AN ANOMALOUSLY LARGE PROTEIN PROVIDES POSSIBLE INSIGHTS IN MOSQUITO SPERM

By

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APPROVED

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Abstract

An unusual protein has been detected in the sperm flagellum of the Culex quinquefasciatus mosquito, a vector for diseases such as West Nile fever, Eastern equine encephalitis, and potentially Zika. This protein has been identified as a massive tubulin, a protein in which significant changes can lead to structural failure of the important axoneme structure and infertility in the organism. Normally, microtubule structures formed from tubulin subunits form the characteristic “9+2” axoneme in the sperm flagella that Microtubule-Associated Proteins (MAPs) can attach to, acting as the molecular machinery that generates an oscillating wave. Modifications to the C-terminal tail on individual tubulin subunits can inhibit MAPs from binding to the C-terminal tail, thus modifying proper waveform generation of the flagella. Culex quinquefasciatus appears to contain a heavy β-tubulin with a novel amino acid sequence extending beyond the normal C-terminal tail. Tubulin isoforms of higher molecular mass are entirely unique, though many tubulin proteins are subject to post-translational modifications to the protein structure. However we have found preliminary evidence that this β-tubulin isoform is wholly transcribed for translation from the genome of Culex quinquefasciatus within the testes: this leads us to believe that the novel β-tubulin is incorporated into the axoneme of the sperm flagella and may play an important role in the regulation of cell motility. By asking how this extension can be contained within the axoneme, we hope to gain key insights as to how quinquefasciatus compares with other species of Culex.

Acknowledgments

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INTRODUCTION

Culex pipiens quinquefasciatus (*C. quinquefasciatus*) is a member of the *Culex pipiens* complex of mosquitoes and a vector of diseases including, but not limited to, West Nile Virus, Japanese Encephalitis, and Dengue Fever (Andreadis, 2012; Benelli, 2018). *C. quinquefasciatus* originated in Africa but has since seen distribution to tropical, subtropical, and temperate climates worldwide, making it a common mosquito in nearly every part of the world (Farajollahi *et al.*, 2011). The distribution of *C. quinquefasciatus* has overlap with other species within the Culex pipiens complex, opening the species to the possibility of hybridization for *Culex* which are genetically close enough to interbreed. A particularly efficient vector of mosquito-mediated disease happens to be the hybrid of *C. pipiens quinquefasciatus* and the cold climate-adapted *C. pipiens pipiens*, a hybrid common to portions of the northern United States and Canada (Farajollahi *et al.*, 2011). The benefit of this hybridization has allowed *C. quinquefasciatus* to live in biomes on the cusp of the colder north and more moderate and coastal climates in North America. California with its Mediterranean climate hosts the most landmass in which the *C. quinquefasciatus* hybrid can be found which can constitute a problem for suburban residents of the densely-packed state (Andreadis, 2012). Mosquito population control has been a major issue not limited to the confines of North America, as population control measures of mosquitoes can be found in every locale about the globe. In developing countries, mosquito vector control methods are being improved upon to achieve similar results in the quest to eradicate malaria (Killeen *et al.,* 2017a, Killeen *et al*., 2017b). Particularly, different chemical methods are being deployed to combat the adults and prevent infection from adult mosquitoes, other methods target still water and aim to prevent the development of larval mosquitoes, and other methods approach the question of mosquito population control by targeting the fertility event and preventing the

development of fertile eggs by cytoplasmic incompatibility (Koval and Vazquez-Prokopec, 2018, Yen and Barr, 1971). Each method has particular benefits which make them more or less effective in their environment, however there are issues with growing pesticide resistance in insects as a whole and even from behavioral changes in insects that render population control methods ineffective (Killeen *et al.,* 2017a, Mnzava *et al*., 2015). This study seeks an alternative control method, preceding even the event of fertilization of egg by sperm; we are seeking a protein target specific to *C. quinquefasciatus* which can be genetically modified to render a certain percentage of *C. quinquefasciatus* sperm immotile.

Sperm is an important target for further study as it is an important part of the C. quinquefasciatus life cycle: without sperm, there can be no fertile eggs. Previous work had been conducted on the sperm of *C. quinquefasciatus* in the Cardullo Lab in Thaler *et al.*, 2013. Within this study, there were several notable findings which garnered further interest in exploring the sperm proteome. The first such finding was that Ca^{2+} was necessary for sperm activation and continued motility in *C. quinquefasciatus*; calcium ions (Ca^{2+}) are an important ion for cellular signaling and for co-regulation of outer-arm dyneins with cyclic adenosine monophosphate (cAMP) which effect flagellar motility in all eukaryotic organisms (Konno *et al.*, 2015, Thaler *et al.*, 2013). Interestingly, this Ca^{2+} dependence had also shown that different levels of Ca^{2+} can cause *C. quinquefasciatus* sperm to swim in predictable ways. Increased phosphorylation was achieved using 20 μM okadaic3 acid, a phosphatase inhibitor, in solution with sperm in a low Ca^{2+} solution. The result of this experiment was a reversal in sperm swimming direction, with the sperm being lead by the tail rather than the head in a form similar to that of the low-energy A-Waveform described in the paper. When Ca^{2+} was increased in solution, Thaler *et al.* found that the sperm would change to a high-energy C-Waveform, suggesting not only that sperm can swim

backwards outside of the male reproductive tract, but that sperm can change between the three sperm waveforms to achieve fertilization in the female reproductive tract. The waveform of *C. quinquefasciatus* has been thought to be tied to Mitogen-Activated Protein Kinase (MAPK) and Phosphodiesterase activity along the axoneme. Thaler *et al.*, 2013 described MAPK targets along the axoneme of the flagella using MAPK substrate antibody and immunofluorescence microscopy; a fluorescent signal can be seen throughout the visible portion of the sperm flagella, extending all the way to the tip of the tail of the sperm. Dynein light-chain proteins are also known to be a phosphorylation target under cAMP-dependent regulation (Inaba, 2003), meaning that they are not outside of the realm of possibility of being the MAPK target observed in Thaler *et al.*, 2013. However, the pathway of flagellar activation in *C. quinquefasciatus* is not well-understood and the presence of the MAPK targets may suggest that there are many more targets than what we may hypothesize. This suggestion of multiple targets located along all parts of the axoneme have led us to investigate a unique tubulin as a possible target of MAPK phosphorylation.

Tubulin is present within all cell types as it is necessary for many important cellular structures and functions. Tubulin is important for organizing the spatial arrangement of organelles by creating a large web-like network of anchoring microtubules and developing structures such as the mitotic spindle, which pull apart chromosomes during meiosis and mitosis, or the flagellar axoneme (Inaba, 2003, Linck *et al.*, 2016, McKean *et al.*, 2001). The function of tubulin proteins is largely dependent on the type of tubulin and post-translational modifications which may occur to the protein. The Tubulin Superfamily includes a wide-array of known Tubulin Family isoforms, notably α-, β-, γ-, δ-, ε-, ζ-, and η-, with a weight of 50 kiloDaltons (kDa) each and different functions within the cell. The most understood families among the

Tubulin Superfamily are the α- and β- varieties as these are most abundant within all cell types and can be seen performing a multitude of important cellular functions (McKean *et al.*, 2001). α-Tubulin(GTP) and β-Tubulin(GTP) dimerize into αβ-Tubulin(GTP), the fundamental subunit of the microtubule, a protein polymer (Inaba, 2003, McKean *et al.*, 2001). αβ-Tubulin(GTP) polymerize in a ring by affixing the α-Tubulin side of αβ-Tubulin(GTP) to the β-Tubulin side of another with stabilization afforded by neighboring tubulin on either side of the added Tubulin. This "polarizes" the microtubule into a Minus Side to which few to no tubulin are added and a Plus Side which almost all of the tubulin are added. β-Tubulin expresses GTPase activity within the microtubule, contributing to the phenomenon of dynamic instability, or the sudden catastrophic depolymerization of the microtubule. From the Minus Side of the microtubule, β-Tubulin begins converting GTP to GDP, altering the structure of the β-Tubulin within the microtubule which threatens to pull the microtubule apart. As long as there are αβ-Tubulin(GTP) subunits to the Plus Side of the microtubule, it will remain stable. However, should all the αβ-Tubulin(GTP) within a microtubule be converted to αβ-Tubulin(GDP), the structure will no longer hold its form and will rapidly depolymerize (McKean *et al.*, 2001). Stabilizing proteins prevent these rapid depolymerization events in microtubules by "capping" the microtubule, thus never letting the αβ-Tubulin(GDP) reach the Plus Side of the microtubule and allowing cells to reliably utilize microtubules in larger, more useful cytoskeletal structures. Within sperm, these microtubules are incorporated into a much larger structure called the sperm axoneme. The axoneme structure is not unique to sperm, it is commonly used to create the organizing cytoskeletal structure of flagella or cilia, and has a structure which can be widely different within different organisms.

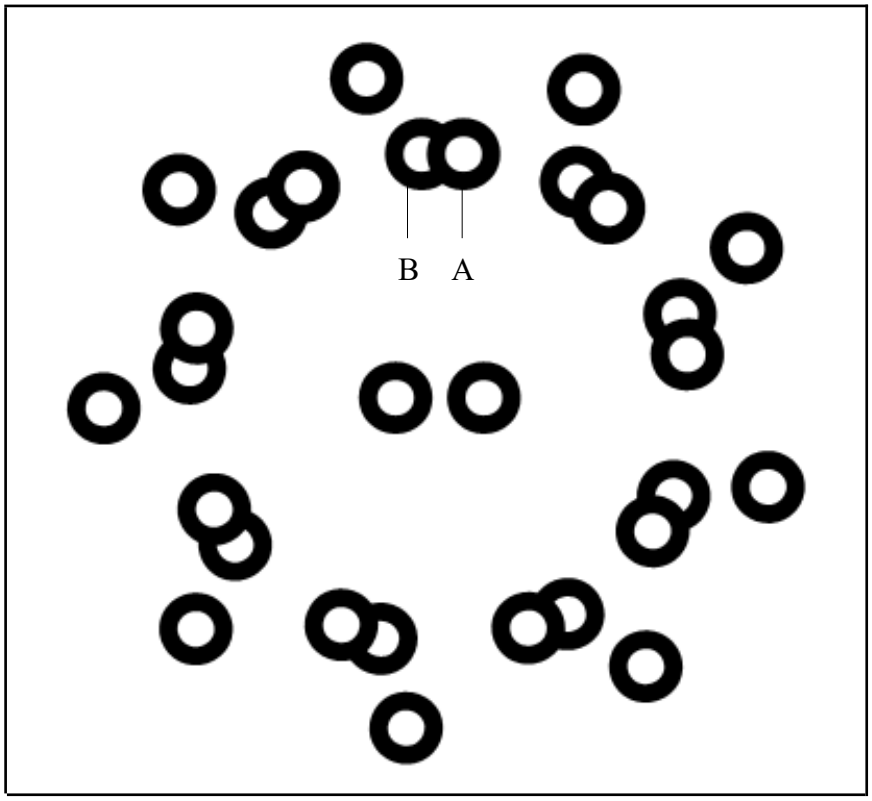

Figure 1: Arrangement of the microtubule doublets in the sperm flagella of *C. quinquefasciatus* with the A and B Rings labeled.

The axoneme is a critical component of the sperm flagella which organizes the approximately 250 different types of proteins within axonemal structures into a functional unit to aid in propelling the cell (Inaba, 2003, Linck *et al.*, 2016). It is the major organizing unit of the flagella which gives both form and function to the sperm flagella and is an important aspect in understanding fertilization events. The axoneme of *C. quinquefasciatus* is found to follow the 9+9+2 microtubule doublet organization, which can be observed in *Figure 1* above (Thaler *et al.*, 2013, Dallai *et al.*, 2006). This doublet is composed of an A Ring, thirteen longitudinal microtubule protofilaments arranged into a perfect tube and a B Ring which is typically composed of ten microtubule protofilaments bound to A Ring Microtubules 1 and 10 (Linck *et al.*, 2016, Mencarelli *et al.*, 2008). The inner nine A/B Ring Doublets are arranged about the central pair of microtubule rings of the axoneme and bound by Microtubule-Associated Proteins

(MAPs), such as dynein (Dallai *et al.*, 2006, Inaba, 2003, Linck *et al.*, 2016, McKean *et al.*, 2001). The outer nine microtubule Rings are not a feature commonly found in Eukaryotic organisms and seem to be something more unique to certain hexapods. It has been documented that the outer nine rings of the 9+9+2 Axoneme are closely associated with the same type of motility displayed in *C. quinquefasciatus* in Thaler *et al.,* 2013, with a complex three-dimensional waveform composed of two superimposed waves (Dallai *et al.*, 2006). The interaction of the outer nine Rings with the inner nine Ring Doublets allows this waveform to be generated along the 9+9+2 Axoneme due to the interactions between the microtubules and the MAPs which connect the individual protein components. The generation of this waveform is still not very well understood, as there are likely many contributions from MAPs which we have yet to understand. As was mentioned with Thaler *et al.*, 2013, there are MAPK targets located about the length of the axoneme of *C. quinquefasciatus* which have not been identified. The regulatory pathway in *C. quinquefasciatus* in which flagellar phosphorylation is achieved to activate cellular motility is still not well understood, much less the pathways involved in reversible alternation of the three-dimensional waveforms. It is possible that a unique tubulin, the 70 kDa β-Tubulin may contribute to this pathway either structurally or functionally.

The 70 kDa β-Tubulin is a unique isoform of β-Tubulin recently identified within *C. quinquefasciatus* by Mauceli, E., 2007 by genome assembly. What makes this β-Tubulin so unique is that it is the first of its kind with a 20 kDa extension from the regular protein; moreover, this extension is not predicted to be a post-translational modification. The extension on the 70 kDa β-Tubulin is predicted to be transcribed from the same gene and translated from the same Messenger Ribonucleic Acid (mRNA) as the 70 kDa β-Tubulin. Typical monomeric tubulin proteins can be thought of as having two major components which all isoforms of tubulin

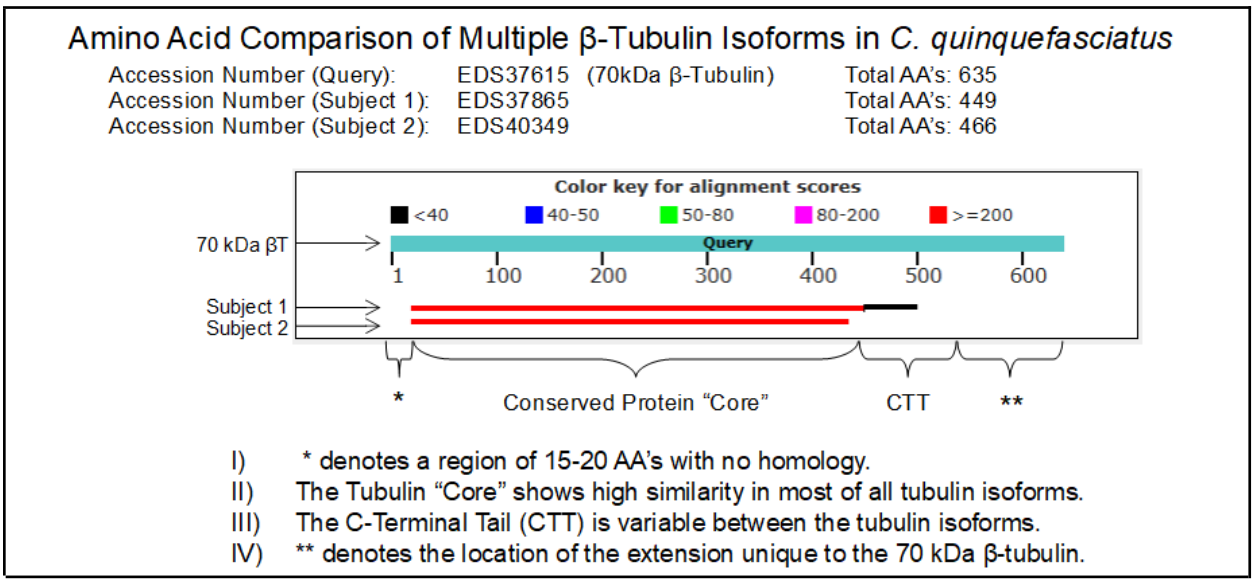

Figure 2: This comparison graphic shows the difference in size between different forms of β-Tubulin present in the proteome of *C. quinquefasciatus*. This comparison image was created using the results of the Protein BLAST software available from www.ncbi.nlm.nih.gov.

share: a relatively conserved "Core" domain which can bind Guanosine Triphosphate (GTP) and a variable-sequence Carboxy-Terminal Tail (CTT) which is available for post-translational modification (McKean *et al.*, 2001). The Core domain is the major structural component of tubulin and can bind to other tubulin in a highly directional manner. Regulation of assembly is partially dependent on the core domain as it needs bound GTP or a GTP homologue to induce proper tubulin binding (McKean *et al.*, 2001). The Core domain is mostly unchanged between tubulin isoforms because of its importance to the cell and cellular function with enough variation to induce proper specific binding. However, the CTT is far more dynamic than the Core domain of tubulin and can have little to no homology between isoforms of the same tubulin type. In Figure 2, the Amino Acid (AA) sequences of two β-Tubulin isoforms in the proteome of *C. quinquefasciatus* were compared to the 70 kDa β-Tubulin. What immediately becomes apparent is the amount of homology shared in the core regions of the proteins, with near-perfect scores. However, the homology stops at the CTT with low or non-existent scores for homology when

comparing the isoforms to the 70 kDa β-Tubulin. The CTT is far more dynamic and can be subject to heavy post-translational modifications, including polyglutamylation and polyacetylation which can alter whether certain proteins bind to microtubules (Lacroix *et al.*, 2010, Mary *et al.*, 1996). This is what makes the 70 kDa β-Tubulin so unique among tubulin: the 20 kDa extension extends beyond the CTT to produce a very large and unexplored protein domain of unknown form and unknown function which could have been detrimental to the survival of a species. Yet, this tubulin isoform possibly persists in the sperm proteome of *C. quinquefasciatus* and may contribute to the structure or function of the sperm axoneme in some way. Most importantly, this tubulin is unique among organisms and may be relegated to the *Culex pipiens* complex of mosquitoes.

The control of *C. quinquefasciatus* fertilization can prove to be a useful and highly effective method of mosquito population control. The survival and growth to maturity of *C. quinquefasciatus* larvae is dependent on the density of the larval population and the abundance, or lack, of resources (Koval and Vazquez-Prokopec, 2018). Adulticidal methods of mosquito population control are not effective in targeting this cache of infant mosquitoes and usually ignore the population of adult mosquitoes targeting avian populations for blood-meals. Larvicidal mosquito population controls can prove to be highly effective in controlling mosquito populations within localized areas (Benelli, 2018, Koval and Vazquez-Prokopec, 2018). These can be useful in preventing mosquito populations from becoming a problem in the area in which larvicidal techniques are utilized. This means that larvicidal techniques will need to be a more involved process compared to chemical adulticidal methods which can be deployed and persist in the environment (Killeen *et al.,* 2017a, Killeen *et al*., 2017b). *C. quinquefasciatu*s preys on a wide variety of animal hosts, thus it does not rely on any animal as its primary food source. For

vector control, this is a problem as *C. quinquefasciatus* can still lay eggs with a blood meal without ever having to come in direct contact with human hosts, breeding and accumulating a larger natural population (Farajollahi *et al.*, 2011). The most effective method of mosquito vector population control lies in mosquito fertility and the prevention of this wild population's accumulation in regions close to humans. Methods like cytoplasmic incompatibility using *wolbachia* bacteria can prove useful and persist within *C. quinquefasciatus* and effectively reduce the mosquito population (Altinli *et al.*, 2018, Yen and Barr, 1971). In a similar vein as the use of *wolbachia* to produce nonviable offspring, we propose the use of genetic engineering to render the sperm of male *C. quinquefasciatus* immotile*,* an infertile phenotype which can be engineered to be recessive in the population. It is our belief that the 70 kDa β-Tubulin, should we prove its existence and role in the sperm axoneme, can be this target which can be manipulated to control populations of *C. quinquefasciatus* regardless of location or animal host.

METHODS

Dissection

Two colonies of autogenous *C. quinquefasciatus* mosquitoes were maintained for dissection within a temperature-controlled 28 Celsius Room. Humidity was controlled with moist towels surrounding each flight cage to provide the optimal environment for both infant and adult *C. quinquefasciatus* such that each colony would lay eggs every two weeks. Larval mosquitoes were fed a blend of 20 grams (g) mouse feed and 1.5 g fish oil every day until pupation when mosquito pupae were transferred to a flight cage for adult emergence. Mosquitoes would be collected at the soonest availability after mating and egg-laying to isolate male specimens for dissection. Breeding-age males were selected and isolated into cups prior to dissection.

Mosquitoes were exposed to 100% Chloroform (Fisher Scientific) under a fume hood and quickly submerged in 1X Phosphate-Buffered Saline (PBS) on a dissection slide under a dissection microscope. 1X PBS had been diluted down using 1 mL 10X PBS in 9 mL nuclease-free diethyl pyrocarbonate-treated and deionized water (DEPC-dH_2O, Fisher Scientific). 10X PBS was prepared using 1.20g 20mM NaH_2PO_4, 7.10 g 100 mM Na_2HPO_4, 39.42 g 1350 mM NaCl, balanced with HCl to pH 7.2 and brought to a total volume of 500 mL with deionized water (dH_2O).

Mosquito testes were extracted by holding the last segment of the abdomen and the thorax with fine-tipped forceps and pulling the abdomen segment from the rest of the body. The gonads, contained in the second-to-last segment of the abdomen, were then exposed and the testicles were cleaned by pulling away excess tissue and separated from the remainder of the gonads before submerging the testes in 0.5 mL Invitrogen™ TRIzol™ reagent (Thermo Fisher Scientific) kept in ice at 0 Celsius. Twenty to thirty mosquitoes were dissected, with forty to

sixty testes in solution per dissection. Testes were homogenized for two minutes in TRIzol™ using a plastic pestle treated with Molecular BioProducts RNase AWAY® Surface Decontaminate (RNase AWAY) before storage at -80 Celsius.

Ribonucleic Acid (RNA) Extraction

The homogenized tissue in TRIzol™ was removed from -80 Celsius and allowed to thaw in an ice bath at 0 Celsius. The testes homogenate was removed from the ice bath and incubated at 23 Celsius (room temperature) for five minutes. 0.2 mL 100% chloroform for every 1 mLTRIzol™ in testes homogenate. The tube was then shaken vigorously for fifteen seconds and incubated for two to three minutes at room temperature. The testes homogenate was then centrifuged at 12,000 g for fifteen minutes at 4 Celsius.

After centrifugation, a clear upper aqueous phase containing the RNA could be selectively removed from the solution via careful pipetting with an RNA-free filter pipette tip into a new, labeled tube. The lower, red, organic phase contained protein and was stored at -80 Celsius for future experimentation.

0.5 mL 100% isopropanol per 1 mL TRIzol reagent used for homogenization was added to the removed aqueous phase and incubated at room temperature for ten minutes. The room temperature aqueous phase was then centrifuged at 12,000 g for ten minutes at 4 Celsius. A semi-translucent, white pellet had formed at the bottom of the tube. The supernatant was removed by quickly inverting the tube after centrifugation on to a Kimtech Kimwipe. The pellet was then washed with 1 mL 75% ethanol (2.5 mL DEPC-dH_2O, 7.5 mL 100% ethanol) per 1 mL TRIzol™ and immediately centrifuged at 7500 g for five minutes at 4 Celsius. The 75% ethanol

wash was removed by quickly inverting the tube after centrifugation on to a Kimtech Kimwipe and the pellet was air dried in the tube.

Once almost dry, 20 µL DEPC-dH_2O was added to the RNA pellet and incubated in a dry heat bath set at 60 Celsius for ten minutes. RNA concentration of the sample was determined using an Invitrogen Qubit™ HS RNA (High Specificity) Assay in an Invitrogen Qubit™ 2.0 Fluorometer. 1 µL of sample was diluted into 99 µL sample assay solution due to the small volume of sample; optimal RNA concentration was empirically determined to be approximately 30 ng/µL or more of stock RNA solution based on success in subsequent RNA Reverse Transcription steps. Remaining sample was stored at -80 Celsius.

RNA Reverse Transcription (RT-PCR)

RNA Reverse Transcription was achieved using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit with a protocol developed by Dr. Kimberly Stephens to improve efficacy with *C. quinquefasciatus* tissue samples. 6.00 µL of the RNA sample, regardless of concentration, was mixed with 2.00 µL DNase, 2.00 µL Reverse Transcription Buffer, and 10.00 µL DEPC-dH_2O and incubated at room temperature for twenty minutes. Following the incubation, 2.00 µL Ethylenediaminetetraacetic Acid (EDTA) was added to the solution and incubated further at 65 Celsius for fifteen minutes.

Two Nuclease-Free 100 µL Eppendorf tubes were labeled and used to hold the sample and the negative control during the reaction. These samples were placed in a thermocycler and denatured at 25 Celsisus for ten minutes, then incubated at 37 Celsius for two hours. A final extension at 95 Celsius preceded an infinite hold at 4 Celsius. Samples were subsequently removed and the DNA concentration was recorded before long-term storage at -20 Celsius.

Deoxyribonucleic Acid Polymerase Chain Reaction (PCR)

Primers which would amplify the 70 kDa β-Tubulin mRNA sequence were designed using the putative sequence available in the National Center for Biotechnology Information (NCBI) Nucleotide Database. The available mRNA sequence for the 70 kDa β-Tubulin (NCBI Reference Accession: XM_001842025, from Mauceli, E., 2007) was chosen as the best available template for primer design currently available. The forward and reverse primers had to anneal to sections of mRNA exons exclusively to produce the required result in the cDNA for sequencing as well as include section of the β-Tubulin Core, Tail, and 20 kDa Extension while producing a product in both the Genomic DNA (gDNA) and cDNA around a maximum of 1000 nucleotide base pairs (bp) to maintain the optimal activity of both Invitrogen Platinum™ Taq DNA Polymerase and BioLine MyTaq™ DNA Polymerase. Invitrogen Platinum™ Taq DNA Polymerase was chosen over BioLine MyTaq™ DNA Polymerase for PCR Extension before DNA Sequencing due to the higher reported fidelity of the polymerase. When developing the protocols for each primer sequence the recommended extension time for each polymerase, 60 seconds per kbp using Invitrogen Platinum™ Taq DNA Polymerase or 30 seconds per kbp using BioLine MyTaq™ DNA Polymerase, had to be taken in to account as well as the size of each putative PCR product.

The designs for the *C. quinquefasciatus* primers were made using Primer3Plus and unintended primer targets were determined using NCBI Primer-BLAST to avoid false-positive results on DNA Agarose Gel Electrophoresis following PCR. To determine if the 70 kDa β-Tubulin was present in the mRNA of *C. quinquefasciatus*, the primers had to anneal to the Core of the tubulin, extend through the tail domain, and anneal to the Extension. Forcing the primers

to anneal to the beginning and ending of the transcript produced primer pairs with primer annealing temperatures with over a 10 Celsisus between the Forward and Reverse primers. The following primer designs were subsequently used: Culex Actin (ACT) – 5'- GCC CAA TCC AAG CGA GGT AT -3' (forward), 5'- TAC GAC CGG AAG CAT ACA GC -3' (reverse); 70 kDa β-Tubulin 1 (CRTLEX1) – 5'- AAT TCG CGA AGA GTA CCC CG-3' (forward), 5'- CGT CGG GAA GCA AAA GTT GG-3' (reverse); 70 kDa β-Tubulin 2 (CRTLEX2) – 5'- CTC GGG GTC TCA AGA TGT CC -3' (forward), 5'- GTT CCA GCG TTT CGT ACT CC -3' (reverse). Per each 20 µL reaction, the following reagents were used: 10.00 µL 2X MyTaq™ Master Mix, 1.00 µL 10 µM Forward Primer, 1.00 µL 10 µM Reverse Primer, 3.00 µL DEPC-H_2O, and 5.00 µL Sample. The cycling conditions used to test the primers against gDNA, cDNA, and Water Control Samples are listed below.

INITIAL DENATURING	$T_{denature}$:	98.0	°C
	Time:	3	minutes
STEP 1		35	cycles
	$T_{denature}$:	98.0	°C
	Time:	15	seconds
	$T_{annealing}$:	58.0	°C
	Time:	15	seconds
	$T_{extension}$:	72.0	°C
	Time:	30	seconds
FINAL EXTENSION	$T_{extension}$:	72.0	°C
	Time:	5	minutes
INFINITE HOLD	T_{hold}:	04.0	°C

Following PCR, a 1% DNA Agarose Gel was made using 0.75g agarose sugar, 2.00 µL Ethidium Bromide (EtBr) and 50 mL 1X TAE (Stock 10X Concentration: 48.4g Tris Base, 11.4 mL Acetic Acid (17.4 M), 3.7g EDTA, DEPC-H_2O to 1 L Final Volume) and submerged in 1X

TAE Buffer. The connecting electrodes ran at 100 Volts for 40 minutes before the DNA Agarose Gel was imaged using an Ultraviolet (UV) Imaging Machine.

Deoxyribonucleic Acid Sequencing

DNA sequencing was accomplished using NextGen Sequencing available at the UCR Genomics Core facility.

RESULTS

This experiment had been replicated over a period of two years, beginning in 2016, to identify a signal which could help determine whether the designed primers had properly targeted the region. An example of a typical gel can be observed in *Figure 3* of this document. Actin primers produced the most consistent signal during DNA Agarose Gel Electrophoresis, which was expected. The Actin primers were designed and optimized prior to this experiment and have been used as the DNA control; some contamination in the Actin primers had been observed when testing primer designs and the stock Actin primers were replaced to remove the contamination. CRTLEX1 and CRTLEX2 primers have been inconsistent in producing DNA Agarose Gel signals. However, CRTLEX1 has been producing the most consistent hints of the presence of the 70 kDa β-Tubulin in the gDNA sample with minimal success when testing the primers with cDNA. CRTLEX1 primer product sizes when the Samples were gDNA and cDNA were 1169 bp and 997 bp, respectively. Samples sent for sequencing did not produce sequences.

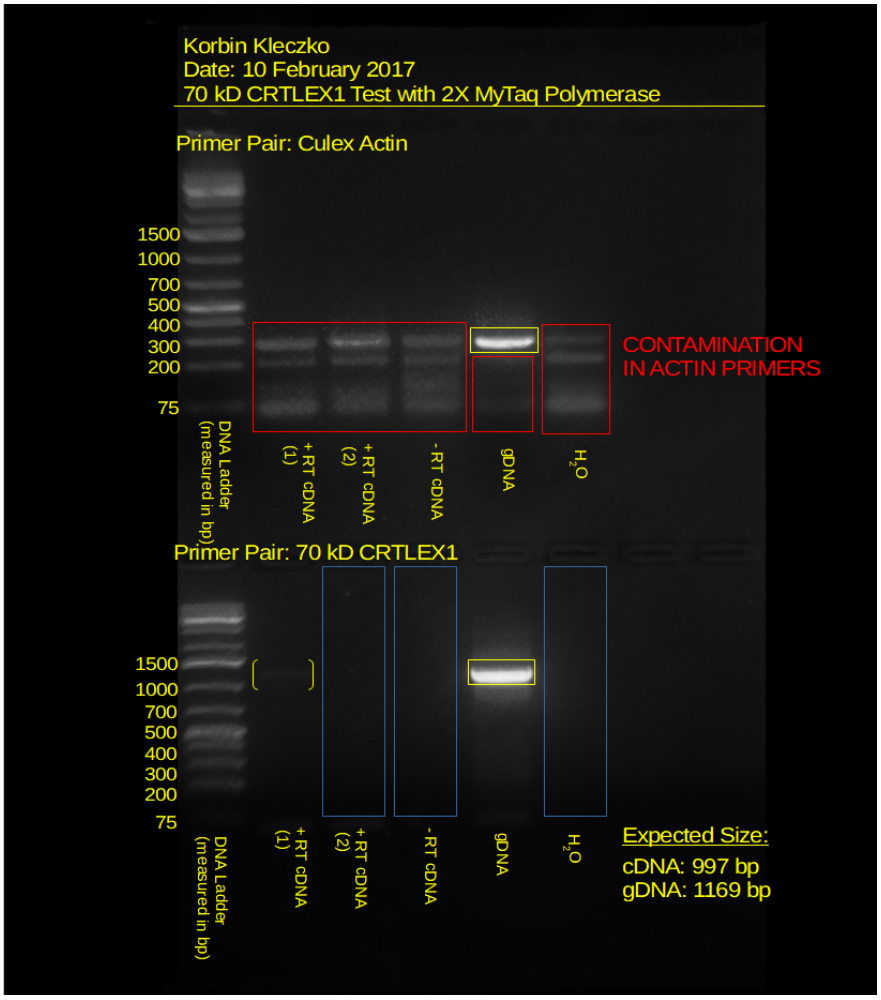

Figure 3: Labeled DNA Agarose Gel showing ACT and CRTLEX1 Primer Pairs. The DNA Molecular Weight Ladder is shown at left.

DISCUSSION

Based on our observations, we cannot conclude with absolute certainty that the 70 kDa β-Tubulin is present in the gDNA of *C. quinquefasciatus*. There is preliminary evidence to suggest the 70 kDa β-Tubulin mRNA scaffolding is correct from circumstantial evidence from DNA Agarose Gel Electrophoresis, but there is not enough evidence to conclusively say this as a fact. Confounding factors may be contributing to the inconsistency of results, the likes of which can include circumstantial contamination of DEPC-H_2O, low concentrations of cDNA, and low concentrations of mRNA. The primers produce PCR products inconsistently at the predicted size of the 70 kDa β-Tubulin mRNA primer target. This requires further testing, though it may indicate that the concentration of sample may be too low to produce a signal. If this is the case, sample must be concentrated prior to PCR to ensure a consistent signal can be achieved from the Actin controls prior to use with CRTLEX1. Additionally, this may indicate a problem with the CRTLEX1 primers. Optimal cycling conditions have not been fully determined for this primer pair and optimal annealing temperatures for cycling could be determined empirically using a temperature gradient PCR. mRNA Extraction may need to occur with higher quantities of *C. quinquefasciatus* testes in a more concentrated volume, such as eighty testes in 500 mL TRIzol™ for mRNA Extraction. However, far more can be done to answer the question of the 70 kDa β-Tubulin outside of genetic analysis.

The failure to say without a doubt that the 70 kDa β-Tubulin is expressed wholly from mRNA have led us to find additional ways at attacking this research question of whether the 70 kDa β-Tubulin can be used as a vector control target. Future experimentation should include proteomic analysis of the 70 kDa β-Tubulin to see if the protein can be isolated wholly from *C. quinquefasciatus* sperm. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-

PAGE) can be used to determine molecular weight of proteins in solution. To study the 70 kDa β-Tubulin, Affinity-Column Chromatography can be used to concentrate tubulin before running it on SDS-PAGE or using the concentrated tubulin for enzymatic analyses. Tubulin concentrated on SDS-PAGE can be subjected to a Western Blot test with tubulin-specific antibodies to determine if a signal appears at 70 kDa. Furthermore, this test can aid in developing antibodies specific to this tubulin at the 70 kDa weight mark for additional applications, including immunofluorescence microscopy. Should this experiment move on to immunofluorescence microscopy, the protocol outlined by Thaler *et al.*, 2013 will be used to ensure success. It is still unknown whether the 70 kDa β-Tubulin does exist in the sperm proteome of *C. quinquefasciatus*.

Whether the sequence is an error of genome assembly or the mRNA is different from our prediction, we do not know; however, the question of how to control the *C. quinquefasciatus* population still stands as an interesting and important one for disease vector control. Methods including cytoplasmic incompatibility are known to be effective in this fight against the spread of mosquito-borne disease in a biologically responsible manner and we urge future scientists to strive for better, manageable methods of vector control. Introducing a genetic variant of tubulin which hinders sperm motility may be one such method of control that can be achieved within our lifetime, a method which can have further implications for not only mosquito motility but the sperm motility of all organisms with or without unique tubulin isoforms.

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