C. ELEGANS RESPONSE TO COMBIEND STRESS

By

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Abstract: Cellular stress has been shown to increase with age, but the human body's ability to respond to this stress decreases with age. Various forms of stress, including osmotic and heavy metal stress, are linked to a broad range of human disease. Age is the leading risk factors for many of these major morbidities, including cardiovascular disease and cancer, among others. Developing a better understanding of stress response pathways will allow targeted therapy for better treatment. Developing a better understanding of stress response pathways will allow targeted therapy for better treatment. Using the roundworm *Caenorhabditis elegans* as a model of study, I investigated the osmotic and heavy metal stress pathways by treating worms with sodium chloride (NaCl) and cadmium chloride ($CdCl_2$), respectively. I first identified a target dose for each stressors that reduced lifespan by 50%, then I combined the stressors in order to observe their interaction. I found that 250mM NaCl and 1mM $CdCl_2$ reduced *C. elegans* lifespan by roughly 50% individually. When these two stressors were combined, I observed an 85% reduction in lifespan, indicating that the stressors are activating different responses within the worms.

Introduction:

Organisms are constantly exposed to stress, both intracellular and extracellular, and stress response is vital to maintain long-term health and prolong survival. Many forms of cellular stress increase with age, and common diseases are often associated with elevated stress levels or the lack of a proper response against that stress, including cancer and heart disease. Many diseases are associated with two or more forms of cellular stress. For example, Alzheimer's disease is associated with high levels of oxidative stress and improper protein folding. Cells respond to stress by activating different response pathways, which create a network of interconnected transcriptional hubs. However, some responses, such as osmotic stress response, are still not well understood. Although there is extensive research on individual stressors and their response pathways, little is known about the interaction of these pathways when an organism needs to respond to simultaneous distinct forms of stress. The nematode *Caenorhabditis elegans* is a model organism that has many evolutionary conserved molecular pathways with mammals—including most major stress response pathways—and is cheap and easy to culture. *C. elegans* also has a short, roughly a three-week lifespan (Rodriguez 2013) that is negatively impacted by cellular stress including oxidative stress, protein misfolding, osmotic stress, Golgi stress, and heavy metal stress. The Sutphin laboratory seeks to profile individual and combined stressors in *C. elegans* in order to define the transcriptional network of their stress-response. While many studies have examined different stress-responses in *C. elegans*, there is wide variation in the specific protocol details, including the dose of stressor employed and the severity of the phenotypic response, the lab aims to identify specific doses to reduce lifespan by 20%, 50%, and 80% for different stress forms, and to use these standardized dose-response relationships to study interactions between stressors. Different drugs or stressors may have different modes of interaction, such as antagonistic, synergistic, and independent. In an antagonistic relationship, one stress sensitizes the organism to the other stress. In a synergistic relationship, one stress makes the organisms more resistant to the second stress. And finally, in an independent relationship, the two stressors have no effect on one another.

Hypertonic stress is a form of osmotic stress that results in the loss of water, causing an intracellular ionic imbalance and disrupting the secondary structure of proteins (Choe 2008). Osmotic stress has been shown to most commonly result in protein aggregation. Loss of water also results in up to a 40% reduction in *C. elegans* volume when worms are placed on 400mM

NaCl agar (standard media contains 51mM NaCl). When volume is reduced, cells become crowded, promoting non-native protein-protein interactions (Hoppe 2011). In worms exposed to hypertonic stress, glycerol-3-phosphate dehydrogenase (GPDH-1) is strongly upregulated, resulting in the de novo biosynthesis of glycerol. This rapid accumulation of intracellular organic glycerol aids in resistance to extracellular osmotic stress by increasing the intracellular osmolarity, allowing the cell to maintain its volume, decreasing unfavorable cell interactions. Expression of GDPH-1 is the rate limiting step for hypertonicity-induced glycerol synthesis in *C. elegans*. GPDH-1 expression is regulated by two GATA transcription factors, ELT-2 and ELT-3, while enzymatic activity is regulated by DOS motif proteins OSM-7, OSM-8, and OSM-11 (**Figure 1A**) (Rodriguez 2013). Additionally, during prolonged hypertonic stress, cells express increased levels of molecular chaperones and ubiquitin conjugates. Molecular chaperones help to stabilize protein structure, inhibit non-native protein interactions, and balance environmental osmolarity, while ubiquitin conjugates target proteins for degradation by lysosomes and proteasomes (Ciechanover 2005). Ubiquitin levels have been shown to increase 2.2 fold within 3 hours in *C. elegans* exposed to 400mM NaCl, revealing a strong role in osmotic stress regulation (Choe 2008). The human renal system is highly studied in regards to its regulatory role of blood osmolarity; however, recent research has shown that osmotic stress is increased in inflammatory responses and is found in common diseases, including diabetes, inflammatory bowel disease, and hypernatremia (Broker 2012).

Cadmium is a heavy metal that in elevated concentrations can cause harm to the human body. Humans can be exposed to cadmium through inhalation and ingestion. Some environments contain high cadmium levels in the soil. Plants take up the cadmium, which results in increased ingestion. Tobacco plants in particular take up increased cadmium from the environment, leaving smokers at increased risk of cadmium inhalation. Additionally, some workplace environments, such as mines, can also have increased cadmium levels ("Environmental Health"). It is a persistent toxicant of occupational and environmental health concern (Waalkes et al., 1992). Numerous adverse health effects, including chronic respiratory disease, osteoporosis, impaired renal function and a variety of cancers are associated with cadmium exposure. Cadmium is known to increase reactive oxygen species (ROS) and decrease expression of the antioxidant glutathione (Nemmiche 2016) as well as activate the Keap1/Nrf2 pathway. Cadmium binds mitochondria and inhibits oxidative respiration. Cadmium is a heavy metal and is believed to activate the oxidative stress response pathway. It has been shown that SKN-1–the Nrf2 ortholog in the nematode *C. elegans*–modulates the responses to oxidative stress by suppressing mitochondrial oxygen production (Zhao 2012). Cadmium results in expression of the *mlk-1* gene, which encodes a kinase. MLK phosphorylates MEK-1, a MAPKK, which phosphorylates downstream protein KGB-1 (Mizuno 2004). Both MLK-1 and MEK-1 mutations have been linked to hypersentivity to heavy metals, such as cadmium (Koga 2000). Additionally, SEK-1 and PMK-1 mutants resulted in enhanced sensitivity in MEK-1 and KGB-1 mutants, revealing that these kinases are part of a a redundant pathway involved in suppressing heavy metal stress (**Figure 1B**) (Mizuno 2004).

Although little is known about the interactions between the osmotic stress response and the heavy metal stress response, **Figure 1** summarizes the individual stress response pathways. This thesis explores osmotic stress response and the interaction between osmotic stress response and heavy metal stress in *C. elegans*. Osmotic stress is less characterized and has not been studied extensively. I first performed a dose responses with the osmotic stressor sodium chloride and identified the 50% reduction in lifespan before combining it with the heavy metal stress. I

found that the osmotic stress induced by sodium and heavy metal stressor induced by cadmium have an antagonistic impact on *C. elegans* when combined, producing a greater reduction in lifespan than observed in individual stressors alone.

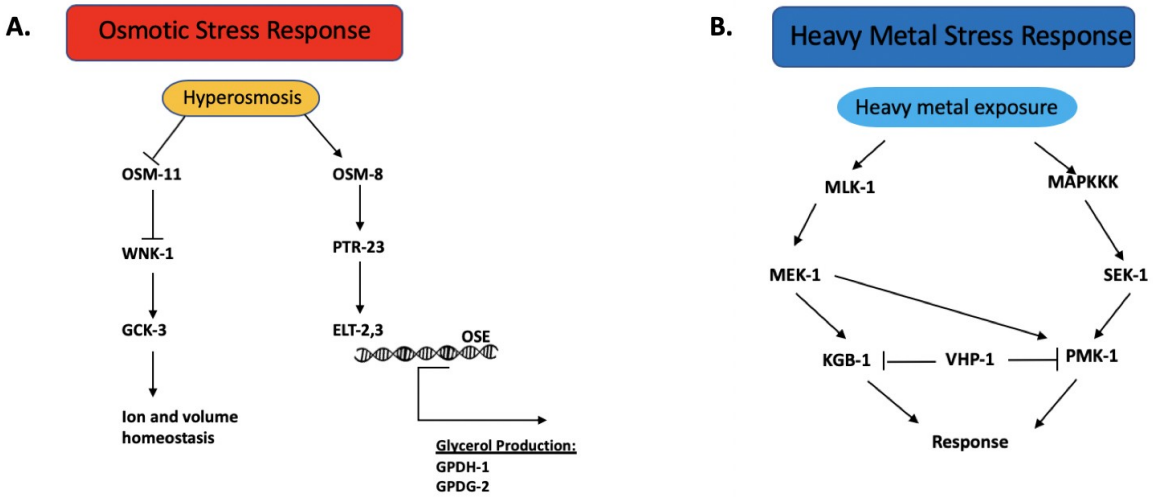

Figure 1. Stress pathways for (**A**) osmotic stress and (**B**) heavy metal stress. The pathway for heavy metal stress is still not fully understood, but these proteins are thought to play a role (Mizuno 2004).

Materials and Methods:

Lifespan assays. Lifespan experiments were conducted according to previously published protocols (Sutphin et al. 2009).

Preparation of plates for stress response assays. Worms were cultured on solid nematode growth media (NGM) plates, which were prepared with varying sodium chloride (NaCl) and cadmium chloride ($CdCl_2$) concentrations in order to determine the concentrations necessary to reduce lifespan by 50%. Combined stressors were tested using 50% lifespan reduction dose. Plates were prepared with normal concentration NGM (51mM NaCl, 0mM $CdCl_2$), with 250mM NaCl, with 1mM $CdCl_2$, and with 250mM NaCl and 1mM $CdCl_2$.

Worms were cultured on 6cm petri dishes with nematode growth media (NGM) agar base containing a minimum of 0.45g NaCl (51mM; standard for NGM), added pre-autoclave. For osmotic stress plates, this amount was increased to 2.19 g (250mM). $CdCl_2$ solution was creating by dissolving 0.1833g of $CdCl_2$ in 5mL of water. For heavy metal stress plates, 750μL of solution was added, making the total $CdCl_2$ plate concentration 1mM. All plates contained 5-fluoro-2′-deoxyuridine (FUdR) and carbenicillin. FUdR is an chemotherapy drug and antimetabolite that inhibits thymidylate synthetase, resulting in inhibition of DNA synthesis, used to prevent the *C. elegans* from reproducing once on the experimental plates, thus maintaining a synchronized population of worms on each plate (Mitchell 1979).

Bacteria Spotting. Once the plates were prepared, they were spotted with 300 μL *Escherichia coli* (strain HT115). The bacteria then grew on the plates, covered and at room temperature, for 5

days before worms were transferred onto the experimental plates. Worms were bleached onto control plates without FUdR and transferred to experimental plates as L4 larva.

Worm Synchronization. Six L4 stage *C. elegans* were passed onto 6cm plates containing the *E. coli* strain OP50 and kept at 20˚C for three days, allowing time for them to lay eggs. These six plates were then bleached to obtain only the eggs. A 10% bleach solution containing 1M NaOH was used to isolate age-synchronized eggs. Distillated and deionized water (ddH_2O) was poured on each of the worm plates and scraped to dislodge the worms. Once all plates were scraped and the water was transferred to the centrifuge tube, the tube was centrifuged at 500 Relative Centrifugal Force (RCF) for 1 minute. Using a vacuum, the supernatant was removed. Bleach solution was then added to the centrifuge tube before rocking it on a shaking for 3 minutes. It was again centrifuged and the bleaching process was repeated. Once the supernatant was removed, 14mL of ddH_2O, was added to the tube. Sample was spun and the supernatant was removed. At this point, only eggs remained. The pellet was gently shaken from the bottom of the tube and the eggs were mixed throughout the remaining water. Using a Pasteur pipette, 1 drop of egg water mixture (300 worms depending on water: egg concentration) was placed onto control plates. Control plates contained regular concentration NGM and were used to synchronize worms. Plates were kept in 20˚C, allowing for eggs to hatch and offspring to grow to L4 stage. 40 L4 worms were then transferred to experimental plates, unless otherwise stated. There were 9 plates per experimental condition. Plates were separated into three repeats and given to three different technicians. For initial experiments to determine the 50% reduction dose, each technician counted their plates on Monday, Wednesday, and Friday for the first two weeks, and then every day for the remainder of the worms' life, marking fatalities. For the combined experiment, technicians counted every day from the day of transfer to experimental plates.

Data Analysis. Data was compiled when all worms were dead and survival data was assessed using a Log Rank Test using the *survival* package in the *R Statistical Programing Language*. Comparisons with $p < 0.05$ were considered significant.

Results:
Given the variability in stressor dose and timing of plate transfer in previous literature, the first goal was to identify a dose for each stress agent that reduced the median lifespan by 50% in order to provide a clear and consistent means of comparing targets between stress experiments. For the osmotic stress pathway, I conducted a survival dose-response study for NaCl and KCl. To determine optimal concentration of NaCl, I conducted a dose-response study, varying concentrations of NaCl from 200mM to 350mM. The doses were selected from previous studies (Lamitina 2004), NaCl is a basic component of NGM plates, so it was unique in that control plates already contained 51mM. For the heavy metal stressor, Emily Turner, a graduate student in the lab, conducted a lifespan dose-response study for $CdCl_2$, ranging the dose from 0.1 to 1mM. We then combined NaCl and $CdCl_2$ doses that resulted in a 50% median lifespan decrease. We chose not to combine KCl and $CdCl_2$ because both NaCl and KCl were osmotic stressors that activated the same response pathway, and displayed similar survival characteristics.

Osmotic Stress. 250mM was found to reduce the lifespan roughly 50% at 20˚C, as shown by the median reduction of 52.4% (**Figure 2**). At a concentration of 250mM, the median lifespan was 10 days, compared to 21 days for the control group. 51mM NaCl was the control condition

because standard NGM pouring protocol contains 51 mM NaCl. There was a dose dependent response, with increasing NaCl resulting in decreased lifespan. Beginning with 250mM NaCl, there was a steep decline in percent alive before it became relatively steady.

I also examined the effects of another osmotic stressor, KCl. As shown in **Figure 2**, as dose increased, there was a steep decline in percentage of worms alive in the beginning days, before it became more constant. 200mM KCl resulted in a 57.1% median reduction in lifespan.

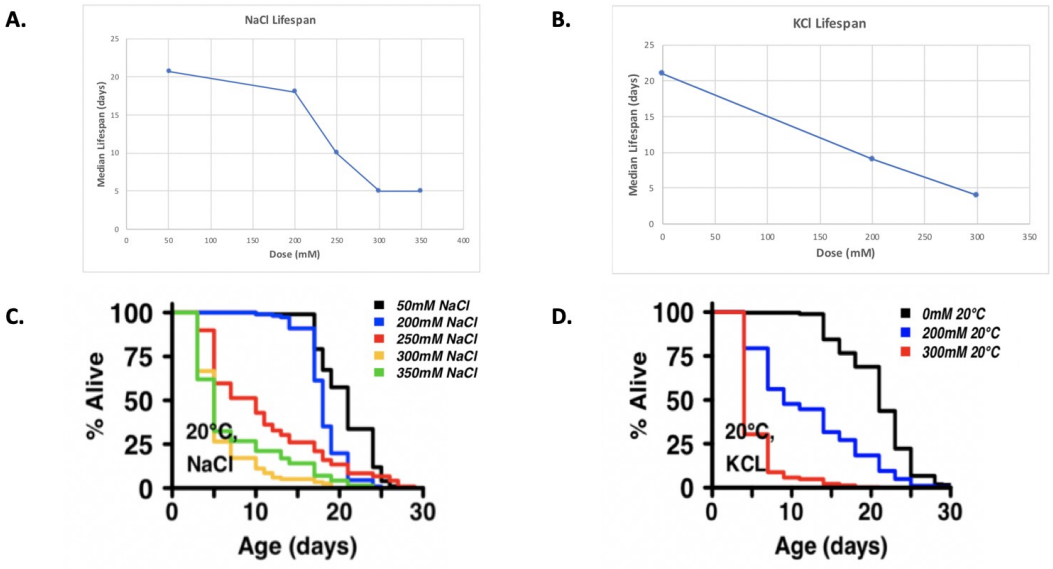

Figure 2. Osmotic Stress. (A) Lifespan dose-response curves for NaCl. NaCl reduces lifespan with increasing dose in the 200-350mM range (control plates contain the standard 51mM NaCl present in NGM). **(B)** Lifespan dose-response curves for KCl. KCl reduces lifespan with increasing dose in the 200-300mM range (control plates contain 0mM KCl) **(C)** Survival curves NaCl **(D)** Survival curves for KCl. Log Rank $P < 0.001$ vs. control mean lifespan for all doses. All differences were determined to be significant.

I ran additional experiments with NaCl in order to examine the effects of varying time of exposure. When the eggs were placed directly onto experimental plates, rather than delaying transfer to the L4 stage, there was no longer a 50% lifespan reduction at 250mM NaCl (**Figure 3A**). Rather there was only a 20% reduction. Worms on 250mM NaCl lived for a median of 19 days, compared to the control worm age of 24 days. This suggests that there was an adaptive effect by plating the worms on the conditions from hatching. Additionally, developmental effects were observed--the worms never fully developed and displayed a dumpy (Dpy) phenotype.

In a third experiment with NaCl, I included a transition plate in order to ensure that the worms were fully developed before being exposed to the stress. After bleaching onto control plates containing no FUdR, worms were transferred to FUdR control plates in order to maintain age synchronization, but delaying transfer to plates containing elevated NaCl until day 2 of adulthood. I again did not observe a 50% lifespan reduction at 250mM. There was only a 32% reduction (**Figure 3B**). Worms placed on 250mM NaCl lived for a median age of 15, compared to the median control worm age of 22 days. Allowing the worms to fully develop before exposing them to osmotic stress allowed them to better adapt to their conditions. These timing

experiments further suggest that L4 worms may be particularly sensitive to osmotic stress relative to other developmental stages.

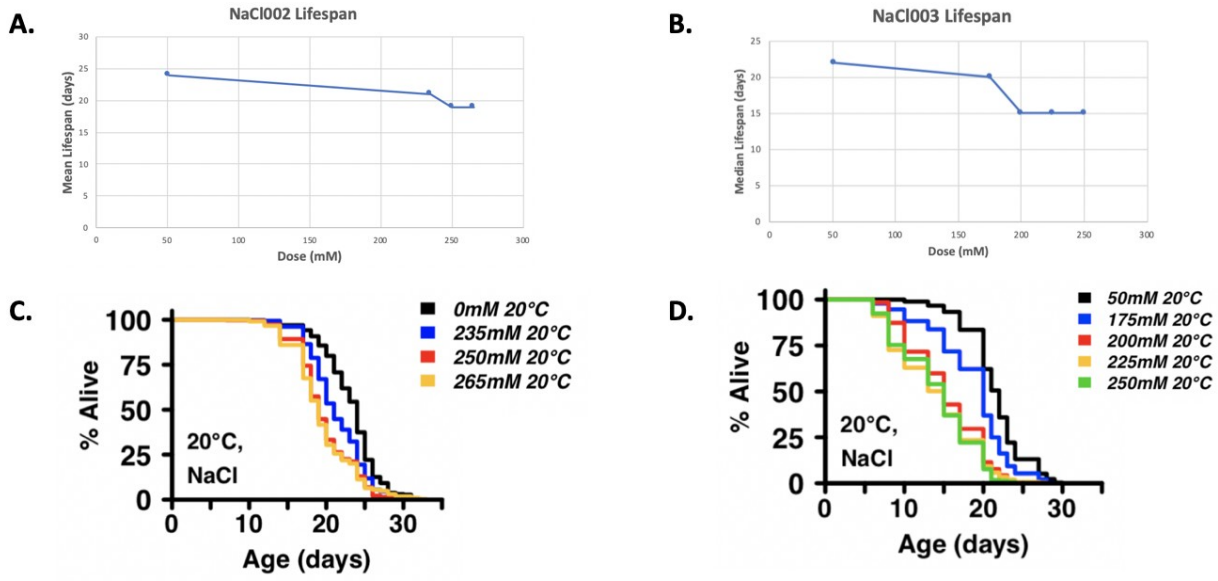

Figure 3. Validation of NaCl. (A) Lifespan dose-response curves for worms exposed to NaCl from egg. (control plates contain the standard 51mM NaCl present in NGM). **(B)** Lifespan dose-response curves for worms exposed to NaCl at day 2 adulthood. **(C)** Survival curves for worms exposed to NaCl from egg. **(D)** Survival curves for worms exposed to NaCl at day 2 adulthood. Log Rank $P < 0.001$ vs. control mean lifespan for all doses. All differences were determined to be significant.

Heavy Metal Stressor. Emily Turner identified the 50% reduction dose for $CdCl_2$ to be 1mM, as shown in **Figure 4.** No previous studies have examined the effect of cadmium on lifespan. However, previous studies on gene expression exposed *C. elegans* to 0.1 mM cadmium to observe the effects on RNA levels. This was used in determining the original concentrations tested.

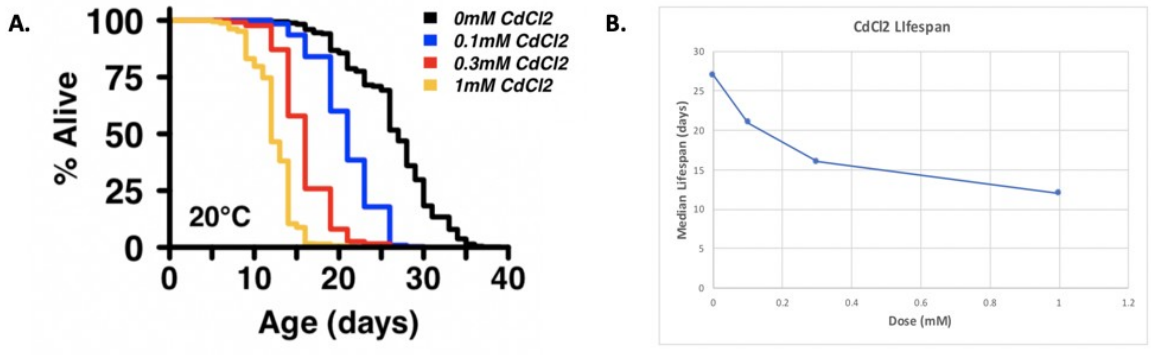

Figure 4. CdCl2 dose response. (A) CdCl2 survival curve. CdCl2 reduces lifespan with increasing dose in the 0.1-1mM range. **(B)** CdCl2 dose-response curve. Log Rank $P < 0.001$ vs. control mean lifespan for all doses. All differences were determined to be significant.

Combined Stressors. After determining doses for reducing the lifespan of *C. elegans* by 50% for NaCl and $CdCl_2$, I looked at the effects of applying these stressors simultaneously. I found that combing the stressors resulted in an 85% reduction in lifespan, as shown in **Figure 5.**

Worms on regular NGM lived for a median age of 27 days. Worms exposed only to 250mM NaCl lived for a median age of 13 days,. Worms exposed only to 1mM $CdCl_2$ lived for a median age of 12 days. Worms exposed to both 250mM NaCl and 1mM $CdCl_2$ lived for a median age of 4 days. While exposure to 250mM NaCl reduced lifespan by 52%, simultaneous exposure to 250 mM and 1mM $CdCl_2$ reduced worm lifespan by 85.2%. This 85.2% reduction in lifespan is also greater than the 55.6% reduction in lifespan observed in worms only exposed to $CdCl_2$, proving that combination of the stressors results in greater lifespan reduction compared to individual shortening.

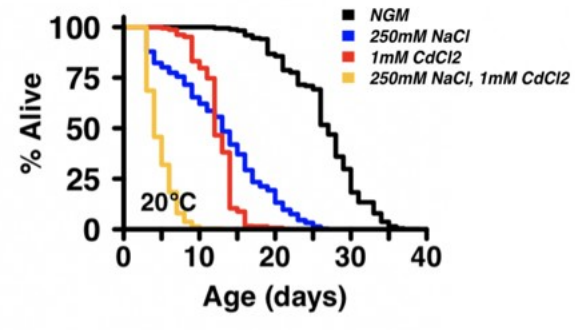

Figure 5. Combined stress response survival curve. Log Rank P < 0.001 vs. control mean lifespan for all doses. All differences were determined to be significant.

Osmotic Stress					
NaCl					
Dose	Mean	Median	Mean % Reduction	Median % Reduction	P-Values
Control	20.7	21			
200mM	18.17	18	-12.3	-14.3	2.30E-10
250mM	10.82	10	-47.8	-52.4	8.80E-13
300mM	5.9	5	-71.5	-76.2	1.40E-48
350mM	7.18	5	-65.3	-76.2	3.30E-32
KCl					
Dose	Mean	Median	Mean % Reduction	Median % Reduction	P-Values
Control	20.8	21			
200mM	11.96	9	-42.9	-57.1	1.50E-29
300MM	5.42	4	-73.9	-81	2.60E-137
Heavy Metal Stress					
CdCl2					
Dose	Mean	Median	Mean % Reduction	Median % Reduction	P-Values
Control	26.4	27			
0.1mM	20.9	21	-21	-22.2	2.30E-81
0.3mM	15.8	16	-40.1	-40.7	5.20E-210
1mM	12.4	12	-53.2	-55.6	7.20E-309
Combined Stress					
Dose	Mean	Median	Mean % Reduction	Median % Reduction	P-Values
Control	26.4	27			
250mM NaCl	12.77	13	-51.7	-51.9	7.80E-219
1mM CdCl2	12.37	12	-53.2	-55.6	7.20E-309
250mM Nacl, 1mM CdCl2	4.78	4	-81.9	-85.2	2.50E-320

Table 1. Summary statistics for all lifespan experiments. Mean and median lifespan data presented in days. Mean % reduction and median % reduction both calculated based on the control. P-values calculated using the log rank test for each group relative to control. P-values vs. control <000.1 for the Log-Rank test, indicating all results significant. N=40 for all experimental groups.

Discussion:

Stress response pathways are complex, but understanding interactions between them could have large implications on human health. Age is the leading factor of the most common diseases, including heart disease and cancer, and cellular stress is known to increase with age. Additionally, resistance to stress decreases with age. Here I have studied osmotic stress and heavy metal stress in an effort to understand how they may interact. I found that the osmotic stress induced by sodium and heavy metal stressor induced by cadmium have an antagonistic impact on *C. elegans* when combined, producing a greater decrease in survival than expected from the lifespan observed from the individual stressors alone (Table 1).

I also found that timing of exposure to sodium had a substantial impact on both survival and development. During preliminary experiments, the time of exposure to NaCl was altered. I exposed worms to NaCl at either egg, L4, or day 2 of adulthood to observe effects on lifespan. Worms exposed to 250mM NaCl at the L4 stage had a 52% reduction in lifespan compared to control worms. For worms exposed to 250mM NaCl at the egg stage, I only observed a 20% reduction in lifespan; however, there was apparent developmental effects, indicated by a dumpy phenotype. Finally, when I first exposed worms to 100mM NaCl at the L4 stage before transferring them to 250mM NaCl at day 2 of adulthood, I observed only a 32% reduction in lifespan. Because NGM contains some sodium (51mM NaCl), this is an example of hormesis- when an organism is exposed to a mild stress and becomes resistant to later exposure from the same stress. Looking more closely at the shape of the survival curves, worms exposed to sodium appear to die off more quickly immediately following exposure to NaCl, but then have a lower "rate of aging", as indicated by a shallower slope to the survival curve (**Figure 2C**). This may indicate that the reduction in lifespan is more driven by the "shock" factor of initial exposure to the stressor. This "shock" factor appeared to be less severe in the worms exposed to NaCl at egg (**Figure 3A**) and at day 2 adulthood (**Figure 3B**), suggesting that worms in the L4 stage may be more shock-sensitive. In preliminary testing, when worms were placed on 400mM KCl, they died within minutes. I did not complete the experiment, as it was clear the worms were dying immediately. This was likely explained by a "shock" factor related to osmotic stress.

Our experiment showed that exposure to combined stress resulted in a greater reduction in lifespan than exposure to the individual stressors, suggesting that the two stress pathways are interacting in some manner. If the pathways were independent of one another we would expect the reduction to be additive, meaning NaCl would reduce lifespan in a similar manner in unstressed and cadmium-exposed worms, and vise-versa. Future studies are needed to understand the mechanisms of interaction between osmotic and heavy metal stress response pathways.

It has been shown that cadmium promotes ROS formation, suggesting an indirect interaction with oxidative stress response pathways (Zhao 2012). This increase in ROS formation is an adaptive response. Exposure to osmotic stress results in increased levels of intracellular glycerol, which does not promote mitochondrial metabolism, resulting in increased ROS. Combining the osmotic stress with oxidative stress may result in increased intracellular glycerol levels, leading to an increase in ROS formation.

Osmotic stress is marked by increases in intracellular glycerol and represents one point of potential molecular interaction between cadmium and osmotic. In the future, it would be beneficial to measure glycerol accumulation in worms after exposure to cadmium. By measuring intracellular levels of glycerol for control worms and worms exposed to either 250mM NaCl, 1mM of $CdCl_2$, or both, one could tell if the genes that are upregulated in osmotic stress to increase glycerol synthesis are also upregulated in worms exposed to heavy metal stress. There may also see protective effects of glycerol accumulation on simultaneous heavy metal exposure. In ongoing work, the Sutphin lab is evaluating the impact of knocking out stress response genes from the heavy metal pathway and the osmotic stress pathway and seeing its effect when exposed to individual and combined stressors. The lab also plans to compare the transcriptional response of the worms when exposed to both combined and individual stressors using RNAseq to identify other pathways that may be unexpectedly impacted by different stress combinations.

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