# Identifying Novel Pair-Rule Genes in *Oncopeltus fasciatus*

Honors Thesis
Department of Cellular Biology and Molecular Genetics

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**Abstract**

Pair-rule genes encode transcription factors that play a significant role in the development of segments during the embryogenesis of insects. In *Drosophila melanogaster*, nine pair-rule genes have been identified. Although many developmental studies have focused on *Drosophila*, the establishment of segments that form sequentially after gastrulation in *Oncopeltus fasciatus*, milkweed bug, make it an important representative model to study. Earlier studies demonstrated that orthologs of the nine *Drosophila* pair-rule genes had no pair-rule like expression in *Oncopeltus*. The discovery of a pair-rule gene, *Ofas-E75A*, in *Oncopeltus* paved the way to looking for more pair-rule genes in this organism. We investigated whether a set of candidate genes exhibit pair-rule like expression, similar to *Ofas-E75A*, in *Oncopeltus*. These genes were selected based on their coexpression with *Ofas-E75A*. The expression patterns of these genes were assessed by synthesizing an antisense RNA probe, labeled with digoxigenin, targeting the gene of interest, then performing an in-situ hybridization using the RNA probe. Thus far, the expression patterns of two of the ten candidate genes have been examined. *Ofas-000545* displayed staining in the thoracic segments and *Ofas-000627* displayed expression along the midline in *Oncopeltus*. Neither displayed pair rule-like expression patterns. Identification of the expression patterns of the remaining eight genes is in progress. Whether they display pair-rule like expression patterns remains to be determined. If a gene is found to have a pair-rule like expression pattern, parental RNAi can be used to determine the function of the gene.

**Introduction**

During embryogenesis in the model insect, *Drosophila melanogaster*, three groups of genes are involved in the proper pattern formation of body segments along the anterior to posterior axis. These genes were identified through genetic screens for embryonic lethal mutants (Nüsslein-Volhard and Wieschaus, 1980). The first set of genes are the maternal-effect genes, the second set are the segmentation genes, and the third set are the homeotic genes. The maternal-effect genes encode proteins that establish concentration gradients across the embryo even before fertilization. The expression of segmentation genes is controlled by the maternal-effect genes. The segmentation genes control the development of segments, repeated regions of the body that make up specific structures. The three main classes of segmentation genes are gap genes, pair-rule genes, and segment polarity genes. Gap genes are expressed early in development in broad regions. Mutations in gap genes lead to loss of several contiguous segments. While the maternal-effect genes have already defined the anterior and posterior ends of the embryo, the gap genes further subdivide the embryo into regions such as the head, thorax and parts of the abdomen. The transcription factors that are encoded by the maternal and gap genes control the expression of pair-rule genes.

The pair-rule genes are necessary for the formation of individual segments. These genes are expressed in an alternating pattern in the primordia of alternate segmental units. The discovery of pair-rule genes suggested that the embryos of *Drosophila* have a repeat unit corresponding to two segments, which are subdivided into individual segments at a later time (Nüsslein-Volhard and Wieschaus, 1980). Blastoderms are organized into parasegments by pair-rule genes. Mutants of the pair-rule genes displayed pattern deletions in every other segment.

Each of these mutants showed deletions that were skipping specific elements in the even- and odd-numbered denticle bands or showed pairwise fusions of the denticle bands (Figure 1). All pair-rule genes encode transcription factors that are expressed in regions that correspond to the gene's domain of function. "During segmentation, the pair-rule genes act as intermediates between the nonperiodic expression of gap genes and the repeated expression patterns of the segment-polarity genes" (Carroll, 1990). The segment-polarity genes create polarity within individual segments. The expression of these genes establishes the anterior and posterior parts of each individual segment. The homeotic genes, controlled by the expression of the segmentation genes, determine the development of the segments into specific body parts of the *Drosophila* (Reviewed in Lewis, 1998).

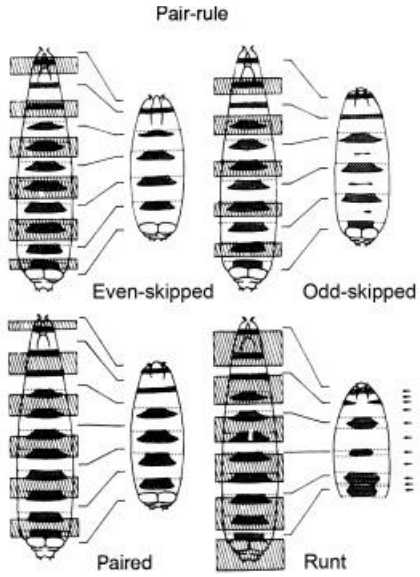


Figure 1. Pattern deletions in an embryo homozygous mutant for pair-rule genes.
The *even-skipped, odd-skipped, paired* and *runt* pair-rule genes' pattern deletions were displayed. (Figure adapted from Nüsslein-Volhard and Wieschaus, 1980).

The model organism I am using for my Thesis work is *Oncopeltus fasciatus*, commonly known as milkweed bugs. *Oncopeltus* are a good model system because they are easy to culture as they only require sunflower seeds and water for maintenance and reproduction. *Oncopeltus* adults live for approximately thirty days and their eggs hatch in about seven days (Feir, 1974). The adults and eggs are handled easily as they are rather sizeable. RNA in-situ hybridization and RNA interference (RNAi) are effective techniques to study gene regulation and function in *Oncopeltus* (Liu et al., 2009).

*Oncopeltus* belong to the order Hemiptera, a group of hemimetabolous insects that are classified by the fact that there is no pupal stage in the metamorphosis. *Oncopeltus* are a useful model system because of their phylogenetic position. "They are a good outgroup for holometabolous insects, positioned as the sister group for holometabolous insects" (Kristensen et al. 1991). *Drosophila and Tribolium castaneum* are holometabolous insects. They have been studied much more extensively for molecular and developmental biology than hemimetabolous insects. Therefore, studying hemimetabolous insects is useful in understanding more about ancestral development and gene regulation.

Furthermore, *Oncopeltus* are a good model system to study development because most of their segments form sequentially after gastrulation. The segmented body plans of insects are

conserved. The number of segments that have been specified by the end of the blastoderm stage, determines the classification of the insect as having a short, intermediate or long germ band development (Sander, 1976). In the long germ band insects, such as *Drosophila,* all the segments are specified in the blastoderm stage.

For the intermediate and short germ band insects, the anterior segments are specified in the blastoderm stage. Then, the posterior segments are added one-by-one from the anterior to posterior end. The posterior segments develop from the segment addition zone (SAZ) (Sander, 1976). *Oncopeltus* have intermediate germband development (Liu et al. 2009). Intermediate germband development means that some of the segments, such as the mandibular, maxillary, labial, thoracic one, thoracic two and thoracic three segments are specified in the blastoderm and the rest are added sequentially from the SAZ as the germband elongates.

The development of *Oncopeltus* embryos has been classified into four stages. The first stage is known as early embryogenesis. The male and female pronuclei fuse near the middle of the yolk mass inside the egg. This is during the first thirty minutes after the eggs are laid (AEL). Following this, the nuclei undergo several rounds of division without cell division. At ~15 hours AEL, the nuclei and surrounding cytoplasm migrate to the outside of the egg. At ~17 hours AEL, the nuclei divide, and the cell membrane forms. This leads to the process of the blastoderm becoming established (Butt, 1949).

The stage of blastoderm formation begins when cell numbers are growing in the blastoderm embryo. Approximately 24 hours AEL, there is a group of cells that develop at the posterior end of the egg. Roughly six hours later, the segment of the blastoderm that is on the ventral side of the egg thickens and the part on the dorsal side begins to thin out. The thickened side of the blastoderm will develop into the germband, the group of germ band layers that form during gastrulation in insects, and the thinner side will develop into the serosa. The mitotic figures have developed two hours later, and the cell numbers continue to increase (Butt, 1949). Towards the end of the blastoderm stage, approximately 34-36 hours AEL, six segments of the *Oncopeltus* body plan have become specified (Liu et al. 2009). These segments are the mandibular, maxillary, labial, thoracic one, thoracic two and thoracic three segments. These constitute the anterior segments, while the remaining segments are added later in development.

The next stage is the germband invagination. Approximately 35 hours AEL, there is the formation of a slight invagination that increases in size. This leads to the formation of a significant invagination in the germband (Butt, 1949).

The final stage of the development of the *Oncopeltus* embryo is germband extension. Following the germband invagination, the germband begins to extend. At this point, the tip of the germband finally reaches the anterior end of the egg and the head of the germband stage embryo reaches the posterior end of the egg (Butt, 1949).

In *Oncopeltus*, genes that are involved in pair rule-like patterning are expected to be expressed first at the blastoderm stage, then throughout germband elongation as the abdominal segments are added. Pair-rule like expression was displayed for the gene *E75A* (Figure 2). After RNAi targeting *E75A*, the neighboring segments of the *Oncopeltus* embryos fused, showing pair-rule like pre-patterning mechanisms (Erezylimaz et al., 2009). Pair-rule expression is defined as stripes of gene expression in the primordia of alternate segmental units. Previous work from our lab asked whether orthologs of the *Drosophila* pair-rule genes also play a role in pair-rule patterning in *Oncopeltus* (Reding et al., 2019). They found that only one of these nine genes

displayed an expression pattern that was pair-rule like, *Ofas-runt* (Reding et al., 2019) (Figure 3). The orthologs of other *Drosophila* pair-rule genes (*Ofas-even-skipped*, *Ofas-ftz-f1*, *Ofas-hairy*, *Ofas-odd-skipped* (*odd)*, *Ofas-odd-paired*, *Ofas-paired*, and *Ofas-sloppy-paired*) have segmental-like expression (Reding et al., 2019) (Figure 3). Many of the *Drosophila* pair-rule gene orthologs (*even-skipped, ftz, hairy, odd, odd-paired,* and *runt*) also show pair-rule expression in the Hymenoptera and Coleoptera orders (Figure 3). These two orders, along with the Diptera order, the order which includes *Drosophila,* form a monophyletic group with each other and together are considered holometabolous insects. Holometabolous insects are characterized by their development, as these insects undergo complete metamorphosis. This is in comparison to the hemimetabolous insects', which include *Oncopeltus*, development. Their development is characterized by the insects undergoing partial metamorphosis. There are two hypotheses presented in Figure 3. One hypothesis suggests that the ancestor of all arthropods exhibited pair-rule expression for the orthologs of the *Drosophila* pair-rule genes, and the pair-rule expression of these orthologs was lost in the lineage leading to the Hemiptera order (depicted by the red tick marks). In contrast, another competing hypothesis suggests that the pair-rule gene orthologs lacked pair-rule like expression in the ancestor of all arthropods while the pair-rule expression developed in the lineage leading to myriapods and the holometabolous insects (depicted by the blue tick marks).

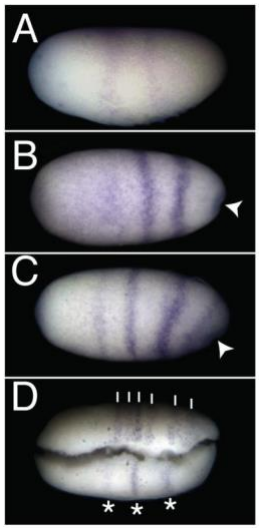


Figure 2. In-situ expression of the *Ofas-E75A* gene in *Oncopeltus* blastoderm stage embryos.
"Each image is a lateral view with the anterior of the embryos to the left. In A, *Ofas-E75A* resolves into two transverse bands. In B, as the invagination pore appears (indicated by the arrowhead), a third stripe of *Ofas-E75A* emerges. In C, a fourth stripe of *Ofas-E75A* expression appears at the onset of germband invagination. And in D, the top half of the embryo was stained with *Ofas-eve*, which marks from anterior to posterior, the mandibular, maxillary, labial, thoracic one, thoracic two and thoracic three segments. Each *Ofas-eve* stripe is indicated with a white tick mark. The bottom half of the embryo was stained for *Ofas-E75A* and each *Ofas-E75A* stripe is indicated by an asterisk. The expression of *Ofas-E75A* is in a pair rule-like pattern compared to the segmental expression of *Ofas-eve*" (Erezyilmaz et al. 2009). (Figure adapted from Erezyilmaz et al. 2009).

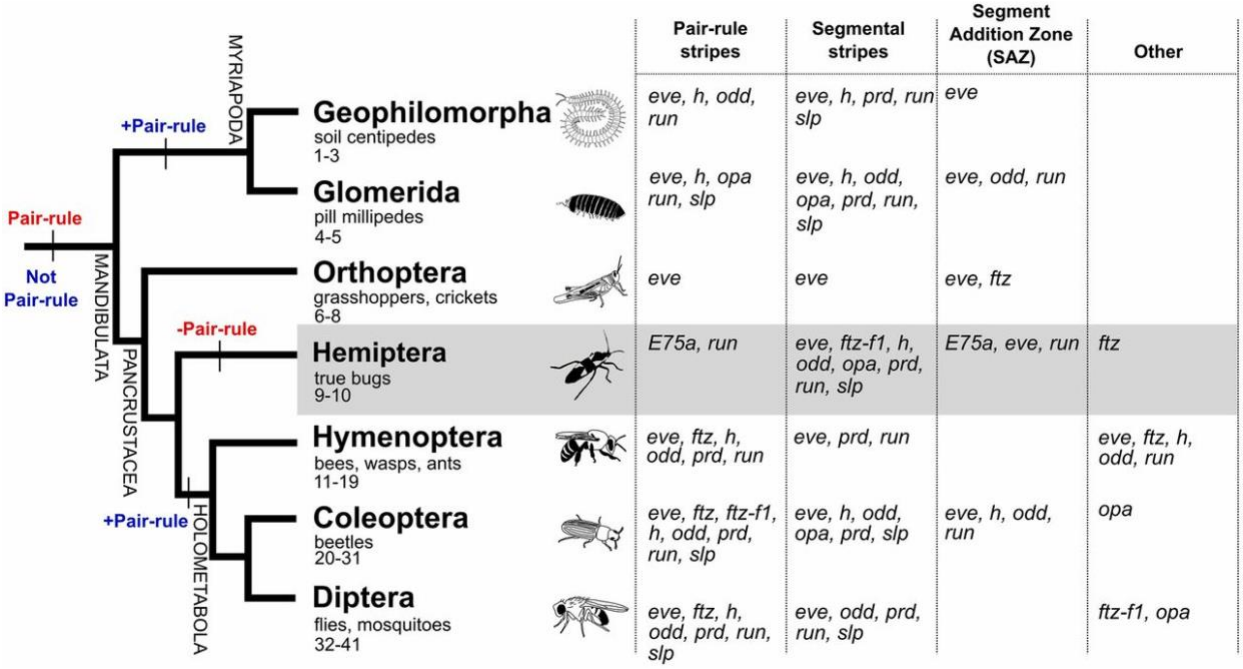


Figure 3. Models for the ancestral origin of pair-rule patterning.
This is a cladogram of insect groups in which pair-rule gene ortholog expression patterns have been studied and annotated on to the figure. Expression patterns are classified as pair-rule like, segmental, or segment addition zone (SAZ). Segmental expression is defined by expression in every segment. SAZ expression refers to broad expression in the segment addition zone. *Oncopeltus fasciatus* is a member of the Hemiptera order (shaded region in figure). (Figure adapted from Reding et al. 2019).

Since most of the orthologs of the *Drosophila* pair-rule genes did not show pair-rule expression in *Oncopeltus* (Reding et al., 2019), an unbiased RNA-seq screen was carried out to identify other genes that have pair-rule expression and function (K. Reding, unpublished). The specific goal of my project is to identify novel pair-rule genes in *Oncopeltus*. It is expected that there are other pair-rule genes in *Oncopeltus* that have not yet been identified because of the number of pair-rule genes in *Drosophila*, as well as the other holometabolous insects. The intricacy of the pair-rule patterning in these insects indicates that there are more pair-rule genes to be discovered in *Oncopeltus.* In-situ hybridizations were used to determine the expression patterns of a set of candidate genes during embryogenesis. If a gene is found to have a pair-rule like expression pattern, parental RNAi can be used to determine the function of the gene. This study may enhance our understanding of how genes regulate segmentation during embryogenesis in *Oncopeltus*, in comparison to *Drosophila*.

**Results and Discussion**

The goal of my project was to identify novel pair-rule genes during embryogenesis in *Oncopeltus*. I investigated whether a set of candidate genes exhibit pair-rule like expression, similar to *Ofas-E75A*, a previously discovered pair-rule gene in *Oncopeltus*. These genes were selected based on their coexpression with *Ofas-E75A* (see below). The expression patterns were

determined by first synthesizing specific RNA probes for the genes of interest, followed by in-situ hybridizations using the RNA probes. This procedure allowed me to identify the expression patterns on the *Oncopeltus* germbands.

Initially, RNA was sequenced from three timepoints during embryonic development and results were analyzed by graduate student Katie Reding, in collaboration with Dr. Julie Dunning-Hotopp's lab at the University of Maryland School of Medicine. Genes were selected as candidate pair-rule genes if they (1) were coexpressed with the known *Oncopeltus* pair-rule gene, *E75A*, and (2) potentially encoded a transcription factor. Genes were parsed into different clusters based upon their relative expression levels. The coexpression cluster that I studied included genes that exhibited high levels of expression at 24-36 hours and 48-60 hours AEL in 26°C (Figure 4, black box). These timepoints were chosen because the highest expression of *Ofas-E75A* was detected at 24-48 hours AEL. The working assumption was that *Oncopeltus* pair-rule genes are coexpressed at high levels at similar timepoints to the known *Oncopeltus* pair-rule gene, *E75A*. Katie Reding selected her coexpression cluster as such so that the genes exhibited low levels of expression at 0-12 and 48-60 hours AEL, but high levels of coexpression from 24-36 hours AEL, similar to *Ofas-E75A* expression. Hence, I selected another coexpression cluster that contained high levels of expression both at 24-36 hours and 48-60 hours AEL. As a result, the coexpression cluster that I studied was independent from Katie Reding's cluster of genes. My coexpression cluster was chosen because some pair-rule genes may be expressed after the expression of *Ofas-E75A* has decreased. These genes may play an additional function later in development, in addition to their role as pair-rule genes. This is the case for some pair-rule genes in *Drosophila,* as they are expressed in a pair rule-like fashion, then later are expressed segmentally.

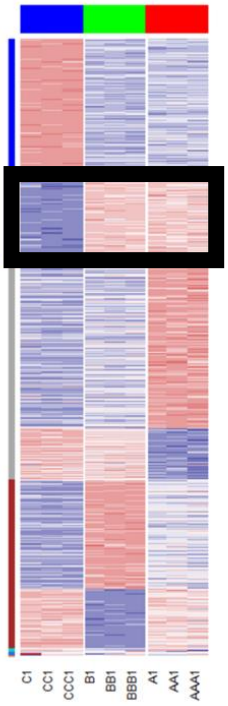


Figure 4. Heat map of expression levels of the different genes at the different time periods being studied.
The columns include nine different samples of *Oncopeltus* embryos. There were three different samples of embryos for each time period. There were three time periods denoted by the grouping of the nine samples into three columns. The first represented the time period of the first 12 hours AEL in 26°C. The second column represented the time period from 24-36 hours AEL in 26°C. The third column represented the time period from 48-60 hours AEL in 26°C. The rows were genes that were expressed in similar levels at the different time periods. These genes were clustered into coexpression modules using weighted correlation network analysis. Weighted correlation network analysis is used to define clusters and analyze the relationship between coexpression modules. The candidate genes that I focused on were from the area inside the black box. They had low expression, indicated by the blue color, in the first 12 hours AEL, they had higher expression from 24-36 hours AEL, and they had high expression from 48-60 hours AEL. (Figure courtesy of Katie Reding and Matthew Chung).

Establishment of Techniques Using *Ofas-odd* as a Control

Once the set of candidate genes were selected, the next step was to examine their spatial expression in embryos to determine if the genes show pair-rule expression. In order to synthesize RNA probes to determine the expression patterns of candidate genes, cDNA needed to be prepared. The cDNA was used as the template for the PCR reaction to amplify the gene of interest. Once the gene was amplified and the product purified, an RNA probe was synthesized. I began by conducting an RNA extraction from *Oncopeltus* embryos in order to make the cDNA.

To practice synthesizing an RNA probe and running an in-situ hybridization, I initially used the *Ofas-odd* gene since the expected results are known (Reding et al. 2019). To begin, a PCR reaction was run to amplify *Ofas-odd*. The annealing temperature was 56°C and the extension time was 10 seconds. The expected product size was 367 bp. The gel that was run after the PCR reaction confirmed the PCR product was the correct size (Figure 5). Katie Reding (KR) also ran the same PCR reaction alongside mine (OT) as a comparison. Both PCR products, KR and OT, were the expected size (Figure 5). Next, the PCR product was purified, and subsequently the RNA probe was synthesized (concentration 249.2 ng/uL, A260/A280 1.83).

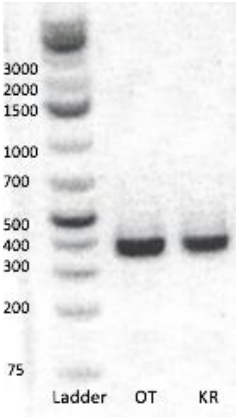


Figure 5. Agarose gel of OT and KR PCR products. Both, OT and KR, PCR products were of the expected size for the *Ofas-odd* gene.

To validate my embryo fixation and RNA probe synthesis techniques, four groups of embryos were tested. The first group of embryos were fixed by KR and the in-situ hybridization was run with the OT-made *Ofas-odd* probe. The second group of embryos were fixed by KR and the in-situ hybridization was run with the KR-made *Ofas-E75A* probe. The third group of embryos were fixed by OT and the in-situ hybridization was run with the OT-made *Ofas-odd* probe. The fourth group of embryos were fixed by OT and the in-situ hybridization was run with the KR-made *Ofas-E75A* probe. All four groups showed the expected expression patterns. The first and third groups that used the *Ofas-odd* probe showed segmental expression (Figure 6), and the second and fourth groups that used *Ofas-E75A* showed pair-rule like expression. This demonstrated that I was able to successfully fix the *Oncopeltus* embryos and synthesize the RNA probe. I then mounted the germbands from the in-situ hybridization to observe the *Ofas-odd* segmental expression pattern on the germband. (Figure 7). I used an AxioCam MRc camera and SteREO Discovery.V12 microscope from Zeiss to take the images of the blastoderm-stage embryos and germbands. If needed, Adobe Photoshop was used to piece the image together (Figure 7).

Figure 6. *Ofas-odd* stained embryo showed segmental expression.
The expression pattern of the *Ofas-odd* stained embryo showed segmental expression. Six *odd* stripes were seen at the blastoderm stage embryo.

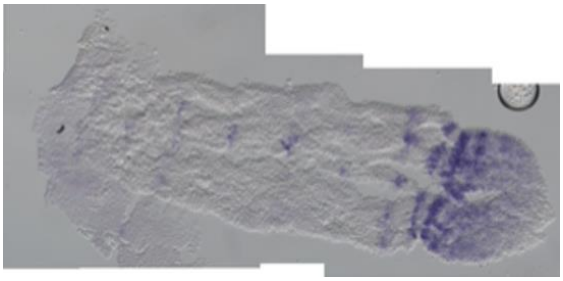

Figure 7. *Ofas-odd* stained germband showed segmental expression.
The expression pattern of the *Ofas-odd* stained germband showed expression in the anterior SAZ and in the mature segments. The striped expression in the mature segments was less apparent.

Establishment of Techniques Using *Ofas-E75A* as a Control

To further practice the process of synthesizing an RNA probe and running an in-situ hybridization, I used the *Ofas-E75A* gene since the expected results are known and provide a comparison for my results to be checked with. To begin, a PCR reaction was run to amplify *Ofas-E75A*; the annealing temperature was 56°C and the extension time was 13 seconds. The expected product size was 413 bp. However, the agarose gel showed no band for the PCR product. The PCR reaction was redone using the same conditions. The second PCR reaction was successful as the agarose gel showed a band at the expected size of the PCR product (Figure 8). Next, the PCR product was purified (concentration 71.2 ng/uL, A260/A280 ratio 1.80), and an RNA probe was synthesized (concentration 548.2 ng/uL, A260/A280 ratio 1.89).

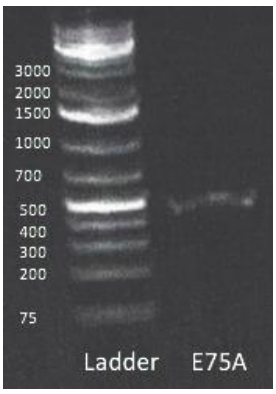


Figure 8. Agarose Gel of *Ofas-E75A* PCR Product.
The *Ofas-E75A* PCR product was the expected size based on the gene model.

Embryos were fixed at the timepoint 24-49 hours AEL. For the in-situ hybridization, there were three groups of embryos: one was run with the *Ofas-odd* probe, one was run with the *Ofas-E75A* probe at a 1:100 dilution of *Ofas-E75A* probe to hybridization buffer, and one was run with the *Ofas-E75A* probe at a 1:200 dilution of *Ofas-E75A* probe to hybridization buffer. This was

done to determine the ideal concentration of the *Ofas-E75A* probe. The *Ofas-odd* probe was used as a positive control. The results of the in-situ hybridization were that the embryos in all three of the groups were overstained. In addition, the number of embryos left in the tubes were low. The embryos were being unintentionally washed away during the washes of the in-situ hybridization protocol. By the end of the in-situ hybridization, there were not enough embryos left. To fix this issue, I began using the P-20 pipette after removing most of the solution inside the tubes with the P-1000 pipette. This way, the embryos would not be taken out of the tubes when going through the washes.

The same in-situ hybridization as previously described was repeated and the embryos did not show any staining this time. To identify if the issue had to do with my embryo fixation, or the probes that were synthesized, the next in-situ hybridization was set-up differently. Four groups of embryos were used: the first group being embryos that KR fixed and using the control *Ofas-odd* probe, the second group being embryos that KR fixed and using my *Ofas-E75A* probe, the third group being embryos that I had fixed and using the control *Ofas-odd* probe, and the fourth group being embryos that I had fixed and using my *Ofas-E75A* probe. The results showed that both probes worked, however there was an issue with my embryo fixation. This was determined because the two groups of embryos that I fixed showed no staining, whereas the two groups that KR fixed showed staining with both probes. I subsequently practiced my embryo fixation technique.

Protocol Optimization

For the following in-situ hybridizations, KR tried four different embryo fixation methods. I ran in-situ hybridizations with the *Ofas-odd* probe to decide which embryo fixation method led to the least background staining, stained the fastest, and stained the most specifically. The fixation methods all began with 3 minutes of boiling followed by placing the embryos on ice for 10 minutes. Then, for the first method of embryo fixation the eggshells were removed manually, followed by being rocked for 20 minutes in 4% paraformaldehyde (PFA). The rationale for this method was to break the chorion of the embryos before the PFA was added, which could help the fixative reach the embryos. In addition, shaking the embryos with heptane may not be needed anymore using this embryo fixation method. The second method of embryo fixation was the method that has been generally used for in-situ hybridizations of *Oncopeltus* embryos in the past. The embryos were shaken for 20 minutes in a 1:1 12% PFA:heptane solution, followed by a quick shake in a methanol:heptane solution. The third method of embryo fixation included a quick shake of the embryos in a solution of methanol and heptane, followed by a 20 minute shake in a 1:1 12% PFA:heptane solution. The rationale for this method was to break the chorion of the embryos before adding the fixative. This could help better fix the embryos. The fourth method of embryo fixation included a quick shake of the embryos in a solution of methanol and heptane, followed by rocking the embryos for 20 minutes in a solution of 4% PFA. For methods 2-4, the embryos were placed in the freezer and the eggshells were manually removed another day. The rationale for the fourth method was to break the chorion before adding the fixative. This could also help better fix the embryos. This method may make shaking the embryos with heptane unnecessary if the chorion was able to be removed. It was a similar method to the first method, except it was faster since the eggshells would not need to be manually removed, like in the first

method. The results showed that all the methods produced specific stains of *Ofas-odd*. Methods two and three stained the fastest, and methods one and two resulted in the lowest background (data not shown). Therefore, method two was determined to be the most optimal protocol for embryo fixation. It was also the method that was being previously used for *Oncopeltus* embryo fixation.

The next part of the embryo fixation protocol that was being optimized was the 4% PFA wash. I wanted to test if the 4% PFA was necessary to lower background staining and result in faster staining of the embryos. I completed an in-situ hybridization using the same *Ofas-odd* probe. I conducted the 1.5-hour 4% PFA wash on one set of embryos, and on the other set of embryos I did not perform the 4% PFA wash. The results showed that the 4% PFA wash was needed for staining of the embryos as the set of embryos that were not washed did not show any staining.

Primer Design and Sequencing

Following the optimization of the embryo fixation protocol, I designed primers to make the RNA probes for the genes of interest. As described above, I have analyzed the expression of two genes. The primers were designed using the guidelines on OligoAnalyzer. I also obtained information on the identity of these genes by using the top tBLASTn hit with the models included, then the models excluded, and finally using the NCBI conserved domain search to identify the DNA binding domain.

The templates for the *Ofas-000545* and *Ofas-000627* were sequenced to confirm that the amplified sequences were the intended sequences. The sequence for *Ofas-000545* had a slightly longer exon 1 than predicted by the gene model. The predicted exons 2 and 3 were not present. Exon 5 was slightly shorter than predicted, and exon 6 was longer than the predicted gene model. The sequence for *Ofas-000627* had slightly shorter exons 1 and 2 than predicted by the gene model. Exon 1 began later in the sequence than predicted and exon 2 ended earlier than expected. Following the sequencing results, the exon boundaries were better defined. Since the gene model predicts sequence information and exons using computer models and genomic data, it may not exactly correspond with the template sequencing result that was the actual sequence of the gene. This explains the slight discrepancies between the template sequencing result and the gene model.

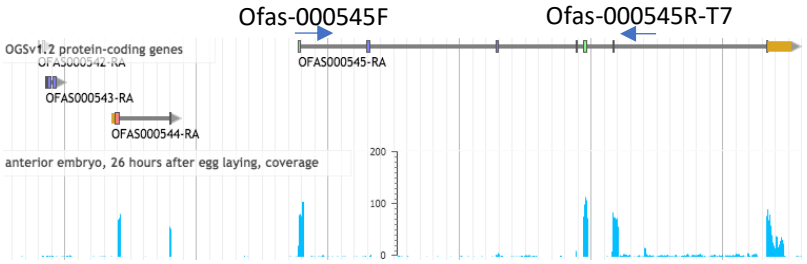


Figure 9. Gene Model for *Ofas-000545*
The model showed RNA expression levels through coverage plots. RNA seq data from Dr. Ariel Chipman's lab showed the RNA expression levels in the anterior of the embryos 26 hours AEL. The forward and reverse primers were represented by the arrows and labeled.

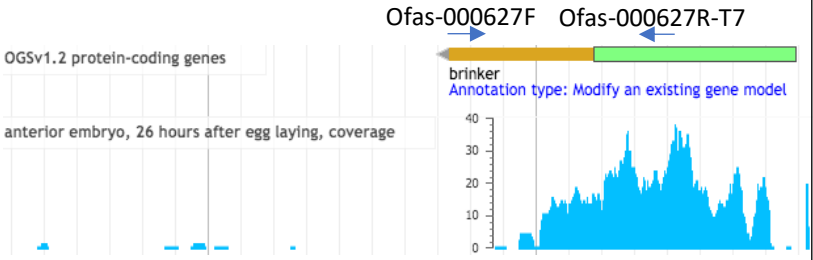


Figure 10. Gene Model for *Ofas-000627*
The model showed RNA expression levels through coverage plots. RNA seq data from Dr. Ariel Chipman's lab showed the RNA expression levels in the anterior of the embryos 26 hours AEL. The forward and reverse primers were represented by the arrows and labeled.

Expression Pattern of *Ofas-000545*

The first gene I chose to study was *Ofas-000545.* This gene was chosen because it has a *TSO1*-like CXC domain which is involved in the development of reproductive tissue and control of cell division. The domain is cysteine rich and binds zinc. In *Arabidopsis*, it has been shown that *TSO1* functions in stopping cell proliferation (Wang et al., 2018). In addition, mutating the gene results in less differentiation and more cell proliferation (Wang et al., 2018). This makes it an intriguing candidate gene that could play an important role in development.

A PCR reaction was run to amplify *Ofas-000545*, the annealing temperature was 58.5°C and the extension time was 23 seconds. The expected product size was around 784 bp. The gel that was run with the PCR product confirmed the product was the correct size, however the band was faint. Therefore, the PCR product from this reaction was used as the template for another PCR reaction to amplify the gene further. The same reaction conditions were used for the second PCR reaction. The gel showed another faint band. Therefore, I got new aliquots of water, Phusion buffer, and dNTPs. I also began to use a positive and negative control for the PCR reactions. The positive control being *Ofas-E75A,* as that appeared as a bright band on the gels. For the negative control I did not add template DNA. Following the next PCR reaction (at the same reaction

conditions as before), the gel showed the positive control had a bright band around the expected size, 413 bp. The negative control showed no bands. The *Ofas-000545* still showed a faint band around the expected product size. I repeated this PCR reaction another time and still had the same issue (only observed a faint bad for the *Ofas-000545* product). For the following PCR reaction, I ran the PCR at a slightly higher annealing temperature, 60°C, this time there was a bright band around the expected product size for *Ofas-000545.* The PCR product was purified (concentration 93.4 ng/uL, A260/A280 ratio 1.80). However, during the RNA probe synthesis, there was no pellet observed after centrifugation.

I decided to repeat the PCR reaction for *Ofas-000545*, with the annealing temperature at 60°C and the extension time being 23 seconds. The results of the controls were as expected and there was a bright band for *Ofas-000545* around the expected product size. Following the PCR product purification (concentration 35.1 ng/uL, A260/A280 1.61), the RNA probe was synthesized (concentration 490.0 ng/uL, A260/A280 1.93). However, the gel showed a band near where the DNA template was expected to be. Therefore, it was suspected that the RNA transcription did not occur properly. It was deduced that there was an issue with the T7 RNA polymerase, and a new T7 RNA polymerase was ordered. The PCR reaction was run again, and there were non-specific bands for *Ofas-000545*. When I repeated the PCR reaction a following time, there was a bright band at the expected product size for *Ofas-000545* (Figure 11). The PCR product was purified (concentration 34.0 ng/uL, A260/A280 ratio 1.80). The RNA probe was synthesized (concentration 1579.6 ng/uL, A260/A280 ratio 1.96) using the newly ordered T7 RNA polymerase. To confirm the aforementioned transcription issue was due to the old T7 RNA polymerase, the RNA probe was also attempted to be synthesized with the old T7 RNA polymerase. This resulted in no pellet again, confirming the issue was due to the old T7 RNA polymerase. Once the in-situ hybridization was run, neither the 1:500 or 1:1000 dilution for the *Ofas-000545* stained embryos showed staining, while the control *Ofas-odd* stained embryos showed segmental expression as expected. The lack of staining was deemed to be due to the DNA concentration, after PCR product purification, being too low. It was measured to be 34.0 ng/uL when I proceeded to the RNA probe synthesis, however for the future, the goal for the concentration was determined to be at least 100 ng/uL.

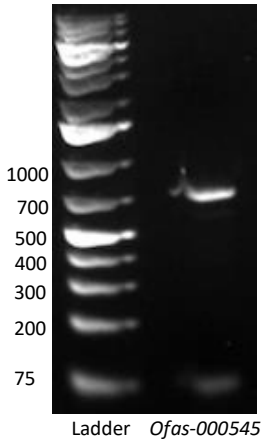


Figure 11. Agarose Gel of *Ofas-000545* PCR Product. The *Ofas-000545* PCR product was the expected size with a bright band.

I ran the PCR reaction again for *Ofas-000545* and observed a faint band on the gel. I used that PCR product, for the template DNA of a following PCR reaction, and observed a bright band.

However, after PCR product purification, the concentration was measured to only be 42.8 ng/uL. So, for the next set of PCR reactions, I ran the controls stated previously, along with three PCR reactions of *Ofas-000545*. This resulted in faint bands for the *Ofas-000545* reactions. I combined them into one tube for the PCR product purification and the concentration was measured to be 99.3 ng/uL (A260/A280 ratio 1.84). I proceeded to the RNA probe synthesis (concentration 418.3 ng/uL, A260/A280 ratio 1.84). The RNA gel showed bands of sizes smaller than the DNA band, indicating the transcription successfully occurred (Figure 12). The in-situ hybridization was run with the *Ofas-odd* probe as a control, and 1:500 and 1:1000 dilutions of the *Ofas-000545* probe. The *Ofas-odd* stained embryos showed segmental expression. Initially, the *Ofas-000545* stained embryos showed ubiquitous expression as the staining was expressed along the entire body plan (data not shown). However, subsequent staining of *Ofas-000545* showed expression in the thoracic segments (Figure 13, one intact mounted germband). Given the discrepancies between the two staining patterns, the in-situ hybridization will be repeated in the near future to determine the spatial expression of *Ofas-000545* in additional *Oncopeltus* germbands.

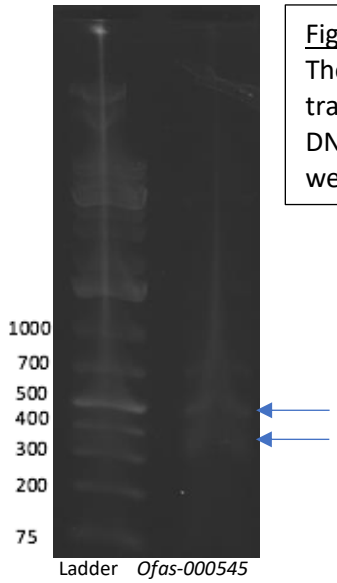


Figure 12. Agarose Gel of *Ofas-000545* RNA.
The *Ofas-000545* RNA bands were of the expected size and demonstrate the transcription occurred. They traveled further down the gel than where the DNA band was expected. This was because the RNA bands were smaller and were able to travel further down the gel.

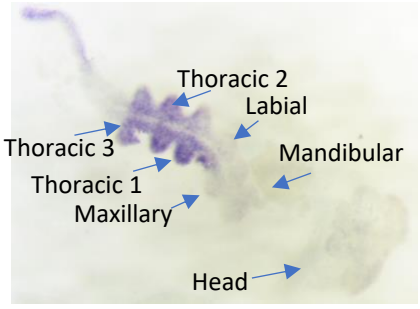


Figure 13. *Ofas-000545* stained germband showed expression in thoracic segments.
The expression pattern of the *Ofas-000545* stained germband showed staining in the thoracic 1, 2 and 3 segments, and no staining along the midline. There was also no staining in the head region and the mandibular, maxillary and labial segments. Previous staining had shown ubiquitous expression. Therefore, the in-situ hybridization will be repeated to verify the expression pattern of *Ofas-000545*. The germband was oriented anterior to the right. Only one mounted germband was intact.

Expression Pattern of *Ofas-000627*

The second gene I chose to study was *Ofas-000627.* The homolog of *Ofas-000627*, the *Brinker* gene, in *Drosophila* embryos had previously shown expression in two broad lateral stripes within the neurogenic ectoderm (Zhang et al., 2001). I expected a similar expression pattern in *Oncopeltus. Brinker* is a sequence-specific transcriptional repressor in *Drosophila* embryos. *Brinker* protein represses decapentaplegic response elements (Zhang et al., 2001). Decapentaplegic plays a role in the development of *Drosophila* (Zhang et al., 2001). It is needed for the correct patterning and development of the early embryo, as well as in regulating the growth and size of the tissues (Zhang et al., 2001). The *Brinker* gene represses *tolloid* transcription (Zhang et al., 2001). Interestingly, in *Drosophila, tolloid* is involved in the process of dorsal-ventral polarity. This all makes *Ofas-000627* an intriguing candidate gene that could have an integral role in development.

A PCR reaction was run to amplify *Ofas-000627*, the annealing temperature was 61°C and the extension time was 19 seconds. The expected product size was 640 bp. The gel that was run with the PCR product showed no bands. Therefore, a new cDNA template was used for the next PCR reaction along with new aliquots of water, Phusion buffer, and dNTPs. The previous reaction conditions were used for the second PCR reaction. *Ofas-000627* showed no bands again, while the positive control showed a band with the expected product size. For the next attempt, I lowered the annealing temperature to 58°C. I repeated the PCR reaction and still did not observe a band on the gel. For the next PCR attempt, I increased the extension time to 30 seconds. The PCR reaction still did not show a band on the gel for *Ofas-000627*, while the controls worked as expected. The following PCR reaction, there was a bright band at the expected product size for *Ofas-000627* (Figure 14). The PCR product was purified (concentration 175.7 ng/uL, A260/A280 ratio 1.87). The RNA probe was synthesized (concentration 1058.8 ng/uL, A260/A280 ratio 1.89). The RNA gel showed bands that were smaller than the expected DNA product size, demonstrating the transcription was successful (Figure 15). The in-situ hybridization was run with the *Ofas-odd* probe as a control, and the *Ofas-000627* probe at 1:500 and 1:1000 dilutions. The results showed that the control *Ofas-odd* stained embryos were overstained, and the *Ofas-000627* stained embryos showed staining along the midline; a representative example of the two mounted germbands was pictured (Figure 16). *Ofas-000627* did not show pair-rule like expression. Our preliminary data suggests that the expression of this gene in *Oncopeltus* may be spatially and temporally different in comparison to *Drosophila.* To test this, I can examine the expression pattern of *Ofas-000627* in *Oncopletus* blastoderm-stage embryos to compare it directly with the expression pattern of *Drosophila* blastoderm-stage embryos reported by Zhang et al., 2001.

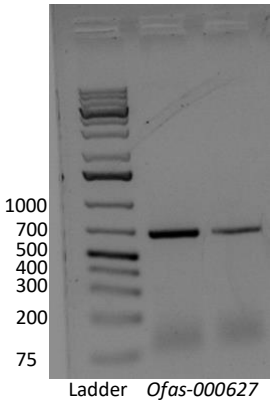


Figure 14. Agarose Gel of *Ofas-000627* PCR Product.
The two *Ofas-000627* DNA bands in wells two and three were the expected size with bright bands.

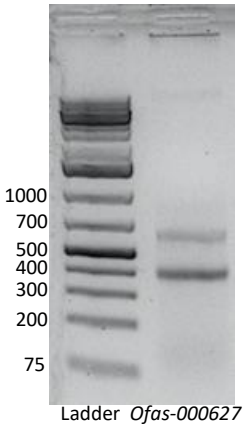


Figure 15. Agarose Gel of *Ofas-000627* RNA.
The *Ofas-000627* RNA band demonstrated it was the expected size and the transcription occurred. The RNA band traveled further down the gel than where the DNA band was expected. There was a faint band where the DNA band was expected to be, however the RNA band was brighter.

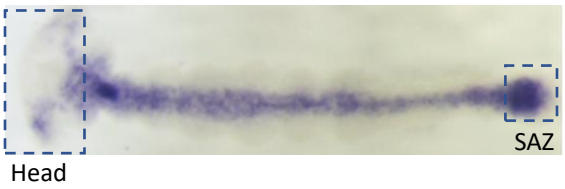


Figure 16. *Ofas-000627* stained germband showed midline expression.
The *Ofas-000627* stained germband showed staining along the midline from the SAZ through the mature segments. There was a small amount of staining in the head region. The germband was oriented anterior to the left.

## Expression Pattern of *Ofas-000926* to be Identified in Near Future

The third gene I chose to study was *Ofas-000926.* This gene was chosen because it is expressed highly at 24-36 hours AEL. *Ofas-E75A*, the known pair-rule gene in *Oncopeltus*, is expressed highly at this timepoint as well, therefore *Ofas-000926* is an interesting candidate pair-rule gene. *Ofas-000926* is also expressed highly from 48-60 hours AEL. Some pair-rule genes may be expressed after the expression of *Ofas-E75A*. These genes could have another role later in development, in addition to their role as pair-rule genes. This is the case for some pair-rule genes in *Drosophila,* as they are expressed in a pair rule-like fashion, then later are expressed segmentally. Therefore, *Ofas-000926* is an interesting candidate pair-rule gene.

A PCR reaction was run to amplify *Ofas-000926*, the annealing temperature was 57°C and the extension time was 25 seconds. The expected product size was 871 bp. Following the PCR

reaction, the gel showed a bright band at the expected product size (Figure 17). The PCR product was purified (concentration 132.1 ng/uL, A260/A280 ratio 1.86). The RNA probe was synthesized (concentration 1372.8 ng/uL, A260/A280 ratio 1.86). The RNA gel showed bands that are smaller than the expected DNA product size, demonstrating the transcription was successful (Figure 18). The in-situ hybridization to identify the expression pattern of *Ofas-000926* will be run in the near future.

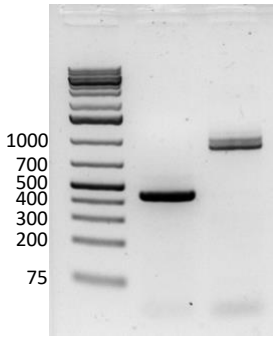


Figure 17. Agarose Gel of *Ofas-000926* PCR Product. The control, *Ofas-E75A,* in well two showed a bright band. The *Ofas-000926* PCR product in well three was the expected size and was a bright band.

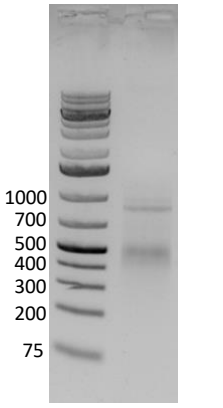


Figure 18. Agarose Gel of *Ofas-000926* RNA.
The *Ofas-000926* RNA band showed it was the expected size and the transcription occurred. The band traveled further down the gel than where the DNA band was expected. There was a faint band where the DNA was expected to be, however the RNA band was brighter.

**Conclusions and Future Directions**

We sought to identify novel pair-rule genes in *Oncopeltus*. Thus far, the expression patterns of two candidate genes were investigated. *Ofas-000545* showed expression in the thoracic segments and *Ofas-000627* showed midline expression in the *Oncopeltus* germbands, however they lacked pair-rule expression. A double in-situ hybridization can be done with a marker gene to specify the spatial expression of *Ofas-000545* and *Ofas-000627* in the body plan with regard to the marker. This would specify the location of the expression of the novel gene in the *Oncopeltus* segments. Furthermore, the RNA probes of the remaining seven candidate genes will also be synthesized, and the in-situ hybridizations will be run to identify the expression patterns of the genes and determine whether they exhibit pair-rule expression. Since the genes encode transcription factors and given that pair-rule genes regulate segment-polarity genes, if the candidate genes do show pair-rule expression, the expression of their targets, segment-polarity genes, can be identified. This can be achieved by using parental RNAi to observe the

effects of perturbing the gene on embryogenesis. In conclusion, this approach has allowed us to investigate the expression pattern of genes in order to identify novel pair-rule genes in *Oncopeltus.*

**Materials and Methods**

Insect Rearing and Embryo Collection

*Oncopeltus* were purchased from Carolina Biological Supply Company (Burlington, NC) and maintained in our lab. They were kept in 30x18x20 cm plastic cages. Their diet consisted of water and raw organic sunflower seeds. They were maintained at 25-26°C with 50% humidity and a photoperiod of 16 hours light and 8 hours dark. At the start of the collection period, a piece of cotton was placed on top of a paper towel inside the plastic cage. At the end of the collection period, the cotton was removed and stored at 25-26°C. When the embryos reached the desired age, they were removed from the cotton.

Embryo Fixation

Embryos were collected from the cotton and transferred (100-200 uL) into a 2 mL tube. DEPC-treated water (600 uL) was added to each tube to completely submerge all the embryos. The tubes were then transferred into a boiling water bath for 3 minutes. Next, they were transferred onto ice for 7 minutes. Water was carefully removed from each tube.

In the fume hood, equal volumes of heptane (600 uL) and freshly thawed 12% PFA (600 uL) were added. The solution was mixed well by gently shaking the tubes until the embryos aligned between the two phases. The tubes were then shaken at 300 RPM for 20 minutes. Subsequently, the PFA was removed from the aqueous lower phase. Methanol (600 uL) was added, and the tubes were shaken gently for 15 seconds by hand to crack the eggshells. The methanol/heptane solution was later removed, and the embryos were washed in 1 mL of methanol three times. Then, stored in methanol at -20°C.

RNA Extraction with TRIzol

TRIzol was used to extract RNA from tissues. 1 mL of TRIzol can be used to extract RNA from 50-100 mg of starting material. One *Oncopeltus* embryo weighed approximately 0.25 mg. Embryos were frozen ahead of time in 150 uL of TRIzol at -80°C. To extract RNA, the TRIzol/embryo mixture was thawed on ice, and the embryos were homogenized with a pestle in TRIzol. If working with fresh embryos, TRIzol (150 uL) was added and then the embryos were homogenized. Additional TRIzol (450 uL) was used to rinse the embryos off the pestle and to completely homogenize the embryos, followed by additional incubation for five minutes at room temperature to allow the complete dissociation of the nucleoproteins complex. Next, chloroform (120 uL) was added to the tube and vortexed, followed by three minutes of incubation, and centrifugation for 15 minutes (11,000 RPM at 4°C). The colorless upper aqueous phase containing the RNA was transferred to a new 1.5 mL tube, and the same volume of the aqueous phase that was removed, was added of chloroform. Then, the tube was vortexed and centrifuged for three

minutes (13,000 RPM at 4°C). The upper aqueous layer was then transferred into a new tube and isopropanol (300 uL) was added. The tube was vortexed and incubated for ten minutes at room temperature. This was followed by centrifugation for 10 minutes at 1,000 RPM in 4°C. The total RNA precipitate formed a white gel-like pellet at the bottom of the tube. The supernatant was discarded, and the pellet was rinsed with 800 uL 75% ethanol. The tube was briefly centrifuged for five minutes at 9,000 RPM in 4°C. Then, the supernatant was discarded, and the pellet air dried. The pellet was resuspended in 50 uL of RNase-free water/DEPC-treated water. The concentration was measured and recorded using the Thermo Scientific NanoDrop Lite Spectrophotometer. The tube was stored in -80°C.

cDNA Preparation

The first stage of cDNA preparation was RNA denaturation. 1 ug total RNA, 1 uL primer mix, and 1 uL dNTPs were mixed into a tube and the tube was filled to 16 uL using milliQ-grade water. Then, the tube was heated to 65°C for five minutes. Next, the tube was placed immediately on ice.

The second stage was reverse transcription. First, 2 uL of 10x M-MLV-RT First-Strand buffer was added with 1 uL of RNase inhibitor to the tube. This was mixed, spun down and incubated at 37°C for two minutes. Next, 1 uL of M-MLV-RT was added and mixed into the solution. Then, this solution was incubated at 37°C for 50 minutes in order for the reverse transcription to occur. The reverse transcriptase was inactivated when the solution was heated at 70°C for 15 minutes. The tubes were stored in the -80°C freezer.

Primer Design and Making Working Dilution for PCR

The design of the primers was based on the standard primer guidelines. For the RNA probe synthesis that occurs after the PCR, a T7 recognition sequence was added to the start of the reverse primer. This allowed for the T7 RNA polymerase to undergo transcription. The primer pairs were also checked to make sure they do not have complementary regions to each other. The lyophilized primers were centrifuged at 13,000 RPM for two minutes. Then, the primers were resuspended with milliQ-grade water to make a final concentration of 100 uM. Using the concentration that the primers arrived in, the appropriate volumes of water were added to get the solution to a concentration of 100 uM. Next, the primers were diluted to a working dilution of 10 uM. This was completed by adding 10 uL of the stock primer to 90 uL of water. Both the stock and working dilutions were kept at -20°C.

PCR

35.7 uL water, 10 uL 5x Phusion buffer, 1 uL dNTPs, 1 uL forward primer (10 uM), 1 uL reverse primer (10 uM), 1 uL cDNA, and 0.3 uL Phusion polymerase were added to a PCR tube. The annealing temperature used for the PCR reaction was 2-3°C higher than the melting temperature of the primer with the lower melting temperature. The extension time for the PCR reaction was determined by the size of the expected product (15-30 second extension time per kb). After the PCR reaction was complete, the product size was checked by running a 1% agarose

gel. If the product size was the expected size, the PCR product was purified using a Monarch PCR product purification kit. Once purified, the product's concentration and A260/A280 ratio were measured and recorded using the Thermo Scientific NanoDrop Lite Spectrophotometer. The expected A260/A280 ratio for pure DNA is 1.8.

RNA Probe Synthesis

MilliQ-grade water, volume dependent on DNA concentration as total reaction volume will be 20 uL, was added to a 1.5 mL tube. 2 uL 10x RNA polymerase buffer, 2 uL dig-labeling mix, 1 uL RNase inhibitor, 200 ng PCR product, 2 uL T7 RNA polymerase were added to the 1.5 mL tube. The solution was mixed, spun down and incubated at 37°C for 2 hours. Then, 1.7 uL 6 M LiCl was added to the tube along with 75 uL cold ethanol. The solution was incubated in -20°C for two hours. Then, it was centrifuged for 15 minutes at 13,000 RPM at 4°C. The pellet was dried, and RNA was dissolved in 20 uL water. The RNA probe's concentration was measured, and a working dilution was made at 100 ng/uL in hybridization buffer. The A260/A280 ratio was also measured to assess RNA purity. The expected A260/A280 for pure RNA is 2.0. Both the stock and working dilution were stored at -80°C.

To check if the RNA was transcribed, the sample was run on a 1% agarose gel. First, 1 ug of RNA was diluted into 10 uL of water. RNA was denatured by heating it to 95°C for 3 minutes using a heat block. Then, the RNA was immediately placed on ice, and the samples were run on the gel.

In-Situ Hybridization

Day 1

Embryos were washed away from 100% methanol as 300 uL methanol was removed and 300 uL Phosphate-Buffered Saline/0.1% Tween-20 (PBST) was added to each tube. After 5-minutes, this wash step was repeated three more times to remove all the methanol. Then, 1 mL of PBST was added to each tube and the embryos were transferred to a dissection plate. The eggshells were manually removed under a dissection microscope, and the embryos were transferred back to the tubes. The PBST was removed, and 1 mL of 4% PFA was added. Embryos were washed for 1.5 hours at room temperature on a rocker before the PFA was removed and they were rinsed three times with PBST. Next, PBST was removed and 1 mL of pre-warmed (at 60°C) hybridization buffer was added to each tube. The embryos were allowed to rock at room temperature for three minutes, or until the embryos sank in the buffer. Embryos were subsequently incubated at 60°C for 4-6 hours. The probe dilution was prepared in hybridization buffer using a dilution of 1:500 and 1:1000. After the removal of embryos from the incubator, they were allowed to sit upright for five minutes as they settled to the bottom of the tube. The hybridization buffer was removed and at least 200 uL of the probe dilution was added to each tube. The tubes were placed vertically in the 60°C incubator.

Day 2

Two wash volumes (one wash volume is equal to one mL per tube of embryos) of hybridization buffer, and one wash volume of 2x saline-sodium citrate (SSC)/0.1% Tween-20 were placed in the 60°C incubator. The probe from the tubes were removed. The embryos were washed and rocked twice for 30 minutes in the pre-warmed hybridization buffer. Then, they were washed and rocked for 30 minutes in the pre-warmed 2x SSC/0.1% Tween-20. This was repeated with room temperature 2x SSC/0.1% Tween-20. The last wash was done with 0.2x SSC/0.1% Tween-20 as described in the previous steps. The embryos were rinsed three times in PBST, and finally the embryos were resuspended in PBST supplemented with 10% sheep serum. They were rocked for 1-2 hours at room temperature. 800 uL of Anti-Digoxigenin-Alkaline Phosphatase (AP), Fab fragments (Sigma-Aldrich) (1:1600 dilution in PBST supplemented with 10% sheep serum) was added to each tube and the embryos were rocked at room temperature for 1-2 hours. Next, the antibody solution was removed, and the embryos were rocked in PBST overnight at 4°C.

Day 3

The embryos were washed in 1 mL of PBST and rocked for 20 minutes at room temperature five times. The embryos were washed in 1 mL of AP staining buffer and rocked for 5 minutes three times. The AP staining buffer was then removed and 500 uL of 5-Bromo-4-chloro-3indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) staining solution was added to the tubes. BCIP was converted to a colored product by the alkaline phosphate that was coupled to the anti-digoxigenin antibodies. The product of BCIP reacted with NBT to form a product that was dark blue to purple in color, which marked the cells expressing the gene of interest. The embryos from the tubes were pipetted into individual wells of a 24-well plate. The plate was covered with aluminum foil and left for staining overnight at room temperature.

Day 4

The NBT/BCIP staining solution was removed to stop the reaction. The embryos were transferred back to the tubes, from the plate. The embryos were rinsed three times in PBST, followed by the embryos being rinsed in PBST and rocked for 10 minutes three times. Next, the embryos were washed in 1:1 PBST:methanol and rocked for five minutes. The embryos were rinsed twice in methanol, followed by an additional rinse in 1:1 PBST:methanol, and rocked for five minutes. The process from cDNA preparation, until the completion of the in-situ hybridization is depicted (Figure 19).

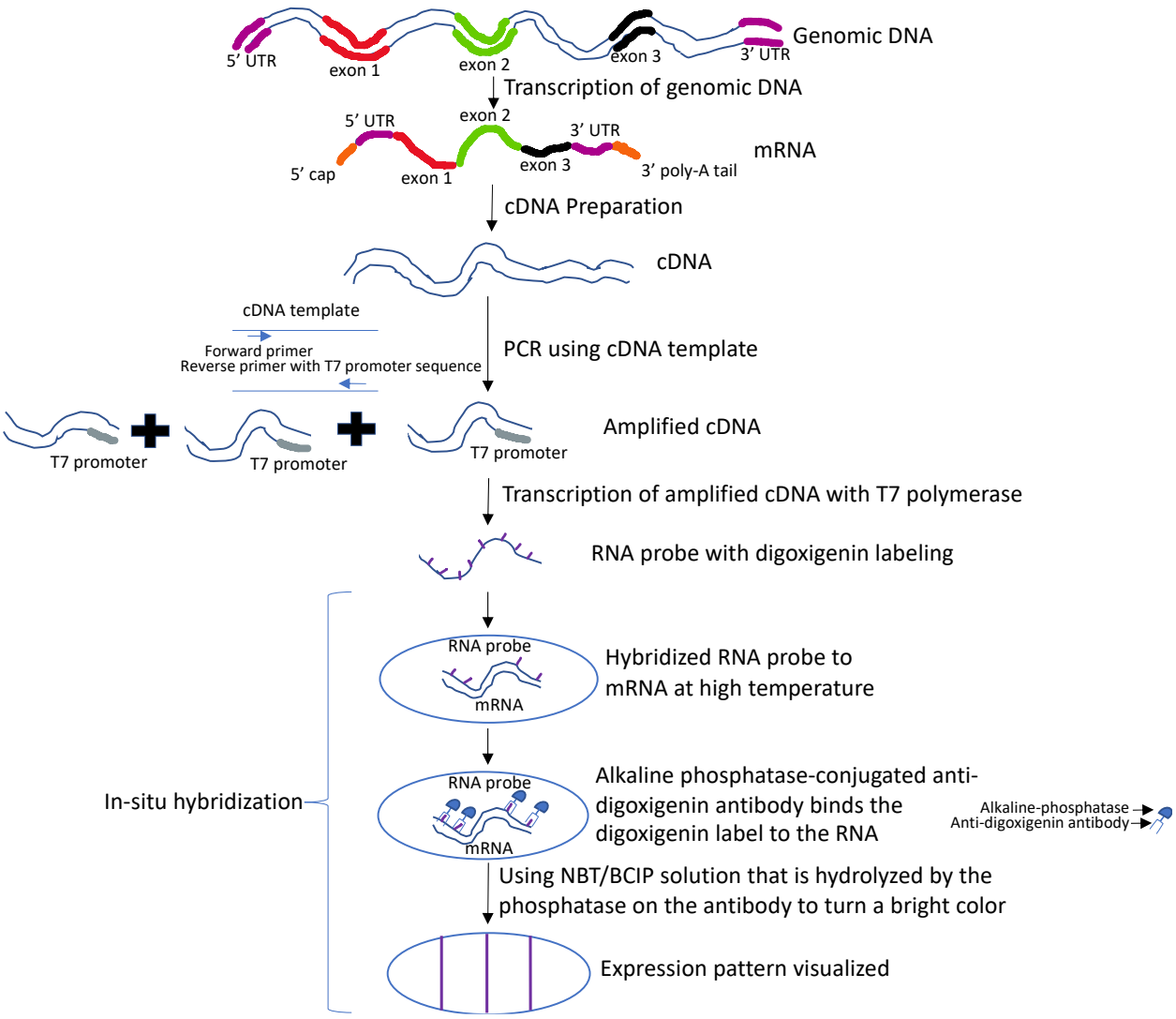


Figure 19. Schematic of Workflow.
The workflow, starting from the cDNA preparation until the in-situ hybridization, is depicted above. The cDNA was prepared from the mRNA. Then, PCR was used to amplify the cDNA. Next, the amplified cDNA was transcribed with the T7 polymerase to create the RNA probe with digoxigenin labeling. For the in-situ hybridization, the mRNA was first hybridized to the RNA probe at a high temperature. Then, the alkaline phosphatase-conjugated anti-digoxigenin antibody bound the digoxigenin label to the RNA. Finally, using the NBT/BCIP solution, that is hydrolyzed by the phosphatase on the antibody, the expression pattern was visualized on the embryos.

## Mounting Germbands of Short- and Intermediate-Germ Insects

The embryos were carefully dissected to remove the yolk from the germbands. The germbands were placed onto a slide, and 25 uL of 70% glycerol in PBST was added onto the slide. The germbands were gently flattened against the slide, and a coverslip was placed over them. The

border of the coverslip was sealed with clear nail polish and allowed to air-dry at room temperature overnight. The following day, the slides were stored at 4°C.

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**Appendix**

Solutions for In-situ Hybridization

Hybridization Buffer

12.5 mL 20x SSC
250 uL heparin (10 mg/mL)
125 uL yeast tRNA (20 mg/mL)
50 uL Tween-20
12.5 mL DEPC-treated distilled water
25 mL formamide (pipet in fume hood)

2x SSC/0.1% Tween-20

1.5 mL 20x SSC DEPC
15 uL Tween-20
Fill to 15 mL with distilled water

0.2x SSC/0.1% Tween-20

150 uL 20x SSC DEPC
15 uL Tween-20
Fill to 15 mL with distilled water

AP staining buffer (pH: 9.5)

300 uL 5 M NaCl
750 uL 1 M $MgCl_2$
1.5 mL 1 M Tris-HCl (pH: 9.5)
15 uL Tween-20
Fill to 15 mL distilled water

BCIP/NBT staining solution (for two tubes, 500 uL per tube)

4.5 uL NBT
3.5 uL BCIP
1 mL AP staining buffer