THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

DETERMINING THE CONTRIBUTIONS OF THE *SPP-1* AND *UMPS-1* GENES ON THE HYPERACTIVE MUSCLE PHENOTYPE

YIXUAN WANG
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Reviewed and approved* by the following:

Wendy Hanna-Rose
Associate Professor of Biochemistry and Molecular Biology
Thesis Supervisor

Ming Tien
Professor of Biochemistry
Honors Advisor

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Many diseases that result in a decrease in muscle function are gene related. In an attempt to develop a better understanding of muscle locomotion and eventually an effective method to increase muscle locomotion in those with muscle weakness, this study aims to identify genes that are potentially related to human muscle function using the model organism *Caenorhabditis elegans*. The *ok2703* allele contains a deletion of parts of the *spp-1* and *umps-1* gene sequences and shows a hyperactive muscle activity phenotype. This indicates that there is a high probability that one or both of the mutated genes were the cause of the increased muscle movement. In order to validate this hypothesis, expression levels of these two genes in RNAi treated and several commercial mutant alleles were studied using qRT-PCR and compared to the muscle mobility of the *C. elegans*. The results are inconclusive and I am not able to identify which gene (or both) is contributing to the hyperactive muscle activity phenotype. Both technical and biological replicates need to be done for further validation.

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ACKNOWLEDGEMENTS

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Chapter 1

Background Information

1.1 – Significance of Study: Muscle Function

Muscle weakness is a common symptom found in people with a variety of illnesses and health conditions such as muscular dystrophy and stroke patients and can severely impact a person's life. One example of muscular dystrophy is Duchenne muscular dystrophy. This disease generally starts during early childhood and is mainly found in boys (Venugopal and Pavlakis, 2018). It is characterized by weakness in the knees and hips, resulting in the need of a wheelchair by age 12 (Venugopal and Pavlakis, 2018). Another example of muscular dystrophy is Oculopharyngeal muscular dystrophy. This disorder often starts around 50 to 60 years old and affects the proximal limbs as well as swallowing (Abu-Baker and Rouleau, 2007). The most common cause of death for such patients is starvation (Abu-Baker and Rouleau, 2007). Motor function defects are also found in patients who have had a stroke in the past (Hatem et al., 2016).

Not only is muscle weakness linked to diseases, it can also be found in the healthy elderly (Papa, Dong, and Hassan, 2017). In addition, those taking certain medications such as Cocaine, Penicillin, Chemotherapeutic agents, or antithyroid agents may also experience muscle weakness (Saguil, 2005).

By identifying genes that can help increase muscle function, one can utilize the new information in translational research. Researchers can attempt to identify any abnormalities of these genes for those who have muscular dystrophy or general muscle weakness. Through gene

therapy, these genes can be targeted in the patients (Karpati and Acsadi, 1993). Hopefully, by targeting the genes known to be responsible for an increase in muscle function in the model organism there will be a similarly observed phenotype in the patients. By helping increase muscle function, symptoms of dysphagia or difficulty in walking seen in muscular dystrophy patients or problems with daily life may be eased.

1.2 – *Caenorhabditis elegans* as a Model Organism

Caenorhabditis elegans is a free-living, self-fertilizing hermaphrodite that has been used for medical-related research. This nematode is well suited as a model organism because of its small size, short generation time and complete genome sequence data (Culetto and Sattelle, 2000). More importantly, the *C. elegans* has about 60-70% of human gene counterparts (Sparrow, Hughes, and Segalat, 2008) and 40% of the counterparts are human disease genes (Culetto and Sattelle, 2000).

Because this study will be focusing on the muscle function in the *C. elegans,* it is important to understand the basic anatomy of the nematode in relation to its muscles. The *C. elegans* has four sets of body wall muscle, which are located underneath the epidermis. These muscles span the length of the *C. elegans* and allow for the wave-like movement of the nematode (Figure 1).

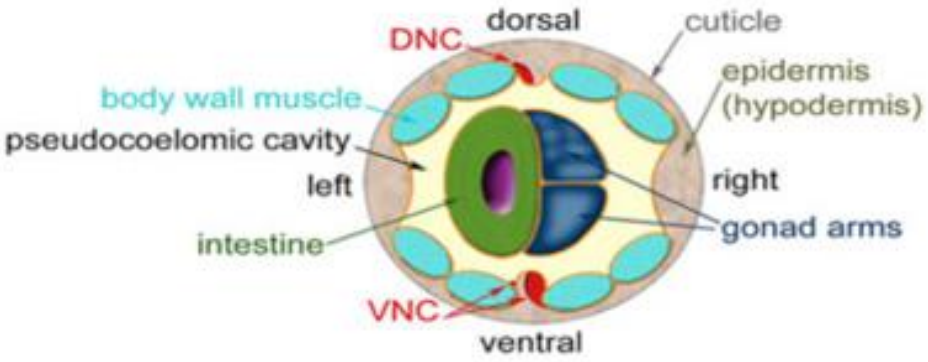

Figure 1: Picture of Epidermis (Image course from wormbook.org, online, http://www.wormbook.org/chapters/www_celegansintro/celegansintro.html#figure3)

1.3 – Pyrimidine Biosynthesis Pathway

The synthesis of pyrimidine is an important role in all organisms and is found in both humans and *C. elegans*. Pyrimidine biosynthesis is a part of the nucleotide metabolism pathway. This study will be focusing on the last part of the pyrimidine pathway in which orotate is converted into OMP and then into UMP (Figure 2). In this pathway, the enzyme used in converting orotate / orotic acid (OA) into Uridine monophosphate (UMP) is uridine-5'-monophosphate synthase (UMPS), which is coded by the *umps-1* gene (Merry et al., 2014). Problems that can arise due to the problems in this pathway are Miller Syndrome and Orotic Aciduria (Merry et al.,

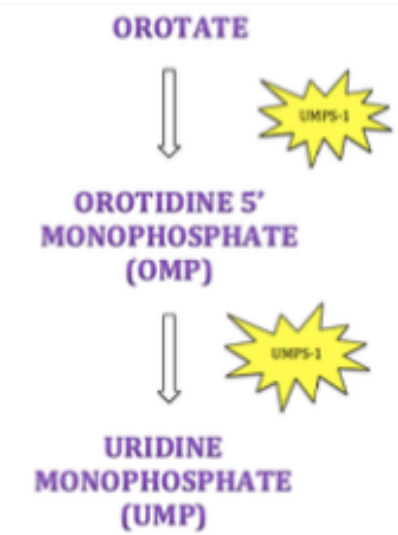

Figure 2: Part of the Pyrimidine Biosynthesis Pathway. UMPS-1 helps Orotate change into OMP and OMP into UMP

2014) (Duley et al., 2016). For Miller Syndrome, this is caused by defects in the dihydroorotate dehydrogenase (DHODH) gene (Duley et al., 2016).

Children may develop facial as well as bone abnormalities (Miller, Fineman, and Smith, 1979). Orotic aciduria is the opposite: the build up of orotic acid is due to the deficiency of the UMP synthase (UMPS) (Wortmann et al., 2017). Symptoms seen in those with this disorder are anemia, cognitive impairment, and physical retardation (Debnath, Aggarwal, and Mittal, 2011)

1.4 – *umps-1* gene and *spp-1* gene

UMPS-1

As stated previously, UMPS is required for two steps of the pyrimidine biosynthesis pathway. A loss of function in this protein results in a disorder that displays symptoms including ones that involve muscle function (Wortmann et al., 2017); (Debnath, Aggarwal, and Mittal, 2011). Uridine MonoPhosphate Synthetase, *umps-1*, codes for the UMPS protein. This protein has two roles, one as the orotate phosphoribosyltransferase and one as the orotate monophosphate decarboxylase (Merry et al., 2014). This gene is located on the third chromosome and is also required for development as well as gut granule size in the *C. elegans* (WormBase: umps-1, n.d.) and (Levitte et al., 2010).

SPP-1

spp-1 codes for a protein in the SaPosin-like Protein family and is involved in antibacterial roles *in vitro* (Hoeckendorf, Stanisak, and Leippe, 2016) and (Bányai and Patthy, 1998). It is also

located on the third chromosome of the *C. elegans* genome, just downstream of the *umps-1* gene (WormBase: spp-1, n.d.).

1.5 – Mutant Alleles

ok2703 is an allele that has a deletion of part of the 5' end of the *spp-1* gene and part of the 3' end of the *umps-1* gene. What makes this mutant unique is that it displays a hyperactive muscle function phenotype. Previous data show that there is an increase in thrashing rate in the ok2703 mutant *C. elegans* compared to that of the N2 wild type control *C. elegans* (Prestipino, 2017).

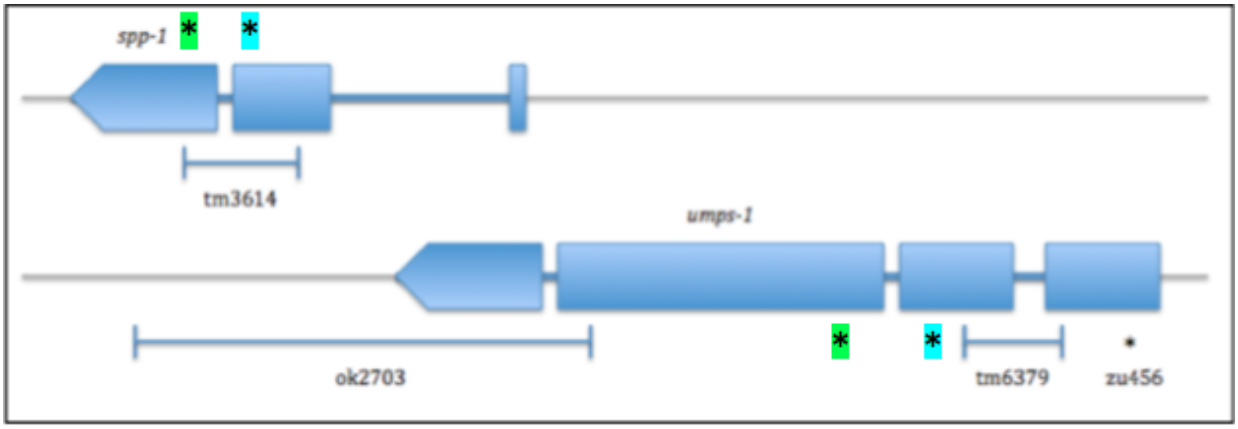

Figure 3: Genetic map of the *spp-1* and *umps-1* genes with the approximate locations of the alleles and *spp-1* and *umps-1* primers used. The green stars are the forward primers; blue ones are the reverse primers.

In addition to the strain carrying the *ok2703* allele, there are several other mutant strains that are expected to impact the *umps-1* gene are used in the experiment. The *zu456* is an allele that contains a nonsense substitution that interferes with the translational initiation site. The next start codon would result in an out-of-frame peptide (Levitte et al., 2010). The tm6379 allele is a knockout deletion affecting parts of the intron and exon of the *umps-1* sequence. The mn160

allele is a reference allele of the *umps-1*, meaning that it is the first allele found within the umps-1 gene sequence (WormBase: mn160, n.d.) and (Hartman and Herman, 1982). The *tm3614* allele is a deletion of part of the *spp-1* gene (Figure 3).

Chapter 2

Methods

2.1 – Thrashing Assay

I used thrashing assay to quantify the muscle movement of the *C. elegans*. First, I grew nematodes on *E. coli* OP50 spotted NGM agar plates (0.25% Peptone, 51 mM NaCl, 25 mM [PO4], 5 ug/ml cholesterol, 1 mM CaCl2, 1mM MgCl2, and 2% Agar) at 20°C. Once the nematode is at the fourth larval stage (L4), I then placed it in a 10 µl M9 buffer solution (42 mM Na2HPO4, 22 mM KH2PO4, 1 mM MgSO4, and 86 mM NaCl) on a NGM agar plate. After waiting 1 minute for the nematode to adapt to the change, I counted the number of thrashes (movement of the nematode back and forth as seen in Figure 4) over a time period of 1 minute. A minimum of 30 nematodes was recorded (the RNAi groups and Empty Vector (EV –nematodes fed bacteria with the plasmid without inserted umps-1 or spp-1 gene) control had 30, mn160 had 40, and the rest had 35 nematodes). I did statistical analysis by using t-test to find the p-value.

Figure 4: Thrashing Assay. Picture (a) shows the *C. elegans* in a NMR plate, suspended in M9 solution. Pictures (b) through (e) depict half of a thrash. For this experiment, one thrash is counted when the *C. elegans* moves its body from one side (b), to the other side (e), and then back again (b). This picture is adapted from a journal from Nature Protocols (Ullrich et al., 2014).

2.2 - RNAi

In order to identify which gene contributed to the observed hyperactive muscle phenotype, I used RNAi to silence either the *spp-1* or the *umps-1* gene. First, I added a bacterial colony containing a plasmid with the RNAi target gene and Tet/Carb antibiotics (10 ug/mL Tet and 100 ug/mL Carb concentration, 1:10 of each antibiotic to LB ratio) to lysogeny broth (LB) and incubated it overnight. I then spotted 5 drops of the culture on each NGM agar plates using an electric pipette pump. The plates were left on the bench for 2 days before use. Approximately 5 adult worms were placed on the agar plates. I then collected L4 progeny for thrashing assays or collected the entire plate of worms and used it for RNA isolation in preparation for qRT-PCR.

2.3 – qRT-PCR

I used quantitative RT-PCR (qRT-PCR) to determine the gene expression levels of the *spp-1* and *umps-1* genes in each of the mutant and RNAi treated strains compared to that of the N2 or EV control group. I placed adult nematodes on either *E. coli* spotted NGM agar plates (mutants strains or N2 wild type control strain) or spotted RNAi agar plates (for RNAi groups and EV). After one or two generations, the RNA was extracted from mixed stage nematodes using TRIZOL reagent. For qRT-PCR, 10 µl of 2 µg of RNA was added to 10 µl of 2x RT master mix to produce cDNA which was further purified. 5 µl of a 1:10 diluted purified cDNA was added to 10 µl of Master Mix and quantified using qPCR. I used SYBR Green and three control primers, *cdc-42*, *tba-1*, and *pmp-3*, for comparative ΔCT. The sequence of the three control primer sets are:

- *cdc-42* F: ctgctggacaggaagattacg; R: ctgggacattctcgaatgaag

- *tba-1 F: gtacactccactgatctctgctgaca; R: ctctgtacaagaggcaaacagccatg*
- *pmp-3 F: gttcccgtgttcatcactcat; R: acaccgtcgagaagctgtaga*

The sequence of the *spp-1* primer set is:

- *spp-1* F: ggtgttttctgtgatgtctgc; R: atagtccagcaaagagttccg

The sequence of the *spp-1* primer set is:

- *umps-1 F: gctcgacatgcaaacagttc; R: agctcgctgttgatttccag*

Chapter 3

Results

3.1 – Thrashing Assay

In order to determine the involvement of the two genes in the hyperactive muscle phenotype, I conducted thrashing assays on *umps-1* mutant strains and compared the results to that of the control N2 wild type group. The results showed that *ok2703* allele had a significant increase ($p < 0.0001$) in thrashing rate, while *mn160* allele had a significant decrease ($p < 0.0001$) in thrashing rate compared to that of the N2 group. The other two alleles *zu456* and *tm6379* showed no significant change ($p > 0.05$) (Figure 5).

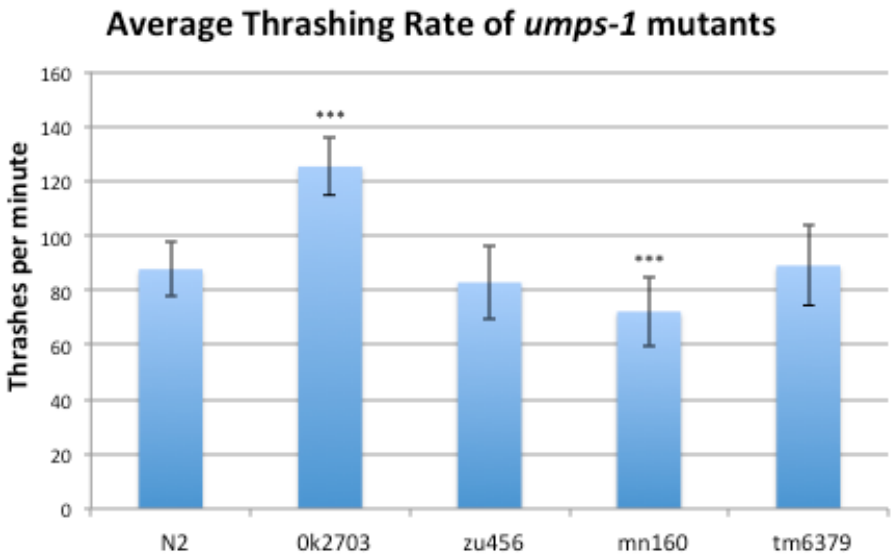

Figure 5: Number of thrashes per minute for *umps-1* mutants of L4 stage nematodes. * is p-value <0.05, ** is p-value 0.01, and * is p-value 0.0001. P-value is calculated using t-test and compared to the N2 average thrashing rate.**

Because *ok2703* deletes part of both *umps-1* and *spp-1*, there is a need to determine the impact of the *spp-1* gene on muscle activity as well. Therefore, I did a thrashing assay on the *tm3614* allele. Both alleles caused a significant increase in thrashing rate ($p<0.0001$) compared to that of the N2 group. However, *tm3614* had a slightly lower thrashing rate than *ok2703* (Figure 6).

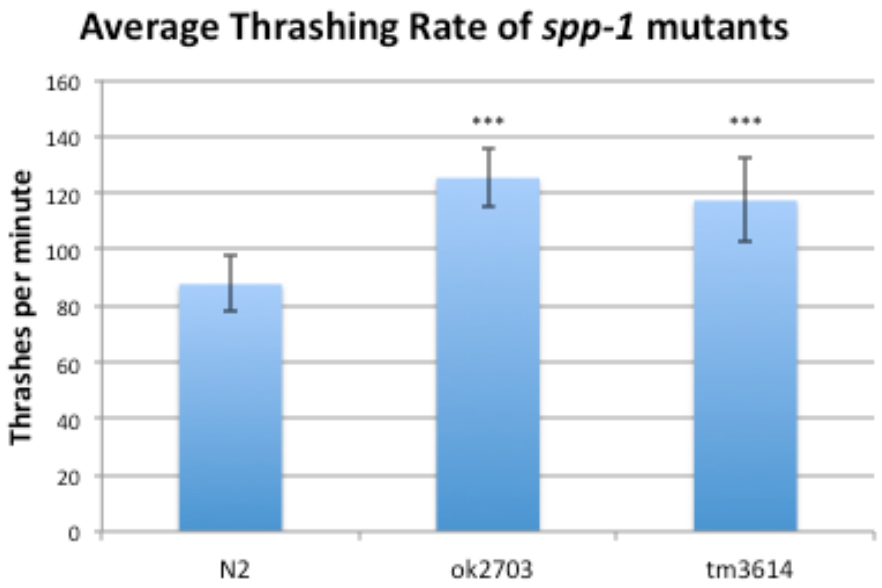

Figure 6: Number of thrashes per minute for *spp-1* mutants for L4 nematodes. * is p-value <0.05, ** is p-value 0.01, and * is p-value 0.0001. P-value is calculated using t-test and compared to the N2 average thrashing rate.**

As another way to determine the contribution of either of the genes to the hyperactive muscle phenotype, I used a gene silencing technique to knockdown either *spp-1* or *umps-1* in a N2 nematode. Only the *umps-1* RNAi group showed a significant increase ($p<0.05$) in thrashing rate compared to the EV control. The *spp-1* RNAi group had no significant impact on thrashing rate (Figure 7).

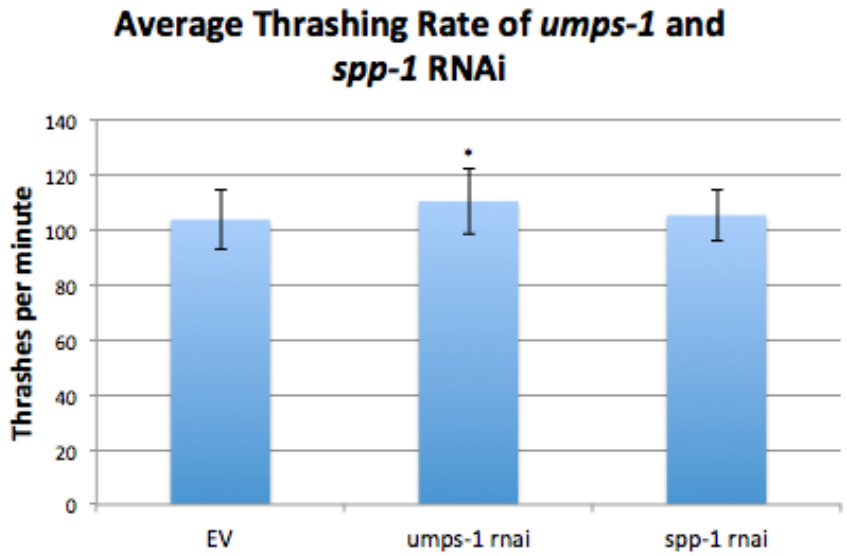

Figure 7: Number of thrashes per minute for *umps-1* and *spp*-1 L4 nematodes. * is p-value <0.05, ** is p-value 0.01, and * is p-value 0.0001. P-value is calculated using t-test and compared to the N2 average thrashing rate.**

3.2 – *ok2703* qPCR Results

In order to understand the impact of the gene mutation in the strains on the actual gene expression levels of *umps-1* and *spp-1*, I used qRT-PCR on RNA extracted from each of the alleles and compared it to that of the N2 control group. Because this was preliminary data, statistics could not be calculated.

The *ok2703* allele, which deletes portions of each gene, has an 84% decrease in *umps-1* expression level compared to that of the N2 wild type control (Figure 8). The primers used for this experiment targets the region within the *ok2703* deletion region, thus rendering it useless. Additionally, the *ok2703* deletes most if not the entire *spp-1* gene. I assume that there is no *spp-1* mRNA or protein present (Figure 3). Therefore, qPCR was not done for the *spp-1* gene expression level for *ok2703*.

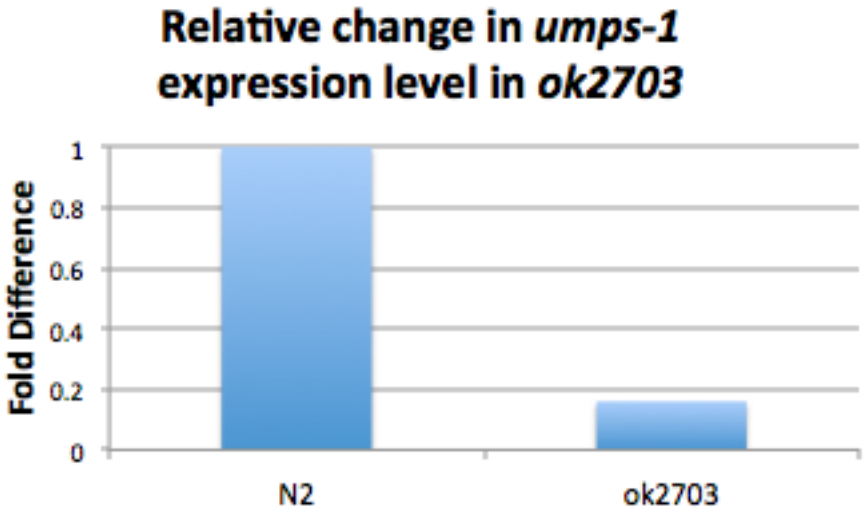

Figure 8: Preliminary data for *ok2703 umps-1* gene expression level compared to that of the N2 group. Fold difference with N2 gene expression levels normalized to 1, calculated using the DDct value of the N2 and *ok2703* for the *umps-1* gene.

3.3 – *tm6379* qPCR Results

The *tm6379* allele, which has a deletion restricted to the region of the *umps-1* gene alone, has a significantly large decrease in *spp-1* expression compared to that of the N2 wild type control (Figure 9a). However, the *umps-1* expression had no change (only 32% decrease) compared to that of the N2 wild type control (Figure 9b).

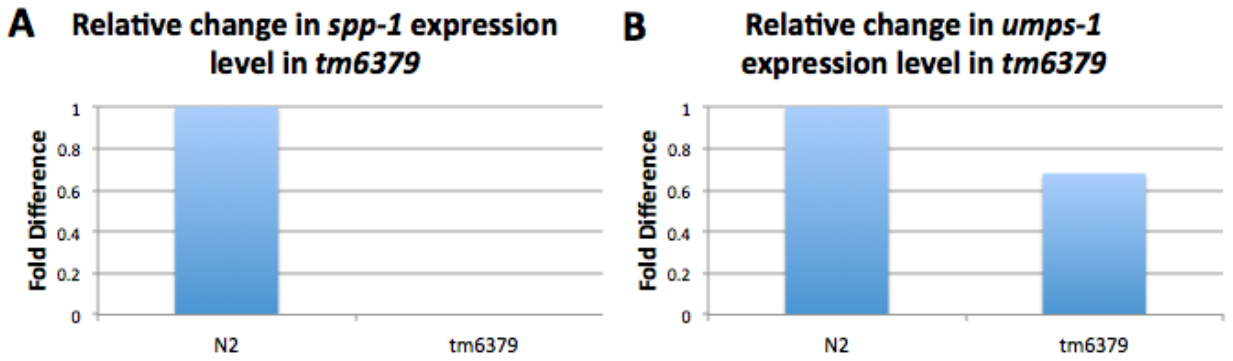

Figure 9: Preliminary data for *tm6379 spp-1* (a) and *umps-1* (b) gene expression level compared to that of the N2 group. Fold difference with N2 gene expression levels normalized to 1, calculated using the DDct value of the N2 and *tm6379* for each gene. Note that in graph a, there is *spp-1* mRNA in *tm6379* but is such a small amount that calculations resulted in a 100% decrease in expression level.

3.4 – *mn160* qPCR Results

The *mn160* allele, which is a reference allele for *umps-1* and whose location is still unknown, has a 87% decrease in *spp-1* expression and a 85% decrease in *umps-1* expression compared to that of the N2 wild type control (Figure 10).

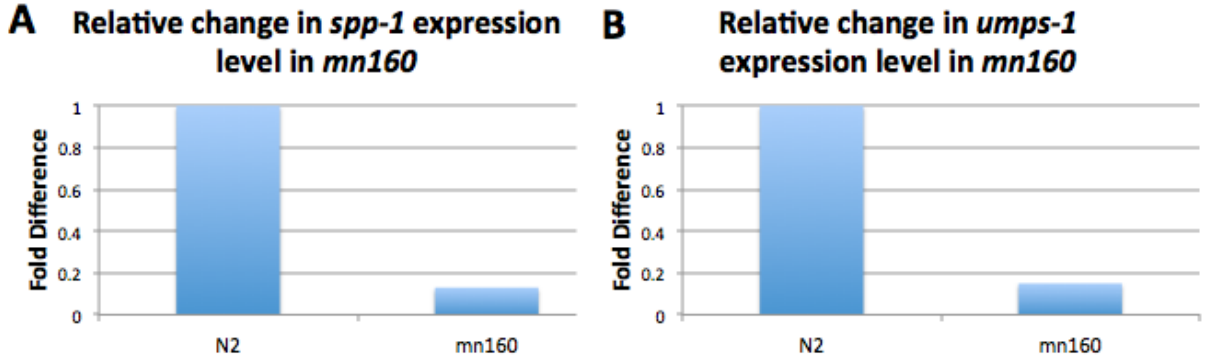

Figure 10: Preliminary data for *mn160 spp-1* (a) and *umps-1* (b) gene expression level compared to that of the N2 group. Fold difference with N2 gene expression levels normalized to 1, calculated using the DDct value of the N2 and *mn160* for each gene.

3.5 – *zu456* qPCR Results

The *zu456* allele, which is a nonsense mutation at the beginning of the *umps-1* sequence, has an approximate 2-fold increase in *spp-1* expression and a 63% decrease in *umps-1* expression compared to that of the N2 wild type control (Figure 11).

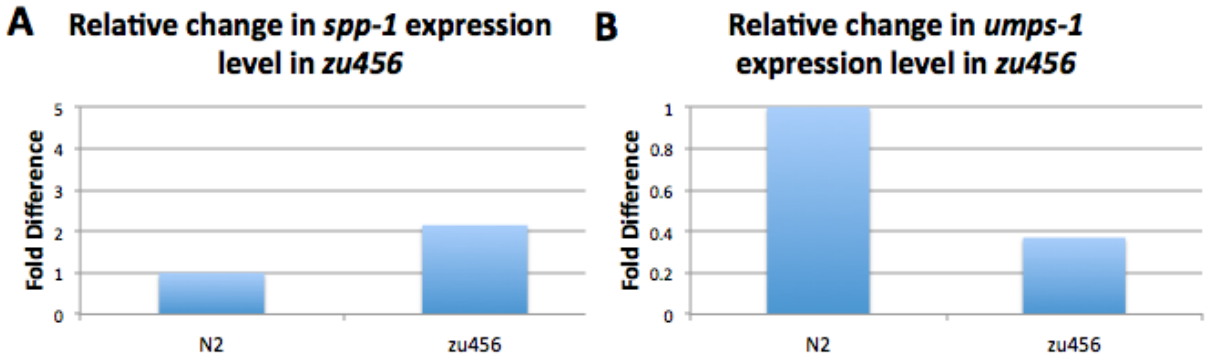

Figure 11: Preliminary data for *zu456 spp-1* (a) and *umps-1* (b) gene expression level compared to that of the N2 group. Fold difference with N2 gene expression levels normalized to 1, calculated using the DDct value of the N2 and *zu456* for each gene.

3.6 – *tm3614* qPCR Results

The *tm3614* allele, which is a deletion restricted to the region of the *spp-1* gene alone, has no change (only 44% decrease) in *umps-1* expression compared to that of the N2 wild type control (Figure 12). For this experiment, the *spp-1* expression level was not found because the *spp-1* primers partially fall within the region of the deletion in this allele (Figure 3). Therefore, any results from qPCR would not be reliable.

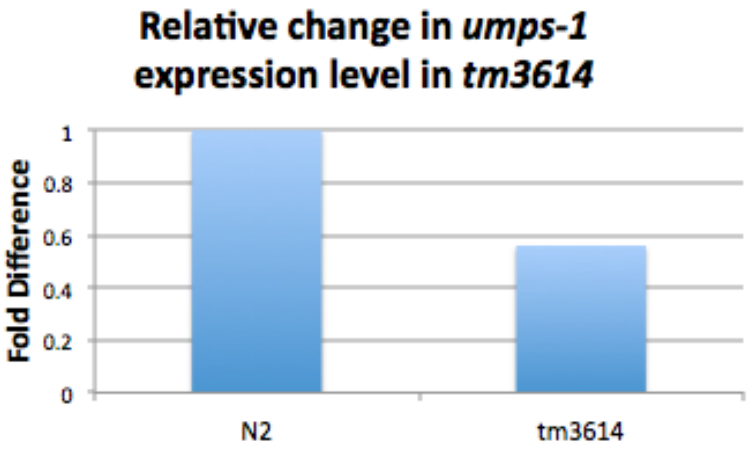

Figure 12: Preliminary data for *tm3614 umps-1* gene expression level compared to that of the N2 group. Fold difference with N2 gene expression levels normalized to 1, calculated using the DDct value of the N2 and *tm3614* for *umps-1*.

3.7 – *spp-1* RNAi qPCR Results

The *spp-1* RNAi has an approximate 9000-fold increase in *spp-1* expression compared to that of the EV empty vector control (Figure 13a). On the other hand, there is no significant change in *umps-1* expression level in the *spp-1* RNAi compared to that of EV empty vector control (Figure 13b).

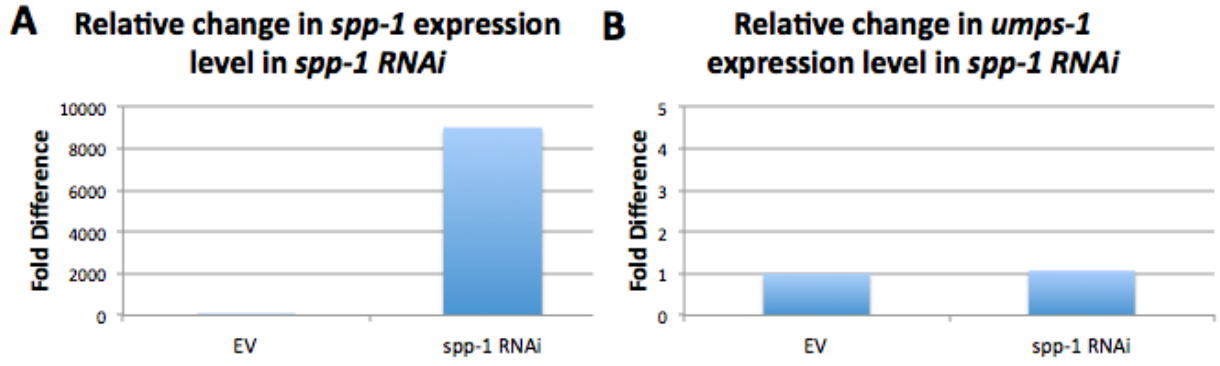

Figure 13: Preliminary data for *spp-1* RNAi compared to that of the EV group, looking at both the *spp-1* (a) and *umps-1* (b) gene expression level. Fold difference with EV gene expression levels normalized to 1, calculated using the DDct value of the EV and *spp-1* RNAi for each gene.

3.8 – *umps-1* RNAi qPCR Results

The *umps-1* RNAi had an 87% decrease in *spp-1* expression compared to that of the EV empty vector control (Figure 14a). On the other hand, there was an approximate 772-fold increase in *umps-1* expression compared to that of the EV empty vector control (Figure 14b).

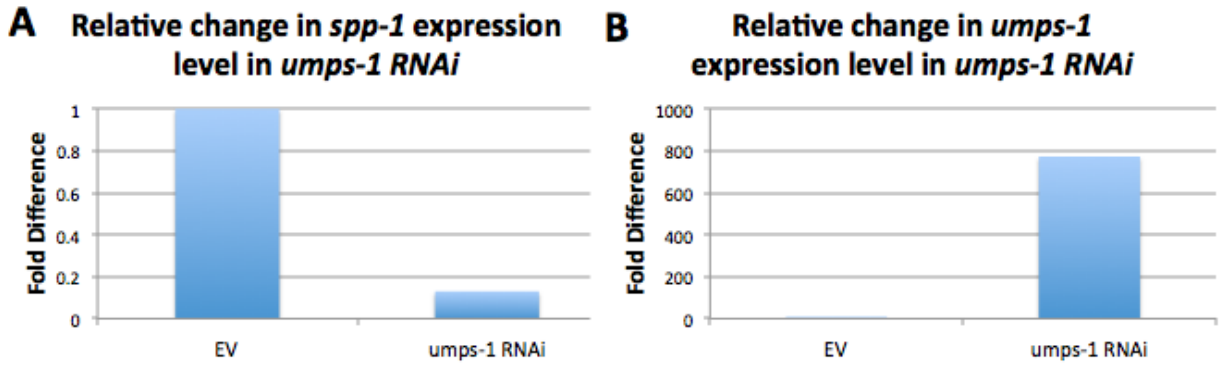

Figure 14: Preliminary data for *umps-1* RNAi compared to that of the EV group looking at both the *spp-1* (a) and *umps-1* (b) gene expression level. Fold difference with EV gene expression levels normalized to 1, calculated using the DDct value of the EV and *umps-1* RNAi for each gene.

3.9 – Table of Overall Results

Figure 15, as seen below, contains the summary of all the results found in this study.

Table 1: Table recording the overall change in thrashing rate, *umps-1* expression level, and *spp-1* expression level of the alleles and RNAi groups compared to that of the N2 wild type or EV empty vector control group. + means that there was an increase. – means that there is a decrease. 0 means that there is no significant change. N/A means that the experiment was not done for that particular group.

	Thrashing Rate	***spp-1* Expression**	***umps-1* Expression**
ok2703	+	N/A	-
tm6379	0	-	0
mn160	-	-	-
zu456	0	+	-
tm3614	+	N/A	0
***spp-1* RNAi**	0	+	0
***umps-1* RNAi**	+	-	+

Chapter 4

Discussion

ok2703 allele led to a significant decrease in the *umps-1* gene expression level and an assumed total decrease of the *spp-1* gene expression level. This allele showed a significant increase in thrashing rate, suggesting that something in the *ok2703* allele is causing an increase in muscle activity.

The *ok2703* data initially suggest that a decrease in both *spp-1* and *umps-1* results in an increase in muscle function. However, the data gathered from the *mn160* allele suggests otherwise. Similar to *ok2703*, *mn160* had a decrease in both the *spp-1* and *umps-1* gene expression level but a significant decrease in muscle function.

tm3614 allele had an unknown change in *spp-1* expression level and no change in *umps-1* expression level. There was an overall increase in thrashing rate for this allele. This data was not similar to that of the *ok2703* data.

umps-1 RNAi showed a significant increase in thrashing rate compared to that of the N2 wild type control. Additionally, there was a decrease in *spp-1* expression and increase in *umps-1* expression level compared to that of the EV empty vector control. This data was not similar to that of the *ok2703* data.

In comparison, there were three groups that had no change in thrashing rate compared to that of the N2 wild type or EV empty vector control. They were: *tm6379, zu456,* and *spp-1 RNAi*. However, there is no similarity between these three groups in terms of the change in *spp-1* or *umps-1* expression level compared to the control. *tm6379* had an increase in *spp-1* expression and no change in *umps-1* expression. *zu456* had an increase in *spp-1* expression and decrease in

umps-1 expression compared to the control. *spp-1* RNAi had an increase in *spp-1* expression and no change in *umps-1* expression compared to the control.

Overall, there was no overall pattern seen between the qPCR and thrashing assay results for the different allele and RNAi groups. There are some possible reasons behind this inconsistency of results, however. One possible explanation is that while the *ok2703* and *mn160* resulted in a decrease in the *spp-1* and *umps-1* mRNA, the *ok2703* produced low levels of a nonfunctional protein while the *mn160* (and potentially other alleles) produced low levels of still active proteins. Because *umps*-1 codes for two different enzymes (Merry et al., 2014), the area in which the mutation occurs may play a role in determining the functionality of the protein. Deletions such as ones in *tm3614* and *tm6379* can either result in an in-frame or out-of-frame peptide. These possibilities, in return, can result in a difference in thrashing rate results. One future experiment would be to try to test the function of the proteins from these alleles.

Another possibility is that the *ok2703* allele has a mutation other than the deletion shown in the gene map provided (Figure 3). If this is the case, the unknown mutation or the combination of the mutations in *ok2703* may have been the contributor to the observed hyperactive muscle activity phenotype. One possible future experiment would be to do crosses with the *C. elegans* to identify if there are any mutations elsewhere in the *C. elegans* chromosomes.

Chapter 5

Conclusion

Overall, the data gathered from this study was inconclusive in identifying which of the two genes (or both) contributed to the observed hyperactive muscle activity phenotype first seen in nematodes with the *ok2703* allele. Possible explanations for such results were discussed and future experiments were suggested. However, it is important to note that due to time-constraints, only preliminary data is collected and presented in this study. Further validation via technical and biological replicates is required.

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Academic Vita

Jane (Yixuan) Wang

Education

Bachelor of Science in Biology - Vertebrate Physiology

- Pennsylvania State University (Main Campus), Schreyer Honors College
- Intend to graduate with honors in Biochemistry and Molecular Biology
- Graduation: May 2018
- Dean's List: Fall 2014 - Fall 2017, all

Advanced coursework: Functional Genomics, Biochemistry, and Molecules and Cells

Research Related Experience

Undergraduate Researcher

Associate Professor Wendy Hanna-Rose's BMB lab Spring 2015 – Present

- Extracted RNA from nematode using RNAi
- Used qRT-PC, thrashing assay, statistical analysis (p-value, t-test), Nanodrop
- Determined the impact of two genes in nematode on hyperactive muscle phenotype

Undergraduate Researcher

Assistant Professor Santhosh Girirajan's BMB lab Fall 2014 – Fall 2015

- Conducted surveys and collected, organized, and analyzed large sets of data from the patients with neurological disorders and their family members
- Attempted to correlate the different phenotypes of the patients and their relatives to their genome

Undergraduate Research Poster Presentation April 2016 and April 2017

Project Proposal Fall 2016 and Fall 2017

Other Activities and Involvement

South Halls Residence Association Diversity Chair Officer Fall 2018

- Planned and implemented events and bulletin boards to increase awareness and respect for people of all cultures, thoughts, and origins.