Investigation of *even-skipped*, a developmentally-regulated gene controlling neural segmentation in dragonflies

By

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ABSTRACT

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A comprehensive understanding of the genetic mechanisms underlying pattern formation and neurogenesis is necessary in order to trace the evolutionary history of insect embryogenesis. One of the most important processes of embryogenesis is the organized pattern formation that allows for proper body segmentation and neural development. Proper segmentation, which relies on a series of specific gene expressions, is necessary for the development of an operational nervous system. *Even-skipped* (*eve*), one such regulatory gene, has been studied extensively in certain model organisms, and theories regarding the evolution of its functional role could be further elucidated by visualizing its expression in adult and larval dragonflies, which has yet to be accomplished.

Through a protocol of immunofluorescence using antibodies raised against the even-skipped protein product (*eve*), this study aimed to visualize the localization of *eve* expression in both adult and larval dragonflies and thereby compare its expression throughout development. However, several methodological limitations were encountered, including a lack of published literature detailing a procedure for immunostaining in dragonflies and subsequent inability to properly permeate the target ganglia. Future research should attempt alternative methods of tissue permeation in order to successfully access the target neurons as well as explore alternative primary antibodies for use in targeting *eve* in tissue samples.

TABLE OF CONTENTS

INTRODUCTION

Physiology and Evolutionary History of Dragonflies

The phylum *Arthropoda* is a diverse group of organisms generally characterized by their jointed appendages and segmented body plan. This phylum includes all of Earth's insects, crustaceans, and arachnids, as well as several other classes, and is the most physiologically and geographically diverse phylum on Earth. In addition to being extremely diverse, arthropods are quite ancient, in evolutionary terms, first appearing in fossil records from the Early Cambrian era over 500 million years ago (Edgecomb & Legg, 2014).

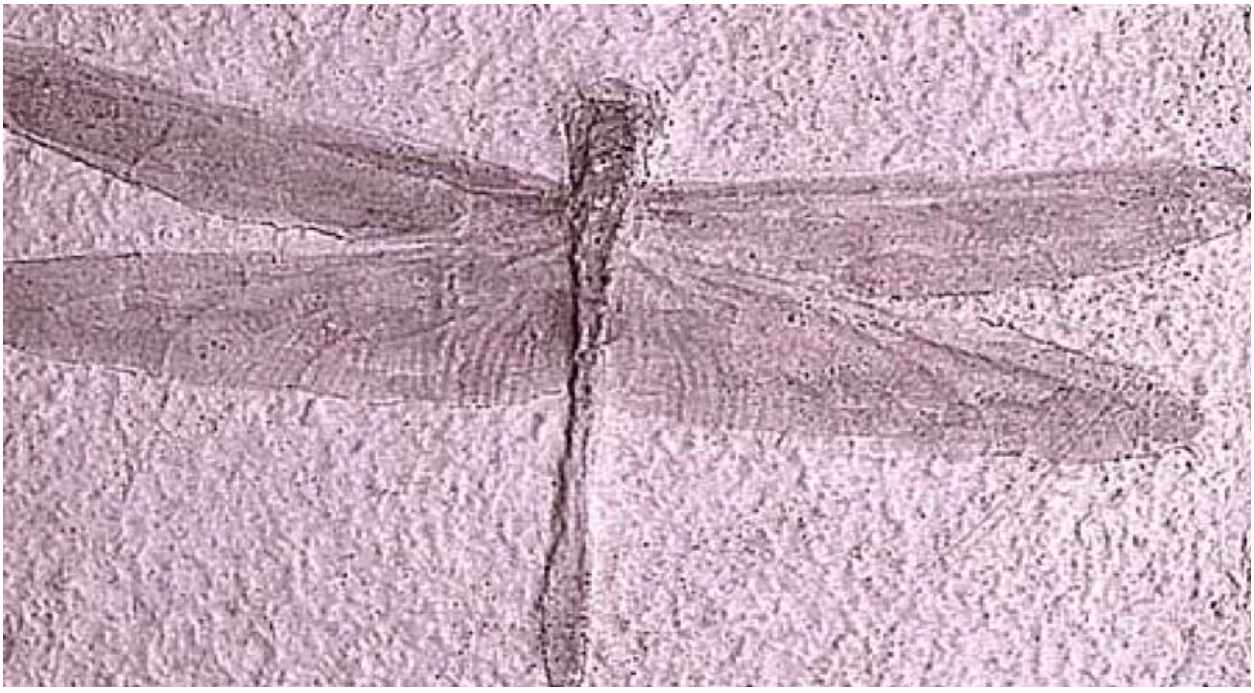

Figure 1. Fossil of *Protolindenia wittei*, found in the Jurassic *Solnhofen* limestones of Bavaria, Germany. With a wingspan of about 15 cm, this fossil is approximately 155 million years old, only half the age of the oldest known fossils of Odonata (Sabet-Peyman, 2000).

The dragonfly (*Anisoptera*) serves as an extant representation of some of the first winged arthropods on Earth. Ancient ancestors of the modern dragonfly are known as *Meganisoptera*

(formerly *Protodonata*) and have been observed in fossil records dating back to the early Permian period (Resh, 2009). *Meganisoptera* appeared almost identical to modern dragonflies except for one key aspect - their wingspan reached up to 75 centimeters (Sabet-Peyman, 2000). Although the dragonfly is not genetically identical to that of the ancient *Meganisoptera*, it is, along with damselflies, their closest known living relative. *Meganisoptera* fossil records show evidence of several traits still exhibited by modern dragonflies, such as complex life cycles and mating systems, and an aquatic nymph stage (Bybee et. al., 2016).

As with all arthropods, dragonflies are extremely versatile and geographically widespread, due in part to a protective cuticle that serves as their exoskeleton. The presence of a cuticle lends to the ability of arthropods to adapt to a variety of extreme environments. Comprised of chitin and covered in a waxy coat, the cuticle waterproofs organisms and protects other layers of tissue from damage or decay (Moussian, 2013). This cuticle, though tough, is segmented, thereby allowing for a high level of mobility which enables arthropods to occupy a large and diverse array of ecological niches, both terrestrial and aquatic. Segmentation of the cuticle is a key characteristic of all arthropods that contributes largely to their versatility and geographical diversity. Moreover, the arthropod nerve cord develops using the same genetic mechanisms as does the segmentation of the cuticle. Due to its durable nature, the cuticle can be a formidable obstacle in immunostaining some arthropods, which will be discussed in later sections.

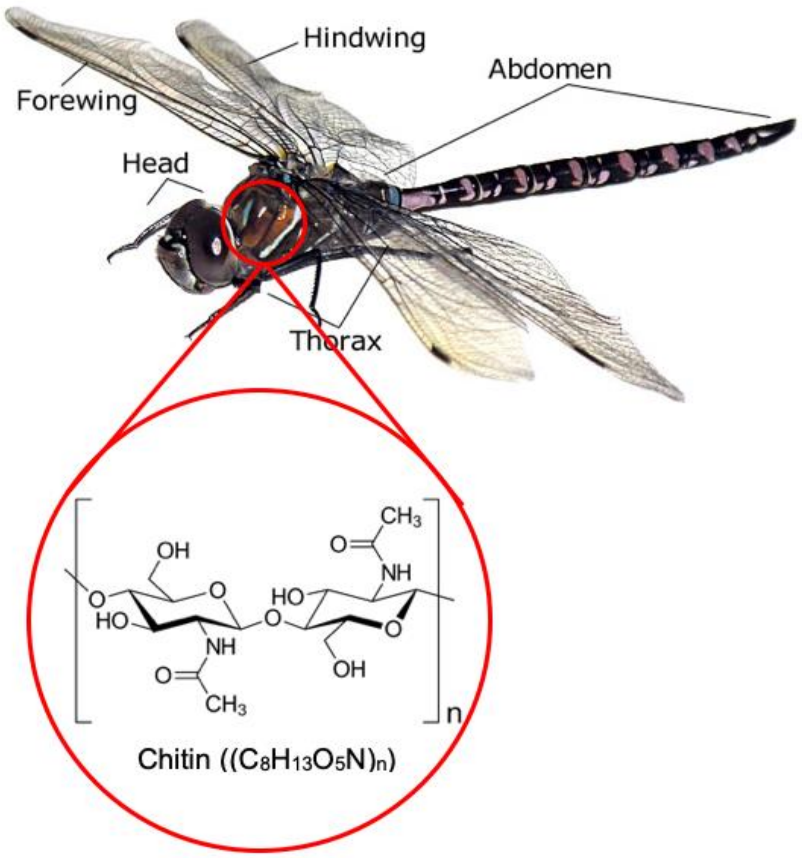

Figure 2. Chemical structure of chitin as found in the cuticle of dragonflies.

Dragonflies spend most of their life as aquatic nymphs. Depending on geographical location, the development of nymphs to their final adult form can take as many as six years (Sabet-Peyman, 2000). During embryonic development, newly forming dragonflies are segmented horizontally along the anterior-posterior axis. As they grow to adults, their body is divided into three distinct segments: the head, the thorax, and the abdomen (Figure 2). Adult dragonflies have six appendages attached to their thorax, three on each side of the body, and two large, elongated wings, which they use to fly for the remainder of their life span (Suhling et. al., 2015).

Early Pattern Formation in Insect Embryogenesis

In the embryonic development of any Arthropod, pattern formation is one of the key factors by which an organism is able to develop a functioning nervous system (Smarandache-Wellmann, 2016). Because body and nerve cord segmentation are controlled by overlapping genetic mechanisms, knowledge of one is necessary to fully understand the other. The basic body plan of all Arthropods consists of several distinct segments with attached appendages. These segments are specialized for function, developing and differentiating during embryogenesis. The arthropod nervous system consists of a pair of ventral nerve cords that run along the anterior-posterior axis of the organism. In each segment, the cords form a pair of ganglia from which sensory and motor neurons extend into the segment; this creates a "ladder-like" appearance of the central nervous system (Smarandache-Wellmann, 2016). Though this structure is generally common to all arthropods, it is difficult to make many generalizations due to the high level of diversity within the phylum. In dragonflies, the nerve cord extends from the brain through the thorax and abdomen and contains five major ganglia: subesophageal, prothoracic, mesothoracic, metathoracic, and abdominal (Figure 3).

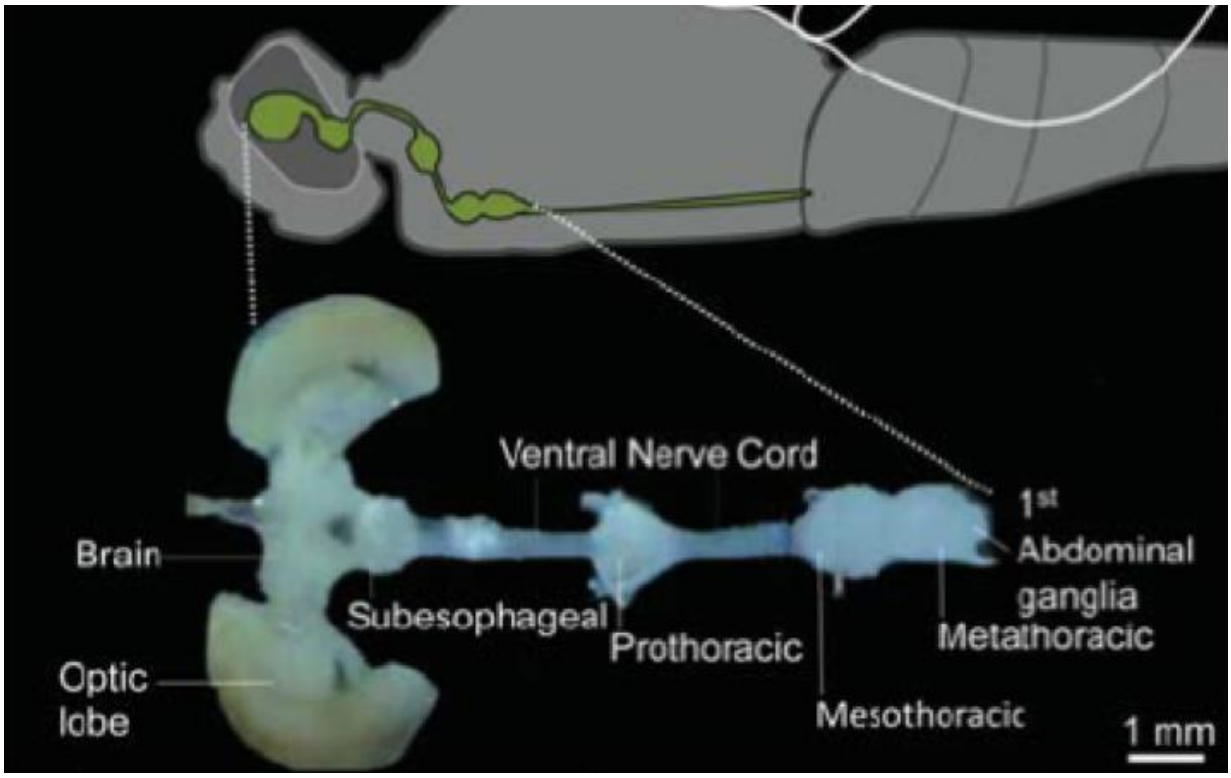

Figure 3. Dragonfly nerve cord anatomy. Prothoracic, Mesothoracic, and Metathoracic ganglia were dissected out and used in this study (Gonzalez-Bellido & Wardill, 2012).

The central nervous systems of all arthropods develop in concert with body segments, and therefore proper segmentation, which relies on a series of specific pattern formations, is necessary for the development of an operational nervous system. This process of pattern formation is controlled by a complex hierarchy of genes expressed throughout development. In order to effectively elucidate the purpose, function, and evolutionary history of these genes, it is necessary to perform comparative studies between model species. As of yet, such studies have been conducted extensively in *Drosophila melanogaster* and *Schistocerca americana*, also known as fruit flies and grasshoppers.

Three families of genes known as *gap, pair-rule and hox,* have been identified in *Drosophila* during embryogenesis. In early embryonic development, the body plan of the embryo

is subdivided into increasingly specific segments under the guidance of this hierarchical cascade of regulatory genes. This cascade begins with the gradiented expression of localized maternal factors from the anterior and posterior poles of the embryo. Expression of these maternal factors then controls the transcription of *Gap* genes, which repress the formation of adjacent body segments. This creates "gaps" in the body plan, thus segmenting the embryo along the anterior-posterior axis. *Gap* genes encode transcription factors that control the expression of *Pair-rule* genes. *Pair-rule* genes are then expressed in alternating segments, thus further subdividing the body plan of the larvae (Brook, 1998). The downstream targets of many *pair-rule* genes are known as *Homeobox* (*Hox)* genes, which regulate the expression of transcription factors that ultimately determine the appendages that will develop on each body segment. Hox genes have a wide array of downstream targets, including genes that promote apoptosis, cell adhesion, cell division, and cell migration - functionalities that serve to promote morphogenesis and cell differentiation (Pearson et. al., 2005).

In summary, maternal factors establish polarity, *gap* and *pair-rule* genes work to divide the embryo into segments, and *hox* genes work to differentiate and specialize those individual segments. In order for an embryo to successfully develop, all of these gene families must be expressed and must work in concert with one another. This level of segmentation control guides neuroblasts of the developing nervous system to orient in a structurally similar manner (Jarvis et. al., 2012). Dozens of genes work in synchrony to create a functional ectoderm and nervous system during embryogenesis, but this study will focus on the function and presence of the *even-skipped* gene in particular, as it serves as a representation of the establishment of regional identity during development (Brody, 1998).

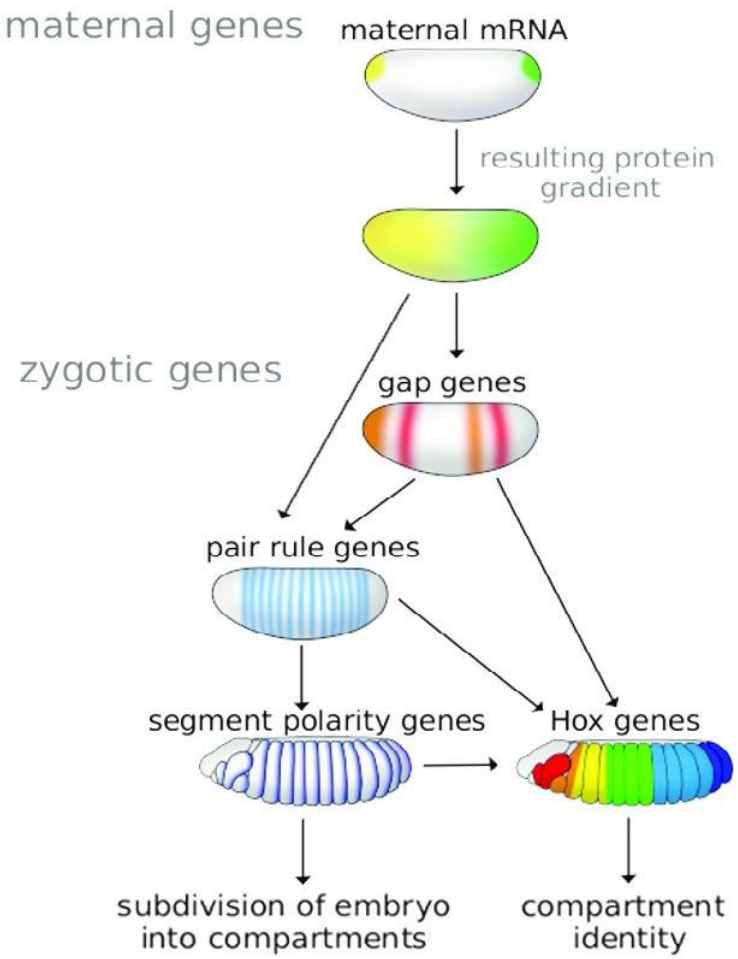

Figure 4. Schematic representation of segmentation during embryogenesis in *Drosophila melanogaster* (Hueber, 2009).

Role of *Even-skipped* in Drosophila and Schistocerca:

Even-skipped (*eve)* is a member of the *pair-rule* gene family and serves as a transcriptional repressor of several downstream targets, thereby fulfilling a key role in segmentation and insect neurogenesis. For instance, one of the primary gene targets of *eve* is Fushi-tarazu *(ftz)*, which, when repressed, allows for an alternating pattern of *eve* protein expression in the developing blastoderm. This *pair-rule patterning*, which is indeed controlled by a number of complex protein expressions and interactions, ensures that each cell of the developing embryo has a unique identity (Brody, 1998).

Nipam Patel's lab at the University of Chicago's Howard Hughes Medical Institute has contributed extensively to the literature on pattern formation in arthropods, and the work done in his lab regarding *even-skipped* has served as much of the foundation for this current study. Patel and colleagues have published several papers using both *Drosophila melanogaster* and *Schistocerca americana* as model organisms in order to investigate the functions and conservation of *Hox* and *Pair-rule* genes, including *eve*. In 2003, Patel, alongside Gregory Davis of Princeton University, published a review of Pair-rule gene conservation among several species of arthropod in order to elucidate a possible history of pair-rule gene evolution. In this review, Patel and Davis concluded that current literature supports the hypothesis that Pair-rule patterning is a mode of segmentation utilized only by insect arthropods, but that this hypothesis must be provisional due to a scarcity of gene expression data (Davis & Patel, 2003).

In looking specifically at *eve* expression patterning, Patel has found that Drosophila express *eve* in seven complementary stripes along the developing blastoderm, while Schistocerca express it in broad posterior domains, suggesting that the protein plays a different role in pattern formation among different insect species (Patel et. al., 1992). The specific functions of *eve* in the developing Drosophila embryo are well-studied; *eve* expression in Drosophila has been found to contribute to the formation and fate of early neural progenitor cells in the developing embryo and to the guidance of motor axons towards the dorsal muscle field (Broadus & Doe, 1995, Landgraf et. al., 1999). The exact purpose of *eve* expression in developing Schistocerca embryos is uncertain, but its differing location during early development as compared to Drosophila suggests that it plays a much different role in pattern formation. In addition, phylogenetic studies indicate that *eve* played a role in axon patterning and guidance in the common ancestor of vertebrates and arthropods, as evidenced by the identification of similar expression patterns in

specific identified neurons between Drosophila and Schistocerca (Patel et. al., 1992). In short, although Drosophila and Schistocerca express *eve* in differing patterns during different stages of development, they do express it identically in specific neural subsets that are involved in axon guidance. This suggests that axon guidance is possibly the most well-conserved function of *eve* across insect species.

In addition to spatial expression, temporal expression of *eve* differs between species and can therefore contribute toward a clearer picture of the evolution of pattern formation in arthropods. One characteristic of insects that can predict temporal *eve* expression is the germ-band length. The terms *short germ-band* and *long germ-band* refer to the initial size of the germ-band during embryogenesis and are often used to distinguish insects with differing patterns of embryonic development. Thus, identification of the length of a species' germ-band can provide clues as to how similar or different its patterns of development might be to those of other species. Therefore, germ-band length can be useful for making phylogenetic hypotheses. Long germ-band insects, such as Drosophila, express *eve* during early neurogenesis, as discussed earlier, while short germ-band insects, such as Schistocerca, have been shown to express *eve* during later neural development in order to direct pair-rule patterning (Patel et. al., 1992, Figure 5). For instance, one study conducted using the locust *Schistocerca gregaria* found that *eve* seems to demonstrate both phasic and consistent expression in segment-specific subsets of neurons throughout the adult lifetime of the insect. The authors suggest that maintained *eve* expression within subsections of the adult CNS helps to maintain neural phenotype by regulating the expression of downstream cell adhesion factors (Bevan & Burrows, 2003). This evidence suggests that as long germ-band insects evolved, *eve* gained an additional function of pair-rule patterning and phenotypic maintenance of the mature CNS.

Dragonflies, as it turns out, are of the intermediate germ-band variety, and thus it is unclear exactly what temporal role *eve* might play in their neural development (Davis & Patel, 2002). Elucidation of the temporal role of *eve* could support the theory that *eve* gained additional functions as long germ-band insects evolved from their short germ-band ancestors.

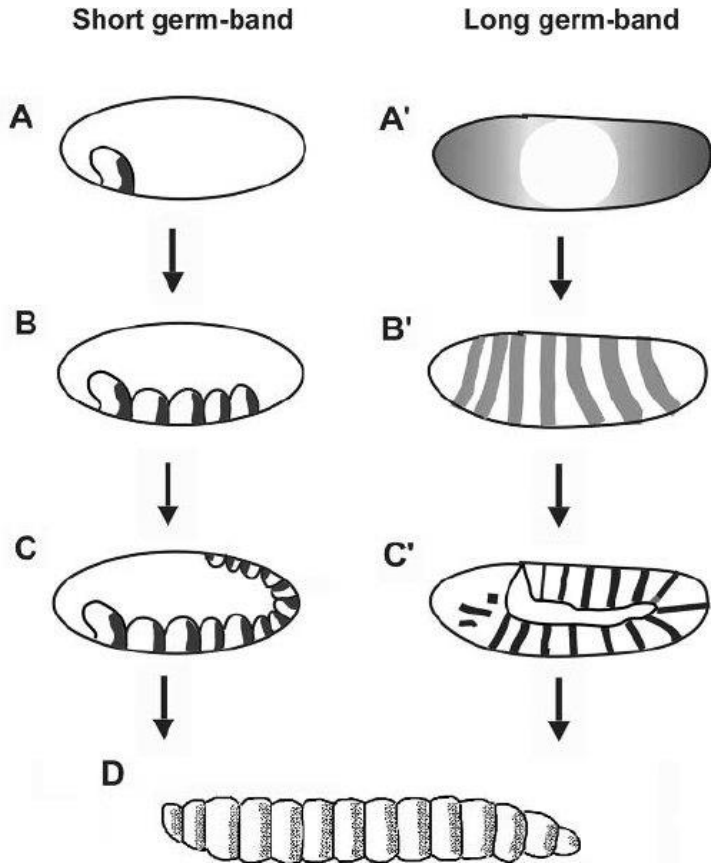

Figure 5. Differential segmentation in short and long germ-band insect embryogenesis. Expression of *eve* is depicted schematically in grey. Note that *eve* is only expressed during embryogenesis in long germ-band insects and not in short germ-band insects (Newman & Forgacs, 2007).

Potential Techniques for Determining Gene Expression

In order to investigate *eve* expression in dragonflies or in any other organism, it is necessary to employ one of several molecular techniques, depending on the needs, limitations, and specific goals of the study. Previous literature on Pair-rule gene expression indicates that a

variety of techniques can be used for measuring *eve* expression either spatially or temporally. Often, mRNA quantification is used to measure gene expression, as this quantity would indicate how much of a particular gene has been transcribed within any cell at any one time. This can be achieved through several techniques, including Northern hybridization and blotting, RNA sequencing using synthesized cDNA, and hybridization microarray. However, because these techniques require isolation of mRNA from the organism of interest via cellular lysate, they can only be used for studying relative temporal gene expression and do not allow for visualization of patterns of expression within the intact specimen. Even so, the temporal information gleaned from such techniques is relatively unspecific, and therefore not always useful. Similarly, techniques of protein quantification such as Western hybridization or spectrophotometric assay reveal little to no information about spatial differences in expression, and instead only provide isolated concentrations of proteins in the sample. In addition, hybridization techniques such as these require a known heterologous or homologous sequence to the gene of interest in order to design primers that can be used for Polymerase Chain Reaction (PCR). This can be an obstacle when investigating gene expression in an organism in which no such known sequence is available.

In addition to these hybridization techniques, it is possible to measure gene expression using immunostaining techniques such as immunohistochemistry or, specifically, immunofluorescence. These techniques take advantage of the binding of antibodies to their respective antigens. When a tissue is treated with a specific antibody, those antibodies will bind wherever their corresponding antigens are present in the sample. Once these primary antibodies are bound to the antigens present, the tissue can be treated with a secondary antibody tagged with a fluorescent dye known as a fluorochrome which binds to the primary antibody. Thus, when

viewed under a fluorescent microscope, the tissue sample will fluoresce wherever the target antigen is expressed. This method is therefore especially useful for detection of protein location within a sample, as it allows one to visualize exactly where in the tissue a target protein is being expressed.

GOALS, MATERIALS, AND METHODS

Goals

This extensive study of *even-skipped* expression in *Drosophila* and *Schistocerca* has provided investigators with a limited understanding of the purposes and functions of the gene, but not much is known yet about its conservation across evolution. In order to demonstrate conservation of the *even-skipped* gene further back through evolutionary history, it is necessary to identify its expression in a more evolutionarily ancient species. Because dragonflies are extant remnants of an ancient species of arthropod, they consistently prove to be valuable tools in the study of insect evolution and phylogeny. By investigating the expression of *even-skipped* in larval and adult dragonflies, this study aims to add to the existing body of knowledge regarding the conservation of developmentally regulated gene expression throughout arthropod evolution. In addition, understanding the temporal role of *eve* in dragonflies could provide evidence for or against the theory described earlier that *eve* gained certain functions during the evolution of long germ-band insects from their short germ-band ancestors.

Specimens of both larval and adult stages will be analyzed in order to give a temporal comparison of *eve* expression across the lifespan of the insect. An immunohistochemical protocol will be employed in order to visualize the localization of *eve* expression within the tissues, as was done previously in studies looking at both Drosophila and Schistocerca (Patel et. al., 1993, Bevan & Burrows, 2003).

Specimens Used

For this experiment, Aeshnid dragonfly larvae were collected throughout the summer and early fall in the nearby area. Because these insects can remain in their larval state for months, they were kept alive in pools of water until the date of dissection. Adult Aeshnid specimens were

kept in petri dishes at 4°C until the date of dissection.

Dissection Protocol

The dissection of both larvae and adult dragonflies aimed to isolate the mesothoracic and metathoracic ganglia located on the anterior ventral surface of the thorax. At both stages, dragonflies were first anesthetized for 15-20 minutes in a freezer at 0°C. When properly anesthetized, dragonflies were transferred to a Sylgard plate and the head was removed at the labium. Next, all legs and wings were removed using the same scissors and the thorax was cut vertically down the cuticle to expose the interior of the specimen. The animal was pinned to the Sylgard plate on both sides of the opened cuticle, and connective tissue and digestive organs were carefully removed using tweezers. When the mesothoracic and metathoracic ganglia were exposed, the nerve cord above, below, and to each side of the ganglia was cut to allow the removal of the ganglia. When removed, the ganglia were placed in a ____ in a 4% formalin solution and placed in a 4°C refrigerator for at least 24 hours.

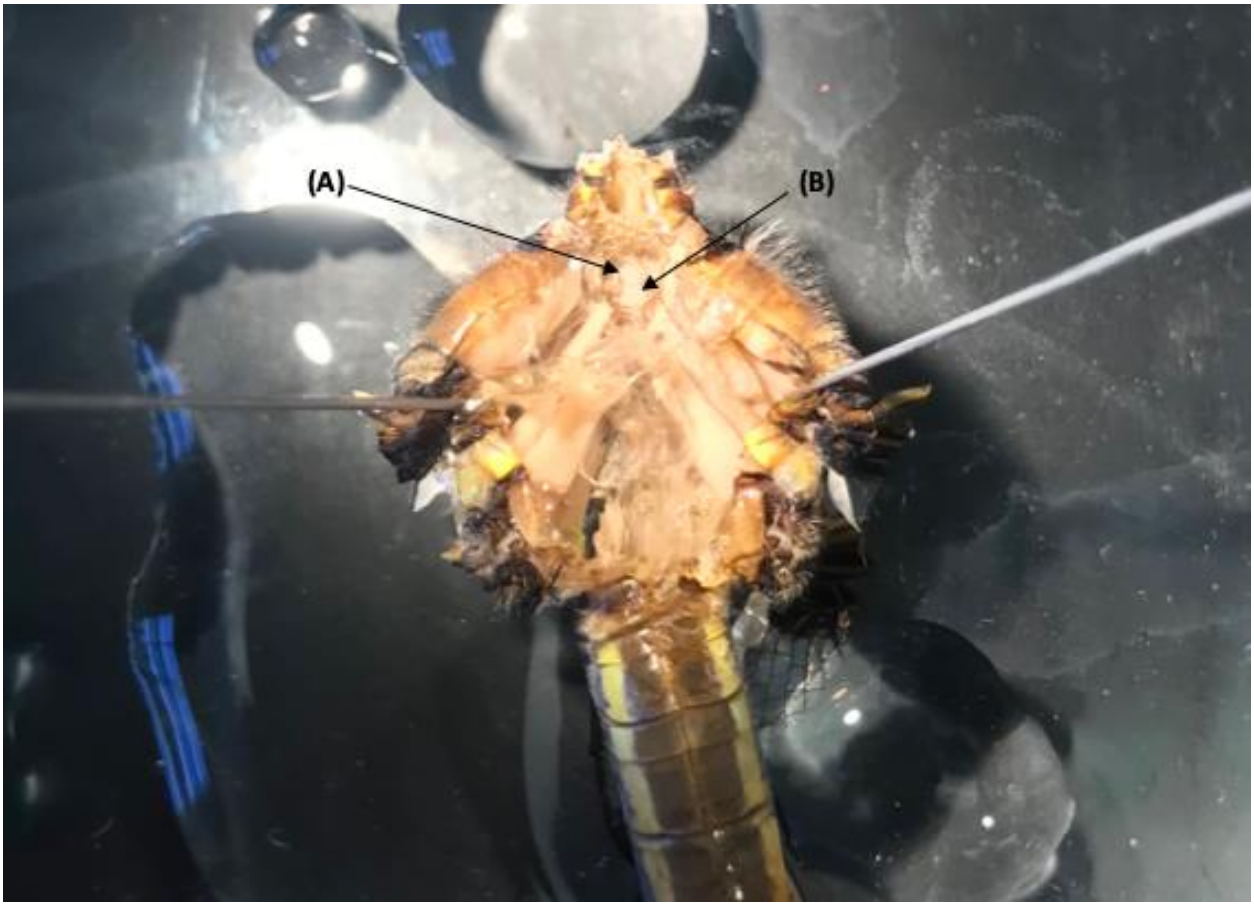

Figure 6. Example dissection setup of an adult dragonfly. (A) and (B) indicate the mesothoracic and metathoracic ganglia, respectively.

Immunohistochemistry and Fluorescent Imaging

The immunohistochemical technique employed for this study was adopted from a protocol developed by Nipam Patel at the University of Chicago Howard Hughes Medical Institute. Ganglia obtained from dissection were rinsed three times for 10 minutes in 0.1% PBT (500mL Phosphate buffered saline solution and 1mL Triton X-100) at room temperature. Samples were then dehydrated in an ethanol series (30%, 50%, 70%, 90%, 100%) for 40 minutes each, and subsequently rehydrated in a reverse series for 15 minutes each. They were then washed in 0.1% PBT for 20 minutes and refrigerated overnight at 4°C. The next day, each specimen was submerged in a permeation solution (3mL PBS per specimen + 30uL papain

(20mg/mL) and 30uL hyaluronidase (10mg/mL) per mL PBS) and incubated for 30 minutes at 37°C. The permeation solution was removed, and all specimens were washed twice for 30 minutes in 0.1% PBT and then submerged in diluted (1:20) Normal Goat Serum (3mL 0.1% PBT and 150uL NGS per specimen) and refrigerated at 4°C for 2 hours. After 2 hours, the NGS was removed and a primary antibody (FAS-II) was diluted 1:50 and added to each specimen. FAS-II was chosen as the primary antibody as recommended by Nipam Patel in his comprehensive manual of antibody staining protocols (Patel, 1994). The specimens were then incubated at 37°C for 3.5 days. Next, the samples were washed twice for 30 minutes in NGS and once for 30 minutes in 0.1% PBT. They were then submerged in a diluted (1:200) secondary antibody (488nm Goat anti-mouse) at 4°C for 2-4 days. After 2-4 days, the tissue samples were washed again twice for ten minutes in PBS and submerged in 50% glycerol for 1-2 days. Finally, the samples were washed again twice in PBS for 10 minutes. Once all samples had been treated with both antibodies and cleared with glycerol, they were placed in PBS in individual dishes and viewed under fluorescent microscopy.

RESULTS AND DISCUSSION

Results

Due to several limitations that will be discussed below, this study was unsuccessful in determining any localization of *eve* expression in either larval or adult dragonfly specimens. Because no existing literature employed immunohistochemical techniques in dragonflies, the limitations and challenges associated with such a task were unknown and proved sufficient to prevent any viable results from being obtained. The level of difficulty associated with using such techniques to study dragonfly genetics may explain the lack of published literature on the subject. However, this study did prove useful in providing information as to how the task at hand could be successfully completed in future studies, as well as what techniques should be avoided.

Methodological Limitations

Due to a lack of published literature on any studies that have been conducted using an immunohistochemical approach to dragonfly genetics, there was little information on which to base the methods employed in this study. Several previous experiments have used antibody staining to investigate developmental gene expression in Drosophila and Schistocerca, as well as various other species of the Arthropod phylum. Thus, it was determined that such an approach could be appropriate as a relatively fast and easy way of determining protein expression in dragonflies.

At least one study, which investigated the presence of the protein Resilin in the developing cuticle of various insects, relied on immunostaining in order to detect protein localization in dragonflies (Wong et. al., 2012). Researchers in this case were successful in binding the anti-resilin antibody they developed to the target protein present in dragonflies.

However, because this experiment aimed to detect protein presence within the cuticle structure of the insect, complete penetration through the cuticle was unnecessary for proper binding of the antibody. Thus, the researchers were able to successfully access the target tissue using a simple dehydration series followed by incubation in a resin mixture. Because the current study aimed to detect protein presence in a subset of neural tissue, it was necessary to completely penetrate through the surrounding tissue of the insect. Even though the target ganglia were isolated via dissection, the cell bodies within each ganglion are, much like the human brain, covered with insulatory cells which protect against penetration of any unwanted foreign substances ("The Insect Brain", Eldefrawi et. al., 1968). Therefore, extra steps may be necessary in order to fully penetrate this layer of cells and access the target neurons.

In addition, this experiment utilized FAS-II as the primary antibody of choice based on the recommendation of Nipam Patel, who detailed the use of various antibodies in a comprehensive manual of immunohistochemical procedures (Patel, 1994). However, Dr. Patel used Drosophila in his research, and thus his recommendations were based on results obtained using the Drosophila model. Although it is reasonably safe to generalize these results to other arthropods such as dragonflies, it is entirely possible that FAS-II is not specific to recognize the dragonfly and therefore cannot be used. In this case, the use of a different primary antibody would be necessary in order to obtain results when using dragonflies as a model specimen. Several alternative antibodies were considered for use in this experiment, but time and availability of specimens did not allow for proper exploration of every available option. For instance, 22C10 is an antibody typically used against *eve* in Drosophila and thus could prove a viable alternative to the use of FAS-II (Patel, 1994).

Future Directions

Future attempts to visualize *even-skipped* expression in dragonflies should explore more efficient methods of tissue penetration and alternative primary antibodies that could better target *eve*. The current study is a useful pilot examination of what it might take to successfully visualize *eve* expression in dragonflies. Moreover, more specimens of varying species of *Anisoptera* should be dissected in future experiments in order to ensure better reliability of results. It is clear from this current study that there is a high probability of visualizing *eve* expression in either dragonfly larvae or adults, but future studies must explore several avenues of change in order to identify a useful procedure for doing so.

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