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Competition Versus Choice: Evolution Along a Narrow Path in *Drosophila* β2 Tubulin

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Abstract

The *Drosophila melanogaster* β2 protein (Dmβ2) has sustained a long evolutionary stasis for the last 60 million years (Nielsen 2006). Even small changes to the protein's primary amino acid sequence render it non-functional, suggesting its stasis may be due to stringency in the structure/function relationship (Nielsen 2001). This project seeks to understand what has prevented Dmβ2 from evolving, with the two main hypotheses being that Dmβ2 either exists as an ideal protein configuration that competitively bests all alternates or that Dmβ2 is the only possible configuration that will support spermatogenesis in *Drosophila melanogaster.* In order to test these hypotheses, the ability of other proteins to rescue β2 function must be assessed. Previous work done to test β2 function used the major, non sperm-generator tubulin (β1) as a backbone to test the function of candidate sperm-generating residues. While sperm-generating residues were identified, none were sufficient to rescue fertility in a Dmβ2 null background (Nielsen 2001, Raff 2000). This project represents a different approach to analyzing the evolutionary stasis of Dmβ2 by testing the ability of a known sperm-generating ortholog from *Glossina morsitans* (commonly known as the tsetse fly) to rescue fertility. This sequence is 96% identical to Dmβ2 and is of particular interest because it is the closest relative to *Drosophila melanogaster* that possesses a variation in β2 sequence. When expressed in a Dmβ2 null background, the tsetse fly β2 (Gmβ2) generates long-tailed, fertile sperm when examined by light microscopy on testis samples and fertility tests between transgenic males and virgin wild-type females. This evidence supports the first of the two hypotheses outlined above, that β2 alternates exist but Dmβ2 is competitively superior. This shows the potential for β2 to participate in the process of evolution, potentially through allelic effects on sperm-tail length, which plays an important role in the retention of sperm in the female reproductive tract. Comparative analyses of outgroups, such as the human β2 ortholog (Hsβ3), will provide further information necessary to assess the roles of generic aspects of β2 such as motility versus more lineage-specific properties such as sperm tail length in the process of spermatogenesis.

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Introduction

Despite the fanfare surrounding the subject, evolution can quite simply be boiled down to one principle: choice plus competition equals evolution. Choice is the presence of alternates in nature, different alleles of a gene that provide different phenotypes each with their own advantages and disadvantages. Competition is the process by which these alternates assert dominance over the other phenotypes through the process of natural selection, with some alternates becoming more present in the gene pool due to their competitive prowess (Nielsen 2002). Many who have studied evolution have sought out what principles drive the process, looking for factors that have caused competition or given rise to more choices. Such approaches have introduced the world to famous case studies in evolution, ranging from the Galapagos finches to the human race itself (Liu 2018). But what if this approach is limited in its scope? What if there is an equally important angle to examine evolution from that has been overlooked? What if, instead of looking for factors that drive evolution, scientists turned their sights to the factors that prevent it? This is a more difficult approach, but it could prove invaluable when assessing proteins that have defied norms set into place as early as Darwin's time and refused to evolve.

One such protein is the *Drosophila melanogaster* Beta 2 Tubulin protein (Dmβ2), which has been notoriously difficult and has not evolved in 60 million years (Nielsen 2006). Dmβ2 is the testis-specific isoform of beta tubulin in *D. melanogaster* that is required for the formation of sperm tails, and it is the sole source of beta tubulin in the post mitotic male germ line. Dmβ2's complement is the *Drosophila melanogaster* Beta 1 Tubulin protein (Dmβ1), which is the major tubulin isoform and performs necessary

functions in the other cells of the body such as microtubule formation (Raff 1990). Previous work seeking to understand the stasis in evolution for Dmβ2 found that Dmβ1 is unable to function in its place to form a sperm tail, despite high levels of sequence similarity. This was shown through the use of TEM, where it was seen that Dmβ1 was unable to express the necessary 9+2 axoneme structure when expressed in testes (Raff 1990, Raff 2000). Other work has shown that certain amino acids from Dmβ2 are able to give Dmβ1 more sperm-generating function, although these alterations do not make a fully functional sperm (Nielsen 2001, Raff 2000). This body of previous work is reflected in both Figure 1 and Table 1, respectively.

Figure 1: Axoneme Structures Generated by Dmβ1 and Dmβ2

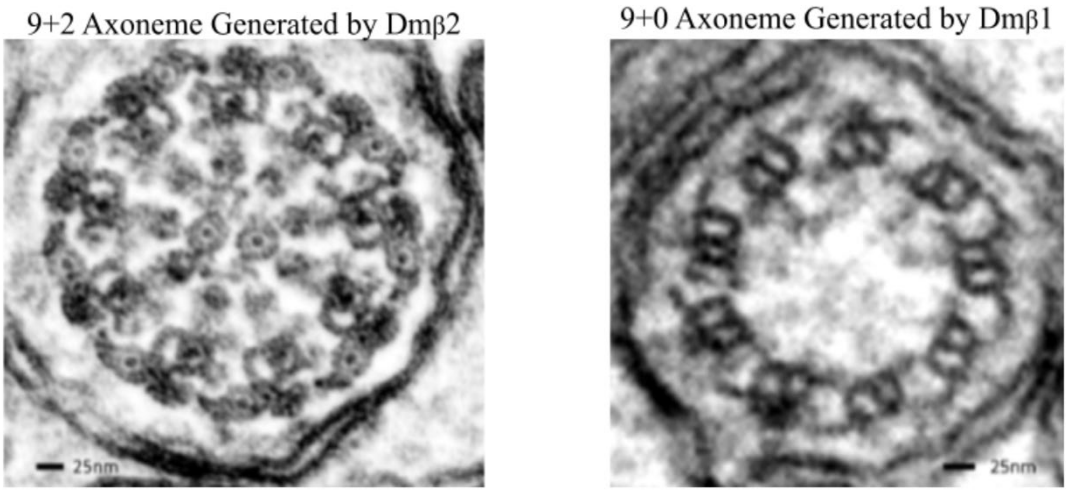


Table 1: Phenotypic Alterations of Dmβ1 by Increasing Dmβ2 Identity

Tubulin Tested	Sperm Tail Phenotype	Percent Identity with Dmβ2
Dmβ1	9+0 axoneme, short, non-motile	94%
Dmβ1 + Dmβ2 amino acids 433-434	9+2 axoneme, longer, non-motile	95%
Dmβ1 + Dmβ2 amino acids 433-446	9+2 axoneme, even longer, non-motile	97%
Dmβ1 + Dmβ2 amino acids 55, 57, and 433-446	9+2 axoneme, short, non-motile	97+%

Considering this body of past work surrounding Dmβ2, and the current understanding that no current functional alternates to Dmβ2 have been shown to function in *D. melanogaster*, the aforementioned evolution equation is more important than ever. If we maintain that choice plus competition equals evolution, then the evolutionary stasis of Dmβ2 must be a cause of either a deficit in competition or in choices. This has led to the formation of two hypotheses to examine this stasis. The first hypothesis states that Dmβ2 must be the best competitive version of beta tubulin sufficient for sperm-generation, therefore while other choices may be present they do not compete with Dmβ2 in a meaningful way. The second hypothesis states that Dmβ2 is the only possible

configuration for beta tubulin that can support spermatogenesis, therefore there is no evolution due to a deficit in choices. The direction of this work was initially created in an effort to determine if spermatogenesis in *D. melanogaster* could be sustained by a beta tubulin configuration other than Dmβ2, since that would prove whether or not evolutionary choices existed and allow for the selection of one of the two potential hypotheses for future examination.

In order to test for the existence of alternate beta tubulin configurations, this project examines known sperm-generating Dmβ2 orthologs. The primary ortholog examined in this project is the beta 2 tubulin from *Glossina morsitans* (which has been dubbed Gmβ2). Gmβ2 possesses a high sequence similarity to Dmβ2, making it a likely candidate for potentially rescuing fertility in a Dmβ2 null background. The ability of Gmβ2 to function as an alternate configuration of Dmβ2 will be determined by experiments meant to study the structure/function relationship.

Discoveries made using Gmβ2 prompted further examination of tubulins from a computational perspective, which provides data that hints at a pattern of beta tubulin structure that exists across multiple evolutionary outgroups. Table 2 shows the tubulins chosen for study, including their organism of origin and the nomenclature used to differentiate them for this project, as well as whether these tubulins function as a major tubulin or a sperm-generator.

Table 2: Tubulins Selected for AlphaFold Analysis

Tubulin	Organism of Origin	Major Tubulin or Sperm-Generator?
Dmβ1	*Drosophila melanogaster*	Major Tubulin
Gmβ1	*Glossina morsitans*	Major Tubulin
Hs Major	*Homo sapiens*	Major Tubulin
Mdβ1	*Musca domestica*	Major Tubulin
Hsβ3	*Homo sapiens*	Sperm-Generator
Hsβ4	*Homo sapiens*	Sperm-Generator
Gmβ2	*Glossina morsitans*	Sperm-Generator
Dmβ2	*Drosophila melanogaster*	Sperm-Generator
Hvβ2	*Heliothis virescens*	Sperm-Generator
Bmβ2	*Bombyx mori*	Sperm-Generator

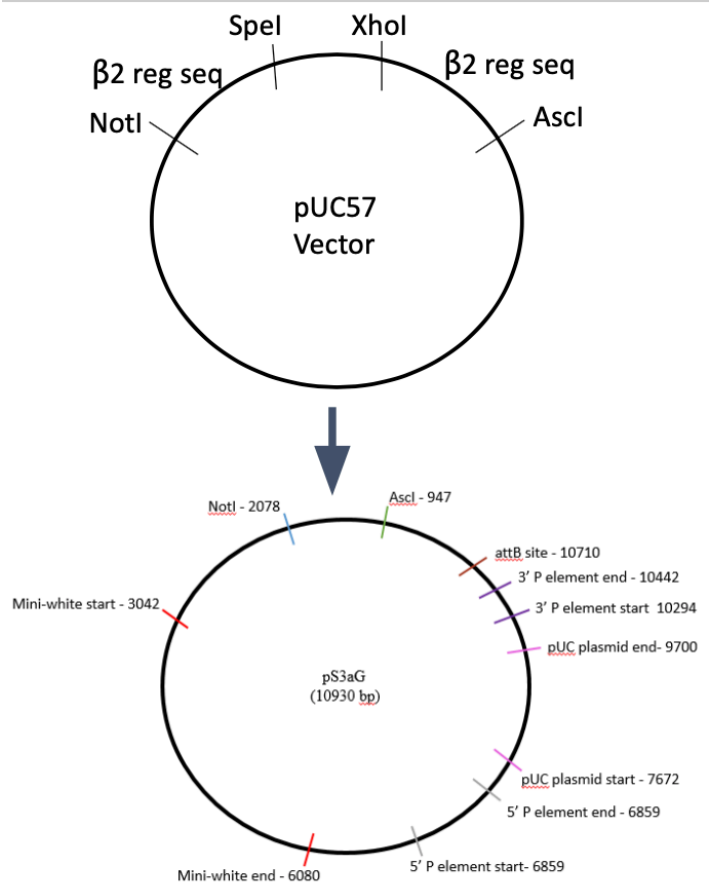
Analysis of these tubulin structures will surely be important in answering multiple evolutionary questions: Is Dmβ2 restricted in evolution due to a deficit in competition or choices? Are other beta tubulins undergoing similar evolutionary constraints? Is there a conserved structure necessary for sperm-generation across animal taxa?

Materials and Methods

Production of Transgenic Flies Using a Testis Vector

Figure 2 shows the testis vector utilized, derived from previous work (Goloconda 2018). Since the Dmβ2 gene rests on the third chromosome in *D. melanogaster*, this insert can be added to the second chromosome at the attP40 site and still express in the testes since it is flanked by Dmβ2 regulatory sequences. These flies were generated under the supervision of Dr. Mark Nielsen and Sarah Goloconda, prior to the beginning of this research project, and the methods undergone can be gleaned from their published work (Goloconda 2018).

Figure 2: Testis Vector



Once the flies containing the transgene insert were produced, they were crossed with existing stocks to produce flies containing the insert without any endogenous Dmβ2. These flies were produced in a white eyed background to better visualize phenotypic markers affecting eye color. Figure 3 shows the process by which the experimental flies were generated via genetic crosses. These crosses show chromosomes one, two, and three (excluding chromosome four since it is inconsequential for this work). While Figure 3 shows the sex of the flies used at different steps of the process, it is not vital for this portion of the research and the use of either sex will work.

Figure 3: Genetic Crosses to Generate Experimental Groups

Step 1: $\frac{w-}{w-}\frac{cyo}{+}\frac{tm3}{+}$ x $\frac{w-}{y}\frac{+}{+}\frac{ser}{cxd}$

a. Desired progeny from this cross: $\frac{w-}{w-}\frac{cyo}{+}\frac{tm3}{cxd}$

Step 2: $\frac{w-}{w-}\frac{cyo}{+}\frac{tm3}{cxd}$ x $\frac{w-}{y}\frac{+}{+}\frac{\beta 2^{-}}{tm3}$

b. Desired progeny from this cross: $\frac{w-}{w-}\frac{cyo}{+}\frac{\beta 2^{-}}{cxd}$

Step 3: $\frac{w-}{w-}\frac{cyo}{+}\frac{\beta 2^{-}}{cxd}$ x $\frac{w-}{y}\frac{Dm\beta 2b}{cyo}\frac{tm3}{+}$

c. Desired progeny from this cross: $\frac{w-}{w-}\frac{Dm\beta 2b}{cyo}\frac{\beta 2^{-}}{tm3}$

Step 4: $\frac{w-}{w-}\frac{Dm\beta 2b}{cyo}\frac{\beta 2^{-}}{tm3}$ x $\frac{w-}{y}\frac{Dm\beta 2b}{cyo}\frac{\beta 2^{-}}{tm3}$

d. Desired progeny from this cross: $\frac{w-}{y}\frac{Dm\beta 2b}{Dm\beta 2b}\frac{\beta 2^{-}}{\beta 2^{-}}$

Fecundity Tests

Fecundity tests were conducted using two male virgins from one of the three experimental groups and one virgin w118 female to test the fecundity of different genotypes when mated to wild type. Once virgins were collected they were isolated and allowed to mature for 5-7 days before being introduced in a new food tube and allowed to mate. Number of pupae were counted 3 days after the appearance of the first pupae and the number of adults were counted 5 days after the first adult hatched.

Phase Contrast Microscopy of Testis Samples

Phase contrast microscopy of testis samples allows for the visualization of sperm production by various experimental groups. Testis samples were obtained via dissection from male virgin flies of different genetic backgrounds. The samples were dissected in TB1 buffer solution and viewed using a Nikon light microscope.

Primary Amino Acid Sequence Analysis

The Molecular Evolutionary Genetics Analysis (MEGA) software was used to align primary sequences from the various tubulins and specific amino acid sites lacking a shared identity between two tubulins were identified in the search for candidate residues. The chemical significance of certain amino acid differences was determined by consulting literature on amino acid chemistry (Bischoff 2012).

Protein Structure Comparisons

Protein structures were derived using the AlphaFold software and its existing database of resolved protein structures (Jumper 2021, Varadi 2021).

Results

Fecundity Tests

A fecundity test between one w-/y; Gmβ2/+; Dmβ2-/ Dmβ2- virgin male and one w-/w-; Gmβ2/+; Dmβ2-/ Dmβ2- virgin female produced 50+ progeny, which is comparable to the positive control's (w-/y; Dmβ2/Dmβ2; Dmβ2-/ Dmβ2-) ability to rescue fertility. Table 3 shows the available data for the experimental group, positive control, and negative control.

Table 3: Fecundity Results of Experimental Groups

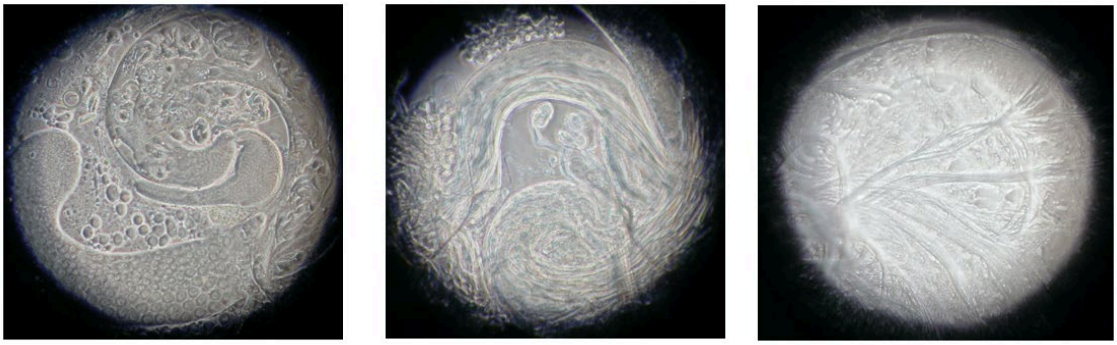
Experimental Group	Genotype	# Pupae	# Adults
Negative Control	w-/y; +/+; Dmβ2-/ Dmβ2-	0	0
Negative Control	w-/y; +/+; Dmβ2-/ Dmβ2-	0	0
Negative Control	w-/y; +/+; Dmβ2-/ Dmβ2-	0	0
Negative Control	w-/y; +/+; Dmβ2-/ Dmβ2-	0	0
Positive Control	w-/y; Dmβ2/Dmβ2; Dmβ2-/ Dmβ2-	34	44
Positive Control	w-/y; Dmβ2/Dmβ2; Dmβ2-/ Dmβ2-	35	48
Positive Control	w-/y; Dmβ2/Dmβ2; Dmβ2-/ Dmβ2-	51	54
Positive Control	w-/y; Dmβ2/+; Dmβ2-/ Dmβ2-	53	62
Positive Control	w-/y; Dmβ2/+; Dmβ2-/ Dmβ2-	36	50
Positive Control	w-/y; Dmβ2/+; Dmβ2-/ Dmβ2-	33	42

Positive Control	w-/y; Dmβ2/+; Dmβ2-/ Dmβ2-	62	81
Experimental Group	w-/y; Gmβ2/+; Dmβ2-/ Dmβ2-	•	50+

Phase Contrast Microscopy of Testis Samples

Figure 4 shows the different testis samples. In these images, the long sperm-tail filaments can be visualized, hinting at the ability of various experimental groups to produce sperm.

Figure 4: Sperm Production



w-/y; +/+; Dmβ2-/ Dmβ2- w-/y; Dmβ2/ Dmβ2; Dmβ2-/ Dmβ2- w-/y; Gmβ2/+; Dmβ2-/ Dmβ2-

Primary Amino Acid Sequence Analysis

Table 4 shows the identity between various tubulins and Dmβ2, which takes into account the total differences within the 446 amino acid long sequences and displays that data as percent identity. Tables 5-7 show specific amino acid changes between different tubulins and briefly comment on the chemical significance of those changes. Chemical significance was determined based on literature reviews, with only changes that affected the chemical conditions of the side chain being noted as significant and changes affecting the size of the residue being noted as not significant (Bischoff 2012).

Table 4: Percent Identity of Various Tubulins with Dmβ2

Tubulin	Percent Identity with Dmβ2
Dmβ1	94%
Gmβ2	96%
Hsβ3	90%

Table 5: Amino Acid Sequence Differences between Dmβ2 and Gmβ2

Site #	Change from Dmβ2 to Gmβ2	Significance of Change
120	V → I	No significant chemical change
124	S → A	No significant chemical change
130	L → F	Shift between aliphatic and aromatic
152	I → L	No significant chemical change
153	S → T	No significant chemical change
165	N → C	Shift from non-sulfur containing to containing sulfur, and from amidic to non-amidic
190	H → Y	Shift from being basic to being neutral
193	V → M	No significant chemical change
202	I → M	No significant chemical change
229	V → I	No significant chemical change
335	N → Q	No significant chemical change
347	N → S	Shift from being amidic to non-amidic
371	S → T	No significant chemical change
381	V → I	No significant chemical change
414	N → S	Shift from being amidic to non-amidic
431	D → E	No significant chemical change
444	G → A	Shift from hydrophilic to hydrophobic

Table 6: Amino Acid Sequence Differences between Dmβ2 and Hsβ3

Site #	Change from Dmβ2 to Hsβ3	Significance of Change
18	G → A	No significant chemical change
29	C → G	Change from hydrophilic to hydrophobic, and loss of sulfur
32	A → P	Potential helix breaking by shift to P
33	T → S	No significant chemical change
35	T → N	No significant chemical change
37	Y → V	Shift from hydrophilic to hydrophobic, and aromatic to aliphatic
48	N → S	No significant chemical change
55	T → S	No significant chemical change
56	G → S	Shift from hydrophobic to hydrophilic
57	A → H	Shift from neutral to basic, and hydrophobic to hydrophilic
83	Q → H	Shift from neutral to basic, and amidic to non-amidic
84	I → L	No significant chemical change
91	V → I	No significant chemical change
124	S → C	Shift from not containing sulfur to being sulfur containing
126	G → N	Shift from hydrophobic to hydrophilic
155	I → V	No significant chemical change
189	V → I	No significant chemical change
218	T → A	Shift from hydrophilic to hydrophobic
239	C → S	Shift from containing sulfur to not containing sulfur
275	S → A	No significant chemical change
315	A → T	Shift from hydrophobic to hydrophilic
316	I → V	No significant chemical change
332	N → A	Shift from hydrophilic to hydrophobic
335	N → S	No significant chemical change
340	F → Y	Shift from hydrophobic to amphipathic
349	C → V	Shift from containing sulfur to not containing sulfur, and hydrophilic to hydrophobic

351	T → V	Shift from hydrophilic to hydrophobic
365	A → S	Shift from hydrophobic to hydrophilic
381	V → I	No significant chemical change
427	E → D	No significant chemical change
431	D → E	No significant chemical change
436	F → M	Shift from hydrophobic to amphipathic
437	D → Y	Shift from hydrophilic to amphipathic, and neutral to basic
440	E → D	No significant chemical change
442	G → E	Shift from hydrophobic to hydrophilic, and neutral to acidic
443	G → E	Shift from hydrophobic to hydrophilic, and neutral to acidic
444	G → S	Shift from hydrophobic to hydrophilic
445	D → E	No significant chemical change
446	E → A	Shift from hydrophilic to hydrophobic, and acidic to neutral

Table 7: Amino Acid Sequence Differences between Dmβ1 and Gmβ2

Site #	Shift from Dmβ1 to Gmβ2	Significance of Change
18	A → G	Shift from hydrophobic to hydrophilic
23	I → V	No significant chemical change
35	A → T	Shift from hydrophobic to hydrophilic
37	H → Y	Shift from being basic to being neutral
64	V → I	No significant chemical change
80	P → A	Loss of helix-breaker status by shifting away from P
120	V → I	No significant chemical change
126	S → G	Shift from hydrophilic to hydrophobic
130	L → F	Shift from aliphatic to aromatic
152	I → L	No significant chemical change
153	S → T	No significant chemical change
165	N → C	Shift from non-sulfur containing to containing sulfur, and from amidic to non-amidic
167	Y → F	Shift from amphipathic to hydrophobic

190	H → Y	Shift from being basic to being neutral
193	V → M	No significant chemical change
202	I → M	No significant chemical change
229	V → I	No significant chemical change
231	L → A	No significant chemical change
335	N → Q	No significant chemical change
340	Y → F	Shift from amphipathic to hydrophobic
347	N → S	Shift from being amidic to non-amidic
349	V → C	Shift from not containing sulfur to containing sulfur, and hydrophobic to hydrophilic
371	S → T	No significant chemical change
414	N → S	Shift from being amidic to non-amidic
431	D → E	No significant chemical change
444	G → A	Shift from hydrophilic to hydrophobic

Protein Structure Comparisons

AlphaFold structures for various tubulins are shown in Figure 5. The tubulins fall into two different groups: the major tubulins and the testis-specific sperm generators. Figure 6 shows one specific structural motif that varies between major tubulins and sperm-generators, known as the H3 helix (Nogales 1999). Figure 6A shows the general location of the motif as modeled on Dmβ1, while Figure 6B shows a comparison of the region between Dmβ1 and Dmβ2.

Figure 7 shows a specific region of interest, the 275 to 281 residues, as seen on the various structures. Figure 7A shows the location of this region modeled on Dmβ1, while Figure 7B shows different structural motifs seen in this region. Table 8 shows a comparison of the primary amino acid sequence, structural motif in the 275-281 region, with a brief commentary in instances where the model confidence was below 70 out of 100.

Figure 5: Overall Structures of Various Beta Tubulins

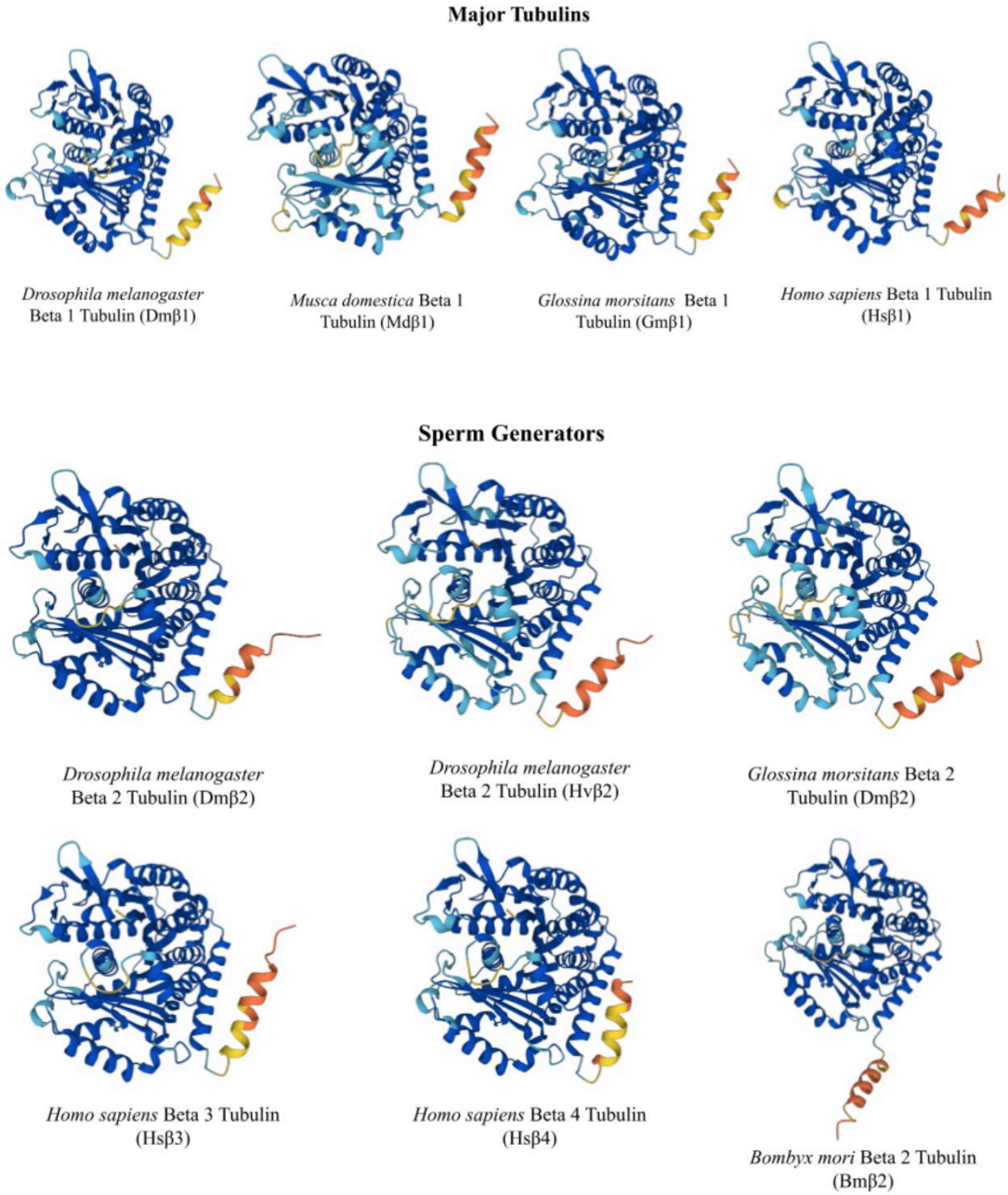


Figure 6: A Closer Look at the H3 Helix

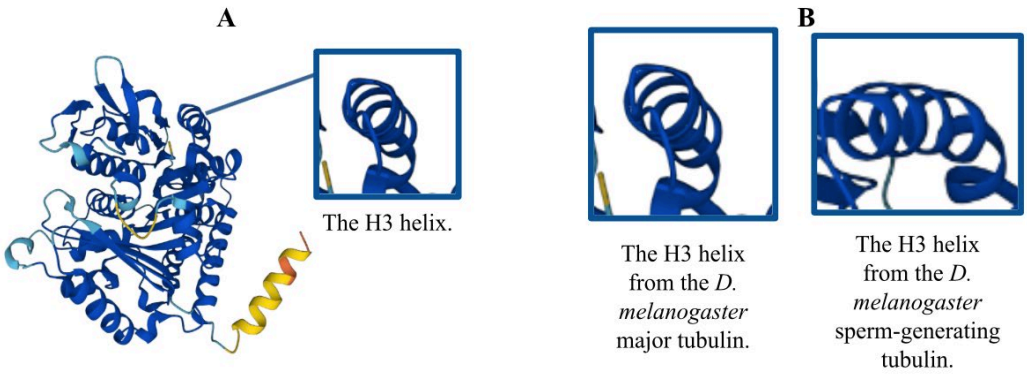


Figure 7: The 275-281 Structural Region

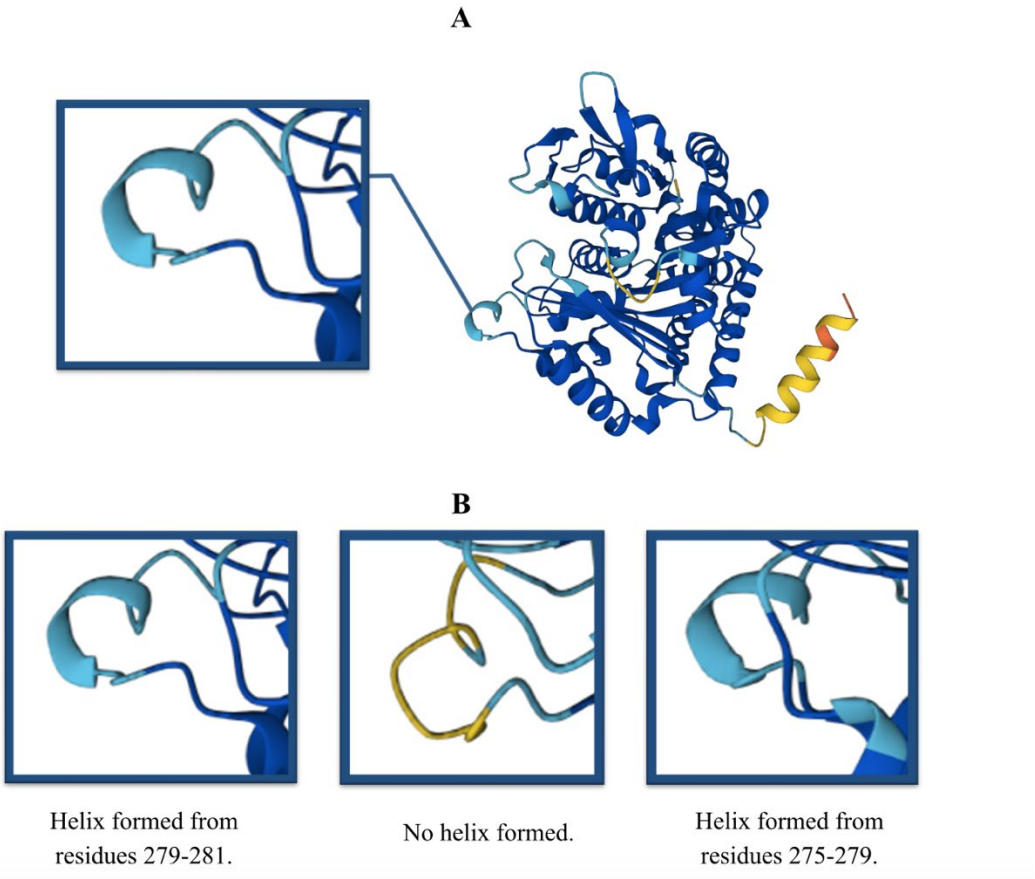


Table 8

	Residue 275	Residue 276	Residue 277	Residue 278	Residue 279	Residue 280	Residue 281	Residues Involved in Helix
Dmβ1	Ser	Arg	Gly	Ser	Gln	Gln	Tyr	279-281
Gmβ1	Ser	Arg	Gly	Ser	Gln	Gln	Tyr	279-281
Hs Major	Ser	Arg	Gly	Ser	Gln	Gln	Tyr	279-281
Mdβ1	Ser	Arg	Gly	Ser	Gln	Gln	Tyr	No Helix (Low Model Confidence)
Hsβ3	Ala	Arg	Gly	Ser	Gln	Gln	Tyr	No Helix (Low Model Confidence)
Hsβ4	Ser	Arg	Gly	Ser	Gln	Gln	Tyr	275-279
Gmβ2	Ser	Arg	Gly	Ser	Gln	Gln	Tyr	No Helix (Low Model Confidence)
Dmβ2	Ser	Arg	Gly	Ser	Gln	Gln	Tyr	275-279
Hvβ2	Ser	Arg	Gly	Ala	Gln	Gln	Tyr	No Helix (Low Model Confidence)
Bmβ2	Ser	Arg	Gly	Ser	Gln	Gln	Tyr	275-279

Discussion

Rescue of Fertility and Sperm Production by Gmβ2 Transgene

The ability of male flies expressing only the Gmβ2 transgene to have progeny when mated with a virgin female shows that the Gmβ2 gene is capable of rescuing Dmβ2 null function. Additionally, the phase contrast microscopy images show sperm production by the Gmβ2 gene. This shows that the first of the two hypotheses is correct, that Dmβ2 alternates exist but the current version is competitively superior.

Sequence Analysis

After results showed that Gmβ2 could function in place of Dmβ2, the next logical choice was to analyze the sequence of various tubulins to potentially identify regions of them that contributed to sperm-generation. Gmβ2 and Dmβ2 were chosen as known-sperm generators, Dmβ1 was chosen as an example of a major tubulin isoform that cannot generate sperm, and Hsβ3 was chosen because of its status as an evolutionary outgroup and its known sperm-generating function in humans. These varying tubulins have high identity with Dmβ2, and initial efforts attempted to identify specific residues that may serve as a smoking gun of sorts that would give a clear indication of what bestows sperm-generating ability on testis-specific tubulins and not major tubulin isoforms. This analysis was not nearly as fruitful as expected, as most changes appeared to convey very little chemical significance and none of the residues appeared to be significant players in intradimer and interdimer tubulin interactions (Nogales 1999, Gaertig 2009). These specific amino acid shifts, while undoubtedly important in some manner, did not lend themselves nicely to the clear identification of candidate residues that would convey sperm-generating function. These results necessitated a switch in

experimental approach from highly focused sequence analysis to a more comprehensive view of tubulin structure on a larger scale than that of primary sequence.

Overall Protein Structure Differences Between Tubulins

AlphaFold allowed for this analysis to occur, and initial results showed a clear difference in the overall structure of sperm-generating tubulins and a major tubulin isoform as seen in Figure 5. A specific region of interest showing this structural change was the H3 helix seen in Figure 6, which appeared to be at a different angle in the major Dmβ1 isoform than it was in the sperm-generators. This larger scale conformational change, and the apparent parallel evolution of the sperm-generators to a highly similar three-dimensional structure despite their dissimilar sequences, shows that the approach of identifying individual amino acids as candidates based on primary sequence alone was flawed because of what is likely a sequence of compensatory evolutionary changes meant to preserve a specific three-dimensional structure necessary for the generation of sperm tails. Since much of this analysis was conducted based on observations of these structures, however, a shift had to be made to more concrete regions of these proteins that could indicate a specific structural change unique to sperm-generators. This portion of the analysis included many more tubulins, which were selected based on the availability of sequence data and whether or not their expression pattern and status as either a major tubulin or a sperm-generator was known.

There seems to be a pattern of sperm-generating tubulin forming a helix with amino acids 275-279 and major tubulin isoforms forming a helix with amino acids 279-281, shown in Figure 7. This variance in secondary structural motifs is fascinating since these tubulins for the most part share a primary sequence and would be expected to fold

in a more homologous manner. Instead, there is a delineation between the secondary motifs of sperm-generators and major tubulin isoforms. However, it should be noted there are a few tubulins (Mdβ1, Hsβ3, Gmβ2, and the major *Homo sapiens* tubulin) that do not form a helix at all. This may be due to long-ranging interactions between various regions of the protein or simple low model confidence. Either way, the pattern of secondary structure formation between the ten tubulins shown in Table 8 shows that despite identical primary sequences, different structural motifs can form. This is likely due to compensatory changes that may be due to the actions of distant residues. The variance in helix formation for residues 395-399 shows a potential region impacting helix formation in the 275-281 region. The tubulins that failed to form a helix in the 275-281 region form a helix with amino acids 396-399, while tubulins possessing a helix in the 275-281 region have a helix with amino acids 395-399. This specific example warrants much more examination; it certainly shows a method of examining potential long-range interactions that could prove useful for the future examination of structural differences between major and sperm-generating tubulins.

The use of AlphaFold 2.0 will elevate this analysis, since the software could obtain predicted structures given a primary amino acid sequence. The future of this research will hinge on the use of AlphaFold 2.0 to identify specific amino acid changes that could alter a tubulin structure to more closely resemble the conserved sperm-generating structure noticed previously. Since the pattern of helix formation in the 275-281 region had already been identified, it served as a great starting point to examine long-range effects of amino acid shifts on a specific structural motif. Using previously

obtained sequence alignment data, specific changes can be studied in a much faster manner than those previously available.

Conclusion

The ability of Gmβ2 to rescue function in a Dmβ2 null background supports the first of the two hypotheses outlined previously, that Dmβ2 alternates exist but Dmβ2 is competitively superior. This shows the potential for Dmβ2 to participate in the process of evolution, potentially through allelic effects on sperm- tail length, which plays an important role in the retention of sperm in the female reproductive tract. Comparison of testis beta forms from different lineages indicate that a degenerative "sperm-motif" may support sperm tail production across animal taxa. Further tests with additional tubulins and AlphaFold 2.0 will determine if the pattern holds across more outgroups and uncover the underlying compensatory changes that conserve shape.

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