EFFECTS OF SODIUM BENOZATE ON FAT DEPOSITION AND GROWTH IN *CAENORHABDITIS ELEGANS*

By: Leia Lewis

July 2019

Director of Thesis: Xiaoping Pan, PhD

Major Department: Department of Biology

Clinical observation has proposed the linkage between the occurrence of obesity and slow weight loss with daily exposure to common food additives such as sodium benzoate. However, well-controlled laboratory experiments on this topic have been lacking; the dose-response relationship of sodium benzoate and obesity occurrence and/or slow weight loss has not been established; the potential mechanism of such linkage has not been explored. The insulin-signaling and fatty acid synthesis pathways in the model organism *Caenorhabditis elegans* (*C. elegans*) are highly conserved with higher organism including humans and thereby has been widely utilized to study obesity and aging related mechanisms. Scientific literature links the insulin pathway of *C. elegans* to growth, development, longevity, behavior, and metabolism in the organism. *C. elegans* is also a perfect model for exploring the genetics of fat storage. Thereby, *C. elegans* was used as a model organism to study the effects of sodium benzoate exposure on fat storage and on the gene expression of major players in insulin signaling and fatty acid synthesis pathway, as well as, key nuclear hormone receptor genes. This study demonstrated that *C. elegans* growth was greatly affected over time with exposure to sodium benzoate. This study could not provide a clear conclusion about the effects of sodium benzoate on fat deposition. Sodium benzoate

exposure also led to the down-regulation of one nuclear hormone receptor gene, and two genes involved in fatty acid synthesis. After 72 hours of exposure, all treatment groups were larger than the control group, with the highest treatment causing significantly more growth. After 72 hours of exposure all treatment groups showed higher fluorescence values than the control group, with the lowest treatment group demonstrating significantly higher fluorescence levels than the control group. Three genes of interest were significantly downregulated after 24 hours exposure: nhr-50, fat-1, and elo-5. These findings indicate that sodium benzoate exposure is significantly impacting the model organism *C. elegans* and could be significantly impacting humans as well.

EFFECTS OF SODIUM BENOZATE ON FAT DEPOSITION AND GROWTH IN *CAENORHABDITIS ELEGANS*

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirement for the Degree

Master of Science in Molecular Biology and Biotechnology

by

Leia Lewis

July 2019

Effects of Sodium Benzoate on Fat Deposition and Growth in *Caenorhabditis elegans*

By: Leia Lewis

APPROVED BY:

DIRECTOR OF

THESIS: _____

Xiaoping Pan, PhD

COMMITTEE MEMBER: _____

Baohong Zhang, PhD

COMMITTEE MEMBER: _____

David Collier, MD, PhD

CHAIR OF THE DEPARTMENT OF BIOLOGY: _____

Cindy Putnam-Evans, PhD

DEAN OF THE GRADUATE SCHOOL: _____

Paul J. Gemperline, PhD

Table of Contents

List of Tables

List of Figures

CHAPTER 1: INTRODUCTION

Obesity is now recognized as a major problem around the world. According to the World Health Organization (WHO) in 2014 39% of the adults, aged 18 and over, were considered overweight, and 13% were considered obese [13]. In 2014 the WHO estimated that 67.3% of adults in the United States of America were considered overweight [8]. According to information published by the Centers for Disease Control and Prevention (CDC), based on information gathered from 2011-2014, it is estimated that 36.5% of adults in the United States of America, aged 20 and older, were considered obese [11]. Individuals are identified as being overweight if their body mass index (BMI) is ≥25, and they are identified as obese if their BMI is ≥30, and BMI is calculated as weight in kilograms divided by height in meters squared [10]. Obesity and being overweight are major risk factors for chronic diseases, such as: cancer, cardiovascular diseases, and diabetes [10]. A “chemical obesogen” hypothesis states that synthetic substances are contributing to the global epidemic of obesity, and intentional food additives are largely unstudied with regards to their effects on overall metabolic homeostasis [14]. Despite growing momentum regarding the chemical obesogen movement, evidence-based research to support the movement is greatly lacking, and tools and models from toxicology should be adopted [14]. A common food preservative that is greatly understudied at this time, regarding its potential effects on obesity, is sodium benzoate.

Sodium benzoate is a colorless powder that is crystal-like in nature. It is commonly used as a food preservative, antiseptic, medicine, in tobacco, in pharmaceutical preparations, as an intermediate for manufacture of dyes, and as a rust and mildew inhibitor [15]. Sodium benzoate is used as a preservative in such foods as sauces, pickles,

cider, fruit juices, wine coolers, syrups and concentrates, mincemeat, margarine, egg powder, fish, bottled carbonated beverages, fruit preservatives, jams and jellies, and it is added directly to human food [15]. The demand of sodium benzoate had increased due to the popularity of diet drinks [15]. Currently, the US Food and Drug Administration limits the amount of sodium benzoate in food to 0.1% [3]. Sodium Benzoate is currently considered generally safe as a food additive [3]. However, there have been few recent studies examining its potential effects on obesity. The U.S. Environmental Protection Agency (EPA) does not currently have an established limit on sodium benzoate [2]. The EPA does have a published oral Reference Dose (RfD) for sodium benzoate, which is 328 mg/day [2]. However, this RfD is "is based on the assumption that thresholds exist for certain toxic effects such as cellular necrosis" [2]. "In general, the RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime" [2]. It should also be noted that the RfD value is based on a report published by the FDA in 1973 and the value has not been adjusted since 1988 [2]. Based on the lack of recent information regarding the potential effects of sodium benzoate on obesity, a model organism should be used to examine potential effects.

Caenorhabditis elegans is considered a model organism for many reasons. It is an inexpensive, and time-efficient model because of its reduced lifespan. Traditionally rodents have been used in obesity studies, however, using the *C. elegans* will reduce the number of rodents and higher animals required, and this model can also serve as in important intermediate step before moving to higher organisms [16]. *C. elegans* is a small

nematode that does not require approval by Animal Care and Use Committees [16]. The Wild-type of this organism reaches adulthood within 38-46 hours after hatching, and the lifespan is approximately 21 days [6]. The worms have a generation time of 3 days and produce large numbers of offspring, with about 300 children per hermaphrodite [16]. Also, *C. elegans* was the first organism to have its genome completely sequenced, and more than 65% of human genes related to disease are conserved in the organism [16]. This organism is also a model for obesity studies due to its transparent body, and fat stores mainly within the hypodermal and intestinal cells that are easily stained with lipid affinity dyes, such as Nile Red [16]. These fluorescent dyes can easily be imaged through the transparent body of the worms [16]. Furthermore, *C. elegans* has highly conserved genes that regulate fat metabolism, and a major example is the sterol response element binding protein (SREBP) [9]. It also has conserved behavioral responses to satiety and starvation, which makes is a reasonable model system to show how feeding behavior is regulated by fat metabolism [9]. The effects of sodium benzoate on this model's fat deposition were not previously known.

Studies about sodium benzoate's effect on obesity are minimal. One study published in the *British Journal of Nutrition* found that food additives, such as sodium benzoate, decrease the release of leptin from adipocytes in a state of chronic inflammation, which is associated with obesity [4]. A decrease in "leptin release during the consumption of nutrition-derived food additives would decrease the overall amount of circulating leptin and could thereby influence food intake and contribute to an obesogenic effect" [4]. Patients who are deficient in leptin cannot control feeding and as a result become severely obese, which indicates that a single gene mutation may be enough to

affect feeding behavior [9]. Leptin is released from adipose tissue and helps to signal the brain to reduce feeding, which indicates a communication between fat stores and the brain, and the communication between fat storage and the brain is likely conserved in other animals [9]. While *C. elegans* does not contain leptin, there is likely a 'fat to brain' communication that is evolutionarily conserved but has not been discovered [9]. *C. elegans* stores fat in the form of triglycerides in the intestine or hypodermis, and this storage method is common in many animals, including humans, and is considered to be an ancient way of storing fat before a designated fat tissue evolved [9]. The organism's conserved genes that regulated fat metabolism, along with its conserved behavioral responses to starvation and satiety make it a good genetic model for studying how fat metabolism regulates feeding behavior [9]. Nuclear hormone receptors (NHRs) are a family of transcriptional factors, and many of these regulate metabolism in response to changes in nutritional inputs, and *C. elegans* contains about 7 times as many NHRs as mammals [9]. Some of the NHRs in *C. elegans* are involved in dauer formation [9]. The dauer is a dormant stage that the worms enter when environmental conditions are not favorable, and an example of this is starvation [9]. The dauer stage of the worms is tightly linked to an animal's metabolic status because the worms enter that stage when it lacks nutrient signals like insulin [9]. Studies show that *C. elegans* NHRs serve conserved functions to regulate metabolism in response to changes in nutritional input, like those of animals [9]. One study showed that some NHRs helped to regulate fat storage and satiety in the worms [9]. The same study showed that a conserved fatty acid synthesis pathway regulates satiety in *C. elegans* [9]. Unlike Leptin the NHRs are conserved in both mammals and *C. elegans* and play critical roles in metabolism, often acting as receptors

for steroid or lipid hormones [9]. The study found that certain NHRs regulate feeding and fat storage which suggests that NHRs might mediate leptin-like response in invertebrate animals, however it should be noted that it is unknown whether NHRs regulate feeding behavior [9].

Based on these previous findings, it was hypothesized that sodium benzoate exposure will induce increased fat storage in the model organism *C. elegans* at specific concentrations, due to an effect on NHRs that mediate leptin-like responses. These concentrations were 0.05% Sodium Benzoate, 0.1% Sodium Benzoate, and 0.5% Sodium Benzoate. Research in this area in extremely important due to a lack of current information on the subject. During this study, *C. elegans* will be exposed to various levels of sodium benzoate, their body fat stores will be stained using Nile Red dye, and the fat stores will be monitored over the course of their lifespan. Nile Red is a lipophilic dye that is derived from Nile Blue [12]. It becomes concentrated when in hydrophobic environments when used invitro or in cell lines [12]. When Nile Red is in a hydrophobic environment the dye undergoes an increase in yellow-gold fluorescence, which makes it a useful indicator of lipid droplets in cells [12]. Gene expression analysis was also performed on 30 genes of interest, including: key genes from the insulin signaling pathway, genes important for fatty acid synthesis, and genes that encode for NHRs previously shown to be upregulated during starvation and downregulated during refeeding [9]. The genes of interest and their known functions are displayed in Table 1 [5].

Table 1. Genes of Interest. The table below shows genes of interest that encode for Nuclear Hormone Receptors, key players in the insulin signaling pathway, and genes involved in fatty acid synthesis. A brief description of functions of these known genes is provided.

Genes	Functions
1. nhr-170 (regulate fat storage and satiety quiescence)	Nuclear hormone receptor that was found to regulate fat storage and satiety quiescence in some manner.
2. nhr-206 (regulate fat storage and satiety quiescence)	Nuclear hormone receptor that was found to regulate fat storage and satiety quiescence in some manner and is an ortholog of human HNF4G (hepatocyte nuclear factor 4 gamma) and HNF4A (hepatocyte nuclear factor 4 alpha); nhr-206 is involved in response to starvation.
3. nhr-21 (regulate fat storage and satiety quiescence)	Nuclear hormone receptor that was found to regulate fat storage and satiety quiescence in some manner.
4. nhr-64 (regulate fat storage and satiety quiescence)	Encodes a conserved nuclear receptor that is a member of the NR2 subfamily of nuclear receptors that contains Drosophila and human HNF4 and a Nuclear hormone receptor that was found to regulate fat storage and satiety quiescence in some manner.
5. nhr-8 (regulate fat storage and satiety quiescence)	Nuclear hormone receptor that was found to regulate fat storage and satiety in some manner.
6. nhr-50 (regulate fat storage and satiety quiescence)	Nuclear hormone receptor that was found to regulate fat storage and satiety in some manner.
7. nhr-18	Nuclear hormone receptor gene encodes a member of the superfamily of nuclear receptors, which is one of the most abundant class of transcriptional regulators.
8. nhr-120 (regulate fat storage and satiety quiescence)	Nuclear hormone receptor that was found to regulate fat storage and satiety in some manner.
9. nhr-49	Nuclear hormone receptor that functions as a key regulator of fat metabolism and lifespan by regulating induction of beta-oxidation genes upon food deprivation and activation of stearoyl-CoA desaturase in fed animals, respectively.
10. nhr-162 (regulate fat storage and satiety quiescence)	Nuclear hormone receptor that was found to regulate fat storage and satiety in some manner.

11. nhr-212 (regulate fat storage and satiety quiescence)	Nuclear hormone receptor that was found to regulate fat storage and satiety in some manner.
12. daf-2	Encodes a receptor tyrosine kinase that is the C. elegans insulin/IGF receptor ortholog and DAF-2 signals through a conserved PI 3-kinase pathway to negatively regulate the activity of DAF-16, a Forkhead-related transcription factor, by inducing its phosphorylation and nuclear exclusion; in addition, DAF-2 negatively regulates the nuclear localization, and hence transcriptional activity, of SKN-1 in intestinal nuclei; amongst the 38 predicted insulin-like molecules in C. elegans, genetic and microarray analyses suggest that at least DAF-28, INS-1, and INS-7 are likely DAF-2 ligands; genetic mosaic and tissue-specific promoter studies indicate that daf-2 can function cell nonautonomously and within multiple cell types to influence dauer formation and adult lifespan, likely by regulating the production of secondary endocrine signals that coordinate growth and longevity throughout the animal; temporal analysis of daf-2 function indicates that daf-2 regulates lifespan, reproduction, and diapause independently, at distinct times during the animal's life cycle.
13. daf-18	Encodes a lipid phosphatase homologous to the human PTEN tumor suppressor (OMIM:601728, mutated in Cowden disease and several cancers); DAF-18 negatively regulates insulin-like signaling mediated by DAF-2/IR and AGE-1/PI3K and thus plays a role in metabolism, development, and longevity; based on sequence and genetic analysis, DAF-18 is predicted to dephosphorylate AGE-1-generated PIP3 in order to limit activation of the downstream AKT-1 and AKT-2 kinases that negatively regulate DAF-16.
14. daf-16	Encodes the sole C. elegans forkhead box O (FOXO) homologue; DAF-16 functions as a transcription factor that

	acts in the insulin/IGF-1-mediated signaling (IIS) pathway that regulates dauer formation, longevity, fat metabolism, stress response, and innate immunity.
15. DAF 7/ TGF-ß	Encodes a member of the transforming growth factor beta superfamily; in C. elegans, DAF-7 functions as part of a signaling pathway that interprets environmental conditions to regulate energy-balance pathways that affect dauer larval formation, fat metabolism, egg laying, pathogen avoidance behavior, and feeding behavior.
16. DAF-3	Encodes a co-SMAD protein that is most closely related to Drosophila Medea and the vertebrate Smad4 proteins; DAF-3 functions as a transcriptional regulator that is required for formation of the alternative dauer larval stage as well as for regulation of pharyngeal gene expression during non-dauer development; DAF-3 activity is antagonized by signaling through the DAF-7/TGF-beta pathway which promotes reproductive growth.
17. AGE-1/ PI3K	Encodes the C. elegans ortholog of the phosphoinositide 3-kinase (PI3K) lying downstream of the DAF-2/insulin receptor and upstream of both the PDK-1 and AKT-1/AKT-2 kinases and the DAF-16 forkhead type transcription factor, whose negative regulation is the key output of the insulin signaling pathway; in accordance with its role in insulin signaling, AGE-1 activity is required for regulation of metabolism, life span, dauer formation, stress resistance, salt chemotaxis learning, fertility, and embryonic development.
18. AKT-1	Encodes an ortholog of the serine/threonine kinase Akt/PKB; akt-1 genetically interacts with the insulin signaling pathway and functions to regulate such processes as dauer larval

	development and salt chemotaxis learning.
19. tub-1	Encodes a TUBBY homolog that affects fat storage across species including C. elegans.
20. FAT-5	Encodes a delta-9 fatty acid desaturase that is predicted to be mitochondrial.
21. FAT-6	Encodes an Acyl-CoA desaturase (also known as delta-9 fatty acid desaturase).
22. pod-2	Encodes an acetyl-CoA carboxylase; by sequence similarity, POD-2 is predicted to catalyze the first step in de novo fatty acid biosynthesis.
23. sbp-1	Encodes a basic helix-loop-helix (bHLH) transcription factor homologous to the mammalian Sterol Regulatory Element Binding Proteins (SREBPs, OMIM:184756, overexpression of nuclear form of SREBP-1c is associated with insulin resistance and features of congenital generalized lipodystrophy); in C. elegans, SBP-1 activity is required for normal lipid metabolism and wild-type levels of lipogenic enzyme expression, as well as for embryonic and larval development; in addition, sbp-1 is required for mediating the lipid accumulation and increased body width/length ratio that occurs in response to oxygen deprivation; sbp-1 expression is detected in the intestine, the major site of fat storage, from early embryogenesis through adulthood and also in the amphid neurons.
24. FAT-2	Encodes a delta-12 fatty acyl desaturase that increases membrane fluidity when expressed in yeast, and that may protect against cold stress; FAT-2's substrate is unknown, but may be acyl-CoA; FAT-2 is required in vivo for normal levels of C18 or C20 polyunsaturated fatty acids (PUFAs).
25. FAT-1	Encodes an omega-3 fatty acyl desaturase that acts on substrates of 16-20 carbons with a preference for omega-6 fatty acids; FAT-1 dehydrogenates its

	substrates 3 carbons from the methyl terminus; FAT-1 is required in vivo for n3 polyunsaturated fatty acid (PUFA) synthesis.
26. FAT-3	Encodes a delta-6 fatty acid desaturase ('linoleoyl-CoA desaturase') containing an N-terminal cytochrome b5 domain that is orthologous to the vertebrate fatty acid desaturases (FADS).
27. ELO-5	Gene encodes a paralog of elo-1 and elo-2, each of which encodes a polyunsaturated fatty acid (PUFA) elongase; ELO-5 is required for normally rapid growth and for normal fatty acid composition.
28. ELO-1	Encodes a component of C-18 polyunsaturated fatty acid (PUFA) elongase that is required for normal elongation of n-6 and n-3 20-carbon PUFA in vivo; ELO-1 is a condensing enzyme that elongates n-6 and n-7 (and, with less efficiency, n-3) series C18 PUFAs.
29. ELO-2	Encodes a palmitic acid elongase, homologous to polyunsaturated fatty acid (PUFA) elongases such as ELO-1, that is required for normally rapid growth, normally large body size, fertility, and for the quantitative regulation of the ultradian defecation rhythm; the joint action of ELO-2 with ELO-1 is strongly required for 20-carbon PUFA production and for general viability.
30. ELO-6	Encodes a paralog of elo-1 and elo-2, each of which encodes a polyunsaturated fatty acid (PUFA) elongase; ELO-6 may be required for a normally high growth rate.

CHAPTER 2: MATERIALS AND METHODS

2.1- Research Design and Methods

The worms were grown on Nematode Growth Medium (NGM), because this is the standard medium for the worms. The worms were grown on 6cm petri plates. Wild-Type, N2, *C. elegans* were used. For treatment with sodium benzoate, 80µL sodium benzoate of various concentrations were added to the top of the growth medium for the worms to consume. These concentrations were of 0.05%, 0.1%, and 0.5%. These percentages were chosen based on the FDA standard limit of 0.1%, with 0.05% reflecting a lower test dosage and 0.5% reflecting a higher test percentage [3]. A positive control of 0.5% glucose was also used because glucose at this level has been shown in previous studies to increase fat storage in *C. elegans* [7]. A negative control plate containing only OP50 was also used. Before the test worms are plated age- synchronization will be performed to ensure all worms are in the same developmental stage. The worms will be fed OP50, a strain of *E. Coli* that is not harmful, which is a standard food source for *C. elegans.* To stain the fat stores of the worms Nile Red was used. This method was chosen because it is a common fat staining method for *C. elegans*. A ZEISS Z1 AxioObserver fluorescent microscope with be used to examine fat deposition. The expression profiles of 30 genes of interest will be examined using qRT-PCR.

2.2- Synchronization

The worms were washed with 7mL of a standard M9 solution from the plate and added to a 15 mL centrifuge tube. The worms were then centrifuged at 2000rpm for 2 minutes.

The supernatant was removed, and the worms were suspended again in 7mL of M9. The worms were then centrifuged again, the supernatant was removed, and 7 mL of M9 was added to the tube. The worms were then centrifuged again. The supernatant was removed and a solution containing 1400 µL bleach, 700 µL sodium hydroxide, and 5mL water was added to a centrifuge tube containing adult worms and eggs. The tube was shaken until the adult worms dissolved, leaving behind only eggs. The eggs were then centrifuged, and the bleach solution was removed. The worms were then rinsed with 7mL of M9 and centrifuged. The M9 supernatant was removed and fresh M9 is added to the tube. More M9 was added to the tube and the washing process was repeated two more times. The worms were then placed on a shaker in the incubator at 20°C for 18 hours to allow the worms to hatch. Since there is no food source in the tube, the worms all remained in the same phase of development, the L1 phase. The worms were then plated on treatment plates, or control plates. This process is important to ensure the worms are all in the same stage of development.

2.3- Sodium Benzoate Treatments

Wild Type N2 worms, that were grown as previously described, were gathered from NGM plates and synchronized, as described previously. Five treatment plates were then prepared using 6 cm NGM plates. To prepare the treatment plates 80 µL of 0.05% sodium benzoate was added to plate 1, 80 µL of 0.1% sodium benzoate was added to plate, 80 µL of 0.5% sodium benzoate was added to plate 3, 80 µL of 0.5% glucose was added to plate 4 to serve as a positive control, and no sodium benzoate was added to plate 5 to serve as a negative control. The plates were allowed to dry and then 80 µL of OP50 was

added to each plate and allowed to dry. Then 80 µL of Nile Red dye with a concentration of 0.25 µg/mL was added to each of the plates and allowed to dry. After the plates dried, the synchronized L1 worms were distributed approximately equally among the 5 prepared plates to begin the exposure. Each treatment plate was replicated three times for a total of fifteen plates. Worms were then chosen at random from each plate and mounted on 2 % agarose slides. The worms were then examined using fluorescence microscopy at 24 hours exposure, 48 hours exposure, and 72 hours exposure. Zeiss ZEN Blue Software was used to determine the body area, length, and fluorescence intensity for each worm. Each worm was manually outlined and measured using the microscope software. After the worm is outlined, the software generates the area and fluorescence intensity value of the worm automatically. To determine the length of each worm, a line was drawn down the center of the worm from its head to its tail. Once the line is drawn the software automatically generates the length measurement.

2.4- Nile Red Staining

Nile Red powder (N-1142 Molecular Probes) was dissolved in acetone at 500 µg/ml, diluted in 1X phosphate buffered saline (PBS) and added on top of nematode growth media (NGM) plates already seeded with OP50, to a final concentration of 0.25 µg/ml [12]. Worms were placed on the treatment plates as starved L1s from the synchronization. The staining phenotypes were then assessed at various exposure times including: 24 hours, 48 hours, and 72 hours using fluorescence microscopy with a ZEISS Z1 AxioObserver microscope. The worms were mounted on 2% agarose slides and photographed. Changes in body fat deposition were examined.

2.5- Calculating Total Fluorescence

Using ImageJ© software, total fluorescence can be calculated. Using an Excel spreadsheet, image fluorescence can be calculated using measurements obtained through ImageJ©. Once the application is open, the area of interest is selected using the drawing tools. From the Analyze menu, set measurements must be selected, and area integrated intensity and mean grey value selected should be selected in that menu. The area of interest in then measured. After the area of interest is measured, 3 separate background areas are also selected and measured. This eliminates any background noise. After the measurements are taken they can be inserted into the following formula in an Excel Spreadsheet: CTCF = Integrated Density – (Area of selected cell *x* Mean fluorescence of background readings). The CTCF was then standardized based on size. The CTCF value was divided by each worm's size to standardize the measurements.

2.6- Isolation of RNA

RNA was isolated using the following protocol. Worms were collected from 3 control plates and 3 0.1% sodium benzoate treatment plates to serve as 3 biological replicates. Worms were then frozen at -80°C until RNA extraction was performed. RNA isolation was then performed using the following protocol. 400 µL Lysis/ Binding Buffer was added into each 2mL centrifuge tube containing worms on ice. Tubes were sonicated for 15-10 seconds using Ultrasonic Convertor on ice. 40 µL of miRNA Homogenate Additive was added to the tissue lysate and mixed well by inverting the tube several times. The mixture was left on ice for 10 minutes. 400 µL of Acid-Phenol:Choloform was then added to each

tube. The tubes were vortexed for 30-60 seconds to mix. The tubes were centrifuged for 5 minutes at 10,000 x g at room temperature. The aqueous phase was recovered and transferred to a fresh tube. 375 µL of room temperature 100% ethanol was added to the aqueous phase and mixed thoroughly. For each sample, a filter cartage was placed into a collection tube. The lysate/ethanol mixture was pipetted into the filter cartridge. Up to 700 µL can be applied at a time. The tubes were centrifuged for 15 seconds at 10,000 x g to pass the mixture through the filter. The flow-through was discarded and the process was repeated until lysate/ ethanol mixture was through the filter. 700 µL miRNA Wash Solution 1 was applied to the filter cartridge and centrifuged for 10 seconds. The flow-through was discarded and the filter cartridge was replaced. 500 µL Wash solution 2/3 was applied and the tubes were centrifuged for 10 seconds. The flow-through was discarded. This was step was repeated. The filter cartridge was replaced, and the tubes were spun for 1 minute to remove residual fluid from the filter. The filter cartridge was transferred to a fresh collection tube and 50 µL of pre-heated (95°C) nuclease-free water was applied to the center of the filter. The cap was closed, and the tubes were spun for 30 seconds at 10,000 x g to recover RNA. The RNA sample was gently mixed, and the RNA concentration was measured using the Nano-Drop 1000. RNA quality of each tube was measured using the NanoDrop ND-1000 Micro-Volume UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and based on the absorbance ratios of 260/280 and 260/230.

2.7- RT-PCR using TaqMan® MicroRNA Reverse Transcription Kit

After NanoDrop analysis of the RNA samples, RT-PCR was performed using a TaqMan® MicroRNA Reverse Transcription Kit. 6 new 0.5 ml microfuge tubes were collected. Several calculations were made to determine how much nuclease free water, RNA sample, and master mix would be added to each the microfuge tubes. 1 µg of total RNA per 15 µL RT reaction was used. This amount is calculated based on the RNA concentration. The master mix contained calculated volumes of components provided by the kit (RNase inhibitor, 100 mM dNTPs, 10X Reverse Transcription Buffer, Multiscribe™ Reverse Transcriptase, and primer mix (not provided in kit)). After each tube received calculated volumes of nuclease-free water, RNA sample, and the master mix, the tubes were mixed gently and centrifuged for 10 seconds (200 rpm). The tubes were then incubated on ice for 5 minutes and loaded into the thermal cycler for reverse transcription. The thermal cycler process includes 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and will hold at 4°C. After RT-PCR, each tube, now containing RT product or cDNA, was removed from the thermal cycler and each tube received 80 µl of DNase-free water. The tubes were then mixed by vortexing and immediately stored at -20°C until needed for qRT-PCR.

2.8- qRT-PCR

Previously made RT-PCR products and purchased SYBR® Green dye were placed on ice to thaw. The previous products were further diluted using Nuclease-free water. Using 6 96-well microplates, following a written template, I loaded each individual well with 7 µL RNase DNase-free water, 10 µL SYBR® Green dye, and 1 µL RT-PCR product. I then loaded each well with 2 µl of primer solution (1 µl forward and 1 µl reverse) specific to the

genes of interest. The gene act-1 was used as a reference gene. For each RT-PCR product there were 3 technical replicates. Once the plate was fully loaded it was covered and sealed tightly with a film to prevent evaporation of any well samples. The plate was then centrifuged to ensure each sample mixture is settled on the bottom of each well. Next, the plate was loaded into the qRT-PCR machine to undergo one 10-minute cycle at 95°C (enzyme activation), and 45 PCR cycles including 15 seconds at 95°C (denaturation of DNA), and 60 seconds at 60°C (DNA annealing and extension).

2.9- Statistical Analysis

For statistical analysis, the IBM SPSS Statistics 25 software for Windows 7 was used. To determine statistical differences between treatment and control groups in area, length, intensity mean fluorescence, corrected total cell fluorescence, and gene expression fold changes the statistical test analysis of variance (ANOVA) was used. Least significant difference (LSD) multiple comparisons were carried out to compare means among groups. The significance level for all analysis performed was $p < 0.05$.

CHAPTER 3: RESULTS

3.1- Impact of Sodium Benzoate on Growth

Sodium Benzoate treated one and a half-day old N2 worms had an average area of: 7832.78 μm^2 for the negative control, 7530.15 μm^2 for the 0.05% treatment, 6175.70 μm^2 for the 0.1% treatment, 9546.90 μm^2 for the 0.5% treatment, and 9537.24 μm^2 for the 0.05% glucose positive control (Fig 1). There was a significant difference in body size when comparing the positive control group to all other treatment groups. The 0.5% sodium benzoate treatment group and the 0.5% glucose group (positive control) were statistically larger than the control group. The 0.1% sodium benzoate treatment group was statistically smaller than the control group and the 0.05% sodium benzoate treatment group was not statistically different in size than the control group($p<0.05$).

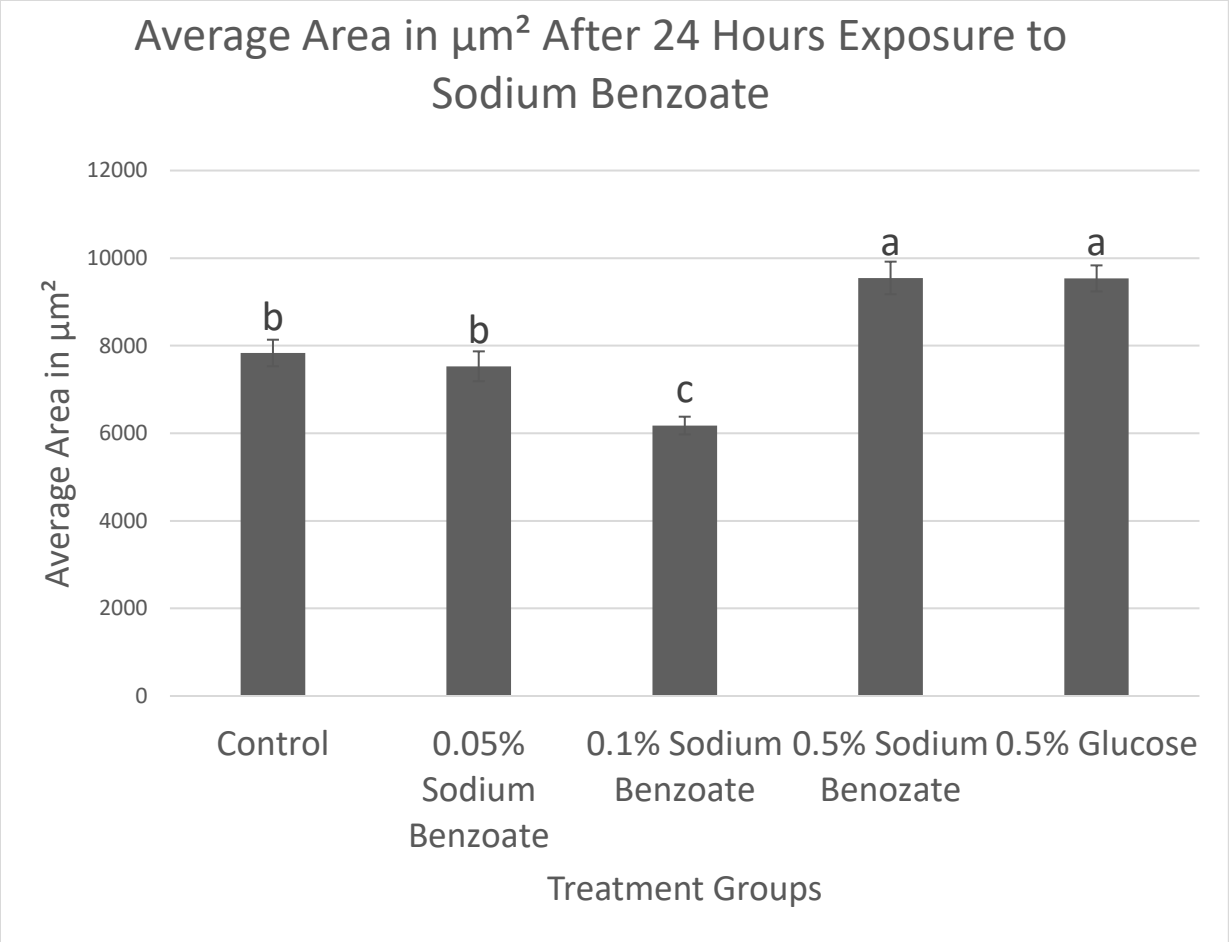


Figure 1. Average size following 24 hours of exposure. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =14, $N_{0.05\% \text{ Sodium Benzoate}}$ =12, $N_{0.1\% \text{ Sodium Benzoate}}$ =18, $N_{0.5\% \text{ Sodium Benzoate}}$ =20, and $N_{0.5\% \text{ Glucose}}$ =32, $p < 0.05$.

Sodium Benzoate treated one and a half-day old N2 worms had an average length of: 377.28µm for the negative control, 360.37µm for the 0.05% treatment, 334.14µm for the 0.1% treatment, 395.17µm for the 0.5% treatment, and 386.20µm for the 0.05% glucose positive control (Fig 2). There was not a significant difference in body length when comparing the negative control group to the 0.05% sodium benzoate group or the 0.5% glucose group. There was also not a statistically significant difference in body length

when comparing the negative control group to the 0.05% sodium benzoate treatment group. However, the 0.05% treatment group was significantly smaller than both the 0.5% sodium benzoate group and the 0.5% glucose group. The 0.1% sodium benzoate group was significantly smaller than all other groups ($p<0.05$).

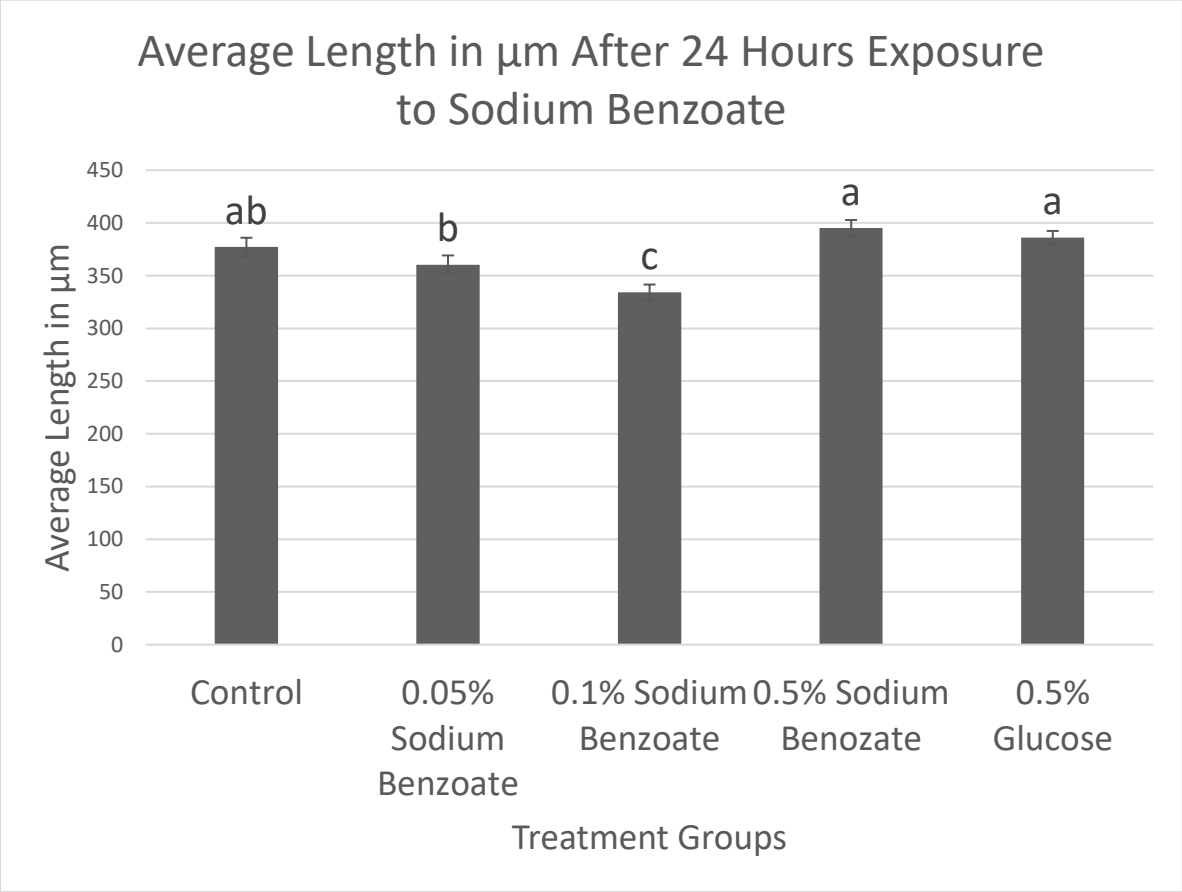


Figure 2. Average length following 24 hours of exposure. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =14, $N_{0.05\%\ Sodium\ Benzoate}$ =12, $N_{0.1\%\ Sodium\ Benzoate}$ =18, $N_{0.5\%\ Sodium\ Benzoate}$ =20, and $N_{0.5\%\ Glucose}$ =32, $p< 0.05$.

Sodium Benzoate treated two and a half-day old N2 worms had an average area of: 20389.50µm^2 for the negative control, 20759.94µm^2 for the 0.05% treatment,

28124.0μm^2 for the 0.1% treatment, 27284.0μm^2 for the 0.5% treatment, and 28535.80μm^2 for the 0.05% glucose positive control (Fig 3). There was a significant increase in body size in the 0.1% sodium benzoate group, the 0.5% sodium benzoate group, and the 0.5% glucose group, when compared the control. There was not a statistical difference in body size between the control group and the 0.05% sodium benzoate group ($p<0.05$).

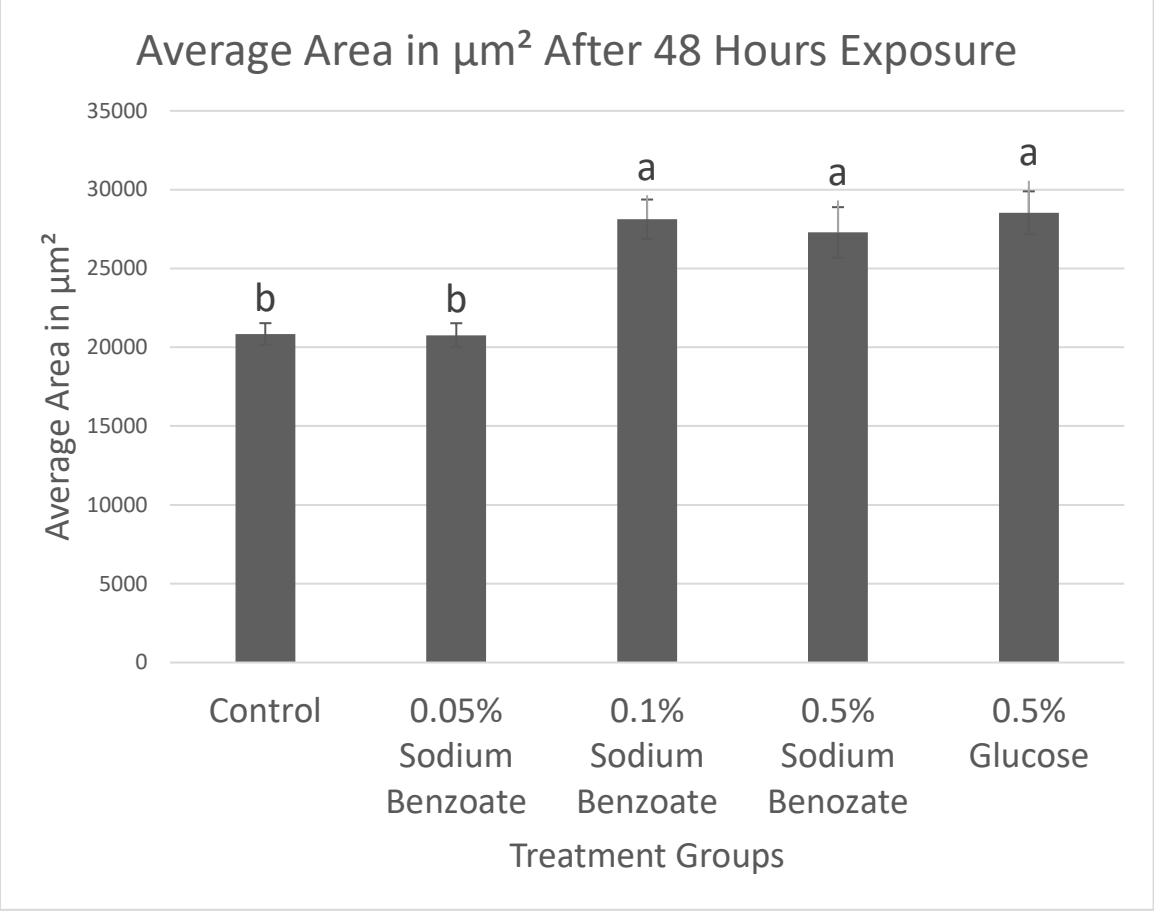


Figure 3. Average body area of N2 worms 48 hours after dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =35, $N_{0.05\%\ Sodium\ Benzoate}$ =33, $N_{0.1\%\ Sodium\ Benzoate}$ =31, $N_{0.5\%\ Sodium\ Benzoate}$ =21, and $N_{0.5\%\ Glucose}$ =33, $p< 0.05$.

Sodium Benzoate treated two and a half-day old N2 worms had an average length of: 564.09µm for the negative control, 605.36µm for the 0.05% treatment, 692.98µm for the 0.1% treatment, 658.54µm for the 0.5% treatment, and 651.70µm for the 0.05% glucose positive control (Fig 4). There was a significant increase in length across all treatment groups when compared to the negative control group. The 0.1% sodium benzoate treatment group, 0.5% sodium benzoate treatment group, and the 0.5% glucose group were also statistically longer than the 0.05% sodium benzoate treatment group ($p<0.05$).

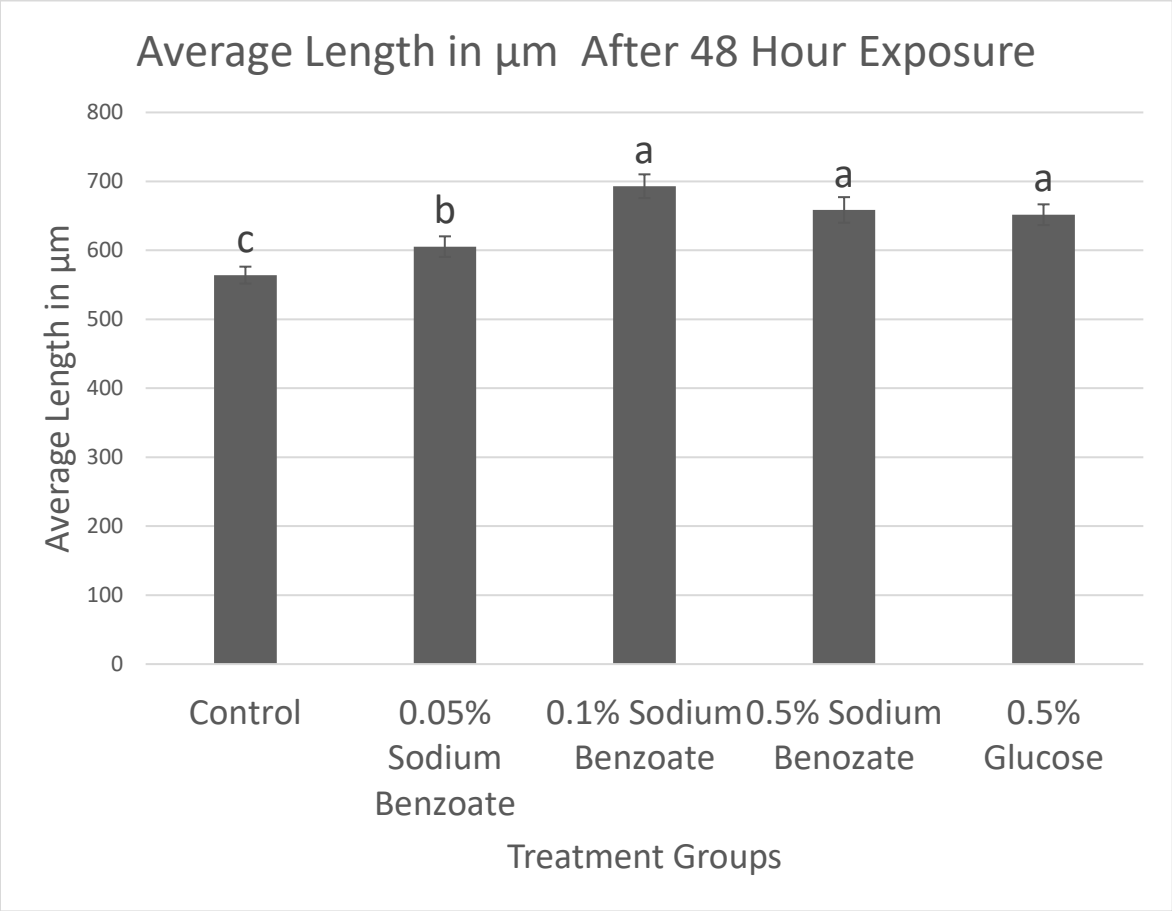


Figure 4. Average body length of N2 worms 48 hours after dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =35, $N_{0.05\% \text{ Sodium Benzoate}}$ =33, $N_{0.1\% \text{ Sodium Benzoate}}$ =31, $N_{0.5\% \text{ Sodium Benzoate}}$ =21, and $N_{0.5\% \text{ Glucose}}$ =33, $p< 0.05$.

Sodium Benzoate treated three and a half-day old N2 worms had an average area of: 44645.34μm^2 for the negative control, 46009.49μm^2 for the 0.05% treatment, 53475.04μm^2 for the 0.1% treatment, 74953.68μm^2 for the 0.5% treatment, and 57614.24μm^2 for the 0.05% glucose positive control (Fig 5). The 0.5% sodium benzoate treatment group was significantly larger than all other treatment groups. The 0.5% glucose treatment group was significantly larger than both the control and 0.05% sodium benzoate treatment groups, and there was not a statistical difference compared to the 0.1% sodium benzoate group. The control group was not statistically different in size when compared to the 0.05% sodium benzoate group or the 01% sodium benzoate group ($p<0.05$).

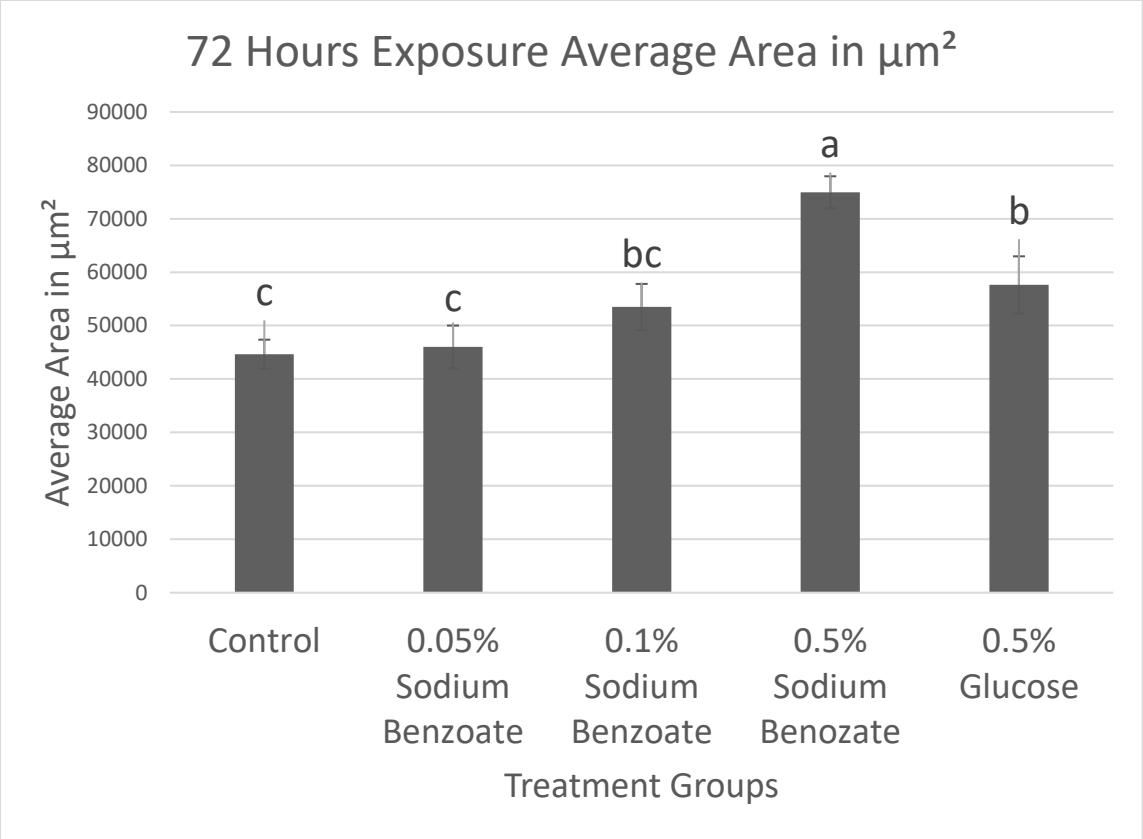


Figure 5. Average body area of N2 worms 72 hours after dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =20, $N_{0.05\%\ Sodium\ Benzoate}$ =32, $N_{0.1\%\ Sodium\ Benzoate}$ =21, $N_{0.5\%\ Sodium\ Benzoate}$ =21, and $N_{0.5\%\ Glucose}$ =18, $p < 0.05$.

Sodium Benzoate treated three and a half-day old N2 worms had an average length of: 785.39µm for the negative control, 816.51µm for the 0.05% treatment, 870.15µm for the 0.1% treatment, 1042.11µm for the 0.5% treatment, and 890.82µm for the 0.05% glucose positive control (Fig 6). There was a significant difference in body length in the 0.5% sodium benzoate treatment group when compared to all other groups ($p<0.05$).

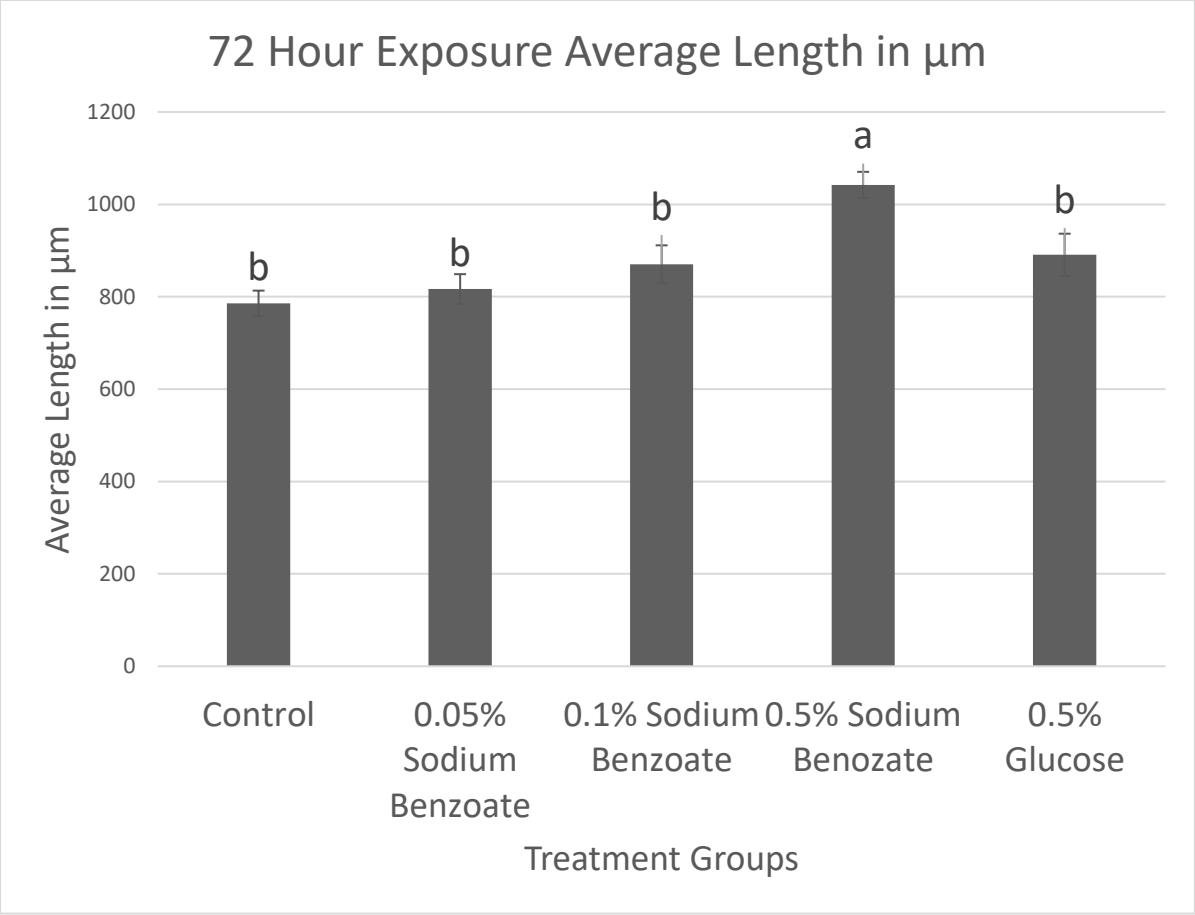


Figure 6. Average body length of N2 worms 72 hours after dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =20, $N_{0.05\%\ Sodium\ Benzoate}$ =32, $N_{0.1\%\ Sodium\ Benzoate}$ =21, $N_{0.5\%\ Sodium\ Benzoate}$ =21, and $N_{0.5\%\ Glucose}$ =18, $p < 0.05$.

Over the period of 72-hour exposure, the 0.5% sodium benzoate group was much larger that the negative control group. While they were not statistically larger, all other treatment groups were also larger than the control worms at the end of 72 hours (Fig 7).

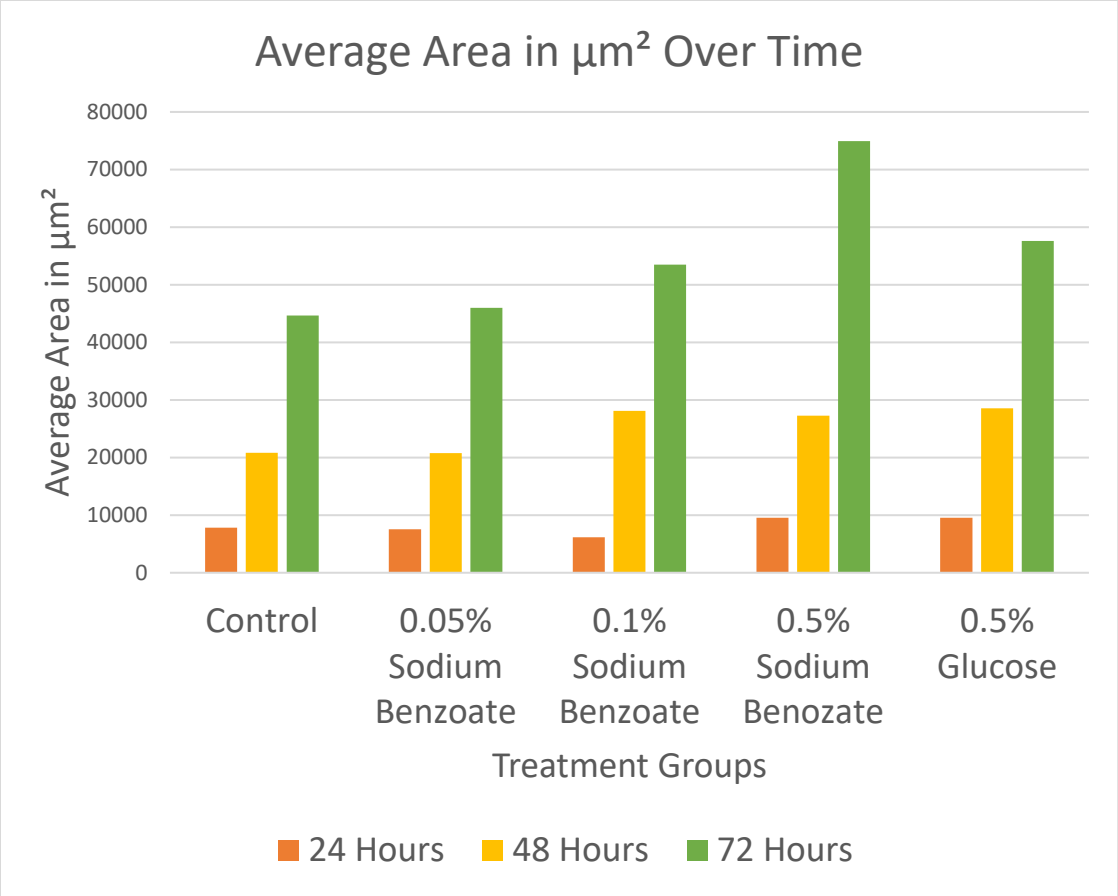


Figure 7. Average body area of the worms over the course of the 72-hour exposure.

The average body length of the worms was similar across all treatment groups at 24 hours with the 0.05% treatment group and the 0.1% treatment group being slightly smaller. At 48 hours the control and 0.05% treatment group had similar lengths, however the 0.1% treatment group, 0.5% treatment group, and the 0.5% glucose groups were all longer. At the end of 72 hours the 0.5% treatment group was much larger than the other groups and all other treatment groups were similar in size (Fig 8).

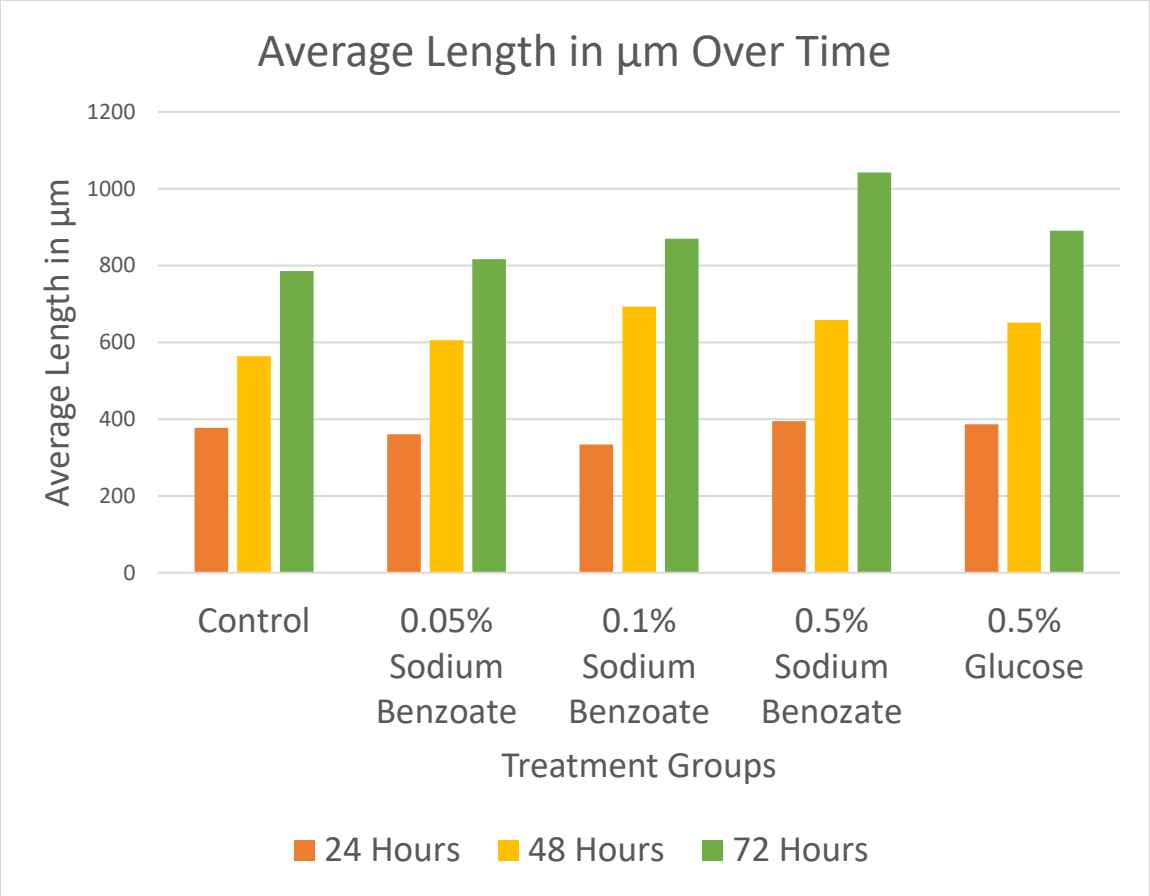
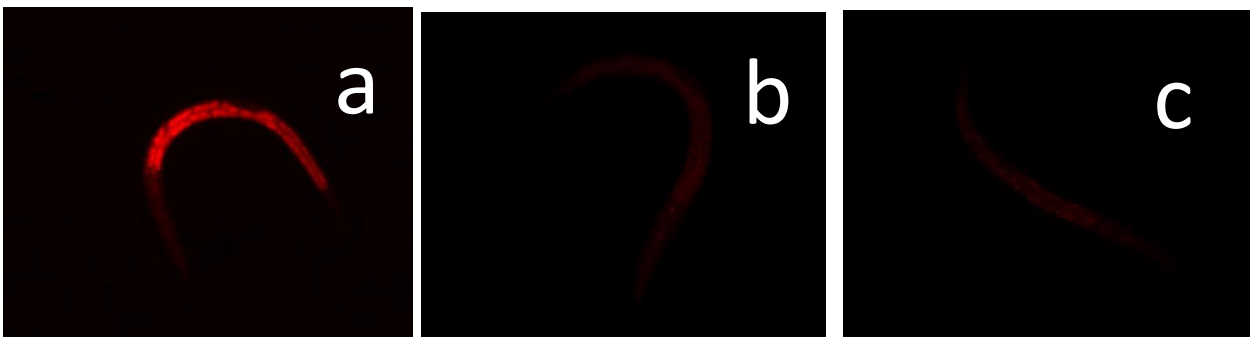


Figure 8. Average body length of the worms over the course of the 72-hour exposure.

3.2- Impact of Sodium Benzoate on Fat Deposition Based on Fluorescence

Sodium benzoate treated worms showed less fluorescence that the control worms after 24 hours of exposure. Glucose treated worms also demonstrated less fluorescence (Fig 9).



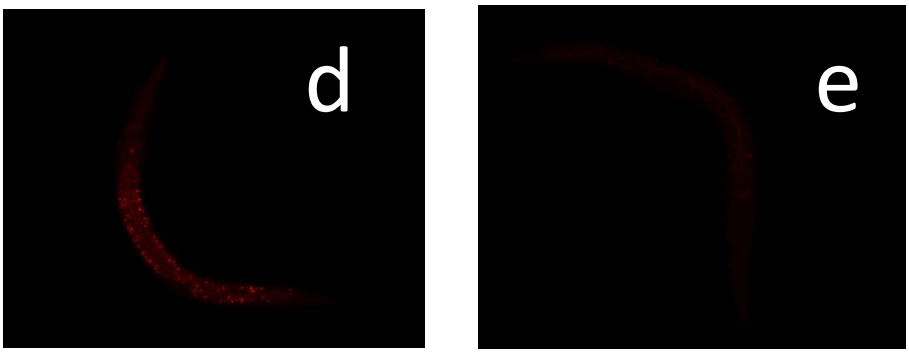


Figure 9. Example of Nile Red dye stained worms at 24 hours exposure. a) Control Worm b) 0.05% Sodium Benzoate c) 0.1% Sodium Benzoate d) 0.5% Sodium Benzoate e) 0.5% Glucose

Sodium Benzoate treated one and a half-day old N2 worms had an average Corrected Total Cell Fluorescence (CTCF) of of: 12.33 for the negative control, 7.04 for the 0.05% treatment, 8.44 for the 0.1% treatment, 8.92 for the 0.5% treatment, and 6.88 for the 0.05% glucose positive control (Fig 10). All treatment groups had significantly less fluorescence than the control group at 24 hours ($p<0.05$).

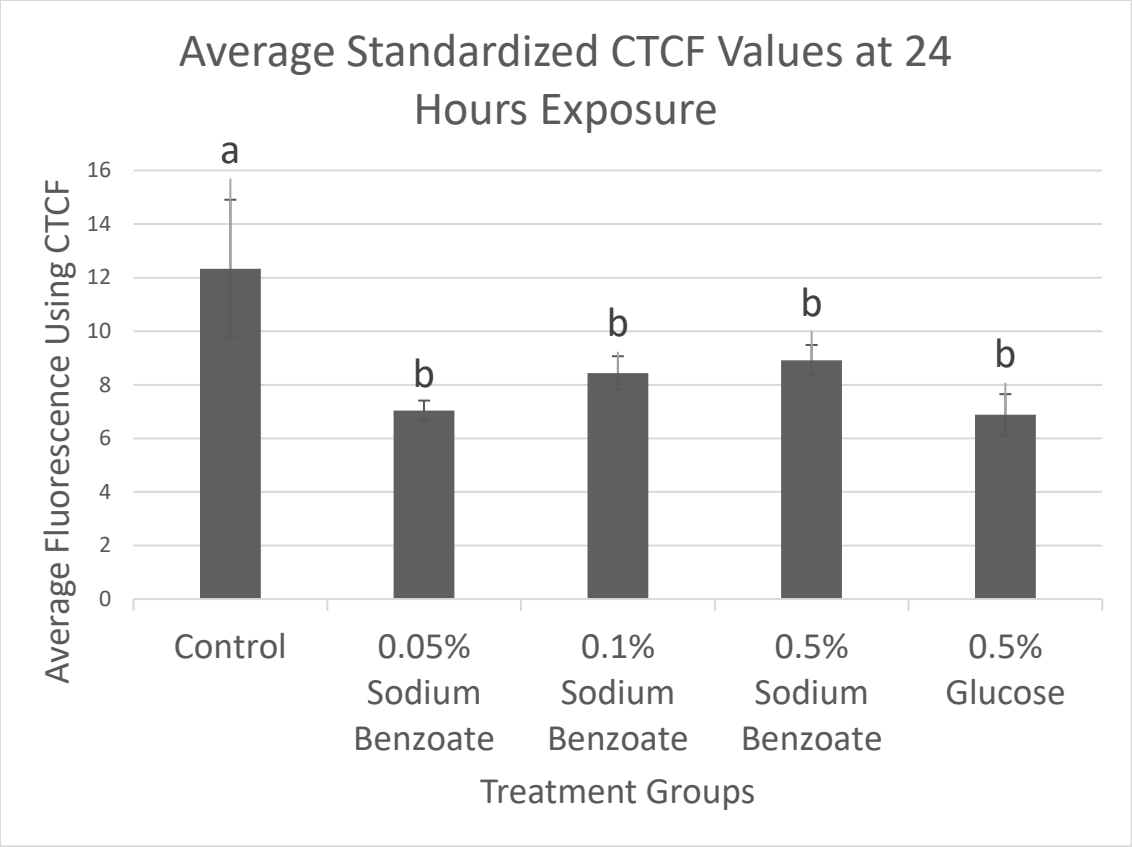


Figure 10. Average Corrected Total Cell Fluorescence of N2 worms 24 hours dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =14, $N_{0.05\%\ Sodium\ Benzoate}$ =12, $N_{0.1\%\ Sodium\ Benzoate}$ =18, $N_{0.5\%\ Sodium\ Benzoate}$ =20, and $N_{0.5\%\ Glucose}$ =32, $p< 0.05$.

Sodium Benzoate treated one and a half-day old N2 worms had an average intensity mean fluorescence of: 2962.69 for the negative control, 1709.86 for the 0.05% treatment, 1838.41 for the 0.1% treatment, 1838.37 for the 0.5% treatment, and 1292.16 for the 0.05% glucose positive control (Fig 11). All treatment groups had significantly less fluorescence than the control group at 24 hours with the 0.5% glucose group showing the least fluorescence ($P<0.05$).

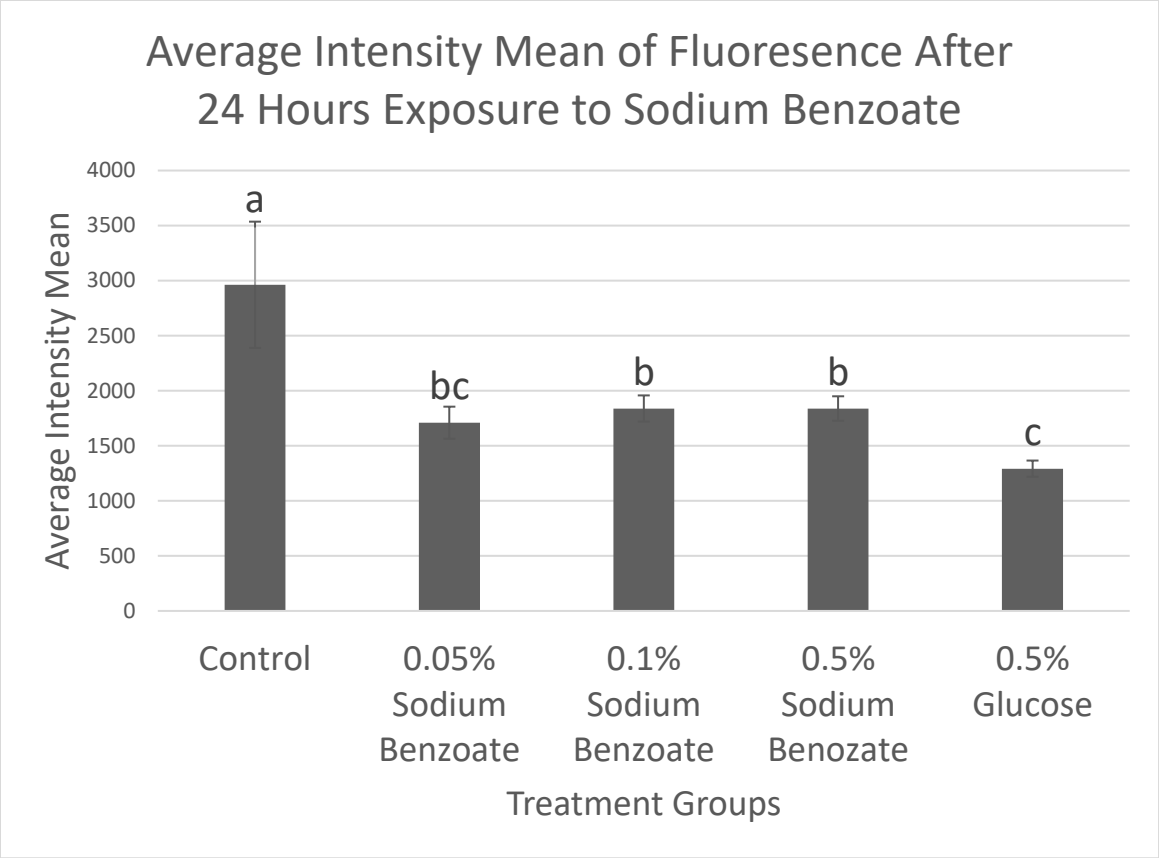


Figure 11. Average Fluorescence based on intensity mean of N2 worms 24 hours dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =14, $N_{0.05\% \text{ Sodium Benzoate}}$ =12, $N_{0.1\% \text{ Sodium Benzoate}}$ =18, $N_{0.5\% \text{ Sodium Benzoate}}$ =20, and $N_{0.5\% \text{ Glucose}}$ =32, $p < 0.05$.

Sodium Benzoate worms showed approximately the same fluorescence at 48 hours exposure, except the 0.1 % treatment group which showed less fluorescence (Fig 12) . Glucose treated worms also showed similar fluorescence to control worms at 48 hours.

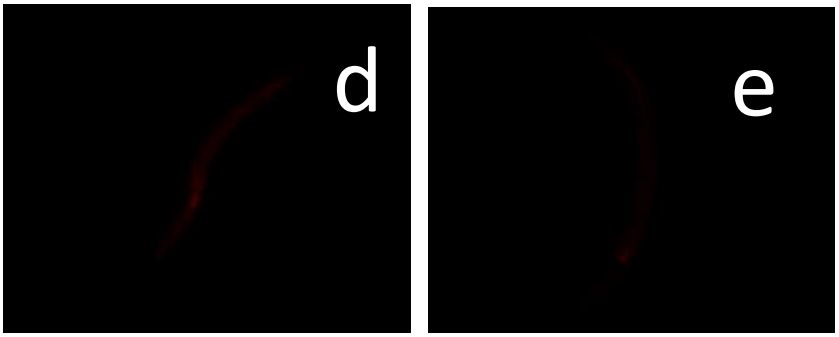


Figure 12. Example of Nile Red dye stained worms at 48 hours exposure. a) Control Worm b) 0.05% Sodium Benzoate c) 0.1% Sodium Benzoate d) 0.5% Sodium Benzoate e) 0.5% Glucose

Sodium Benzoate treated two and a half-day old N2 worms had an average Corrected Total Cell Fluorescence (CTCF) of: 2.92 for the negative control, 2.33 for the 0.05% treatment, 1.93 for the 0.1% treatment, 2.68 for the 0.5% treatment, and 2.51 for the 0.05% glucose positive control (Fig 13). When comparing the treatment groups to the control groups, only the 0.1% treatment group was statistically different. Total Fluorescence was not impacted by treatment after 48 hours of exposure in most groups. Fluorescence decreased in the 0.1% treatment group ($P<0.05$).

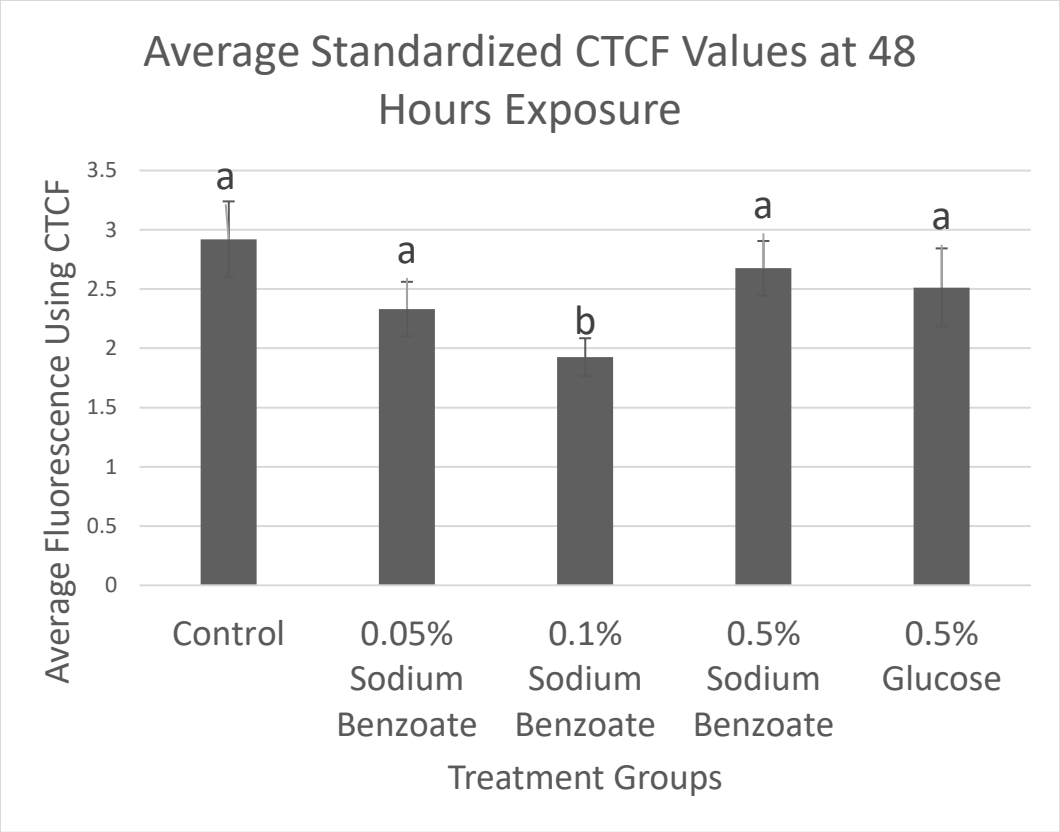


Figure 13. Average Corrected Total Cell Fluorescence of N2 worms after 48 hours of dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =35, $N_{0.05\% \text{ Sodium Benzoate}}$ =33, $N_{0.1\% \text{ Sodium Benzoate}}$ =31, $N_{0.5\% \text{ Sodium Benzoate}}$ =21, and $N_{0.5\% \text{ Glucose}}$ =33, $p < 0.05$.

Sodium Benzoate treated two and a half-day old N2 worms had an average intensity mean fluorescence of: 653.53 for the negative control, 568.66 for the 0.05% treatment, 490.29 for the 0.1% treatment, 633.30 for the 0.5% treatment, and 549.18 for the 0.05% glucose positive control (Fig 14). When comparing the treatment groups to the control groups, only the 0.1% treatment group was statistically different. Intensity mean fluorescence was not impacted by treatment after 48 hours of exposure in most groups. Fluorescence decreased in the 0.1% treatment group ($P<0.05$).

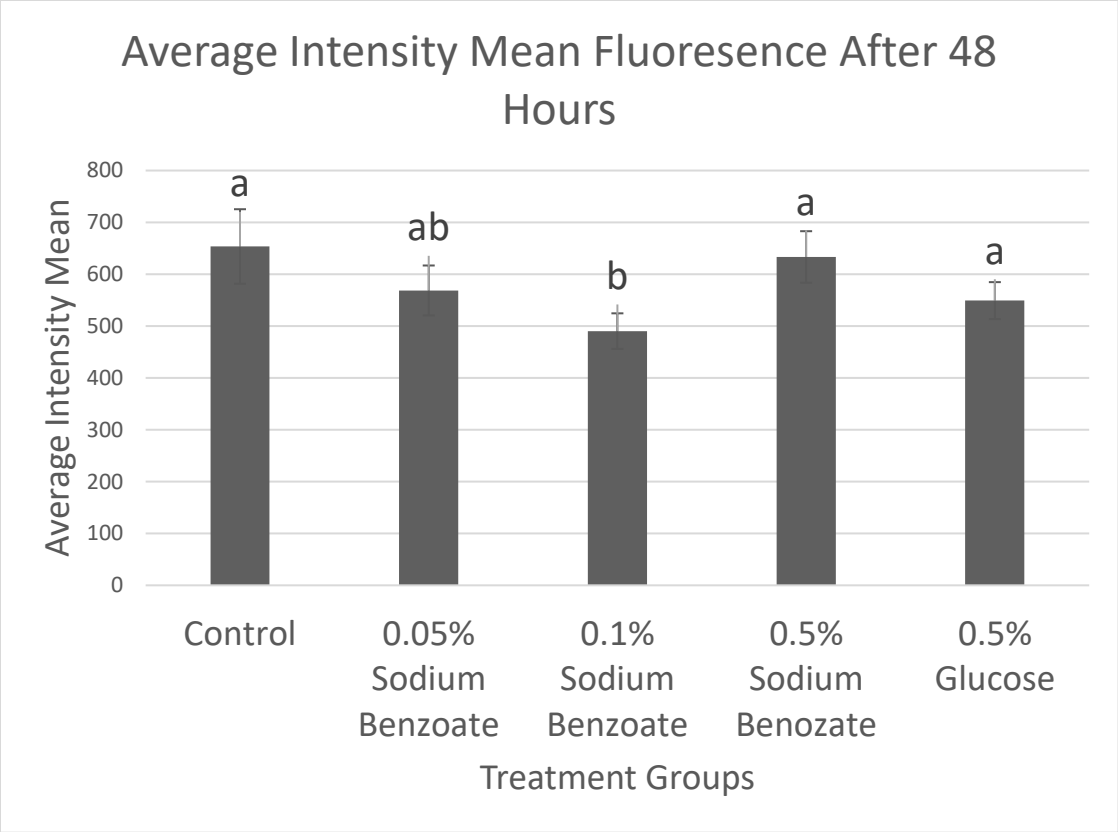
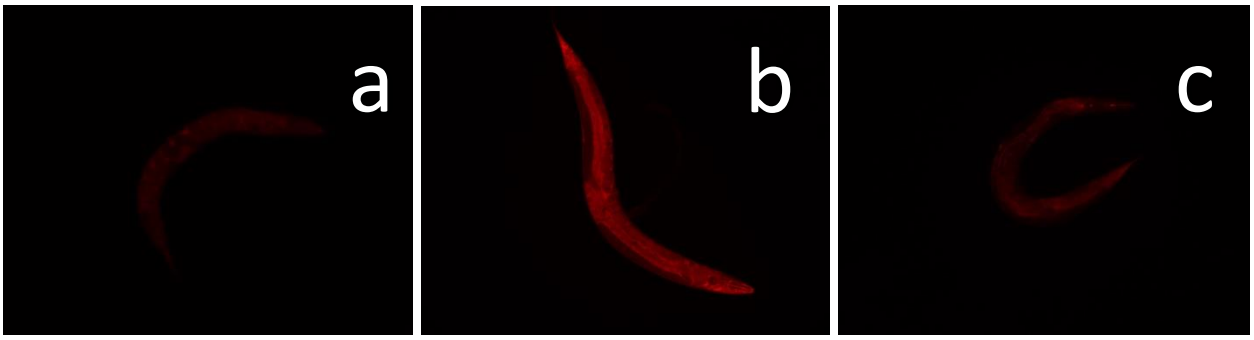


Figure 14. Average Corrected Total Cell Fluorescence of N2 worms after 48 hours of dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =35, $N_{0.05\% \text{ Sodium Benzoate}}$ =33, $N_{0.1\% \text{ Sodium Benzoate}}$ =31, $N_{0.5\% \text{ Sodium Benzoate}}$ =21, and $N_{0.5\% \text{ Glucose}}$ =33, $p < 0.05$.

Sodium Benzoate treated worms in the 0.05% group, and the glucose treated group, showed the most fluorescence at 72 hours. The control group showed the least fluorescence at 72 hours.



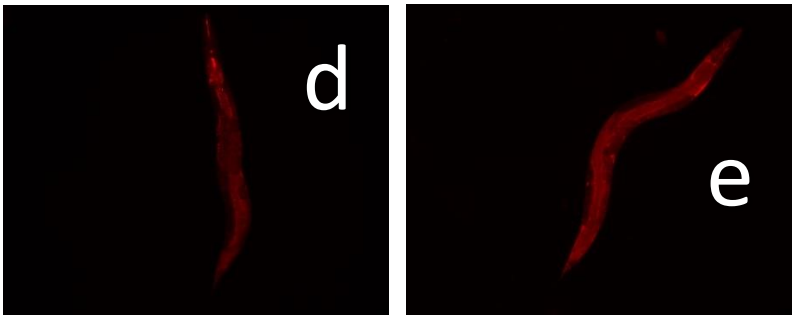


Figure 15. Example of Nile Red dye stained worms at 72 hours exposure. a) Control Worm b) 0.05% Sodium Benzoate c) 0.1% Sodium Benzoate d) 0.5% Sodium Benzoate e) 0.5% Glucose

Sodium Benzoate treated three and a half-day old N2 worms had an average Corrected Total Cell Fluorescence (CTCF) of: 9.08 for the negative control, 14.79 for the 0.05% treatment, 11.19 for the 0.1% treatment, 12.55 for the 0.5% treatment, and 15.3 for the 0.05% glucose positive control (Fig 16). When comparing the treatment groups to the control group, the 0.05% sodium benzoate and 0.5% glucose treatment groups were statistically more fluorescent than the control group. However, despite not being statistically more fluorescent, all treatment groups were more fluorescent that the control group at 72 hours ($P<0.05$).

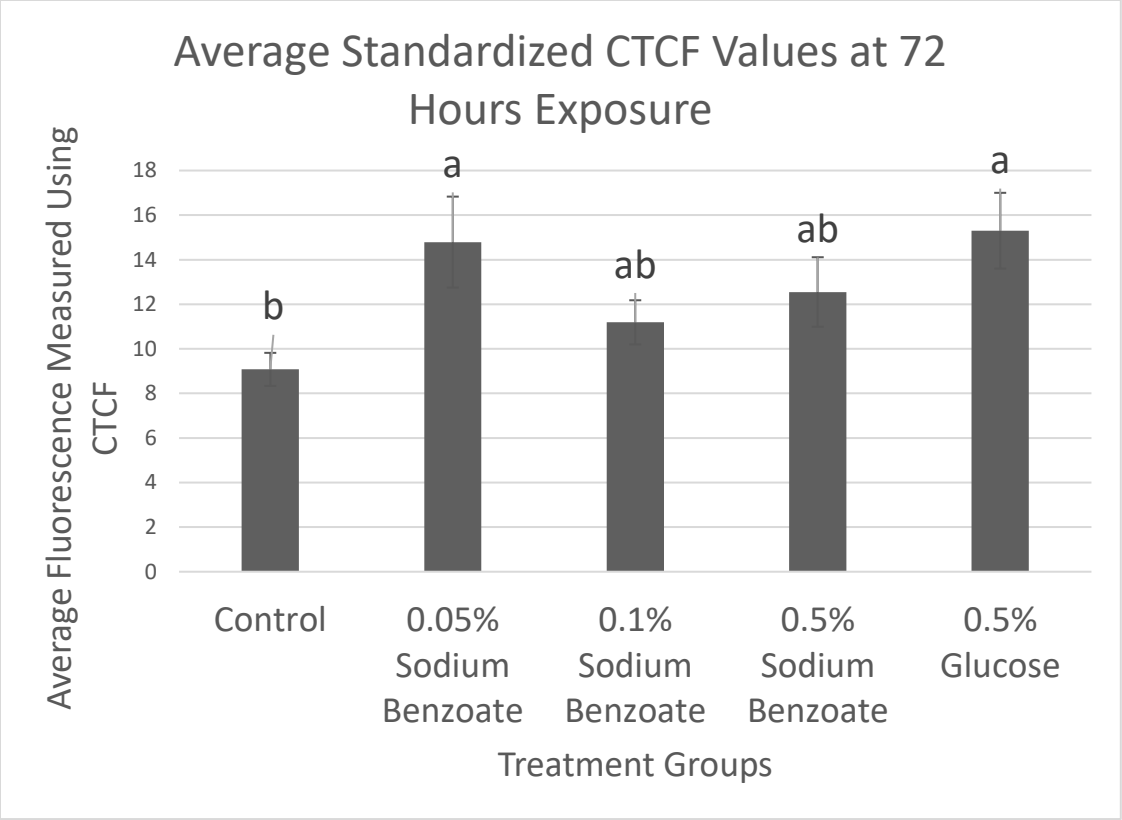


Figure 16. Average Corrected Total Cell Fluorescence of N2 worms after 72 hours of dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =20, $N_{0.05\%\ Sodium\ Benzoate}$ =32, $N_{0.1\%\ Sodium\ Benzoate}$ =21, $N_{0.5\%\ Sodium\ Benzoate}$ =21, and $N_{0.5\%\ Glucose}$ =18, $p< 0.05$.

Sodium Benzoate treated three and a half-day old N2 worms had an average intensity mean fluorescence of: 9.08 for the negative control, 14.79 for the 0.05% treatment, 11.19 for the 0.1% treatment, 12.55 for the 0.5% treatment, and 15.3 for the 0.05% glucose positive control (Fig 17). When comparing the treatment groups to the control group, the 0.05% sodium benzoate and 0.5% glucose treatment groups were statistically more fluorescent than the control group. However, despite not being

statistically more fluorescent, all treatment groups were more fluorescent that the control group at 72 hours ($P<0.05$).

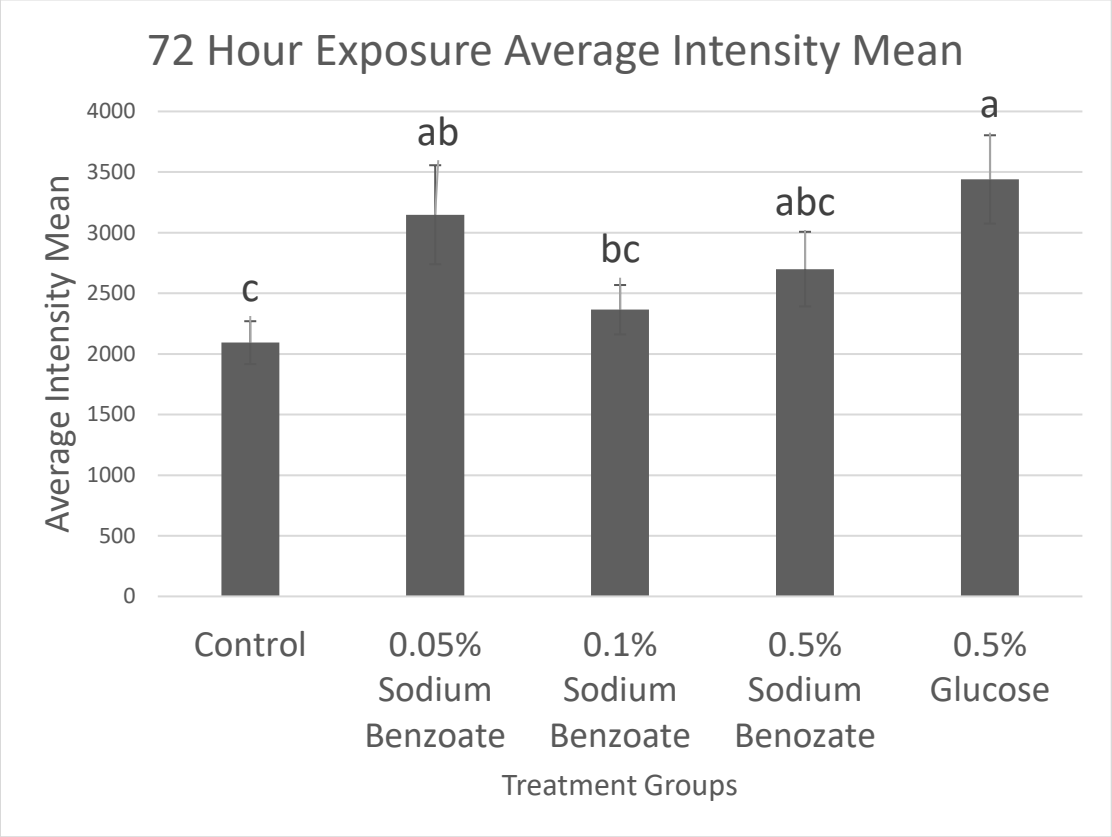


Figure 17. Average Intensity Mean fluorescence of N2 worms after 72 hours of dosing. Different letters denote statistically significant differences. Error Bars indicate the standard error of 3 replicate plates. $N_{control}$ =20, $N_{0.05\%\ Sodium\ Benzoate}$ =32, $N_{0.1\%\ Sodium\ Benzoate}$ =21, $N_{0.5\%\ Sodium\ Benzoate}$ =21, and $N_{0.5\%\ Glucose}$ =18, $p< 0.05$.

The average corrected total cell fluorescence decreased from 24 hours to 72 hours in the control group. In all other treatment groups, the fluorescence increased from 24

hours to 72 hours. At 48 hours fluorescence decreased in all experimental groups (Fig 18).

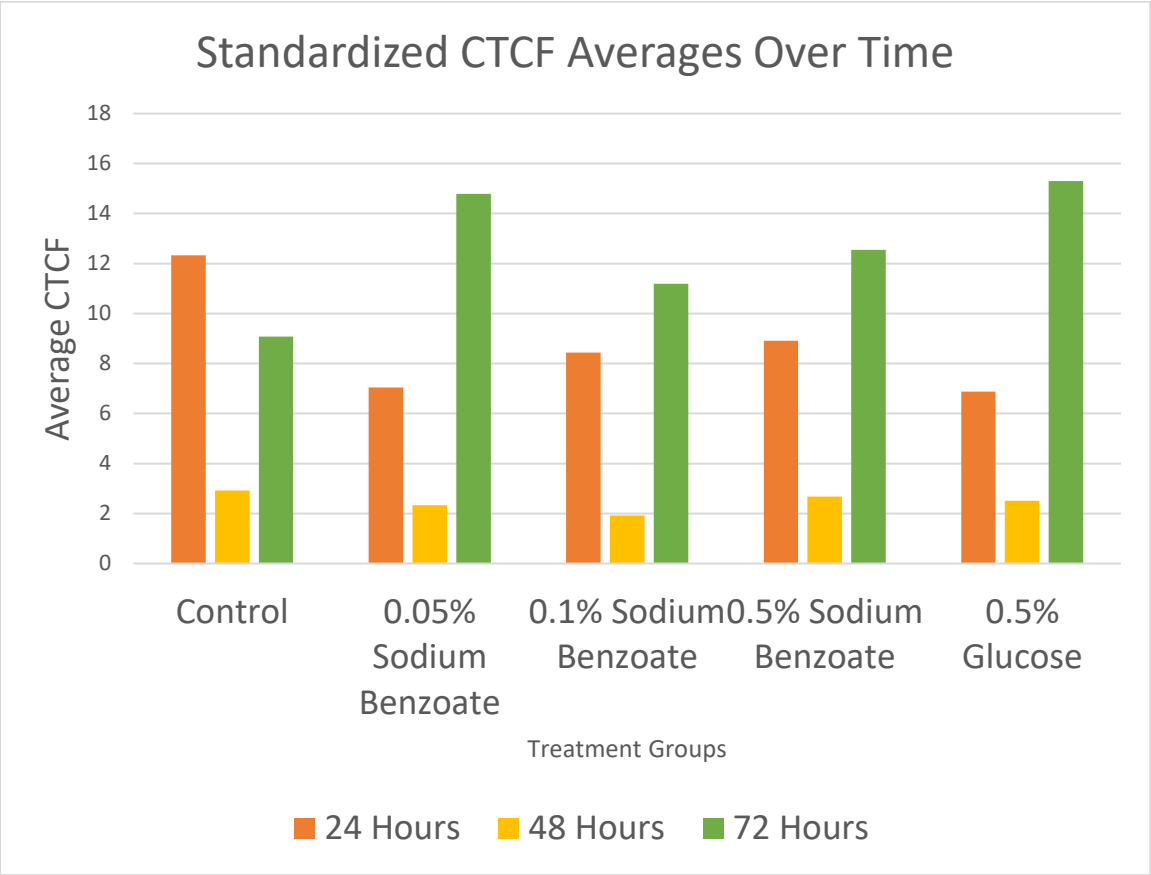


Figure 18. Standardized corrected total cell fluorescence over the course of the 72-hour exposure period.

The average intensity mean fluorescence decreased from 24 hours to 72 hours in the control group. In all other treatment groups, the fluorescence increased from 24 hours to 72 hours. At 48 hours fluorescence decreased in all experimental groups (Fig 19).

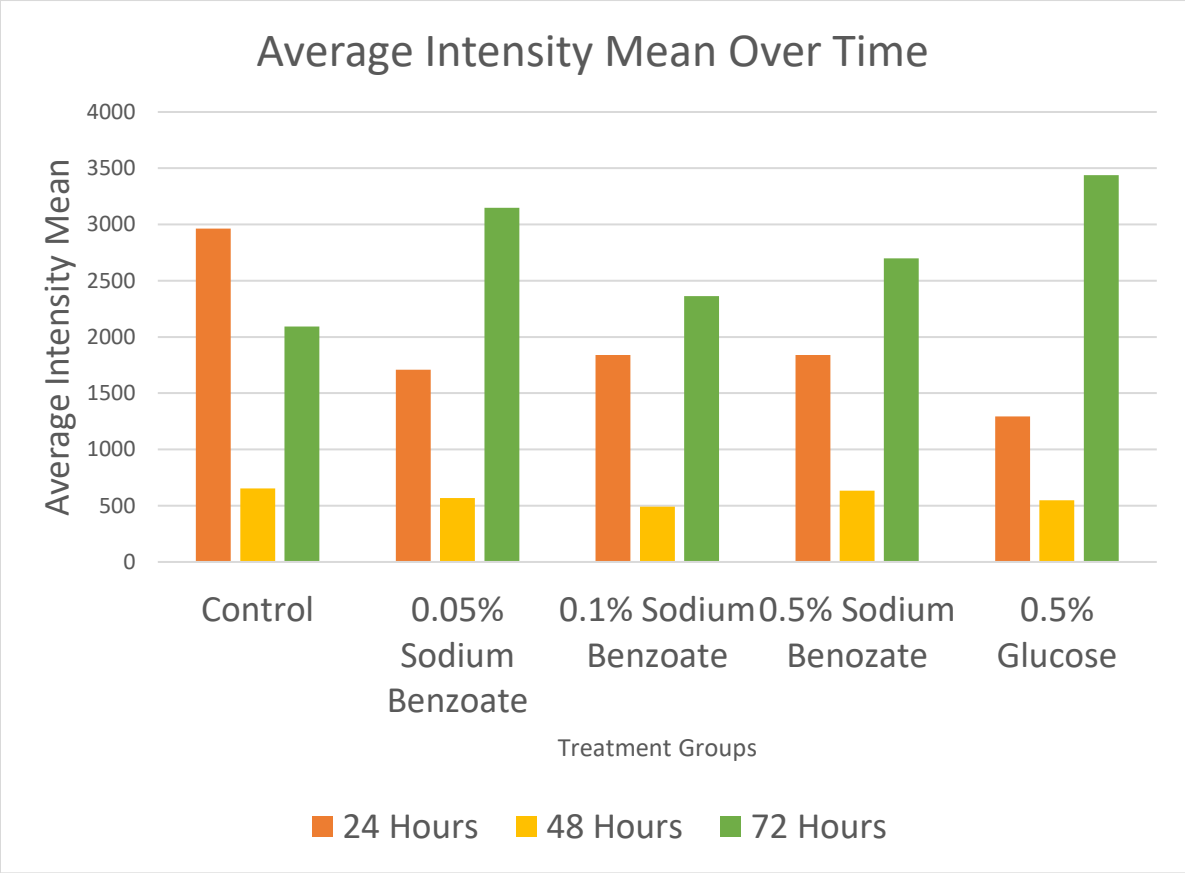


Figure 19. Standardized corrected total cell fluorescence over the course of the 72-hour exposure period.

3.3- Gene Expression Analysis

Gene expression analysis using qRT-PCR revealed one significant fold change in the selected nuclear hormone genes of interest. Table 2 summarizes the fold changes in the nuclear hormone receptor genes of interest. NHR-50 had a significant down-regulated fold change of 0.348 (Fig. 20).

Table 2. Summary of fold changes in nuclear hormone receptor genes: Fold changes after 24 hours exposure. Asterisk (*) indicates a significant fold change, $p < 0.05$.

Gene	Average Fold Change	Direction of Fold Change
NHR-170	8.624760794	UP
NHR-206	0.241247257	Down
NHR21	1.197954682	Up
NHR-64	0.678496003	Down
NHR-8	0.587936262	Down
NHR-50	0.347675237 *	Down
NHR-18	0.775429635	Down
NHR-120	0.573403785	Down
NHR-49	0.494181265	Down
NHR-162	0.058721574	Down
NHR-212	9.010774477	Up

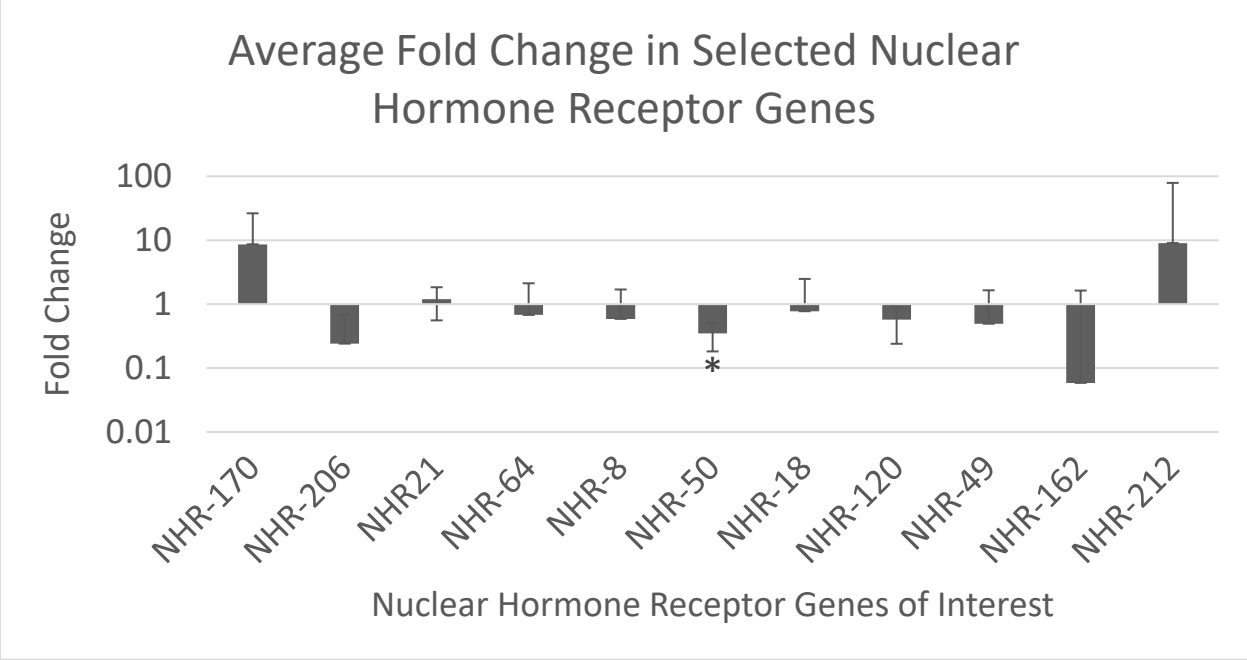


Figure 20. Fold changes in nuclear hormone receptor genes. Visual representation of fold changes discovered using gene expression analysis in selected nuclear hormone receptor genes after 24 hours exposure. Error bars represent standard error of the 3 biological replicates. Asterisk (*) indicates a significant fold change, $p < 0.05$.

Gene expression analysis using qRT-PCR revealed no significant fold changes in the genes of interest in the insulin signaling pathway (Fig 21). Table 3 summarizes the fold changes in the insulin signaling pathway genes of interest.

Table 3. Summary of fold changes in insulin signaling pathway genes: Fold changes after 24 hours exposure. An asterisk (*) indicates a significant fold change, p< 0.05.

Gene	Average Fold Change	Direction of Fold Change
DAF-2	1.064834256	UP
DAF-18	0.440961111	Down
DAF-16	15.32656215	Up
DAF-7	0.589453989	Down
DAF-3	0.808632821	Down
AGE-1	46.38538251	Up
AKT-1	0.467218005	Down

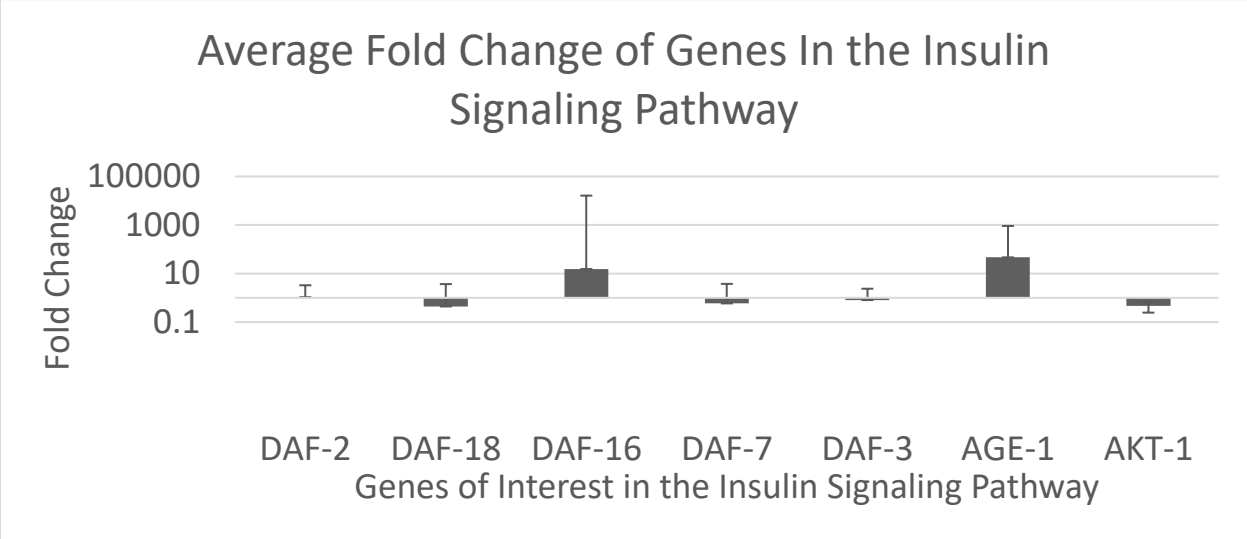


Figure 21. Fold changes in insulin signaling genes. Visual representation of fold changes discovered using gene expression analysis in selected insulin signaling pathway genes after 24 hours exposure. Error bars represent standard error of the 3 biological replicates. An asterisk (*) indicates a significant fold change, p< 0.05.

Gene expression analysis using qRT-PCR revealed two significant fold changes in the genes of interest involved in fatty acid synthesis. Table 4 summarizes the fold changes in

the insulin signaling pathway genes of interest. The genes FAT-1 and ELO-5 both had significant fold changes (Fig 22).

Table 4. Summary of fold changes in fatty acid synthesis genes: Fold changes after 24 hours exposure. An asterisk (*) indicates a significant fold change of p< 0.05 and a double asterisk (**) indicates a significant fold change of p< 0.01.

Gene	Average Fold Change	Direction of Fold Change
TUB-1	0.732861368	Down
FAT-5	1.001287772	Up
FAT-6	0.64524944	Down
Pod-2	2.02272629	Up
Sbp-1	1.002935543	Up
Fat-2	1.242499745	Up
FAT-1	0.072001073 **	Down
FAT-3	0.345381382	Down
ELO-5	0.747112981 *	Down
ELO-1	1.476702175	Up
ELO-2	2.02272629	Up
ELO-6	0.372347953	Down

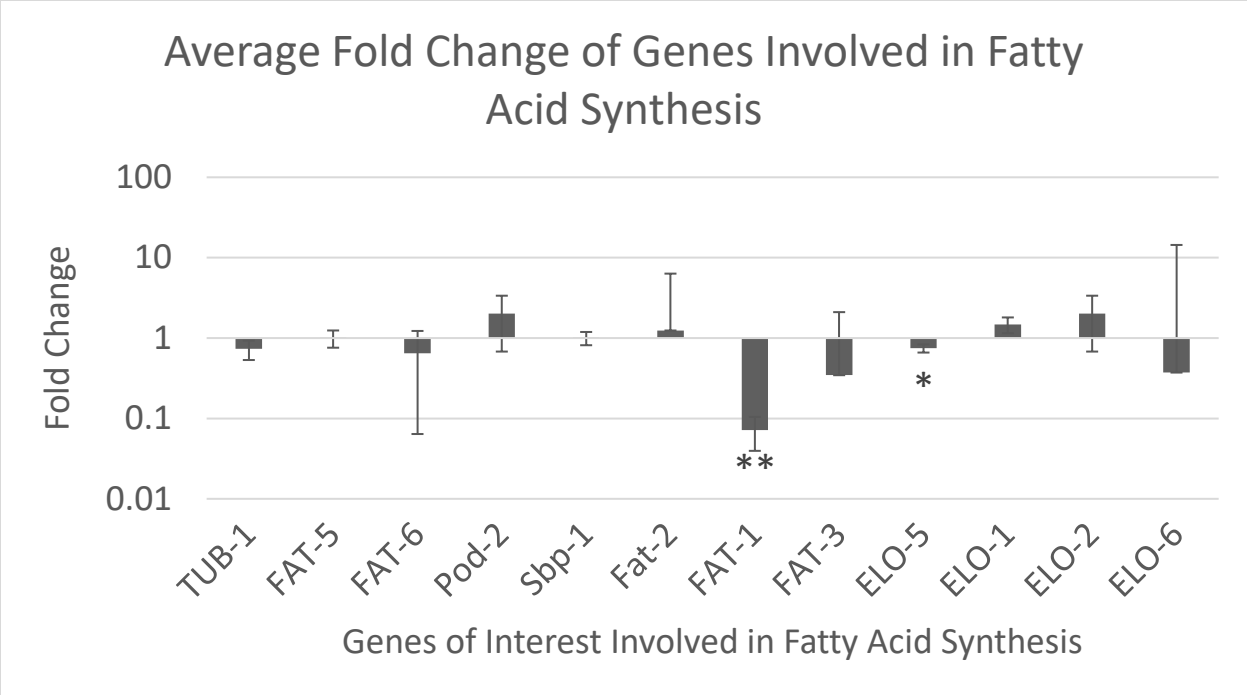


Figure 22. Fold changes in fatty acid synthesis genes. Visual representation of fold changes discovered using gene expression analysis in selected genes involved in fatty acid synthesis after 24 hours exposure. Error bars represent standard error of the 3 biological replicates. An asterisk (*) indicates a significant fold change of $p < 0.05$ and a double asterisk indicates a significant fold change of $p < 0.01$.

CHAPTER 4: DISCUSSION

This research demonstrates that sodium benzoate greatly effects the growth of *C. elegans* at various concentrations. After only 24 hours of exposure, the highest treatment group was significantly larger in size when compared to the negative control group. After 48 hours of exposure, both the middle and high treatment groups were significantly larger in size, and longer, than the negative control group. Lastly, after 72 hours of exposure, the highest treatment group was significantly larger in size, and longer, than both the negative and positive control groups. This evidence shows that sodium benzoate exposure has a large impact on growth in *C. elegans*. The mechanism behind this altered growth was not discovered during this research. Further research should be conducted to examine the effect of sodium benzoate on growth over time to discover a mechanism this effect.

The results for the effect on fat deposition were somewhat inconclusive. After 24 hours of exposure, fluorescence decreased across all treatment groups, when compared to the control group. After 48 hours of exposure, the fluorescence was much lower in all test groups. The reason for this major decrease in fluorescence across all groups was not discovered. For this reason, this experiment should be repeated in the future to make sure these results can be replicated. Lastly, after 72 hours of exposure fluorescence increased in all treatment groups, with a significant increase in the lowest treatment group. This increase in fluorescence suggests that sodium benzoate is increasing fat deposition in *C. elegans*, based on previous studies using Nile Red to stain major fat storage in the organism [1]. However, after this research was conducted, a study was discovered that demonstrated that Nile Red may not actually stain the

major fat storage vesicles in *C. elegans* [12]. This study showed that Nile Red stained lysosome related organelles and that *C. elegans* major fat stores are contained in independent specialized neutral lipid-containing vesicles [12]. Their study indicated that the lysosome related organelles may play an important role in metabolism and lipid degradation [12]. The study also noted increased Nile Red staining of lysosomes may be a predictor of decreased resistance to stress, and a shortened life span [12]. Based on these previous findings, my research suggests that sodium benzoate may be decreasing the lifespan of *C. elegans*. If the Nile Red stained lysosomes in my study, then the increased fluorescence could indicate a reduced resistance to stress, and to probability of a shorter lifespan. This could also explain the overgrowth in the treatment groups in this study. However, in the previous study they also state that under certain conditions, Nile Red dye could stain neutral lipids as well [12]. Due to the findings in the previous study, a conclusion about the effects of sodium benzoate on fat deposition cannot be drawn. Further research must be performed. The previous study validated the use of the fat staining dye Oil Red O to stain neutral lipid stores in *C. elegans* [12]. In the future, Oil Red O should be used along with Nile Red so the results from the two stains can be compared. Since Oil Red O was shown to stain neutral lipids, it should demonstrate the effects on fat deposition in *C. elegans*. However, it should be noted that the procedure involved in fixative Oil Red O staining is very detailed and lengthy. It could be challenging to use that staining method in this specific experiment.

Lastly, the gene expression analysis revealed a significant decrease in the expression of three genes of interest, elo-5, fat-1, and nhr-50. The gene elo-5 encodes a polyunsaturated fatty acid elongase, and elo-5 is required for normal fatty acid

composition [5]. The significant decrease in the gene expression shows that the *C. elegans* demonstrates that after 24 hours exposure, fat composition is affected in the middle treatment group. This finding matches the fluorescence data for the middle treatment group at 24 hours, because fluorescence is lower compared to the control group at this time. This suggest less lipids are being produced overall. The gene fat-1 is required in vivo for n3 polyunsaturated fatty acid synthesis [5] . The significant decrease in gene expression for this gene also demonstrates that fatty acid synthesis is being affect at 24 hours exposure in the middle treatment group. This matches with the data from elo-5, as well as, the fluorescence data. The gene nhr-50 is still greatly understudied. However, gene expression was shown to increase in response to starvation and decrease in response to satiety [9]. The significant decrease in the expression of this gene demonstrates that after 24 hours of exposure the worms in the middle treatment group were satiated, based on the previous findings. However, it should be noted that only one treatment group underwent gene expression analysis in this study, and only at one time point. In the future, all treatment groups should be analyzed at all time points. The 72-hour time point appears to be the most significant timepoint based on the data in this research. Sodium benzoate's effects on *C. elegans* increase over time. This increase demonstrates that long term exposure has the greatest effect on the organism

References

1. Ashrafi, K., Chang, F. Y., Watts, J. L., Fraser, A. G., Kamath, R. S., Ahringer, J., & Ruvkun, G. (2003). Genome-wide RNAi analysis of Caenorhabditis elegans fat regulatory genes. Nature, 421(6920), 268-272. doi:10.1038/nature01279
2. Benzoic acid; CASRN 65-85-0. EPA. 1988 Jul 9 [accessed 2016 Nov 6]. https://cfpub.epa.gov/ncea/iris/iris_documents/documents/subst/0355_summary.pdf
3. CFR - Code of Federal Regulations Title 21. accessdata.fda.gov. 2016 Apr 1 [accessed 2016 Nov 3]. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1733
4. Ciardi C, Jenny M, Tschoner A, Ueberall F, Patsch J, Pedrini M, Ebenbichler C, Fuchs D. Food additives such as sodium sulphite, sodium benzoate and curcumin inhibit leptin release in lipopolysaccharide-treated murine adipocytes in vitro. British Journal of Nutrition. 2011;107(6):826–833. doi:10.1017/s0007114511003680
5. European Bioinformatics InstituteProtein Information ResourceSIB Swiss Institute of Bioinformatics. European Bioinformatics Institute. European Bioinformatics InstituteProtein Information ResourceSIB Swiss Institute of Bioinformatics. [accessed 2019 Apr 12]. https://www.uniprot.org/
6. Explore Worm Biology. WormBase. [accessed 2019 Apr 12]. http://www.wormbase.org/
7. Garcia, A. M., Ladage, M. L., Dumesnil, D. R., Zaman, K., Shulaev, V., Azad, R. K., & Padilla, P. A. (2015). Glucose Induces Sensitivity to Oxygen Deprivation and Modulates Insulin/IGF-1 Signaling and Lipid Biosynthesis in Caenorhabditis elegans. Genetics, 200(1), 167-184. doi:10.1534/genetics.115.174631
8. GHO | By category | Prevalence of overweight among adults, BMI ≥ 25, age-standardized - Estimates by country. World Health Organization. [accessed 2016 Nov 6]. http://apps.who.int/gho/data/node.main.A897A?lang=en
9. Hyun M, Davis K, Lee I, Kim J, Dumur C, You Y-J. Fat Metabolism Regulates Satiety Behavior in C. elegans. Scientific Reports. 2016;6(1). doi:10.1038/srep24841
10. Obesity. World Health Organization. 2014 Sep 5 [accessed 2016 Nov 6]. http://www.who.int/topics/obesity/en/
11. Ogden CL, Carroll MD, Fryar CD, Flegal KM. Prevalence of obesity among adults and youth: United States, 2011–2014. NCHS data brief, no 219. Hyattsville, MD: National Center for Health Statistics. 2015.
12. O'Rourke EJ, Soukas AA, Carr CE, Ruvkun G. C. elegans major fats are stored in vesicles distinct from lysosome-related organelles. Cell Metabolism. 2009;10(5):430–435. doi:10.1016/j.cmet.2009.10.002.
13. Overweight and obesity. World Health Organization. 2018 Dec 27 [accessed 2016 Nov 6]. https://www.who.int/gho/ncd/risk_factors/overweight/en/

14. Simmons, A. L., PhD, Schlezinger, J. J., PhD, & Corkey, B. E., PhD. (2014). What Are We Putting in Our Food That is Making Us Fat? Food Additives, Contaminants, and Other Putative Contributors to Obesity. Current Obesity Report, 3(2), 273-285. doi:10.1007/s13679-014-0094-y.
15. Sodium Benzoate. U.S. National Library of Medicine. 2016 Oct 25 [accessed 2016 Oct 31]. https://toxnet.nlm.nih.gov/cgi-bin/sis/search/r?dbs hsdb:@term @rn @rel 532-32-1
16. Zheng J, Greenway FL. Caenorhabditis elegans as a model for obesity research. International Journal of Obesity. 2011;36(2):186–194. doi:10.1038/ijo.2011.93