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## Preliminary Analysis of $\beta$ -Methylamino-L-Alanine Interactions With Cu/Zn Superoxide Dismutase in Amyotrophic Lateral Sclerosis Zebrafish Models

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PRELIMINARY ANALYSIS OF  $\beta$ -METHYLAMINO-L-ALANINE INTERACTIONS  
WITH CU/ZN SUPEROXIDE DISMUTASE IN AMYOTROPHIC LATERAL  
SCLEROSIS ZEBRAFISH MODELS

by

Elizabeth Pflugradt

A Thesis Submitted in Partial Fulfillment  
of the Requirements for a Degree with Honors  
(Molecular and Cellular Biology)

The Honors College

University of Maine

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## Abstract

Amyotrophic Lateral Sclerosis (ALS) is a terminal neurodegenerative disease with symptoms including limb-onset muscle wasting, difficulties swallowing and breathing, leading to death. Sporadic ALS occurs in 90% of patients, while 10% of cases are familial (FALS). Twenty percent of FALS cases are a result of mutation in the copper-zinc superoxide dismutase (*SOD1*) gene, leading to the activation of the mitochondrial apoptosis pathway. Meanwhile, a prominent cause of sporadic ALS is exposure to neurotoxins, such as  $\beta$ -methylamino-L-alanine (BMAA). BMAA has been suggested to induce selective motor neuron death, which is observed in ALS patients. While research has been done to how BMAA may impacts organisms on the cellular level, not much has been performed on the genetic or epigenetic level. This research investigates how BMAA and mutations in the *SOD1* gene affect the regulation of well-known neurological genes. Within the year, zebrafish from four different genotypes were subjected to three different BMAA concentrations, and spinal samples from these fish were collected. RNA was extracted from these and cDNA was produced from the resulting RNA. RNA concentrations varied significantly in concentration and purity, while cDNA concentrations were more pure and higher in concentration. The spinal samples also displayed that fish exposed to 2.5  $\mu\text{g/L}$  of BMAA had the lowest levels of actin transcripts, reflected in previous work that these fish would have the shortest motor neuron axons. These samples can now be used to perform transcriptome analysis to detect the regulation of neurological genes depending on BMAA concentration and *SOD1* mutation.

## Acknowledgements

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## Introduction

### **Amyotrophic Lateral Sclerosis:**

ALS is the fourth most prevalent neurodegenerative disease, following Alzheimer's, Pick's, and Parkinson's disease.<sup>1</sup> Symptoms of ALS typically (70% of cases) start with limb-onset muscle wasting, and lead to difficulties in swallowing, respiratory weakness, unclear speech, depression, and eventually death.<sup>2</sup> Research looking into the association with ALS and depression focuses on the prevalence of depression in ALS patients more often than the biological cause of depression in said patients<sup>3</sup>. While one-fifth of patients live up to five to ten years after symptoms begin, half of all patients diagnosed with ALS die within 30 months of symptom onset.<sup>2</sup> There are no current cures for the disease, although there is one treatment; Riluzole. However, it only prolongs life for patients for a few months.<sup>4</sup> Only between 5-10% of ALS are familial (FALS)<sup>5</sup>, indicating that between 90 and 95% of cases are sporadic (SALS). Of the FALS cases, 20% of them involve mutations in the copper-zinc superoxide dismutase gene (*SOD1*).<sup>6</sup>

SOD1 is an enzyme found in the inner mitochondrial space and the nucleus of cells. It is a dimer made of two identical constitutively expressed polypeptides, bound by hydrophobic interactions. Each polypeptide is 153 amino acids long, and dimerization is stabilized and catalyzed by a zinc and copper ion. Its function is to convert superoxide into hydrogen peroxide.<sup>7,8</sup> *SOD1* mutations, have generally been shown to induce a toxic-gain of function, rather than a loss of function.<sup>9</sup> This toxic gain of function is caused by the aggregation of mutant SOD1 protein, and binding to B-cell lymphoma (Bcl) proteins on the outer membrane of mitochondria.<sup>10</sup>

### **Use of zebrafish as an ALS Model:**

The zebrafish is a useful model for studying ALS because it develops quickly, produces numerous offspring multiple times a day, and is more cost effective than other models such as a mouse or rat. More importantly, the physiology of the zebrafish neuromuscular system is similar to humans. This neuromuscular system develops rapidly, and is well characterized.<sup>12,13,14,15</sup> . Also, since the zebrafish breeds by laying eggs in shallow water, its embryos are externally developed, and absorb any toxins placed into their environment through their derma.<sup>16</sup> . We use several transgenic zebrafish models in our experiment. The models are non-transgenic SOD1 zebrafish littermates (nTG-SOD1-wt), transgenic wildtype SOD1 zebrafish (SOD1-wt), littermates of mutant SOD1 zebrafish (nTG-SOD1-G93R), and transgenic mutant SOD1 zebrafish (SOD1-G93R). Models labeled as transgenic overexpress the SOD1 allele they possess.

### **$\beta$ – Methyamino-L-alanine:**

$\beta$  – Methyamino-L-alanine (BMAA) is a non-proteogenic amino acid discovered in high concentrations (627  $\mu$ g BMAA/g brain tissue) in the brains of the Chamorro people, native to Guam. This is an approximately fifteen times higher concentration than that found in brains of people serving as neurological controls (41  $\mu$ g BMAA/g brain tissue). This is significant because the Chamorro people had a 100x fold higher rate of developing ALS than the average population.<sup>10</sup> The source of the BMAA for the Chamorro is cycad seeds. The cycad plants have a symbiotic relationship with cyanobacteria, which reside in the coralloid roots. The BMAA resulting from the cyanobacteria accumulates into the seeds.<sup>17</sup> The Chamorro people would not only grind

cycad seeds into flour, but would also consume animals which eat cycad seeds mainly flying foxes. Thus, these people can acquire the amino acid through multiple sources of bioaccumulation.<sup>18</sup>

BMAA does not only exist in these plants; it has been found in many fresh and salt water sources, including lakes in the United Kingdom<sup>19</sup> and New Hampshire<sup>20</sup>, and the Baltic Sea<sup>21</sup>. People exposed multiple times to cyanobacterial harmful Algal Blooms (HABs) have between a 10 to 25 times chance of developing ALS than those not near these events.<sup>22</sup> Also, while BMAA is primarily noted in HABs globally,<sup>10</sup> it is also produced by other organism including diatoms<sup>23</sup> and dinoflagellates<sup>24</sup>. Fish and shellfish predate on these organism, providing another avenue for people to be exposed to BMAA through bioaccumulation<sup>10</sup>.

There are multiple mechanisms proposed as to how BMAA affects motor neurons, depending if the amino acid is free floating or incorporated into proteins. When outside of the cell, free-floating BMAA acts as an agonist for glutamate receptors such as NMDA and AMPA/kainate. When introduced to mixed spinal cord culture at 30  $\mu$ M, BMAA was shown to induce selective motor neuron death, demonstrating it as a potential mechanism for ALS development<sup>10</sup>. There are also suggestions that it can be incorporated into proteins, which can cause proteins to become misfolded<sup>25</sup>, and these proteins act as BMAA reservoirs<sup>26</sup>. Since BMAA is a fairly well known neurotoxin that is prevalent worldwide, and has evidence of causing symptoms of ALS, BMAA is the contending neurotoxin used in this research.

### **Experimental Design:**

In a previous experiment in the Sher Lab, performed by Samantha Powers [personal communication], zebrafish with and without the G93R SOD1 mutation were exposed to different concentrations of BMAA. Then the axons of the motor neurons located in the lumbar spines of each fish were measured. Fish without the mutation were observed to have shorter motor neuron axons as the concentration of BMAA exposure increased. Fish with the mutation and exposed to the BMAA had the shortest axons at 2.5 µg/L BMAA concentration, but demonstrated longer axons at higher and lower BMAA concentrations, indicating a recovery mechanism existed to compensate for the short axons. This led into curiosity about how genes were expressed when the fish had the mutation, and when they were exposed to different BMAA concentrations at the start of development.

In past parts of this current experiment, performed by Samantha Powers, the seventy-two zebrafish were distributed evenly into the four genotypes of zebrafish, and were exposed to three different concentrations of BMAA. After six months of living in non-toxic water, their lumbar spinal cords were extracted, frozen in liquid nitrogen, and stored at -80°C.<sup>27</sup> Half of each sample group (3 fish per genotype per dose) were sent for proteomic analysis to be performed by Dr. Randall Moon, University of Washington.<sup>28</sup> The remaining spines, in this current research, went through RNA extraction, cDNA production, and subsequent RNA expression analysis. While this current research was supposed to continue with microarray analysis of the spinal samples within this research, time restraints prevented this from occurring.

**Hypothesis:**

It is known that BMAA has detrimental effects on the phenotype of motor neurons, from selective motor neuron death in cell culture to decreased axon length in motor neurons. From previous research, phenotypic change was observed in the length of motor neuron axons, indicating possible changes in the transcriptome and/or the proteasome. Through the microarray analysis, we wanted to observe the genetic regulation patterns exhibited in zebrafish when exposed to different concentrations in BMAA and when they had the SOD1-G93R mutation. Specifically, the genes involved in neurodevelopment would decrease when exposed to BMAA, and increase in fish with the G93R mutation when exposed to 5.0 µg/L BMAA.

## Materials and Methods

**Spine extraction:** Seventy-two zebrafish of four different genotypes (nTG-SOD1-wt, SOD1-wt, nTG-SOD1-G93R, and SOD-G93R) were exposed to three different doses of BMAA (0.0, 2.5, and 5.0 ug/L). The fish developed for 188 days, and were euthanized with Tricane-S (300mg/L) (Western Chemical INC, Ferndale, WA). Each fish was dissected, and the lumbar region was isolated. Spinal cords were pulled from exposed column. Individual spines were frozen in liquid nitrogen, and stored at -80°C.<sup>27</sup> Half of the samples were sent for proteomics, while the others were saved for RNA extraction.<sup>28</sup>

**RNA extraction:** RNA was individually extracted from 35 zebrafish lumbar spinal cords using a phenol-chloroform protocol. The frozen spines were homogenized using a frozen pestle, and TRIzol (800 µL) and 1mg/mL glycogen (200 µL) were added to each sample. Samples incubated in Phase Lock Gel Tubes (5 Prime, Gaithersburg, MD). Chloroform was added. Samples were centrifuged at 12000xg for twenty minutes at room temperature. The resulting pellets were washed twice, with isopropanol and 75% ethanol, and centrifuged after each wash. Pellets were left to dry, and the RNA were suspended in 15 µL of nuclease-free water. RNA samples were analyzed using a NanoDrop spectrophotometer (NanoDrop 2000; Thermo Scientific; Rochester, NY).

**cDNA production:** cDNA was produced in 20 µL reactions using the Applied Biosystems cDNA Reverse Transcription kit (Applied Biosystems; Carlsbad, CA). Primers used were a 1:1 ratio of Random primers, which came with the kit, and Oligo-DT nucleotides (Thermo Scientific, Waltham, MA). The protocol that utilizes the RNase

inhibitor was followed in the procedure. The amount of RNA added per reaction was dependent on the yield of RNA for any given sample; samples with yields below 1000ng either contained 250 ng (samples 11 and 42) or 500 ng (samples 16, 22, 28, and 35) of RNA, while samples that had yields above 1000 ng contained 1000ng of RNA, in the reactions. cDNA production occurred in the PCR cycler under the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. cDNA samples were analyzed using a NanoDrop spectrophotometer (NanoDrop 2000; Thermo Scientific; Rochester, NY).

**Primer quality control:** The zebrafish primers were tested for quality on AB wildtype cDNA (Table 1). Primers were designed to target mRNA transcripts of both muscle and nervous tissue specific genes observed in the proteomic studies (Integrated DNA technologies, headquarters). 20 µL reactions were run using the protocol with the Taq PCR Master Mix Kit (QIAGEN Inc; Valencia, CA). PCR ran under the following conditions for 39 cycles: 94°C for denaturing, 55°C for annealing, and 72°C for extension. PCR samples were then run in a 2% agarose gel for evaluation of target amplification.

**qPCR:** 50 ng of each cDNA were tested in duplicate to SsoAdvanced Universal SYBR Green Supermix (BioRad Laboratories; Hercules, CA) with actb1 zebrafish primers. qPCR ran under the following conditions for 39 cycles: 95°C for denaturing, and 60°C for annealing and extension. Samples were analyzed with CFX Manager™ Software (BioRad Laboratories; Hercules, CA).

## Results

### **RNA:**

To analyze the transcriptome of the zebrafish samples, RNA must first be collected specifically mRNA. RNA extractions were performed on the individual spines of the zebrafish, then analyzed the RNA samples concentrations and purity using spectrophotometry. Before figuring out the optimal procedure, we tested co-precipitants glycogen and linear acrylamide. RNA samples extracted with glycogen were ten times higher than those extracted with linear acrylamide. The concentration of RNA samples ranged from 26.3 to 725.2 ng/ $\mu$ L. The average concentration of RNA is 334.1 ng/ $\mu$ L (Figure 1A). Purity was measured using the  $A_{260}/A_{280}$  ratio. None of the RNA samples have the optimal ratio of 2.0. Samples between 276 and 426 ng/ $\mu$ L have the highest  $A_{260}/A_{280}$  ratios. The average purity of the RNA samples 1.62 (Figure 2A).

### **cDNA:**

To ensure that the spinal samples would last, cDNA was produced from RNA through reverse transcription. DNA is a more stable molecule than RNA, so conversion of RNA to cDNA would ensure that the samples would be viable when we could analyze them. After reverse transcription, the samples were analyzed using spectrophotometry. The average concentration of the cDNA samples was 2758 ng/ $\mu$ L. There were fewer cDNA samples with significantly lower yields than their counterparts, as there were fewer cDNA samples with significantly lower yields than their counterparts, as there was only one sample that was below 1500 ng/ $\mu$ L (54) and five samples that were above 4000 ng/ $\mu$ L (53, 59, 65, 66, and 72) (Figure 1B). The  $A_{260}/A_{280}$  of the cDNA samples,

measuring purity, are approximately 1.8 for samples between 1928 and 3200 ng/ $\mu$ L. The  $A_{260}/A_{280}$  of these samples range from 1.67 to 1.82, with an average of 1.77 (Figure 2B).

### **qPCR:**

Before samples could go through transcriptome analysis, they must first be tested to make sure they are compatible with the SYBR master mix that will be used in said analysis. This was done by testing 50 ng of cDNA from each of all thirty-five samples with the same primer pair, *actb1*, and checked for consistent amplification. However, variation of the levels of *actb1*, or *actin*, transcripts were observed between samples. The average relative fluorescent unit (RFU) measurement was 2025 RFU. Samples with the G93R mutation had higher RFU measurements than their wildtype counterparts (Figure 3A). Samples exposed to 2.5  $\mu$ g/L of BMAA had lower RFU measurements than other samples (Figure 3B).

## Discussion

Overall, the results illustrated several important points about the protocols involved collecting samples for transcriptome analysis. Even when RNA samples were extracted with glycogen as the co-precipitant, the concentration of samples varied significantly, with less than optimal purity. These samples, when converted to cDNA, increased in yield and purity. When samples were tested for compatibility with the SYBR master mix, it was found that there was variation in *actb1* levels between different samples.

Several modifications had to be made to the original RNA extraction procedure to increase the RNA yield as well as the purity of each RNA samples. The modification that increased the yield of RNA was changing the co-precipitant from linear acrylamide to glycogen. The changes made to protocol to make the samples purer were doubling the time samples were centrifuged, and increasing the temperature of centrifuging samples from 4°C to room temperature.

. When testing RNA extraction methods using linear acrylamide, it was noticed that the RNA yields were averaging less than 50 ng/μL, so less than half of the minimum yield required to use in future experiments. We looked up some similar extraction procedures that successfully used glycogen instead of linear acrylamide as the co-precipitant.<sup>29</sup> From this, it was hypothesized that using glycogen as a co-precipitant would increase the RNA yield more than linear acrylamide. When RNA from wildtype AB zebrafish was extracted with both 250x linear acrylamide, and 1 mg/ mL glycogen, the samples extracted with glycogen exhibited higher yields. However, a possible reason why there might be a higher yield with glycogen than with linear acrylamide is because

the glycogen is added in with the TRIzol reagent before the chloroform step. This is the opposite of using linear acrylamide, as linear acrylamide is added after the chloroform in procedures that use that co-precipitant. By providing the glycogen more time to contact the RNA, it increased the odds of the glycogen pelleting the RNA.

Next, it was thought that the purity of RNA samples would increase if the centrifuging stages in the extraction procedure were performed for double the time at 25°C than the time suggested at 4°C. This was from consultation with Dr. Roger Sher [personal communication], which suggested that the salts from the TRIzol were precipitating into the RNA pellet when centrifuging the samples at 4°C. At room temperature, it was thought that these salts would not precipitate, thus increasing the purity of the RNA samples. This was in an attempt to prevent the salts from the TRIzol reagent from precipitating into the pellet, which had been previously seen to contaminate the RNA samples. While samples were still not found to be at the optimal purity, they were sufficiently purified for the RNA to be produced into cDNA for the purposes of expression analysis.

With the thirty-five RNA samples extracted, samples varied in overall yield. This is contradictory to how the procedure was predicted to work, as a similar amount of RNA should come from similar sample sizes. The main source of error from the extraction procedure is most likely human error. This is due to multiple people with different levels of experience performing the procedure involved in the collection of RNA samples (Figure 1A).

There is a noticeable trend that samples with relatively low or high RNA have lower  $A_{260}/A_{280}$  ratios than samples with more average concentrations (Figure 2A). While

low  $A_{260}/A_{230}$  ratios were also observed, they were expected to be low for two reasons; glycogen was being used as the co-precipitant, and the main component of TRIzol is phenol. Residual phenol and glycogen are both known to lower the purity of nucleic acid samples, especially lowering  $A_{260}/A_{230}$  ratios.<sup>30</sup> Due to the very low  $A_{260}/A_{230}$  ratios, and the relatively normal  $A_{260}/A_{280}$  ratios, the  $A_{260}/A_{280}$  ratio was considered the more accurate measurement of purity. There was consideration to precipitate RNA samples with poorer purity and rewash samples, but this additional procedure would decrease the yield enough that it would be insufficient for producing cDNA from.

The variation of RNA concentrations between the samples was a concern for the next step of the procedure, cDNA production. According to the protocol that came with the Applied Biosystems cDNA Reverse Transcription kit (Applied Biosystems; Carlsbad, CA), 1  $\mu\text{g}$  of RNA was required to produce cDNA. For samples with low yields, 250 ng (11 and 42) or 500 ng (16, 22, 28, and 35) of RNA were used (Figure 1A). However, the resulting cDNA concentrations from these samples were sufficient for future analysis, even with smaller yields than other samples. All of the cDNA samples that were of a high concentration (greater than 3000 ng/ $\mu\text{L}$ ) came from samples in which 1  $\mu\text{g}$  of the sample's RNA was added to the reaction mixture (10, 17, 18, 23, 34, 40, 48, 53, 66, 67, and 72) (Figure 1B). These results show that a substantial amount of cDNA can be produced with a quarter of the RNA stated the protocol, but not necessarily an optimal amount. This is good to know for producing cDNA samples from very small pieces of tissue in the future. Overall, enough cDNA was produced to perform qPCR analysis on all of the sample.

The purity of the cDNA samples was more optimal than the purity of the RNA samples. Unlike the RNA, the optimal  $A_{260}/A_{280}$  ratio for cDNA is 1.8. Most of the cDNA samples have an approximate ratio of 1.8. Samples with cDNA concentrations higher than 3500 ng/ $\mu$ L are less pure, with  $A_{260}/A_{280}$  ratios between 1.6 and 1.8 (Figure 2B). These cDNA samples are more pure than the RNA counterparts because it is suspected that there is less of the residual phenol in the samples. Not all of the RNA is necessary for most of the cDNA samples, so when the small amount of RNA is transferred to the reaction mixture, only some of the contaminants come with it. The small amount of contaminant dilutes into the 20  $\mu$ L reaction mixture, and thus has less of an effect on the purity of the resulting cDNA sample.

Once all the samples were collected, it was hypothesized that the gene for actin could be used as a housekeeping gene for qPCR quality control. This idea stems from the fact that actin filaments comprise of globular actin, and these filaments are part of the cytoskeletons on all cell types, not just neurons. While the research did suggest that glycogen increased the RNA yield of samples, and purity did increase when the samples were centrifuged at 25°C, the levels of actin transcripts varied among samples (lowest for samples exposed to 2.5  $\mu$ g/L BMAA.) Since this is a constitutive gene, it is expected that all samples should have similar relative fluorescent unit (RFU) measurements, since the same amount of cDNA was added into each reaction. As can be seen in the results, the measurements vary greatly. The measurements ranged from 75 to 3655 RFU. To observe whether these difference could be due to one of the experimental variables, the samples were color coded by genotype and BMAA exposure. There were significant differences between the RFUs of genotype groups, displaying greater RFU averages for transgenic

samples than non-transgenic samples, and samples with the G93R mutation have higher RFU's than those without the mutation (Figure 3A). More analysis will have to go into how a constitutive gene is influenced by the amount of wild type SOD1-wt transcript and the G93R mutation, since these results are contradictory to what was expected. There is also a significant decrease of actin transcript when samples were exposed to 2.5 ug/L of BMAA as opposed to 0.0 ug/L and 5.0 ug/L (Figure 3B). This result is understandable, because previous studies in this lab have shown that the length of motor neuron axons decrease the most when zebrafish are exposed to a dose of 2.5  $\mu$ g/L of BMAA. Actin is a prominent cytoskeletal protein found in actin filaments, especially in the axon, so it makes sense that there would be less actin transcripts in the neurons of fish exposed to this dosage of BMAA.

For future directions, there are multiple methods of studying the cDNA samples that we have collected. First, if possible, there should be an alternative housekeeping gene found to be used as a control. This way, we can observe the levels of non-constitutive genes in comparison. While qPCR analysis was attempted with *gapdh* primers before *actb1* primers were used, it was observed that the reverse primers showed amplification in a negative control without cDNA added. The most likely possibility of this observation is contamination. New primers can be designed and ordered for *gapdh*, and tested again to observe their levels in these samples. After a second useful control primer is found, the next step will be to test each cDNA sample with the ten primers mentioned in table 1. This way, we can observe basic upregulation and downregulation patterns of target neurological and muscle genes. These results would help scientists look at genetic regulation when it comes to exposure of BMAA, the G93R mutation, the

overexpression of the wildtype and SOD1 genes, and a combination of these factors.

Also, we can perform microarray analysis using twelve 96 well plates from Bar Harbor Biotechnology containing 94 genes involved with neurotoxicity and neurodegeneration (and two control genes). These plates have a primer pair for distinct neurological gene in each well. Each plate would be used for a sample type with a genotype and an exposure of BMAA. For example, one plate would have cDNA from a sample(s) from non-transgenic SOD-wt littermates exposed to 5.0  $\mu\text{g/L}$  of BMAA during early development. This way, we can get a comprehensive look at genetic regulation of neurological genes depending on these conditions.

## Conclusion

This research provides several conclusions that are useful for future research. From modifying the procedure, it is demonstrated that not only using glycogen as a co-precipitant for extraction increases the RNA yield of a sample, but also that centrifuging the samples at 25°C for double the recommended time increases the purity of the RNA samples. These observations will be important for extracting RNA from nervous tissue in the future. The data also verifies the short axons measured in Samantha Power's work; a correlation can be made between the fewer actin transcripts in the cell, and the shorter length of the axons. This means that *actb1* is not a good gene to analyze if looking for a good housekeeping gene in the near future. It is also an indicator that the samples used in this experiment are consistent with zebrafish with the same genotypes used in previous experiments. Overall, the samples collected through this research are pure enough, and are at a high enough concentration, that they can be used for future transcriptome analysis, using microarrays to perform said analysis. The transcriptome analysis can then be compared with the performed proteomics. This information will then contribute toward the summation information toward how neurotoxins in the environment can influence the development of ALS, and thus lead to possible treatments for the disease.

Tables and Figures

Primer Pair Number	Gene Target	Forward Primer Sequence	Reverse Primer Sequence
1	actb1	5' - TCC TCC CAC CAT GAA GAT CA - 3'	5' - CTT GCT GAT CCA CAT CTG CT - 3'
2	aldoaq	5' - CAT CCT TGC TGA TGA GT - 3'	5' - TAC AAC CTC CGG TTC TCC TC - 3'
3	chata	5' - ATT GCC AAG GAG CTG AAA CT - 3'	5' - GAC TGG ACC ATA GCA GCA GA - 3'
4	gapdh	5' - GCT GGT ATT GCT CTC AAC GA - 3'	5' - CTA CTC CTT GGA GGC CAT GT - 3'
5	myhb	5' - CCA TGA TCA GTC AGG GTG AG - 3'	5' - AAG CCC AGA ATA TCA ATG GC - 3'
6	myh2	5' - CAA CAA GAA GGC CAG TGA GA - 3'	5' - TCA AGG GTA CCC AGA AGA CC - 3'
7	spg20b	5' - ACC TGA CCA TTT CCT ATG GC - 3'	5' - CCA GAC TAC TCC CAG CAG GT - 3'
8	tpm1	5' - GCC GTA GGA ACC TTG ACA TT - 3'	5' - AGG GCA CCA TCA AAC CTA AG - 3'
9	tubla	5' - GGA GGA CAG GAG CCA ACA GT - 3'	5' - GAG CTT CTC TTC GAC GCT CT - 3'
10	vars	5' - GGT AAC GTC ATC GAC CCT CT - 3'	5' - GTC GGA TTT CTG TCC CTG TT - 3'

Table 1. Zebrafish primers that target muscle and nervous system related genes.

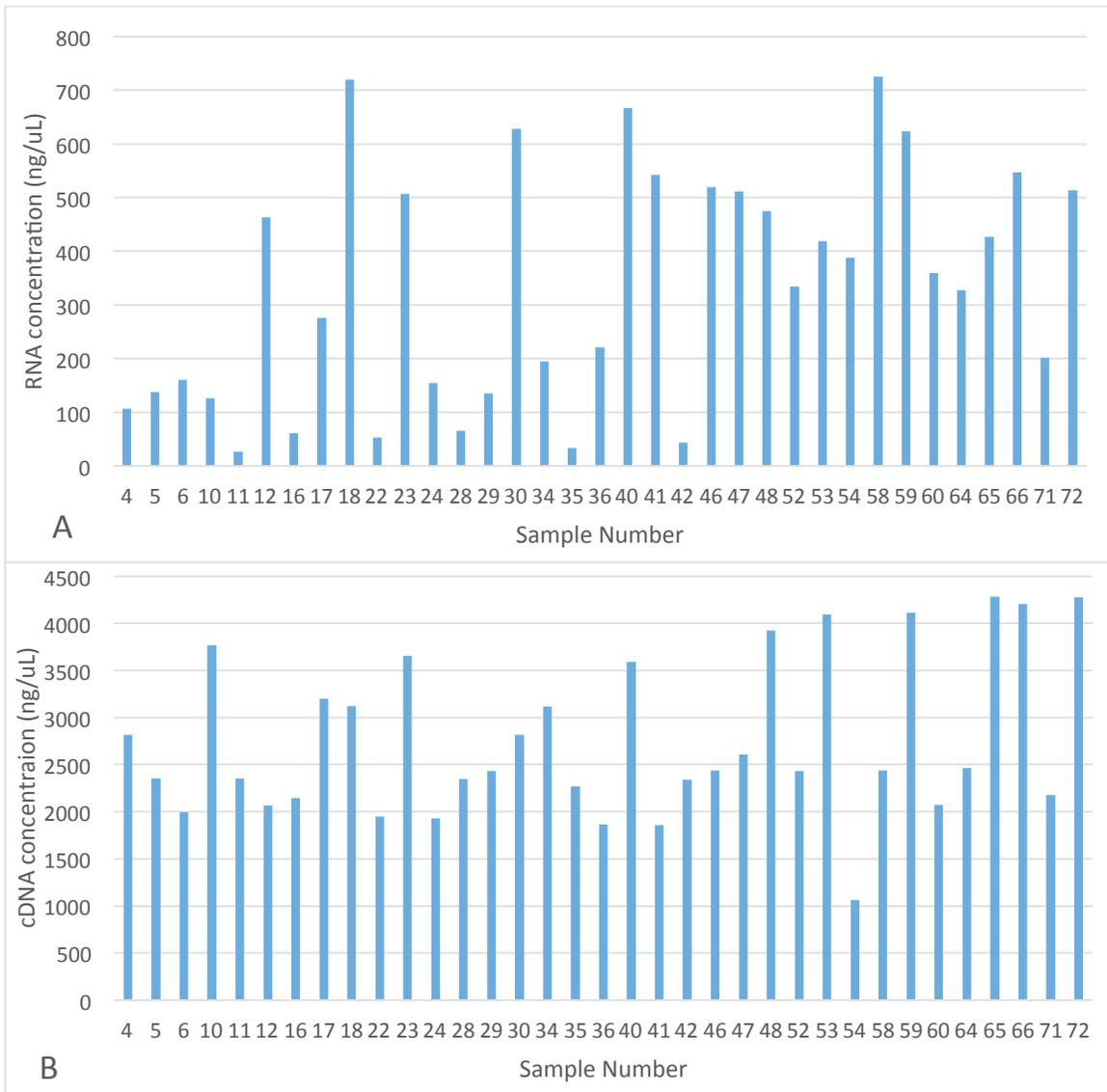


Figure 1. The concentration (ng/μL) for A) RNA samples and B) cDNA products, varies from sample to sample.

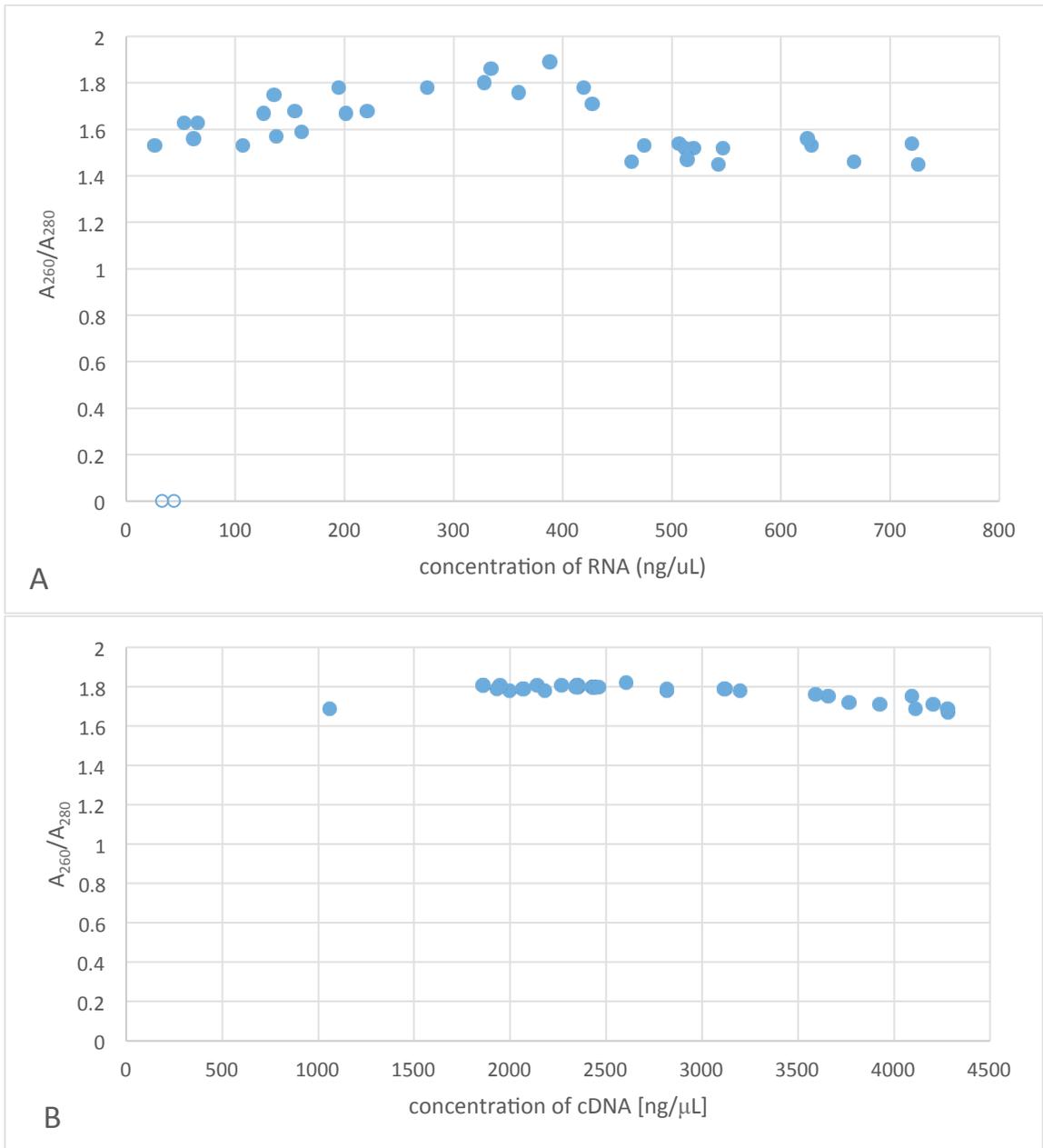


Figure 2.  $A_{260}/A_{280}$  ratios are dependent on the nucleic acid concentration. A)  $A_{260}/A_{280}$  ratio dependent on RNA concentration (ng/ $\mu$ L). B)  $A_{260}/A_{280}$  ratio dependent on cDNA concentration (ng/ $\mu$ L).

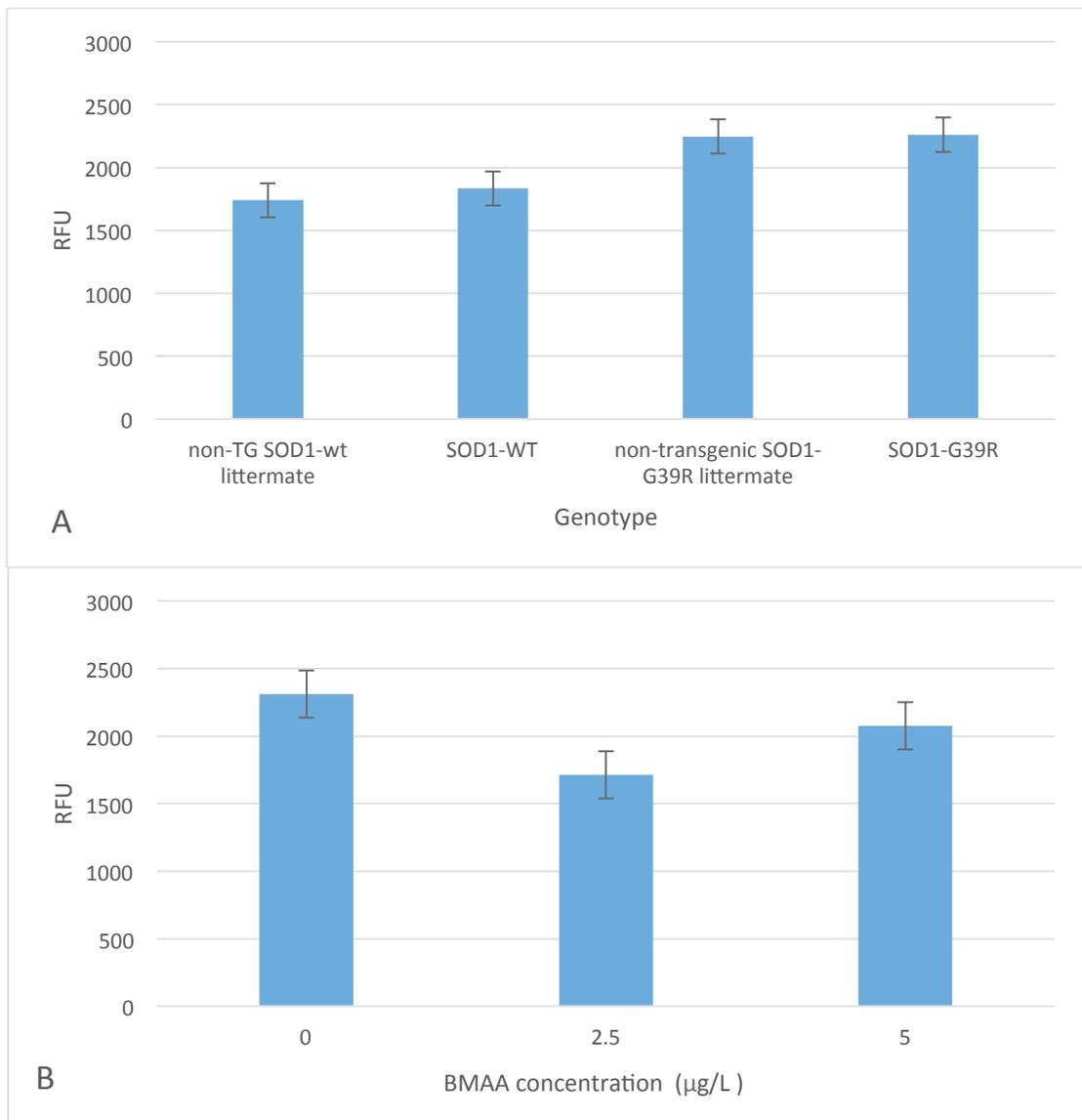


Figure 3. RFU measurements of *actb1* transcripts are different depending on genotype and BMAA concentration zebrafish were exposed to. A) RFU depending on genotype. B) RFU depending on BMAA concentration.

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Appendix: IACUC Approval Letter

UNIVERSITY OF MAINE  
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE 581-1498

**PLEASE DISPLAY ON OR NEAR ANIMAL CAGE**

INVESTIGATOR: Sher, Roger  
TITLE OF PROTOCOL: Effects of Environmental Toxicants on  
Amyotrophic  
PROTOCOL NUMBER: Lateral Sclerosis Zebrafish Models  
APPROVAL PERIOD: A2014-03-02  
4/4/2014 - 4/3/2017

SPECIES	# APPROVED	LOCATION
Zebrafish Adults	3600	Hitchner Hall, Rm 180 (Zebrafish Facility)

UNIVERSITY OF MAINE  
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**PLEASE DISPLAY ON OR NEAR ANIMAL CAGE**

INVESTIGATOR: Sher, Roger  
TITLE OF PROTOCOL: Effects of Environmental Toxicants on Amyotrophic Lateral Sclerosis  
Zebrafish Models  
PROTOCOL NUMBER: A2014-03-02  
APPROVAL PERIOD: 4/4/2014 - 4/3/2017

SPECIES	# APPROVED	LOCATION
Zebrafish embryos/larvae	4896	Hitchner Hall, Rm 310

### Author's Biography

Elizabeth Pflugradt was born and raised in New Gloucester, Maine on August 11, 1994. She graduated from Gray-New Gloucester High School on June 9, 2012 in the top ten percent of her class. She majors in Molecular and Cellular Biology, and minors in Mathematics. She is a member of Phi Beta Kappa, and a recipient of the Mary Rumpho Kennedy Scholarship.

After graduation in May, 2016, she plans on attending the PhD. program at the University of Connecticut in Molecular and Cellular Biology. In the future, she hopes to be conducting genetic research in her own laboratory.