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Early Embryology of The Insect Oligotoma Saundersii (Embiidina) Using Targeted Genes as Developmental Landmarks

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EARLY EMBRYOLOGY OF THE INSECT *Oligotoma saundersii* (EMBIIDINA) USING TARGETED GENES AS DEVELOPMENTAL LANDMARKS

A Thesis

by

OMAR DORIA

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ABSTRACT

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Embiidina, or "webspinners", is a small, sexually dimorphic, polyneopterous insect order and is the only order to spin silk throughout their life cycle. Unique morphological features and the phylogenetic position of Embiidina make it an attractive subject for study of the evolution and development of the insect body plan and the origin of novel morphological features. *Oligotoma saundersii* is easily cultured and a viable laboratory organism. It has a fairly standard short-germ embryogenesis process requiring 453 hours at 28°C with progressive development beginning in the anterior region. Preliminary work on targeted developmental genes has yielded small portions of seven genes. ARACED-PCR is a novel, efficient method to amplify larger pieces of target genes using degenerate primers. This research represents the first detailed investigation of Embiidina embryology and one of few among the polyneopterous insects; therefore, it represents an important addition to our understanding of the evolution and development of insects.

DEDICATION

The completion of my graduate studies would not have been possible without the love and support of my family. My mother, Rosa Maria Doria, my father, Omar Doria Sr., my siblings, Rosa Doria, Joshua Doria, and Daisy Doria. I would not be here without all of them. They supported me and encouraged me to complete degree. Thank you for your love and patience.

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CHAPTER I

INTRODUCTION

Insect Phylogenetics and Evolution

Despite advances in techniques in the field of evolutionary developmental biology and the ability to apply these techniques across a wide range of organisms, most available data is concentrated among a small group of species (Patel 1994a; Mito et al. 2010). Most of the work has been done on *Drosophila melanogaster,* and few other derived holometabolous insects. There have been numerous studies of limited scope exploring the genetic underpinnings of development across some of insect ordinal diversity (Driever and Nussleinvolhard 1988; Patel 1994b; Lynch and Desplan 2003) this has given us an general understanding of some of the remarkable similarities in development across very divergent groups (Whiting et al. 1997; Yoshizawa 2007) Because we know so much about *Drosophila* embryonic development it has been used as the ground plan model for insect development as a whole, however in many ways *Drosophila* represents a highly derived state that is not indicative of insects as a whole or their ancestral developmental patterns (Angelini et al. 2005; O'Donnell and Jockusch 2010); see discussion below regarding germ band development as one example). Another example of *Drosophila*'s derived status among insects is the arrangement of its Hox gene cluster and duplication of portions of this cluster (Hennig 1981). To understand the evolutionary changes that have shaped insect diversity it is critical that we expand both our depth and breadth of understanding about developmental processes across key insect taxa.

Our understanding of hexapod phylogeny continues to be revised as both taxonomic sampling and genetic data sets increase and there are some key orders whose placement among the other insect

groups remains inconclusive (Bitsch et al. 2004). Despite this phylogenetic uncertainty for some orders, there are many relationships within Hexapoda that are nearly unanimously accepted among workers in the field and whose support increases as more data is added. Early hypotheses regarding the phylogeny of arthropods supported the Atelocerata (a.k.a. Tracheata) hypothesis, which assumes a sister group relationship of Hexapods and Myriapods (Budd and Telford 2009). Recent studies, including a preponderance of the genetic data support a Pancrustacea hypothesis, with Hexapoda as a derived group nested within the crustaceans (Terry and Whiting 2005), although its sister group remains uncertain with both Remipedia and Branchipoda supported under different analyses. Presently there is also wide agreement in regards to the monophyly of Hexapoda, although a small number of studies have supported entognathous hexapods as a group outside of the true insect orders (Heming 2003), thus rendering Hexapoda paraphyletic.

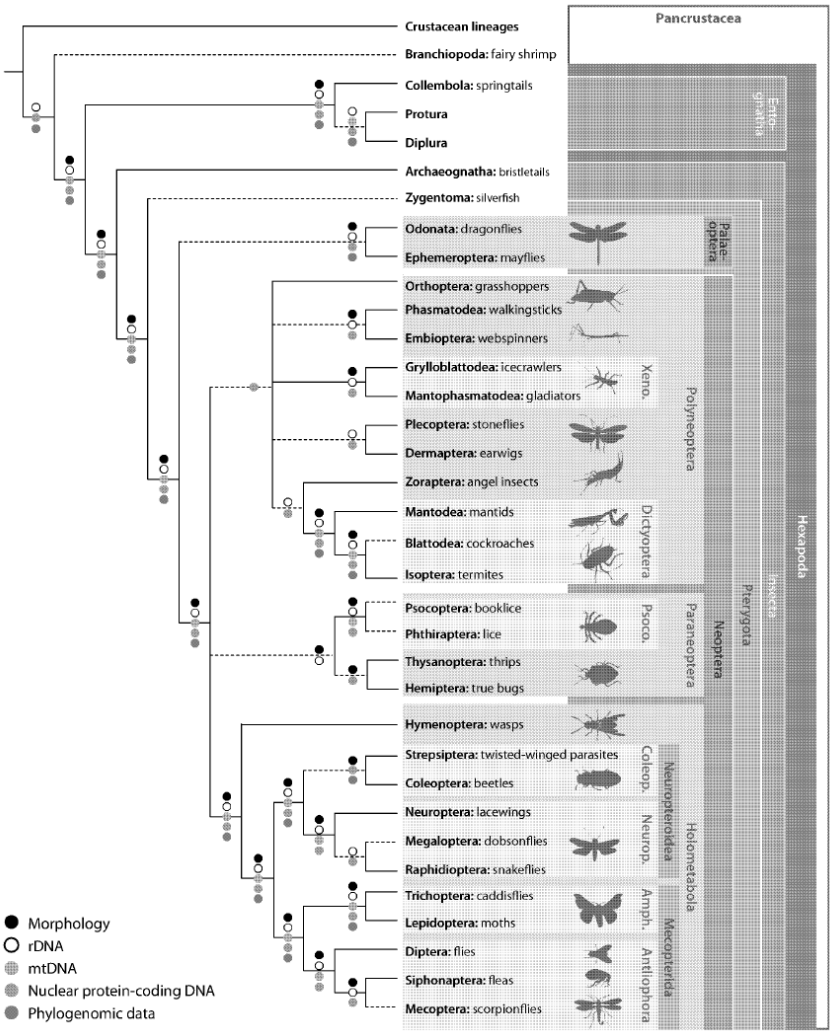

Figure 1. Summary of the Phylogenetic Relationships within Hexapoda (After Trautwein et al. 2012). Embiidina is part of the Polyneoptera assemblage and most recent studies support a sister group relationship with Phasmatodea.

Insecta can be divided into two assemblages: the "Apterygota" which are the wingless hexapods and a paraphyletic assemblage (Bitsch and Bitsch 2004; Cameron et al. 2004; Simon et al. 2009; Grimaldi 2010); and the Pterygota which have a winged imaginal stage (Sander 1996). Pterygota is composed of two groups: Paleoptera, consisting of Odonata (dragonflies) and Ephemeroptera (mayflies); and Neoptera, which is made up of the remaining insect orders and is characterized by an ancestral condition of complex wings with associated pteralia and musculature that allow the wings to be folded away when not in use. All neopterous insects can be placed into one of three groups: **1)** ''Polyneoptera'' or the ''orthopteroid" insects, a name applied to a paraphyletic assemblage of 11 insect orders comprising the lower neopterous insects (Stjohnston and Nussleinvolhard 1992; Patel 1994a; Patel 1994c; Heming 2003); **2)** Paraneoptera, true bugs and their kin; and **3)** Holometabola, insects that have complete metamorphosis. Our study organism, *O. saundersii*, is a member of the polyneopterous assemblage and not only represents a unique morphology among insects, but also serves as an example of a group that is exhibits a more ancestral insect body plane and is underrepresented in evolutionary developmental studies. This study will lay the groundwork to fill a key hole in our understanding of the evolution of the insects.

Insect Development

Morphology

Embryogenesis is the process by which a larva or a juvenile develops from a single cell. The fertilized egg divides to produce hundreds of cells that grow, move, and differentiate into all the organs and tissues required to form a larva or juvenile (Patel 1994c). Prior to tissue differentiation these cells form a germ band that extends along the anterior to posterior axis of the embryo. Arthropod embryos can be defined by the size of this initial band of tissue relative to the total size of the egg and have been placed in one of three classes: short-, intermediate- or long-germ band (Patel 1994b); although there is no clear line of demarcation between the

classes, and modern workers have developed a simpler system that only distinguishes between long and short-germ developers (Patel 1994a). In *Drosophila*, and other long-germ developers, the germ band is comprised of cells that span the entire anterior to posterior axis of the egg (Fig. 2). Segmentation and subsequent differentiation of the germ band cells begins after formation of this long germ band and segment polarity genes, which pattern the boundaries of each segment, are expressed nearly simultaneously, with a slight anterior to posterior order (Bate and Arias 1991). By contrast, the short-germ developing insects have a germ anlage that is limited to a small portion of the anterior to posterior axis of the egg (Fig. 2) and differentiation of the individual segments occurs serially with a clear and extended anterior to posterior pattern (Bentley et al. 1979).

In addition to this dramatic difference in the basic formation of individual segments, there are also differences between long- and short-germ band developing insects in the temporal order of major morphogenetic events (Lewis 1978; McGinnis and Krumlauf 1992). One such difference is the timing of gastrulation. In long-germ developers the

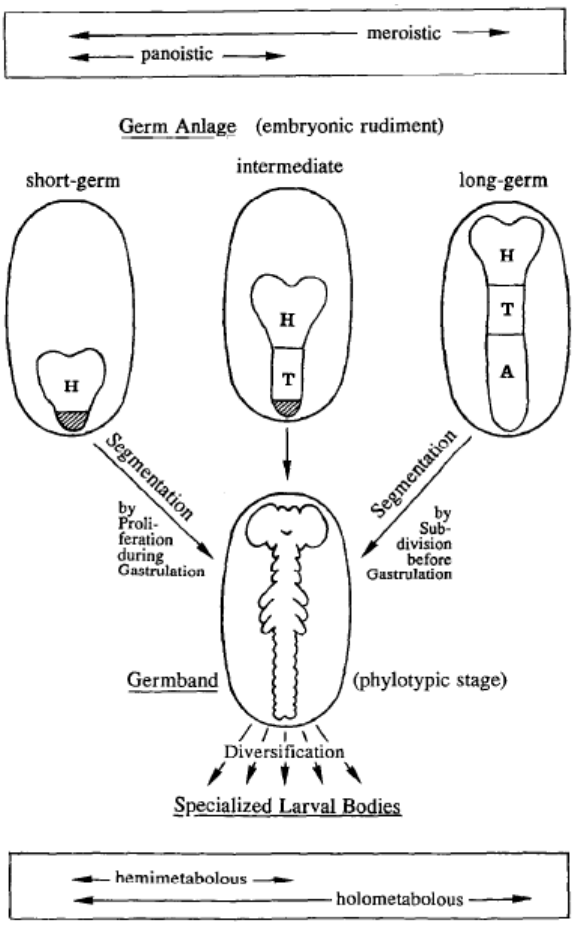

Figure 2. Long v. Short Germ Band Development. Drosophila demonstrated the derived condition of long germ band development while most insects, including nearly all basal lineages are short germ band developers. (Sander 1996)

pattern of body segmentation is established first and then gastrulation begins (Gehring 1985). In insects with short germ-band development gastrulation begins at an early stage when the germ anlage consists of only the embryonic head and a small extension of posterior cells that are largely undifferentiated (Wada et al. 1999; Monteiro and Ferrier 2006). The remainder of the organism along with the continued patterning of the digestive tract is generated and then patterned sequentially by cell proliferation at the posterior end of the insect (Wakimoto et al. 1984). This difference in timing between the two types of insect development also applies to the expression of the Hox genes, which are the primary determinants of segmental identity in arthropods (McGinnis and Krumlauf 1992). (For a detailed discussion of the role of Hox and pair-rule genes in insect development see next section.)

Drosophila, and most other long-germ insects, are phylogenetically derived insects (Trautwein et al. 2012) and the vast majority of insect orders, including a majority of those comprising the basal insect lineages are short-germ developers. The appendages of *Drosophila* are formed from imaginal discs that are localized after the germ band is differentiated into individual segments. These specialized groups of epithelial cells develop inside the larva and originate from small groups of precursor cells that invaginate from the epidermis of the nearly mature embryo (Beutel and Gorb 2001). These imaginal disks then stop any further differentiation and remain quiescent until the onset of pupal development when they are reactivated and develop into adult features. However, flies have a highly derived form of development and most insects, both long- and short-germ, demonstrate a much more direct sequence of development from undifferentiated cells to adult appendages (Wheeler et al. 2001). In short-germ insects the appendages appear slightly after the segmental boundaries are visible,

```
Consensus   ?RRGRT?YTR?QTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMK?KKE
Lab         NNSGRTNF.NK.LT..............A......NT.Q.N.T.V..........Q..R
pb          P.RL..A..NT.L..........K..C.P......AS.D.....V.V........H.RQ
Dfd         PKRQ..A...H.I........Y..............T.V.S..............W..D
Scr         TKRQ..S...Y.............................................W...
Antp        RKR..QT...Y.............................................W...
Ubx         R.R..QT...Y..........T.H.........M......................L...
AbdA        R.R..QT...F............H...............................L...
AbdB        V.KK.KP.SKF.........L..A.VSKQK.W.L.RN.Q.....V..........N..N
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Figure 3. Alignment of Amino Acid Sequences for *Drosophila* Hox Genes. First line indicates consensus of sequence for amino acid residues with greater than or equal to 50% majority. Dots in the gene alignment represent agreement with consensus strand. Notice the remarkable degree of conservation, particularly in the middle region.

but in the same order with the antennae appearing first, followed by the mouthparts and then the appendages of the thorax and abdomen (Yoshizawa 2007)

A complete review of the genetic mechanisms of insect segmental and appendage development is beyond the scope of this thesis; however a general overview of segmental specification during development is given in the following section. For a more complete understanding of how this developmental process has evolved it is critical that we gather more data from insect groups that occupy key phylogenetic positions.

Homeotic Genes

Hox genes encode a family of transcription factors that are expressed in the developing embryo and specify body region identity (Ross 2000; Edgerly et al. 2006; See Fig. 5). Differential Hox gene expression is the signal that directs anterior cells to become head segments and develop eyes, antennae, and mouthparts; other cells in the middle of the anterior to posterior axis to make wings and legs; and it signals for development of the limbless abdomen and reproductive structures at the posterior end of the developing embryo. The Hox genes belong to

a larger class of genes that are identifiable as paralogous gene duplications by the conserved region called the homeodomain (or homeobox); a 180 base pair sequence that encodes an amino acid sequence that recognizes and binds to DNA the DNA promoters of its target genes (Fig. 3). This region is largely conserved across the Hox genes and across a wide range of organismal diversity and is essential to the Hox genes' role as developmental fate determinants.

Homeotic genes are shared by all animals, but most in-depth work has been done on the model organism *Drosophila melanogaster*. Not only do the Hox genes play a critical role in development, but they are also found in close proximity to each other in the genome; a condition known as colinearity. The ancestral insect Hox cluster is made up of eight genes with canonical Hox function: labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr), Antennapedia (Antp), Ultrabithorax (Ubx), abdominal-A (abd-A) and Abdominal-B (Abd-B). There are also two other genes that are part of the ancestral insect Hox cluster that are the result of ancient gene duplications: zerknult (zen) and fushi tarazu (ftz). These genes no longer perform standard Hox function, but still have an important, although somewhat divergent, role in insect development. Unlike most insects the *Drosophila* Hox cluster is broken up into two groups: the Antennapedia complex, labial (lab), proboscipedia (pb), Deformed (Dfd), Sex comb reduced (Scr), and Antennapedia (Antp) (Kaufman et al. 1990); and the Bithorax complex which includes Ultrabithorax (Ubx), abdominal-A (abd-A) and Abdominal-B (Abd-B) (VonAllmen et al. 1996).

Original discovery of the Hox genes was due to Antennapedia homeotic gene mutations in *Drosophila* which caused legs to grow on the head in place of antennae, and gave early clues as to the critical role of these genes in development (Fig. 4). Mutations to other Hox genes lead to similarly dramatic homeotic transformations, such as transformation of the metathoracic

halteres into an extra pair of wings identical to those on the mesothorax via loss of function of the *Ultrabithorax (Ubx)* gene (Fig. 3a, Garciabellido and Lewis 1976) or transformation of the proboscis into thoracic legs via mutation of proboscipedia (pb) (Fig. 3b, Kaufman et al. 1990).

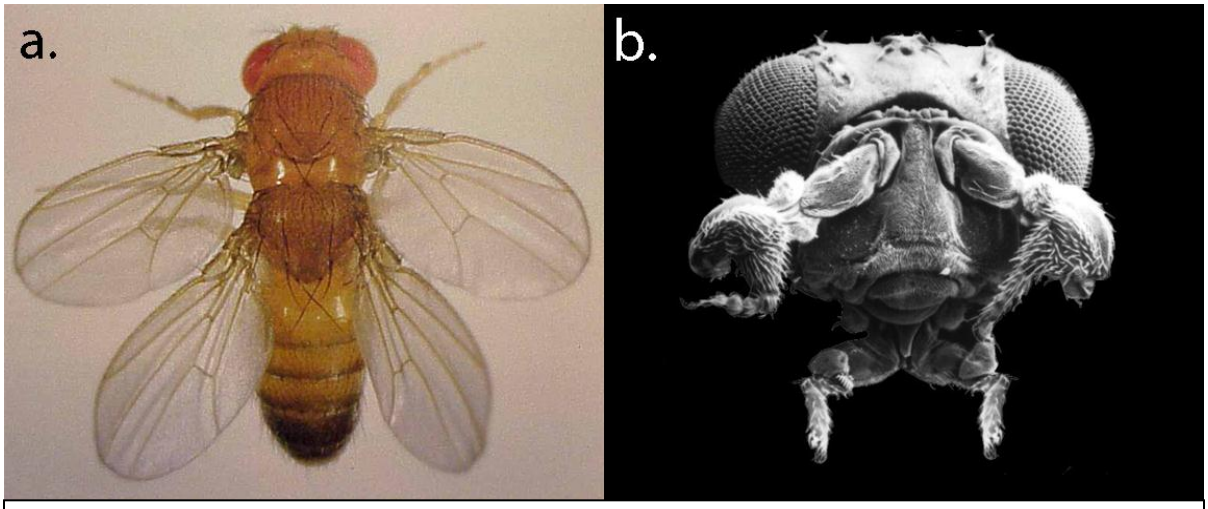

Figure 4. *Drosophila* Mutant Phenotypes of Hox Genes. a. Ultrabithorax (Ubx) and b. proboscipedia (pb).

Distal-less (Dll) and engrailed (en)

For limb development no other gene plays as vital a role as the *Distal-less (Dll)* gene. The initiation of Dll expression in the embryonic leg primordia (imaginal discs) represents the first specific marker for *Drosophila* leg formation (Cohen 1990; Goto and Hayashi 1997) and for the formation of antennae, mouthparts, and wings (Dong et al. 2000; Beermann et al. 2001). It is the repression of *Dll* by products of two Hox genes, Ultrabithorax (Ubx) and abdominal-A(abd-A), that results in the limbless insect abdomen (Cohen 1990; Vachon et al. 1992; Lewis et al. 2000), a transition representing one of the key innovations of the insect body plan. The *engrailed (en)* gene is conserved gene throughout Metazoa and in arthropods functions as a segment polarity gene (Holland et al. 1997) that designates the anterior boundary of each developing segment. Because of their critical role in the patterning of the early insect embryo, *Dll* and *en* can serve as key developmental landmarks that signify the initiation of major morphological features.

CHAPTER II

Oligotoma saundersii AS A LABORATORY ORGANISM

Embiidina

Embiidina (Embioptera, “webspinners,” or “embiids”) are elongate, small, or moderately sized insects that live gregariously in silk tunnels. The head bears filiform antennae, compound eyes, and mandibulate mouthparts but lacks ocelli. They are endemic throughout the world and have about 200 described species, including 13 species from North America and 65 from Australia (Ross 1970). As a group, the Embiidina physiology is fairly homogeneous, but they exhibit strong sexual dimorphism: males of nearly all species have fully functional wings while females are wingless (Fig. 5). The tarsal segments of both sexes have about 200 internal silk glands that secrete the silk via exterior hair-like ejectors that can each spin a strand of silk with each stroke of the leg. The silk is used to form into narrow galleries which serve as protective tunnels or create passages and galleries to a food supply— mostly consisting of weathered bark, lichens, moss, or leaf litter. In arid regions the galleries extend deep into soil and there serve as refuges from heat and desiccation. The most common Embiidina habitat is the side of tree trunks where rough bark can provide protection and substrate for silken galleries. Female webspinners will lay eggs in clusters from a few to a few dozen and, some species with cover their eggs with a protective layer of masticated plant matter.

Although males of most species have functional wings and are capable of flight, they are weak fliers, with flight used almost exclusively as a method for dispersal from their home

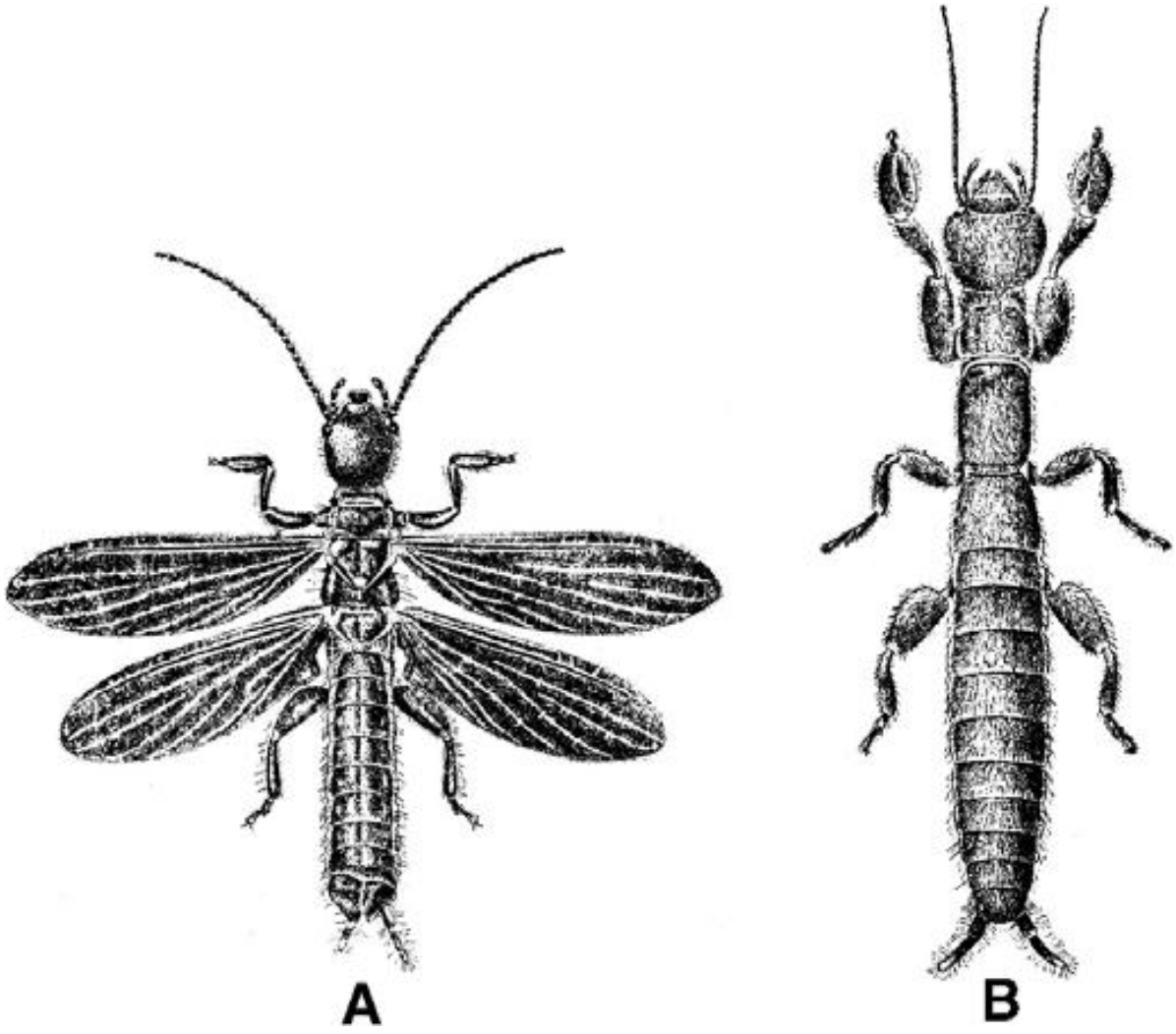

Figure 5. External Morphology of Embiidina (*Embia major*). (A) Male; and (B) female. (After Imms 1913). Demonstrating the strong sexual dimorphism of Embiidina.

colony. The wings are flexible and able to fold long a lateral point of weakness in the wing veins to facilitate backward movement along the silken tunnels. Male cerci are usually asymmetrical, and the remaining morphology is that of the generalized ground plan of the insects.

Although Embiidina is widely accepted as a member of the unresolved polyneopteran radiation, the precise position of the order remains controversial. Recent analyses have posited a close relationship for the Embiidina with Dermaptera, Plecoptera , and Zoraptera ; however a growing consensus of analyses, particularly those including genetic data place Embiidina as

sister group to Phasmatodea (Terry and Whiting 2005; Kjer et al. 2006; Ishiwata et al. 2011; Friedemann et al. 2012). While other hypotheses state a monophyly of Neoptera excluding Plecoptera and Embioptera are weakly supported statistically. An autapomorphic condition occurred in female Embiidina a secondary modification caused the absence of wings. Hairy soles of tarsomeres have evolved several times independently. However in Embiidina, the presence of hairy tarsomeres was considered a synapomorphy of Embiidina and Dermaptera (Beutel and Gorb 2001).

Husbandry Methods

Laboratory colonies were originally established with wild captured *Oligotoma saundersii* from Edinburg, Hidalgo Co., Texas in August of 2009. This genus is an introduced species from the Middle East that has established itself across the Southern United States and Northern Mexico beginning in the mid-20th century (Ross 1984). Adults and juveniles of both sexes were collected via aspirators from established colonies in leaf litter from forested areas near the University of Texas, Pan American. Once collected the webspinners were transfer to a 1L Mason jar with a mesh seal in the top for oxygen exchange (Fig. 6). Autoclaved dry live oak leaves (*Quercus virginiana*) where placed inside the jar and served as a substrate for silk galleries and oviposition (Ross 2000; Edgerly et al. 2006). About four individuals of either sex were used to establish initial individual

Figure 6. Embiidina Laboratory Cultures. Notice the extensive silk galleries in the lower half of each culture jar.

cultures and these were maintained in an incubator with a constant temperature of 28°C. A pan with water was placed inside the incubator to regulate humidity. Romaine lettuce was placed in the colonies at least weekly and served as a food and water source. These colonies remain viable and productive nearly three years after their initial establishment.

Staging of Embryos Methods

To precisely determine the age of individual eggs four to eight female embiids were removed from laboratory cultures and placed between two 100mm petri dish lids that were separated by a 2 mm foam barrier around the outer edge (Fig. 7). This barrier prevented escape of the ovipositing females while still allowing ample air flow. A small amount of romaine lettuce was provided as a food source, a numerical grid was drawn on the outer surface of one side, and the petri dish was placed vertically inside the 28°C incubator. A Nikon D80 SLR camera was placed in time-lapse mode and pictures were taken at 15-minute intervals over a period of between 12-48 hours. An the end of this oviposition period the location of individual eggs was marked on the surface of the petri dish and then the time series photographs were used to identify oviposition time to within a 15 minute interval.

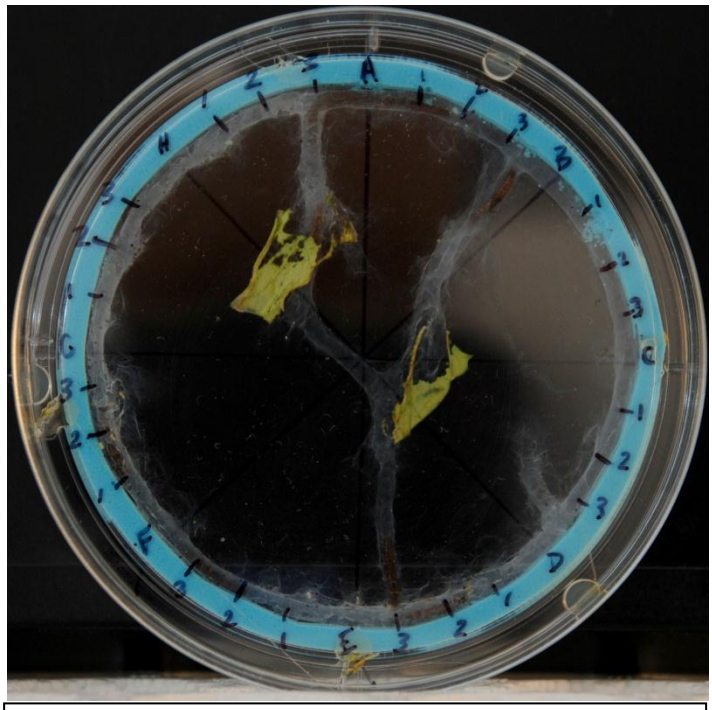

Figure 7. Apparatus for Staging *O. saundersii* eggs.

Eggs with known oviposition times were then either fixed at various developmental stages or allowed to proceed to eclosion. As eggs neared eclosion the time lapse images were taken at 15 minute intervals to precisely measure total hatching time.

Embryo Fixation Methods

Embryos collected from the colonies of unknown oviposition date were gathered in large batches by carefully scraping them from the leaf substrate and transferring them to a 1.7 ml microcentrifuge tube. Eggs from the petri dish staging apparatus were placed on a moistened plaster of paris substrate with a labeled grid. Individual eggs were placed in their own grid cell and time of oviposition was recorded. All following fixation protocols were identical for both large batches of eggs and for individual eggs of known oviposition.

Eggs were treated with a solution composed of 50% embryo wash buffer (0.04% Triton, 100 mM NaCl) and 50% commercial bleach (6% hypochlorite) for three minutes at room temperature with gentle agitation. This treatment removed most of the silk and detritus attached to the eggs and also removed a portion of the chorion. Embryos washed several time in embryo wash buffer to remove residual bleach, followed by a wash solution of 5X PBS and 0.25M EGTA. At this point *O. saundersii* embryos in the large batches floated to the surface and could be separated from the remaining detritus. Embryos were transferred to fix solution (4X PBS, 200 mM EGTA, 10% formaldehyde) and individual eggs were partially dissected in small batches by removing the operculum and perforating the vitellin membrane with a microprobe. After partial dissection a volume of heptane equal to that of the fix buffer was added and the embryos were incubated at room temperature with vigorous agitation for 30 minutes. After

removal of the aqueous layer (bottom) a volume of 100% methanol equal to the remaining heptane was added, followed by vigorous shaking for 15 seconds. The heptane layer was then removed and the embryos were washed three times in 100% methanol and one time in 100% ethanol. Embryos can be stored indefinitely in 100% ethanol at -20°C or rehydrated and used immediately. Rehydration was accomplished by removing half of the ethanol solution and adding an equal volume of embryo wash buffer until the embryos were in a 25% ethanol solution at which point the entire ethanol solution was removed and the embryos were washed three times in embryo was buffer. After rehydration individual embryos were dissected from the remainder of the chorion using microprobes.

For fluorescent visualization embryos were then incubated in embryo wash buffer with one part per thousand DAPI for 10 minutes, washed twice in buffer and mounted for imaging on a Leica MZ10F stereoscope with fluorescent capability. For this study 168 individual embryos at various stages since oviposition time were fixed, stained and imaged. Figure 8 shows an embryo at 26% of development when all head and thoracic appendages have begun developing and two abdominal segments are externally visible.

Embryogenesis Results

At 28°C, the time from oviposition to eclosion of *O. saundersii* eggs is 453 hours (sample size 8 embryos, SD ±8.95 hours). Germ band elongation proceeds in a manner identical to that described from other hemimetabolous insects (Bentley et al. 1979; Tojo and Machida 1998; Uchifune and Machida 2005) beginning at the anterior region. The early germ band is visible at 12% of development, and can be seen as a well formed anlage at 16% of development (Fig. 9.1).

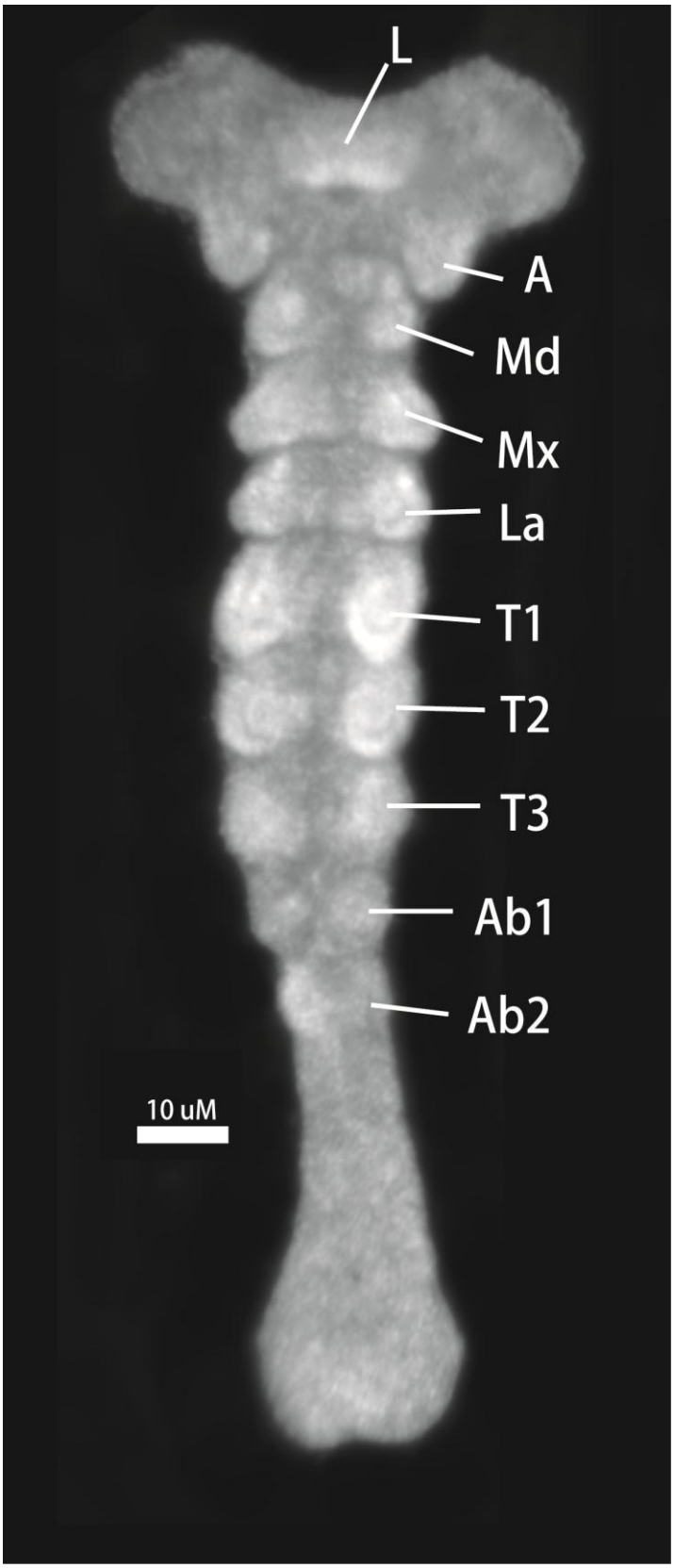

Figure 8. DAPI Stained *O. saundersii* Embryo. Embryo is at 26% of development. Head appendages: L, Labrum; A, Antenna; Md, Mandible; Mx, Maxilla; La, Labium. Thorax appendages: T1, Prothoracic leg; T2, Mesothoracic leg; T3, Metathoracic leg. Abdominal segments: A1, Abdominal segment 1; A2, Abdominal segment 2 (remaining abdominal segments have not yet differentiated)

By 17% of development the head region is clearly differentiated from the remainder of the embryo in both its overall width and by the fact that an anterior furrow begins to separate it into two distinct lateral lobes (Fig. 9.4). From 17% to 22% of development the germ band continues to elongate posteriorly without any major morphological differentiation. At 22% of development the antennae begin to form as small buds along the posterior margin of either side of the head (Fig. 9.10) and by 22.5% of development the mandibles and the maxillae are evident as lateral appendage buds in the two segments just below the head (Fig. 9.11); despite multiple embryos sampled at this stage it was impossible to see mandibular development prior to that of the maxillae. Development of these two appendages may happen simultaneously and the labial lobes form shortly thereafter.

The prothoracic and mesothoracic limb buds (T1 and T2) also form nearly simultaneously commencing at 23% of development (Fig. 9.12) and then the metathoracic limb buds begin to form at 23.5% of development (Fig. 9.13). External differentiation of the first three abdominal segments is visible in and anterior to posterior wave at 25% , 26%, and 26.5% of development (Fig. 9.15, 9.17 and 10.1, respectively).

Bifurcation of the maxillae can be seen at 26.5% of development and is followed by division of the labia at 27% of development (Fig. 10.3-4). Also at 27% of development the embryo initiates katrepsis; during this process the ventral side of the embryo pushes toward the center of the egg, beginning near the posterior end of the abdomen. This process is completed by

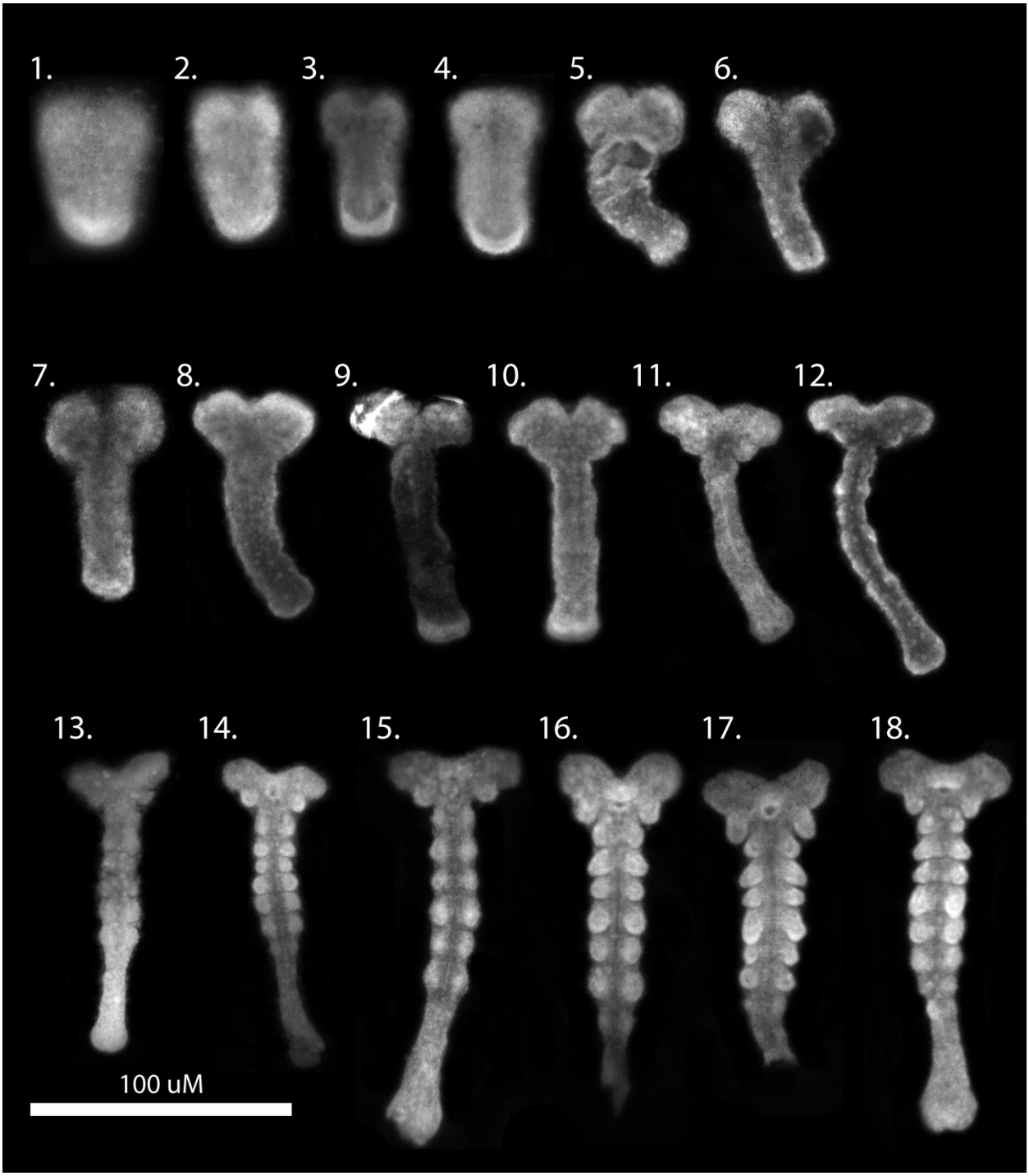

Figure 9. *O. saundersii* from 16% to 26% Total Development Time. Anterior aspect at top, earliest to most mature embryos from left to right. Percent of total development as follows: 1. 16%, 2. 16.3%, 3. 16.5%, 4. 17%, 5. 17.5%, 6. 18.5%, 7. 19%, 8. 20%, 9. 21%, 10. 22%, 11. 22.5%, 12. 23%, 13. 24%, 14. 25%, 15. 25.3%, 16. 25.6%, 17. 25.7%, 18. 26%.

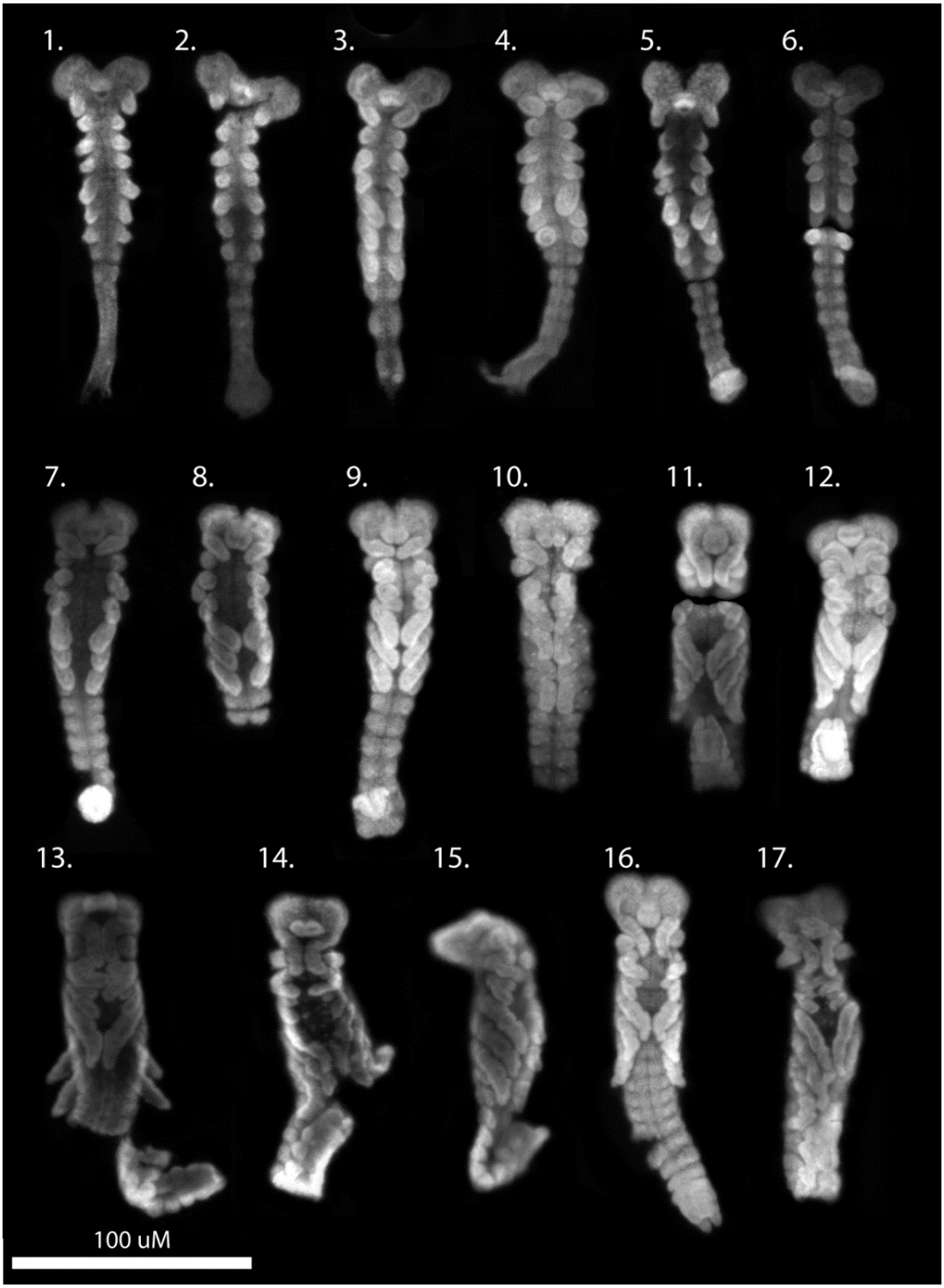

Figure 10. *O. saundersii* from 26% to 40% Total Development Time. Anterior aspect at top, earliest to most mature embryos from left to right. Percent of total development as follows: 1. 26.5%, 2. 26.7%, 3. 27%, 4. 30%, 5. 31%, 6. 32%, 7. 33%, 8. 33.3%, 9. 33.5%, 10. 35%, 11. 36%, 12. 36.4%, 13. 36.8%, 14. 37%, 15. 37.3%, 16. 37.5%, 39.7%.

32% of development and results in an embryo curving around the egg with the dorsal surface facing towards the outside of the egg and the ventral side in the center. From 27% of development through 32% of development the remaining abdominal segments is completed (Fig. 10.3-6) and cercal development commences at 33% of development (Fig. 10.7). Visible external leg segmentation begins at 33.5% of development and progresses through 40% of development, although the segmentation of the antennae is not externally visible until 37% of development is complete (Fig. 10.14).

CHAPTER III

ARACED PCR

Introduction

One of the challenges of evolutionary developmental biology is to identify and clone large portions of target genes that are of interest to researchers. This is particularly difficult for the Hox genes, a focus of many developmental studies. Although the homeodomain of each gene is largely conserved across Metazoa (Greer et al. 2000; Lemons and McGinnis 2006), and even more so within the arthropods(Averof 2002; Hughes and Kaufman 2002), this represents only a small portion (~180) bp of each gene and other parts of the Hox genes are much less conserved. This makes it more difficult to obtain larger portions of these genes for effective investigation of gene expression, functional assays using gene knockdown via RNAi, or for detailed study of molecular evolution.

One approach to obtaining larger portions of target genes when only a small portion of the gene is known is RACE-PCR (Frohman et al. 1988; Jain et al. 1992). RACE-PCR is short for Rapid Amplification of cDNA Ends and relies on a gene specific internal primer and a terminal primer designed to match a sequence common to all cDNA molecules in the target pool. Using this method much longer pieces of target genes spanning the area from the known sequence to either the 3’ or the 5’ end can be amplified. While very useful, this method has two disadvantages: first, you need to have a gene specific primer for each target gene; and second,

because of the universal nature of the binding sequence near the end of every cDNA there are often large amounts of non-specific product in the resulting amplified DNA (Borson et al. 1992; Russinova et al. 1995). We have developed a methodology called ARACED-PCR that is a novel combination of PCR techniques designed to overcome both of the disadvantages of traditional RACE-PCR.

Methods

Available DNA sequences for insect and crustacean genes Distal-less (Dll) and Ultrabithorax (Ubx) were downloaded from the NCBI (http://www.ncbi.nlm.nih.gov/) website and aligned in the program MEGA5 (Tamura et al. 2011) using the ClustalX algorithm(Larkin et al. 2007). Sequences were chosen to provide a wide range of organismal diversity so that globally conserved regions of these genes could be identified. Once these regions were identified the program CODEHOP (Chakravorty and Vigoreaux 2010; Staheli et al. 2011) was used to design internal degenerate primers that provide a match for all aligned sequences. cDNA for three target arthropods (*Oligotoma saundersii*, Embiidina; *Xenylla pseudomaritima*, Collembola; and *Tramea onusta*; Odonata) was created via standard methods using reverse transcriptase and a PolyT primer that includes an adapter sequence on the terminal end with a primer binding sites. This yields a pool of cDNA wherein each member has a specific primer binding site on the 3' terminus.

PCR was performed in two separate phases. The first phase is an asymmetric amplification of the target genes using only the internal degenerate primers (Gyllensten and Erlich 1988; Mazars et al. 1991). This step enriches the cDNA mixture for the target gene

without any non-specific amplification of untargeted genes. This PCR was performed using the following cycling profile: 95°C 5 min., [95°C 30 sec., 60°C 30 sec., 72°C 90 sec.] X 30. After this first round of PCR the 3' adapter primer and additional dNTPs was added to the mixture and the original cycling profile was repeated. This procedure was performed for both target genes for all target taxa and also for control reactions to which no DNA was added. In addition, controls containing both the internal and the terminal primers were subjected to a single round of cycling to determine the effectiveness of this approach. The reactions were assayed for the presence of PCR product via standard agarose gel electrophoresis. Reactions with strong bands were cloned using Invitrogen's TOPO® TA cloning system and positive transformants were subjected to another round of standard PCR using vector specific primers to identify inserts of target length. PCR product of putative clones with an insert size matching that of the ARACED-PCR product were sequenced and BLAST searched to verify identity of the cloned genes.

Results

Alignments for both genes revealed a relatively small region of conservation corresponding with the homeodomain of each gene. In Abd-B this region covered 210 bp and in Dll it covered 321 bp. Gel electrophoresis revealed strong PCR products for all taxa for the Abd-B gene (Fig. 11, estimated length: 291bp for *X. pseudomaritima*; 428 bp for O. *saundersii;* 503 bp for *T. onusta*) and strong products for both *X. pseudomaritima* and *O. saundersii* for the Dll gene (Fig. 11, estimated length 245 bp and 176 bp, respectively). The reaction for Dll for *T. onusta* yielded a smear spanning the region between 50 and 200 bp without any clear bands. There were also weaker bands representing secondary and tertiary products in all reactions, but

these were much weaker than the primary bands (Fig. 11). Sequencing and BLAST search of clones containing inserts corresponding to the above product sizes all yielded matches to the orthologous genes with the first several matches coming from other arthropod species. Both of the controls including not added DNA resulted in solution free of any significant PCR product. The controls consisting of a standard RACE-PCR approach using the degenerate primers (Fig. 11 lanes 2, 4, and 6) show only a moderate amount of PCR product of much smaller size and they also have much more non-specific product as demonstrated by the darker smear of larger DNA sizes.

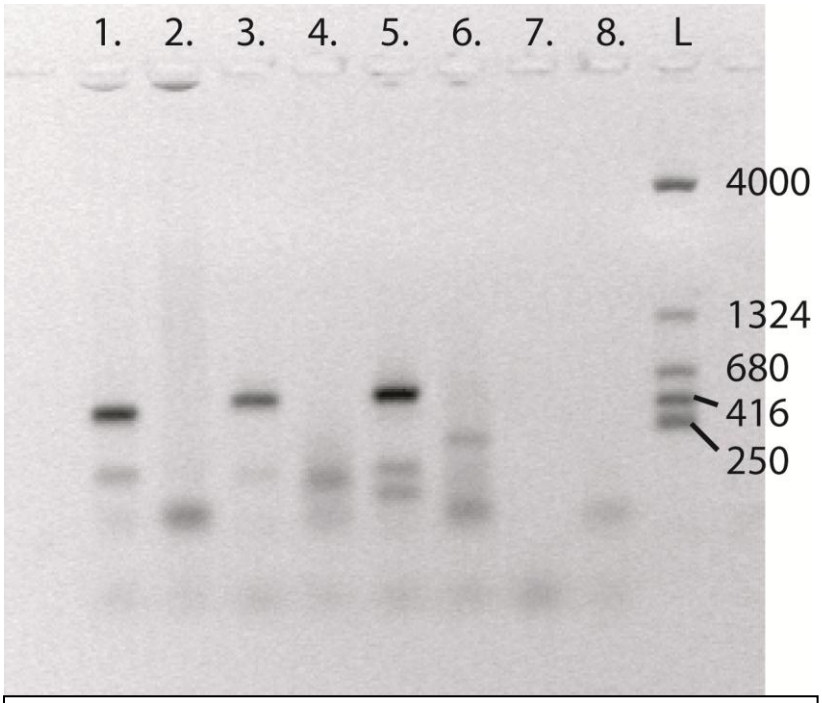

Figure 11. ARACED-PCR Products. Lanes 1, 3, 5, 7: ARACED-PCR for *X. pseudomaritima, O. saundersii, T. onusta* and control without DNA, respectively. Lanes 2, 4, 6, 8: Conventional RACE-PCR using our degenerate primers for *X. pseudomaritima, O. saundersii, T. onusta* and control without DNA, respectively. Lane L: DNA ladder with sizes indicated in base pairs

Discussion

This new approach overcomes the drawbacks of traditional RACE-PCR and provides a significant tool for the identification and cloning of large fragments of genes of interest. This

approach will be particularly useful for evolutionary/developmental studies that take a broader phylogenetic sampling approach in an attempt to describe and document the evolutionary shifts between major lineages (Minelli 2009). All of the resultant products from this study were relatively small, although larger than fragments that were previously available after traditional PCR using degenerate primers targeted to the conserved homeodomain (see section below for results of more traditional PCR approaches). This was not unexpected as the conserved regions for both of these genes are very near the 3' terminus. The specificity of PCR products for these two genes and the dramatic difference between ARACED-PCR and a more traditional approach indicates that this procedure should work equally well for longer pieces of genes. It is also possible to use this approach to amplify the 5' end of cDNA transcript as well; one would only need to use a cDNA synthesis procedure that adds a known primer binding site and then utilize a reverse internal primer designed in the same manner as described above. This approach will greatly increase our ability to examine and utilize large fragments of expressed genes.

CHAPTER IV

IDENTIFICATION OF TARGET GENES

Methods

RNA was isolated from mixed stage *O. saundersii* embryos using Trizol reagent and manufacturer's instructions. First strand cDNA was synthesized via SMARTScribe® reverse transcriptase (Clontech) by incubation at 42°C for 1 hour in the presence of two oligonucleotides (Gubler and Hoffman 1983). The first is a polyT primer with an adapter sequence that selectively transcribes complete mRNAs and includes a non-palindromic cut site for SfiI restriction endonuclease. The second oligonucleotide creates an adapter on the 5' end of the transcript that includes a separate non-palindromic SfiI cut site and a primer binding site (Fig.12). Double stranded cDNA pools were then made via LD PCR using Advantage 2 Polymerase® and primers specific for both adapters.

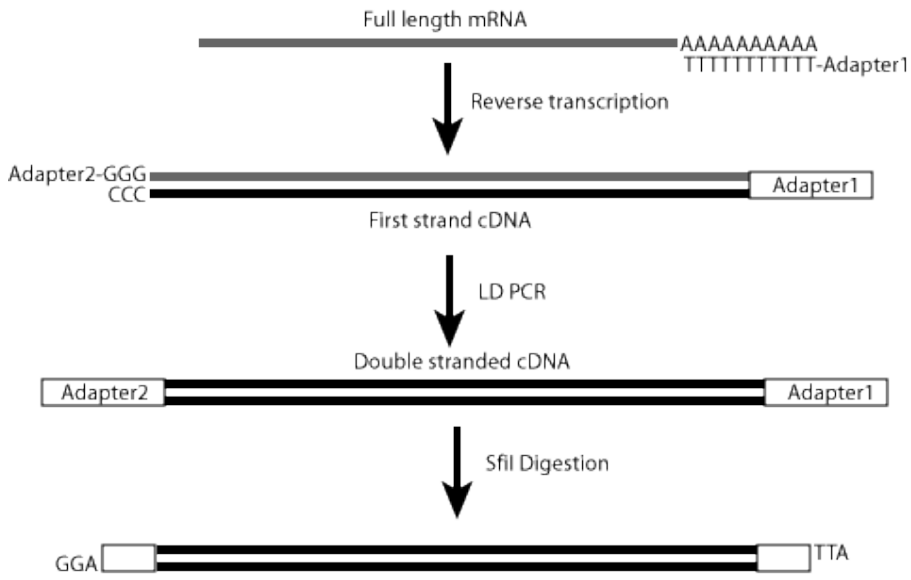

Figure 12. Outline of Preparation of cDNA for Library. Unique, non-palindromic restriction site create different overhangs on either end of the cDNA to create more efficient ligation reactions.

The cycling sequence was 95°C, 1 min. (95°C, 15 s.; 68°C, 6 min.) x 22 cycles. Following LD PCR the sample was treated with protein kinase at a final concentration of 200 µg/ml and incubated at 45°C for 20 min. The cDNA was then purified via phenol: chloroform extraction followed by ethanol precipitation, resuspended in 60 µl ddH_2O and concentration of cDNA was measured using a Nanodrop® 1000 spectrophotometer. A portion of this cDNA pool was set aside for PCR and the remainder was digested with SfiI endonuclease at 50°C for 2 hours to create specific ligation sites at either end. Column fractionation of the final cDNA pool was done using a Sephacryl ® column equilibrated with TEN buffer (10 mM Tris pH 7.5, 0.1 mM EDTA, 25 mM NaCl). 18 separate fractions were collected in separate microcentrifuge tubes and assayed for DNA via agarose gel electrophoresis. The first two fractions with visible DNA represent a portion of the cDNA pool containing large cDNA transcripts now separated from all smaller fragments of DNA. These two fractions were purified via ethanol precipitation, and resuspended in 12 µl ddH_2O.

A suitable ligation vector was created by removing a small sample of the raw cDNA pool prior to restriction digest and treating with T4 DNA Polymerase for 15 minute at 37°C in the presence of dNTPs. This creates blunt ended double stranded DNA. This blunted DNA was then ligated into a blunted vector, transformed into *E. coli* and then plated onto selective media. Twelve colonies were selected from the resultant transformants, cultured overnight in selective liquid media, plasmid purified and then amplified via PCR using vector specific primers to verify the presence of the SfiI cut sites. An appropriate plasmid was then selected, and a larger overnight culture was performed followed by standard plasmid extraction and purification. The purified plasmid was then digested with SfiI endonuclease at 50°C for two hours then subjected to agarose gel electrophoresis to separate the vector (~4000 bp) from the insert (~1450 bp). The

vector band was cut from the agarose gel using a sterile razor blade and the vector was removed from the gel by placing the sample in a filter column and then centrifuging at 3000 xg for 10 minutes into a sterile microcentrifuge tube. The resultant sample containing the vector was then purified via two rounds of phenol: chloroform extraction followed by ethanol precipitation and resuspension in ddH_2O. This vector solution was then assayed as used for ligation reactions with the prepared cDNA pools.

Four ligation reactions were performed using small amounts of the cDNA pool and the vector at estimated ratios of 1:1, 1:2, 1:4 and 1:8 vector to cDNA molar concentrations. cDNA molar concentrations were estimated using 2.5 kb as an average insert size. After transformation into competent *E. coli* by electroporation and plating onto selective media the ratio with the highest number of transformed colonies was noted and 10 random colonies were cultured overnight in selective liquid media, plasmid purified and then subjected to restriction digest to determine the average insert size. After this assay a larger ligation reaction was performed using the molar ratio that yielded the most transformants. Total transformed bacteria were pooled and then assayed for unique colony forming units (cfu) via serial dilution and plating onto selective media.

For standard PCR reactions degenerate primers were designed using alignments generated by downloading available insect and crustacean sequences for our target genes from the NCBI website. Alignments were done at the amino acid level using the ClustalX (ref) algorithm at default settings as implemented in the program MEGA5 (ref). Target genes included all eight of the insect Hox genes (labial, lab; proboscipedia, pb; Deformed, Dfd; Sex-combs reduced, SCR; Antennapedia, Antp; Ultrabithorax, Ubx; abdominal-A, abd-A; Abdominal-B, Abd-B) and the developmental genes Distal-less (Dll) and engrailed (en).

Conserved sections of each alignment were used to generate primers using the CODEHOP (Staheli et al. 2011)program. These primers were then used in standard PCR reactions with annealing temperatures ranging between 50 and 60°C. PCR reactions yielding a clear product were cloned using Invitrogen's TOPO® TA cloning system and assayed for the appropriate insert size via standard PCR using the vector specific primers M13F and M13R and a small sample of the colonies of putative colonies of *E. coli* containing the cloned gene. PCR reactions of the correct target size, which varied from gene to gene, were then sequenced and subjected to BLAST searches to verify gene identity.

Results

cDNA synthesis of 1.5 µg of total *O. saundersii* RNA yielded a strong smear of visible DNA ranging from over 10 kb to below 200 kb. After fractionation there was a clear separation based on size in the separately collected fractions (Fig. 13), showing good removal of smaller contaminating DNA strands. The final prepped cDNA pool had a measured concentration of 110 ng/µl. The 1:6 estimated molar ratio (vector: insert) had the most transformants and also had an estimated average insert size of 2.1 kb. The larger ligation reaction and subsequent transformation at this molar ratio yielded an estimated 1.3 million unique colony forming units. Future work will use this full length cDNA library to isolate complete target genes for expression assays and molecular evolution analyses.

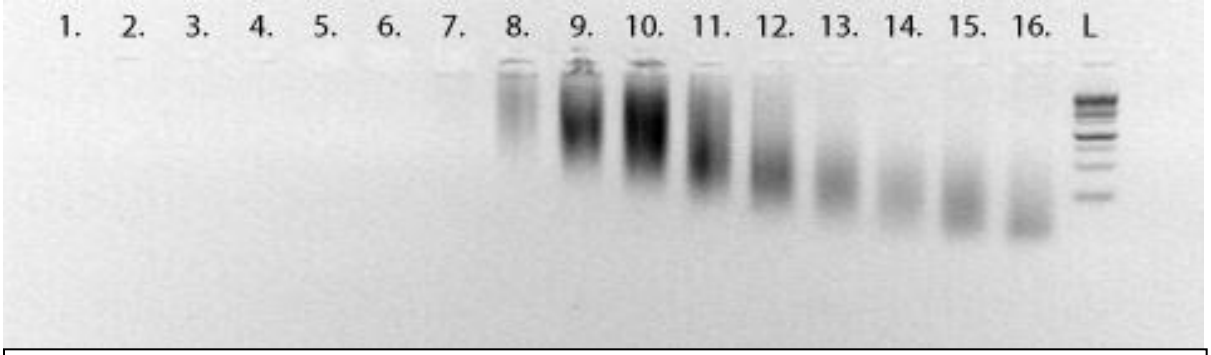

Figure 13. Fractionation of *O. saundersii* cDNA. Largest pieces of cDNA are found in the early fraction, first visible faintly in fraction 7. Fractions 7, 8, 9 were pooled and cleaned up via ethanol precipitation for ligation into library vector.

amino acid level, ranging from 200 to 260 bp. In all cases this conserved region corresponded to the homeodomain and its immediate surrounding sequence. CODEHOP primers designed from these regions yielded PCR products for six of the eight HOX genes (lab, Dfd, Antp, Ubx, abd-A, Abd-B) and both Dll and en. Subsequent cloning, sequencing, and BLAST search positively identified small portions of all of these genes except Abd-B. Although all these sequences are less than 300 bp they provide a valuable evidence for the Hox genes in *O. saundersii* and a resource that will allow for isolation and identification of larger portions of the target genes.

CHAPTER V

DISCUSSION

This work makes several valuable contributions to our understanding of Embiidina development specifically and also, generally, to our ability to describe and understand the evolutionary history of insect morphology. *O. saundersii* can be cultured for multiple generations in the laboratory and will provide ample embryos with only minimal maintenance. This makes it an ideal laboratory organisms as both a representative of Embiidina and as a model for polyneopteran insects. Embiids unique morphology also make this species a valuable opportunity for the study of the origin and evolution of novel features. Study and description of the morphology and genetics of the development of the Embiidina tarsal silk glands will provide valuable insight into the evolution of silk, a feature that has evolved multiple times across the arthropods (Craig 1997; Collin et al. 2011). Examination of Embiidina silk formation has only been cursory (Collin et al. 2005; Okada et al. 2008; Collin et al. 2009). Our work demonstrates that Embiidina have an early embryology that is typical of hemimetabolous insects. Their development takes place via germ band elongation in an anterior to posterior pattern.

We anticipate that expression data for Embiidina Hox genes, and both Dll and en will exhibit a typical insect pattern corresponding with the conservation seen in the development of the embryos. Whether, the unique morphology of the fore tarsi is controlled by the same genes as in other insects or by novel or co-opted genes is still undetermined. We anticipate a fairly typical pattern of leg development during early stages and divergence and expression of as yet

unknown genes during the fine patterning of the distal elements of the forelimbs. Understanding the conserved and divergent morphologies underlying unique taxa such as Embiidina is essential for addressing questions about the evolutionary diversification of insect limbs (Jockusch and Ober 2004)

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