Chronic Low Dose Toxicity of Acrolein in *C. elegans* Mitochondria

By

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Abstract:

Acrolein (ACR) is a chemical of interest in the pathology and progression of mitochondrial disease and disorder. Chemical exposure to ACR may occur exogenously due to the use of herbicides or burning of fossil fuels, or endogenously as a by-product from various metabolic pathways. Several studies focus on the impact of high dose acute exposure of ACR with various model organisms, including live rats and mice, human spermatozoa, and several cell culture models. These models do not address the consequences of long term or chronic, low dose exposures and impacts on progeny. For these reasons, the model *C. elegans* was ideal because of its established genome, ability to self-fertilize or sexually reproduce, ability to yield three hundred progenies in its lifetime, and its short and understood lifespan. We hypothesized that *C. elegans* would experience mitochondrial dysfunction when chronically exposed to low doses of ACR, particularly with the 15 μM concentration. Based on previous research indicating another electrophilic aldehyde, 4-hydroxynonenal (4-HNE) inhibits SDH activity by forming a protein adduct with the redox subunit of SDH, we predicted that ACR may inhibit complex II (SDH) activity through protein adduct formation. We also predicted that ACR may also decrease lifespan and fecundity across generations of nematodes. To test our hypothesis and aims, we utilized *C. elegans* in experiments incorporating lifespan and fecundity assays, performed a histochemical stain to qualitatively measure SDH activity, used RT-PCR targeting specific genes

related to oxidative stress and SDHA-1, and attempted to isolate mitochondria to perform SDH activity assays measured with spectrophotometry. Our results from the lifespan and fecundity assays suggested that low dose, chronic ACR exposure may decrease lifespan and fecundity in *C. elegans*. We also learned that *C. elegans* will attempt to leave the agar plate at ACR concentrations of 75 μM and 100 μM. Our RT-PCR results showed an upregulation in SDHA-1 and SOD-3 transcription in *C. elegans* after a 24-hour exposure to 100 μM ACR. These results suggest that higher doses of ACR may cause oxidative stress or damage to the mitochondria and SDH. Further experimentation may help better determine the interaction between ACR and the flavonoid subunit of SDH.

CHRONIC LOW DOSE TOXICITY OF ACROLEIN IN *C. ELEGANS* MITOCHONDRIA

A Thesis

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In Partial Fulfillment of the Requirements for the Degree

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Table of Contents

List of Tables……..….... Pg vi

List of Figures…….....… Pg vii

List of Abbreviations……...……..……..….... Pg viii

List of Graphs……..……..…….……...…..... Pg xi

Chapter I: Introduction

1.1. Background Information……. Pg 1

1.2. Previous Research with Acrolein Toxicity or SDH Health……..... Pg 8

1.2.A. Aitken et al.'s study: Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase……... Pg 8

1.2.B. Woodhouse et al.'s study: Mitochondrial succinate dehydrogenase function is essential for sperm motility and male fertility……..… Pg 11

1.2.C. Hong et al.'s study: Acrolein promotes aging and oxidative stress via the stress response factor DAF-16/FOXO in Caenorhabditis elegans……..…. Pg 13

1.2.D. Simmon's Thesis: Developing alternatives to methyl bromide: A focus on acrolein (2-propenal)…….…. Pg 14

1.3. Knowledge Gap…… Pg 15

1.4. C. elegans as the Model Organism……... Pg 16

1.5. Hypothesis and Aims……. Pg 17

Chapter II: Methods and Materials

2.1. *C. elegans maintenance*…… Pg 20

2.2. *Acrolein Treatment*……..... Pg 21

2.3. *Sexual Reproduction and Hermaphroditic Reproduction Fecundity Assays of Worms Exposed to Chronic, Low Dose Acrolein*…… Pg 25

2.4. *Lifespan Assays of Worms Exposed to Chronic, Low Dose Acrolein*……... Pg 26

2.5. *SDH Activity Histochemical Stain*……..... Pg 27

2.6. *Spectrophotometric Assay of SDH*…… Pg 28

2.7. *Reverse Transcription Polymerase Chain Reaction (RT-PCR)*…… Pg 29

2.8. *Statistical Analysis*…… Pg 30

Chapter III: Results

1. *Lifespan Assays*

3.1.1. Trial One Lifespan Assay……... Pg 31

3.1.2. Trial Two Lifespan Assay……... Pg 35

3.1.3. Trial Three Lifespan Assay…… Pg 36

2. *Fecundity Assays*

3.2.1.A. Trial One Sexual Reproduction Fecundity Assays……. Pg 37

3.2.1.B. Trial One Hermaphroditic Reproduction Fecundity Assays…….. Pg 38

3.2.2.A. Trial Two Sexual Reproduction Fecundity Assays……..... Pg 40

3.2.2.B. Trial Two Hermaphroditic Reproduction Fecundity Assays…….. Pg 41

3.2.3.A. Trial Three Sexual Reproduction Fecundity Assays……... Pg 42

3.2.3.B. Trial Three Hermaphroditic Reproduction Fecundity Assays…… Pg 43

3. *SDH Activity Histochemical Stain*……..…. Pg 45

4. *Spectrophotometric SDH Activity Assay*…….. Pg 46

5. *Real-Time Polymerase Chain Reaction (RT-PCR) Results*……. Pg 48

Chapter IV: Discussion

1. *Lifespan Assays*……..... Pg 52

2. *Fecundity Assays*…….… Pg 54

3. *SDH Activity Histochemical Stain*…….. Pg 54

4. *Real-Time Polymerase Chain Reaction (RT-PCR) Results*……. Pg 55

5. *Spectrophotometric SDH Activity Assay*…….. Pg 57

Chapter V: Future Directions……... Pg 60

References……... Pg 62

List of Tables

1. The forward and reverse primers used for the genes SDHA-1, CTL-1, CTL-2, and SOD-3 RT-PCR reaction tubes……. Pg 29

2. Impacts of low dose chronic ACR exposure on *C. elegans* fecundity from trial one…..… Pg 43

3. Impacts of low dose chronic ACR exposure on C. elegans fecundity overview of all three trials……..……..… Pg 44

4. Absorbance table from spectrophotometric SDH activity assay results……..… Pg 46

List of Figures

1. Cartoon depiction of the electron transport chain …….....Pg 2

2. Modes of action of nonselective herbicides that impact mitochondrial health……..... Pg 3

3. Cartoon of acrolein DNA adduct formation mechanism…….....Pg 4

4. Cartoon of acrolein protein adduct formation via Michael's addition…….....Pg 5

5. Mitochondrial dynamics depicting fusion and fission, along with proteins involved in both steps……....Pg 7

6. 2-D structures of 4-hydroxynonenal and acrolein…….....Pg 8

7. Western Blot results confirming a 4-HNE and SDHA protein adduct formation …….....Pg 10

8. Polyunsaturated fatty acid undergoing lipid peroxidation, yielding 4-HNE, and 4-HNE's biochemical reactions to protein and lipids……..Pg 11

9. Ribbon model of *C. elegans* succinate dehydrogenase and all four subunits (A-D) generated via I-TASSER software……....Pg 12

10. Dissecting microscope image of *C. elegans* attempting to leave the MYOB agar plate at 75 μM ACR concentration…….Pg 51

List of Abbreviations Used (In Alphabetical Order)

4-HNE: 4-hydroxynonenal

ACR: acrolein

ATP: adenosine triphosphate

CDC: Center for Disease Control

cDNA: complementary DNA

C. elegans: *Caenorhabditis elegans*

CoQ: coenzyme Q

COX: Cytochrome C Oxidase

CTL-(1,2): catalase 1 or 2

DAF-16: dauer formation-16 gene

DNA: deoxynucleic acid

EPA: Environmental Protection Agency

ETC: electron transport chain

FAD: flavin adenine dinucleotide

FDA: Food and Drug Administration

FOXO: Fork head box protein O1

GFP: green fluorescent protein

HSP: heat shock protein

HSR: heat shock response

IMM: inner mitochondrial membrane

JC-1: 5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimi-dazoylcarbocyanine iodide dye

L1-L4: larval stage 1-4

MDA: malondialdehyde

mtDNA: mitochondrial DNA

mtUPR: mitochondrial unfolded protein response

MYOB: Modified Youngren's Only Bactone-Peptone agar media

$NADH_2$: nicotinamide adenine dinucleotide

NBT: nitro blue tetrazolium

nDNA: genomic DNA

NGM: nematode growth media

OMM: outer mitochondrial membrane

OP-50: *Escherichia coli* bacterial strain

Redox: reductive-oxidative

RET: reverse electron transport

RNA: ribonucleic acid

ROS: reactive oxygen species

rt-PCR: reverse transcription polymerase chain reaction

SDH: succinate dehydrogenase

SDHA: succinate dehydrogenase subunit A

SOD-(1,2,3): superoxide dismutase 1, 2, or 3

TCA: citric acid cycle

List of Graphs

1. Chronic, low dose acrolein exposure may act as a worm repellent, or coupled with heat stress may decrease the lifespan of *C. elegans*…….. Pg 30

2. Worm survival after malfunctioning incubator issues were resolved during trial one…… Pg 33

3. Chronic 15 μM ACR exposure does not impact lifespan in *C. elegans* during trial two… Pg 34

4. Chronic 15 μM ACR exposure does not impact C. elegans lifespan during trial three…... Pg 35

5. Low dose ACR exposure and possible heat stress may decrease fecundity in *C. elegans* sexual reproduction in trial one…….. Pg 36

6. Low dose ACR exposure and possible heat stress may decrease fecundity in *C. elegans* hermaphroditic self-reproduction in trial one…….. Pg 38

7. Chronic 15 μM ACR exposure decreases fecundity in C. elegans sexual reproduction in trial two……... Pg 39

8. Chronic 15 μM ACR exposure does not impact hermaphroditic reproduction in C. elegans for trial two…… Pg 40

9. Chronic 15 μM ACR exposure decreases fecundity in C. elegans sexual reproduction in trial three……. Pg 41

10. Chronic 15 μM ACR exposure decreases fecundity in C. elegans hermaphroditic reproduction in trial three…… Pg 42

11. SDH activity decreases as ACR concentration increases with the histochemical stain…. Pg 45

12. SDHA-1 is upregulated in ACR treated worms compared to untreated worms…… Pg 47

13. There was no downregulation of CTL-1 in ACR treated worms compared to untreated worms…….. Pg 48

14. There was no downregulation of CTL-2 in ACR treated worms compared to untreated worms…….. Pg 49

15. SOD-3 is upregulated in ACR treated worms compared to untreated worms……... Pg 50

Chapter I. Introduction

1.1 Background Information

Mitochondria hold critical roles in an organism's life, being at the crux of various metabolic pathways and being the second organelle to contain heritable DNA. The most important function of mitochondria is the generation of ATP. ATP synthesis in eukaryotes involves oxidative phosphorylation, which incorporates enzymes and protein complexes located within the mitochondria involved with the citric acid cycle (TCA) and electron transport chain (ETC), and both metabolic reactions are depicted in figure 1. Disruption of mitochondrial function may lead to devastating consequences to an organism, such as an imbalance in ATP generation or progression into mitochondrial disorders. (Gonzalez-Hunt et al., 2016; Meyer et al., 2013; Jovaisaite et al., 2014; Tilokani et al., 2018; Bora et al., 2013; Zheng. H, et al., 2021)

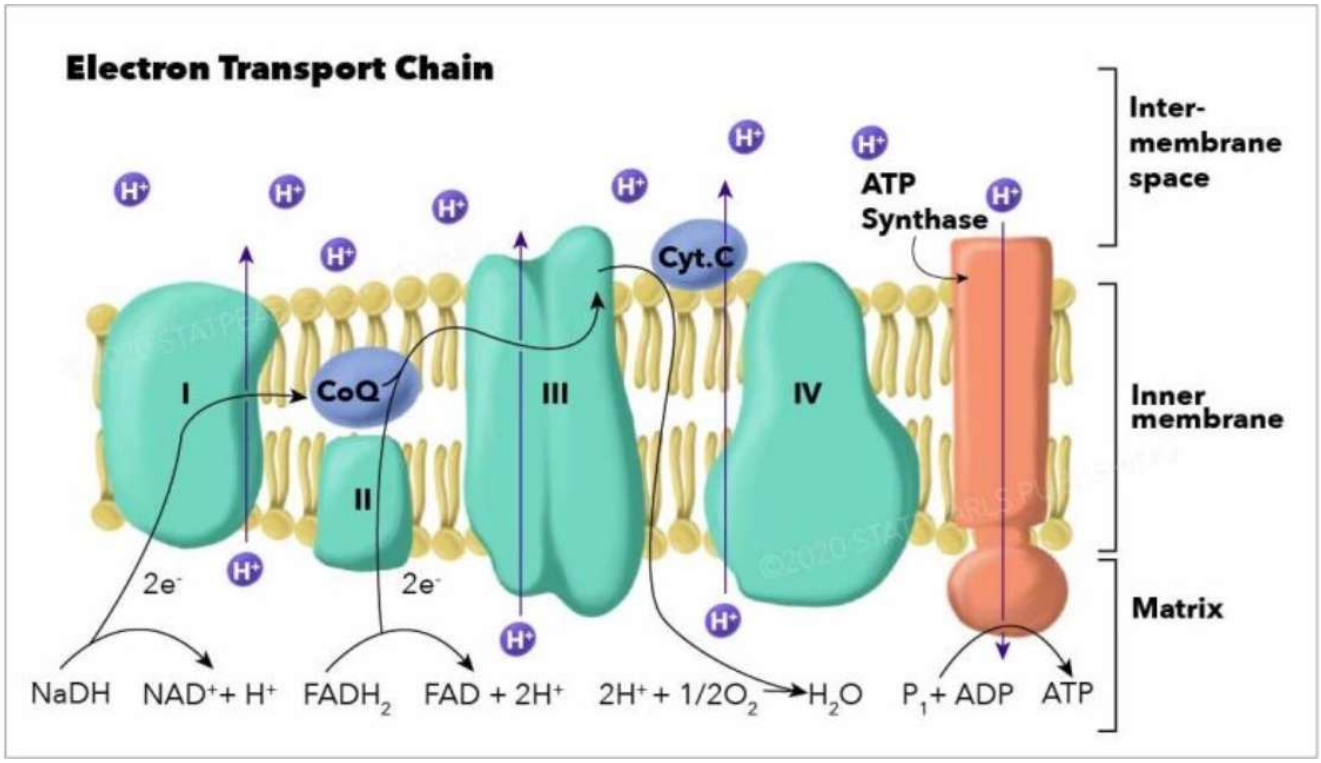


Figure 1: Cartoon depiction of the electron transport chain embedded within the mitochondrial membrane. The ETC is powered by protons being pumped from the mitochondrial matrix into the intermembrane space by Complexes I, III, and IV, in a process known as the proton motive force. Electrons are passed along the chain by Complexes I-IV through redox centers. Image from: Gregory, 2023. (https://www.ncbi.nlm.nih.gov/books/NBK526105/figure/article-20982.image.f1/)

Previous toxicity screenings have shown that nonselective herbicides, such as Gramoxone© and Roundup©, have active ingredients, paraquat and glyphosate respectively, that are redox agents which interfere with mitochondrial protein complexes, lipids, or signaling pathways. (Bora et al., 2013; Meyer et al., 2013; Tsai, 2013) The National Center for Biotechnology Information (NCBI) has stated on their official forum that paraquat is disallowed in European agricultural use, and alternatives such as glyphosate and diquat, have picked up popularity in American agriculture. Another commercially used herbicide is Magnicide H©, with the active ingredient of acrolein, which is used to remove submersed plants in aqueducts. According to the EPA's Magnicide H© application and safety manual, the mode of action on plants consists of "general cellular toxicity impacting various enzyme systems and appears to be toxic to all submerged weeds and algae."

Acrolein (ACR) is a highly reactive α, β-unsaturated aldehyde. Exogenous exposures may come from an individual's diet, medications, lifestyle choices, or environmental living conditions. (Yue et al., 2023) The World Health Organization (WHO) deemed human oral intake of ACR to be no more than 7.5 μg/kg of body weight per day. (Hong et al., 2022) This chemical may occur endogenously as a degradation product from lipid peroxidation or amino acid degradation. (Evans et al., 2021; Desai et al., 2016; Craig et al., 2012)

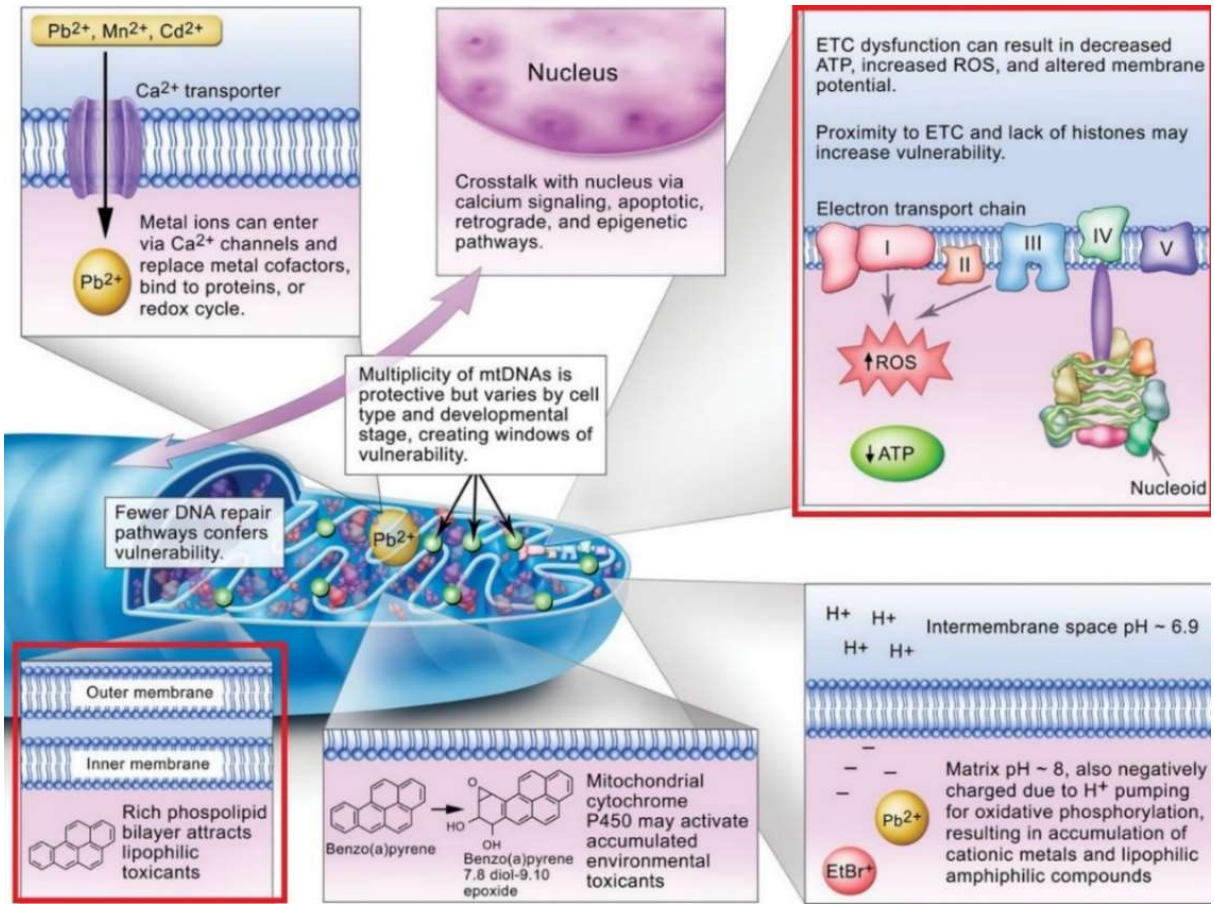


Figure 2: Modes of action of nonselective herbicides that impact mitochondrial health. From: Meyer, et al., 2013 **(**https://doi.org/10.1093/toxsci/kft102**)**

As shown in figure 2, there are different mitochondrial targets that nonselective herbicides impact. However, the impacts on the electron transport chain and mitochondrial membranes, boxed in red, are the primary foci of our research. Environmental toxicants, such as ACR, interact with the lipid rich outer and inner membranes of the mitochondria. This may impact the surface area in which biochemical processes occur or the integral structure of the organelle. ACR may even impact the membrane potential generated from the ETC protein complexes if the toxicant binds to a protein complex or lipids within the inner mitochondrial membrane peroxidize. Membrane potential also impacts other mitochondrial proteins involved with support

functions, such as proteins involved with mitochondrial dynamics. (Gonzalez-Hunt et al., 2016; Tilokani et al, 2018; Twig & Shirihai, 2011; Myers et al., 2013)

ACR and other redox agents also target mitochondrial DNA (mtDNA). mtDNA lacks DNA repair mechanisms recognized in genomic DNA, such as nucleotide excision repair or non-homologous end joining pathways. mtDNA also lacks maintenance proteins, such as nucleosomes or histones, to keep mtDNA bound to a specific shape. There may be consequences to an organism because the damaged mtDNA may be replicated. ACR can form two bulky DNA adducts via nucleophilic attack of the deoxyguanosine nucleotide at positions 1 and N2, yielding α-hydroxy-1,N2-propanodeoxyguanosine and γ-hydroxy-1,N2-propanodeoxyguanosine, which readily reacts with other guanine bases in the DNA strand, sketched in figure 3. (Mohge et al., 2018; Yue et al., 2023; Gonzalez-Hunt et. al, 2016)

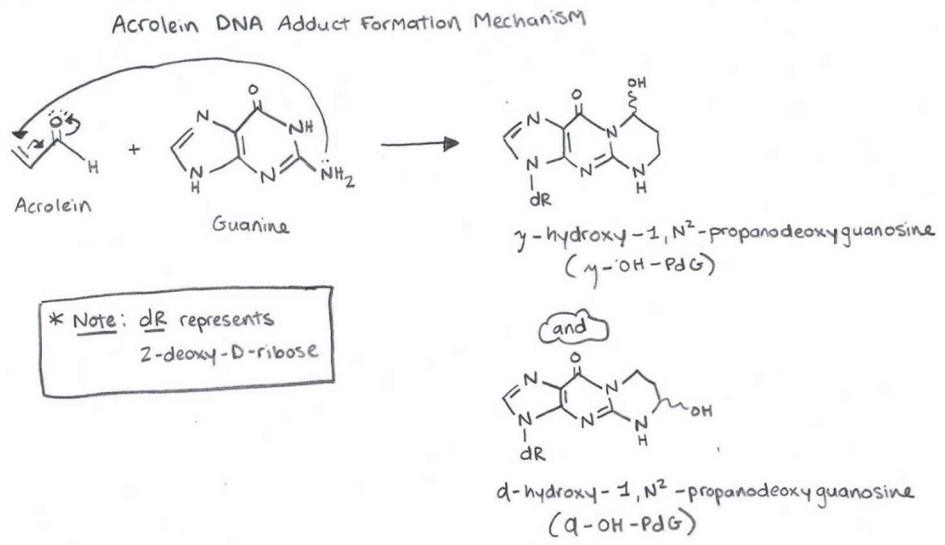


Figure 3: Cartoon of acrolein DNA adduct formation mechanism.

Another target of redox agents within the mitochondria are the amino acids of proteins, particularly if the protein is rich with cysteine, lysine, or histidine. ACR covalently bonds with those residues via Michael's addition, sketched in figure 5. The amino acid becomes an unstable intermediate and will react with other nucleophilic amino acids within the polypeptide chain, forming a crosslink which will alter the protein's shape and function. (Yue et al., 2023; Mohge et al., 2018; Stevens & Maier, 2008)

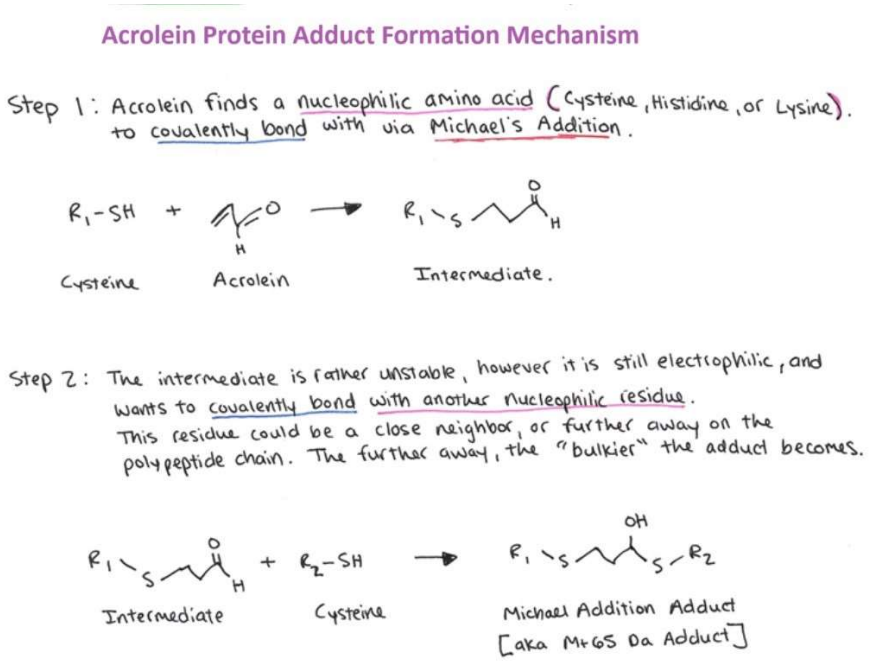


Figure 4: Cartoon of acrolein protein adduct formation via Michael's addition.

Mitochondria are not defenseless organelles. Under stress, sensor proteins may respond and activate the antioxidant pathway. After the stress is neutralized, the mitochondria may participate in repair mechanisms known as mitochondrial dynamics. (Zheng et al., 2023; Weydert & Cullen, 2011; Hunter et al., 1997)

Eukaryotic organisms have defensive antioxidant mechanisms that monitor ROS levels within the cytoplasm and mitochondria to prevent excessive amounts of ROS from accumulating. These chaperone and monitor proteins participate in the heat shock response and DAF-16/FOX pathways. Once activated, these pathways trigger the transcription of ROS scavenging enzymes, superoxide dismutase and catalase, to convert ROS into less harmful species. Superoxide dismutases (SODs) scavenge superoxide anions and convert them into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Catalase (CTLs) scavenge hydrogen peroxide molecules, converting them into water and molecular oxygen. SODs and CTLs exist in three different cellular compartments: the mitochondrial matrix, the cytosol, and the peroxisomes. SOD-2, SOD-3, and CTL-1 are in the cytosol and mitochondrial matrix. SOD-1 and CTL-2 are in the peroxisomes. (Zheng et al., 2023; Weydert & Cullen, 2011; Hunter et al., 1997)

To remove mtDNA damage or to repair proteins and components, mitochondria participate in mitochondrial dynamics. Mitochondrial dynamics consists of three stages: fusion, fission, and mitophagy. Mitochondrial fusion and fission are sketched in figure 5 below, along with the proteins involved in these steps. (Liu et al., 2020; Meyer et al., 2019; Byrne et al., 2018)

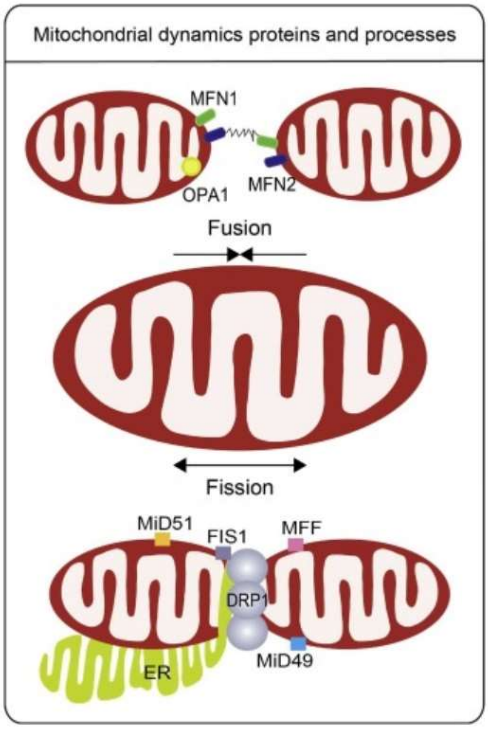


Figure 5: Mitochondrial dynamics depicting fusion and fission, along with proteins involved in both steps. Image from: Liu et al., 2020. https://doi.org/10.1016/j.mad.2020.111212

Mitochondrial fusion occurs between two damaged mitochondria joining together. Both organelles mix and retain healthy components to form one healthy mitochondrion, while simultaneously removing damaged components in a process called functional complementation. Fusion helps minimize the accumulation of mtDNA mutations and damaged proteins. (Byrne et al, 2018)

Mitochondrial fission occurs when damage to one mitochondrion is extensive, so the mitochondrion divides into two unequal daughter mitochondria. One of the daughters inherits a copy of undamaged components and mtDNA. However, the other daughter inherits the most extensively damaged components and gets tagged for mitophagy. (Byrne et al., 2018; Meyer et al., 2019)

Mitophagy, or mitochondrial autophagy, consists of the removal of an entire organelle or the mitochondrial components removed during fission or fusion. The organelle or components are tagged with ubiquinone to recruit peroxisomes for removal. (Twig & Shirihai, 2011)

Failure to maintain healthy mitochondria with regulation of mitochondrial dynamics leads to the pathology and/or progression of mitochondrial disease and disorder.

1.2 Previous Research with Acrolein Toxicity or SDH Health

Section 1.2.A. Aitken et al.'s study: Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase.

Aitken et al.'s research focused on the impact of ACR and 4-hydroxynonenal (4-HNE) on human and hamster spermatozoa mitochondria. During experimentation, the researchers exposed spermatozoa to synthetic electrophiles (such as ethyl vinyl ketone and iodoacetamide) as well as the naturally occurring electrophiles from lipid peroxidation (ACR and 4-HNE).

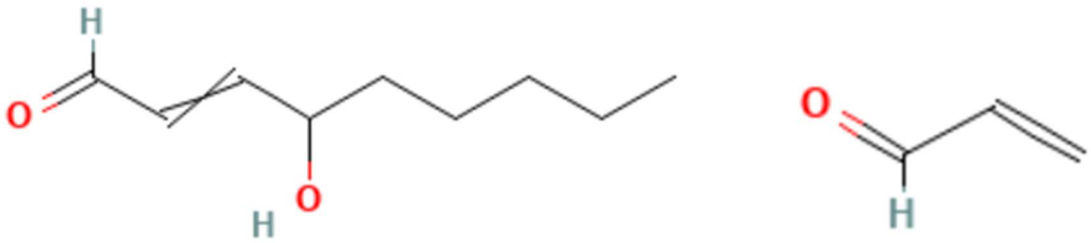


Figure 6: 2-D structures of 4-hydroxynonenal (left) and acrolein (right). Structures generated from Pubchem. 2024. https://pubchem.ncbi.nlm.nih.gov/compound/4-Hydroxynonenal and https://pubchem.ncbi.nlm.nih.gov/compound/7847#section=2D-Structure

The results indicated that the lipid peroxidation electrophiles generated more mitochondrial ROS and decreased spermatozoa motility and vitality compared to the synthetic electrophiles. ACR generated more ROS and loss in cellular vitality than 4-HNE exponentially, so the researchers focused on 4-HNE rather than ACR for the rest of the study to determine the mechanism behind ROS generation and apoptosis within the spermatozoa mitochondria.

ROS, such as superoxide anion and hydrogen peroxide, tend to target redox proteins involved in the TCA cycle and ETC, such as fumarate reductase and pyruvate dehydrogenase. (Messner & Imlay, 2002) Aitken et al. hypothesized that pyruvate dehydrogenase's ubiquitin subunit, dihydrolipoyl dehydrogenase, was targeted by 4-HNE. However, after measuring the ROS and mitochondrial stress signals generated after exposure to both 4-HNE and a known inhibitor of pyruvate dehydrogenase, 5-methoxyindole-2-carboxylic acid, there was no significant increase in ROS. This suggested that there was a different target involved from the ETC, such as succinate dehydrogenase (Complex II) or cytochrome c oxidase (Complex III).

The team's results suggested that 4-HNE generated more ROS and activity inhibition in succinate dehydrogenase than cytochrome c oxidase. After performing Western blots, the researchers discovered that 4-HNE forms a protein adduct with SDHA, shown in figure 7 below. This caused the protein to lose functionality within the ETC. Instead of passing electrons along to Complex III, SDHA passed the electrons instead to the molecular oxygen present in the mitochondrial matrix in a process known as autooxidation.

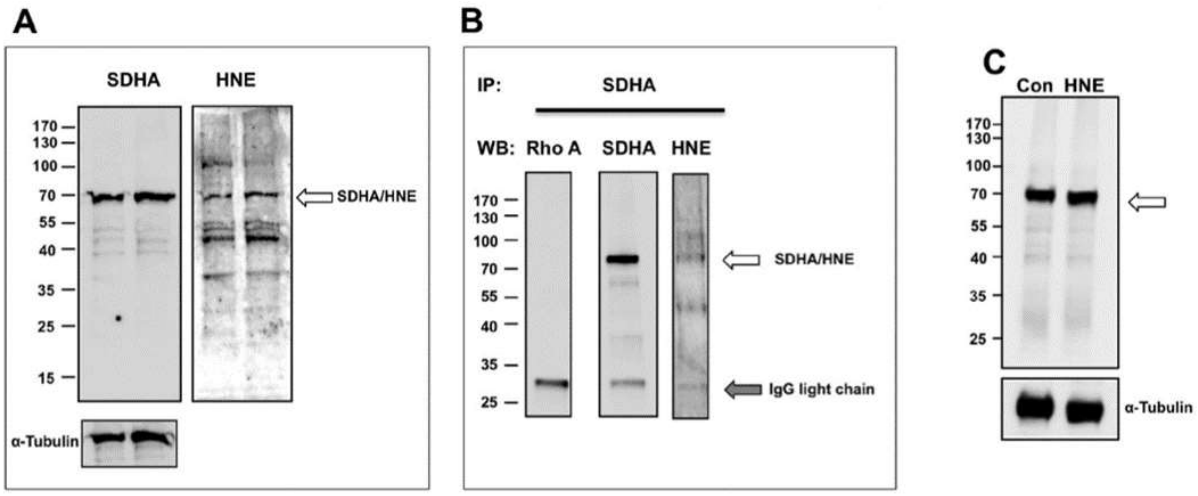


Figure 7: Western Blot results confirming a 4-HNE and SDHA protein adduct formation. Figure edited from: Aitken et al., 2012. (https://doi.org/10.1074/jbc.M112.366690)

4-HNE and ACR both share similar functional groups which form adducts with proteins via Michael's addition with nucleophilic amino acids, depicted in figure 8 on the bottom left-hand side. ACR may form a protein adduct with SDHA through Michael's addition and cause SDHA to autoxidize electrons like 4-HNE.

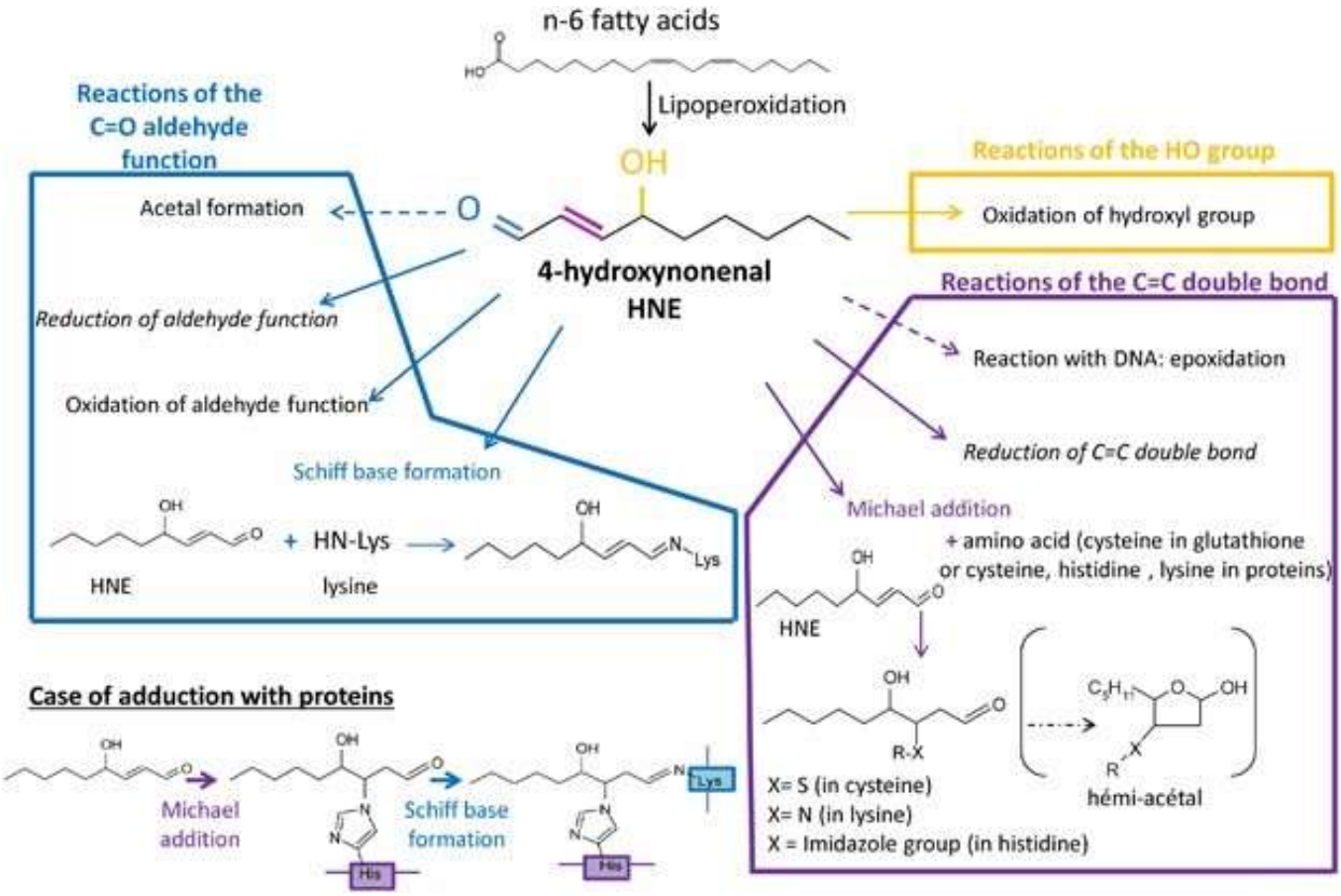


Figure 8: Polyunsaturated fatty acid undergoing lipid peroxidation, yielding 4-HNE, and 4-HNE's biochemical reactions to protein and lipids. On the bottom left-hand corner of this image depicts 4-HNE forming a protein adduction through Michael's addition. Image from Dalleau et al., 2013. (https://doi.org/10.1038/cdd.2013.138)

Section 1.2.B. Woodhouse et al.'s study: Mitochondrial succinate dehydrogenase function is essential for sperm motility and male fertility.

Woodhouse et al.'s study focused on SDH function and the enzyme's impact on sperm motility and fertility in *C. elegans*. The SDH enzyme for *C. elegans* shares approximately 71% homology to human SDH. A ribbon model of *C. elegans'* SDH is depicted in figure 9 below. Both human and *C. elegans* SDH possess four subunits, with the SDHA subunit being the flavoprotein, or catalytic subunit, for extracting electrons. There are two *C. elegans* genes that code for SDHA:

SDHA-1 and SDHA-2. Homozygous SDHA-1 mutations are lethal in *C. elegans*, so the researchers made two SDHA-2 mutant strains. Both strains carried a mutation in the FAD-binding site; one mutation was a single nucleotide variation, while the other mutation was an amino acid variation.

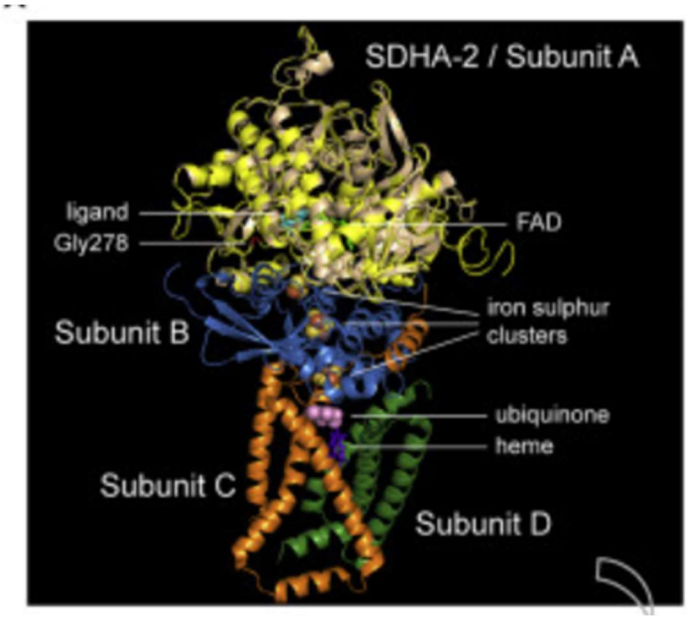


Figure 9: Ribbon model of *C. elegans* succinate dehydrogenase and all four subunits (A-D) generated via I-TASSER software. Image from: Woodhouse et. al, 2022 **(**https://doi.org/10.1016/j.isci.2022.105573)

During various assays focusing on brood size and brood viability assays utilizing male reproduction and hermaphroditic self-reproduction, the SDHA-2 mutant strains yielded smaller and less viable brood sizes, more unfertilized oocytes, and more defective sperm compared to the wildtype under normal lab conditions with both reproductive methods.

To measure mitochondrial membrane potential and observe mitochondrial morphology, they stained the worms with the dye, JC-1. The mitochondrial membrane potential in the SDHA-2 and wildtype strains showed no significant difference. However, the mitochondrial morphology in

the SDHA-2 mutants displayed longer and more filamented mitochondria compared to the wildtype mitochondria, suggesting that SDH plays a role in mitochondrial dynamics and maintenance.

When measuring the mitochondrial unfolded protein response with a GFP reporter strain for both SDHA-2 mutants and wildtype, the fluorescence intensity measured was not significantly different in all the strains, suggesting that a mutation in SDH does not upregulate heat shock response.

To further investigate the significance of SDH function has on sperm motility and viability, the team induced different stress tests with chemicals, heat, starvation, oxidation, and pathogens on mutant and wildtype strains. Oxidative stress was induced with paraquat, and they utilized a GFP reporter for SDHA-2 mutants and wildtypes. The SDHA-2 mutant strains yielded less intense fluorescence compared to wildtypes, and the researchers implied this was due to further generation of ROS.

Section 1.2.C. Hong et al.'s study: Acrolein promotes aging and oxidative stress via the stress response factor DAF-16/FOXO in Caenorhabditis elegans.

Hong et al.'s research focused on the impacts and recovery of single-dose, acute ACR exposure on *C. elegans*. They exposed wildtype and DAF-16 (CF1038) mutant worm strains to concentrations of 12.5 μM, 25 μM, 50 μM, and 100 μM ACR for 48 hours on an NGM plate. After the 48-hour exposure, they transferred the worms to untreated NGM plates to evaluate lifespan, fecundity, progeny development, oxidative stress levels, and DAF-16/FOXO pathway regulatory genes. (Hong et al., 2022) The DAF-16/FOXO pathway engages in other processes of

the *C. elegans*, including growth development, aging, stress response, and the immune response. (Senchuk et al., 2018)

Their results implied that lifespan was impacted based on ACR dosage and was shortened by approximately 19% with 50 μM ACR exposure and 40% with 100 μM ACR compared to the control. Reproductive assays showed a decrease in brood size at the 50 μM and 100 μM ACR concentrations compared to the control as well.

They qualitatively analyzed ROS by measuring fluorescent intensity with DCF fluorescence, and the levels of ROS generated increased as ACR concentration increased. They also discovered that the enzymes involved in the antioxidant pathway, superoxide dismutase (SOD) and catalase (CAT), had decreased activity and transcription levels with the 100 μM ACR exposure compared to the control worms and lower concentration ACR exposed worms. Malondialdehyde (MDA) levels, which is an end-product of lipid peroxidation, were increased at the 50 μM and 100 μM concentrations.

The researchers utilized a GFP-reporter mutant strain of DAF-16 as well to determine the cytosolic localization of DAF-16 in gene regulation. They claimed that at the lower concentrations of 12.5 μM and 25 μM, ACR decreased cytosolic localization of DAF-16, thus triggering an immune response and activation of the antioxidant pathway. However, worms exposed to 100 μM ACR had lower transcription levels of DAF-16, CTL-1, and CTL-2, suggesting that an immune response cannot be triggered with excessive ROS.

Section 1.2.D. Simmon's Thesis: Developing alternatives to methyl bromide: A focus on acrolein (2-propenal).

In a different scope of study, an ecological doctoral student, Simmons, studied ACR as a replacement nematicide for methyl bromide against the parasitic nematode species, *Rotylenchulus reniformis* and *Meloidogyne spp.* Parasitic nematodes are detrimental to agricultural crops, causing thousands of dollars of waste and food shortages nationwide in the United States.

The researcher used two techniques to apply ACR to crop soil: either directly or using drench application. He used a variety of ACR concentrations to evaluate population growth and control of different nematode species. When ACR was applied directly to crop soil in increments of 25 mg/kg up to 300 mg/kg, populations of parasitic nematodes were decreased in all treatments compared to the control. There were no parasitic nematodes in the 175 mg/kg – 300 mg/kg ACR treatments. The microbivore nematodes were not present in ACR treatments of 100 mg/kg or more.

Simmons did not observe cytotoxicity in the soybean plants of all treatments. However, Simmons did not evaluate molecular mechanisms for ACR's mode of action as a nematicide.

1.3 Knowledge Gap

Previous studies focus on the acute toxicity of ACR as well as the recovery of an organism after an acute, high dose ACR exposure. There is a knowledge gap about the impacts of chronic, low dose exposure of acrolein throughout an organism's lifespan.

There are also studies that utilize model organisms, such as live animals and cell cultures. However, there are limitations with these model organisms, such as recognizing the

consequences of chronic exposure over an organism's entire lifespan or the impacts on progeny, particularly over generations.

It is also unclear if the ETC complex, SDH, is a target of acrolein. Acrolein may interact with SDH's flavoprotein subunit like 4-HNE. However, there may be other targets that acrolein may interfere with in the mitochondria, such as a different ETC complex, the inner mitochondrial membrane, mtDNA, or a different protein embedded in the mitochondrial membrane.

1.4 C. elegans as the Model Organism

Caenorhabditis elegans (*C. elegans*), or more commonly called nematodes or worms, make an excellent model organism to study *in vivo* chronic toxicity and impact on progeny. Bora et al.'s team has already demonstrated the effectiveness of studying chronic toxicity of paraquat in nematode lifespan and progeny. Hong et al.'s team has performed a recovery assay of 48-hour ACR exposure with 12.5 μM to 100 μM ACR concentrations. *C. elegans* have been used in a variety of studies involved with mitochondrial health and with toxicity screens of potential environmental toxicants. (Pan & Zhang, 2021; Bora et al., 2013; Hong et al., 2022)

The *C. elegans* genome is extensively studied, and approximately 42% of their proteins are either homologous or orthologous to human proteins. The SDH enzyme for *C. elegans* shares approximately 71% homology to human SDH. *C. elegans* mitochondria are homologous to mammalian mitochondria, sharing genes of interest involved with mitochondrial function and disease. The nematode mitochondrial membrane is comprised of approximately 44% phosphatidylcholine (PC), 34% phosphatidylethanolamine (PE), and 14% cardiolipin (CL), along

with trace amounts of sterols and other phospholipids. (Pan & Zhang, 2021; Woodhouse et al., 2022; Luz et al., 2017; Bora et al., 2013; Tsang et al., 2003; Koyiloth, M. & Gummadi, S., 2022)

There are limitations utilizing *C. elegans* as a model organism. Mitochondria differ between the organs of eukaryotic organisms, such as heart or stomach cell mitochondria, which are organs that *C. elegans* lack. Spermatozoa, which are haploid cells that contain mitochondria, also differ between eukaryotic species. Previous mitochondria toxicity work has demonstrated that toxicant mechanisms may differ between cell types, and this may be due to factors such as pH and local oxygen availability based on cell type and function. However, it is important to gather as much information as possible to help organizations, such as the FDA and EPA, when determining toxicity thresholds for ACR, especially since this carbonyl accumulates endogenously through various metabolic pathways and environmental exposures. (Kuznetsov & Margreiter, 2009; Fisher, Roldan, Avidor-Reiss & Rowe, 2022; Aitken et al., 2012; Picklo et al., 1999)

1.5 Hypothesis and Aims

We hypothesized that *C. elegans* would experience mitochondrial dysfunction when chronically exposed to low doses of ACR. Based on previous research, ACR may inhibit complex II (SDH) activity by protein adduct formation. ACR may also decrease lifespan and fecundity across generations of *C. elegans.*

Aim One

Our first aim examined inhibition of SDH activity after a 24-hour exposure to 15 μM and 100 μM ACR. Given the mode of action of impacting various enzymatic systems in plants, ACR may potentially be an SDH inhibitor. SDH activity was measured with spectrophotometry and

histochemical staining assays. Transcription levels of SDHA-1 mRNA were taken with reverse-transcription polymerase chain reaction (rt-PCR).

Spectrophotometry measures the changes in light intensity of a reaction solution while a specific wavelength of light passes through it over a set period. Light may either be absorbed or scattered by the reaction solution, and a high absorbance is attributed to high enzyme activity. For complex II and SDH activity assays, the higher absorbance levels correspond to cytochrome c reduction, which means that electrons are being passed along the electron transport chain.

An SDH histochemical staining assay was performed on treated and untreated worms for qualitative analysis. The worms were stained following a protocol, transferred to a fresh 60 mm x 15 mm MYOB plate, and examined under light microscopy. When the dye NBT gets reduced by SDH, the chemical changes color from yellow to blue. Activity levels correlated with a darker blue intensity, whereas a lighter blue intensity correlated with less SDH activity. Therefore, a darker blue intensity would show that SDH is able to reduce NBT.

RT-PCR was performed investigating the regulation of the SDHA-1 and CTL-1 genes. If SDHA was damaged or inhibited, we would expect to see upregulation of SDHA-1 transcription. If the mitochondria were under oxidative stress, we would expect to see an upregulation in the cytosolic antioxidant enzyme, CTL-1, to scavenge excessive ROS in the mitochondria and cytosol.

Aim Two

Our second aim focused on lifespan and fecundity across generations of chronically exposed worms. Since ACR has been demonstrated to be a mitochondrial toxin and redox agent, it may

potentially decrease the lifespan and fecundity of worms across generations. To determine the impact of chronic low dose ACR exposure on *C. elegans* lifespan and reproduction, survival and fecundity assays were performed.

Lifespan assays were conducted similarly to methods performed by Bora et. al. with the use of solid MYOB agar plates. Fecundity assays followed methods from Kwah and Jaramillo-Lambert to determine the brood size of chronically exposed worms after hermaphroditic or sexual reproduction. All low-dose concentrations of ACR for lifespan and fecundity were 10 μM, 15 μM, 25 μM. The primary focus of these concentrations was determined to be the 15 μM ACR concentration.

Chapter II. Methods and Materials:

2.1. C. elegans maintenance

This study only utilized the N2 Bristol (wildtype) *C. elegans*. Dr. Pan's lab at East Carolina University has cultured and provided this strain for our experiments.

The worms were maintained at 22°C incubation on nematode growth medium (NGM) or Modified Youngren's Only Bacto-Peptone (MYOB) in a 100 mm x 15 mm Petri plate seeded with 60 µL Escherichia coli (*E. coli*) strain OP-50 as a food source.

Synchronized worm populations were obtained by following a sodium hypochlorite treatment, then allowing worms to grow for three days until adulthood was reached on an NGM agar plate seeded with 60 µL of OP-50. Worms were synchronized with a hypochlorite treatment for each trial so that they were the approximately the same age when experiments, such as the lifespan and fecundity assays, were conducted.

The synchronization process began with washing worms off NGM agar plates with fresh M9 buffer and gathering them up with a serological pipette to be transferred into a 15 mL conical. The worms were pelleted through centrifugation at 2000 RPM for two minutes at room temperature. This process was repeated until the supernatant was clear, and pipetting was carefully performed to not disturb the worm pellet at the bottom of the conical. 5 mL of synchronization solution [17.5 mL dH_2O, 5 mL premade bleach solution, and 2.5 mL 10N NaOH] was added to the worm pellet and gently inverted up and down for five minutes to harvest the eggs from the worms. The eggs and worm detriment were separated via centrifugation at 2000 RPM for two minutes at room temperature. The supernatant was removed

and 10 mL fresh M9 buffer was added to the eggs, and centrifugation was performed again at 2000 RPM for two minutes at room temperature. This step was repeated three more times to wash the eggs and to remove any trace of bleach solution. On the third rinse, the supernatant was not removed. The conicals were placed on a flat surface with the lids of the conicals twisted open and taped down to allow air flow while the eggs were allowed to hatch for eighteen hours. The lids were closed tightly and the conicals were centrifuged at 2000 RPM for two minutes at room temperature. All but 200 µL of supernatant was removed. The 200 µL of supernatant should contain L1 *C. elegans*, so it was pipetted carefully onto a fresh NGM agar plate with 60 µL of OP-50. Worms were allowed to grow for three days until adulthood was reached. Once the worms were confirmed to be adults under a dissection microscope, worms were allowed to be transferred to the ACR treated MYOB agar plates via stainless steel wired worm pick.

2.2 Acrolein Treatment

Stock solutions of 50 mM ACR were prepared from a 0.18 M vial of ACR (Brand: Spex Certiprep, Part Number: ECS-A-CAN; Total Volume: 1.5 mL) with dH_2O and stored in a freezer. To make the 50 mM ACR stock solutions, 5.4 mL dH_2O was added to the 1.5 mL 0.18 M ACR vial, for a total volume of 6.9 mL of 50 mM ACR stock. 1 mL of 50 mM ACR stock was added to Eppendorf tubes, so a total of seven 50 mM ACR stock Eppendorf tubes were stored in the freezer.

The 50 mM ACR stock solutions were further diluted down to a 25 mM ACR concentration with dH_2O and stored in a freezer. To make 1 mL of 25 mM ACR stock from a 1 mL 50 mM ACR stock, 500 µL dH2O and 500 µL of the 50 mM ACR stock were added to an Eppendorf tube.

ACR has a boiling point of 53°C and a flash point of 23°C and may violently polymerize at temperatures greater than 50°C, which were limiting factors for adding ACR directly to the media. Therefore, MYOB agar plates were prepared without ACR by serological pipetting 5 mL of MYOB agar to Petri dishes that had dimensions of 60 mm x 15 mm, or 10 mL of MYOB agar to Petri dishes that had dimensions of 100 mm x 15 mm, then allowed to cool overnight before storing in a 4°C freezer. The recipe for MYOB agar is listed below in *Section 3.3: Making NGM or MYOB Agar Plates*.

MYOB plates were treated with ACR daily before worms were transferred onto the plates. To make the plates a specific concentration, the MYOB agar media had to be 5 mL for the 60 mm x 15 mm Petri dishes, or 10 mL for the 100 mm x 15 mm Petri dishes. Then the ACR may be added to achieve the following concentrations:

For 10 µM ACR treatments used in lifespan and fecundity assays: if the plate had 5 mL of media, then 2 µL of the 25 mM ACR stock solution was pipetted onto the plate and spread evenly across the agar surface with a glass rod. If the plate had 10 mL of media, then 4 µL of the 25 mM ACR stock solution was pipetted onto the plate and spread evenly across the agar surface with a glass rod.

For the very first lifespan and fecundity assays with higher concentrations of ACR (such as 75 µM or 100 µM), the experiments were conducted with 60 mm x 15 mm MYOB agar plates with 5 mL of media. The 75 µM and 100 µM ACR plates had either 7.5 µL or 10 µL of 50 mM ACR stock solution pipetted onto the plate, then spread evenly across the agar surface with a glass rod. The ACR was allowed to dry on the agar surface for approximately 30 minutes. Then 20 µL of the *E. coli* strain, OP-50, which served as the *C. elegans'* food source, was spread evenly across

the agar surface. Worms were then transferred to the agar plate and allowed to grow and lay eggs on the treated plate for 24 hours before being transferred to a new freshly prepared ACR treated plate. This process continued until the worms died or ceased laying eggs. The worms were transferred to the solid MYOB plates with a worm pick and stainless-steel wire that was changed frequently between ACR treatment groups. It must be noted that the higher ACR treatments were not continued due to the worms escaping the plates at these concentrations. The worms were located either on the plate lid or on the outside in the parafilm that was used to keep the plates sealed and moist. Further details of higher dose ACR exposures on *C. elegans* are mentioned below in the discussion section.

The following low dose ACR treatments for lifespan and fecundity assays also followed the same methods as the 10 µM ACR concentration mentioned above, just with different amounts of the 25 mM ACR stock solution were added: for 15 µM add 3 µL of 25 mM ACR stock solution to a 5 mL MYOB agar plate or 6 µL of 25 mM ACR stock for a 10 mL MYOB agar plate; for 25 µM add 5 µL of 25 mM ACR stock solution to a 5 mL MYOB agar plate or 10 µL of 25 mM ACR stock for a 10 mL MYOB agar plate. The ACR was allowed to dry on the agar surface for approximately 30 minutes. Then 20 µL of the *E. coli* strain, OP-50, which served as the *C. elegans'* food source, was spread evenly across the agar surface. Worms were then transferred to the agar plate and allowed to grow and lay eggs on the treated plate for 24 hours before being transferred to a new freshly prepared ACR treated plate. This process continued until the worms died or ceased laying eggs. The worms were transferred to the solid MYOB plates with a worm pick and stainless-steel wire that was changed frequently between ACR treatment groups.

For the 100 µM or 200 µM ACR treatments that were used for the SDH spectrophotometric activity assays and SDH histochemical stain, the volume of media used for both types of reaction tubes was a total volume of 1 mL. The 100 µM ACR treatment used 2 µL of the 50 mM ACR stock solution and the 200 µM ACR treatment used 4 µL of 50 mM ACR stock solution.

3.3 Making NGM or MYOB Plates

Nematode growth media (NGM) agar plates and Modified Youngren's Bacto-Only Peptone (MYOB) agar plates were used during experimentation. The NGM plates were used for maintenance N2 Bristol worms and for allowing synchronized worms to grow to Day-3 adult. The MYOB agar plates were used during the lifespan and fecundity assays.

To make the NGM agar plates, 8.5 g Bacto agar, 1.5 g NaCl, and 1.25 g peptone were added to 500 mL dH_2O and autoclaved, then allowed to cool to 55°C in a bead bath. After cooling the following were added to the flask while on a stir plate: 500 µL of 1 M $CaCl_2$, 500 µL $MgSO_4$, 500 µL of 5% cholesterol solution, and 12.5 mL of KPO_4 buffer. 5 mL of media was serologically pipetted into 60 mm x 15 mm Petri dishes and allowed to cool overnight. Unused plates were stored in a 4°C freezer.

To make the MYOB agar plates, 10 g Bacto agar, 1 g NaCl, 0.28 g Tris-HCl, 0.12 g Tris(base), 1.55 g peptone, and 0.8 mL 20% cholesterol/ethanol were added to 500 mL dH_2O and autoclaved. 5 mL or 10 mL of MYOB agar media was serologically pipetted into 60 mm x 15 mm or 100 mm x 15 mm Petri dishes, respectively, and allowed to cool overnight. Unused plates were stored in a 4°C freezer.

Plates were parafilmed during the lifespan and fecundity assays to prevent the worms from escaping, prevent contamination, and to retain moisture on the MYOB agar.

2.3 Sexual Reproduction and Hermaphroditic Reproduction Fecundity Assays of Worms Exposed to Chronic, Low-Dose Acrolein

To determine if ACR impacted the fecundity of *C. elegans*, two types of fecundity assays were performed. One fecundity assay focused on sexual reproduction between hermaphroditic and male worms. The second fecundity assay focused on the hermaphroditic, or self-reproduction method. Three independent biological trials were conducted with three replicates per trial.

For the fecundity assays, the brood from sexual and hermaphroditic plates was scored via grid and counted with a cell counter every third day and recorded. The data collected was entered into Excel then analyzed with the XLMiner Toolpak add-in. Three independent biological trials were conducted with three replicates per trial. The first trial was conducted to narrow down a low dose concentration of ACR from the concentrations of 10 μM, 15 μM, and 25 μM. The 15 μM concentration was chosen based on trends noticed in the fecundity assays suggesting that brood counts were lower in worms exposed to 15 μM ACR concentration.

For the hermaphroditic reproduction assays, one age-synchronized Day-3 adult worm was transferred to an MYOB plate treated with the desired amount of ACR and seeded with 20 μL OP-50, parafilmed, then incubated at 22°C. The worm was allowed to lay eggs for 24 hours before being transferred with a stainless-steel wire worm pick to a new freshly treated ACR MYOB plate following the process listed under the *Acrolein Treatment* section in methods. Plates were retained in a 22°C incubator and scored for brood three days later by grid under a

dissecting microscope. This procedure was repeated until the worm lost fertile embryo production. The brood size obtained for each treatment was compared to the untreated control.

For the sexual reproduction assays, two age-synchronized Day-3 adult worms were transferred to an MYOB plate treated with desired ACR concentration and seeded with 20 µL OP-50, parafilmed, then incubated at 22°C. The worms were allowed to lay eggs for 24 hours before being transferred with a stainless-steel wire worm pick to a new freshly treated ACR MYOB plate following the process listed under the *Acrolein Treatment* section in methods. Plates were retained in a 22°C incubator and scored for brood three days later by grid under a dissecting microscope. This procedure was repeated until the worms lost fertile embryo production. The brood size obtained for each treatment was compared to the untreated control.

2.4 Lifespan Assays of Worms Exposed to Chronic, Low-Dose Acrolein

Worms from the fecundity assays were utilized to determine the lifespan of chronic, low dose ACR exposure. This totaled to be nine age-synchronized Day-3 adult worms (six from the sexual reproduction method, and three from the hermaphroditic reproduction method).

Worms were transferred with a stainless-steel wire pick to a new freshly treated ACR MYOB plate following the process listed under the *Acrolein Treatment* section in methods. Worms were exposed to the treated plates for 24-hours. Alive worms were transferred to a new MYOB plate treated with the desired ACR concentration, provided above in the *Acrolein Treatment* section, and seeded with 20 µL OP-50 for plates with the dimensions of 60 mm x 15 mm or 40 µL OP-50 for plates with the dimensions of 100 mm x 15 mm, parafilmed, then incubated at 22°C.

Worms were considered dead if they did not respond to gentle prodding by worm pick, or if the worm was visibly vivisected. Dead worms were not transferred to fresh MYOB plates.

2.5 SDH Activity Histochemical Stain

This is a procedure from Grad et al. Approximately ten fixed and permeabilized Day-3 adult N2 Bristol nematodes are added to 1 mL of SDH activity assay solution [5 mM EDTA, 1 mM KCN, 0.2 mM PMS, 50 mM sodium succinate, 0.25 mM NBT in 10 mL PBS] and incubated for fifty minutes at 37°C in the dark on a rotator, then washed three times with 1.5 mL H_2O to remove excess stain. The nematodes were then transferred to a fresh 60 mm x 15 mm NGM plate and observed under the dissecting microscope and a handheld light microscope with imaging capabilities. ETC protein complexes were inhibited besides Complex II (SDH). Since SDH was the only active enzyme, electrons that were being passed from the TCA cycle could not get passed along to other ETC proteins. Thus, SDH would pass electrons along to molecular oxygen or ROS species if they were in proximity, which is a process known as autoxidation. This would lead to a buildup of superoxide anions in the mitochondrial matrix. Nitro blue tetrazolium chloride (NBT) measures redox potential in respiring cells and organelles. If NBT is reduced by superoxide anion or SDH into diformazan, then the stain will change from a yellowish color to a dark blue, signifying that SDH is still active and autoxidizing. SDH activity appears blue, with darker stains of blue representing higher levels of SDH activity, whereas light blue stains represent lower levels of SDH activity. A lack of a blue color (or clear) represents no SDH activity, or possibly a dead worm. There may be residual background staining from the process. The negative control used was no treatment with ACR or sodium malonate. The positive control used was 20 mM sodium malonate.

Blue intensity was measured qualitatively with a dissecting microscope and a count conducted with a cell counter.

2.6 Spectrophotometric Assay of SDH

Mitochondria were isolated following a method modified from Hench et. al, 2011.

Age synchronized worms were collected from NGM or MYOB agar plates with M9 buffer, then pelleted through centrifugation at 2000 RPM for two minutes at room temperature. The supernatant was carefully removed, and the pellet was resuspended with 1 mL of MSE buffer (pH 7.4). MSE Buffer consisted of 220 mM mannitol, 70 mM sucrose, 10 mM Tris, and 2 mM EDTA. The pellet was manually homogenized with a glass homogenizer for ten minutes, or until the mixture was homogenized. Subtilisin A was then added to the homogenate (10 mg/gram of wet mass of worm pellet), then incubated for twenty minutes at 28°C. The homogenate was homogenized a second time by hand with a glass homogenizer for ten minutes. The remaining steps for mitochondrial isolation were performed in a 4°C cold room. The homogenate was centrifuged at 10,000 x G for five minutes. The supernatant was carefully removed, and the pellet was resuspended in 1 mL of 0.4% BSA-supplemented MSE buffer and centrifuged at 1,000 x G for five minutes. The supernatant was then transferred to a fresh Eppendorf tube and centrifuged at 10,000 x G for five minutes. The pellet formed after this step are the mitochondria, which may be diluted with 0.1 M Tris-SO_4 buffer or stored in a freezer until ready for spectrophotometry.

All spectrophotometric assays were performed at room temperature with wavelength 550 nm.

SDH activity was assayed as phenazine methosulphate (PMS)-mediated reduction of cytochrome c in 1 mL cuvette containing: 0.1 M Tris-SO_4 buffer (pH 7.4), that also contained: 0.165 mg PMS, 0.8 mg cytochrome c, 1 mM sodium cyanide, 20 mM sodium succinate, against a reference of 20

μL of 20% sodium malonate. The reaction was started by adding 20 μg mitochondria and activity was measured for 5 min. Cytochrome c reduction was measured by the increase in absorption at 550 nm, with the molar extinction coefficient of (e = 21.0 $mM^{-1}cm^{-1}$). The molar extinction coefficient of mitochondria was estimated to be e = 10.0 $mM^{-1}cm^{-1}$.

2.7 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Age synchronized Day-3 adult *C. elegans* were collected from MYOB agar plates and washed three times before being transferred to a 24-wells plate with 1 mL M9 media buffer and 1 μL OP-50. Worms were exposed to ACR concentrations of 0 μM, 15 μM, or 100 μM for twenty-hours before being collected and washed three times before RNA extraction.

C. elegans RNA was extracted utilizing Invitrogen TRIzol© reagent and procedures. *C. elegans* cDNA synthesis was conducted using the Takara PrimeScript© RT reagent kit with gDNA Eraser©. Each reaction tube consisted of the following: 5 μL of ABclonal© 2X Universal SYBR Green Fast qPCR mix, 2 μL of forward and reverse primers (see supplementary data for a list of used primers), 2 μL of synthesized 100 ng or 200 ng cDNA template, and 1 μL of nuclease-free water.

The CFX96 Realtime System© and Thermal Cycler 1000© from BioRad© were used in conjunction to perform the final RT-PCR reaction. Results from RT-PCR were pulled using the CFX BioRad© Manager and analyzed with Excel.

The endogenous control used was ACTIN, also known as the *C. elegans* ACT-2 gene. This gene was chosen because it should not have changed between the control group and the other ACR concentration treatments. A table of the primers used is displayed in table 1 below.

	5' --> 3' Sequence	
SDHA-1:	Forward Primer:	TTCACGTGATCCTTTTCGCC
	Reverse Primer:	GTGCCTAATAGGAGCGGACC
CTL-1:	Forward Primer:	GCGGATACCGTACTCGTGAT
	Reverse Primer:	GTGGCTGCTCGTAGTTGTGA
CTL-2:	Forward Primer:	TCCGTGACCCTATCCACTTC
	Reverse Primer:	TGGGATCCGTATCCATTCAT
SOD-3:	Forward Primer:	GGGATATTGCAAGAAAGACAAAAT
	Reverse Primer:	GATATTCTTCCAGTTGGCAATCTT

Table 1: The forward and reverse primers used for the genes SDHA-1, CTL-1, CTL-2, and SOD-3 for RT-PCR gene amplification.

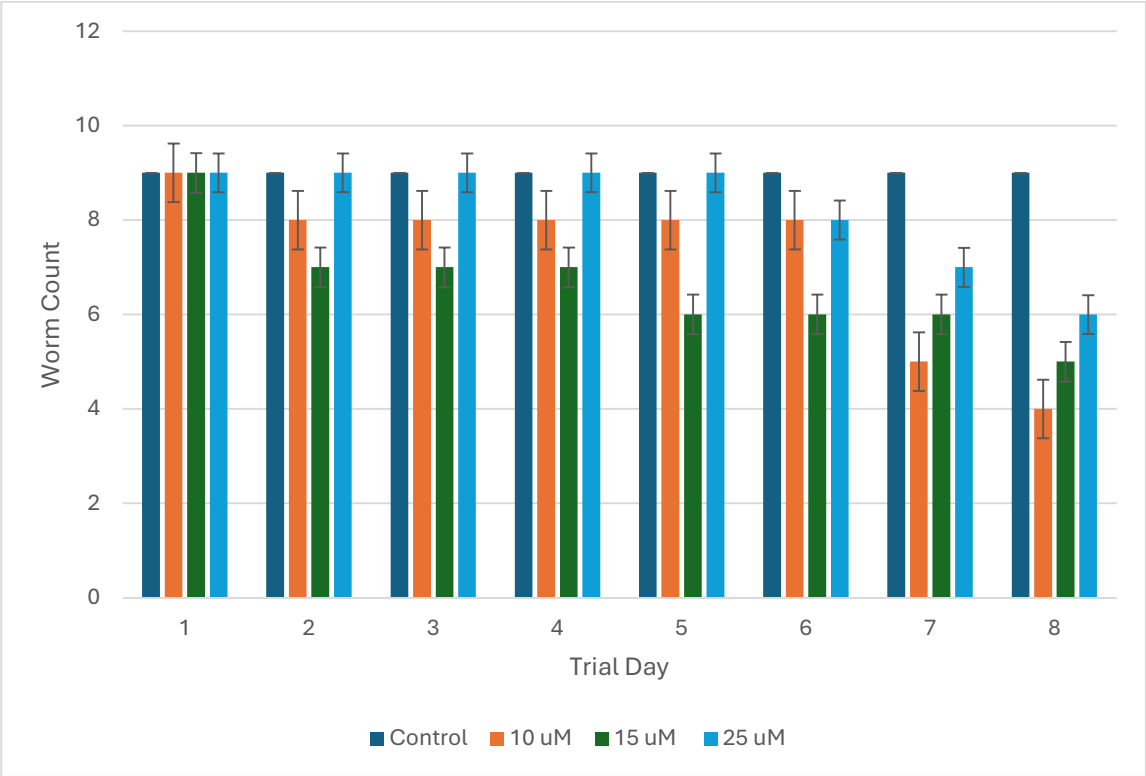
2.8 Statistical Analysis

Unless mentioned in the figure legends, at least three biological replicates and three independent trials were performed for all lifespan and fecundity experiments. All p-values were calculated by one-tailed t-test or one-way ANOVA and graphs were plotted using Excel XLMiner Analysis Toolpak.

Chapter III: Results

3.1 Lifespan Assays

3.1.1. Trial One Lifespan Assay



Graph 1: Chronic, low dose acrolein exposure may act as a worm repellent, or coupled with heat stress may decrease the lifespan of *C. elegans*. This graph displays trial one's lifespan assay of *C. elegans* in the presence of ACR at concentrations of no treatment, 10 µM ACR, 15 µM ACR, and 25 µM ACR treatments during days one through eight. A total of nine age-synchronized Day-3 adult *C. elegans* were exposed to the different concentrations of ACR for a 24-hour period before being transferred to a freshly treated ACR MYOB plate with a stainless-steel wire worm pick. During trial one, an incubator malfunction occurred which may have induced heat stress on worms. Worms were considered dead if they were immobile after gentle prodding with the worm pick and were not transferred. After performing a one-way

ANOVA test in Excel, the p-value obtained was 0.003, which is considered statistically significant, however, this may be due to such a small population observed.

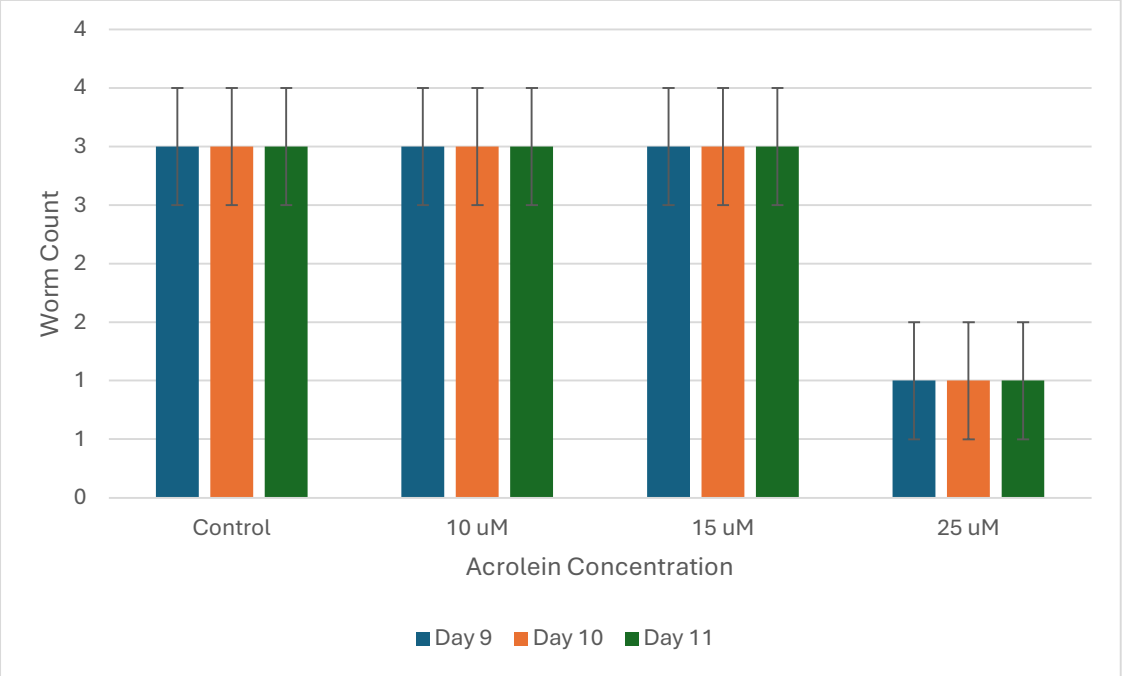
On day 2, some of the worms could not be found on the MYOB agar plates. For the 10 μM ACR treatment worms, one worm could not be located on the MYOB agar plate. Two worms were missing from the 15 μM ACR treatment plates. Because worms could not be found on the plate, this may imply that low doses of acrolein may serve as a nematode repellent. During day 5, a 15 μM hermaphroditic reproduction plate with one worm was lost and could not be found in the incubators or within the lab space. Day 6, one 25 μM ACR exposed worm was found dead on the plate. Day 7, three 10 μM and one 25 μM ACR treated worms were found dead on the plates. Day 8, one 10 μM, one 15 μM, and one 25 μM ACR treated worms were found dead on the plates.

During day eight of trial one, there was an incubator malfunction where the MYOB plates and the OP-50 food source were stored. To resolve this issue, the plates and OP-50 were transferred to a different incubator that held a temperature of 22°C. The malfunctioning incubator was unplugged and allowed to reach room temperature of approximately 23°C and was monitored with a glass bulb thermometer. Once the temperature was verified to be less than 25°C, only brood count plates were stored in that incubator, while the experimental plates and OP-50 were maintained in the 22°C incubator.

The temperature in the malfunctioning incubator reached 55°C for an undetermined amount of time despite the gauge reading of 20°C. *C. elegans* cannot tolerate temperatures above 25°C, or else they endure heat stress. OP-50 strain *E. coli* cannot survive temperatures over 37°C, so the food source that was given to the experimental *C. elegans* had perished as well. This may have

impacted the results gathered during the first trial for lifespan and fecundity of *C. elegans*. It is possible that the incubator temperature may have gradually increased from day six until it was noticeable by day eight, which would explain the trend seen in Graph 1 of a decrease in worm count from days six to eight. Heat stress coupled with low dose ACR exposure may have decreased *C. elegans* lifespan. (Gómez-Orte et al., 2017)

Due to limited space in the incubator from maintaining brood count plates, we decided to take one plate from both reproductive methods (sexual and hermaphroditic) on day nine. For example, the control had one plate from the sexual reproduction method and one plate from the hermaphroditic self-reproduction method, for a total of three worms for the control to examine during a lifespan assay. The 25 μM ACR group only had one plate for hermaphroditic self-reproduction, so only one worm was evaluated for lifespan analysis. For three days, all the worms tested survived chronic low dose ACR treatment as depicted in Graph 2, suggesting that heat stress and possible starvation may have compounded the effects of ACR exposure in *C. elegans* lifespan.



Graph 2: Worm survival after malfunctioning incubator issues were resolved during trial one. To determine if the lifespan of *C. elegans* was impacted from heat shock or a combination of heat shock and ACR exposure, one plate from each treatment and reproduction method was randomly selected to continue in the lifespan assay. We were unable to gather data for the 25 µM ACR treated worms' sexual reproduction method and could only gather data for the hermaphroditic reproduction method. This was due to worm deaths that the 25 µM ACR concentration group had accrued during the first eight days. No p-value was obtained through a one-way ANOVA test in Excel because there was no statistically significant trend observed, nor was there variance calculated between data points collected.

Since all three ACR treated worm groups survived for three days, it may be implied that ACR exposure coupled with heat stress may have decreased *C. elegans* lifespan. Further experimentation may be necessary to explore the mechanisms behind how higher temperatures and low dose ACR exposures impacts *C. elegans* lifespan.

The first trial helped determine which low dose ACR concentration to focus on for trials two and three lifespan and fecundity assays. Based on trends observed in the fecundity assays for sexual reproduction and hermaphroditic self-reproduction assays, the brood counts were lowest for the 15 µM ACR concentration, which was why this dose was selected for further experimentation.

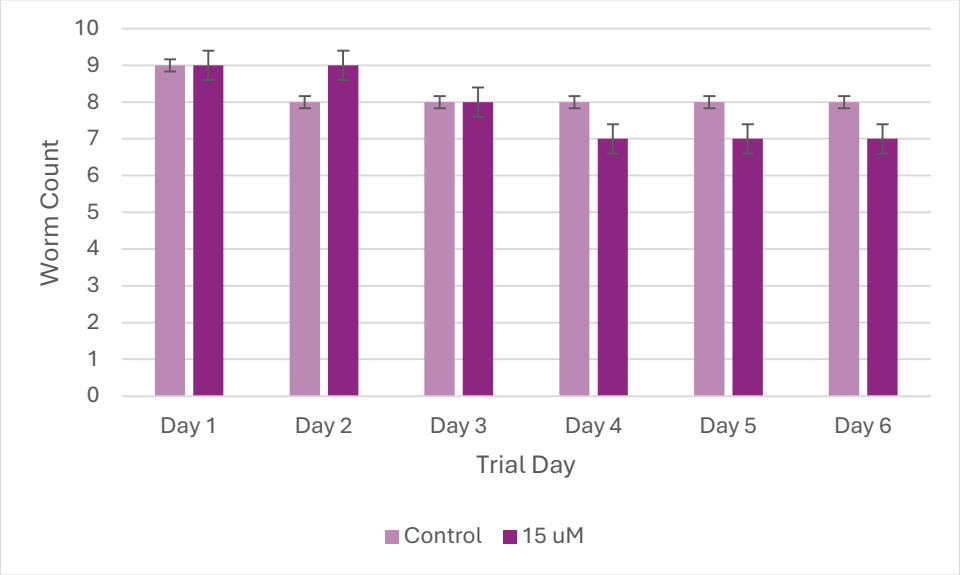
3.1.2. Trial Two Lifespan Assay

Graph 3: Chronic 15 µM ACR exposure does not impact lifespan in *C. elegans* during trial two. This graph depicts the trial two lifespan assay of *C. elegans* in the presence of no ACR exposure or 15 µM ACR exposure for nine days. A total of nine age-synchronized Day-3 adult *C. elegans* were exposed to 15 µM ACR for a 24-hour period before being transferred to a freshly treated acrolein MYOB plate. Worms were considered dead if they were immobile after gentle prodding with a stainless-steel wired worm pick. After performing a one-tailed T-test in Excel, the p-value obtained was 0.14, which is considered not statistically significant, however, this may be due to such a small population observed.

During trial two, it appeared that chronic exposure to 15 µM ACR did not impact *C. elegans* lifespan.

On day 3, a control hermaphroditic plate worm was not transferred over to the new plate based on a lack of worm tracks in the agar and OP-50 lawn. Day 7, one 15 µM ACR treated worm was found dead on the plate. Day 8, two control worms and two 15 µM ACR exposed worms were found dead on their plates. This may have been due to a hot worm pick used on the worms during the transfer process to new plates.

3.1.3. Trial Three Lifespan Assays

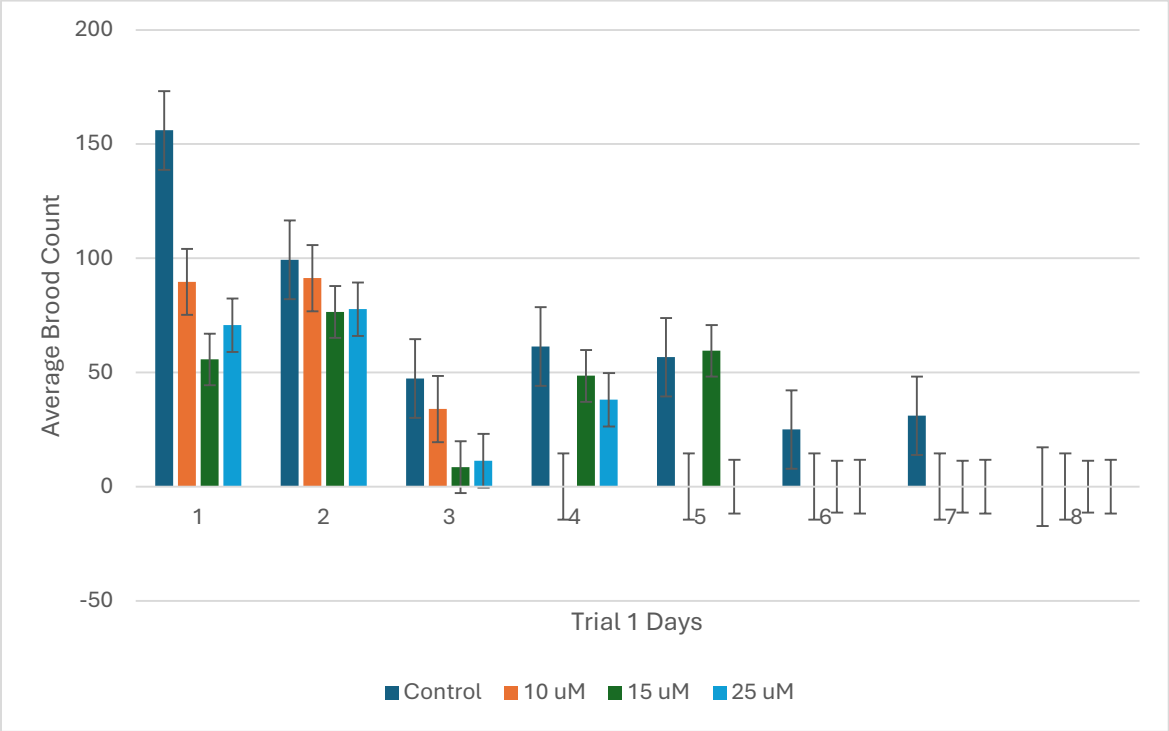


Graph 4: Chronic 15 µM ACR exposure does not impact C. elegans lifespan during trial three. This graph depicts trial three lifespan assay of *C. elegans* in the presence of no ACR exposure or 15 µM ACR exposure for six days. A total of nine age-synchronized Day-3 adult *C. elegans* were exposed to 15 µM ACR for a 24-hour period before being transferred to a freshly treated acrolein MYOB plate. Worms were considered dead if they were immobile after gentle prodding with a stainless-steel wired worm pick. After performing a one-tailed T-test in Excel, the p-value obtained was 0.46, which is not statistically significant, however, this may be due to such a small population observed.

On day two, we ran out of MYOB plates and had to make some more. Unfortunately, we were missing one plate to transfer worms onto. We decided to take out one of the hermaphroditic control plates. On day three, one 15 µM ACR worm was dead on the plate. Another hermaphroditic 15 µM ACR worm was found in the parafilm of the plate.

3.2 Sexual and Hermaphroditic Fecundity Assays

3.2.1.A Trial One Sexual Reproduction Fecundity Assay



Graph 5: Low dose ACR exposure and possible heat stress may decrease fecundity in *C. elegans* sexual reproduction in trial one. Depicted is trial one's sexual reproduction fecundity assay average brood counts from days one through eight. Two age-synchronized Day-3 adult worms were allowed to lay eggs for 24 hours before being transferred to a new MYOB plate. The plate was retained in a 22°C incubator and scored for brood three days later by grid under a dissecting microscope. Since three replicates were tested during this trial, six worms were examined per treatment. This procedure was repeated until the worms lost fertile embryo

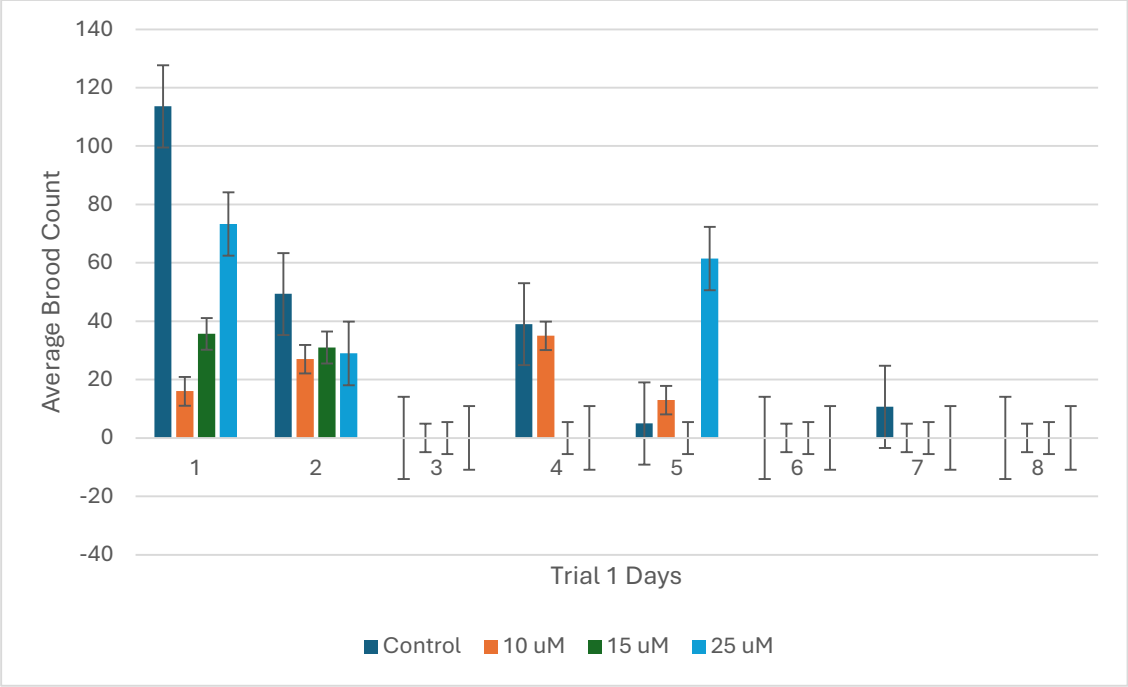
production, which was around day eight for the control group. Brood counts were averaged out between the plates of each treatment. The brood size average obtained for each treatment was compared to the untreated control. After performing a one-way ANOVA test in Excel, the p-value obtained was 0.27, which is not statistically significant, however, this may be due to such a small population observed.

As mentioned before in the trial one lifespan assay, there was an incubator malfunction that may have induced heat stress on the experimental *C. elegans,* or impacted the plates left to be scored. Heat stress or heat stress coupled with low dose ACR exposure may have decreased *C. elegans* fecundity with sexual reproduction. (Gómez-Orte et al., 2017)

However, it was estimated that the incubator malfunction may have occurred as soon as day six or as late as day eight. Our results suggest a trend that low dose ACR exposure may decrease fecundity in *C. elegans,* particularly with the 15 μM ACR concentration as seen with days one through three in Graph 5.

According to the toxicity screenings performed by Bora et al. with paraquat, their control's brood counts stopped around day seven, which is the trend observed in Graph 5 with our control group. (Bora et al., 2021)

3.2.1.B. Trial One Hermaphroditic Self-Reproduction Fecundity Assay



Graph 6: Low dose ACR exposure and possible heat stress may decrease fecundity in *C. elegans* hermaphroditic self-reproduction in trial one. Depicted is trial one hermaphroditic self-reproduction fecundity assay results from days one through seven. One age-synchronized Day-3 adult worm was transferred to ACR at a low dose of 10 µM, 15 µM, or 25 µM allowed to lay eggs for 24 hours before being transferred to a new MYOB plate. The plate was retained in a 22°C incubator and scored for brood three days later by grid under a dissecting microscope. Since three replicates were tested during this trial, three worms were examined per treatment. This procedure was repeated until the worms lost fertile embryo production, which was around day eight with the control group. Brood counts were averaged out between the plates of each treatment. The brood size average obtained for each treatment was compared to the untreated control. After performing a one-way ANOVA test in Excel, the p-value obtained was 0.50, which is not statistically significant, however, this may be due to such a small population observed.

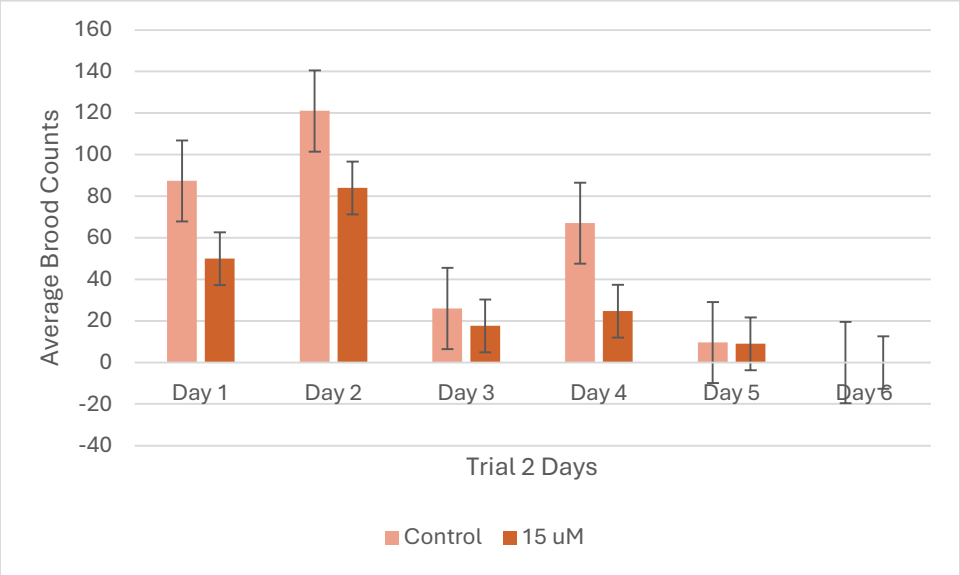
As mentioned before in the trial one lifespan assay, there was an incubator malfunction that may have induced heat stress on the experimental *C. elegans,* or impacted the plates left to be scored.

Heat stress or heat stress coupled with low dose ACR exposure may have decreased *C. elegans* fecundity with sexual reproduction. (Gómez-Orte et al., 2017)

However, it was estimated that the incubator malfunction may have occurred as soon as day six or as late as day eight. Our results suggest a trend that low dose ACR exposure may decrease fecundity in *C. elegans,* particularly with the 15 µM ACR concentration as seen throughout the entire trial in Graph 6. On day 3 of the hermaphroditic fecundity assay, no broods were found on any of the fecundity plates, including the control.

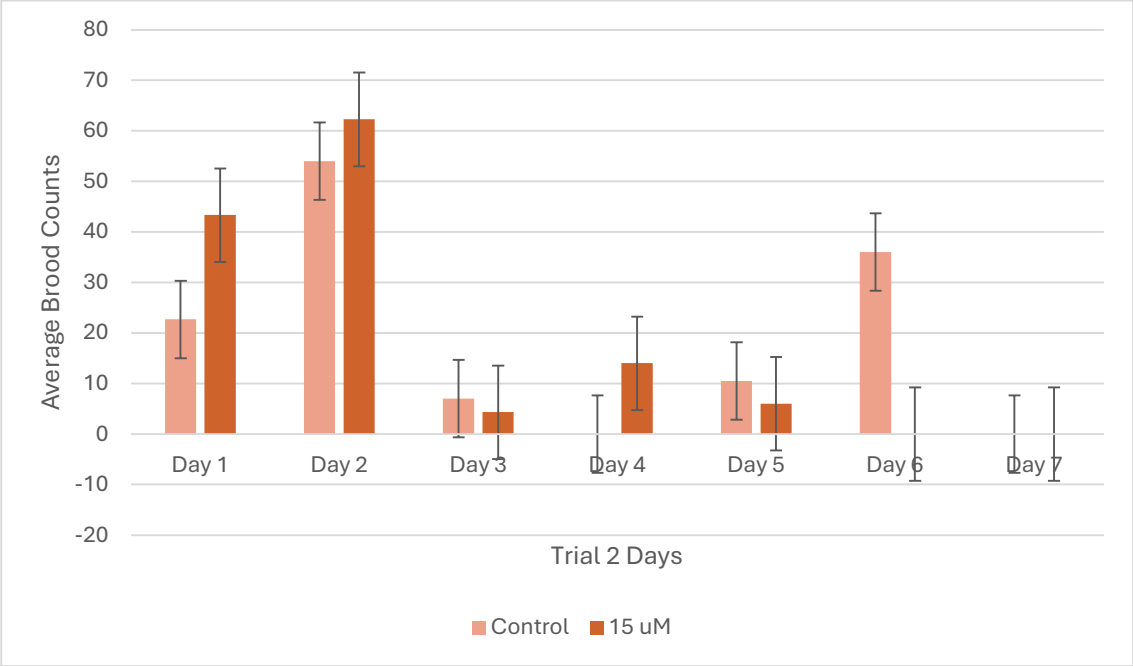
According to the toxicity screenings performed by Bora et al. with paraquat, their control's brood counts stopped around day seven, which is the trend observed in Graph 6 with our control group. (Bora et al., 2021) Bora's research group did not focus on hermaphroditic fecundity assays, but rather sexual reproduction between a hermaphrodite and male worm.

3.2.2.A. Trial Two Sexual Reproduction Assay



Graph 7: Chronic 15 µM ACR exposure decreases fecundity in C. elegans sexual reproduction in trial two. This graph depicts trial two sexual reproduction fecundity assay results from days one through six with a focus on 15 µM ACR treatment. Two age-synchronized Day-3 adult worms were allowed to lay eggs for 24 hours before being transferred to a new MYOB plate. The plate was retained in a 22°C incubator and scored for brood three days later by grid under a dissecting microscope. Since three replicates were tested during this trial, six worms were examined per treatment. This procedure was repeated until the worms lost fertile embryo production, which was around day six for the control group. Brood counts were averaged out between the plates of each treatment. The brood size average obtained for each treatment was compared to the untreated control. After performing a one-tailed T-test, the p-value obtained was 0.39, which is not statistically significant, however, this may be due to such a small population observed.

3.2.2.B. Trial Two Hermaphroditic Self-Reproduction Fecundity Assay

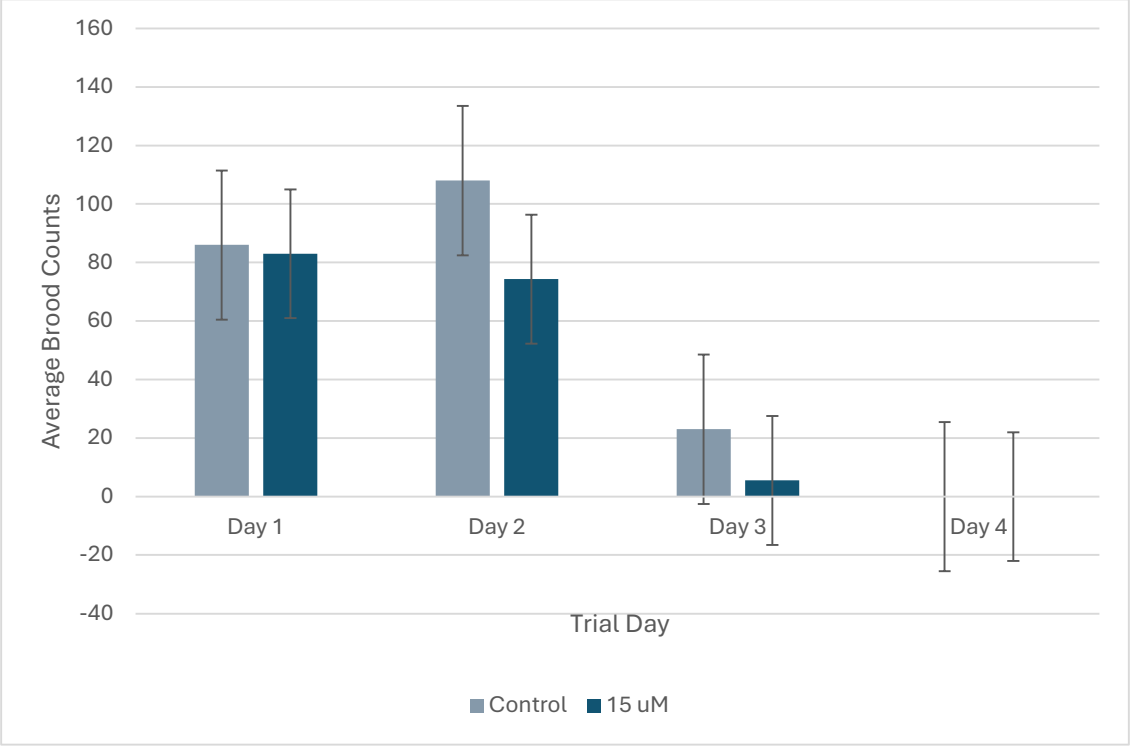


Graph 8: Chronic 15 µM ACR exposure does not impact hermaphroditic reproduction in C. elegans for trial two. Depicted is trial two hermaphroditic reproduction fecundity assay results from days one through seven with a focus on 15 µM ACR treatment. One age-synchronized Day-3 adult worm was transferred to a 15 µM ACR treated or untreated plate and

allowed to lay eggs for 24 hours before being transferred to a new MYOB plate. The plate was retained in a 22°C incubator and scored for brood three days later by grid under a dissecting microscope. Since three replicates were tested during this trial, three worms were examined per treatment. This procedure was repeated until the worms lost fertile embryo production, which was around day seven for the control group. Brood counts were averaged out between the plates of each treatment. The brood size average obtained for each treatment was compared to the untreated control. After performing a one-tailed T-test, the p-value obtained was 0.99 and therefore not statistically significant, however, this may be due to such a small population observed.

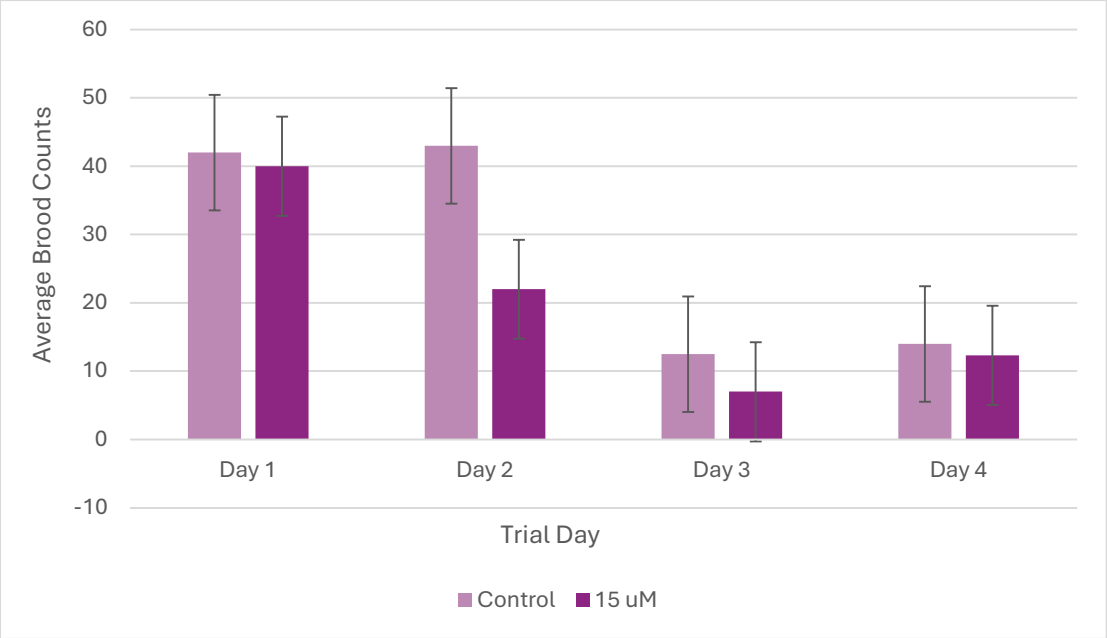
During trial 2, one control worm was not transferred from the hermaphroditic plate, which would explain the trend seen in Days 1 and 2 in Graph 8 that implies the control group has less brood than the 15 µM ACR treated group.

3.2.3.A. Trial Three Sexual Reproduction Fecundity Assay



Graph 9: Chronic 15 µM ACR exposure decreases fecundity in C. elegans sexual reproduction in trial 3. This graph depicts sexual reproduction fecundity assay results from days one through four with a focus on 15 µM ACR treatment. Two age-synchronized Day-3 adult worms were allowed to lay eggs for 24 hours before being transferred to a new MYOB plate. The plate was retained in a 22°C incubator and scored for brood three days later by grid under a dissecting microscope. Since three replicates were tested during this trial, six worms were examined per treatment. This procedure was repeated until the worms lost fertile embryo production, which was around day four. The brood size obtained for each treatment was compared to the untreated control. After performing a one-tailed T-test in Excel, the p-value obtained was 0.35, which is not statistically significant, however, this may be due to such a small population observed.

3.2.3.B. Trial Three Hermaphroditic Self-Reproduction Fecundity Assay



Graph 10: Chronic 15 µM ACR exposure decreases fecundity in C. elegans hermaphroditic reproduction in trial 3. This graph depicts trial three hermaphroditic reproduction fecundity assay results from days one through four with a focus on 15 µM ACR treatment. One age-synchronized Day-3 adult worm was transferred to a 15 µM ACR treated or untreated plate and allowed to lay eggs for 24 hours before being transferred to a new MYOB plate. The plate was retained in a 22°C incubator and scored for brood three days later by grid under a dissecting

microscope. Since three replicates were tested during this trial, three worms were examined per treatment. The brood size obtained for each treatment was compared to the untreated control. After performing a one-tailed T-test in Excel, the p-value obtained was 0.26, which is greater than 0.05 and therefore not statistically significant, however, this may be due to such a small population observed.

4.2.3.A. C. elegans Reproductive Method Impacts with ACR Based Off of Trial One Results

Comparisons of Brood Count Averages				
	Control	10 uM ACR	15 uM ACR	25 uM ACR
Sexual Reproduction	25	12	15	11
Hermaphroditic	23	10	14	18

Table 2: **Impacts of low dose chronic ACR exposure on *C. elegans* fecundity from trial one.** ACR exposure does not seem to impact the reproductive method used by *C. elegans* in trial one. The top row displays the average brood counts rounded to the nearest whole number yielded by *C. elegans* that had the potential to sexually reproduce. The bottom row displays the average brood count rounded to the nearest whole number yielded by *C. elegans* that did not have a partner, and therefore could only self-reproduce.

These results in table 2 suggest that acrolein does not impact the method of sexual reproduction between *C. elegans*. There is a seven-worm difference between the 25 μM acrolein treatments sexually reproductive and hermaphroditic reproductive worms.

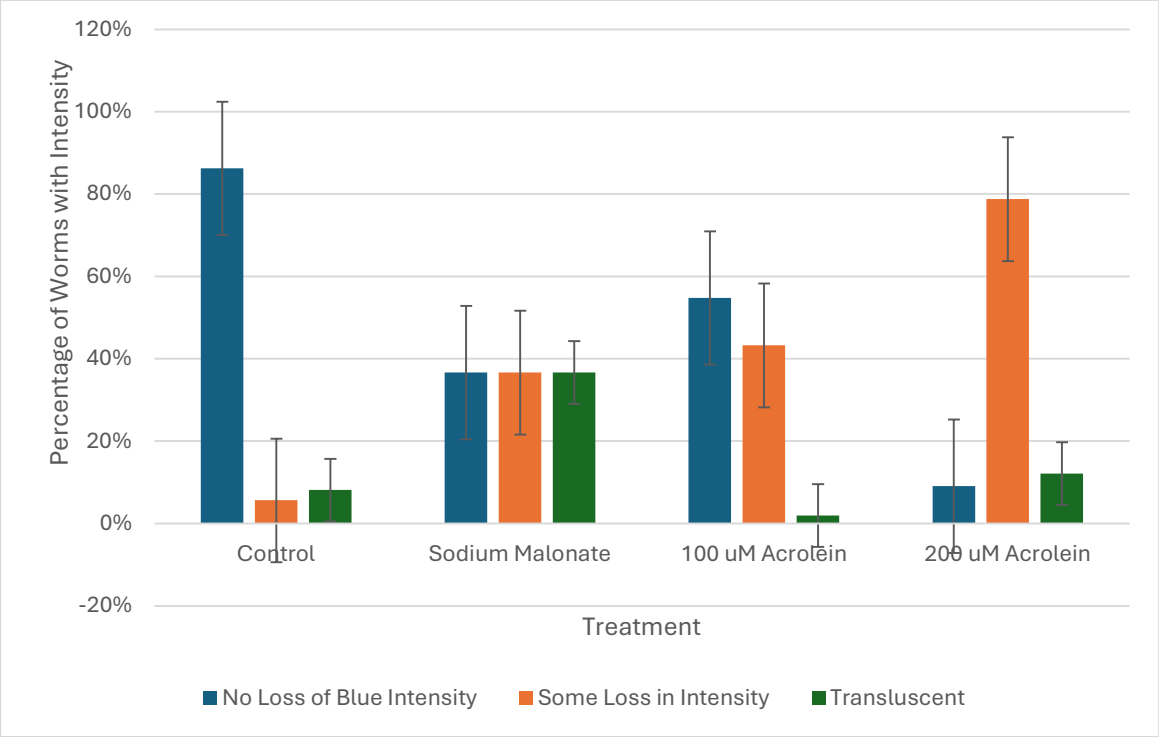
4.2.3.B. C. elegans Reproductive Method Impacts with ACR Based Off of All Three Trials

Comparisons of Brood Count Averages				
	Control	10 uM ACR	15 uM ACR	25 uM ACR
Sexual Reproduction	31	15	18	14
Hermaphroditic	27	13	16	23

Table 3: **Impacts of low dose chronic ACR exposure on *C. elegans* fecundity overview of all three trials.** ACR exposure does not seem to impact the reproductive method used by *C. elegans* comparing all three trials. The top row displays the average brood counts rounded to the nearest whole number yielded by *C. elegans* that had the potential to sexually reproduce. The bottom row displays the average brood count rounded to the nearest whole number yielded by *C. elegans* that did not have a partner, and therefore could only self-reproduce.

These results in table 3 suggest that acrolein does not impact the method of sexual reproduction between *C. elegans*. There is a nine-worm difference between the 25 μM acrolein treatments sexually reproductive and hermaphroditic reproductive worms.

3.3 SDH Activity Histochemical Stain



Graph 11: SDH activity decreases as ACR concentration increases with the histochemical stain. SDH activity histochemical stain blue stain intensity after addition of 20% sodium malonate, 100 µM ACR, and 200 µM ACR, or no additional chemical at all. Worms were qualitatively analyzed under a dissecting microscope with a grid and counted with a cell counter. If the worm were completely blue and did not lose blue color throughout its body, it would fall under the blue column of "no loss of blue intensity." If color were lost in any portion of the worm's body, it would fall under the "some loss in intensity" orange column. If no color were observed at all and the worm was completely translucent, the worm would fall under the "translucent" or gray column. Worms in the translucent category were dead before a sodium malonate or ACR treatment was administered.

Based on these results in graph 11, all three treatment groups worms exposed to either 20 µM sodium malonate or 100 µM or 200 µM ACR had some loss in blue color intensity compared to the control worms. The 200 µM ACR treatment had the most worms that lost some blue intensity compared to the other treatment groups.

3.4 SDH Activity Measured through Spectrophotometry

Sample (λ=500 nm)	Absorbance
Isolate Only (Start)	0.40
100 μM Acrolein (Start)	0.44
20% Sodium Malonate (Start)	0.43
100 μM Acrolein (5 Min.)	0.44
20% Sodium Malonate (5 Min)	0.44
Isolate Only (New RXN)	0.44
20% Sodium Malonate (New RXN)	0.44

Table 4: Absorbance table from spectrophotometric SDH activity assay results. The left-hand column "Sample" describes the group of worms, such as the control group with no reagent exposure, and treatment groups exposed to 20% sodium malonate or 100 μM ACR. Absorbances were taken from the time the treatment was administered, and these samples have the additional label of (start). The same samples were allowed to react for an additional five minutes before absorbance measurements were taken again, and these samples have the additional label of (5 min.) A new reaction tube was started for the control of isolate only and the 20% sodium malonate treatment, and these samples have the additional label of (New RXN). The right-hand column reads the absorbance of each sample's reaction cuvette at the wavelength of 550 nm.

Before the SDH activity assay was performed, a sample from the isolate collected from subcellular fractionation was ran through a Nanodrop to determine a protein concentration. The concentration was 2.784 mg/mL, with an A280 of 2.78, and an A260/A280 of 0.64.

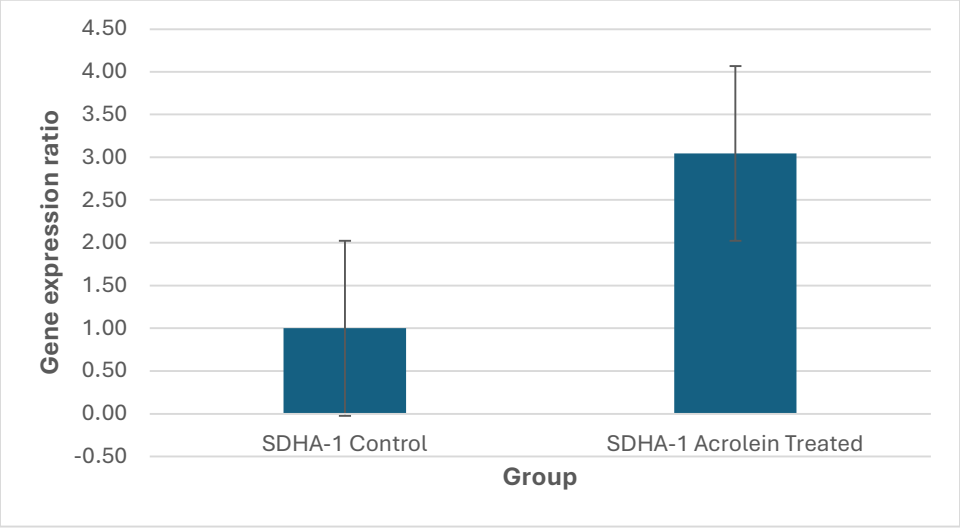
The first three samples had 10 μL of freshly prepared isolated mitochondrial protein, which would be rounded to 27 μg of protein as per the recommended method by Hench et al. The first sample prepared was the negative control of protein only. The second sample was the 100 μM ACR treatment after immediate addition of ACR. The third sample prepared was the positive control of 20% sodium malonate after the immediate addition of sodium malonate. Sample four

is the absorbance measurement for 100 µM ACR treatment after five minutes have passed. Sample five is the absorbance for 20% sodium malonate after five minutes have passed. Sample six is the absorbance of protein isolate only. Sample seven is the absorbance of a freshly prepared negative control of 20% sodium malonate.

3.5 Reverse Transcription Polymerase Chain Reaction (rt-PCR)

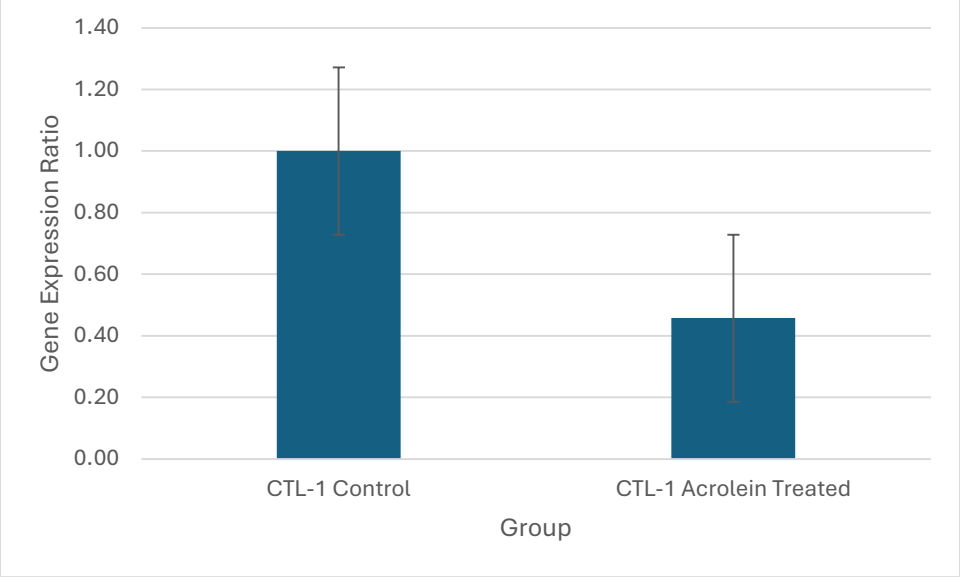
CTL-2 and SOD-3 were run together on a separate 96-well plates using 100 ng of cDNA in the RT-PCR reaction tubes. SDHA-1 and CTL-1 were run together on one 96-well plate using 200 ng of cDNA in the RT-PCR reaction tubes.

The Ct and ΔCt values for the genes, SDHA-1, CTL-1, CTL-2, and SOD-3 were collected from the BioRad CFX Manager© program and inserted into Excel for analysis to obtain a gene expression ratio, also called a relative fold change. A gene is considered significantly upregulated when the gene expression ratio is a positive value and has a difference in value of at least 2.0. A gene is considered significantly downregulated when the gene expression ratio is a negative value and has a difference in value of at least 2.0.



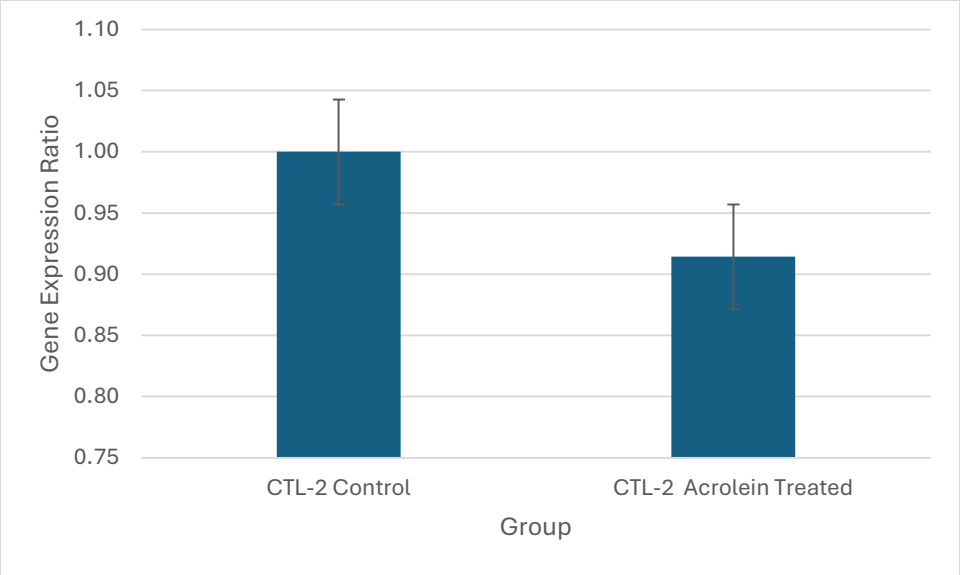
Graph 12: SDHA-1 is upregulated in ACR treated worms compared to untreated worms. The untreated control worms have a gene expression ratio of 1.00 while the ACR treated worms have an average gene expression ratio of 3.05. Not depicted are the individual SDHA-1 gene expression ratios for 15 µM ACR and 100 µM ACR treatments which were 2.11 and 3.98, respectively.

The control's average cycle threshold (Ct) value was 27.92, the 15 µM ACR treatment's average Ct value was 24.35, and the 100 µM ACR treatment's average Ct value was 20.92.



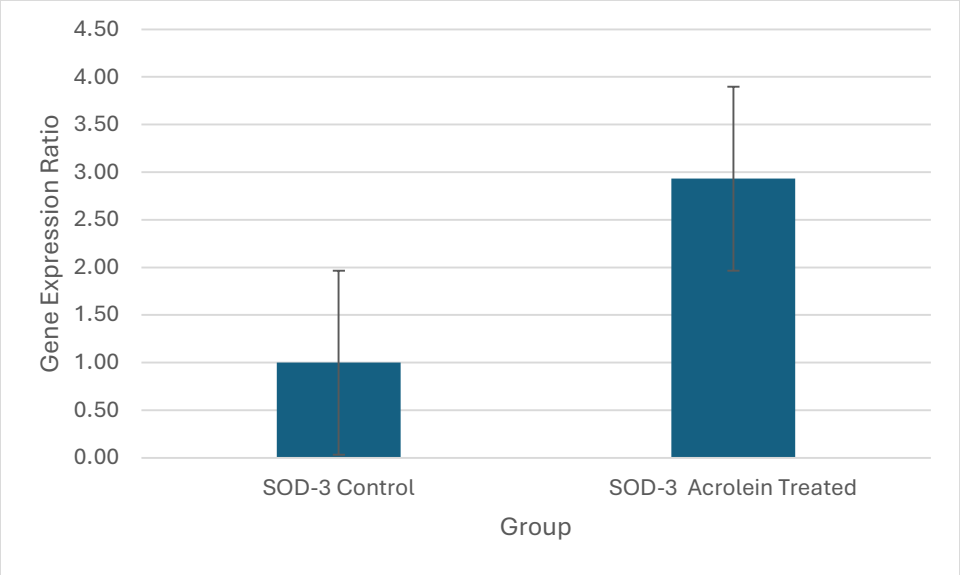
Graph 13: There was no downregulation of CTL-1 in ACR treated worms compared to untreated worms. The untreated control worms have a gene expression ratio of 1.00 while the ACR treated worms have an average gene expression ratio of 0.46. Not depicted are the individual CTL-1 gene expression ratios for 15 µM ACR and 100 µM ACR treatments which were 0.88 and 0.03, respectively.

The control's average Ct value was 32.24, the 15 µM ACR treatment Ct value was 31.24, and the 100 µM ACR treatment average Ct value was 36.19.



Graph 14: There was no downregulation of CTL-2 in ACR treated worms compared to untreated worms. The untreated control worms have a gene expression ratio of 1.00 while the ACR treated worms have an average gene expression ratio of 0.66. Not depicted are the individual CTL-2 gene expression ratios for 15 µM ACR and 100 µM ACR treatments which were 0.25 and 1.58, respectively.

The control's average Ct value was 32.24, the 15 µM ACR treatment Ct value was 29.85, and the 100 µM ACR treatment average Ct value was 25.38.



Graph 15: SOD-3 is upregulated in ACR treated worms compared to untreated worms. The untreated control worms have a gene expression ratio of 1.00 while the ACR treated worms have an average gene expression ratio of 2.93. Not depicted are the individual SOD-3 gene expression ratios for 15 µM ACR and 100 µM ACR treatments which were 0.40 and 5.46, respectively.

The control's average Ct value was 32.24, the 15 µM ACR treatment Ct value was 33.14, and the 100 µM ACR treatment average Ct value was 26.84.

Chapter IV: Discussion

We hypothesized that *C. elegans* would experience mitochondrial dysfunction when chronically exposed to low doses of ACR, which may lead to SDH inhibition as well as a decrease in *C. elegans* lifespan and reproduction. Based on the results of our fecundity assays, the SDH histochemical assay, and rt-PCR run, our findings support our hypothesis. Based on results obtained from the lifespan assays, chronic 15 μM ACR exposures do not impact lifespan in *C. elegans*. However, a combination of heat stress and chronic low dose ACR exposures in the concentration ranges of 10 μM and 25 μM ACR may decrease *C. elegans* lifespan.

4.1 Lifespan Assays

There was an overall trend that chronic 15 μM ACR exposures do not impact the lifespan of *C. elegans.* However, our first trial raised questions about the impact of heat stress induced by higher temperature and chronic, low dose ACR exposure impacting *C. elegans* lifespan. The incubator malfunction only occurred during the first trial and was not repeated.

There was no statistical significance with our lifespan assay results either, but this may have been due to such a small population observed for this toxicity screening. There should have been at least 50 worms per replicate observed. However, this would make transferring the worms between plates difficult, especially when fecundity assays required us to keep the plates for three days to allow *C. elegans* to hatch. Methodically, lifespan and fecundity assays should have been conducted separately.

Before the low dose lifespan assays were conducted, higher ACR concentrations were considered, such as 50 μM, 75 μM, and 100 μM. After transferring worms to these concentrated

plates, it was difficult to find them. Worms tend to burrow into agar, seeming to disappear from the plate. However, these plates were deliberately poured thin and with a thicker agar concentration to accommodate for burrowing worms and to maintain eggs at the agar surface for easier brood counts for the fecundity assays. Originally, ten worms were transferred to each plate via worm pick and stainless-steel wire, then parafilmed to secure the plate and protect against contamination. However, these higher concentration worms were difficult to account for the next day during lifespan counts. After closer inspection of the plate, worms could be found on the sides of the plate trying to escape from the 75 μM and 100 μM concentrations, as depicted in figure 10 below.

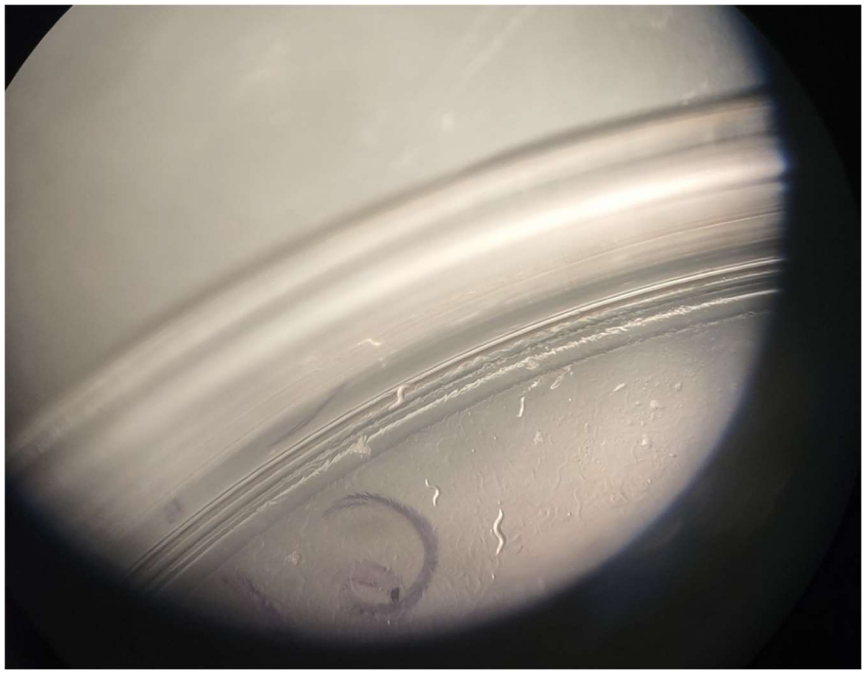


Figure 10: Dissecting microscope image of *C. elegans* attempting to leave the MYOB agar plate at 75 μM ACR concentration.

This supports the research Simmons conducted in 2008, considering ACR as a nematocidal replacement for methyl bromide. Soil that was treated with ACR concentrations of 100 mg/kg or

higher lacked the herbivorous *C. elegans* strains of nematode. With Hong et al.'s recovery experiments exposing worms to ACR concentrations ranging from 12.5 μM to 100 μM for 48-hours, their results suggested that lifespan was impacted based on dosage and was shortened by approximately 19% and 40% in the 50 μM and 100 μM concentrations compared to the control, and reproduction was also decreased in worms exposed to 50 μM and 100 μM concentrations around day 20 of their 30-day trials. Within the first 24-hours of our trial, worms were already clinging to the plate walls, the lid, or even trapped within the parafilm that was wrapped around the outside of the agar plate. Since it was a small-scale trial, with only ten worms, it was easy to notice the worms trying to escape. There are chemicals that may be used to treat plates to prevent worms from leaving, such as palmitic acid. However, it is not recommended to treat plates with other chemicals while performing toxicity screenings.

4.2 Fecundity Assays

There is a trend observed with our data that low dose ACR exposure decreases the fecundity of *C. elegans* in both reproductive methods compared to the untreated control worms. It does not appear to impact which method of reproduction, such as hermaphroditic self-reproduction or sexual reproduction between a male and hermaphrodite.

Another trend observed that for trial one and two, *C. elegans* stopped yielding brood after day seven, and if trial 3 continued, may have stopped reproducing by day seven as well. This is very similar to the data collected from the low dose paraquat studies conducted by Bora et. al, with their control yielding brood until day seven.

4.3 SDH Activity Histochemical Stain

The SDH activity stain utilized histochemistry and colorimetry to offer qualitative data. Nitro blue tetrazolium chloride (NBT) measures redox potential in the *C. elegans* mitochondria. If NBT is reduced by superoxide anions or SDH into diformazan, then the stain changed from a yellowish color to a dark blue, which meant that SDH was active. Activity levels correlated with a higher blue intensity, whereas a lower blue intensity correlated with less SDH activity. It was observed that the 20 μM sodium malonate and 100 μM ACR treatments had the most worms that held a less intense blue color compared to the negative control of no treatment.

Brightfield light microscopy may have detected blue intensity in the *C. elegans* that were deemed "translucent". The light source of the dissecting microscope used shined light onto the worms, rather than through the worms, to help detect differences in color intensity. This may have impacted the results for a loss of intensity instead of considered dead.

4.4 RT-PCR

RT-PCR helped us determine the regulation of genes involved in the transcription of the antioxidant pathway or the ETC protein complex, SDHA-1. A gene expression ratio, also known as the relative fold difference, tells us about the upregulation and downregulation of a gene. A gene is considered upregulated when the gene expression ratio is a positive value greater than 1.5. A gene is considered downregulated when the gene expression ratio is a negative value greater than 1.5. The ΔCt value tells us the difference in gene expression between a control and a treatment.

Our rt-PCR results show upregulation in the SDHA-1 gene when exposed to 15 μM and 100 μM ACR for a 24-hour period. The 15 μM ACR treatment had a gene expression ratio of 2.11, and

the 100 μM ACR treatment had a gene expression value of 3.98. This upregulation suggests that SDHA may have been damaged or inhibited during the 24-hour period of ACR treatment.

CTL-1 and CTL-2 are genes that transcribe catalases 1 and 2, which are enzymes involved in the antioxidant pathway. Under oxidative stress, it may be presumed that these genes would be upregulated to scavenge hydrogen peroxide in the cytosol or within the mitochondria. For CTL-1, the control's gene expression ratio was 1.00, the 15 μM ACR treatment's ratio was 0.88, and the 100 μM ACR's treatment gene expression ratio was 0.03. These gene expression ratios are not greater than 1.5 and are therefore not considered downregulated. For CTL-2, the control's gene expression ratio was 1.00, the 15 μM ACR treatment's ratio was 0.25, and the 100 μM ACR's treatment gene expression ratio was 1.58. These gene expression ratios are not greater than 2.0 and are therefore not considered downregulated.

However, our findings support the results from Hong et al.'s ACR recovery research, which show a decrease in CTL-1 and CTL-2 transcription with 48-hour 100 μM ACR exposure, as seen in figure 35 below. However, their calculated gene expression ratios were not significant with a value of 2.0 or greater. They speculated that high dose exposure to ACR generated excessive ROS that disrupted DAF-16 and the antioxidant defense system and impacted CTL-1 and CTL-2 enzyme transcription to scavenge the excessive hydrogen peroxide in the cytosol, which may have induced more ROS generation.

It may also be speculated that the main ROS generated from ACR may not have been hydrogen peroxide, but rather superoxide anion, which is neutralized by SOD rather than CTL. Our results show that there was an upregulation of SOD-3 in our ACR treated worms compared to the

untreated control worms. The untreated control worms have a gene expression ratio of 1.00 while the ACR treated worms have an average gene expression ratio of 2.93.

Superoxide anions are not commonly generated from Complex II under normal physiological conditions. However, if Complex II is already damaged from oxidative stress or inhibited, the solubility of this protein complex increases due to its location in the IMM. The chance of superoxide generation would occur when complex II's FAD site is fully reduced, and the CoQ site is unable to bind electrons because it is already fully saturated. The electrons may react with molecular oxygen or other ROS present in the mitochondrial matrix, which may set off a chain reaction to generate more superoxide anions through lipid peroxidation, especially since the IMM consists of polyunsaturated fatty acid tails. (Craig et al., 2012; Stuart, 2009)

Reverse electron transfer (RET) between complex I and complex II may be the highest source of ROS generation in the mitochondria. RET occurs when succinate is the substrate, adenosine diphosphate (ADP) levels are low and ubiquinol levels are high, which reduces the electron flow. The electrons will reverse their flow from complex II back to complex I's CoQ binding site to react with the ubiquinol present. The reversal of electron flow will in turn reduce SDHA, as well as reduce the electron carrier NAD+ to NADH. With both SDHA's and NADH's redox centers reduced, if enough molecular oxygen is present within the mitochondrial matrix, superoxide anion will be generated and may further propagate ROS by catalyzing lipid peroxidation in the inner mitochondrial membrane. (Craig et al., 2012; Stuart, 2009)

4.5 Spectrophotometric SDH Activity Assays

For the spectrophotometric assay that was conducted during our research, SDH (Complex II) activity was measured by the reduction of cytochrome c. Succinate was used to inhibit SDHA from reducing the citric acid cycle electron carrier, FAD, and instead pass electrons along the ETC to cytochrome c. Cyanide was used to inhibit Complex IV activity, so the reaction measured was focused on SDH's activity and ability to pass electrons along to complex III. PMSF was added to the 0.1 M Tris-Sulfate Buffer (pH 7.4) to maintain the membrane potential so that the electron transport chain could continue to pass on electrons despite inhibited protein complexes within the chain. Higher absorbance readings correlated with higher reduction levels of cytochrome c, therefore a higher level of activity in complex II with passing electrons along to complex III.

Our SDH activity assay results in figure 12 are inconclusive based on the absorbance levels being similar in the presence of the positive control with protein isolate only, the positive control of 20% sodium malonate, and the 100 μM ACR treatment. With the Nano Drop concentration reading of 2.784 mg/mL, it is plausible that different organelles may have been isolated out, such as the endoplasmic reticulum or the Golgi apparatus. Endoplasmic reticulum contamination is common when utilizing differential centrifugation to isolate mitochondria. (Williamson et al., 2015) Unfortunately, the yield of protein isolate is small if only utilizing less than ten plates of *C. elegans*. This makes protein concentration determination and purification steps difficult, if not possible when performing the activity assay after isolation.

Since chemical lysis with subtilisin A was utilized, there was a high chance that the isolate collected was a mixture of cellular proteins. Subtilisin A is a serine protease that hydrolyzes peptide bonds between serine residues of any protein, and not selectively mitochondria. (Barrett,

Rawlings, & Woessner, 2012) The higher mechanical force of ultracentrifugation at 100,000 x G may be a preferred method over chemical lyses for mitochondrial isolation, however, the sample collected is not guaranteed to be a pure mitochondrial sample. Once the cell is lysed, the cellular components separate out based on molecular weight when centrifugal force is applied. Kuzmin, Levchenko, & Pliss utilized micro-Raman-BCA analysis to quantify the molecular weights of the nucleolus, mitochondria, and endoplasmic reticulum in HeLA and WI-38 cell lines. They discovered molecular weights varied not only between both cell lines, but within each cell line as well. They estimated the molecular weights of the nucleolus proteins to range between 50 mg/ml to 120 mg/ml, the mitochondrial proteins to range between 35 mg/ml to 110 mg/ml, and the endoplasmic reticulum proteins to range between 20 mg/ml to 120 mg/ml. Based on their findings, there is an overlap in molecular weight between these organelles. Cellular heterogeneity, which is the dynamics and interactions between the different cell types in a eukaryotic organism, is essential for life and is a key reason cells and cellular components vary between organisms. (Kuzmin, Levchenko, & Pliss, 2017)

Chapter V: Future Directions

Further investigation into ACR's interactions with SDHA may help determine if protein-adduct formation occurs between ACR and SDHA, or if ACR induces oxidative stress which results in damage or impairment of SDHA function. A Western blot performed on lysed *C. elegans* after an acute high dose exposure of ACR may help determine if protein-adduct formation occurred between SDHA and ACR. This is based on results and images from the Western blot obtained from Aitkens' lab consisting of 4-HNE forming a protein adduct with human spermatozoa SDHA. The suspected functional group involved with protein adduct formation between 4-HNE and SDHA is the carbonyl group. Both ACR and 4-HNE contain a highly reactive α, β-unsaturated aldehyde, and since ACR is a smaller molecule, it could be possible that ACR would have a higher affinity compared to 4-HNE to bind to the SDHA's FAD site. A flavonoid colorimetric assay kit may help measure oxidative stress induced on a flavonoid protein.

If spectrophotometric assays of mitochondrial isolates were to be conducted again, it would be beneficial to try and use multiple plates to yield at least five grams of wet worm pellet mass. This may yield higher chances of mitochondrial proteins in isolates. Ultracentrifugation with speeds of 100,000 x G may be a preferred method over chemical cell lysis for subcellular fractionation.

If lifespan and fecundity assays were to be performed again, some modifications recommended include, but are not limited to: focusing fecundity assays to the sexual reproduction method and testing several worms per replicate; utilizing liquid culture rather than solid agar plates because of how volatile ACR is; and including mutant strains of *C. elegans*, such as an SDHA-2 mutant strain to see if there is any sensitivity to the ACR treatment. Lifespan and fecundity assays should be performed separately and not with the same plates of worms. Use a larger population

of worms, such as at least 50 worms per replicate, to account for any random error that may occur during experimentation.

It may be beneficial to see the impact higher temperatures and low dose acrolein exposure have on C. elegans lifespans as well. Our first trial of lifespan assays left questions as to whether heat stress and low dose ACR exposure compounded decreases the *C. elegans* lifespan.

RT-PCR has offered valuable insight into gene regulation of SDH and the antioxidant enzyme, CTL-1 after high dose, acute exposure of acrolein. It may be beneficial to investigate the impacts acrolein may have on the *C. elegans* second copy of SDHA, which was SDHA-2. It may also be worth repeating the RT-PCR experiment at different time intervals to narrow down an exposure time to acrolein that causes CTL-1 to be downregulated and SDHA to be upregulated.

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