Effects of Antioxidants on *Caenorhabditis elegans* under Oxidative Stress

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CONTENTS

I. INTRODUCTION

A significant factor affecting overall health is oxidative stress. A biological system or an organism is said to undergo oxidative stress when an irregular amount of reactive oxygen species are present. Reactive oxygen species (ROS) are highly reactive chemicals formed as byproducts from the metabolism of diatomic oxygen (O_2), and include a vast amount of molecules, such as superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), among other molecules [1]. Oxygen toxicity and other numerous loss of function conditions can result from uncontrolled production of insufficient elimination of ROS. Thus, oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and an organism's ability to detoxify the ROS or repair the damage incurred [2]. In a study by Kumsta et al. [3], age-synchronized *Caenorhabditis elegans* (*C. elegans*) are exposed to H_2O_2 to induce oxidative stress, resulting in dramatic behavioral and physiological changes, such as loss of mobility, reduction in growth rate and ATP levels. The effect of oxidative stress can be thoroughly studied in *C. elegans*.

To investigate effects on lifespan, health, and development, researchers often employ a model organism to conduct experiments on. For our pur-

poses, *C. elegans* serves as the perfect one, being relatively easy to acquire, fast-growing with short lifespans, producing a multitude of offspring, and harboring many genes with functional homologs in humans [4]. The benefits a model organism, such as *C. elegans*, can provide are indispensable when it comes to efficient and effective research. Modern science relies on model organisms to understand much of how a variety of treatments, such as vaccines, antibiotics, and diets, can affect numerous aspects of health, including lifespan, motility, rate of development, and size [5].

Commonly studied treatments to combat oxidative stress, include a diverse repertoire of antioxidants, which are known to regulate ROS levels in *C. elegans*. Polyphenols, a category of plant compounds found naturally in plant foods, act as dietary antioxidants that neutralize ROS by promoting natural resistance. Polyphenols often favor indirect mechanisms, including inhibiting pro-oxidation mechanisms and increasing activity of antioxidant enzymes. Some types of polyphenols can also act directly as ROS scavengers [2]. *C. elegans* holds up as a superior model organism to study oxidative stress because the antioxidant mechanisms and pathway they employ are homologous to humans [2].

In our research we explore the effects of two commonly consumed drinks, pomegranate juice and green tea, in combatting oxidative stress. These drinks are often praised as high in antioxidant capabilities for the high levels of polyphenols they contain. Pomegranate juice is rich in two categories of polyphenols: tannins and flavonoids. Flavonoids demonstrate antioxidant activity with indirect inhibition of inflammatory markers that induce oxidative stress and lipid peroxidation. Tannins inhibit lipid peroxidation and the Fenton reaction, which produces ROS [6], [7]. The second treatment, green tea, contains the highest polyphenol content of all teas and includes a sub-category of flavonoids, called catechins. Catechins have a strong reduction ability [8] and defend against lipid oxidation [9]. However, research has shown pomegranate juice to have three times the antioxidant activity as green tea, as pomegranate juice containts a significantly larger pool of antioxidants [7]. Through a variety of assays, we observe the effects of these two treatments on *C. elegans* in oxidative stress.

We examine the motility and survivability of *C. elegans* in two separate assays, which are carried out with various concentrations of each treatment to understand if antioxidants affect motility or survivability concentration-wise. In our first assay, we characterize the motility of *C. elegans* supplemented with antioxidant treatments and compare to a negative control. The next assay measures the survival rate of *C. elegans* receiving antioxidants with and without hydrogen peroxide, which induces oxidative

stress. We hypothesize both antioxidants to enhance the motility of *C. elegans*, and improve the survivability of *C. elegans* under oxidative stress. We expect the effect on both motility and survivability to increase as concentration of antioxidants increase. Finally, we predict pomegranate juice to affect *C. elegans* at a higher capacity compared to green tea.

II. Results

A. Motility Assay

To grasp the effects of antioxidants on *C. elegans*, we performed a motility assay. Research by Kumsta et al. demonstrated *C. elegans* exposed to a short treatment of H_2O_2, inducing oxidative stress, suffered a significant loss of mobility [3]. ROS exist at a smaller capacity in *C. elegans* without inducing oxidative stress. In this experiment, we hoped to employ antioxidant treatments to scavenge and prevent the formation of ROS in *C. elegans*. We explored the effect of antioxidant treatments on motility of *C. elegans* promoting these direct and indirect mechanistic pathways. Before we observed the specific interaction between antioxidant treatments and oxidative stress in *C. elegans*, we investigated the impact of solely antioxidants on *C. elegans* through a motility assay.

The average motility for the negative control *E. coli* OP50 group is measured to be approximately 60 head turns per minute (Fig. 1). The findings indicate only the antioxidant treatment of pomegranate juice at a concentration of 0.1 mg/mL of polyphenols to have an effect in improving motility of *C. elegans* compared to the negative control (Fig. 1). We performed a One-Way ANOVA with Post-Hoc Tukey HSD to test the significance of our results. The 0.1 mg/mL pomegranate juice demonstrated a significant increase in motility compared to the negative control *E. coli* OP50 ($N = 2$, $p \approx 0.006$) and all other treatment groups (Fig. 1).

Between the concentrations administered within the pomegranate juice treatment type, we saw a general trend of motility decreasing as concentration increased from 0.1 mg/mL to 0.5 mg/mL to 2.5 mg/mL of polyphenols. ANOVA showed a significant difference between 0.1 mg/mL and 0.5 mg/mL ($N = 2$, $p \approx 0.004$), as well as a significant difference between 0.1 mg/mL and 2.5 mg/mL ($N = 2$, $p = 0.001$; Fig. 1).

None of the green tea treatments improved motility compared to the *E. coli* OP50 ($N = 2$, $p > 0.05$; Fig. 1). The average motility values for each of the green tea treatments were lower than the average motility seen for the negative control. There was no significant difference between concentrations administered within the green tea treatment type ($N = 2$, $p > 0.05$). Additionally, the 0.1 mg/mL pomegranate juice treatment saw a significant increase in motility compared to the 0.1 mg/mL green

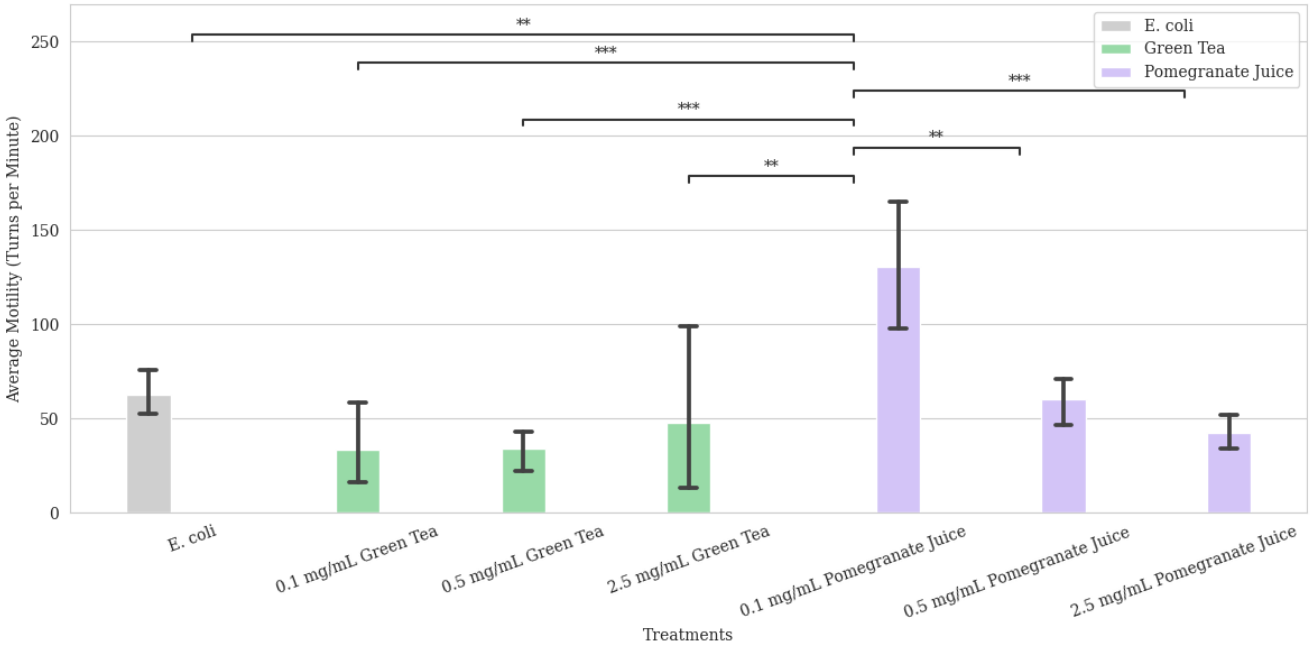

Fig. 1: Pomegranate juice at 0.1 mg/mL increases motility of *C. elegans*. Motility of *C. elegans* was measured by counting head turns per minute after 2 - 4 hours of exposure to green tea and *E. coli* OP50 at three concentrations (green bars), pomegranate juice and *E. coli* OP50 at three concentrations (purple bars), or just negative control *E. coli* OP50 (gray bar). One-Way ANOVA with Post-Hoc Tukey HSD was performed. *Error bars* represent confidence interval of 95%, $N = 2$ replicates and $n = 3$ nematodes sample size per treatment; statistical differences between treatments are indicated by $*$ where $0.01 < p \leq 0.05$, by $**$ where $0.001 < p \leq 0.01$, and by $***$ where $0.0001 < p \leq 0.001$.

tea ($N = 2$, $p = 0.001$), 0.5 mg/mL green tea ($N = 2$, $p = 0.001$), and 2.5 mg/mL green team ($N = 2$, $p \approx 0.002$; Fig. 1).

B. Survival Assay

We performed a survivability assay on *C. elegans* under neutral conditions and under oxidative stress to further elucidate the effects of antioxidants. Survival rate of *C. elegans* has been shown to deteriorate when under oxidative stress [10]. Because ROS exist in *C. elegans* without inducing oxidative stress, we first hoped to elongate lifespan of *C. elegans* by supplementing their diets of *E. coli* OP50 with antioxidant treatments (Fig. 2 A & B). Next, we carried out the same experiment but, additionally, induce oxidative stress unto *C. elegans* and fed them antioxidants (Fig. 2 C & D). Through this survivability experiment we investigated the impact on survival rates of *C. elegans* fed antioxidant treatments with and without inducing oxidative stress.

Our findings illustrate that the survival rate of *C. elegans* fed *E. coli* OP50 decreased when under oxidative stress (Fig. 2). The rate of decrease in survival rate for this group was significantly more dramatic under oxidative stress. The survival rate for the negative control group decreased to 5% survival

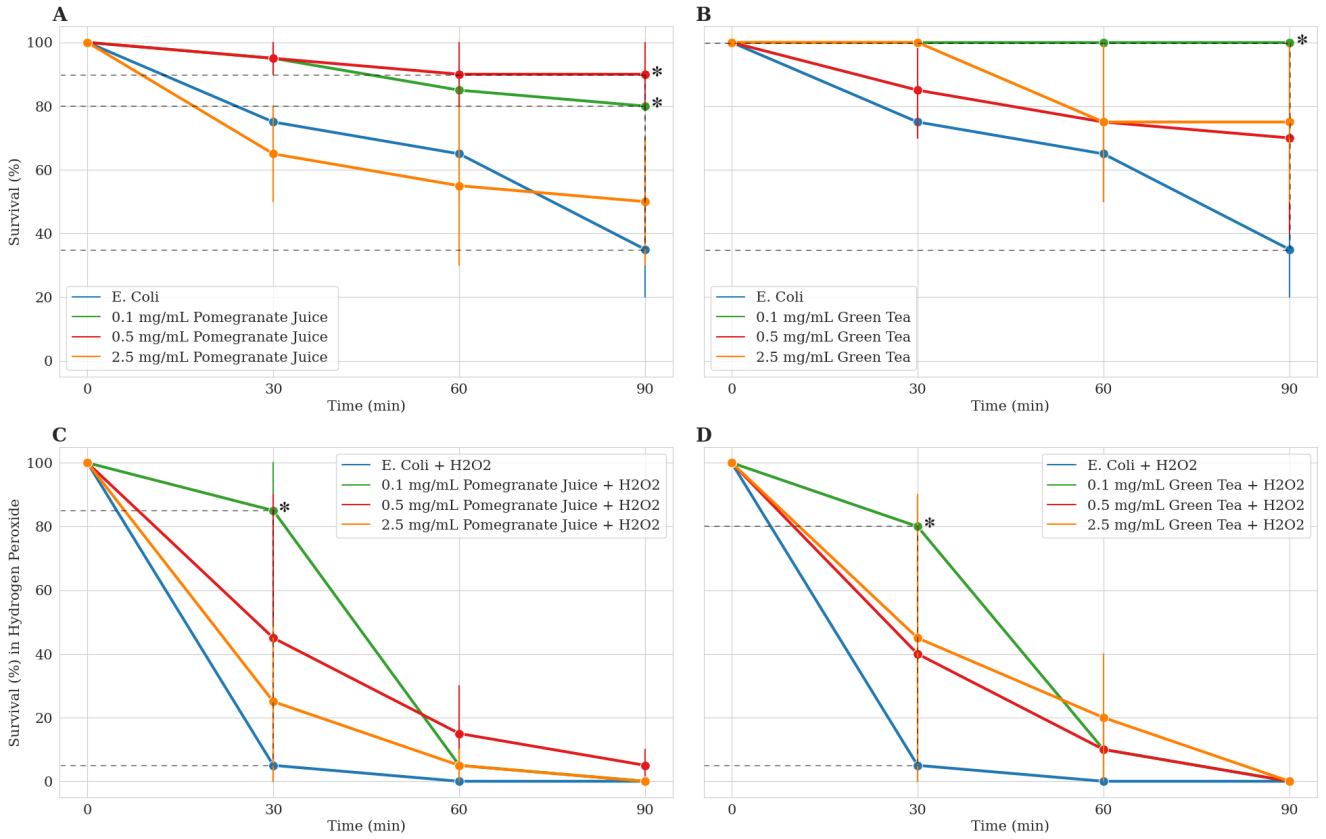

Fig. 2: Antioxidants heighten the survivability of *C. elegans*. Survival rate was measured by counting nematodes at 30 minute intervals for **a** *C. elegans* fed pomegranate juice, **b** *C. elegans* fed green tea, **c** *C. elegans* in H_2O_2 fed pomegranate juice, and **d** *C. elegans* in H_2O_2 fed green tea. One-Way ANOVA with Post-Hoc Tukey HSD was performed at each 30 min time interval for all sub-figures. *Error bars* represent confidence interval of 95%, $N = 2$ replicates and $n = 10$ nematodes sample size per treatment; statistical differences between treatments are indicated by $*$ where $0.01 < p \leq 0.05$.

at 30 minutes under oxidative stress compared to 75% survival at 30 minutes under neutral conditions (Fig. 2).

Our results for survivability of *C. elegans* fed pomegranate juice in neutral conditions demonstrated increased survival rate with pomegranate juice at a concentration of 0.1 mg/mL ($N = 2$, $p \approx 0.042$) and 0.5 mg/mL polyphenol content ($N = 2$, $p \approx 0.04$) compared to the negative control group at the 90 minute interval (Fig. 2A). The treatment of pomegranate juice at 2.5 mg/mL shows no significant difference in survivability of *C. elegans* compared to the control group.

Our measurements for the survivability of *C. elegans* fed green tea in neutral conditions highlight the effect of green tea at 0.1 mg/mL to significantly boost survival rate compared to the negative control group ($N = 2$, $p \approx 0.026$; Fig. 2B). The green tea treatments at 0.5 mg/mL and 2.5 mg/mL show a similar trend in moderately increasing survival rate

of *C. elegans* when also compared to the negative control (Fig. 2B).

Our findings indicate antioxidants to exhibit differing effects at different time intervals on the survival rate of *C. elegans* under oxidative stress fed pomegranate juice (Fig. 2C). At the 30 minute mark, we observe the most significant differences between the effects of the antioxidant treatments. 0.1 mg/mL pomegranate juice has the largest effect on increasing survivability under oxidative stress ($N = 2$, $p \approx 0.031$), 0.5 mg/mL shows the second largest effect, and 2.5 moderately increases survival rate (Fig. 2C). However, this pattern fails to continue for the 60 minute and 90 minute measurement intervals, as the survival rates converge for all groups.

Green tea treatment shows similar results to pomegranate juice of increasing survivability of *C. elegans* under oxidative stress at the 30 minute interval (Fig. 2D). The green tea treatments at 0.5 mg/mL and 2.5 mg/mL produce a similar trend in moderately improving survival rates of *C. elegans* at 30 minutes. 0.1 mg/mL green tea has a significant effect on increasing survivability under oxidative stress at the 30 minute interval ($N = 2$, $p = 0.038$; Fig. 2D).

III. Discussion

To combat oxidative stress, antioxidants destroy and prevent the formation of reactive oxygen species (ROS) in *C. elegans*. Utilizing two distinct antioxidant-heavy treatments, pomegranate juice and green tea, we carried out a motility and survival assay to elucidate the effects of antioxidants on *C. elegans*. The treatments were administered at three different concentrations based on mg/mL of polyphenol content. We hypothesized antioxidants would raise the motility of *C. elegans*, and would heighten the survivability of *C. elegans* under neutral conditions and oxidative stress. Through our motility assay, we identified the 0.1 mg/mL concentration of pomegranate juice to increase the motility of *C. elegans*. Our survival assay revealed a trend across our lower concentrations of antioxidant treatments to increase survivability of *C. elegans* in neutral conditions. However, antioxidants improved *C. elegans* survivability for only a short interval when under oxidative stress.

The results from our motility assay provide the most straightforward effect of the antioxidant treatments we tested. Our findings highlighted pomegranate juice to improve motility of *C. elegans* at the lowest concentration of polyphenol content at 0.1 mg/mL. However, none of the green tea treatments seemed to have any effect on the motility of *C. elegans*, which was unexpected but not inconceivable. Green tea has been shown to have lesser effects as an antioxidant treatment compared to pomegranate juice, due to a three times as large

pool of polyphenols in pomegranate juice [7].

However, the effects of green tea as an antioxidant treatment differ when contrasting with the findings from our survivability experiment. In this assay, we saw green tea treatments produce a similar effect to pomegranate juice treatments under neutral conditions and oxidative stress. The results of the motility assay, regarding green tea, don't correlate with its effects on survivability, as the antioxidant capabilities of green tea may boost survivability but have no effect on motility. Pomegranate juice, however, demonstrate effects on enhancing survivability at lower concentrations. These data substantiate the evidence gathered in the motility assay, regarding the strong effect of pomegranate juice at 0.1 mg/mL improving motility of *C. elegans*.

Our findings presented a significant interaction between oxidative condition and antioxidant treatments at the 90 minute interval for the survivability assay. This implies that the effect of antioxidants on survival rate after 90 minutes will differ depending on whether *C. elegans* are in neutral conditions or are under oxidative stress. After 90 minutes without inducing oxidative stress upon *C. elegans*, some antioxidant treatments increased survival rate. However, antioxidants are unable to affect *C. elegans* under constant oxidative stress for 90 minutes.

Polyphenols have a wide range of antioxidant capacity on *C. elegans'* lifespan and stress resistance. Research by Saul et al. revealed the effects of catechins (found in green tea) and tannic acid, consisting of tannins (found in pomegranate juice), to vary across concentration levels. Tannic acid was found to increase survival rate of *C. elegans* in neutral conditions at $100\,\mu\mathrm{M}$ but decrease survival rate at $300\,\mu\mathrm{M}$. These results confirmed tannic acid to behave in a hormetic manner by displaying a relatively narrow beneficial concentration range, with toxic effects observed at higher concentrations. Catechins did not display hormetic properties, as they were found to increase survival rate across concentrations from $100\,\mu\mathrm{M}\,\mathrm{to}\,800\,\mu\mathrm{M}$ [11]. These findings corroborate our results for the survivability assay that we conducted. We found pomegranate juice, which contains tannins, to increase survivability of *C. elegans* in neutral conditions at lower concentrations. We found green tea, which contains catechins, to show a significant increase in survival rate at the lowest concentration, but there was no significant effect on survival rate for other concentrations due to high variance in survival rate across experiment replicates.

Saul et al. further highlighted tannic acid at lower concentrations of $100\,\mu\mathrm{M}\,\mathrm{to}\,200\,\mu\mathrm{M}$ to slightly boost survival rate of *C. elegans* under oxidative stress, while catechins greatly increased survival rate at all observed concentrations from $100\,\mu\mathrm{M}\,\mathrm{to}\,800\,\mu\mathrm{M}$ [11]. While our results for

pomegranate juice treatment match what Saul et al. discovered, we found green tea treatment to not greatly increase survivability of *C. elegans* under oxidative stress. We hypothesize that this difference in results comes from the difference in how *C. elegans* were treated. Saul et al. pre-treated *C. elegans* with polyphenols, then transferred them to plates with H_2O_2 while we only fed *C. elegans* our treatments after picking them onto plates with H_2O_2. We theorize that the limited supply of antioxidant treatments, pomegranate juice or green tea, given only while on plates with H_2O_2 was not enough to combat the constant oxidative stress.

The research Saul et al. conducted, uncovering the hormetic properties of tannins, correlates well to our motility assay where we saw pomegranate juice prove beneficial at the lowest tested concentration of 0.1 mg/mL but not at 0.5 mg/mL or 2.5 mg/mL. However, green tea was unable to increase motility at all. Tian et al. conducted research on the effects of green tea catechins on textitC. elegans, which justify our unexpected results. Their findings revealed catechins to cause a transient drop in ATP levels, accompanied by temporary hampering of mitochondrial respiration [12]. Research by Xu et al. found that a decrease in ATP levels correlates to a reduction in motility of *C. elegans* [13]. These findings taken together validate our results of green tea treatment to not escalate motility of *C. elegans* because of the short-term drop in ATP levels by green tea catechins. Xu et al. also discovered the same partial inhibition of mitochondrial activity that caused lower ATP levels and reduced motility to concurrently increase lifespan of *C. elegans* [13]. This research allows us to understand why the green tea treatment was able to enhance the survivability of *C. elegans* in neutral conditions, despite the temporary inhibition of mitochondrial activity.

Our findings do not correlate with our original hypotheses. We predicted all antioxidant treatments to enhance the motility of *C. elegans*, but our results demonstrated only pomegranate juice to do so. Along with this, we hypothesized an increase in survival rate from both antioxidants in neutral conditions and when *C. elegans* is under oxidative stress. Our findings refuted this prediction, as our antioxidant treatments heightened survival rates of *C. elegans* only when in neutral conditions. The results for our motility and survivability assays demonstrated concentration of antioxidant treatment to only affect pomegranate juice, however, in a different way than expected. We expected effect of antioxidant treatments to increase with concentration, but we found pomegranate juice to have a lesser effect with higher concentrations. Finally, we saw similar results for the effect of pomegranate juice and green tea on survival rate, but green tea failed to improve motility at all. Thus, our final hypothesis

that pomegranate juice would have a larger effect than green tea on motility and survivability was disproven, as pomegranate juice only had a larger effect on motility.

Our current results address observable changes to *C. elegans*, but we hope to understand fully the mechanism that our antioxidants treatments use to cause these changes. We suggest utilizing a qPCR assay to quantify expression of the *sod* genes, as they correlate to superoxide dismutase, an enzyme known to be activated by antioxidants and the presence of ROS. If we could observe an up-regulation of the *sod* genes in treated *C. elegans*, we can more strongly indicate that the treatments are affecting *C. elegans* because of the antioxidants within them and not other substances they might contain, such as caffeine or sugar.

The research we conducted indicated various significant effects of antioxidant treatments on the motility and survivability of *C. elegans* in neutral conditions and under oxidative stress. The assays we conducted can bridge the gap in knowledge on how antioxidants and oxidative stress can affect organisms. Our research may support further investigation into how incorporating antioxidants into a regular diet can affect the lifespan and mobility of humans. We investigated how inducing oxidative stress, causing an increase in ROS levels, affects the survival of *C. elegans*. As humans share a homologous pathway to reduce ROS levels with *C. elegans*, our experimentation utilizing oxidative stress can further support future research into how we might aid human survival under oxidative stress [4].

IV. Acknowledgments

This endeavor would not be possible without equal contribution from my teammates. We worked on the planning and carrying out of this research project as a whole, as well as each individual assay. As a result, we successfully completed our research while broadening our knowledge on the field. I would also like to express my deepest appreciation to Dr. Schlichting for incredible instruction and advising throughout the research process. Without the hours of laboratory time Dr. Schlichting held for us and the instruction on laboratory equipment and techniques, this research would not be possible. Special thanks to the lab staff and UAs for assisting with difficult tasks and advising along the way as well. Lastly, I'd like to mention my classmates for insightful feedback throughout the writing process and for making this endeavor a wholly enjoyable experience.

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