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Early Embryology in Collembola with an Emphasis on Wing Development

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EARLY EMBRYOLOGY IN COLLEMBOLA WITH AN EMPHASIS ON WING DEVELOPMENT

A Thesis

by

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ABSTRACT

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The primary objective of this study was to further the understanding of the wing origin in insects. Currently there are several theories which are popular such as the tergum theory, the pleural plate theory, and the dual theory of wing origin. Studies for these theories have been done on crustaceans, the ancestors to modern day insects. By utilizing a pre insect hexapod, it may be possible to better understand the transition to insect wings. Members of the Collembola species Hypogastrurid are one of these pre-insect hexapods which may provide a clue into origins of wing development. During this study, the expression of *Engrailed, Distal-less, Dachshund, Abdominal – A, Abdominal – B, Cut, Notch, Ultrabithorax, Wingless, and Sex – Combs Reduced* were studied through antibody stains.

DEDICATION

This paper is dedicated to my friends and family. These are the people in my life who acted as constant support and active encouragement during the challenges that came about while attempting to complete a master's degree as the world succumbed to a global pandemic. During every doubt and joke about dropping out, these people stood by and told me I could anything I set my mind to. Thank you for the motivation to continue and finish this project.

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CHAPTER I

INTRODUCTION

Insects are one of the most diverse and abundant organisms on Earth. Their large numbers and diversity can be partially attributed to the development of wings and flight within their class, approximately 350 - 400 million years ago (Medved *et al.*2015). Insects were the first organisms capable of achieving flight, a somewhat unique ability which has only independently evolved three more times across history in pterosaurs, birds, and bats (Engel *et al.* 2013).

Wings have been studied extensively in insects. Insects that have wings or had ancestors with wings at some point, are part of the subclass Pterygota. This group is monophyletic and comprises the vast majority of all insect species. Insects without any history of wings or ancestral wings form a paraphyletic assemblage sometimes termed 'Apterygota' and are composed of two major linages: Zygentoma and Archaeognatha. Many members of the Pterygota have been used to study and understand the patterns of wing development and morphogenesis, and although understandings of morphogenesis and development have been made clearer, however we still have much work to do to fully understand the evolution and origin of the insect wing.

The origin of wings in insects, and more particularly the structure from which they are derived, has been an elusive topic. In vertebrates there is no debate; and although we may not know all of the evolutionary steps along the way, the homology of all three vertebrae wings to

forelimbs is clear and understood (Engel *et al.*2013). Most current knowledge of wing development in insects has come from more recent and highly derived orders of insects, particularly members of Holometabola. Even with the use of fossil evidence, there's a lack of transitional data (Smith and Jokusch 2020). Because of this, millions of years of evolution and development is missing.

Early theories on wing development were divided between the possibilities of wings being the result of a co-option and modification of ancestral morphology or that wings evolved *de novo*. Currently, it has been somewhat agreed upon that a co-option event took place for wing development to occur, with current research focusing on determination of which morphological feature allowed for the evolution of wings in insects. To complete this work investigation of extant lineages most closely related to Pterygota is critical. Prior theoretical and applied work has used more distant ancestors to winged insects, the Crustacea, as the primary subject to understand if there was a possibility for wings to have evolved from some ancestral trait.

1.1 Current Theories of Wing Origins

Two theories which have been presented are the tergal and the pleural plate hypothesis. In the tergal hypothesis of wing evolution, insect wings are posited to have developed from the dorsal body wall. This hypothesis postulates that wings originated from an extension of the thoracic tergum, which initially formed paranotal lobes and then further evolved into fully articulated wings (Elias-Neto and Belles, 2016). The second hypothesis, the pleural plate hypothesis, posits that wings were thought to have evolved from modification of ancestral proximal leg structures. These could have also evolved from crustacean exites, which could be something such as gill located on the proximal region of the legs. (Bruce and Patel, 2020; Clark-Hachtel and Tomayasu, 2020).

Recently a third hybrid theory has gained support amongst the community studying insect wing origin. This hypothesis acts as a sort of combination between the two which were originally mentioned, the pleural plate and the tergal theories. It has come to be known as the dual hypothesis of wing evolution. This dual origin hypothesis is able to overcome some of the limitations of the pleural and tergal hypotheses. For example, the tergal hypothesis is missing vital information which does not account for the musculature and articulations which are necessary for flight, however the pleural hypothesis is able to offer some explanation for the origins of the muscles and articulations (Elias-Neto and Belles 2016). On the other hand, where the pleural hypothesis is strong in supporting the origin of the muscles and articulations by stating they are within the leg branch limb, it becomes difficult bridge the positional gap of how a leg branch located at the ventral junction of the pleura is capable of evolving into a wing located at the dorsal junction of the pleura (Elias-Neto and Belles 2016).

Whereas the dual origin hypothesis for wing evolution is able to account for limitations of each individual hypothesis, the contribution each individual hypothesis holds in wing origins is debated. Recent studies conducted on *Paryhale hawaiiensis*, a crustacean somewhat distantly related to insects, have provided some confirmation towards this. For one, the lateral body wall of insects may be homologous to the proximal – most leg segment of the Paryhale (Clark-Hachtel and Tomayasu, 2020; Bruce and Patel 2020; Smith and Jokusch 2020). Secondly, gene expression evidence supports a mechanism of body wall component derivation of the insect pleura from crustacean legs (Clark- Hachtel and Tomayasu, 2020; Bruce and Patel, 2020; Smith and Jokusch 2020). These new developments give more insight and provide additional avenues of investigation as we move towards a more comprehensive theory of insect wing origin.

There is no doubt that studies on crustaceans have provided new insights and understandings to the origins of insect wings. However, studies with crustaceans have their own limitations, such as the possibility of convergent evolution or in the case of gene expression studies, that the expression is not definitive (Requena *et al.*2017). Data for taxa that can act as a bridge between terrestrial, wingless arthropods and fully winged insects is critical. Collembola are a group of non-insect hexapods and they represent one of the critical links in the taxonomic chain that must be completed to provide a better understanding of this question.

CHAPTER II

REVIEW OF LITERATURE

2.1 Collembola as laboratory species (Hypogastruridae)

Collembola; or more as they are commonly known, springtails; are a widespread and nearly ubiquitous terrestrial wingless arthropod which play important roles in soil development and wellbeing (Fountain and Hopkin, 2005). Collembola also have several unique and specialized structures distinct from other members of the Hexapoda subphylum, which form along the ventral side their abdominal segments. One such structures is the collophore, or the ventral tube, which forms on the ventral surface of the first abdominal segment. This structure plays a role in osmoregulation for Collembola (Konopova and Akam 2014). They also have the furca, which may be considered the defining feature of the species as it is the "spring" from which their common name is derived. Found on the ventral midline of the fourth abdominal segment, it functions as a type of catapult and allows Collembola to "leap" into the air to heights several times the length of their body (Konopova and Akam 2014; Schaeper *et al.*2013). The retinaculum is another unique feature which secures the furca while the structure is at rest (Konopova and Akam 2014). Together, the retinaculum and the furca allow for the collembola to jump through the rapid release of the furca from the retinaculum allowing the species to catapult itself, thus the name springtail (Schaeper *et al.*2013).

Phylogenetically, Collembola are members of the subphylum Hexapoda, a type of arthropod that includes insects and the non-insect hexapods: collembolans, proturans, and diplurans (Konopova and Akam 2014). Therefore, collembolans represent the most successful and diverse descendants of primitively wingless hexapods.When conducting genetic and phylogenetic analysis a number of species of Collembola have been used. Two commonly used species are *Folsomia candida* and *Orschesella cincta*. For our work we have established a laboratory colony of *Xenylla pseudomaritima*, established originally from populations collected in the environs of UTRGV. This species is a member of family Hypogastruridae and is our representative in both genetic analysis and gene expression of the class Collembola.

2.2 Collembola Morphology

The overall body plan of the Collembola is similar to insects which is why studies based on morphology classed the group with insects for so many years. Collembola have a distinct head with a pair of segmented antennae and a three segmented thorax which will develop three pairs of legs (Hilsenhoff 2001). Collembola abdomens are composed of six segments, three of which develop appendages (Konopova and Akam, 2014). These unique appendages form on either side of the lateral margins of the ventral side of abdominal segments A1, A3, and A4, but migrate and fuse along the medial ventral line as the embryo develops. These fusions are responsible for the unique appendages the collembola are known for having. The collophore is a result of the fusion on the A1 segment. The retinaculum is a result of the A3 segment fusion and the furca is due to the fusion on abdominal segment A4 (Konopova and Akam, 2014). There have been previous proposals that the fused proximal parts of the abdominal segments may be homologous with coxopodites whereas those on the distal ends may be homologous to

telopodites in other arthropod appendages (Konopova and Akam, 2014). Figures 1 and 2 demonstrate where the abdominal segments are as well as these specific structures formed.

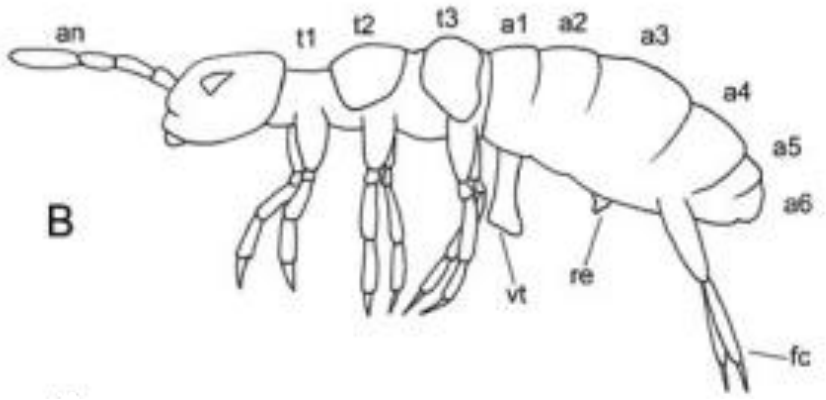

Figure 1. General overview of the morphology and body plan of an adult Collembola (from Shaeper et al.2013).

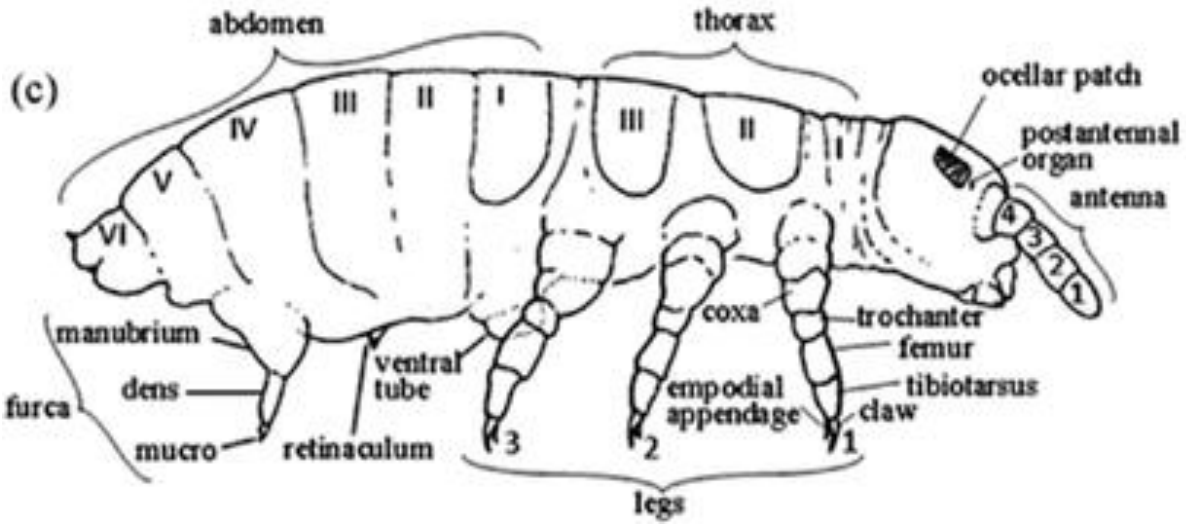

Figure 2. A generalized morphology of a hypogastrurid. In this diagram more specific morphological structures are specified (from Greenslade et al.2014).

2.3 Genes of Interest in Wing Development

Engrailed (en)

Engrailed belongs to a family of genes which encodes homeodomain containing transcription factors (Peel *et al.*2006). Expression of engrailed can be seen clearly in *Drosophila melanogaster*, where it a repeated lateral stripe pattern that defines the posterior portions of the limbs and wings. This is because the gene is necessary for the formation of segment borders and establishment of the positional information in those segments (Jockusch and Nagy 1997, Peel *et al.* 2006).

Engrailed is also known to have roles in wing development in *Drosophila melanogaster.* *Engrailed* patterns the wing margin and is involved in cell interactions that maintain the expression of other genes such as *decapentaplegic* and *hedgehog*, which have roles in patterning and morphogenesis of the wing (Hidalgo, 1994). It is necessary for maintaining the anteroposterior compartment border in wing cells as well.

Distal-less (Dll)

Distal-less (Dll) is a gene which regulates leg development and has a conserved expression pattern, typically found expressed in the distal leg of members of the hexapod phylum. It is crucial for development of most if not all ventral appendages in the Drosophila, such as the legs and antennae and also has roles in development of components of the peripheral nervous system. However, there have been observed differences in is expression amongst more basal lineages of the hexapod, such as members of the collembola. In the species *F. candida*, *Dll* is expressed in the regulates leg development and has a conserved expression pattern, typically found expressed in the distal leg of members of the hexapod phylum, inner endites of the maxilla

and within the labium (Schaeper *et al.*2013). The expression occurs as a continuous domain which extends from the trochanter to the distal tip of the leg (Schaeper *et al.*2013). *Distal-less* plays important roles in appendage development. *Dll* knockdowns have demonstrated failure to develop legs and antennae. In *Drosphila melanogaste*r, *Dll* has roles in differentiation of the wing margin (Campbell and Tomlinson, 1998). It is theorized that the roles of *Dll* are a late requirement in wings.

Abdominal A (abd-A) / Abdominal B (abd-B) / Ultrabithorax (Ubx)

Abdominal A (abd-A), Abdominal B(abd-b), and Ultrabithorax (Ubx) are the three genes that make up the Drosophila bithorax complex. *Abdominal B* specifies the identify of posterior abdominal segments. In Collembola, these genes are necessary for determining the developing morphology of the thoracic and abdominal segments. Knockdown experiments conducted on the collembola species *Orchesella cincta* determined the function of *Ubx* and *abd-A*. *Ubx* in *Orchesella cincta* leads to the development of the collophore. Whereas *abd-A* in the same species leads to development of the furca. Both of these genes are necessary for development of the furca. (Konopova and Akam 2014).

Regarding wing development, the bithorax genes may have abilities to regulate tissue formation in segments. *Ubx* and *abd-A* may be able to repress abdominal wing and leg primordial development (Engel *et al.*2013). *Ubx* is involved in development of the halteres, or hindwings, through repressing some wing development genes (Tomoyasu *et al.* 2005). Knockdown of *Ubx* results in the formation of 4 wings in Drosophila, rather than a set of forewings and hindwings. RNAi experiments done on Tribolium have demonstrated the possibility that *abd-A* may play a greater role in development of the serial homolog to tergal

wings through suppressing wing identity and allowing for the distribution of the wing serial homolog cells across the anterior – posterior axis of the insect (Linz and Tomoyasu 2013).

Dacshund (dac)

Dachshund, or *dac*, regulates eye, leg, gonad, and brain development (Flybase 2021) *Dac* has also been found to have roles in leg development. In loss of function experiments, flies without functioning *dac* developed with legs which were severely shortened compared to their wild type counterparts (Mardon *et al.*1994). Findings have determined that it is very likely that *dac* may act downstream to some sort of proximal – distal patterning signal in order to determine and establish segment boundaries and relationships (Mardon *et al.*1994). For *Folsomia candida*, *dac* expression in the legs occurs as a broad medial ring with a similar but thinner ring expression in the furca (Shaeper *et al.*2013).

Cut (Ct)

Cut, or *ct*, has roles in differentiation of the wing disc, muscle, oocytes, and sense organ cells in Drosophila (Flybase). In wings, *cut* is expressed in isolated groups of cells on the prospective wing blade, wing hinge region, and the notum (Blochlinger *et al.*1993). In *ct* mutants, flies will exhibit a scalloping pattern along the entirety of the wing margin.

Sex- Combs Reduced (Scr)

Sex-Combs Reduced is a part of the Antennapedia complex. It is expressed in the embryonic labial and first thoracic segments and has roles in controlling the identify of segments which contribute to the head and anterior thorax (Flybase 2021). *Scr* has roles in repressing wing development in the T1 segment of *Drosophila melanogaster* as without the gene, embryos begin to develop ectopic flight appendage primordia in that segment (Carroll *et al.*1995)

Notch (N)

Notch encodes a signaling protein with many roles in the developmental process. It regulates the differentiation of the ectoderm and development of the central and peripheral nervous systems, the eye, the wing disk, muscles, and appendages such as the antennae and the legs. It does this through lateral inhibitions or inductions (Flybase, 2021). *Notch* works alongside *wingless* in order to promote tissue growth in the wing (Giraldez and Cohen 2003).

Wingless (wg)

Wingless is a segment polarity gene which is a part of the Wnt family (Flybase, 2021). *Wingless* is necessary for the survival, proliferation, and tissue growth of the developing wing of *Drosophila melanogaster*. It is also responsible for the development of pattern elements along the wing margin. *Wingless* also has roles in maintaining the expression of other genes such as engrailed by encoding signals involved in cell interactions. (Cuoso *et al.*1994). In older embryos of the same species, *wingless* takes on more complex roles (Giraldez and Cohen 2003). For example, *wg* plays several roles in development of the eye and has also been suggested to have roles in patterning the nervous system (Cuoso *et al.*1994).

CHAPTER III

MATERIALS AND METHODS

3.1 Fixation

Collembola embryos were fixed on a regular basis. This occurred approximately every 36 to 48 hours in order to ensure that embryos at the proper stages were found. 12 unique colonies of Hypogastruridae were raised on plaster habitats and provided with yeast and water at three-to-four-day intervals for nutrition. Eggs from these colonies were collected by wetting the tip of a small paintbrush and using it lightly in order to pick up the eggs and place them into microcentrifuge tubes. The embryos collected were then incubated for 3 minutes in a 1 : 2 ratio of bleach and embryo wash solution with gentle mixing. Following the incubation period, the solution was washed 5 times with embryo wash buffer and then decanted. 500 µl of heptane and 500 µL paraformaldehyde mix were added into the microcentrifuge tube. The solution was then incubated with vigorous mixing for 20 minutes to allow for the breakdown of the vitelline envelope. A pulled Pasteur pipette was then used to remove the aqueous layer after the mixing period and 100% methanol was added to fill the tubes. This was then mixed vigorously for 10 – 15 seconds. The remaining heptane and methanol solution was removed, and the embryos were washed again in methanol three times. The embryos were store in methanol indefinitely at 20 °C

3.2 Antibody Staining

For the purpose of this procedure and for the best results, the antibody staining took place over the course of three days. To begin, the embryos were serially rehydrated. The first rehydration step involved 3:1 MeOH to PbTween ratio, where it was left to rock gently at room temperature for three minutes. The next rehydration step was a 1:1 ratio of MeOH to PbTween left to rock gently at room temperature again, for three minutes. The next step was a series of three washes, two minutes each in PbTween.

Following the rehydration series, the embryos were left gently rocking in a solution of PbTween + 2% BSA for two hours. Once the two hour period was over, the solution was decanted and incubated in a 1:500 dilution of the primary antibody to PbTween + 2% BSA overnight.

On the second day a series of washes occurred. The embryos left in the primary antibody overnight were washed three times quickly in PbTween + 2% BSA and then three times for 10 minutes each in the same solution. They were then decanted and incubated in a 1:200 dilution of the secondary antibody and PbTween + 2% BSA. This would be left to incubate overnight.

On the third day the embryos were washed again three times quickly in PbTween + 2% BSA. They were then DAPI stained in a 1:1000 ratio of DAPI to PbTween. After the DAPI stain the embryos were washed twice, ten minutes each in PbTween. From this point they were mounted onto microscope slides in a PbTween solution and sealed using nail polish. They were then visualized on a fluorescent confocal microscope.

Table 1. The primary antibodies used in the antibody staining procedures.

Primary Antibodies

Genes	Abbreviation	Species with Positive Tested Reactivity
Distal-less	Dll	
Engrailed	En	Arthropods/ Vertebrate/ Mollusks
Dachshund	Dac	*Drosophila melanogaster*
Notch	N	*Drosophila melanogaster*
Cut	Ct	
Abdominal-B	abd-B	*Drosophila melanogaster*
Wingless	Wg	Arthropods/ Vertebrate/ Paryhale
Sex-Combs Reduced	Scr	*Drosophila melanogaster*
Ultrabithorax	Ubx	*Drosophila melanogaster*
Ultrabithorax/ Abdominal-A	Ubx/ abd-A	Many arthropods/ Onychophoran

Table 2. The secondary antibodies used in the antibody staining procedure.

Secondary Antibodies

Alexa Fluor 647	Goat Anti-Mouse
DAPI	

CHAPTER IV

RESULTS

X. pseudomaritima embryos collected at 3-4 days were viewed under an Olympus FV10i Confocal microscope following Antibody Staining protocol. Nuclei were stained using DAPI and the antibodies were stained using Alexa Fluor 647. Positive results were observed in *X. pseudomaritima* in *Ubx*/ *abd-A* and *Wingless*, however results could not be confirmed in *Distal-less, Engrailed, Dachshund, Notch, Cut, Abdominal-B, Sex-Combs Reduced,* and *Ultrabithorax*. Experiments with Drosophila embryos were conducted either simultaneously or alongside *X. pseudomaritima* embryos to act as one method of control and to create a base understanding for expected expression patterns. Positive results were determined in the Drosophila controls for all antibodies except the *Ubx*/*abd-A* antibody. Other control methods utilized were running the experiment with either no primary antibody or no secondary antibody to ensure that what was being seen was the result of specific staining and binding versus staining of artifacts or something similar.

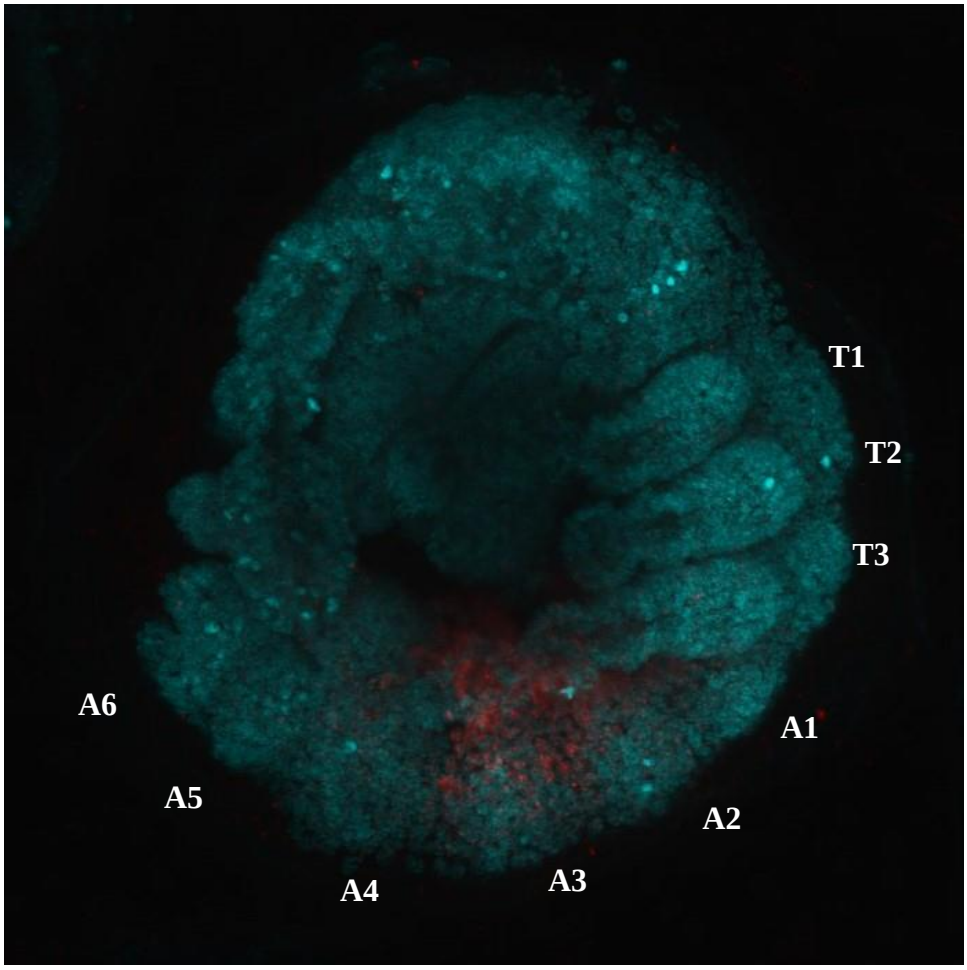

Figure 3. Ubx/abd-A expression in Hypogastrurid. The embryo is in a lateral position with the head towards the top and furca curved in towards the bottom. DAPI staining is seen in cyan and Alexa Fluor 647 is seen in red targeting the primary antibody Ubx/abd-A. Expression can be observed in the abdominal segments of the Hypogastrurid. Image displayed is a stacked image of several slices taken at 60x magnification.

Ultrabithorax/*abdominal-A*

Expression of *Ubx*/ *abd-A* was detected in older embryos of Hypogastrurid, however positive results could not be determined in younger embryos. Younger embryos displayed either no expression or nonspecific expression. Expression in the older embryos can be seen in the abdominal segments A1-A4 similarly to results seen previously in *Orchesella cincta* (Konopova and Akam 2014).

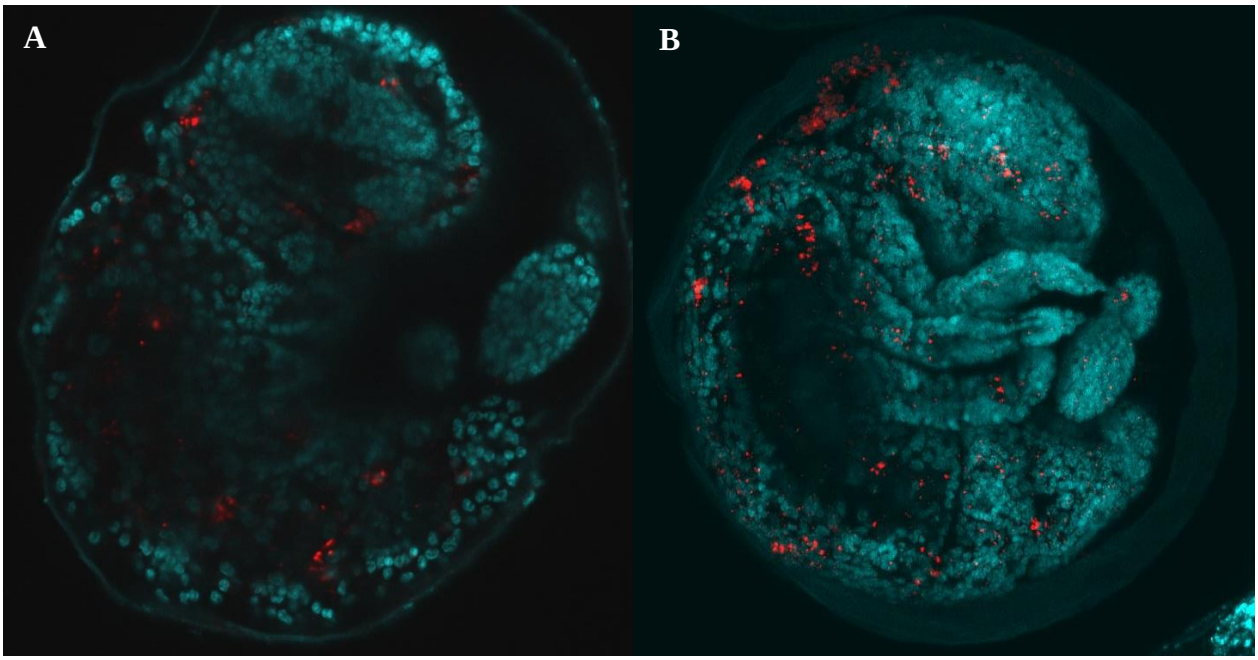

Figure 4. Wingless (Wg) expression in the Hypogastrurid. Images are shown from a lateral view with the head of the organism on top and the furca curved inwards towards the bottom. Expression is seen throughout the Collembola, in isolated groups of cells. DAPI staining is seen in cyan and Alexa Fluor 647 staining of the wingless antibody is seen in red. A) a single image taken at a magnification of 60x. B.) A stacked image composed of multiple slices taken at a magnification of 60x.

Wingless

Positive expression of the wingless protein was observed in older collembola embryos but not in younger embryos similar to *Ubx/abd-A* and likely for similar reasons. Because of this, early expression of *wg* was not observed. The expression pattern of *wingless* observed in the *X. pseudomaritima* does not appear similar to expression patterns seen before in Drosophila, however the pattern was observed multiple times during different trials. Expression in Drosophila typically occurs as a repeated striped pattern. *Wingless* takes on more complex roles in older embryos, for example, it may take on roles in appendage development, development of the peripheral nervous system, and roles in eye determination. Since the embryos which showed positive expression are older, this may be the reason for the observed expression pattern. Expression of *wingless* can be observed in the head, the thorax, and the abdominal regions in isolated groups of cells throughout the organism.

CHAPTER V

DISCUSSION

5.1 Ultrabithorax/ abdominal – A

Seeing that *Ubx/abd-A* expressed as was expected is a step forward in understanding the abdominal segments of the Collembola. Based on studies previously done on *Orchesella cincta*, it is likely that *Ubx/abd-A* has the same conserved function in *X. pseudomaritima*. *Ubx* allows for development of the collophore, *abd-A* allows for development of the furca, and a combination of both genes allows to development of the retinaculum (Konopova and Akam 2014). However, since the antibody itself used for the experiment is a combination of the two genes allowing for a cross reaction between *abd-A* and *Ubx*, it is not possible to tell from antibody staining alone which gene is expressed where, only that some combination of the genes gets expressed in the abdominal segments of the organism.

Regarding wing theories, it is interesting to inquire about what *Ubx* and *abd-A* expression in the Collembola is able to bring to the table. Typically, as in most insects, these genes repress abdominal wing and leg primordial development (Engel *et al*. 2013). *Ubx* silencing seems to have the same effects on Collembola as it would for most insects, in that the abdominal segment develops leg like structures on A1 (Konopova and Akam 2014). In Drosophila, for example, *Ubx* is responsible for the haltere, or hindwing, development in the Drosophila. Silencing this gene would result in the formation of four forewings instead (Engel *et al*. 2013). More recent studies

have discussed if *Ubx* is important for the T2/T3 wing divergence in winged insects. It has been theorized that changes to *Ubx* function may have arisen in early pterygote evolution because these changes can be seen in Paleoptera and Neoptera as well (Requena *et al.*2017).

On the other hand, *abd-A* may have different functions in collembola versus most other insects. It has been argued that *abd-A* does not repress appendage development in Collembola, however, that the furca itself in the species is homologous to a full-length leg (due to previously observed *dac* expression which is normally expressed in thoracic legs) , therefore *abd-A* acts more as a modifier of this appendage versus a repressor (Konopova and Akam 2014).

Complete knockdown experiments of both of these genes have led to the formation of leg like structures in all abdominal segments except for A2 in the Collembola (Konopova and Akam 2014). This is interesting to note because complete knockdown experiments of the same genes in *Tenebrio molitor*, had a different affect. This led to the formation of wings on all abdominal segments of that insect, although they may not have been completely developed (Engel *et al.* 2013).

5.2 Wingless

Interpretation of observed gene expression patterns for the wingless gene is more complex than that of *Ubx* and *abdominal-A. Wingless* is not a gene which has been studied in Collembola species and the observed expression patterns from this experiment do not appear that similar to those seen in Drosophila nor species of Crustacea. Typically, *wingless* is expressed as a repeated striped pattern at the prospective wing margin in Drosophila. In crustaceans, such as Daphnia, *wingless* is coexpressed with *vestigial* and *scalloped* on the carapace margin and may have roles in carapace formation (Shiga *et al.*2017).

In regard to wing development and wing theory, it has been suggested that wings may have evolved through an integration of different developmental models active in different parts of the crustacean body (Shiga *et al.*2017). It is possible that there was a co-option event associated with *wingless* and its co-expressed genes in the flat body wall outgrowths into onto the epipodites, possibly suggesting some form of dual wing origin hypothesis.

It is possible that *wingless* along another one of its co-expressed genes, such as *vestigial*, together may play a more important role in understanding where wings may have developed. A more recent study done on a combination of this genes tested these genes particularly because they were trying to determine if insects incorporated two of their leg segments into the body wall. This was tested by examining the expression of an exite on the seventh leg segment. When *wingless* and *vestigial* underwent RNAi knockdowns, it led to reductions in the wing and the lobe, implying that the lobe and the wing are exites (Bruce and Patel 2020).

5.3 Antibody Troubleshooting

Early trials of antibody staining were unsuccessful. The biggest issue being non-specific binding. Many of the original trials just demonstrated antibody staining everywhere or antibody staining which looked like a "ghost image" of the embryo. Staining tended to be weak when it did occur as well. Originally, the antibody trials were based upon methods found in the protocol by Legendre *et al.*2013 however evolved immensely as the project continued. Initially, it was difficult to determine whether issues with the antibody staining were the result of methodology, the protocol itself, or issues with the collembola/ collembola fixation. In order to rule out some of these suspects, several trials occurred.

Collembola fixed in formaldehyde solution, the initial fixation method utilized, underwent the antibody staining process as described above in the same tube as drosophila embryos fixed in a similar process. The results from this trial demonstrated successful antibody stains in the drosophila embryos but not the Collembola embryos. The next trials dealt with using Collembola embryos fixed in paraformaldehyde, with Drosophila acting as a control once more. Since both fixation methods yielded similar results in the Drosophila, it was determined that the issue did not lie with the fixation methodology.

Further trials were done using collembola fixed in paraformaldehyde as these collembola were fixed and stored more recently than their formaldehyde counterparts. These trials were done with slight changes made to the initial antibody protocol. The main changes in these trials were changes in the duration of each of the main steps in the process. These new durations were based on protocol by Hachtel and Tomoyasu done in Paryhale. In these new trials, the duration of the blocking process was one hour rather than two, the primary antibody stayed the same, being incubated overnight, and the secondary antibody was left for two hours rather than overnight. Then the mounting and visualizing process took place. With these trials, results were better however not all the genes which were being studied were able to be seen and staining was not as strong as would have been preferred. Antibody staining on the Drosophila controls were still much clearer and much more affective, with staining being weak or absent for the Hypogastrurid counterparts. Later antibody trials would determine that using a blocking period of one hour versus two hours did not have that much of a difference on expression with antibodies that did end up working well in *X. pseudomaritima*.

Further literature review and investigations revealed more troubleshooting tips. One of the steps in the original procedure called for a two-hour incubation period with the PbTween + 2

% BSA with gentle rocking, which at this point had already been lowered to one hour. Troubleshooting antibody staining protocol for Drosophila warned against rocking or any forms of agitation during the blocking , primary, and secondary antibody incubations steps because it led to greater instances of nonspecific binding. (Manning and Doe, 2016). Instead, during these incubation periods, the tubes would now be laid on its side.

Further trials exposed that the PbTween + 2% BSA solution was not being made appropriately, and instead double the concentration was being used than originally thought. Experiments were run with a now proper concentration of PbTween + 2% BSA on the collembola, using Drosophila embryos as a control as well as having controls where no primary antibody was used and others where no secondary antibody was used.

One last attempt to try and get results out of *X. pseudomaritima* involved changing the solution which the organisms underwent the antibody staining protocol. For this trial the antibody *Ubx/abd-A* was tested, due to it having the best results with positive expression, in 2 simultaneous batches of *X. pseudomaritima* embryos which were run in PbTween versus PbTriton with 2% PbTween and 2% PbTriton being the blocking agents for their respective trials. This trial concluded with similar observations being seen in each of the solutions, however the PbTriton was a little more destructive on the embryos based on what was seen when it was observed in the confocal.

Fixing these issues and implementing the procedures discussed above, allowed for results to be obtained from some of the targeted genes. It should still be noted that not all of the genes would come to yield antibody stains. Antibodies continued to work well in the Drosophila counterparts throughout these trials. Unfortunately, the genes targeted by the antibodies may just not have been conserved enough to work on the *X. pseudomaritima* embryos.

5.4 Hypogastruridae in the lab

The use of Collembola, particularly *X. pseudomaritima*, in this experiment had its pros and cons. Husbandry of the species was quite easy, however, the collembola seemed to switch between periods where they would provide an excess number of embryos to periods where finding embryos was almost impossible due to sparse egg production from the colony. It is unknown whether or not this is something that is to be expected of lab grown colonies or whether it is something that happened with these particular groups.

Embryos of the *X. pseudomaritima* were easy to collect and fix following the given protocol, however they were quite a bit more difficult to work with when conducting experiments, versus that of other species such as Drosophila. As mentioned prior, the embryos produced by this species are very small, only easily visible when they are bunched together. While the Collembola embryos are in tubes, they do not behave the same as those of Drosophila embryos. The Collembola embryos tend to float in solutions, making washes and decanting difficult. The best way to get around this was to utilize microcentrifuge tubes which could be easily spun down between washes to force the embryos to bunch together at one end, making the aforementioned processes easier. Another issue was that the collembola tended to "stick" to the pipette tips, particularly when using PbTween. It is unknown whether this was an issue with the use of PbTween or the result of the long experiments with the insect embryos, however when *Drosophila melanogaster* embryos underwent the same procedures, they did not have this issue.

5.5 Future studies

Although the antibody staining of the earlier discussed genes did not work as well as anticipated, that does not mean that there isn't potential for future studies or experiments. Most

literature on Collembola discusses the organism's role in soil and how they can be used to determine soil health. There is not a lot of morphology or phylogenetics done on Collembola, meaning there is still much to learn about these organisms. Collembola are very unique in the position they are in. For many years they were thought to be members of the insect class but now separated and moved into a class of their own within the Hexapoda. They still have potential to reveal information regarding wing origins and much more.

In Situ Hybridizations and RNAi

While conducting literature review, many papers used knockdown or RNAi experiments to determine functionality of the genes that were being tested, on top of *in situ* hybridizations. These same experiments could be conducted on the *X. pseudomaritima*, or other species of Collembola such as *Orchesella cincta* and *Folsomia candida*. These types of experiments can be used to determine whether the genes discussed previously have some co-option in functionality with wings or appendage development.

5.6 Another Approach to Wing Theory

Targeted Genes for future work on Wing Development

Vestigial (vg)

Vestigial is a critical wing gene. It has unique functions in the ectoderm in development and organization of wings (Hachtel and Tomoyasu, 2020). Experiments conducted on Drosophila embryos on early *vestigial* expression have shown that the wing discs originate from the leg discs and then separate to migrate dorsally (Engel *et al.*2013).

Nubbin (nub)

Nubbin is responsible for encoding the POU/ homeodomain transcription factor which is required for wing formation in Drosophila (Flybase 2021). *Nubbin* also has similar expression patterns in the leg epipodites of Artemia to that of insect wings.

Pannier (pnr)

Pannier is expressed in a single dorsal stripe in Drosophila. During experiments to determine whether or not insects incorporated ancestral proximal leg regions into the body wall, pannier was chosen due to the fact that it was hypothesized to represent the true body wall in Drosophila and be expressed in dorsal most tissues (Bruce and Patel 2020).

Araucan (ara)

Araucan has roles in encoding the homeodomain containing protein of the TALE subfamily which is a part of the Iroquois gene complex. It helps with cell fate specification and pattern formation. (Flybase 2021). Similar to *pannier, araucan* has been used previously in experiments in order to determine whether insect incorporated part for the proximal leg into the body wall. *Araucan* was chosen because it is expressed in the lateral body wall and specifies the body wall around the wing (Bruce and Patel 2020).

There are many genes involved in the development of wings in insects. There have been several studies which have tested a multitude of these genes in attempts to determine which wing theory holds to most weight. By focusing on the dual hypothesis of wing origins, we can determine more genes of interest that can be tested in the Collembola.

Wing genes have been found in proximal leg components in the crustacean *Paryhale* but have different functions (Hachtel and Tomoyasu, 2020). The genes that have been tested are v*estigial (vg), nubbin (nub),* and *apterous (ap)*. From these experiments, the strongest tissues

which could possibly serve as origins of wings, or some sort of wing homologues, are the coxal plate and the basis, with some possibility for the gill as well. Since development and formation of the tergal edge in the same organism relied on all of the tested genes, there is also the possibility that some sort of pre-wing network operates in that location, making the tergal edge another strong candidate to be a wing homolog (Hachtel and Tomoyasu 2020). Other experiments done on *ap* and *nub* have supported the pleural theory hypothesis as transcription factors for those genes have been seen expressed in the wings of insects and epipodites of crustaceans (Averof and Cohen 1997). Therefore, wings may have originated from a combination of the tergal and pleural plates. Other experiments done on the same hypothesis have proposed that wings are homologous to the tergum of the crustacea, however the tergum itself has parts derived from the pleural plates of crustaceans (Bruce and Patel 2020).

Since members of the Collembola serve as a middle ground to crustaceans and insects, testing members of any one of the species can serve as a better insight on whether the pleural, tergal plate, or dual hypothesis of wing origins provides a clearer understanding of wing development in insects.

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