WHY IS NATURE ABLE TO MOLD SOME PHENOTYPES MORE READILY THAN OTHERS? INVESTIGATING THE STRUCTURE, FUNCTION AND EVOLUTION OF BETA-2 TUBULIN IN DROSOPHILA MELANOGASTER

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ABSTRACT

WHY IS NATURE ABLE TO MOLD SOME PHENOTYPES MORE READILY THAN OTHERS? INVESTIGATING THE STRUCTURE, FUNCTION AND EVOLUTION OF BETA-2 TUBULIN IN DROSOPHILA MELANOGASTER

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Some phenotypes have more ability to evolve than others, captured by the term “evolvability.” While some traits can evolve rapidly, such as the shape, color and size of a butterfly wing, the Drosophila testis specific beta-2 (β2) tubulin protein, a fundamental component of the spermtail axonemes, has not evolved in over 60 million years. This protein is a main element of the microtubules within the axoneme which supports the motility of the sperm cell. There is a 9+2 configuration of microtubules, nine doublets of microtubules arranged along the outer edge of the structure with two central microtubules. Each microtubule consists of tubulin dimers of β2 tubulin and the major alpha tubulin isoform 84B which is present in most cells of the body. Previous studies have shown that substitutions of the of beta-1 tubulin, a 95% identical paralog of beta-2 expressed in somatic cells, and chimeric tubulins composed of beta-1 and beta-2 tubulin sequence are unable to support a motile axoneme, indicating the axoneme is highly sensitive to beta tubulin structure. From these findings, evolutionary conservation and highly sensitive structure/ function relationship, two hypotheses tested here were developed for the long conservation of β2 tubulin. The first, stabilizing selection: nature is constantly selecting a particular sequence even though other sequences may work due to differences in the quality of
sperm produced. Or, it may be that there is no alternative sequences that function, and a co-evolutionary event with another protein found within the axoneme is required to release beta-2 tubulin to evolve. These hypotheses were tested using the substitution of a beta-2 ortholog, the gene in a different species which evolved from a common ancestor, was examined to determine its ability to produce a functional sperm in the *Drosophila melanogaster* model. If able to produce a functional sperm, stabilizing selection is supported; if unable, a co-evolutionary event has occurred. Through database searches the orthologous gene from the closest relation to *Drosophila* with a different amino acid sequence was *Glossina morsitans morsitans* (Tsetse fly). *Glossina* beta-2 was able to support a functional sperm in *Drosophila melanogaster*. The transgenic flies were fertile and able to produce progeny, TEM cross-sections of the spermtail revealed a 9+2 axoneme and the testes showed normal meiosis, spermhead shaping, and alignment, all beta-2 tubulin supported functions. These results suggest the protein is under stabilizing selection, another sequence available in nature is able to produce a functional product but for 60 million years, nature has been constantly selecting the same wildtype sequence. This indicates the opportunity to evolve for such alternative functional proteins may be rare and along a narrow path such as that which maintains beta-2 tubulin function.
Dedicated to my family
ACKNOWLEDGMENTS

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<th>Full Form</th>
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<tbody>
<tr>
<td>β2</td>
<td>Beta-1</td>
</tr>
<tr>
<td>β2</td>
<td>Beta-2</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Sequencing Tool</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxyl terminus</td>
</tr>
<tr>
<td>D. mel</td>
<td>Drosophila melanogaster</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>Drosophila melanogaster</td>
</tr>
<tr>
<td>G.m.m.</td>
<td>Glossina morsitans morsitans</td>
</tr>
<tr>
<td>G. morsitans</td>
<td>Glossina morsitans morsitans</td>
</tr>
<tr>
<td>IVR</td>
<td>Internal Variable Region</td>
</tr>
<tr>
<td>M. domestica</td>
<td>Musca domestica</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center of Biotechnology Information</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
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</table>
CHAPTER 1
INTRODUCTION

Nature is able to mold some phenotypes more readily than others. Many features in today’s world come in multiple forms. This indicated that choices are available in the feature that may then participate in a competition, and the winner will be the option that will thrive within that particular population. This is the adaptive process. These features have multiple forms due to their tolerance to variation; their modification has no effect on whether the organism itself will survive past conception or not- they are heritable variants. However, nature may not have a choice in certain circumstances. These particular phenotypes appear to show no functional alternatives to the current structure and form, remaining unchanged for tens of millions of years. An important consequence is that such features cannot participate in adaptation, or only under rare circumstances. This type of stasis provides a basis for an exploration into what conditions nature is able to mold some phenotypes and not others.

1.1 Examples of Adaption

The butter wing is an example of a phenotype which is able to change and, therefore, to participate in the adaptation process. Wing patterns serves multiple functions: predator avoidance, mate attraction, as well as thermoregulation. [1, 2] The Bicyclus species was used to study a signal partitioning hypothesis which could accommodate these multiple functions on this one appendage. This theory states that an animal may separate the developmental signals spatially and temporally. This separation would better integrate the contrasting functions of the wings by creating the different patterns of eye spots and simple bands of each surface, dorsal and ventral as well as forewing and hindwing. Each surface was thought to accommodate a particular function
based on wing surface exposure during certain behaviors: dorsal and forewing patterns were likely to attract mates while the ventral and hindwing patterns were utilized in predator avoidance. [2] The eye spots and banding patterns on each surface provide variation within this species (Figure 1). The eyespots may diversify in location and size on the perimeter of each surface. Although these wing patterns differ among individuals within the *Bicyclus* species, the ability to integrate conflicting functions is necessary in order for an organism to survive and be successful reproductively in this population.

![Figure 1. Butterfly wings come in a variety of shapes and colors. Species and individuals within a species may differ in patterning and eye spot location and size. [Adapted from 2](image-url)](image-url)

The ability to maintain multiple functions in one pair of appendages could be understood. Through building the phylogenetic relationships based on evolutionary rates of change in these visual characteristics, it was thought the patterns on the wing surfaces would experience the same rate of change if the same signals are utilized in both surface patterns. However, it was shown the dorsal and forewing patterns displayed a higher rate of evolution that the ventral and hindwing patterns. These unequal rates provide evidence of the signal portioning hypothesis—signals developing phenotypes differently in time and space. It was found the traits on the dorsal wing
are sexually selected for while the ventral patterns are under strong stabilizing selection based on the evolutionary rates calculated. Sexually selected traits tend to have higher rates of change than those under stabilizing selection. [2] The ability to attract mates or participate in fertilization may change frequently over time while stabilizing selection acts to conserve a particular phenotype for its predator-avoiding benefits.

Dorsal wing patterns attract mates while ventral wing patterns are used to evade enemies. When on the marginal ventral surface of the wings, these eyespots are thought to not only avoid predators, but to divert the attacks away from vital organs. The butterfly will escape an attack with only parts of the wing lost and minimal damage elsewhere as the predator will attack the wing thinking it is a much bigger animal than a butterfly. [3] The ventral wing characters are thought to evolve slowly as to ward off natural enemies, such as birds and lizards, in the forests and savannahs of Africa and Madagascar while, in comparison, those on the dorsal exterior are evolving relatively quickly. [3, 4] The eyespots on the ventral surface do not affect mate acceptance; females have shown no discrimination against missing or incomplete patterns. Both sexes of this species expose the dorsal wing patterns to potential mates, and intact eyespots and banding patterns on this surface increases chances to mate. [2] This ability of a wing to sort out two conflicting functions, to avoid and to attract, provides an example of a highly flexible feature that can be finely tuned through adaptation.

The Peppered moth of London provides another well-known example of a feature showing standing variation able to adapt to changes in the environment. In the early 1800s, lichens on trees provided camouflage for the pale form of this moth (Biston betulariai) as the lichens displayed a pale grey color. The contrasting melanic form of this moth became easy prey for birds in this time as they were easily detected on the lichen background. However, as industrial pollution worsened between the 1840s and 1890s, the lichens were destroyed by sulfur dioxide. [5] The loss of these lichens equates to the loss of concealment for the pale form of moths. Furthermore, the smoke produced aided in the camouflage in the melanic form, and the
darkened tree trunks, due to the pollution, provided a likeness to the color of the darker form. This increased the frequency of the melanic phenotype from less than 1% to 98% in the population. [5, 6]

In these examples discussed, there is standing variation for traits in the population. Standing variation is defined as more than one allele at a locus within a population. [7] Multiple variations can occur within a select phenotype that are heritable, these traits can tolerate a range of alteration without devastation to the organism itself. The wing patterns and moth colors examined are able to change within and throughout generations in response to environmental pressures, which can allow for adaptation to occur, a process best expressed in the Syllogism of Evolution by Natural Selection as established by Charles Darwin:

If there is heritable variation in phenotypes,
And individuals differ with respect to their abilities to survive and reproduce, based on their phenotype
Then, there will be a natural selection for individuals that possess those phenotypes best able to survive and reproduce. [8]

The ability to evolve is not only possessed by exterior features. This ability to participate in the process of adaption is also present in gametes. They may experience a type of selection referred to as sexual selection as mentioned earlier. This selection is the evolution of traits which are used to attract mates or increase the chance of fertilization post-copulation. [9] For example, sperm competition is a form of post-copulation sexual selection which contributes to the high phenotypic variance in sperm morphology exhibited across all sexual organisms, particularly insects. Certain external features may indicate stronger, longer sperm cells within a male, a means to flaunt their superior sperm to females in order to increase their chances of mating. This relationship was examined within the Passerine bird population in Norway and Germany. Lifjeld et. al examined dorsal plumage coloration and arrival date, and their correlation to sperm length. Females were shown to prefer darker plumage, which ranges from true brown to deep black, and
early arriving males for parental care and territorial acquisition, respectively. However, it was found the males with preferred external phenotypes did not correspond to sperm length or speed. Although the plumage exhibited a diverse range of colors: German males displayed a browner dorsal plumage than Norwegian males, there was no correlation between color and sperm length. Early arriving males at the lek were found to preferentially mate females, but these males did not exhibit significantly longer sperm, thus invalidating the hypothesis of associated arriving date and sperm length phenotypes. The notion of longer sperm swimming faster was also negated. The longer sperm did not have a higher speed; in fact, the longer sperm decreased in speed after a short amount of time compared to the shorter sperm. [10] Although the authors did not find correlated adaption on the part of these internal and external phenotypes, variation was available for these traits to evolve in response to environmental pressures, it may be the particular pressure, if any, has not yet been identified.

1.2 Selection Acting on Drosophilid Sperm

My research is focused on sperm evolution in insects in the genus Drosophila. Spermtail length vary among Drosophilids from 0.32 mm in D. persimilis to 1.9 mm in D. melanogaster to 58.29 mm in D. bifurca; they are the longest axonemes on the planet. [11] These are extravagant features, the peacock feathers of the sperm world, and their length requires an evolutionary explanation, given shorter sperm, humans have 0.04 mm long sperm, work perfectly well and require less energy to generate. [12] As this genus experiences a uniquely highly diversity in length, there is opportunity for sexual selection via sperm competition to underlie the evolution of their incredible lengths.

D. melanogaster females are able to mate with multiple males during a period of fertilization; they are able to store sperm of the multiple males as well. [13] Sperm competition occurs between sperm cells as well as between different males in the same female tract. [11, 13] Studies of this competition provide evidence for the relationship between sperm length and the preferred paternity post-coupling.
Sperm length is positively related to individual body size: the larger the body, longer the sperm. However, longer sperm does not always “win” in the sperm competition between males in the same female tract. The seminal receptacle, the primary sperm-storage organ of female fruit flies, has a capacity to hold sperm from multiple ejaculations, and it has been found longer sperm tend to be in the proximal end of the seminal receptacle and the shorter sperm nearer the distal end of a singular ejaculation. [11] However, following a period of waiting time after the first ejaculation, when a female re-mates to a second male, the second male’s sperm takes precedence over the first male’s sperm. The second male’s seminal fluid, no matter the presence or length of the sperm, incapacitates and displaces the first male’s sperm from the seminal receptacle of the female. [13] This proves, although sperm length may vary within a species, the male-female tract interaction follows an established pattern of second mating predominance despite this variation.

It also has been shown that there is a cost to generating long sperm; longer sperm delay male sexual maturity. The resources to assemble such a large component require more time during development than a shorter sperm. This will prolong the period of time the male will spend in a non-reproductive phase as a positive relationship between sperm length and male age at sexual maturity was found. [11] In contrast, it has been found that longer sperm tails do not correlate with faster swimming sperm nor are they more fertile. [11, 14] The necessity of more energy and time to be developed does not benefit long sperm in mating success.

Long sperm may result from co-evolution with the female reproductive tract. As stated earlier, the longer sperm are more proximal in the seminal receptacle of the female as compared to the shorter sperm. The length of the female seminal receptacle, which shows intra- and interspecific variation, may correlate to sperm length. The longer the seminal receptacle, the higher chance of fertilization from a longer sperm. Conversely, shorter seminal receptacles did not exhibit such a preference or any preference at all; neither long nor short sperm showed an increased chance of fertilization in this phenotype. [13] The hypotheses concerning fruit fly sperm evolution are summarized in Table 1.
Table 1. Conclusions of previous tests of *Drosophila* sperm competition and sperm length preference

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Supported or Not</th>
<th>Major Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longer sperm always “win” in sperm competition</td>
<td>Not supported</td>
<td>• The order of entry into the female tract matters; second matings take precedence in fertilization regardless of sperm length</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• The length of sperm exhibits no correlation to reproductive success when females mate with multiple males</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Sperm length correlates with delayed sexual maturity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• No correlation to higher speed of sperm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• No correlation to higher fertility</td>
</tr>
<tr>
<td>Seminal receptacle length correlates with sperm length</td>
<td>Supported</td>
<td>• Longer seminal receptacles within females demonstrated a preference to longer sperm for fertilization</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Short seminal receptacles showed no preference to size of sperm for fertilization</td>
</tr>
</tbody>
</table>

1.3 *Drosophila* Spermtail Components

The protein beta-2 (β2) tubulin is implicated in the generation of the spermtail which entails the majority of the sperm length in *D. melanogaster*. The spermtail provides locomotion to propel the cell through the female reproductive tract. The cross-section reveals a 9+2 (outer-central) microtubule arrangement of the axoneme, composed of β2 tubulin and alpha tubulin
isoform 84B. [15] (Figure 2) Previous tests show that the axoneme is sensitive to change in beta-2 sequence. Small changes in beta-2 render the spermtail short and immotile. [16] Thus, this consequential protein holds considerable control over the nature of the sperm in both length and function.

![Schematic of cross-section of the spermtail axoneme of D. melanogaster.](image)

Figure 2. Schematic of cross-section of the spermtail axoneme of *D. melanogaster*. Note the 9+2 arrangement of microtubules which consist of β2 tubulin and alpha tubulin isoform 84B dimers. [Adapted from 17]

1.4 Review of Previous Studies

Microtubules consist of a beta and alpha tubulin heterodimers. These structures are essential for intracellular transport and cell division in all eukaryotes. Each tubulin consists of three domains: N-terminus domain, internal variable region (IVR), and the C-terminus domain. The N-terminus is thought to participate in nucleotide binding as it contains a pattern of beta strands and alpha helices of typical proteins. The IVR, involved in strong later contacts, is a drug-binding domain and the target of microtubule stabilizing drugs used in chemotherapy, while the C-terminus helices are most likely involved in binding of microtubule associated proteins (MAPs) and motor proteins. This domain is the most variable among the α and β isotypes. [17]

Previous tests of β2 tubulin proved it is sensitive to change. The gene which encodes beta-1 (β1) tubulin has been substituted for the β2 tubulin gene in *D. melanogaster* in previous
β2 tubulin evolved from β1 tubulin a paralogous genes, genes related by a gene duplication event, and the two differ in 25 amino acid sites. [18,19] The majority of differences occur in the carboxyl terminus, the 3’ end of the protein, and the internal variable region (IVR), middle of the protein that forms a portion of the nucleotide-binding Rossman fold. When the D. melanogaster β1 tubulin gene was substituted for β2 tubulin in its entirety, the spermtail was shortened and not functional compared to the wildtype β2 tubulin; the axoneme lacked the central pair of microtubules as well producing a 9+0 configuration (Figures 3a and 3b). [18, 20] β1 tubulin alone is unable to support the spermtail of the fruit fly, thus there must be an axoneme-generating capability within these 25 differing amino acids which is specific to β2 tubulin in the fruit fly.

Through further investigation, the substitution of a subset of the 25 amino acids of β2 tubulin into β1 tubulin was studied. The amino acids among the C-terminus was a particular interest since a majority of differences between the two proteins is located in this region. β1 tubulin with β2 tubulin C-terminus amino acids 433-434 were first tested. Cross-sections of these spermtails revealed the central pair of microtubules was rescued and the spermtail was longer.
than that supported by β1 tubulin alone, but was still short when compared to wildtype spermtails and was non-motile. (Figure 3a and 3e) The substitution of β1 tubulin with the complete C-terminus β2 tubulin amino acid sequence was then examined. Once again, the spermtail was non-motile, although longer than previous substitution tests. (Figure 3d) These C-terminus substitution experiments provided evidence this region of the protein carries function in forming the central pair of microtubules in the 9+2 arrangement of the axoneme.

Next tested were amino acids within the IVR at sites 55 and 57 as well as the full C-terminus sequence. This chimera produced the correct 9+2 arrangement, but the spermtail was, again, not motile and shorter than wildtype spermtail as well as the previous substitution test of β1 tubulin with β2 tubulin C-terminus. (Figure 3f) [18] The β2 tubulin C-terminus amino acids alone rescued some length to the spermtail; however, when additional amino acids from the IVR were added, the progress went backwards. There was more β2 tubulin identity in the chimeric gene than previously tested constructs but less spermtail generating function. This provided insight into the evolution of certain regions of the protein. One region, the C-terminus, acted independently of the rest of the protein and could rescue more sperm-generating functions as compared to other IVR amino acids which could not improve these functions. (Table 2) These amino acids require additional β2 tubulin amino acids in order to function properly; the function in a beta-2 specific synergism. Synergism has the effect that the order of their evolution matters, in contrast to the C-terminus substitutions that showed additive behavior, by virtue of carrying spermtail function into the novel, beta-1 context. Evolution of synergistic sites would be slower than additive sites given the order of change matters, which could contribute to the long evolutionary stasis of the β2 tubulin protein.
Table 2. Descriptions of phenotypic outcomes of β1 and chimeric tubulin amino acid sequences in place of β2 tubulin; No alternative protein sequence rescued a viable spermtail in previous studies

<table>
<thead>
<tr>
<th>Protein Substitution</th>
<th>Phenotypic Outcomes</th>
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<tbody>
<tr>
<td>β1</td>
<td>9+0; Short; Non-motile</td>
</tr>
<tr>
<td>β1+β2 433-434</td>
<td>9+2; Longer than previous study; Non-motile</td>
</tr>
<tr>
<td>β1+β2 433-446</td>
<td>9+2; Longer than previous study; Non-motile</td>
</tr>
<tr>
<td>β1+β2 55, 57, 433-447</td>
<td>9+2; Shorter than previous study; Non-motile</td>
</tr>
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</table>

These past studies have shown the removal or replacement of D. melanogaster β2 tubulin with paralogous proteins or with chimeric genes shortened the spermtail and excluded accessory proteins within the axoneme. This rendered the sperm nonfunctional. [18, 19, 20] Therefore, the expression of β2 tubulin controls the entirety of length and function of the sperm cell in these fruit flies.

1.5 Current Experiment

This long fruit fly spermtail can be considered as the peacock feather of the fly word; there is an extraneous amount of energy endowed for the development of such a sizeable phenotype under sexual selection. For one of the most diverse cell-types in nature and energy consuming in the fruit fly, one critical component, β2 tubulin, within the sperm cell to remain unchanged for over 60 million years in the spermtail is considered a rare feature and deserves an explanation. Its conservation could be explained by two evolutionary phenomenon. The first is stabilizing selection; other variants of β2 tubulin could produce a functional product, but the current amino acid sequence is only being selected. There is heritable variation (the first premise of the Syllogism as stated earlier), but there is only one “winner” of the competition (the second
premise) among these variants. This particular sequence was the only one passed on to the next generations for 60 million years. The second pathway involves a co-evolutionary event with an accessory protein, meaning there is no other probable sequence which will give a functional product without a compensatory change in the axoneme itself. Essentially, an accessory protein would have also had to change along with β2 tubulin.

These hypotheses can be distinguished from one another by substituting an orthologous gene for D. melanogaster β2 tubulin. Orthologous genes evolved from a common ancestral gene by a speciation event. [21] These types of genes may or may not have the same function between species. Previous studies have substituted a paralogous gene for β2 tubulin. The two genes are descended from the same ancestral gene by a gene duplication event in the course of evolution. [21] By expanding on previous studies and examining an orthologous gene substitution, we will be able to note evidence of the evolutionary pathway for β2 tubulin. If the orthologous gene will produce a functional product when substituted into D. melanogaster in its entirety, the pathway for this protein within the fruit fly follows a stabilizing selection pattern. Other sequences may function, but this particular sequence of β2 tubulin is essentially winning the competition of variants during natural selection. However, if the orthologous gene does not provide a functional sperm with the correct configuration of the axoneme, then this would provide evidence of a co-evolutionary event which occurred between β2 tubulin and an accessory protein within the axoneme. Fundamentally, no other configuration of β2 tubulin would be compatible with the configuration of the axoneme presently. No other variants are acceptable in replacement of this particular sequence of β2 tubulin relating to the first premise of the Syllogism. No viable variants are available, thus heritable variation is not applied to this protein.
CHAPTER 2
SEARCH FOR OTHOLOGOUS SPECIES

2.1 Introduction

Previous studies examined paralogous genes in place of β2 tubulin in Drosophila melanogaster. These studies showed this protein is sensitive to change but that certain amino acids were able to carry partial sperm generating function while others depend on other amino acids evolving in a specific way in order to maintain function. This study examined the effects of substituting an orthologous gene of D. melanogaster β2 tubulin. The similar gene of a different, but closely related species will provide insight into the evolutionary pathway this protein is experiencing. If these aspects of the protein, which were tested before to show functionality, are conserved among β2 tubulin of related Drosophilids, then the ability to have an orthologous gene substitute rescue function is considered. However, if these properties are not conserved among those species, the hypothesis would shift to indicate other β2 tubulin genes may not rescue function of the spermtail in part or in whole.

By examining those species which are most closely related to D. melanogaster, the extent of the conservation of β2 tubulin will be evident through how many millions of years the common ancestor which possessed this particular sequence must have existed. The nucleotide sequence of β2 tubulin of available sequenced Drosophilids was investigated; however, due to the degeneracy of the genetic code, amino acid sequences will be compared among β2 tubulin proteins to ensure a dissimilar protein sequence will be substituted. This bioinformatics screening of DNA and protein alignment and analysis revealed the species of interest for the study.
2.2 Materials and Methods

2.2.1 Utilizing BLAST

The BLAST (Basic Local Alignment Sequencing Tool) program from the NCBI (National Center of Biotechnology Information) website contains many genomes of species which have been sequenced and annotated. The bioinformatic tool searches these genomes for a sequence similar to the one inputted by the user. [22] More specifically, FlyBase.org utilizes this BLAST program for fly genomes exclusively. The nucleotide sequence of β2 tubulin of D. melanogaster was obtained from previous studies and verified through NCBI. This sequence was compared among all Drosophilids which have an available genomic sequence and species outside of the genus as well. Retuned undefined but similar partial sequences within a species were combined to assemble the entire β2 tubulin sequence of that species and compared to D. melanogaster.

2.2.2 Utilizing MEGA

Following nucleotide sequence acquisition, the amino acid sequences were resolved using MEGA (Molecular Evolutionary Genetics Analysis). This software allows users to compare DNA sequences between multiple species at one time. It will also translate the DNA into its amino acid sequence. The program marks differences between species sequences using an asterisk above the amino acid site. [23] Each fly species nucleotide sequence was converted to its amino acid sequence and compared to that of D. melanogaster at homologous sites with an asterisk indicating an amino acid difference between the species.

2.3 Results

*D. albomicans,* and *D. grimshawi.* Inquiring beyond this genus, the house fly, *Musca domestica,* also contained a 100% similar amino acid sequence. The Tsetse fly, *Glossina morsitans morsitans (G.m.m.)*, amino acid sequence of β2 tubulin revealed 17 differences from that of *D. melanogaster.* The alignment of sequences from both species is displayed in Figure 4. Table 3 exhibits the amino acid sequence differences at specific sites as well as the type of amino acid change. All but one amino acid difference retains the same type of amino acid (Non polar, Neutral polar, Acidic polar, Basic polar). The one which differs, amino acid site 190, is polar in both the fruit fly and Tsetse fly; however, in *D. melanogaster,* it is basic while in *G. morsitans* it is neutral.

Figure 4. Amino acid alignment of *D. melanogaster (D.mel)* and *G. morsitans (G.m.m).* Highlighted amino acids represent differences between the species with 17 differences total.
2.4 Discussion

Through the comparisons of the β2 tubulin amino acid sequences between *D. melanogaster* and the Drosophilids which have an available genomic sequence, the 60 million year conservation of β2 tubulin was confirmed. The sequence must have existed in a common ancestor in order for the current amino acid sequence to be passed on to the species discussed previously. This common ancestor existed approximately 60 million years ago. Further evidence is noted in the amino acid sequence of *M. domestica*. The common ancestor of the Drosophilids and this species existed about 60 million years ago as well. [24] Interestingly, the common ancestor of the Tsetse fly and the house fly occurred more recently than the common ancestor of all three flies: the fruit fly, Tsetse fly, and house fly, according to Figure 5. This implies some change in the β2 tubulin protein must have occurred to make the Tsetse β2 tubulin more different than the fruit fly and house fly.

<table>
<thead>
<tr>
<th>AA SITE</th>
<th>D.m. AA, TYPE</th>
<th>G.m.m. AA, TYPE</th>
</tr>
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<tr>
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<td>Isoleucine</td>
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<td></td>
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<td>Non polar</td>
</tr>
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<td>Phenylalanine</td>
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<tr>
<td></td>
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<td></td>
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<td>Threonine</td>
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<tr>
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<td>Cysteine</td>
</tr>
<tr>
<td></td>
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<td>Polar</td>
</tr>
<tr>
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<td>Alanine</td>
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<tr>
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</tr>
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</table>
Figure 5: Phylogenetic tree concerning *D. melanogaster*, *G. morsitans*, and *M. domestica*. [Adapted from 24] All three species derived from a common ancestor more than 60 million years ago. *G. morsitans* and *M. domestica* diverged along the same branch then deviated about 55 million years ago.

However, the amino acid differences between *G. morsitans* and *D. melanogaster* did not warrant any concern of dramatic differences between the two species proteins. The 17 differences were not congregated to one particular region of the protein and were not implicated in sperm-generating functions as in previous studies except for amino acid 444. This amino acid is within the carboxyl terminus but the nature of the amino acid, its charge and properties, are retained. This type of change, such that the type of amino acid is not altered, is present in 16 of the 17 differences. Although the side chains of the amino acids may vary, the fundamental properties of the amino acids do not digress between species. Amino acid 190 may differ in charge but not in polarity. The location of this protein subunit, also, did not generate much interest as it is concealed in the foldings of the protein. [25]

The comparison of *G. morsitans* and *D. melanogaster* β2 tubulin amino acid sequence provided a small but meaningful insight into the evolutionary pathway this protein is experiencing. It pointed the hypothesis towards stabilizing selection. This orthologous gene appeared to contain the capacity to generate a functional sperm within the fruit fly model as the Tsetse protein does not dramatically differ from that of the fruit fly. This implied other sequences could function in place of *D. melanogaster* β2 tubulin. The proceeding steps and experiments would either verify or disprove this claim.
CHAPTER 3

SUBSTITUTION OF GLOSSINA MORSITANS BETA-2 TUBULIN INTO THE DROSOPHILA MELANOGASTER MODEL

3.1 Introduction

β2 tubulin within D. melanogaster is surrounded by regulatory sequences to promote translational activity in the proper manner. These sequences flank the protein coding portion and are unique to β2 tubulin of the fruit fly. They were constructed to flank the Tsetse β2 tubulin in order to be correctly expressed in the fruit fly model to test its functionality to create a sperm. Tsetse β2 tubulin was isolated and amplified using PCR and ligated through a series of vectors. The first vector, pGEM, was utilized to keep a substantial amount of Tsetse β2 tubulin copies readily available. The second vector, pUC57, was previously constructed to incorporate the regulatory sequences to flank the insert. The third vector, pS3aG, was then ligated with Tsetse β2 tubulin and the flanking D. melanogaster sequences to be incorporated into the second chromosome of fruit fly embryos. These fruit flies also express wildtype β2 tubulin. In order to ensure Tsetse β2 tubulin was the sole β2 tubulin being expressed in these flies, the transgenic flies were mated to flies that contain a null copy of the wildtype β2 tubulin. The progeny were then be mated continually following the crosses noted in Methods until flies that only express Tsetse β2 tubulin were obtained.

Functionality of Tsetse β2 tubulin was analyzed through fertility tests. If progeny were produced from mating virgin transgenic flies with wildtype flies, then the hypothesis of stabilizing selection would be supported. Other different sequences may be functional in place of
the *D. melanogaster* β2 tubulin. If no progeny were viable, the Tsetse β2 tubulin gene did not provide a sperm-functioning motif in the fruit fly background; thus, an accessory protein has co-evolved with *D. melanogaster* β2 tubulin to make other differing sequences nonfunctional. β2 tubulin is also implicated in the microtubule functions of formation of meiotic spindle, chromosome movement, and cytokinesis as provided by previous research. Null mutations of β2 tubulin in the fruit fly causes defects in these as the microtubules associated process failed to form precisely. [26] Potential defects may be viewed under phase contrast light microscopy. The nuclei within the normal developing early round spermatids are circular and paired with a nebenkern. The nebenkern are composed of fused mitochondrial derivatives. The nuclei and nebenkern are similarly shaped and sized structures present in these cells which can contain more than one of each within a cell. If there is odd-shaped nucleus with a missing or additional nebenkern present, there is errors occurring in the microtubule functions discussed earlier. For example, malfunctioning microtubules responsible for essentially pulling the chromosomes apart may create unequal segregation of chromosomes and, thus, unequal shape and size of nuclei within the cells. [27] A cross-section of the spermtail axoneme which expresses Tsetse β2 tubulin within the microtubules was viewed as well. The correct configuration of the 9+2 microtubule arrangement should be present if the transgene can support sperm function. If Tsetse β2 tubulin produces defects in these stages of spermiogenesis, stabilizing selection is not possible for the evolution of β2 tubulin in *D. melanogaster*, and, therefore, a co-evolutionary event must have occurred with an accessory protein.

3.2 Materials and Methods

3.2.1 PCR and Tsetse β2 tubulin Gene Isolation

Genomic *G. morsitans* DNA was obtained with Tsetse β2 tubulin-specific primers from BEI Resources. These were utilized for the amplification of the Tsetse β2 tubulin gene using PCR (Polymerase Chain Reaction). The primers contained restriction cut sites for SpeI and XhoI to digest the Tsetse β2 tubulin gene (Table 4). 10 μL of 5X Pfusion High Fidelity buffer, 4 μL of
2.5 mM dNTP, 10 μL each of the forward and reverse primers, as well as 1 μL of *G. morsitans* genomic DNA, 0.5 μL of Pfusion Polymerase enzyme, and 29.5 μL Milli-Q Water were added to each PCR reaction tube. The thermocycler performed 40 cycles of denaturation, annealing, and extension with temperatures of 98°C for 1 minute, 58.5°C for 1 minute, and 72°C for 3 minutes, respectively.

Table 4: Primers designed to isolate Tsetse β2 tubulin gene from genomic Tsetse DNA; in red indicates Spe I cut site in forward primer; Xho I in reverse primer

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>5'- ATA TAC TAG TAT CAA AAT GCG TGA AAT CGT GCA TGT ACA AGC AGG -3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Primer</td>
<td>5'- ATA TCT CGA GAT ATT ATT CAT CAG CTC CAC CCT CTC CG -3'</td>
</tr>
</tbody>
</table>

The Tsetse β2 tubulin gene was purified using the QIAquick PCR Purification Kit. 5 volumes of buffer PBI were added to 1 volume of the PCR sample which was then added to a QIAquick spin column and centrifuged for 60 seconds at 13,000 rpm. 750 μL of buffer PE was added to the column and centrifuged for 60 seconds and for one minute after the flow-through was discarded. 30 μL of elution buffer was added to the center of the membrane within the spin column and remained undisturbed for one to five minutes and centrifuged for 1 minute. The flow-through from this step contained the Tsetse β2 tubulin gene in the elution buffer.

Once the Tsetse β2 tubulin gene was amplified and purified, 12.5 μL gene segments underwent further cycles in the thermocycler with 0.5 μL EconoTaq DNA Polymerase, 5 μL 10X Reaction Buffer, 3 μL 25 mM MgCl₂, and 4 μL dNTPs. This enzyme adds a single adenosine to both 3’ ends of the DNA strands to create an overhang on both sides of the gene. [28] This was tested using gel electrophoresis in 1X TAE buffer with the presence of a 1.5 kb band.
3.2.2 Vector Cloning

These gene segments were then ligated to the vector pGEM obtained from Promega Corporation. The reaction consisted of 1 μL DNA Ligase, 1 μL pGEM, 1 μL DNA, 2 μL Ligation Buffer, and 1 μL H₂O at 4°C overnight. pGEM with the Tsetse β2 tubulin gene was purified using gel extraction by excision and melting of the appropriate band with 3 volumes of Buffer QG added and incubated at 50°C for 10 minutes. One gel volume of isopropanol was added and placed in a QIAquick spin column to bind the DNA; it was centrifuged for 1 minute and washed with 700 μL Buffer PE. 50 μL Buffer EB (10 mM Tris-Cl, pH 8.5) was used to elute the DNA from the QIAquick membrane for 1 minute then centrifuged for 1 minute. The flow-through contained the isolated vector. The vector was then transformed using a mixture of DH5α cells and 5 μL vector incubated on ice for 30 minutes. The cells were then heat shocked for 20 seconds in a 42°C water bath and placed on ice for 2 minutes. 950 μL of warmed liquid media was added and incubated at 37°C at 225 rpm for 1 hour. 20 μL was spread on pre-warmed agar plates and incubated overnight at 37°C.

A second vector, pUC57, contained *D. melanogaster* β2 tubulin regulatory sequences which ensured the correct expression of the Tsetse β2 tubulin gene in the testes of *D. melanogaster*. It also contained restriction enzyme cut sites for Spe I and Xho I (Figure 6). This vector was created by GeneScript and transformed using the previously described protocols. pGEM with the Tsetse β2 tubulin gene and the pUC57 were digested using Spe I and Xho I with each reaction containing 10 μL DNA, 10 μL Cutsmart Buffer, 2 μL of each restriction enzyme, and 76 μL Milli-Q water. The Tsetse β2 tubulin gene and the pUC57 vector samples were then purified using gel extraction as previously described. The Tsetse β2 tubulin gene was then ligated into pUC57 vector (Figure 6A).

The pUC57 vector also contained restriction enzyme cut sites for Not I and Asc I outside the regulatory sequences. Another digestion occurred using the same protocols but with these restriction enzymes in place of Spe I and Xho I to release the Tsetse β2 tubulin gene with
A third attB transformation vector as a gift from Thomas Williams (Addgene plasmid # 31171), pS3aG, also contained restriction sites for Not I and Asc I. This vector was used in the transformation into *D. melanogaster* embryos. It was prepared and digested using methods described earlier. The Tsetse β2 tubulin gene with flanking regulatory sequences was then ligated into this final vector (Figure 6B). All ligation reactions were confirmed using gel electrophoresis.

![Vectors designed to incorporate the Tsetse β2 tubulin gene into *D. melanogaster* embryos. A) Tsetse β2 tubulin gene and pUC57 were digested using SpeI and XhoI and ligated; B) Tsetse β2 tubulin with flanking regulatory sequences and pS3aG were digesting using NotI and AscI and ligated](image)

**3.2.3 Drosophila Strains and Crosses**

The final transformation vector with the Tsetse β2 tubulin gene and regulatory sequences was sent to the company, Genetivision. There it was injected *D. melanogaster* embryos. The Tsetse β2 tubulin gene was incorporated into the second chromosome of the flies, and subsequently balanced by Cyo, which provided a visual of curly wings. [29] A balancer halts recombination by crossing over, or when two chromatids switch parts of their DNA, to occur in meiosis. [29, 30] If recombination was to occur, Tsetse β2 tubulin gene may be broken during this
process. The Tsetse β2 tubulin gene was also linked to another gene which will give the flies red eyes. This allowed the Tsetse β2 tubulin gene to be traced with an easily visible phenotype through the crosses. The third chromosome, which contained the endogenous copy of *D. melanogaster* β2 tubulin, was wildtype. These flies were mated with balancer flies gifted from Dr. Amit Singh’s Lab. This and the following matings were conducted at room temperature in standard media. These balancer flies contained four balancers on chromosomes 2 and 3: CyO, Sco, TM3, and Tb. *D. melanogaster* flies which are null for the endogenous β2 tubulin gene on the third chromosome (represented by K). The third chromosome also contained a balancer, TM3, which was seen through as an ebony abdomen when expressed with K as well as short hairs/bristles on the thorax of the flies in this strain. These flies were also mated with the balancer flies. The progeny from this cross and the first cross were mated. The offspring from this final cross produced flies which are homozygotic for Tsetse β2 tubulin on the second chromosome and K on the third chromosome when mated to the same genotype. They showed the phenotypes of red eyes, long bristles on the thorax, and white abdomens. Each mating consisted of 5 day old virgin male or female flies. The crosses are depicted in Figure 7.

**Figure 7**: Crosses applied in order to produce transgenic fruit flies which only express Tsetse β2 tubulin
3.2.4 Analyzing Tsetse β2 tubulin Functionality

RNA was isolated and purified from 5 day old virgin transgenic testis using RNeasy Mini Kit from Qiagen. After dissection, the testis was homogenized in Buffer RLT. The mixture was lysated for 3 minutes at full speed. The supernatant was obtained and 1 volume of 70% ethanol was added and immediately mixed by pipetting. 700 µl of the sample was placed in an RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. 700 µl Buffer RWI was added to the spin column and centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. 500 µl Buffer RPE was then added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. This step was repeated except centrifuged for 2 minutes. The RNeasy spin column was transferred to a new collection tube and 30 µl RNase-free water was applied directly to the spin column membrane and centrifuged for 1 minute at 10,000 rpm to elute the RNA.

One step Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was then utilized on the eluted RNA to confirm Tsetse β2 tubulin expression. Primers were designed to specifically target *G. morsitans* β2 tubulin RNA sequence and made by BEI Resources. (Table 5) The RT-PCR reaction composed of 10 µl 5x QIAGEN OneStep RT-PCR Buffer, 2 µl dNTP mixture, 2 µl of each primer, 2 µl QIAGEN OneStep RT-PCR Enzyme Mix, and 30 µl RNase-free water. 2 µl of the purified RNA was added as well. A control mixture was also prepared with no RNA. The thermal cycle conditions were as followed: 30 minutes at 50°C (Reverse Transcription), 15 minutes at 95°C (Initial PCR activation step), then PCR proceeded normally as described previously, as well as a final extension step for 10 minutes at 72°C. These primers were also utilized in a PCR utilizing genomic *D. melanogaster* and *G. morsitans* to ensure the primers are specific to the latter. Gel electrophoresis confirmed these results.
Table 5. Primers designed to target Tsetse β2 tubulin RNA in transgenic *D. melanogaster* testis for RT-PCR. The forward primer targets an intron of *G. morsitans* specifically while the reverse primer targets the XhoI cut site sequence which is placed between Tsetse β2 tubulin and the regulatory sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Forward Primer</td>
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</tr>
<tr>
<td>Reverse Primer</td>
<td>5'- CCT CGA GCT CAT CGG CTC CG -3'</td>
</tr>
</tbody>
</table>

3.2.4.1 Fertility Tests

Five 5 day old female wildtype fruit flies from a Canton-S stock courtesy of the Dr. Kango-Singh lab and five 5 day old male virgins which express Tsetse β2 tubulin were kept together for five days in standard media. Vials were kept at 27° and checked every day for 15 days. Progeny were counted and noted for vitality.

3.2.4.2 Sperm Development

Testes of 5 day old virgin transgenic males were stained with Orcein and observed under light microscopy to view its structure and the alignment of the spermheads. After dissection of the testis in 200 µl buffer TBI, the testis was placed in a well containing 200 µl Aceto-Orcein for 10 to 15 minutes, then transferred to a microscope slide with a drop of acetic acid on it. A drop of Propo-Lactic stain on a coverslip was inverted onto the testis. A squash was applied to the coverslip in order for the membrane of the testis to rupture. The developing sperm were released and easily visible utilizing the Nikon Eclipse TE200 inverted microscope. Pictures were taken using Nikon CoolPix 4500 under 20x and 40x objective lens. Any alterations to the cell division process or the alignment of the sperm heads were noted. Testes of 5 day old virgin transgenic males were dissected and viewed utilizing the Nikon Eclipse TE200 inverted microscope to note the development of the nucleus and mitochondrial derivative. Pictures were taken using Nikon CoolPix 4500 under 20x and 40x objective lens. Both protocols were also performed for 5 day old virgin wildtype males.
3.2.4.3 Axoneme Morphology

The testes were fixed using a standard fixation and embedding protocol. After dissection of Testes of 5 day old virgin transgenic males in 200 µl buffer TBI, primary fixation of the testes occurred in 200 µl 2.5% glutaraldehyde in 100 mM phosphate buffer (PB) for four hours, and after a wash step in 200 µl 200 mM PB, postfixation occurred in 1% osmium tetroxide in 100 mM PB for two hours at 4°C and washed five times in 100 µl distilled water. The testis was placed in 200 µl lead citrate at 4°C. Following these steps, the testes were dehydrated through a series of increasing concentrations of ethanol solutions for a duration of 15 minutes in each: 30%, 50%, 70%, 90%, and 100%. The testes were then embedded in a mold using the SPI-Pon Embedding Resin Kit from SPI Supplies. The resin consisted of SPI-PON 812 (9.8 ml), DDSA (3.3 ml), NMA (6.9 ml), and DMP-30 (0.3 ml). The block was polymerized for 24 hours at 65°C. The resin hardened to allow for thin sections of the block to be made. [31] These sections were viewed under TEM to note the axoneme arrangement.

3.3 Results

3.3.1 Gene Amplification and Vector Constructs

Tsetse β2 tubulin gene was successfully cloned into the series of vectors and confirmed by gel electrophoresis (Figure 8). The final transformation vector consisted of 10 kbp of original vector and the 3 kbp bands represent the Tsetse β2 tubulin gene with flanking regulatory sequences.
3.3.2 Fly Mutation

The K/TM3 fly cultures developed a lethal recessive mutation. K/K flies were not viable, and the experiment continued with Tsetse β2 tubulin/ Tsetse β2 tubulin; K/TM3 genotypic flies. These were considered 2:1, two copies of the variant β2 tubulin and one copy of wildtype. Previous studies have shown the results of studies utilizing 2:1 transgenic β2 tubulin genes are similar to 2:0 flies expressing only external β2 tubulin. [19] The expression of Tsetse β2 tubulin was confirmed using RT-PCR. (Figure 9)
Figure 9. Gel electrophoresis confirming the expression of Tsetse β2 tubulin in transgenic fruit flies. The ladder was present in well 1. PCR utilizing the primers in Table 5 and genomic DNA of *D. melanogaster* was present in well 2, note no band is present. PCR utilizing the primers in Table 5 and genomic DNA of *G. morsitans* was present in well 3, note the presence of a strong band. The RT-PCR mixture containing purified RNA of the transgenic fruit fly testis was present in well 4, note the presence of a band within the black outline. A control of RT-PCR with no RNA was present in well 5, note the presence of no band.

### 3.3.3 Fertility Tests

Fertility tests showed seven of ten trials containing wildtype virgin females and transgenic virgin males produced progeny. This indicates the Tsetse β2 tubulin gene contains a sperm-regulating function which can perform in the *Drosophila* model. The offspring were observed with no major physical malformations. The relative fertility (compared to the control) from each mating is described in Figure 10.
3.3.4 Sperm Development

β2 tubulin is implicated in the microtubules associated with the development of sperm and spermhead alignment. [26] The spermhead alignment of the transgenic flies resembled that of wildtype. (Figure 11A-B) The heads in each bundle of spermatids were linearly arranged and exhibited regular nuclear shaping. Irregular shaping would have included moon-shaped and condensed nuclei of spermatid as seen in previous studies.

Figure 10. Relative fertility of 5 day old virgin transgenic male mated with 5 day old virgin wildtype female conducted using fertility tests. Each trial compared to control mating of 5 day old virgin wildtype male and 5 day old virgin wildtype female.

Figure 11. Testis squash with Aceto-Orcein of A) 5 day old virgin wildtype male and B) 5 day old virgin transgenic male: spermatid heads aligned in a manner similar to wildtype spermhead.
Wildtype spermatogenesis includes the formation of nebenkern, hyper-fused mitochondria, paired with individual nuclei within meiotic cells. [32] D. melanogaster expressing Tsetse β2 tubulin exhibits regular formation of both the nuclei in developing cells as well as regular formation of the paired nebenkern as compared to wildtype flies. Wildtype nuclei may also be misshapen in some cases as noted in Figure 12A; however, the majority of nuclei are circular and appear in equal numbers with the nebenkern. The cells expressing Tsetse β2 tubulin also appeared similar to wildtype development in both normal nuclei and nebenkern shaping and number but also in a number of cells with misshapen nuclei and unequal number of nebenkern seen in Figure 12B.

![Figure 12](image)

Figure 12. Testis squash of A) 5 day old virgin wildtype male and B) 5 day old virgin transgenic male. Black arrow represents area containing cells with wildtype-like nuclei (white circles) with respective mitochondrial derivatives (blue circles); White arrow represents area of abnormal cellular development of nuclei (white circles) with respective mitochondrial derivatives (blue circles).

3.3.5 Axoneme Morphology

The 9+2 arrangement of the spermtail axoneme was present in the transgenic flies. It appears without any disruption to this organization. When compared to the wildtype schematic,
the microtubules are arranged in a similar fashion along the outer rim of the tail with the central pair present (Figure 13).

Figure 13: A) Schematic of 9+2 arrangement of microtubules within the spermtail axoneme. The inner green-lined circles represent the central pair of microtubules. B) Cross-section of transgenic *D. melanogaster* axoneme shown under TEM, note the presence of the 9+2 arrangement

### 3.4 Discussion

The Tsetse β2 tubulin gene was able to provide a functioning sperm in the *Drosophila melanogaster* background. 7 out of 10 fertility tests produced progeny with no general defects within the offspring. Spermhead alignment, nuclei shaping, and nebenkern number all also resemble wildtype development. These results support the hypothesis of stabilizing selection acting on this specific protein. Other sequences are available in nature currently; however, nature is constantly selecting this specific sequence for the past 60 million years. Relating to the Syllogism of Evolution by Natural Selection as established by Charles Darwin, there is heritable variation available for this protein, and, concerning the premise of competition, the current sequence is essentially “winning” the limited resources now and for the past 60 million years. These could participate in the process of adaption but this process is following a narrow path. The ability for Tsetse β2 tubulin to carry a sperm-function in to the *D. melanogaster* model is evident
there are heritable variants and a competition is occurring in nature for the amino acid sequence of β2 tubulin.

The ability of the Tsetse β2 tubulin sequence to provide a functional sperm brings about the notion of a spermtail paradigm consistent in the β2 tubulin across these species. Certain amino acids which differ between β2 tubulin and β1 tubulin mentioned previously were unable to support sperm development. However, when an orthologous gene was substituted, there is evidence that there may be a “spermtail paradigm,” a sequence motif hidden within sperm – generating beta tubulins. This is apparent due to the amino acid sequence of Tsetse β2 tubulin being less similar to *D. melanogaster* β2 tubulin than *D. melanogaster* chimeric β1 tubulin with substituted domains of β2 tubulin, yet able to generate a functional sperm. The chimeric fruit fly β1 tubulin contained 9 different amino acids while Tsetse β2 tubulin contained 17 differences from *D. melanogaster* β2 tubulin. The changes between the Tsetse and fruit fly β2 tubulin amino acid sequences do not appear as detrimental as those differences between wildtype β1 tubulin. The capacity for the gene from Tsetse to create the functional spermtail can provide insight into which amino acids carry the sperm-function of β2 tubulin.

Based on the result of stabilizing selection and the notion of the spermtail paradigm, future directions of this hypothesis testing include substituting β2 tubulin from other species with differing amino acid sequences into the *D. melanogaster* model until a species does not provide a functional sperm. This will indicate when a co-evolutionary event occurred in the evolutionary timeline of this protein. Once this timeline is set, differing *D. melanogaster* amino acids will be substituted within the indicated species until a functional sperm is produced in order to precisely note the amino acids needed to bring about the sperm function paradigm as mentioned earlier.
REFERENCES


