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# Evolutionary Development Study of Firebrat *(Thermobia domestica)*

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# EVOLUTIONARY DEVELOPMENT STUDY OF FIREBRAT (*THERMOBIA DOMESTICA*)

A Thesis

by

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ABSTRACT

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As most evolutionary development studies regarding wing origin take place using insects taxonomically closer to model organism, *Drosophila melanogaster*, there is a need of expanding studies to lower clades. While *D. melanogaster* is higher phylogenetically, basal clades would allow for a historical perspective of what occurs within the gene flow of conservative biomarkers. Further understanding of wings and flight would allow for a clearer understanding of how the migration of insects from sea to land has allowed them to become one of the most speciose taxa. Through the study of *Thermobia Domestica*, part of the Zygentoma, we expand on comprehension of biomarker conservation and developmental mechanisms within their embryology process. Developmental HOX genes analyzed include *Distal-less, Wingless, Engrailed 1*, *Engrailed 2*, and *Ultrabithorax*. Genes were sequenced at various developmental stages and used for further analysis through antibody staining and fluorescent *in-situ* hybridization. This study aids in expanding a new model organism within basal clades, while also gaining further genetic information on the novel structure, wings.

# DEDICATION

To my parents, Luis M. Cantu and Maria Aracely ‘Shelly’ Cantu. Thank you for all that both of you have done for me. Thank you for allowing me to explore and learn what I’m passionate for. This has allowed me to attain a perspective of the world which is invaluable to me. You both have instilled so many life lessons into me that this is a testament of all of it. Guardame un riconcito en el cielo, papa. It was a privilege to have been raised by you.

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# CHAPTER I

# INTRODUCTION

## Firebrat (*Thermobia domestica*)

*Thermobia domestica*, commonly known as the firebrat, is a non-model organism that is critical for our understanding or the origin and evolution of the Hexapoda. It belongs to the family Lepismatidae and is within insect order Zygentoma along with Silverfish (*Lepisma sacharrina*) and other lesser studied species (Bai et al., 2021; Truman & Riddiford, 2019). Composed of approximately 370 species, Zygentoma are found within dark, wet environments such as caves or under dry conditions feeding off of organic composed materials in close proximity to humans (synanthropic) (Belles, 2020). They tend to feed off of starchy foods, but studies have also shown they have cannibalistic behaviors. Both the nymph stage and adult *T. domestica* will feed readily on eggs, dead bodies, or even weakened individuals unable to defend from attack. Preferentially, the firebrat tends to be more widespread in tropical and temperate regions. It received its common name for being found near outdoor ovens in England, receiving this name before its scientific nomenclature was assigned. It is one of the more common household pests, typically nocturnal, as well as quite active (Belles, 2020; Sweetman, 1938) . Significantly, it is part of the wingless apterygote insects, and this phylogenetic position makes it a key lineage for understanding the morphological and genetic precursors to the origin of more complex insect body-plans, including wings. A more in depth understanding of *Thermobia*

*domestica* will help fill in the gaps between outgroups and model organisms regarding our understanding of the origin of the group Insecta. *T. domestica* is part of the ametabolous group of insects and can be easily maintained and reared in culture, therefore it has potential to become a more useful organism for insect morphology and genetic studies (Bai et al., 2021). Modern techniques allows us to understand their genome and compare transcriptomes to more commonly used model insects, the increase of genomic information available allows in depth functional analysis of genes (Ohde et al., 2009).

## Phylogenetic Relationships of *T. domestica* within Insecta

Hexapoda is comprised of two subgroups: the non-insect hexapods (including Diplura, Protura, and Collembola) and Insecta. Insecta can also be divided into two main groups: the non-pterytoge or wingless insects including, Archaeognatha and Zygentoma; and Pterygota, or winged insects the most speciose and biodiverse group on earth (Thomas et al., 2013; Truman & Riddiford, 2019). As it currently stands, there is a strict division within phylogenetic systemics between Apterygota (paraphyletic) and a monophyletic Pterygota. Recent advances in DNA sequencing and analysis of large data sets has led to a much more resolved consensus among major insect lineages (Labandeira, 2006; Mendes, 2002; Misof et al., 2014). Insects have three distinct body regions, a head, three segmented thorax, and an abdomen with up to eleven segments. The distinction between the thorax and (mostly) appendageless abdomen is synapomorphy that is the key characteristic for this taxon.

Classification within Zygentoma places *T. domestica* alongside *L. sacharrina*. Zygentoma serves as an intermediate lineage between binding Archaeognatha and Pterygota. Historically Hexapoda was thought to be closely related to Myriopoda (composed of Chiopoda and Diplopoda), however modern analyses have firmly established that they are more closely

related to the crustacean. In fact, the hexapods are more closely related to some lineages of crustaceans than other rendering “crustacea” paraphyletic (Labandeira, 2006; Misof et al., 2014).

Now that we are beginning to reach consensus regarding phylogenetic relationships among Hexapoda it is key that we understand the early development of Zygentoma and its import to the origin and evolution of the insects. This serves to differentiate the various pathways and to distinguish, which are more conserved than others. Due to the limitation of fossil evidence, the wealth of molecular data is crucial in understanding the origin of adaptative novel structures (Klein, 2001; Poprawa & Rost, 2004). As two species diverge from their ancestor, the accumulation of genetic differences marks the origin of general body plan and key innovations that define different taxa. Phenotypes are derived from a complex network of early regulatory genes. We can think of each distinct morphology as a modification of the genes and regulation of key propagating network that guide the epigenesis of complex organisms from simple, undifferentiated cells (Davis et al., 2010).

## Origin of Wings

Wings are perhaps the most important novel morphological structure that allowed for the adaptive radiation and massive biodiversity of insects. The earlies fossils place the single origin of insect wings between the Devonian and Carboniferous period (Thomas et al., 2013). This time period occurred around 370 to 330 million years ago (mya). Historically, relying heavily on model organisms, such as *Drosophila melanogaster*, have supported two hypotheses regarding the origin of wings. However, there is currently only scant fossil evidence to help guide our understanding of the sequential evolutionary steps traversed to acquire the morphological structure of present-day wings. Without the transitional structures to the modern-day insect wing, a developmental comprehension by studying taxa that minimally span the evolution of wings

could help provide great insight into the origin of these critical structures (Dudley et al., 2007; Ohde et al., 2018).

The first hypothesis is named the **tergal origin hypothesis**, and proposes that wings originated as the lateral extensions of the notum, the dorsal component of the insects exoskeleton. This theory provides the potential for a transitional form of gliding organisms eventually leading to the origin of full, powered insect flight. These paranotal lobes can be seen in Paleozoic insect fossils and have been investigated in some molecular studies (Wheat & Wahlberg, 2013; Wheeler et al., 2001).

The second hypothesis is called the **pleural origin hypothesis**, and proposes that the legs, or elements of ancestral legs, fused onto the abdominal wall and formed modern day wings. This would give our current understanding of ancestral legs segments, such as the leg attachment muscle epicoxa, further import when it came to evolutionary development. This also reinforces the idea of migration of cells and exites dorsally, with their proximal legs fusing onto the abdominal wall.

For many decades there has been no clear consensus among insect evolutionary biologists regarding supporting one or the other of these hypotheses. Recent work, relying heavily on genetic data has proposed a third hypothesis: the **dual origin hypothesis**, which is something of an amalgamation of two known hypothesis. While the opposing tergal and pleural origin explain the origination of wing tissues placed on the dorsal body wall and pleural plates – there has been molecular evidence showing that wing tissue homologs are expressed in non-pterygote insects (Clark-Hachtel et al., 2013). Evidence of gene expression has shown that wing orthologs (wingless, wg; apterous, ap; vestigial, vg) are found and expressed in Archaeagnatha (Niwa et al., 2010). This is supports the idea of an evolutionary shift causing the migration of cell through

the tergal and pleural regions to fuse together to form a new structure . . . the wing. As previously proven by both Averof and Cohen, ap and POU domain protein (pdm), are more functional proteins critical for both the development of *D. melanogaster* wings and crustacean limb branches (Averof & Cohen, 1997).

The innovation of new phenotypic occurrences within species is something studied not only through evolutionary biologists but also ecologists. This allows for better comprehension of similar ecologically localized species coexisting (Clarke et al., 2017; Ord et al., 2020). For example, if we are to comprehend the movement from water to land to elucidate *T. domestica* physiology relatedness to crustaceans, we need to be understanding of the adaptation at cost. As gliding insects radiated for purposes of exploitation of resources in new environments, the potential for co-option evolution increases alongside new arboreal available ecosystems (Ord et al., 2020). Novel phenotypes such as flight could then also be attributed to competition found in new environments. Competition tends to be sympatric species that are acquiring evolutionary differences, producing trait dispersion across phylogeny (Clarke et al., 2017). As previously proven through statistical inference, data has suggested that the earliest insects formed ~479 mya (Misof et al., 2014). This would place the radiation of insects at the same as the expansion of plants, also supportive of co-option evolution occurring around the same time influencing flight.

## Embryology, Development, and Morphology of *T. domestica*

Adaptations accumulated gradually over time led to the current morphology shown in the wingless Zygentoma (silverfish) and Archaeognatha (jumping bristletails). These taxa have similar morphology, however modern phylogenies support Zygentoma as the closest extant relative of the pterygote insects, thus it plays a key role in our understanding of both the morphology of the early wingless insects and the potential precursor structure for the origin of

winged insects (Masumoto & Machida, 2006). Silverfish are ametabolous, meaning that nymphs look similar to the adults and they change gradually from one stage to the next without any dramatic transformations (Truman & Riddiford, 2019). During the adult stage, there is a consistent molting with bouts of reproduction. In pterygote insects the formation of wings represents the final, adult stage and there are no subsequent molts. All juvenile stages, however. lack wings and external reproductive structures. A common characteristic for the Zygentoma are three filiform appendages at the distal part of the abdomen; the two lateral ones are the cerci and the middle one is called the appendix dorsalis (also known as the paracercus). Their body is also covered in scales after the first three molts composed of continuous membranes, longitudinally reinforcing ribs running along the side of them (Belles, 2020). The scales appear after the second or third molt and serve a mechanistically protective role.

Reproduction of the *T. domestica,* as well as other Zygentoma occurs through indirect transfer of sperm. The males package their sperm and drop it on the substrate, the female then picks up the spermatophore and inserts it into her genital opening to be released in her reproductive system and thus completing fertilization. The egg of *T. domestica* has also been previously characterized as soft, white, and opaque when initially laid. The chorion hardens and tints the egg with a slight yellow (Woodland, 1957). Development of Zygentoma differs greatly from that of *D. melanogaster* where the initial embryo is formed by cells around the yolk that initiates the development process of segmentation. In comparison, *Thermobia domestica*, forms the initial embryo by a small germ disc on the edge of yolk. Anatrepsis begins with the folding of nuclei cells until sinking into the yolk. Then slowly, segmentation occurs from the posterior until fully enveloped around the yolk filling the egg (Masumoto & Machida, 2006). This form of

development is called germ band elongation and is the ancestral form of insect embryo development.

Early embryonic development begins with the primordium which is situated at the posterior egg pole. Previous to this there is gastrulation that differentiates ectoderm, mesoderm, and endoderm. The growth zone is localized at the posterior end of the germ band forming the mesoderm. During the blastokinesis stage, the band sinks into the yolk, with the amniotic cavity unable to close. It's through the further aggregation of amnioserosal folds that the embryonic pore is able to close to complete fusion. This trait is an ancestral characteristic of Archaeognatha, and proceeds in an identical manner in Zygentoma (Masumoto & Machida, 2006). After gastrulation, organogenesis begins and development of antennae, mouthparts, legs and eyes is completed. As this process continues the germ cells become more specialized and express specificity after cascading cellular signaling (Farris, 2005).

# CHAPTER II

# REVIEW OF LITERATURE

## Homeotic Gene Complex

Through the differentiation of a diverse morphology along body axes, there is a positional cue to each specific region. The positional identity along the anteroposterior (AP) axis is based off of the conserved, Homeobox (Hox) genes (Hueber et al., 2007). This is a commonality among all bilaterian animals, and these genes serve to regulate specific domains along the anterior-posterior axis (von Allmen et al., 1996). Organization of the genomic sequences and their outcome along the AP axis is known as the principle of colinearity (Maeda & Karch, 2009). For *D. melanogaster*, these Hox genes are displayed through defined patterns along the AP axis, distinguishing differing morphologies at the different body segments (Hueber et al., 2007). They are expressed from the 3' to the 5' directionally (Mallo et al., 2010). Expression of neighboring hox genes can be overlapping and expression of their domains can be controlled both individual or, in some instances, through co-regulation. In cases of co-regulation, there may occasionally be a loss of function with a particular Hox gene which can lead to the loss of a neighboring Hox gene as shown by Ed Lewis (Garcia-Bellido & Lewis, 1976; E. B. Lewis, 1978). Changes during this regulation process of Hox genes leads to significant understanding of underlying development and evolutionary comprehension of serial homologs. Additionally, small changes in these critical genes or in their regulation can lead to large shifts in the morphology of organisms.

Homeobox genes contain a highly conserved DNA-binding domain, the homeodomain, which, in their final protein product, serves to regulate and direct morphogenesis. Homeodomains are classified by these protein specific domains with a recognizable 60 amino acid DNA-binding domain (Bodmer, 2008; Garcia-Bellido & Lewis, 1976; Garcia-Fernàndez, 2005). Our current understanding and knowledge of these genes are limited through focused studies to better comprehend specificity and co-factors through interactions. It was over 30 years ago that Antonio Garcia-Bellido proposed a hierarchy of three classes of genes, activators, selectors known as modern day homeobox genes, and realizators that aid in cell differentiation (Garcia-Bellido, 1975; Hueber et al., 2007). Garcia-Bellido postulated that once a gene was activated, the selector genes would send a downstream gene, the realizators, that would encode the differentiator (Garcia-Bellido, 1975; Hueber et al., 2007; Kappen, 1996). Every gene is going to either activate or repress a possible development. It has been established that Hox genes encode specific morphogenesis functionality and shapes shown by Lewis and Garcia-Bellido (Garcia-Bellido & Lewis, 1976; D. L. Lewis et al., 2000). Molecular analyses support the proposition that these Hox genes are localized in the nuclei of cells in which they are expressed (McGinnis et al., 1984).

It is on this experimentally proven basis and understanding that we can discuss the clusters within *D. melanogaster* Hox genes, which are unique in their own evolution, and are divided into two distinct clusters on one chromosome: the Antennapedia (Antp) and Bithorax (Bx-C) complexes (Bodmer, 2008; Duncan, 1987; Furukubo-Tokunaga et al., 1993; Garcia-Fernàndez, 2005; Lemons, 2006; McGinnis et al., 1984). These two complexes are known as their own distinct Homeotic complex (HOM-C). The Antennapedia complex is composed of *Labial* (*lab*), *Proboscipedia* (*Pb*), *Deformed* (*Dfd*), *Sex-Combs reduced* (*Scr*), and *Antennapedia*

(*Antp*) (Furukubo-Tokunaga et al., 1993; Regulski, 1985). The Bithorax complex is composed of three distinct genes: *Ultrabithorax* (*Ubx*), *Abdominal-A* (*Abd-A*), and *Abdominal-B* (*Abd-B*) (Duncan, 1987; Gehring et al., 2009; Lemons, 2006). These 8 genes are the ancestral genes that have led to what we now understand as a complex networking pathway.

Among entomologists as well as evolutionary studies, genetic components that play a role in the development of appendages are integral to our understanding of the origin of modern insects. Appendage patterning genes have been studied in all four major groups of arthropods: chelicerates, crustaceans, myriapods, and insects (Hughes et al., 2004). Since Hox clusters can be found in all major superphyla, Hox clusters arose before the Cambrian explosion and are an ancestral part of the genetic toolkit of all animals (Gehring et al., 2009). Due to the growing breadth and depth of development of molecular data sets and expansion beyond model organisms, such as *Drosophila melanogaster*, it is now possible to identify paralogous and orthologous members of the Hox gene family and study expression patterns that are fundamental in understanding of ancestral patterns of genetic encoding (Force et al., 1999).

## Targeted Homeobox Genes and Downstream Genes

To better comprehend the possible ancestral patterns and mechanisms of key target developmental genes in *T. domestica*, we performed antibody studies and fluorescent *in-situ* hybridizations with the intent to characterize wing genes known from previously studied model organisms, and better understand their expression patterns in an apterygote insect.

### Engrailed (en1) and Engrailed 2 (en2)

Among homeodomain transcription regulatory factors, engrailed (en1) and engrailed 2 (en2), are involved in the development of segmentation among all species of Arthropoda. en2 is also known as invected (inv). En1 is a segment polarity gene, that is vital for segment

differentiation. Its function became apparent as it is expressed in stripes, in portions of the head, in the posterior spiracles, fat bodies, as well as in the hindgut. It is a key gene in the development of appendages and segmentation, where it differentiates the posterior portion of the body (Gustavson et al., 1996; Morgan, 2006; Peel et al., 2006; Simmonds et al., 1995). En1 plays a role at various stages, whether transcriptional or translational. It also plays a role in the development of both mid and hindbrain; en1 protein will also play a role in the compartmentalization of wings. Similarly, both en1 and en2 are needed for development in 14 stripes that will eventually associate to posterior compartments (Peel et al., 2006).

**Dacschund (dac)**

With a novel factor in development of neuroectodermal tissue and mesenchyme, dac plays a critical role in embryo development. This gene is commonly known for being part of the retinal determination gene network (RDGN), knockdown of dac expression alters eye morphology and causes wing specific defects (Zhou et al., 2010). It is also present in vertebrates suggesting an evolutionary conserved network, with potential to be traced to earliest evolution of metazoan development (Popov et al., 2010). Expression studies demonstrate that this gene is found in cellular proliferating areas such as limb buds (Caubit et al., 1999). Dac also plays a role within mushroom bodies found in densely packed fibers to organize neuronal structures within the brain (Kurusu et al., 2000). Other roles of dac include leg development within the proximodistal axis, and it is a key transcriptional regulator specifying the fate and segmentation of the legs.

**Abdominal-A (abd-A) and Abdominal-B (abd-B)**

Together alongside, ultrabithorax (ubx) they are going to form the Bithorax Complex (BX-C). Abd-A is required for the development of thoracic parasegments, encoding for a single

protein composed of 330 amino acids (Cumberledge et al., 1990). It is initially detected during germband extension but can be detected earlier through mRNA. At a gradient it shows strong expression with the most amount at the anterior fading to the posterior from segments 7 through 13 (Karch et al., 1990; Macias et al., 1990). Accumulation of abd-A can also be found in the CNS extending laterally through the embryo. Abd-B patterns posterior abdominal segments, similarly first detected in ectoderm during germ band extension (Kuziora & McGinnis, 1988). It is shown to have expression, also in gradient, during early embryogenesis in the ventral nerve cord. The expression gradient is strongest in posterior segments and weakens anteriorly (Tremml & Bienz, 1989). Within *T. domestica*, abd-B has been shown to be expressed in the cercal appendage primordia which is unlike other insects where it has been indicative in solely posterior segmentation (Peterson et al., 1999).

**Wingless (wg)**

There is conservation within wingless as it can be found conserved through patterning alongside en in a mutual activation interaction. For imaging purposes, it will be expressed as stripes of the segment that can connect historical fundamental mechanisms for most arthropods (Hughes & Kaufman, 2002). One of the first indications of its influence on wing development was a mutation displaying lack of one or both wings; first discovered in 1976 (Sharma & Chopra, 1976). This gene can first appear during blastoderm phase, while progressing to a stronger posterior band. As embryogenesis continues, it is going to extend expression migration along germband. Throughout embryogenesis it takes on distinct roles, such as during organogenesis when it aids in hindgut development or during blastoderm when it is primarily active in signaling (Vorwald-Denholtz & de Robertis, 2011).

**Sex-Combs Reduced (scr)**

First detected during gastrulation and present throughout embryogenesis; scr controls segment identities within two distinct portions: the labial portion as well as the first thoracic segment (Martinez-Arias et al., 1987). It accumulates within the midgut mesoderm as the beginning of the embryo contracts, while some of it can also be found accumulating within the first thoracic leg disc (Mahaffey & Kaufman, 1987). Its signal weakens as you get to the second and third thoracic leg disks. If imaged microscopically, scr can be seen in the dorsal region for fruit flies as well as the basal apterygote, *T. domestica* (L. K. Robertson & Mahaffey, 2017).

**Ultrabithorax (ubx)**

As the most anterior gene in BX-C, it is a critical role in thoracic and abdominal development, while repressing appendage growth (Castelli-Gair & Akam, 1995). With a genomic range of approximately 76 kilobases, encoding for five different protein variants in *Drosophila* (O'Connor et al., 1988). It can first be found expressed during blastoderm stage, with accumulation occurring in gastrulation phase of embryogenesis. It has a strong expression in the posterior and weakening expression through the anterior (Akam, 1998; Castelli-Gair & Akam, 1995). Expression occurs in both the somatic and visceral mesoderm as the germband expands. Studies manipulating the ectopic expression of ubx demonstrate partial wing structure to haltere phenotype transformation (Bender et al., 1983).

**Notch (N)**

Unlike the previously discussed target genes, Notch is distinct in conservation through metazoan serving as a transmembrane receptor alongside its perspective ligand. It is first noted during embryogenesis for cell fate regulation (Mishra et al., 2021). It is important to distinguish and develop imaging where it is expressed to better comprehend regulation of the pathway

(Trylinski et al., 2017). There are currently limitations to imaging for signaling. Notch also plays a maintenance role in neuroepithelial cells while inhibiting medulla neuroblast formation (Wang et al., 2011).

## Study Objective and Hypothesis

To fully understand embryonic development and evolutionary shifts between taxa we must understand these critical genetic components and their functional purpose. This serves to differentiate the various pathways and aids to distinguish which are more conserved than others. Due to the limitation of fossil evidence and lack of extant transitional forms, molecular data is crucial for understanding the facilitation of new adaptative novel structures that arise during embryogenesis. The first step in this process is to identify target gene homologs in T. domestica and begin to gather data regarding the spatial and temporal patterns of gene expression during embryogenesis. By targeting the genes above and using gene expression visualization methodology we can begin to more fully understand the underlying genetic framework that led to the origin of insect wings.

# CHAPTER III

# MATERIALS AND METHODS

## Culture of *Thermobia domestica*

Adult *Thermobia domestica* were reared within a maintained 37°C, humidified tank with a heated blanket in order to comfort the adults to a suitable mating environment. They were given wooden chips and water gel for dietary consumption which was changed every three days. In order to collect eggs, cotton balls were placed within the tank in order for them to deposit eggs. Eggs were then collected every 72 hours in order to capture eggs at the early stages of their segmentation and cell migration processes.

## Embryo Fixation

Eggs of *T. domestica* were placed in a 1.5 milliliter (mL) centrifuge tube to be bleach treated with 500 microliters (µl) of commercial bleach with 500 µl of embryo wash buffer for 5 minutes at room temperature. After the time was allotted, the bleach treatment was then removed from the centrifuge tube and washed with solely embryo wash buffer in order to remove any residual bleach from the tube. After decanting and adding of 1 milliliter of embryo wash, embryos were removed and placed on a watch glass under a dissecting microscope (Leica MZ10F). The eggs were partially dissected, which entailed a puncturing of the egg wall with small forceps and fine electro-sharpened wire dissection tools under the microscope. After all the eggs had been partially dissected, they were then placed into a clean 1.5 mL centrifuge tube with

400 µl of PBTween Solution, 100 µl of Formaldehyde, and 500 µl of Heptane. Eggs were left with slight agitation in the centrifuge tube at room temperature. The partial dissection served as a way for the fixative mixture to penetrate the egg. After 30 minutes of agitation and submersion in the fixative, the top layer of Heptane is decanted. Afterwards, 500 µl of methanol is added in order to prevent shock. The remaining solution is decanted and replaced with 100% methanol for 3 washes. The eggs are then ready to be frozen at -20˚C until needed to be used.

During the rehydration process, eggs that are stored in the -20˚C freezer are added with an equal amount of embryo wash to the 100% methanol to make it 1:1 for approximately 2 minutes. After the allotted time, the fixative is decanted, and they are then added into a 2:1 embryo wash to methanol fixture for another 2 minutes. The eggs were then washed in a 3:1 embryo wash to methanol fixture for 2 more minutes. After, they were then quick washed with 100% embryo wash. This process had to be done in small batches, in order to reduce error in dissections performed on watch glass under the microscope.

There were two distinct dissections performed throughout the process, partial dissection and full dissection. Partial dissection was completed in the beginning of the fixation process in order for the fixative to absorb through the vitelline layer. This was through poking a hole through the vitelline layer using forceps and fine electro-sharpened tools. Yolk from within the vitelline layer would escape distinguishing it had been properly partially dissected. Later when imaging and further experimentation was necessary, full dissection was completed which involved the removal of the vitelline layer from the opaque egg (Poprawa & Rost, 2004). This step is essential in order to view the differentiating mechanisms of segmentation and development through staining.

For staining purposes, DAPI (4′,6-diamidino-2-phenylindole) was used to determine cell morphology through the methanol fixation process. It allowed for fluorescent tagging for prolonged periods, which enabled microscopy to detect nuclear DNA due to the molecular structure of the stain, DAPI (Tarnowski et al., 1991). After the completion of full dissections and fixation, embryos were treated in 1µl of DAPI stain in 1000 µl of embryo wash buffer. Embryos were left to sit in darkness with slight agitation for 5 minutes before being rinsed with embryo wash buffer. Embryos could then be mounted on microscope slides with embryo wash buffer, covered with a coverslip sealed through nail polish. Imaging was completed through Olympus Fluoview FV10i confocal laser scanning microscope.

## RNA Extraction

For collection of *Thermobia domestica* transcriptome, eggs were collected within 24-72 hours of deposit. This method was meant to isolate and determine what transcripts were active within the beginning stages of morphogenesis. Embryos were homogenized within a sterile mortar using 200 µl of Trizol reagent and a 1.5 mL centrifuge tube. Another additional 800 µl of Trizol reagent was added for further homogeneity. Extraction of RNA was done using the standard protocol of the manufacturer. After the completion of the extraction of total RNA, quantification was done via spectrophotometry. The RNA is then separated into individual aliquots to be stored in -80°C until needed further.

## Bioinformatic analysis

In order to attain efficient transcriptome reconstruction assembly, *de novo* assembly allows for the ability of high specificity in identification of transcript structures (G. Robertson et al., 2010). Illumina TruSeq Stranded mRNA Library Prep kit was used for preparation and generation of library through the conduction of the manufacturer's standard protocol. After

acquiring the data from the generation, database library was then quantified for precision of RNA measurements. The library was pooled using “FastQC.exe” for possible problematic detection of partial sequences alongside CyVerse Discovery Environment, an online database and cloud. It was through this process that they can then be pipelined through a *de novo* assembly for the following software (Birger et al., 2017; Joyce et al., 2017). For the generation of reads, Trinity RNA-seq assembly goes through three distinct modular software components: Inchworm, Chrysalis, and Butterfly.

**Trinity RNA-seq Software**

During the first program of Trinity, Inchworm is able to assemble unique reading frame sequences of transcripts by using a greedy *k* nucleotide (*k*-mer) (M. G. ; Grabherr et al., 2013; Haas et al., 2013). Inchworm goes through six step processes in order to reconstruct linear contigs. It begins with a construction of *k*-mer dictionary from all the readings, followed by a removal of high error containing *k*-mers from the dictionary. Inchworm then attains the most frequently *k*-mer within the dictionary in order to further contig assembly(M. G. Grabherr et al., 2011; M. G. ; Grabherr et al., 2013; Haas et al., 2013). This is then extended to either direction until overlapping, further extending the sequence until it can no longer extend. Inchworm then begins with the next most frequent k-mer until it goes through all, deleting them from the dictionary as it goes through the process. This methodology allows for the recovery and selection of the best variant that shares *k*-mers.

In the second program, Chrysalis, there is a clustering of the overlapping contigs into componentry sets in order to construct de Brujin graphs (Haas et al., 2013). It is highly likely that each component is derived from closely related paralogs or alternatively spliced forms. There are three steps within Chrysalis. First, it must group inchworm components into overlapping

connections between them. Secondly, it begins to build de Brujin graphs based on each component using a *k*-1 for representation of nodes (Haas et al., 2013). The *k* is used to identify edges connecting, weighing each one with the number of original *k*-mers that support it. After this step, there is an assigning of each read to a specific component of which it shares the largest number of *k*-mers. This is in order to better determine which read contributes to the specific *k*-mer (M. G. Grabherr et al., 2011).

With the tertiary and final program, Butterfly is reconstructing full-length linear transcripts through the merger of the de Brujin graphs generated via Chrysalis. It is able to construct unique transcripts of differing spliced isoforms and paralogous genes. There are two parts to the methodology of Butterfly. The first is known as graph simplification, Butterfly goes between merging consecutive nodes in the de Brujin graph in order to form longer sequences (Haas et al., 2013). Simultaneously, Butterfly is also employing a pruning mechanism in order to rid of sequential errors in the lengthy transcriptomes. This mechanism is due to the sequencing errors, but also due to the diploid polymorphism which are more likely to occur and be kept maintained throughout the process (Haas et al., 2013). The second part of Butterfly is known as plausible path scoring, it is a dynamic methodology to read potential pathing that is supported. This helps in reduction of possible paths to a small number of actual transcripts by resolving ambiguities (M. G. ; Grabherr et al., 2013; Haas et al., 2013).

After assembly a local blast database was created using the program makeblastdb, target wing genes were then used as query sequences and homologous T. domestica genes were identified via a BLASTX search. These homologs were then verified by BLASTX search against all available insect gene sequences. Verified wing gene homologs were amplified via PCR with

primers generated through the program Primer3, the identifiable genes were chosen by the maximum size of the targeted product length sequence that was available.

**TOPO TA Cloning**

PCR reactions that yielded a distinguishable clear product were cloned into an expression vectored using the Invitrogen TOPO TA Cloning kit. Beginning with the removal of the antibiotic plates from the 4 fridge and placing them at room temperature to warm. While the plates warm, remove the chemicompetent bacteria from the -80˚C freezer, placing it directly on ice. Usage of 25 µl of bacteria will be used for each cloning reaction. Removal of 150 µl of SOC Medium (composed of: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) per reaction from the 4˚C refrigerator in order to warm at room temperature. To set up each cloning reaction: 2 µl of PCR Product, along with ½ µl of salt solution and ½ µl of the vector. Mix it gently and incubate at room temperature for approximately 5 minutes. After, place it on ice for 2 to 3 minutes. Add 25 to 50 µl of competent bacteria and mix gently, then place it on ice for 30 minutes. Heat shock is subsequently followed at 42˚C for 30 seconds and then incubated in ice for 5 minutes. Followed with transferring of 150 µl of SOC Medium into each reaction tube, transferring the entire mix into 0.65 µl centrifuge tubes. Incubate the centrifuge tubes at room temperature with slight mixing for agitation. Spread the reaction onto X-Gal (5-Bromo-4-Chloro-3-Indolyl-beta-D-Galactoside) and IPTG (isopropyl beta-D-1-thiogalactopyranoside) mixed plates (40 µl of X-Gal and 8 µl of IPTG). After, incubate the plates at room temperature overnight before analysis.

**Fluorescent *in-situ* Hybridization**

The protocol used for this procedure was taken from Legendre and team with modifications in order to better suit the organism, *T. domestica*. Previously, it was based on *D.*

*melanogaster* but due to differing embryology there was a necessity for adjustment (Legendre et al., 2013). The final procedure conducted took course over three days, with the final day allowing for the confocal imaging as well. As a model for the experiment, the set control was using *Drosophila melanogaster* as the basis of comparison. At the start of the first day, there will be a 500 µl of the fully dissected *T. domestica* embryos that are being held in a freezer with methanol into a clean 1.65 µl centrifuge tube. To prevent shock to the embryos tissue, they underwent several dilution washes. The first two washes within the new tube was decanting the methanol and replacing it with 1.5 µl of 100% methanol with 5 minutes each for incubation in solution. It underwent a 1:1 PBTween Solution to methanol fixative, single wash for 5 minutes. The last of the initial washes was for 5 minutes, with decanting the former fixative and replacing with 1.5 µl of pure PBTween Solution. This final wash occurred twice.

As the initial washes had taken place, the intermediate solution was prepared by the addition of 2 µl of proteinase K (PK) stock solution (20mg/ µl) to 1000 µl of the PBTween solution and stored at -20°C. After the washes had been completed, a working solution of the PK solution was made through the addition of 100 µl of the intermediate solution to 1.4 µl of PBTween. The embryos sat in the centrifuge tube incubating in the working PK and PBTween solution at room temperature for 15 minutes with gentle inversions. The centrifuge tube was then incubated for an hour on ice without further mixture. After this second incubation period, there was another series of washes. They began with decanting of the PK solution and washed it twice for 2 minute incubations with 2 mg/ml of glycine. After, it was removed and then replaced for three quick washes with PBTween Solution.

After the quick washes, embryos were incubated for approximately 20 minutes within a postfix solution with gentle mixing. The postfix solution was composed of the addition of 10 ml

of paraformaldehyde (PFA) to 30 ml of PBTween solution. Following the incubation period, embryos were then washed 3 times for 2 minutes each time with solely PBTween solution. The T. domestica embryos were then washed with a 1:1 ratio of hybridization solution (50% formamide, 5x SSC, 0.1% Tween-20, 100 g/ml of sheared salmon sperm DNA, and 100 µl/ml Herparin) and PBTween solution. For the purposes of this particular study, we did 500 µl of each solution. The hybridization buffer was activated through boiling for 5 minutes and then cooling in ice for another 5 minutes. Embryos were washed with 500 µl of solely the hybridization buffer twice.

Once the embryos were incubated within the hybridization buffer, they were separated into estimated equal amounts within smaller PCR tubes that would continue to be used for the remainder of the procedure. During the transfer process of the embryos to the PCR tubes, the tip of the micropipette was cut slightly above due to the narrowness and roughness on the tissue. The amount of tubes used was dependent on the amount of genes chosen to be studied, in this particular study those chosen genes were: Distalless (Dll), engrailed 1 (en1), and engrailed 2 (en2). After the transfer into the PCR tubes, they were decanted and replaced with 100 µl of hybridization solution. PCR tubes were then incubated for 3 hours at 56˚C. Succeeding this, 5 µl of the previously prepared RNA fluorescent probe was added to 100 µl of the hybridization buffer. Incubation then took place at 80˚C for approximately 5 minutes and then cooled on ice. The last step of day one was decanting of the contents within the PCR tubes and replacement of 100 µl of the Probe and PBTween Solution to be incubated overnight at 56˚C.

At the start of the second day, the continuation begins with dilution preparatory washes for each of the PCR tubes. The required fixative solution was hybridization solution mixed with PBTween solution at 56˚C with 100 µl for each of the PCR tubes reaction. For the first wash, it

was at a 4:1 ratio of hybridization buffer to PBTween solution at 56˚C for 15 minutes. The second wash was at a 3:1 ratio at 56˚C for 15 minutes of hybridization buffer to PBTween solution. The third wash was at a 1:1 ratio also at 56˚C for 15 minutes of hybridization buffer to PBTween solution The fourth and final initial long wash was at a 1:3 ratio of hybridization buffer and PBTween solution at 56˚C for 15 minutes. These four long washes are followed with three quick washes of pure PBTween solution for 5 minutes each wash.

During the beginning washes, antibody solutions and Streptavidin-HRP conjugate as well as HRP-conjugated mouse monoclonal anti-DIG are prepared. There is 100 µl of antibody solution prepared for each of the PCR tube reactions with a concentration ratio of 1:400 of PBTween solution to 2% BSA solution (500 ml of PBTween solution/1 g of BSA). For this particular study, AlexaFluor 647 Anti-Mouse Antibody was used. This antibody was stored at 4˚C until it was time to be used in the procedure. The Streptavidin-HRP conjugate was prepared through the addition of 1 µl of stock Streptavidin-HRP conjugate to 1000 µl of the PBTween solution with 2% BSA.

After the three quick washes and the solutions had been prepared, the embryos within the PCR tubes were blocked through PBTween and 2% BSA for 20 minutes. During this blocking incubation period, tubes were gently rocked through the process. The PCR tubes were then decanted and an addition of 100 µl of the previously prepared antibodies were added, this incubation period was for 2 hours on the rocker at a gentle mixing speed. After the 2 hour incubation time was allotted, the embryos were washed in a series of washes with PBTween solution and 2% BSA. The first wash after the 2 hours was a quick wash, followed with 3 washes for 5 minutes each, then 5 washes for 10 minutes each. The embryos were then incubated within 100 µl of Streptavidin-HRP conjugate and incubated for 1.5 hours in the dark with gentle

mixing on the rocker. The PCR tubes were wrapped in layers of aluminum foil in order to block any light from the reaction. After the time and incubation period, embryos were taken out of the aluminum foil and washed twice for 5 minutes each with PBTween solution and 2% BSA.

At the end of the second day in the procedure, there is a DAPI stain step. It is prepared through the ratio of 1:1000 which is correspondent of DAPI to PBTween solution. Under the laboratories standardized DAPI staining protocol, there was 100 µl of DAPI into each of the reactions within the PCR tubes. They protocol states for the reactions to incubate and be gently mixed for 15 minutes. The PCR tubes were decanted and washed 4 times for 10 minutes each in solely PBTween solution. Embryos were ready to be stored overnight within the 4°C

At the start of the third day of the fluorescent in situ hybridization, the protocol begins with 3 washes within PBTween solution for 5 minutes each wash. As the washes took place, the C3-Tyramide activation buffer as well as working solution were prepared. For the preparation of the activation buffer, we used 0.006% $H_2O_2$ with a concentration ratio of 1:500 of $H_2O_2$ to PBTween solution. The working solution was made for 100 µl per each reaction tube. After the initial 3 washes, the PCR tubes were decanted and 100 µl of the Tyramine working solution was inserted to each of the tubes for 2 hours of incubation. The tubes were wrapped in multiple layers of aluminum foil and kept in the dark for the incubation period. After the time had been allotted, the embryos were then washed four times quickly in PBTween for 5 minutes each. Then after those washes, the PCR tubes were decanted and were added PBTween solution for 6 washes at a time of 10 minutes per wash. Following this it was the last set of three washes at a time of 5 minutes each with PBTween solution. After the set of washes of the third day, the embryos within the PCR tubes were ready to be mounted on the microscope slide and ready to be sealed for imaging to occur at the Olympus Fluoview FV10i confocal microscope.

**Synthesis of RNA Fluorescent Labeled Probes**

First, begin with linearizing plasmid DNA through the performance of PCR with M13 Forward and Reverse Primers of the cloned gene. After this step, clean up the PCR reaction via ethanol precipitate and resuspend the DNA in 25 µl double distilled $H_2O$. There will be an addition of 1 µl of 0.1 M Dithiotreitol (DTT) as well as 9 µl of nuclease free $H_2O$. For the production of each probe, there will be: 20 µl of Nuclease free $H_2O$, 4 µl of 5x transcription buffer, 1 µl of T7 RNA polymerase promoter or 1 µl of Sp6 RNA polymerase promoter to those perspective tubes, 2 µl of 10x RNA nucleotide, 200 ng of linearized DNA, 1 µl of RNAse OUT, and then 1 µl of RNA polymerase. Mix the various components with slight agitation and incubate at 40°C for approximately 2 hours for the Sp6 probes, with the T7 probes being incubated at 37°C for 1 or 2 hours.

After there would be an addition of 1 µl Deoxyribonuclease (DNase I), swiftly mix the components, and then incubate at 37°C for 15 minutes. Add 79 µl of nuclease free $H_2O$ and vortex for at maximum 10 seconds. After, there will be additional 400 µl of binding buffer with mixing. This is when you add the entirety of the contents to the spin column and centrifuge at 10,000 revolutions per minute (RPM) for 1 minute. Wash the column with 650 µl of wash buffer, spin the contents, and discard the flow through. Centrifuge the remaining contents at 10,000 RPM for 1 minute in order to dry the column. Transfer into the collection tube, add 55 µl of elution buffer and incubate at room temperature for a minute. Centrifuge at 10,000 RPM for a minute in order to attain and collect the purified RNA. After, there will be an addition of 10 µl of 3M NaAc (pH 5.2), 1 µl glycogen, 39 µl of nuclease free $H_2O$, 300 µl of 100% ethanol. Incubate this at -20°C for approximately 30 minutes. Centrifuge at 10,000 RPM at 4°C for 10 minutes, afterwards decanting through the removal of the supernatant. Rinse the pellet that remained with

cold 70% ethanol, remove the residual ethanol, and air dry for 5 minutes. Add on an additional 11 µl of nuclease free $H_2O$, incubating at 37˚C for 5 minutes.

Vortex it in order to resuspend the contents. It can then be stored at -20˚C for up to two weeks, otherwise there is a continuation of the process. Incubate 5 µl of the 200 ng/µl RNA at 65˚C for 5 minutes, then place on ice for an additional 3 minutes. Probes concentrations were then measured using the Thermo Scientific Fisher Nanodrop 1000 Spectrophotometer in order to calculate size. Agarose gel electrophoresis with a standard DNA ladder were run using a 5 µl sample of the probe for verification of synthesis for probe size. Probes were then diluted using DEPC water to a concentration of 100 ng/µl and stored in -80˚C freezer until needed for the fluorescent *in situ* hybridization.

**Antibody Staining**

After obtaining *T. domestica* embryos that have been fully dissected, there is a rehydration process in mixtures of Methanol and PBTween Solution. There is a wash of frozen eggs, in order to rehydrate them beginning with a 75% methanol with 25% PBTween Solution for 2 minutes. Consecutively, then it becomes a 50% methanol with 50% PBTween Solution wash for 2 minutes. After, a 25% methanol and 75% PBTween Solution for another 2 minutes. It then gets decanted and wash 3 times using PBTween Solution. Embryos are then blocked using a PBTween with 2% Bovine serum albumin (BSA) solution, incubating for 1-2 hours at room temperature. This is followed with an incubation within the primary antibody for either 2 hours at room temperature or overnight within the 4˚C refrigerator (Table 2). After this incubation, there is then 3 quick washes using PBTween and 2% BSA Solution followed with 3 slow washes that need to be incubated for 30 minutes each.

It can then either be incubated for 2 hours at room temperature or overnight within the 4°C refrigerator while the embryos sit in the secondary antibody. The secondary antibody is diluted in 1 µl of the studied antibody per 200 µl of AlexaFluor 647 Anti-Mouse Antibody. After this secondary antibody incubation, there is 3 quick washes in PBTween Solution following with a 10 minute incubation where there is 1 µl of DAPI per 500 µl of PBTween. Subsequently, there are 3 washes of 30 minutes each with solely PBTween Solution before mounting and visualizing using the Olympus Fluoview FV10i confocal microscope.

# CHAPTER IV

# RESULTS

## Transcriptome Assembly and Sequencing

Searching the local T. domestica transcript database identified 36 target genes. Biomarkers identified were as followed: ultrabithorax (ubx), Caudal (Cad), engrailed 2/invected (en2/inv), vestigial (vg), apterous (ap), Dachshund (Dac), decapentaplegic (dpp), homothorax (hth), hedgehog (hh), tiptop (tio), teashirt (tsh), serrate (ser), abdominal-A (abd-A), abdominal-B (abd-B), antennapedia, Distalless (Dll), engrailed (en), wingless (wg), fringe (gng), dorsal (dl), Notch (N), scalloped (sd), aristaless (al), spitz (spi), escargot (esg), snail (sna), sex combs reduced (scr), extradenticle (exd), bifid (bi), cut (ct), and scute (sc).

Following the identification of the various transcripts, the usage of MEGAX software was used in order to further analyze the sequences at an amino acid level (Kumar et al., 2018). Length of the identifiable biomarkers were: *Ubx* 1170 bp, *Cad* 1284 bp, *Inv* 1731 bp, *Vg* 1362 bp, *Ap* 1410 bp, *Dac* 3219 bp, *Dpp* 1767 bp, *Hth* 1419 bp, *Hh* 1416 bp, *Tio* 3075 bp, *Tsh* 2865 bp, *Ser* 4224 bp, *Abd-A* 993 bp, *Abd-B* 813 bp, Antennapedia 894 bp, *Dll* 1044 bp, *En* 1659 bp,*Wg* 1411 bp, *Fng* 1239 bp, *Dl* 2034 bp, N 8112 bp, *Sd* 1113 bp, *Al* 1227 bp, *Spi* 1653, *Esg* 3082, *Sna* 1668, *Scr* 4140, *En* 2465, *Exd* 2386, *Bi* 4048, *Ct* 10437, and *Sc* 1438 bp (Table 2).

## Fluorescent *in-situ* Hybridization

After multiple *in situ* trials using *Distal-less* (*Dll*), *Enrailed 1* (*En1*), and *Engrailed 2* (*En2*) as probes, we were unable to have a successful trial. This prevented us from attaining

positive signals from the targeted genes through confocal imaging. Probes were successfully produced and verified through gel electrophoresis.

## Antibody Morphology

For imaging purposes, the DAPI staining worked to indicate the nucleus within developing embryonic tissues. We were able to acquire various developmental stages as well as seeing the initial formation of the head and abdominal segmentation. With the inclusion of antibody staining, we were able to visualize cellular movement pertaining to their specific targeted gene. Initial formation of the embryology and appendages is supported and indicated through previously studied organisms and conserved within *D. melanogaster*. Genes including: ultrabithorax (ubx), abdominal-A (abd-A), abdominal-B (abd-B), wingless (wg), spitz (spi), sex combs reduced (scr), Dachshund (Dac), and Notch (N) were all found to be present through imaging.

# CHAPTER V

# DISCUSSION

## Transcriptome and Bioinformatics

By studying the genome in various development stages to comprehend the growth and metamorphosis of the Zygentoma, we can better understand the interactions occurring to better understand conservation within the genome to aid in the formation of wings. With so few studies done using *T. domestica*, there is a major gap in the phylogenetically critical group Apterygota. If we are to better comprehend the novel structure, we need to understand the ancestral genes and gene expression that could have been the precursor to the origin of wings. Zygentoma's relationship as the sister taxon to the Pterygota makes them important in comprehending early movement to land and significant in the acquirement of wings. As most phenotypic changes can often be traced to specific changes within their pertaining gene regulatory network. Programs such as Trinity and MEGA gave insight to suitable references within the genome that allowed for comparison among more popularly studied insects.

Through the bioinformatic portion, we were able to comprehend historically foundational genetic networking pathways and shed light to precursors of the wing. While we were able to identify targeted developmental genes, some of the transcriptomes generated were incomplete. This indicates that while some presence of Hox sequences were present in the genome, the missing portions could be due to various reasons. This could mean that there may have been degradation or potential error when doing the initial RNA sample, but there could have also been

miscalculation during the generation of the library database, as well as insufficient depth when it comes to recovering the target genes within the genome. In order for the comparison of the transcriptome generated to be conducted, we have to use software readily available as well as online databases. While simultaneously another potential for error, we must also be aware that the sequences generated are going to misalign potential motifs of targeted genes. Through the portions of the Hox genes that were generated through Trinity, we have found historical biomarkers and valuable transcripts that indicate homologs of all major wing development genes are present in *T. domestica*. It is through the relatedness of the genomes compared that we could distinguish divergent portions of the sequence but also analytically visualize the amino acids. Sequences that were able to be linked to other closely related homology are essential as the growth in molecular data gives insight to historical data that has been unattainable through fossil records.

### ***in situ*** **Troubleshooting and Antibody Imaging**

When compiling previously studied evolutionary development studies, there are more commonly used model organisms where the majority of the data comes from. As molecular techniques improve, insects such as *D. melanogaster* remain well studied and established popularly, while most of the extant species remain widely overlooked. Basal clades, especially when studying wing origination, within hexapod phylogenies are key in understanding historical gradualism occurrence that would have shaped the evolutionary diversification of Insecta. It's important to begin including extant species, such as those within *Zygentoma,* to data sets for better understanding ancient characteristics to their specific subsets, especially through their developmental stages.

Through the *in situ* experiment, we failed to visualize the targeted genes with our primary issue being excessive non-specific staining across the embryo. Often, it was too bright and would not allow us to measure the specificity. As a way to mediate this issue, blocking has been tested at different allotted times to modify the protocol. With *Thermobia domestica*, there is limited information on the imaging process. In order to help mitigate the loss of *T. domestica* eggs, modification of the standardized protocol through *D. melanogaster* will aid in resolving non-specific binding. Once they are resolved, this will allow for better description and visualization of the spatial and temporal distribution of genes during the various stages of development. Importantly, it will give comprehension in narrowing down specific pathways that would lead to the development of the novel wing structure.

The inclusion and development of protocol for new organisms presents a challenge, however it is critical if we are to elucidate the origin of key insect structures. As an organism that has very little methodology and generated data, it is important to establish new protocols to accommodate the differences of the species. All species are going to require differences with their methodology, but by being able to modify previously studied organisms, one can distinguish their perspective challenges. With *T. domestica* for instance, the dissection process of the egg is difficult to prepare and fix. It requires meticulously discarding the separating the hardened portion of the egg to the mesenchyme tissue within it to properly image it. Otherwise, the hardened egg will not allow for imaging and proper visualization. Through the antibody staining, we were able to identify gene localization of critical body planning. DAPI gave a generalize perspective, but we were able to localize patterns through antibody staining. Imaging through this process was supportive of co-option as a way to historically comprehend regulation to generating novel phenotypes through their similar development segmentation.

## Progression of the Evo-Devo Approach

To further study this, one would need to do further expansive studying of their sequences by attaining a bigger data set. There needs to be a bigger phylogenetic analysis to understand clades and their respective genetic divergences. Furthermore, there also needs to be more statistical analysis to indicate if there is a significant relationship of common motifs. In order to understand this further, there needs to be further breakdown of the network of transcription activation as well as repression of limb formation.

In order to fully understand the embryogenesis, there has to be an investigation of repressing as well as creating mutant versions of genes found in Arthropoda to distinguish them from one another for their features. There also is evidence of a natural selection due to fitness being correspondent to the alteration of the body plan; Since *T. domestica* are closely related to Crustaceans, it is important to widely study the potential variations throughout the phylogenies and keep it monophyletic from an evo-devo standpoint of limb development. It's through the innovation of further evolutionary development studies that there can be better comprehension of early transitional fragments of insect wings.

**Table 1. Antibody Genes tested for distinguishable patterning.** Genes listed are those that were targeted among *T. domestica* for this studies purpose. Also listed alongside are the serial names of the specific primary antibodies used in order to acquire signaling, as well as the host organism for primary and secondary blocking of AlexaFluor 647 Anti-Mouse Antibody.

| ***T. Domestica* Targeted Gene** | **Antibody Serial** | **Host** |
|---|---|---|
| dachshund (dac) | mAbdac 1-1 | Mouse |
| abdominal-A (abd-A) / ultrabithorax (ubx) | FP6.87 | Mouse |
| abdominal-B (abd-B) | 1A2E9 | Mouse |
| ultrabithorax (ubx) | FP3.38 | Mouse |
| sex-combs reduced (scr) | 6H4.1 | Mouse |
| notch (n) | C17.19C6 | Mouse |
| wingless (wg) | 4D4 | Mouse |
| cut (ct) | 2B10 | Mouse |
| engrailed (en) / invected (inv) | 4D9 | Mouse |

**Table 2. Length of identifiable biomarkers.** The genes listed on the table below display conserved genes that can be seen through various species and their perspective gene network. While not all of the genes are pertaining to the acquisition of wings, they play a role within the development of the embryo.

| **Identifiable biomarker of *T. domestica*** | **Basepair (bp) length of sequence** |
|---|---|
| ultrabithorax (ubx) | 1,170 bp |
| caudal (cad) | 1,284 bp |
| invected (inv) | 1,731 bp |
| vestigial (vg) | 1,362 bp |
| apterous (ap) | 1,410 bp |
| dachshund (dac) | 3,219 bp |
| decapentaplegic (dpp) | 1,767 bp |
| homothorax (hth) | 1,419 bp |
| hedgehog (hh) | 1,416 bp |
| tiptop (tio) | 3,075 bp |
| teashirt (tsh) | 2,865 bp |
| serrate (ser) | 4,224 bp |
| abdominal-A (abd-A) | 993 bp |
| abdominal-B (abd-B) | 813 bp |
| antennapedia (antp) | 894 bp |
| distalless (dll) | 1,044 bp |
| engrailed (en) | 1,659 bp |
| wingless (wg) | 1,411 bp |
| fringe (fng) | 1,239 bp |
| dorsal (dl) | 2,034 bp |
| notch (n) | 8,112 bp |
| scalloped (sd) | 1,113 bp |
| aristaless (al) | 1,227 bp |
| spitz (spi) | 1,653 bp |
| escargot (esg) | 3,082 bp |
| snail (sna) | 1,668 bp |
| sex-combs reduced (scr) | 4,140 bp |
| engrailed (en) | 2,465 bp |
| extradenticle (exd) | 2,386 bp |
| bifid (bi) | 4,048 bp |
| cut (ct) | 10,437 bp |
| scute (sc) | 1,438 bp |

**Table 3. Regulator genes and their prospective HOM-C.** As displayed on the table below, the regulatory genes studied for this analysis are going to correspond to specific HOM-C ancestral genes in either repression or activation mode. Their biological function is shown through various methodologies as previously proven. Verification of function was based off of http://www.flybase.com/ database and also attained from L. K. Robertson & Mahaffey, 2017

| Regulator Gene | HOM-C gene | Biological function |
|---|---|---|
| Involved in activation | | |
| engrailed (en) | deformed (dfd), labial (lab) | Segmentation |
| wingless (wg) | labial (lab) | Imaginal and embryonic developmental |
| sex-combs reduced (scr) | proboscipedia (pb) | Identify and express within the head and anterior thorax |
| distalless (dll) | proboscipedia (pb), deformed (dfd), sex-combs reduced (scr), ultrabithorax (ubx), abdominal-A (abd-A), abdominal-B (abd-B) | Dorsal appendage development |
| Involved in repression | | |
| engrailed (en) | ultrabithorax (ubx) | Segmentation |
| wingless (wg) | labial (lab) | Imaginal and embryonic developmental |
| abdominal-A (abd-A) | labial (lab), proboscipedia (pb), deformed (dfd), sex-combs reduced (scr), antennapedia (antp), ultrabithorax (ubx) | Anterior abdominal segmentation |
| abdominal-B (abd-B) | labial (lab), proboscipedia (pb), deformed (dfd), sex-combs reduced (scr), antennapedia (antp), abdominal-A (abd-A) | Posterior abdominal segmentation |
| sex-combs reduced (scr) | labial (lab), deformed (dfd), proboscipedia (pb) | Identify and express within the head and anterior thorax |
| ultrabithorax (ubx) | labial (lab), proboscipedia (pb), deformed (dfd), sex-combs reduced (scr), antennapedia (antp) | Cell fate and organ identification |
| distalless (dll) | proboscipedia (pb), deformed (dfd), sex-combs reduced (scr), ultrabithorax (ubx), abdominal-A (abd-A), abdominal-B (abd-B) | Ventral appendage development |

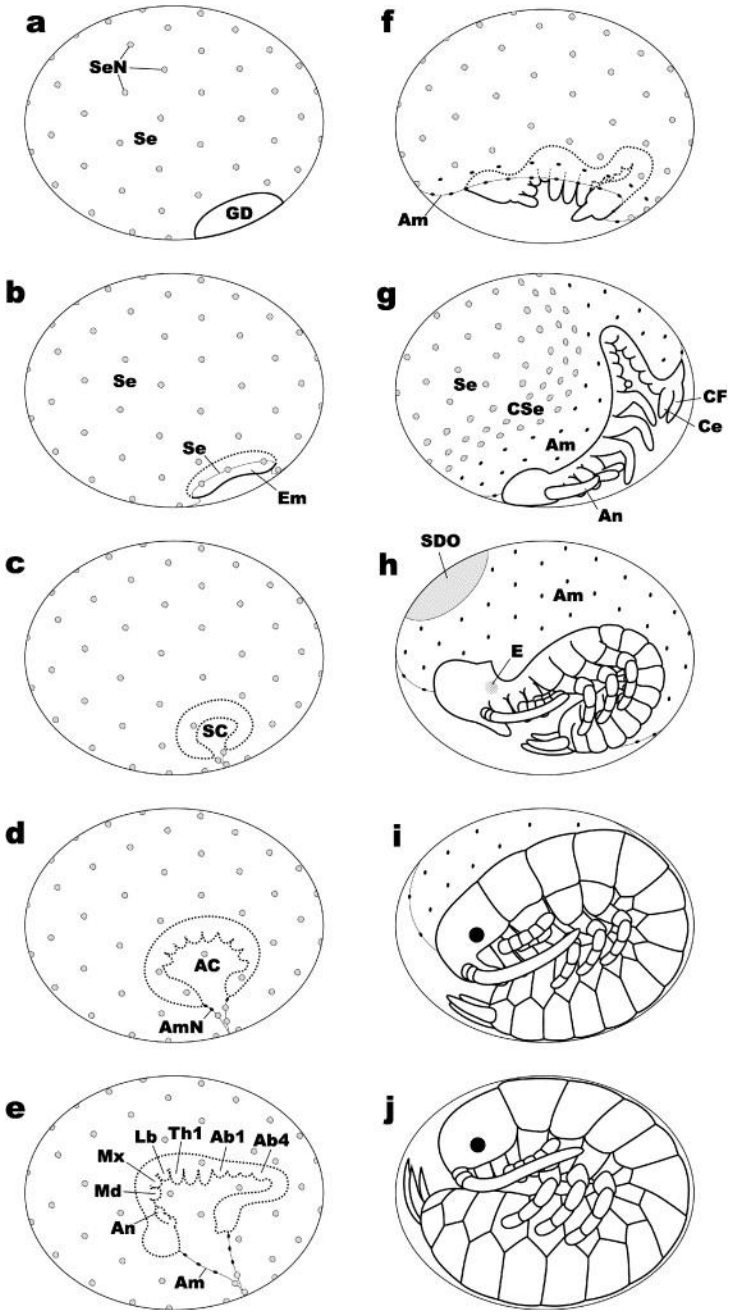


**Figure 1. Embryology of Zygentoma.** Displaying a diagram representing embryogenesis of *Zygentoma* from a lateral view. Ab 1, 4, corresponding to abdominal segments; AC, amniotic cavity; Am, amnion; AmN, amniotic nucleus; An, antenna; Ce, cercus; CF, caudal filament; CSe, condensed serosa; E, eye; Em, embryo; GD, germ disc; Lb, labium; Md, mandible; Mx, maxilla; SC, serosal cavity; SDO, secondary dorsal organ; Se, serosa; SeN, serosal nucleus; Th 1, first thoracic leg. Credit for image and information: Masumoto & Machida, 2006

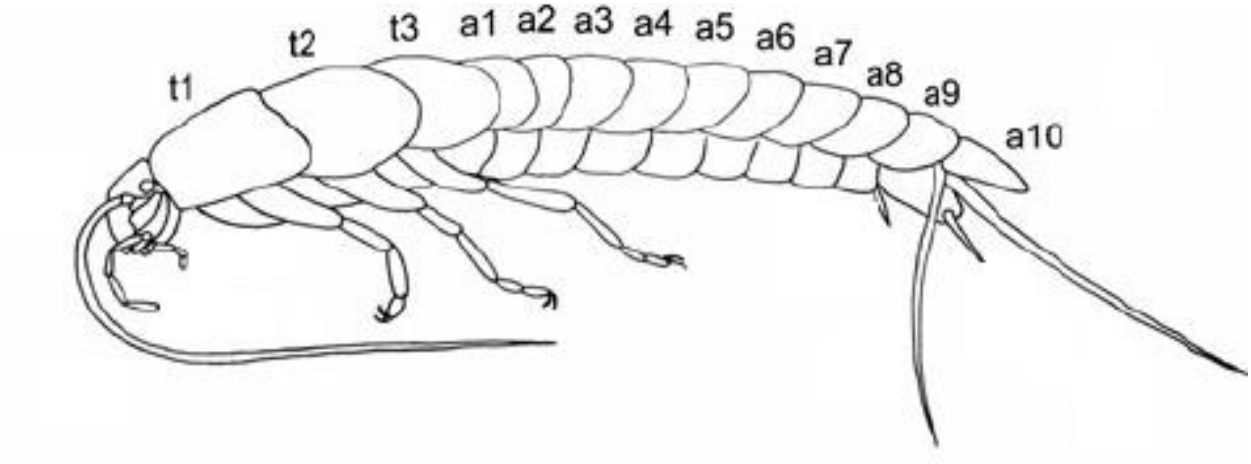


**Figure 2. Overview of body segmentation for Firebrat.** Lateral view of classic *Zygentoma* body plan, *Thermobia Domestica*. T is for the thoracic segments; A is for abdomen segments. Credit for Image: Schaeper et al., 2013

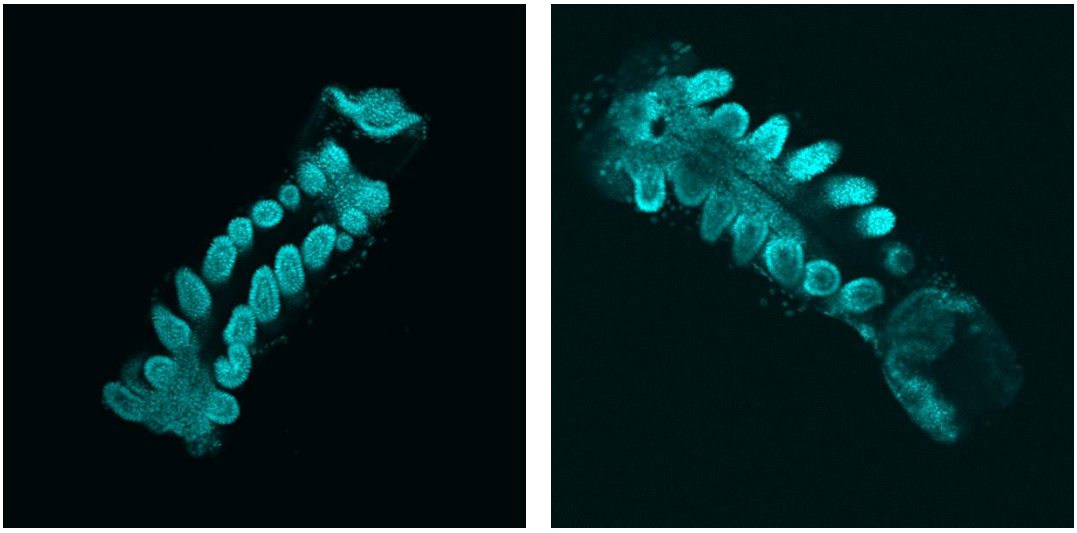

**Figure 3. DAPI staining of studied organism, *Thermobia domestica*.** In the figures above, *T. domestica* is fluorescently displayed using DAPI (4′,6-diamidino-2-phenylindole) at a late stage of development from a ventral view.

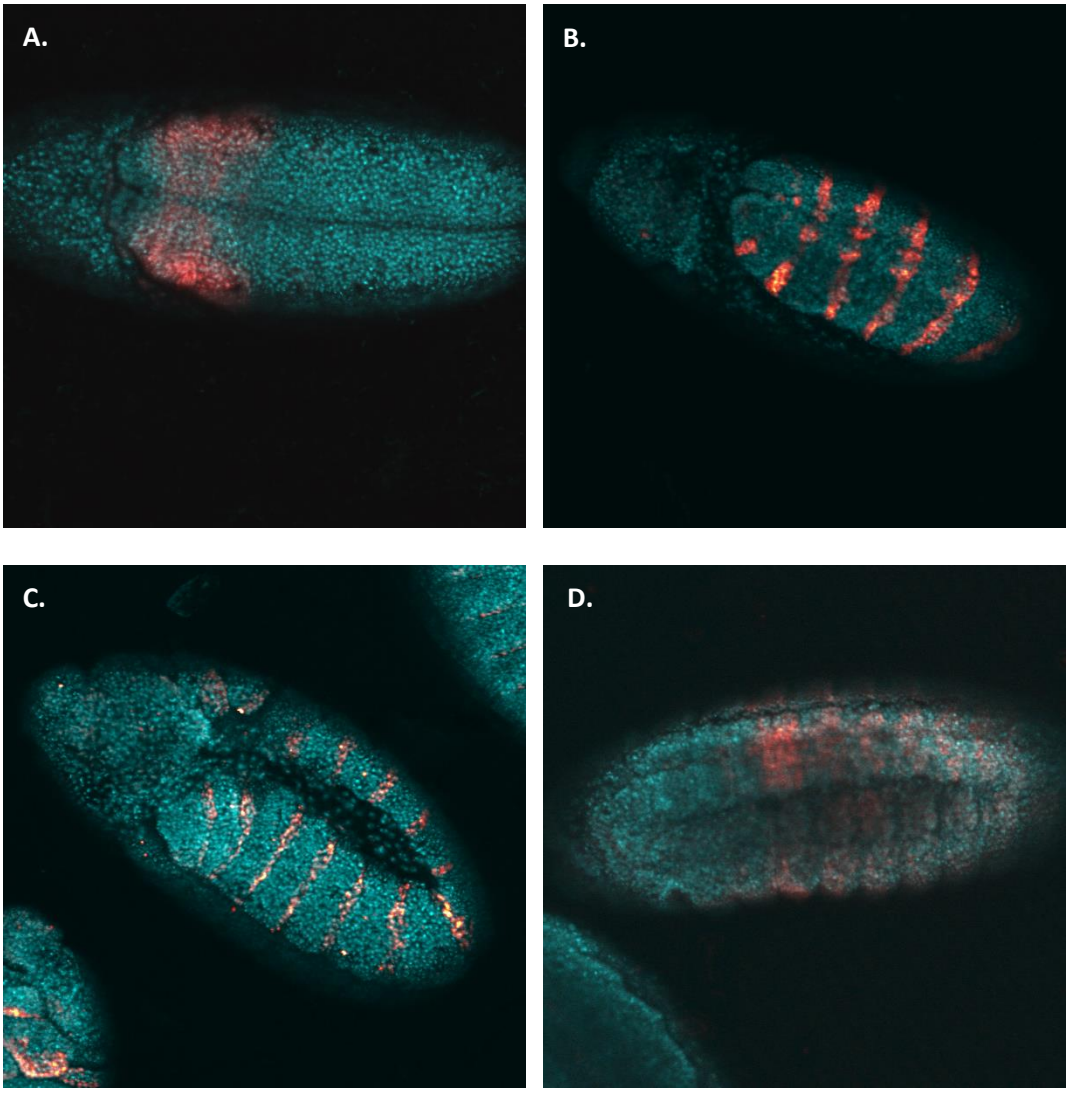


**Figure 4. Control anti-mouse trials of potential target genes.** Before beginning initial antibody trials, as a control *Drosophila melanogaste*r embryo were taken through the methodology. As displayed above, *D. melanogaster* is shown to have activation of regulator genes *Abd-A*, *En*, and *Ubx;* A, *Abd-A;* B, *En;* C, *En;* D, *Ubx*

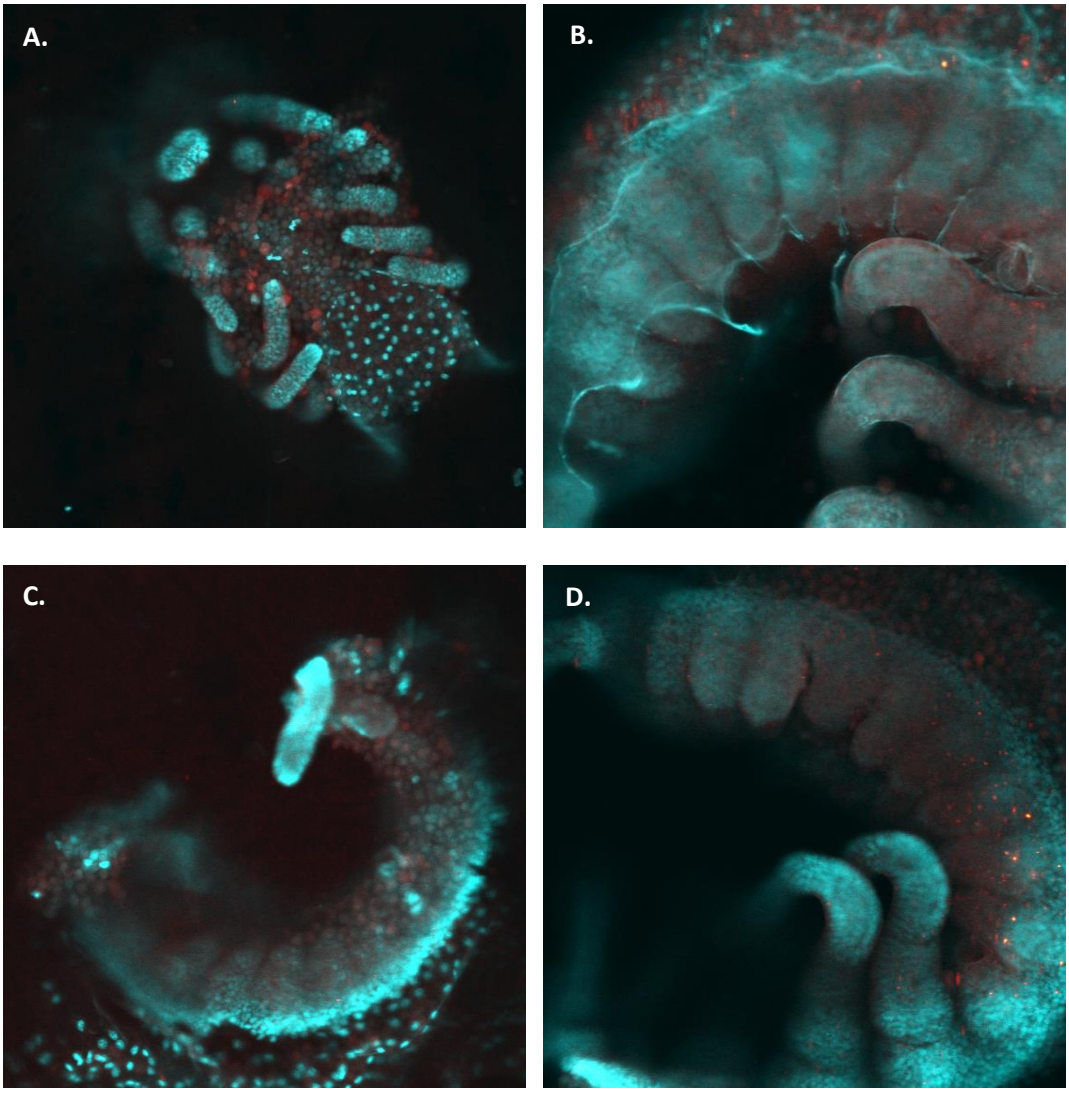


**Figure 5. Target Genes shown to be present through microscopy.** Through conduction of antibody Anti-Mouse studies using *T. Domestica*, we were able to distinguish expressional patterns and presence of gene regulatory pathways. A, *Abd-A*; B, *Wg*; C, *Scr*; D, *Spi*

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# BIOGRAPHICAL SKETCH

Luis Cantu Jr. is a graduate from the University of Texas Rio Grande Valley with a Masters of Science in Biochemistry and Molecular Biology earned in the Fall of 2021. He obtained his Bachelor of Science in Biology with a Secondary Major in Psychology earned in Fall 2017 from the University of Texas Rio Grande Valley. Shortly after graduation, he found himself working full time at South Texas College as a Laboratory Specialist II for the Biology Department. This allowed him to work with individuals from various educational backgrounds which motivated him to pursue further studies. During his time as a graduate student, he had the opportunity to work as both a Graduate Research Assistant under Dr. Matthew Terry, as well as a Graduate Teaching Assistant.

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