

The effect of neuroinflammation and oxidative stress on the recovery time of seizures

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SUMMARY

Epilepsy is a neurological disorder in which neuronal activity in the brain is disturbed resulting in recurrent seizures and is the second most common neurological disorder. Epilepsy has large impacts on patients as well as the healthcare system. Neuroinflammation and oxidative stress are both known to play a role in the occurrence and severity of seizures. When neuroinflammation occurs, oxidative stress occurs simultaneously. Oxidative stress has been shown to accelerate aging and reduce reproductivity in *Caenorhabditis elegans*. We tested effects of oxidative stress from seizures by evaluating the longevity, egg-laying, and electroshock resilience of *C. elegans*. *Pseudomonas aeruginosa* was used as an oxidative stressor in this experiment, resulting in neuroinflammation in *C. elegans*. When neuroinflammation occurs, three anti-inflammatory genes, *pmk-1*, *elt-2*, and *skn-1* are activated in order to help reduce inflammation. We blocked the translation of the anti-inflammatory genes using RNAi, to assess oxidative stress without the *C. elegans* anti-inflammatory response when neuroinflammation was induced by adding *P. aeruginosa* into the *C. elegans* diet. Our study revealed that oxidative stress and neuroinflammation diminish longevity and reproductivity while also increasing recovery time after seizures in *C. elegans*. Further study backs up these findings, indicating that neuroinflammation and oxidative stress both worsen seizure severity. This research can help lead to future studies and may also lead to finding new therapeutics for epilepsy.

INTRODUCTION

Epilepsy is the second most common neurological disorder in the United States. Around 2.2 million Americans and 50 million people worldwide have been diagnosed with epilepsy (1). Every year, around 5 million new cases of epilepsy are diagnosed around the world. According to the World Health Organization, 1 in every 26 people will develop epilepsy during their lifetime (2). One in every 100 people has at least had a series of seizures or has been diagnosed with epilepsy (3). A seizure occurs when there is a burst of uncontrolled electrical activity in the brain that disrupts normal brain signals. When a person experiences two or more unprovoked seizures it is known as epilepsy (4).

Neuroinflammation has been known to affect the occurrence and severity of seizures. Neuroinflammation

is an inflammatory response that occurs in the brain and spinal cord. This is mediated by cytokines, chemokines, reactive oxygen species, and secondary messengers. These mediators are created by microglia and astrocytes during immune responses (5). Uncontrolled neuroinflammation can lead to many degenerative disorders of the brain, such as Alzheimer's, Parkinson's, and multiple sclerosis. Neuroinflammation contributes to the formation of individual seizures and can result in cell death. These, in turn, activate more inflammation, which further causes seizures (6).

Cytokines are a large group of proteins that are secreted by the immune system. They are important in cell immune and inflammation responses. Inflammatory cytokines are also involved in nerve injury and neurodegenerative diseases (7). This is relevant to the project because cytokines are the factors produced by inflammatory genes, *pmk-1*, *elt-2*, *skn-1*, and will be used as a marker for inflammation.

We chose to use *Caenorhabditis elegans* as the model organism for this experiment. *C. elegans* are transparent nematodes that are about 1 mm long. In optimal environments *C. elegans* have a lifespan of approximately 12-18 days which consists of the embryonic stage, four larval stages (L1-L4), and adulthood (8). *C. elegans* are used as model organisms for several experiments including those involving seizures as their nervous system consisting of 302 neurons is well characterized and various proteins required for mammalian neurological function are conserved in *C. elegans*. There are also many electrophysiological techniques that were developed to study individual neurons in nematodes, making *C. elegans* an attractive organism to study abnormal electrical discharge that is seen in epilepsy. Automated systems for recording simple behaviors in *C. elegans* have also been developed (9).

Pseudomonas aeruginosa is a bacterium found in soil and water. It is known to cause infections in humans, and it affects many parts of the body, including the brain for humans and *C. elegans* (10). Every year, there are about 32,600 infections and 2,700 deaths in the United States due to *P. aeruginosa*. It spreads when people are exposed to contaminated water or soil and can even spread through touch and surfaces (11). When *C. elegans* are exposed to *P. aeruginosa*, they initiate an immune reaction to eliminate these bacteria. However, this immune reaction stimulates oxidative stress inside the *C. elegans* which then stimulates neuroinflammation and decreases the life span and egg laying ability in *C. elegans* (12).

Oxidative stress is a disturbance within the balance

between the production of free radicals and antioxidant defenses. Free radicals damage proteins, lipids, and nucleic acids within the cell. Antioxidants are molecules that donate electrons to free radicals and cause them to stabilize. When there are more free radicals than antioxidants or vice versa it results in oxidative stress (13). Oxidative stress has been known to hasten the aging process and cause neuroinflammation in *C. elegans*. This is often because when there are lower oxygen concentrations the lifetime becomes shorter. Oxidative stress also reduces the reproduction of *C. elegans*. It is known that after oxidative stress treatment, there will be a severe decline in egg laying in the first three days. Research shows that this might be because of the lower levels of ATP (14). To recover from the oxidative stress caused from the exposure to *P. aeruginosa*, *C. elegans* go through a three-step recovery process to recover from the symptoms. This recovery process causes neuroinflammation, which is what we hypothesized will increase seizure recovery time. The three steps within the recovery process include resolution of inflammation, elimination of any harmful molecules, and repair of damaged tissue. Using this information, it is often concluded that when *C. elegans* are exposed to *P. aeruginosa*, inflammation occurs. Three proteins that are produced during this recovery process are mitogen-activated protein kinase (PMK-1), ectodermally restricted POU domain transcription factor (ELT-2), and skinhead-1 (SKN-1) (15). PMK-1 and ELT-2 are both necessary to get over the inflammation caused by the bacteria. PMK-1 is a part of the p38 MAPK pathway which regulates the oxidative stress response by phosphorylating SKN-1. SKN-1 is an anti-inflammatory protein which when activated helps lower neuroinflammation in *C. elegans* (16). We knocked down these three genes using RNAi to allow neuroinflammation and oxidative stress to occur while the *C. elegans* were exposed to *P. aeruginosa*. Without these genes, the *C. elegans* will not be able to phosphorylate the proteins needed to recover from the oxidative stress and neuroinflammation. Along with these three genes, we also knocked down *rol-6* as a positive control to make sure that the RNAi was successful.

We hypothesized that by incorporating neuroinflammation and oxidative stress, the recovery time from seizures would increase. A longevity assay was used to measure lifespan which can be correlated to oxidative stress in *C. elegans*. Our results show that there was oxidative stress occurring because the lifespan of the *C. elegans* significantly decreased. An egg-laying assay was used to determine if oxidative stress is happening. It was also used to see if *P. aeruginosa* affects the reproduction of *C. elegans*. The results show that oxidative stress was occurring due to the decreased amount of egg laying. This research could help lead to finding a way to shorten recovery time for seizures.

RESULTS

P. aeruginosa increases oxidative stress

We conducted an egg laying assay in *C. elegans* to determine if oxidative stress was occurring. We knocked down *skn-1*, *elt-2*, *pmk-1*, or *rol-6*, and wild-type with or without an empty RNAi vector. *Rol-6* was used to verify our construct did knock down expression as it produces a known, easily visible phenotype. *P. aeruginosa* was added to the diet of *C. elegans* in each group. All experimental groups that had been exposed to *P. aeruginosa* showed a 66% decrease in reproductivity measured in the egg-laying assay (**Figure 1**). This shows that oxidative stress has occurred because of the decrease in reproductivity. All the RNAi-knockout worms had a decrease in egg laying compared to the wild type worms, but it was not statistically significant ($p = 0.058$, two-way ANOVA), indicating that neuroinflammation does not play as big of a role in egg laying as oxidative stress. However, in all cases, *C. elegans* exposed to *P. aeruginosa* still had decreased reproductivity. It is possible that the observed phenotypes may not be caused by oxidative stress ($p = 0.05$, two-way ANOVA, **Figure 1**).

Neuroinflammation and oxidative stress increase recovery time of seizures

We conducted the electroshock assay to determine how the recovery time from seizures was affected by neuroinflammation and oxidative stress. The same groups used in the egg laying assay were used for this assay. *C. elegans* were shocked in a tube filled with M9 buffer, a solution with no nutrients and that inhibits egg-laying behavior of wild-type animals. The electrical shock causes seizures in *C. elegans* and the time to recover (stop seizing) was measured. The recovery time was measured by recording the amount of time it took for the *C. elegans* to return to its sinusoidal shape using a stopwatch. All groups exposed to *P. aeruginosa* in their diets had significantly higher recovery times than their respective control groups ($p < 0.05$, two-way ANOVA, **Figure 2a**). All the variable groups that were not exposed to *P. aeruginosa* had significantly lower recovery times. All the control groups expressed a shorter recovery time than the variable which is what we expected (**Figure 2b**). All the RNAi worms had a little more increase in recovery time, but it was not statistically significant. It is possible that the observed phenotypes may not be caused by oxidative stress ($p = 0.05$, two-way ANOVA, **Figure 2**).

P. aeruginosa increases neuroinflammation

Next, we conducted a longevity assay to determine whether neuroinflammation is having an effect on the lifespan of the worms. Again, we tested the same groups as previously mentioned. All the RNAi worms had significantly more decline in lifespan, indicating that the knocking out of the genes causes there to be an increase in neuroinflammation ($p < 0.001$, Log-rank test, **Figure 3**). The result demonstrated that *P. aeruginosa* alone can significantly lower the lifespan

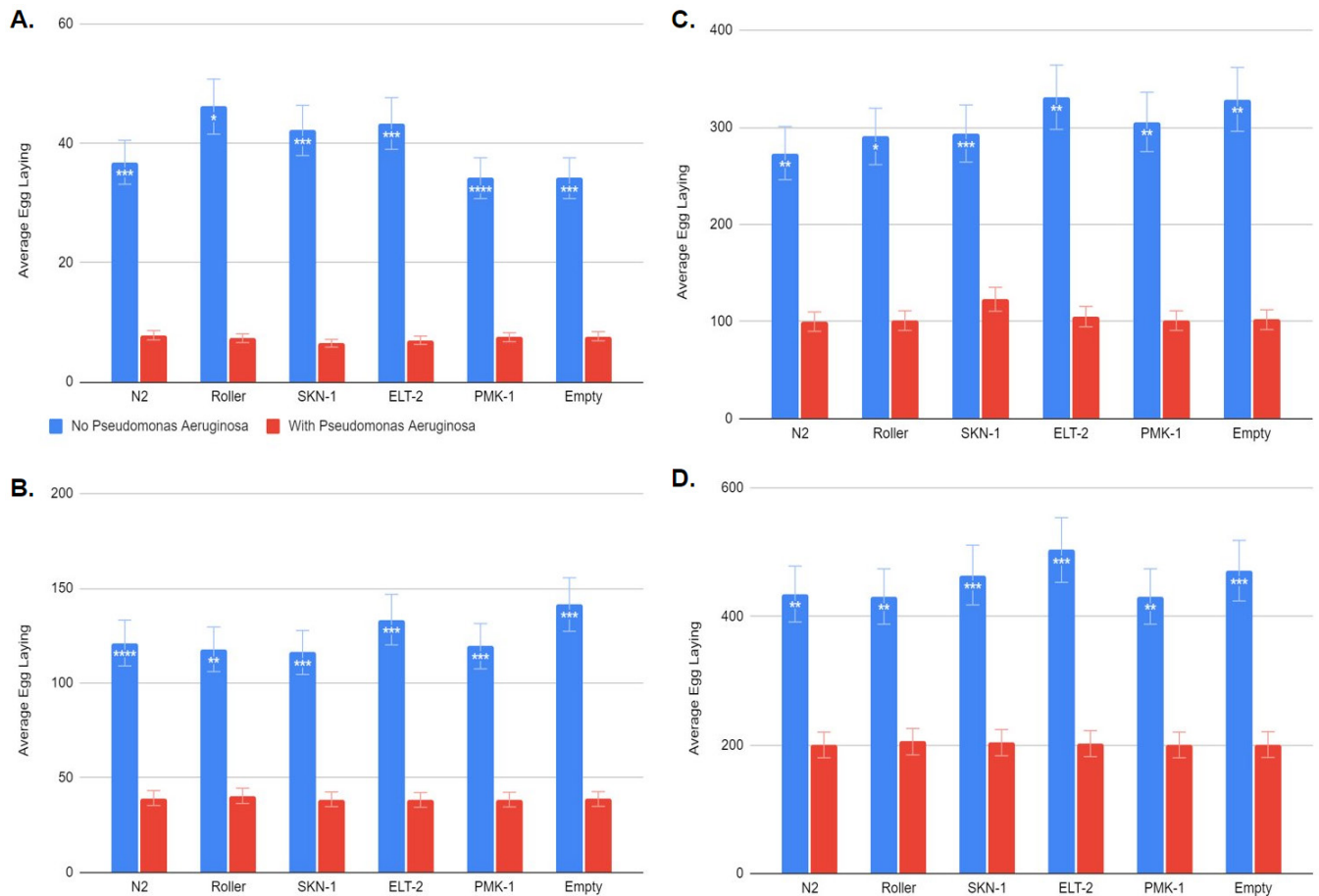


Figure 1. Addition of *P. aeruginosa* reduces egg laying. Average egg laying after (A) 24 hours, (B) 48 hours, (C) 72 hours, and (D) 96 hours. N2 stands for wild type. Data shown as mean \pm SD, $n = 10$ for all timepoints and all groups. Two-way ANOVA, $***p < 0.05$.

of the worms from 18-20 days to 3-6 days. Overall, these results support the hypothesis, indicating that exposure to *P. aeruginosa* increases oxidative stress and shortens lifespan. It is possible that the observed phenotypes may not be caused by oxidative stress ($p = 0.05$, Log-rank test, **Figure 3**).

DISCUSSION

The purpose of this study was to observe the effects of oxidative stress and neuroinflammation on the recovery time for seizures in *C. elegans* that were exposed to *P. aeruginosa*. *P. aeruginosa*, a pathogenic bacterium, was used to cause oxidative stress and neuroinflammation, which have also been linked with seizures (11). The worms were exposed to these bacteria in their diet and/or were genetically manipulated to block PMK-1, SKN-1, and ELT-2, anti-inflammatory markers that are used in the recovery process against oxidative stress and neuroinflammation. We hypothesized that RNAi-mutant and wild-type worms exposed to *P. aeruginosa* would have a significantly less life span compared to the worm group that was not exposed to *P. aeruginosa*. We also hypothesized that *P. aeruginosa* exposure would result in lower reproductivity and longer recovery time from seizures compared to worms not exposed to *P. aeruginosa*. Additionally, we believed

that the RNAi mutants would have worse effects because of the lack of recovery from neuroinflammation compared to wild-type worms with the same exposure. We tested our hypotheses with a longevity, egg-laying, and electroshock assay. Our data supported our hypotheses that impaired neuroinflammation and oxidative increased recovery time from seizures, decreased lifespan, and egg laying.

In future experimentation, the specific stressor that is used to instigate oxidative stress can be different. For example, instead of using *P. aeruginosa* as the stressor for oxidative stress, other stressors could be used, such as starvation and elevated temperatures. This project can also be done with different concentrations of *P. aeruginosa* to analyze which concentration the *C. elegans* have a longer recovery time. In addition, other seizure induction assays could be used. One drug that has been known to induce seizures in *C. elegans* is pentylenetetrazol (17). This project can also be redone using the SD66 Grass Simulator to deliver a better shock to the *C. elegans* as it would allow for more control of the voltage of the shock compared to the 9V batteries we used. Another limiting factor is that the phenotypes shown may not be due to the oxidative stress and could be due to other factors. The egg laying assay was done as a confirmation of oxidative, but we

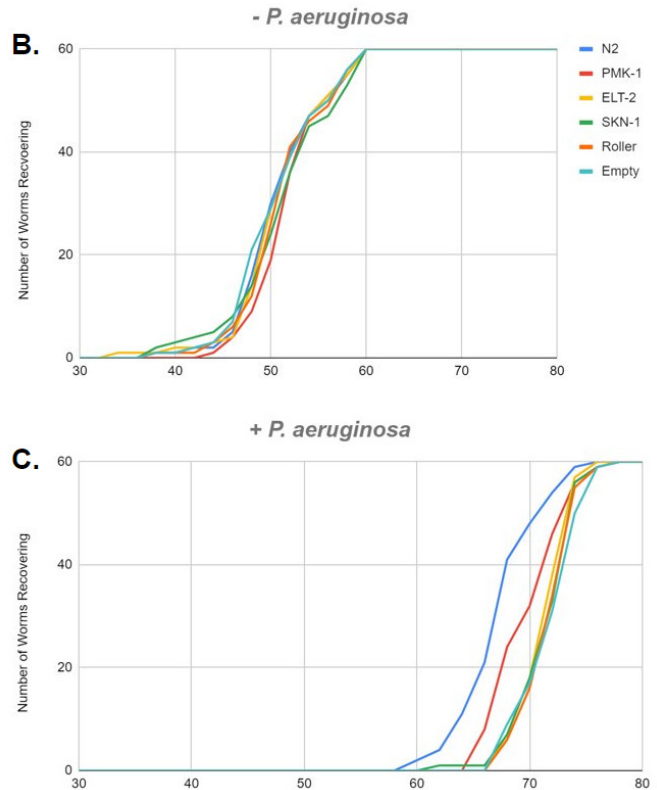
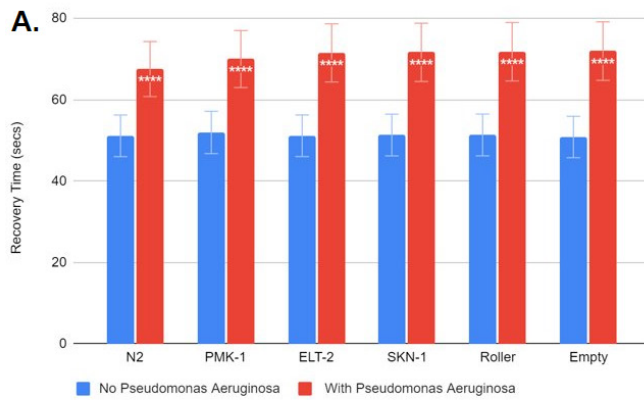


Figure 2. Electroshock assay results. (A) Average recovery time. Recovery time for groups (B) without and (C) with *P. aeruginosa*. Incubation with *P. aeruginosa* increased seizure recovery time for all groups. N2 stands for wild type. Data shown as mean \pm SD, $n = 10$ for all timepoints and all groups. Two-way ANOVA, $***p < 0.05$.

still could not be one hundred percent sure the results were due to oxidative stress.

Our results show that oxidative stress and neuroinflammation play important roles in seizures and recovery from seizures. Better understanding how oxidative stress and neuroinflammation contribute to seizures and other neurodegenerative diseases could result in the development of new treatments for affected individuals.

MATERIALS AND METHODS

C. elegans growth and maintenance

Nematode Growth Medium (NGM) agar from Sigma Aldrich was dissolved in ddH₂O and heated until the agar dissolved. The agar was autoclaved for 15 minutes at 121°C then cooled to 58°C. After cooling, 25 mL of 1M PBS, 1 mL of 1M CaCl₂, and 1 mL of 1M MgSO₄ were added into the agar and plates were poured. After the agar solidified, plates were seeded with *E. coli* OP50, a standard food source *C. elegans*.

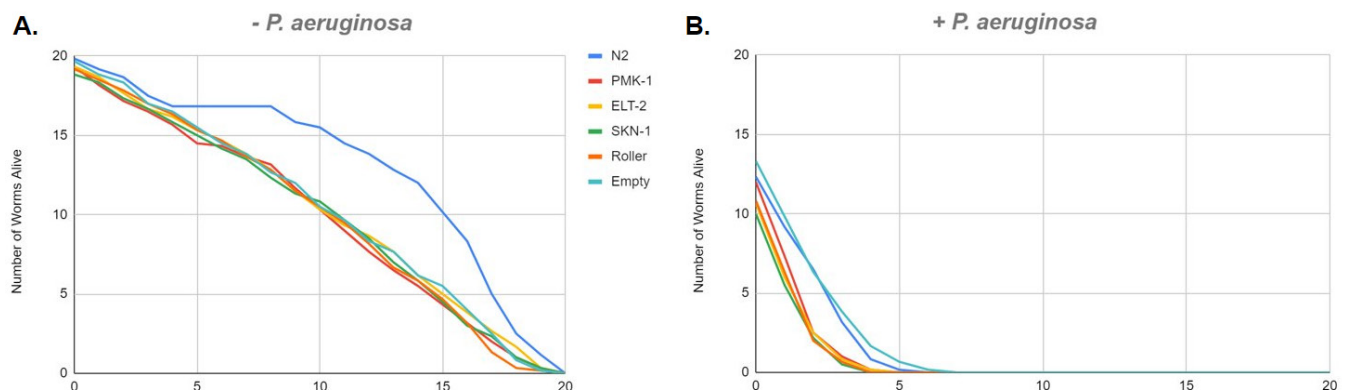


Figure 3. Longevity assay results. Average mortality rate when *C. elegans* (A) were not or (B) were exposed to *P. aeruginosa*. N2 stands for wild type. Data shown as mean \pm SD, $n = 10$ for all timepoints and all groups. Log-Rank Test, $***p < 0.05$.

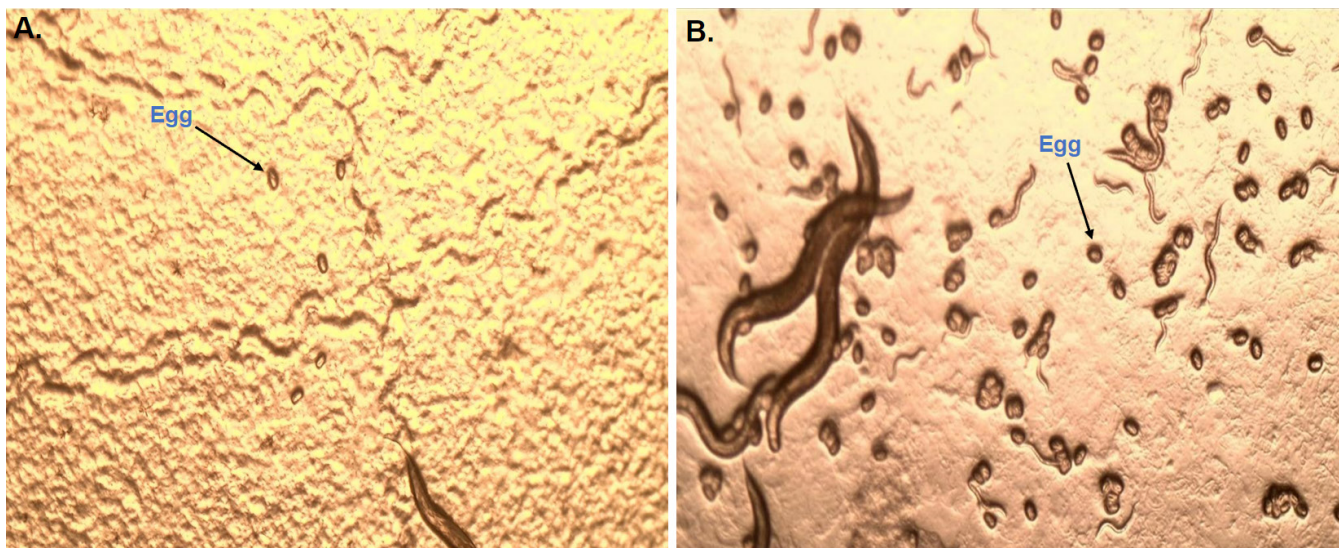


Figure 4. Egg laying assay. Plates (A) without and (B) with exposure to *P. aeruginosa*. Plates with exposure to *P. aeruginosa* in egg laying assay show egg laying for the plates without *P. aeruginosa*.

C. elegans were transferred to new NGM plates using a sterilized scalpel to move a chunk of agar from an old plate to a new plate. The *C. elegans* crawled out of the chunk onto the new plate every 3 days.

M9 buffer was prepared by mixing 3g KH_2PO_4 , 6g Na_2HPO_4 , 5g NaCl, 1 mL 1M MgSO_4 into 1L of H_2O . The solution was sterilized by autoclaving for 20 minutes at 121°C . This buffer was used as the medium for age synchronizing and the electroshock assay because the *C. elegans* can survive in it and it gives them no nutrients to age.

Age synchronization

Worms were grown until the adult stage. The plates were checked for eggs. M9 buffer was pipetted onto the plate and a cell scraper was used to scrape all the *C. elegans* into one place. More M9 buffer was pipetted onto the plates. M9 buffer with the *C. elegans* was pipetted off the plate and put into a micro centrifuging tube. Worms were pelleted at 800 rpm for 15 seconds at 36°C . The supernatant was discarded. The pellet in the tube was washed with the M9 buffer 1-3 times until it did not have any more bacteria. A 5% bleaching solution was added to the worm pellet and placed on a shaker for one minute. The reaction was stopped by adding an M9 buffer to the tube. The tube was centrifuged one more time for 15 seconds and the supernatant was discarded. The pellet was washed with M9 buffer 3 more times. Eggs were placed onto unseeded NGM plates and incubated overnight at room temperature with agitation.

RNAi plates preparation

RNAi vectors were obtained from Horizon Discovery Biosciences. Vectors were maintained on LB + Amp (0.1 g/ml) and were added into each batch of LB broth after cooling. A concentrated solution of tetracycline (0.1 g/mL) was made. 9 μL of 0.1 g/mL tetracycline was added into each batch of LB

broth after cooling to allow the vectors to be activated. Each of the 5 RNAi vectors were added to the broth: rol8, skn-1, elt-2, pmk-1, and empty vector. The broth was then incubated overnight at room temperature with agitation. Then the NGM plates were seeded with this broth, allowing the RNAi vectors to turn "on" once *C. elegans* ingested the tetracycline.

Egg laying assay

Eggs of age-synchronized *C. elegans* were placed onto unseeded NGM plates and incubated with agitation for 48 hours at room temperature. The number of eggs laid by those *C. elegans* were counted (Figure 4). The assay was repeated ten times to ensure accurate results. A two-way ANOVA and a t-test were conducted with an alpha level of 0.05 between the complex moduli of all groups.

Longevity assay

Worms were age synchronized. The synchronized eggs were placed onto unseeded NGM plates and incubated with agitation. A hot platinum wire was used to determine if the worms were alive and to count them. The age, the number of alive worms, and the number of dead worms were recorded for 20 days (Figure 5). The assay was repeated ten times to ensure accurate results. A two-way ANOVA was conducted with an alpha level of 0.05 between the complex moduli of all groups.

Electroshock assay

C. elegans were transferred and maintained at 20°C overnight on NGM. Tygon microbore tubing was cut into 9 mm segments and filled with 15 μL M9 buffer. Thirty minutes before stimulation, ten *C. elegans* adults (Day 1) were taken and transferred to the Tygon tube using a pick. Two 4" single gauge insulated copper wires with 1 mm diameter were inserted into either end of the plastic tube 2 mm deep.

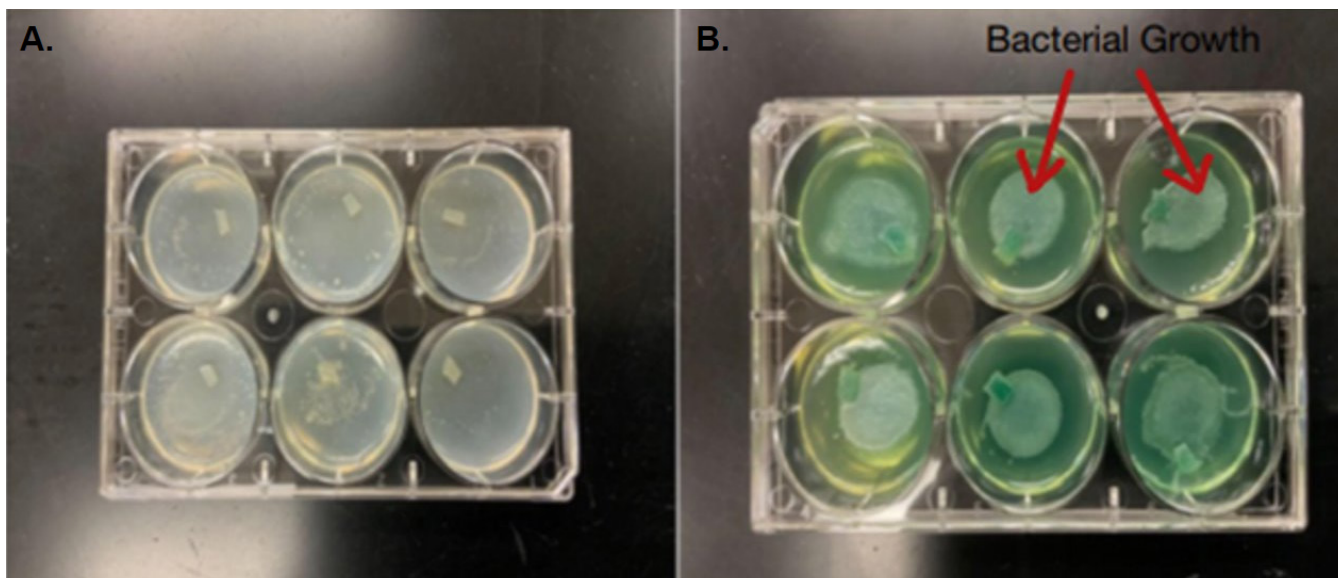


Figure 5. Plates used for longevity assay. Plates (A) without and (B) with exposure to *P. aeruginosa*. Plates with exposure to *P. aeruginosa* show clear *P. aeruginosa* growth.

2 alligator clips were attached clips to the copper wires and connected to the 5 9V batteries. The shocks, or electrical impulses, were delivered for 3 seconds (200 Hz, 3.5 ms, 47V). The assay was repeated ten times to ensure accurate results. A two-way ANOVA was conducted with an alpha level of 0.05 between the complex moduli of all groups.

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