g of yeast powder.

Preparation of Egg-Collection Flour (EF)

Composition: 1 kg of white flour with 50g of yeast powder was mixed

Reagents and Solutions

30% probe hybridization buffer (for 40mL)

30% formamide (12mL) , 5x SSC (10mL of 20X) . 9mM citric acid pH 6 (720uL of 0.5M) , 0.1% Tween20 (40uL), 50ug/mL heparin (200uL of 10mg/mL) , 1x Denhardt's solution (800uL of 50X) , 5% dextran sulphate (4mL 50%). 40mL was filled with MQ water.

30% probe wash buffer (for 40mL)

30% formamide (12mL), 5xSSC (10mL of 20X) , 9mM citric acid pH 6 (720uL of 0.5M), 0.1% Tween20 (40uL) , 50ug/mL heparin (200uL of 10mg/mL heparin), Fill to 40mL with MQ water.

Amplification buffer (for 40mL)

5X SSC (10mL of 20X), 0.1% Tween20 (40uL), 5% dextran sulphate (4mL of 50%), The solution was filled to 40mL with MQ water.

5X SSCT (for 40mL)

5X SSC (10mL of 20X), 0.1% Tween20 (40uL), 40mL was filled with MQ water.

50% dextran sulphate (for 40mL)

20g dextran sulphate powder. 40mL was filled with MQ water.

PBT

1x PBS, 0.1 % Tween20

Tribolium Embryo Fixation

In a glass scintillation vial, fixative (4 ml 1x PBS and 600 µl 37 % formaldehyde) and 5 mL of heptane were combined and mixed well. The mixture was separated into two phases. In the mixture, the fixative was on the bottom. Embryos were placed in a mesh basket (modified 50-mL tube) and immersed in a beaker containing 50% bleach, moving the basket up and down for ~2 min. When dechorionated, the embryos floated on the surface. The embryos were rinsed well with water for ~1 minute to remove all traces of bleach and move them to the center of the mesh. The basket was disassembled, and the mesh was blotted on a paper towel to remove excess water. Using forceps, the mesh was grabbed and submerged in the heptane layer of the previously prepared vial from Step 1. The eggs fell off the mesh and were moved to the fixative/heptane interface. If eggs were stuck to the mesh, they were transferred gently with a paintbrush. The vial was attached to a platform rocker and agitated at 250 rpm for 1 hour. The bottom layer (fixative) was removed using a Pasteur pipette, 5 mL of methanol was added, and the vial was quickly shaken by hand for 30 sec. Devitellinized eggs (usually young embryos) sunk to the bottom. The fixative was disposed of in an appropriate hazardous waste container. Sunk embryos were transferred to a clean collection tube. Still, vitellinized embryos were passed through a 20-gauge 1.5-in. needle which was attached to a 1-mL syringe. This helped devitellinized older embryos and separate germband from yolk cells. Thus, Devitellinized (germband) embryos sunk to the bottom. Sunk embryos were transferred to a clean

collection tube. The steps were repeated until most eggs were devitellinized. Heptane and methanol were disposed of in an appropriate hazardous waste container. Embryos were washed in a collection tube three times for five minutes each with Methanol and stored in Methanol at -20°C.

Sample preparation

Embryos were washed in 50% MeOH/ 50% PBT for 5 min. After that, they were washed in PBT 3x5 min. Then again, they were washed in PBT 3x30 min. Then, the embryos were post-fixed by washing in 4% formaldehyde in PBT (891 µl PBT + 108 µl 37% formaldehyde) for 30 min. They were washed in PBT 3x5 min.

Detection stage

The embryos were prehybridized in 100 µl of 30% probe hybridization buffer, incubating them for 30 minutes at 37 °C. In parallel, the probe solution was prepared by mixing 0.8 µl of a 1 µM stock solution into 3.2 µl of 30% probe hybridization buffer for each probe of each probe into 100 µl of 30% probe hybridization buffer. Then, the probe solution was warmed at 37 °C for 30 minutes. After the prehybridization period, the prehybridization solution was discarded and replaced with the prepared probe solution. The samples were then incubated overnight at 37 °C. 100 µl 30% probe wash buffer was washed (preheated) at 37 °C for 4x15 min. Samples were washed for 3x5 min in 5x SSCT at room temperature (RT). Meanwhile, amplifiers were prepared (see amplification stage).

Amplification stage

To prepare 30 pmol of each fluorescently labeled hairpin, snap cooling was begun for 2 µl of a 3 µM stock solution. To do this, 2 µl of H1 and H2 hairpin solutions for each linker (B1, B2, etc.) in PCR tubes were first thawed. Then, the solutions were heated in a thermocycler at 95 °C

for 90 seconds. Following heating, the solutions were allowed to cool to room temperature in the dark for 30 minutes. Then, the embryos were preamplified in 100 µl amplification buffer for 30 min at Room temperature. Next, the hairpin solution was prepared by adding all snap-cooled hairpins to 100 µl amplification buffer at Room temperature. The preamplification solution was removed, and the hairpin solution was added. Then, the samples were incubated overnight in the dark at Room temperature. The excess hairpins were removed by washing with 5x SSCT at RT for 2x5 min, 2x 30 min and 1x 5 min, and embryos were stored in slowfade gold.

CHAPTER III

RESULTS

Following three hours of egg laying, the eggs were subsequently placed in an incubator set at a temperature of 24 degrees Celsius. The eggs remained in the incubator until they reached incubation times (ITs) ranging from 14h to 23h, with increments of three hours. After that, the eggs were conserved through fixation and subsequently stored in a freezer. The HCR (Hybridization Chain Reaction) technique was used to amplify and detect RNA molecules and was then employed to stain the embryos. Embryos were stained for each IT, and mRNA staining was performed for the pair-rule genes of the three observed genes. The embryos were analyzed under a confocal microscope to gain insight into the staining process. Figure 4 displays the captured images for each Incubation Timed (IT) and the observed genes (eve, run, and odd) related to pair-rule genes. The imaging process commenced at the IT14 position for the gene types. Prior to this, no detectable gene expression pattern was seen in the experiment.

Pair rule Gene expression pattern formation

In this experiment, several ITs were analyzed. The embryos were categorized into two main stages. The blastoderm stage represents the initial phase of embryonic development. The final day of IT23 was the last day of the blastoderm stage. During the blastoderm stage, each of the pair-rule genes produced three distinct stripes that were only observable on the entire egg. Subsequently, the germband stage commenced. Additional stripes emerged on the embryo during the germband

stage, like the ones observed in the blastoderm. An analysis of the comparison between blastoderm and germband (Figure 4) unveiled a consistent expression pattern that undergoes a transition from blastoderm to germband.

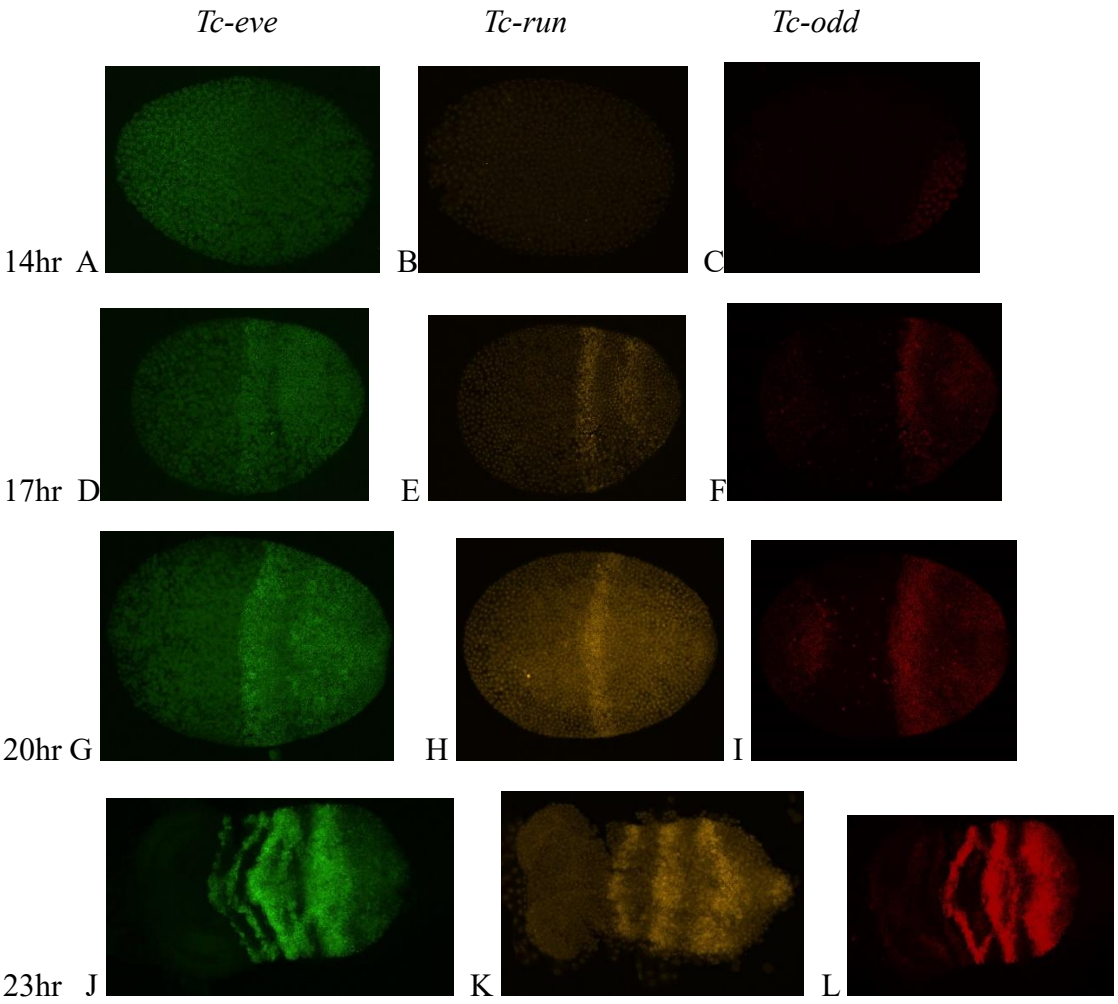

Figure 4: Expression of pair-rule genes eve, run, and odd in Tribolium. The genes are displayed in colors green: eve, yellow: run, red: odd. Posterior to the right. Blastoderm stages are from 14 to 20 h (A-I), and the germband stage is during 23h (J-L) after egg laying.

Figure 4, panels A-C, demonstrate that the signals eve was observed initially. The run and odd genes were only detected at the posterior end towards the end of the process. After three hours (IT17) panels D-F, the wavefronts resulting from the expression of all three genes exhibited a consistent pattern, which ultimately formed three distinct stripes. Based on the figure and evidence,

it can be found that Eve was the earliest and most anterior 1st expression of the stripe. The second stripe run was found to create a stripe that aligns directly with the posterior edge of the first-eve gene pattern. Moreover, the third one, the odd gene, was distinctive and ultimately appeared as a stripe right after the first two gene expressions. In the figure, the expression of Eve and Odd ensures that they do not coincide with each other.

Figure 4D-F clearly illustrates the emergence of a new wavefront in the Eve gene. The appearance of its initial stripe characterizes this pattern. Eve and odd genes do not intersect, and This phenomenon is clearly visible in Figure 1 G and I, where it is clearly seen that a new odd genes wave did not emerge until the eve expression had disappeared entirely from the posterior end and moved towards the anterior to form a distinct band. After approximately twenty-three hours, the embryos had progressed to the germband stage. The presence of typical embryo features, such as head structures and a symmetrical, elongated body distinguishes this stage.

During this stage, a fourth wavefront of eve was observed, which maintained expression levels like those seen in the blastoderm stage. Furthermore, the expressions emerged as wavefronts in the posterior part of the growth zone, and subsequently, they developed into sparsely distributed stripes at the anterior of the growth zone. Moreover, the embryo exhibited an increase in length as additional stripes formed along the anterior-posterior axis. The initial stripe on the Eve gene started to divide into two narrower stripes (Fig.4 J: 1), whereas the three-run and odd stripes remained singular. Every evolved stripe of Eve genes separated among their stripes apart one after another.

CHAPTER IV

DISCUSSION

It was believed that the development of segmentation was attributed to two distinct systems. The first one refers to an expression that relies on information received from nearby cells or morphogen gradients. This situation influences the surrounding cells. Scientist Wolpert (1969) proposed that the French Flag model executes this situation in nonelongating tissue. The cells' impact on the fate of their neighboring cells is determined by a concentration gradient that is dependent on the local threshold of the slope. This model is more common in non-elongating tissues, such as the blastoderm in Tribolium than in elongating tissues. There is another system that is applicable to elongating tissues. Each cell rhythmically exhibits synchronized gene expression waves, which resemble the movement of a segmented clock or clock-like pattern. The elongated tissues of the germband in Tribolium can be demonstrated through this model.

The pair-rule genes show periodic expressions in a consecutive set of genes. In this research, Eve consistently appeared as the initial expression. After that, we detected "run" and, finally, "odd" genes. The pair-rule genes demonstrate oscillatory expressions and are arranged in a hierarchical sequence of genes. Eve consistently precedes run. On the other hand, odd consistently follows as the final expression. Due to this hierarchical arrangement, a pattern composed of three stripes reoccurs consistently. The researchers found that Eve consistently takes the lead in the cascade and acts as an activator for the pair-rule cascade. When the gene expression goes on, Eve and Odd are seen as indistinguishable counterparts. Both genes' expressions were clearly visible without overlapping each other in this research. In Figure 4D/F, it was seen that the unusual wave has

reached the anterior end of the growth zone and began the formation of a stationary stripe. Simultaneously, the eve expression is starting to appear from the growth zone's posterior end. Once the eve expression has passed the posterior pole, the odd wave begins to emerge from the growth zone, as depicted in Figure 4 G/I. This explanation elucidates a gene-activating cascade, wherein Eve initiates the activation of the run expression. This situation subsequently triggers odd gene's activation, which activates Eve. This situation prompts the gene to move once again. The hierarchy produces a recurring arrangement of three stripes.

One characteristic of the expression pattern of the pair-rule gene is the conversion of dynamic wave expressions into static stripes. This result considers both the French Flag model and a clock-based segmentation. During the initial stage of development, known as the blastoderm stage, the embryo does not commence the process of elongation. However, the waves of the forthcoming pair-rule expression exhibited a distinct and well-structured striped pattern. Although the expression pattern remained consistent during the germband stage, it is evident by the researchers that the pair-rule genes are self-regulating in an oscillatory fashion, guided by a segmentation clock. Due to the consistent expression pattern between the germband and the blastoderm, the segmentation clock operates continuously throughout both stages.

REFERENCES

Briscoe, J., Pierani, A., Jessell, T. M., & Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell*, *101*(4), 435–445. https://doi.org/10.1016/S0092-8674(00)80853-3

Briscoe, J., & Small, S. (2015). Morphogen rules: design principles of gradient-mediated embryo patterning. *Development*, *142*(23), 3996–4009. https://doi.org/10.1242/DEV.129452

Brown, S. J., Parrish, J. K., & Denell, R. E. (1994). Genetic Control of Early Embryogenesis in the Red Flour Beetle, Tribolium castaneum. *Integrative and Comparative Biology*, *34*(3), 343–352. https://doi.org/10.1093/ICB/34.3.343

Choi, H. M. T., Beck, V. A., & Pierce, N. A. (2014). Next-generation in situ hybridization chain reaction: Higher gain, lower cost, greater durability. *ACS Nano*, *8*(5), 4284–4294. https://doi.org/10.1021/NN405717P/SUPPL_FILE/NN405717P_SI_002.AVI

Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., Cunha, A., & Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction: Multiplexed, quantitative, sensitive, versatile, robust. *Development (Cambridge)*, *145*(12). https://doi.org/10.1242/DEV.165753/48466

Choi, J., Routenberg Love, K., Gong, Y., Gierahn, T. M., & Love, J. C. (2011). Immuno-hybridization chain reaction for enhancing detection of individual cytokine-secreting human peripheral mononuclear cells. *Analytical Chemistry*, *83*(17), 6890–6895. https://doi.org/10.1021/AC2013916/SUPPL_FILE/AC2013916_SI_001.PDF

Cooke, J., & Zeeman, E. C. (1976). A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. *Journal of Theoretical Biology*, *58*(2), 455–476. https://doi.org/10.1016/S0022-5193(76)80131-2

Dalessi, S., Neves, A., & Bergmann, S. (2012). Modeling morphogen gradient formation from arbitrary realistically shaped sources. *Journal of Theoretical Biology*, *294*, 130–138. https://doi.org/10.1016/J.JTBI.2011.10.014

Davis, G. K., & Patel, N. H. (2002). Short, long, and beyond: Molecular and embryological approaches to insect segmentation. *Annual Review of Entomology*, *47*(Volume 47, 2002), 669–699. https://doi.org/10.1146/ANNUREV.ENTO.47.091201.145251/CITE/REFWORKS

Dirks, R. M., & Pierce, N. A. (2004). Triggered amplification by hybridization chain reaction. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(43), 15275–15278. https://doi.org/10.1073/PNAS.0407024101/ASSET/B86174D6-86E5-49CA-8212-4BA2A737F118/ASSETS/GRAPHIC/ZPQ0450463730002.JPEG

Driever, W., & Nüsslein-Volhard, C. (1988a). A gradient of bicoid protein in Drosophila embryos. *Cell*, *54*(1), 83–93. https://doi.org/10.1016/0092-8674(88)90182-1

Driever, W., & Nüsslein-Volhard, C. (1988b). The bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner. *Cell*, *54*(1), 95–104. https://doi.org/10.1016/0092-8674(88)90183-3

Dubnau, J., Struhl, G., Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W. J., Jäckle, H., Treisman, J., Gönczy, P., Vashishtha, M., Harris, E., Desplan, C., Hanes, S. D., Riddihough, G., Ish-Horowicz, D., & Brent, R. (1997). Sequence-specific RNA binding by Bicoid. *Nature 1997 388:6643*, *388*(6643), 634–634. https://doi.org/10.1038/41692

El-Sherif, E., Averof, M., & Brown, S. J. (2012). A segmentation clock operating in blastoderm and germband stages of Tribolium development. *Development*, *139*(23), 4341–4346. https://doi.org/10.1242/DEV.085126

El-Sherif, E., Zhu, X., Fu, J., & Brown, S. J. (2014). Caudal Regulates the Spatiotemporal Dynamics of Pair-Rule Waves in Tribolium. *PLOS Genetics*, *10*(10), e1004677. https://doi.org/10.1371/JOURNAL.PGEN.1004677

Green, J. B. A., & Sharpe, J. (2015). Positional information and reaction-diffusion: two big ideas in developmental biology combine. *Development*, *142*(7), 1203–1211. https://doi.org/10.1242/DEV.114991

Hartenstein, V., & Campos-Ortega, J. A. (1985). Fate-mapping in wild-type Drosophila melanogaster - I. The spatio-temporal pattern of embryonic cell divisions. *Wilhelm Roux's Archives of Developmental Biology*, *194*(4), 181–195. https://doi.org/10.1007/BF00848246/METRICS

Ikbal, J., Lim, G. S., & Gao, Z. (2015). The hybridization chain reaction in the development of ultrasensitive nucleic acid assays. *TrAC Trends in Analytical Chemistry*, *64*, 86–99. https://doi.org/10.1016/J.TRAC.2014.08.014

Lindstrom, M. R., Nyanney, E., Inam Chowdhury, J., & Dave Philips. (2023). *Ice in the rough: isolating neutron scattering intensities of ice*. https://doi.org/10.17605/OSF.IO/46SGM

Little, S. C., Tkačik, G., Kneeland, T. B., Wieschaus, E. F., & Gregor, T. (2011). The Formation of the Bicoid Morphogen Gradient Requires Protein Movement from Anteriorly Localized mRNA. *PLOS Biology*, *9*(3), e1000596. https://doi.org/10.1371/JOURNAL.PBIO.1000596

Liu, P. Z., & Kaufman, T. C. (2005). even-skipped is not a pair-rule gene but has segmental and gap-like functions in Oncopeltus fasciatus, an intermediate germband insect. *Development*, *132*(9), 2081–2092. https://doi.org/10.1242/DEV.01807

Niessing, D., Blanke, S., & Jäckle, H. (2002). Bicoid associates with the 5′-cap-bound complex of caudal mRNA and represses translation. *Genes & Development*, *16*(19), 2576–2582. https://doi.org/10.1101/GAD.240002

Palmeirim, I., Henrique, D., Ish-Horowicz, D., & Pourquié, O. (1997). Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell*, *91*(5), 639–648. https://doi.org/10.1016/S0092-8674(00)80451-1

Patel, N. H., Condron, B. G., & Zinn, K. (1994). Pair-rule expression patterns of even-skipped are found in both short- and long-germ beetles. *Nature 1994 367:6462*, *367*(6462), 429–434. https://doi.org/10.1038/367429a0

Rivera-Pomar, R., & Jäckle, H. (1996). From gradients to stripes in Drosophila embryogenesis: filling in the gaps. *Trends in Genetics*, *12*(11), 478–483. https://doi.org/10.1016/0168-9525(96)10044-5

Rogers, K. W., & Schier, A. F. (2011). Morphogen gradients: From generation to interpretation. *Annual Review of Cell and Developmental Biology*, *27*(Volume 27, 2011), 377–407. https://doi.org/10.1146/ANNUREV-CELLBIO-092910-154148/1

Sarrazin, A. F., Peel, A. D., & Averof, M. (2012). A Segmentation Clock with Two-Segment Periodicity in Insects. *Science*, *336*(6079), 338–341. https://doi.org/10.1126/science.1218256

Schroeder, M. D., Greer, C., & Gaul, U. (2011). How to make stripes: deciphering the transition from non-periodic to periodic patterns in Drosophila segmentation. *Development*, *138*(14), 3067–3078. https://doi.org/10.1242/DEV.062141

Sharpe, J. (2019). Wolpert's French flag: What's the problem? *Development (Cambridge)*, *146*(24). https://doi.org/10.1242/DEV.185967/223052

Vadde, B. V. L., Roeder, A. H. K., & Wellmer, F. (2020). Can the French flag and reaction–diffusion models explain flower patterning? Celebrating the 50th anniversary of the French flag model. *Journal of Experimental Botany*, *71*(10), 2886–2897. https://doi.org/10.1093/JXB/ERAA065

Vallstedt, A., Klos, J. M., & Ericson, J. (2005). Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. *Neuron*, *45*(1), 55–67. https://doi.org/10.1016/j.neuron.2004.12.026

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *Journal of Theoretical Biology*, *25*(1), 1–47. https://doi.org/10.1016/S0022-5193(69)80016-0

Xie, J., & Hu, G.-H. (2016). Hydrodynamic modeling of Bicoid morphogen gradient formation in Drosophila embryo. *Biomechanics and Modeling in Mechanobiology*, *15*, 1765–1773. https://doi.org/10.1007/s10237-016-0796-z

Zhu, X., Rudolf, H., Healey, L., François, P., Brown, S. J., Klingler, M., & El-Sherif, E. (2017). Speed regulation of genetic cascades allows for evolvability in the body plan specification of insects. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(41), E8646–E8655. https://doi.org/10.1073/PNAS.1702478114/SUPPL_FILE/PNAS.1702478114.SM10.MP4

Zhuang, P., Zhang, H., Welchko, R. M., Thompson, R. C., Xu, S., & Turner, D. L. (2020). Combined microRNA and mRNA detection in mammalian retinas by in situ hybridization chain reaction. *Scientific Reports 2020 10:1*, *10*(1), 1–10. https://doi.org/10.1038/s41598-019-57194-0

VITA

Hasan Mahmud graduated from Shahjalal University of Science and Technology, Bangladesh, with a Bachelor of Science (B.Sc.) and Master of Science in Biochemistry and Molecular Biology in 2016 and 2018, respectively. He started his graduate studies in the Program of Biochemistry and Molecular Biology at the University of Texas Rio Grande Valley (UTRGV) in January 2023 under the supervision of Dr. Ezzat El-Sherif. He was awarded the Master of Science in Biochemistry and Molecular Biology from the University of Texas Rio Grande Valley in August of 2024. His research interests include Immunology, virology, cancer biology, and developmental biology. Hasan was the recipient of the Graduate Teaching Assistantship and the BCMB Graduate Scholarship at UTRGV. He is starting his Ph.D. in Biomedical Science at the University of Nebraska Medical Center in the Fall of 2024. He can be reached at hmahmud.bmb@gmail.com