TRANSGENERATIONAL INHERITANCE OF STARVATION EFFECTS ON LIFESPAN AND REPRODUCTIONS IN *CAENORHABDITIS ELEGANS*

by

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A THESIS

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Approved: _____

Dr. Patrick C. Phillips

It is imperative to study the effects of stressors on future generations to understand how we can mitigate negative effects that transcend generations. Transgenerational effects occur when the fitness of successive generations is affected by environmental stress that is not directly experienced by the progeny. Currently, there is no literature on the transgenerational effects of adult starvation in *Caenorhabditis elegans.* This investigation aims to address the following research question: Is 24 hours of early adult starvation in *C. elegans* significant enough to cause transgenerational effects in progeny lifespan and reproduction? Using the novel technique of microfluidics, *C. elegans* were starved for 24 hours and phenotypes of lifespan, brood size, and viability were assessed for the presence of a transgenerational effect. Data indicated there are transgenerational effects of starvation. In some trials, there was a decrease in lifespan of successive progeny while other trials showed an increase in lifespan. Additionally, in the reproduction assay, data indicated that starved individuals continued to lay more eggs than their fed counterparts on the last days of reproductive viability. Upon further review of collected data, a striking bimodal effect was seen in lifespan, independent of starvation. Some worm cohorts showed high early life mortality while others exhibited long lifespans. Our tightly regulated experiment

suggests that investigator, maintenance of cohort, overall temperature or humidity, and food availability are not factors that influenced the bimodal effect; thus, the results of this experiment have brought about an unexpected result that requires additional mechanistic investigation. In search for reproducible ageing interventions, these results highlight promising leads to be explored in future experiments that can work to uncover what epigenetic changes cause lifespan extension.

Acknowledgements

I want to sincerely thank and acknowledge Dr. Patrick Phillips, who has had unyielding faith in me while I have worked in his lab for the past two years. I am also forever grateful to my mentor Alex de Verteuil for her endless support, guidance, patience, and trust. Under their guidance I have come to learn more about science and research than any other avenue taken in my undergraduate career. I would also like to thank my other thesis committee member, Dr. Helen Southworth, for serving as representative of the Robert D. Clark Honors College. A special thank you goes out to Anna Coleman-Hulbert and Christine Sedore who so graciously helped me in the lab with every question I had. I would also like to thank all other members of the Phillips Lab Group for creating such a supportive work environment which fostered my growth. Lastly, thank you to my incredible family who continue to support me in all my endeavors whole heartily.

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Introduction

Every organism endures **stress** on a day to day basis. Stress is a physical, chemical or biological constraint that affects fitness. **Fitness** referrers to an organism's ability to survive and reproduce in a given environment. Often times, environmental stressors are the culprit that can impact multiple generations. **Environmental stressors** are external stimuli found in organism's habitat and can create variances in species fitness. One type of environmental stressor is deprivation of nutrients. In its extreme forms (starvation and malnutrition) nutrient deprivation can have major impacts on species fitness. The timing in which the stressor is induced can also impact species fitness in various ways. For example, research has shown that the stress a mother is subjected to can have adverse consequences on her offspring. Maternal malnutrition can impact an unborn fetus and potentially cause life-long health issues for the offspring (progeny). The severity of the impact on the offspring varies depending on the stage of **embryological development** during which the stress occurs (Painter, Roseboom, & Bleker, 2005).

Studies conducted in humans demonstrate how environmental stressors in parents can cause a problem for offspring. For example, a longitudinal study that examined the effects of the Dutch famine concluded that maternal malnutrition in utero increased the risk of offspring developing type 2 diabetes, obesity, cardiovascular disease, and renal disease (Painter et al., 2005). Similarly, Pembrey, *et al.* used the Avon Longitudinal Study of Parents and Children (ALSPAC) to determine that pre-adolescent parental smoking is linked to an increased **body mass index** (BMI) in their sons (Pembrey et al., 2006). Notably, there was no link correlated with the daughters of the pre-adolescent parental smokers. Additionally, they identified a link between the paternal grandfather's

nutrition in pre-adolescent age, and the mortality risk of their grandsons. They identified the same link between the paternal grandmother and their granddaughters.

Importantly in these cases, phenotypic changes were observed in the progeny whom did not directly endure the environmental stressor. Therefore, the observed changes in the progeny are an indirect effect of the environmental stressors. In contrast, a change observed due to an environmental stressor within the same generation (early and/or late life stressors) would be a direct effect. Overall, this study provides evidence that environmental factors can sustain generational change.

Our current understanding of environmental stressors allows us to understand how both a direct and indirect effect can potentially impact an unborn **fetus** within a generation. However, for specific environmental stressors, the impact can be sustained for multiple generations resulting in a transgenerational effect**.** A **transgenerational effect** is when the fitness of successive generations is affected by environmental stress that is not directly experienced by the progeny. In other words, progeny characteristics or **phenotypes** are incongruent with their current environment. One way to examine if an environmental stressor can sustain a transgenerational effect indirectly would be to present an environmental stress (for example starvation) to the mother early in the reproductive cycle. This stressor has the potential to impact the germline indirectly and transgenerational effects can be measured through successive generations. If the impact of a transgenerational effect is strong enough, it can affect progeny fitness for multiple subsequent generations (Kaeberlein et al., 2006). In contrast, an effect observed due to starvation of progeny within a generation, would not be classified as a transgenerational effect.

Stress can affect the way genes are expressed. Therefore, by studying transgenerational effects, we can better understand which environmental stressors can persist over multiple generations. Such understanding can work to help us ensure future generations do not suffer or endure the effects of these stressors by breaking the cycle and ensuring a better future for our offspring.

To facilitate my study of transgenerational effects I use the model organism *Caenorhabditis elegans* (*C. elegans*). *C. elegans* are nematode roundworms that live in soil in several climate zones (McDonald, 2014). They feed on bacteria and can be grown and maintained in a lab setting. Specifically, I use C. *elegans* to study the transgenerational effects of maternal starvation on the lifespan of their offspring. *C. elegans* are hermaphrodites that have a short life span, a well-defined stress response network, and distinguishable larval stages. A plethora of research has uncovered this organism's stress network and therefore, we understand their pathways to stress response (Kenyon, 2010). Since *C. elegans* are hermaphrodites that reproduce via self-fertilization, their offspring will have the same genetic makeup as the parent, allowing us to observe changes in the genome. Similarly, because the progeny will have the same genetic makeup as its parent, changes in phenotype are more easily distinguishable. Moreover, *C. elegans* develop to be a **gravid adult** within three days and their short life span permits for rapid experimentation. Additionally, they are easily visible with a microscope and easily maintained.

C. elegans have been classified as a great research model to study stress paradigms due to their well defines stress response network and tractable, measurable, phenotypic changes (Kaeberlein et al., 2006). Currently a large body of research identifies the direct

effect of starvation within the life span of an organism. This work also observes that the direct effects of starvation can cause transgenerational effects. In a study conducted on *C. elegans*, Rechavi *et al.* observed transgenerational effects from *C. elegans* that has been subjected to early-life starvation (Rechavi et al., 2014). The starvation consisted of six days in **L1 arrest,** an arrest in development during the first larval stage. They noted that these worms produced great-grandprogeny (G_3) with an increased lifespan. Therefore, they identify that early-life starvation is a stressor that was able to withstand multiple generations and caused a transgenerational effect. However, this study does not identify whether stressors that occur later in life can cause transgenerational effects to the offspring. This is an important distinction to make given many animals, such as humans, are born with a complete and developed germ line. Thus, to determine if maternal stressors later in life can affect offspring, late-life stressors must be studied.

Project Purpose and Hypothesis

In my study, I was interested in researching transgenerational effects of the starvation of maternal adults. Starvation within early-life development and starvation of fully developed worms differ. This difference is due to the fact that adult starvation implies there needs to be a somatically induced change in the germ line of the adult in order to see a change in the progeny. This is in contrast to a worm in early development which is still developing its germ line. The aim of my research was to address the following question: Is 24 hours of early adult starvation in *C. elegans* significant enough to cause transgenerational effects in progeny lifespan and reproduction?

I collected lifespan data on generation one, two and three offspring of maternally starved adults to assess whether a transgenerational effect was observed. I hypothesized that starvation in a maternal adult would cause an initial decrease in lifespan of the first generation (G1) and a subsequent increase in lifespan of G2, and G3. I believed we would see an initial decrease in lifespan due to indirectly endured stress. I also expected to see a transgenerational effect, indicated by an increased lifespan, that is similar to results conducted in early-life starvation experiments which found an increase in lifespan in the great-grand progeny (Rechavi et al., 2014). Reproduction of generation one progeny of starved maternal adults was completed to investigate whether starvation caused a direct effect on generation one progeny. For the reproduction assay, I hypothesized that starved progeny would have a decreased brood size and decreased viability of their offspring.

Background

C. elegans Applicability to Humans

The entire C. elegans **genome** was sequenced in 1998 and it became the first organism to have its entire genome sequenced. Among the 18,452 C. elegans protein sequences, 83% of those sequences have human homologous genes (Lai, Chou, Ch'ang, Liu, & Lin, 2000). Only about 11% of the genome contains genes that are only specific to nematodes (Lai et al., 2000). Additionally, C. elegans are one of the simplest organism which have a nervous system (McDonald, 2014). One third of their cells are neuronal cells (McDonald, 2014). Therefore, they are ideal for studying cell regulatory and signaling pathways. The findings can then be investigated in humans.

C. elegans Developmental Life Cycle

C. *elegans* have a short developmental time making them a prime choice for experimentation. As seen in the image below, a worm's entire developmental cycle from an egg to a day one adult is about 55 hours. Their development consists of four larval stages.

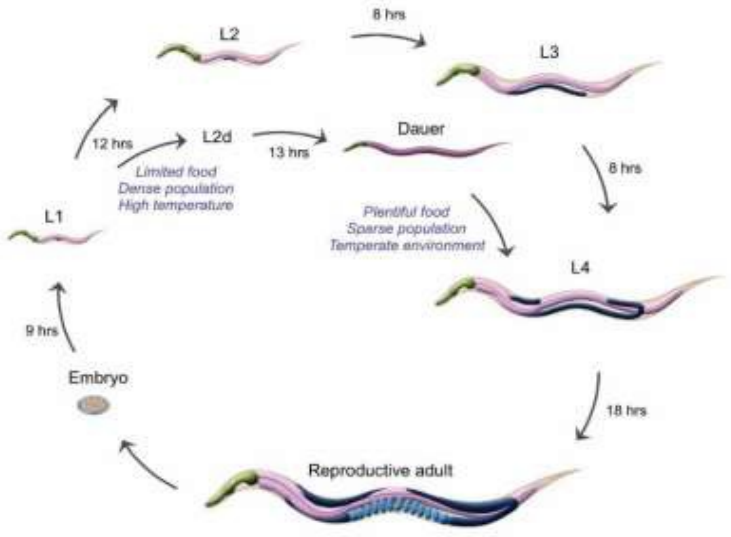

Figure 1: Depicts the developmental life cycle of C. *elegans*. Image resource is Worm Atlas.

Dauer Formation

Dauer is a dormancy state that can occur at the end of L1 or L2 of when the environmental conditions are unfavorable (McDonald, 2014). If worms are exposed to poor environmental conditions early in life, development is halted in the Dauer state. Environmental conditions that can induce Dauer formation include and are not limited to lack of food, high temperature, or overcrowding. Genetically, worms induce the Dauer state when they are there is no ligand present to bind to daf-2 (an insulin/IGF-1 receptor) and age-1 (a kinase) is not signaled (McDonald, 2014). Therefore, age-1 does not phosphorylate daf-16 and it does not signal growth and development (McDonald, 2014). See figure 2 below for signaling cascade in normal conditions and with Dauer formation. The worm can survive in this state for several months and does so by limiting protein synthesis and surviving on stored fat (McDonald, 2014). The Dauer state enables the worm to be resistant to extreme stressors and can lead to lifespan extension. Once environmental conditions return to a favorable state, the worm continues is developmental cycle. They then become of reproductive age and since C. *elegans* are **hermaphrodites**, they are able to self-fertilize. They can self-fertilize because they contain both sperm and eggs. From there they are reproductively active for about 4-5 days.

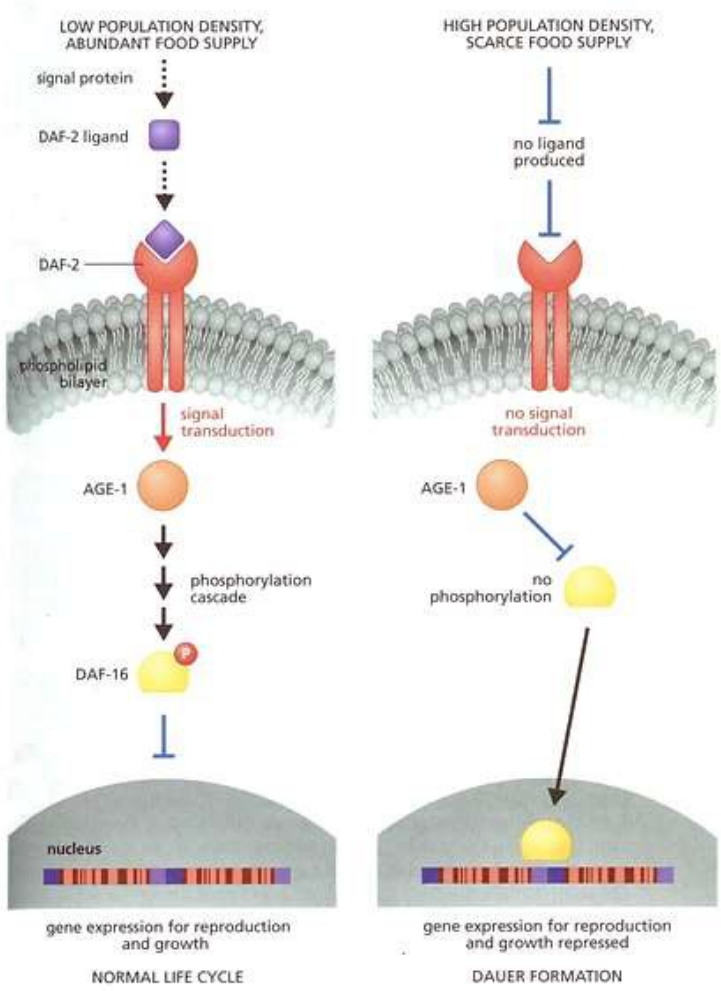

Figure 2: Image describing the signaling cascade in a normal life cycle and in Dauer formation (McDonald, 2014).

Early-Life Starvation

Many studies have identified transgenerational effects in subsequent generations following early-life starvation. Jobson *et al*. conducted a study with *C. elegans* that investigated the effects of starvation on individuals in early larval development and the effect on future generations (Jobson et al., 2015). The study found that *C. el*egans that were starved in their early life, grew at a slower rate, remained smaller as adults, and had delayed fertility. Additionally, they identified that individuals that were starved for

8 days took longer to lay their eggs and were shorter in length than individuals that were starved for 1 day. The study also observed that the progeny, great progeny, and great-grand were smaller and had a reduced **brood size**. Therefore, transgenerational effects were identified with parental early-life starvation. Furthermore, Rechavi *et al*. found that early-life starvation caused increased lifespan in the G3 generation. However, there is still no systemic characterization of transgenerational effects or long-term phenotypic consequences of adult starvation on progeny in *C. elegans*. This thesis aims to identify the consequences of adult starvation on subsequent generations.

Lifespan

Lifespan is an observable phenotype which is influenced by complex interactions between genetic and environmental factors (Stroustrup et al., 2013). In *C. elegans* heat stress, starvation in early-life, and oxidative stress have all shown an increase in lifespan (Kenyon, 2010). Phenotypical changes such as lifespan can occur in response to environmental stressors and allow the organism to protect its cell proliferation and bodily health. Kaeberlein *et al.* conducted a study which indicated that removal of bacterial food from adult worms increase their lifespan (Kaeberlein et al., 2006). A greater increase in lifespan was seen with complete starvation in contrast to partial reduction of food. Houthofood *et al.* found similar results which also showed increased lifespan within one generation when food was reduced and with complete starvation (Houthoofd & Vanfleteren, 2006). The study also identified that the Ins/IGF-1-like (IIF) signaling pathway is involved in life span extension in *C.* e*legans*. Overall, lifespan is a phenotype that has been observed in relation to dietary restriction.

However, currently, there are no present trends that observe lifespan transgenerationally following maternal starvation.

Automated Lifespan Machine (ALM)

The automated lifespan machine (ALM) allows for ease in measuring the phenotype of lifespan (Stroustrup et al., 2013). First, worms are placed on ALM scanners. The ALM then utilizes image processing and data validation software to determine lifespan. It measures lifespan by calculating the age at death based on worm movement (Stroustrup et al., 2013). ALM monitors the movements of each worm to determine when it dies. The machine highlights active worms that are moving frequently in purple. Once the worm begins slowing its rate of activity, the ALM recognizes the change in movement and highlights the worm as yellow on its software. When the ALM senses that a worm is no longer moving, it highlights the worm in red indicating the worms is deceased. All the detected deaths are then compiled into a storyboard which has to be analyzed by the experimenter. The experimenter looks at the images, excluding objects detected that are not worms and verifies that each worm death is marked accurately. Through this analysis, the ALM generates survivorship curves to represent the lifespan of the worms.

Adult Starvation

There is only one existing study in which adult starvation of *C. el*egans has shown an effect in the progeny. Hibshman *et al.* found that starved maternal adults produced less offspring that were bigger in size (Hibshman, Hung, & Baugh, 2016). Additionally, when the progeny were starved in early-life, they recovered better if their mothers had also been starved. These findings facilitated the authors discovery of

genetic pathways that transfer information from the mother to the offspring about the condition of maternal nutrition. The specific pathways identified include, insulin-like signaling, *pha-4/FoxA*, *skn-1/Nrf,* and *nhr-49/Hnf4*. While this study indicates adult starvation does cause an effect in the progeny and identified the specific pathways at play, it did not provide evidence for transgenerational changes. Since the study failed to identify a continual observed change in multiple generations following initial maternal starvation, the results cannot be classified as a transgenerational effect. My research aims to identify a transgenerational effect on lifespan of progeny following maternal starvation.

Microfluidics

Microfluidics is an emerging mode of phenotypic observation and sorting for *C. elegans*. The traditional method to observe longitudinal trends in *C. el*egans is labor intensive and has been utilized for 30 plus years. It involves the maintenance and observation of individual worms on agar plates. These worms would have to be transferred to new plates every day to prevent starvation and to be able to differentiate between generations of progeny. Microfluidics is an emerging practice which has eliminated many of the nuances present in the traditional method. The advantages of microfluidics include compartmentalized chambers which can separate individual worms, channels that can easily deliver food, channels that can flush wastes, and the chips transparency allows for imaging (Chung, Crane, & Lu, 2008). A special microfluidic chip was designed for this experiment by Alex de Verteuil. This chip differs from other microfluidic chips because it includes an egg collecting chamber. The chip design is depicted in figure 1. The egg capture chamber will ensure that only eggs

are collected from the adult starvation. Flushes with **buffer** occur every 30 seconds. Worms will be loaded in the chip and will aggregate in the arena. Once the worms begin laying eggs, the eggs will fall through the filter and collect in the egg capture chamber as depicted in figure 2. Subsequent flushes can be done to flush eggs from the egg capture chamber and into a collection tube. These eggs can then be used for experimentation.

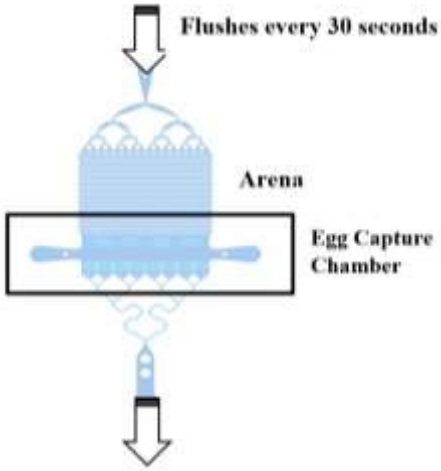

Figure 1: Microfluidic chip with egg capture chamber present. Alex de Verteuil in the Phillips lab designed this chip.

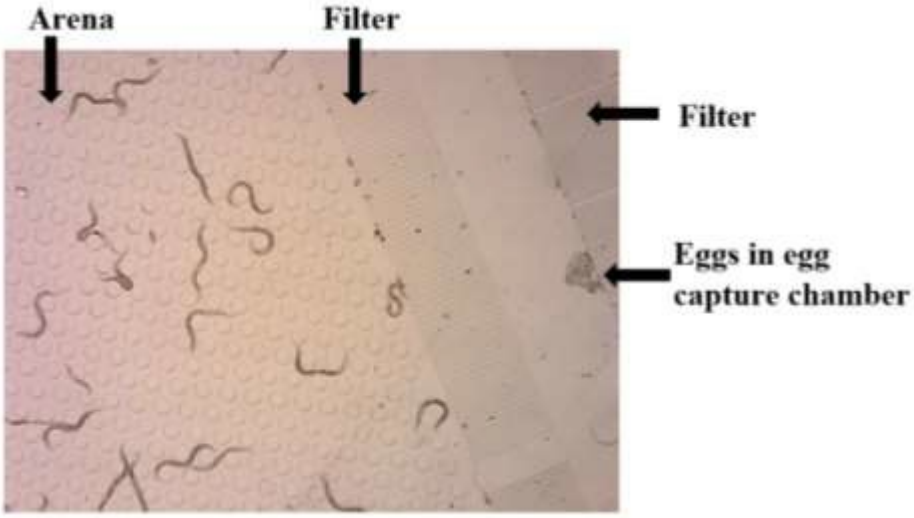

Figure 3: Worms in the arena of the microfluidic chip. Eggs are seen in the filter and the egg capture chamber.

Methods

Strain Use and Maintenance for Lifespan Assay

Research was conducted with wild-type JU775 strain. The *C. el*egans were maintained on agar plates composed of standard nematode growth medium (NGM) and seeded with HB101 food source. The worms were kept in 20 degrees Celsius prior to experimentation. Animals were chunked to different plates throughout maintenance to avoid starvation and overcrowding prior to experimentation. Additionally, cohorts of worms were bleached every week to synchronize the population by age. This was done through a 'hatch off' protocol where bleach and NaOH is added to a cohort of worms. The adult worm carcasses dissolve, and the remaining eggs are rinsed thoroughly of any bleach residue. The hatch off protocol ensures a large age synchronized population for starvation set up. It was ensured that the worms remained well fed prior to experimentation, to prevent Dauer formation. Some of the genes involved in L1 arrest/Dauer formation also affect adult lifespan and therefore, we controlled for this variable to ensure it would not confound the results (Hibshman et al., 2016).

Microfluidic Chip Set-Up

All microfluidic chips were treated in Pluronic detergent. This detergent is toxic in high concentration, but a diluted concentration was used to coat the channels within the chip. Pluronic was used to create a smooth surface within the channels of the chip to prevent bacteria from aggregating. S Basal Complete was flushed through the chip to rinse it of any residue prior to introducing animals into the chip. S Basal Complete was used throughout the entire methodology that required microfluidic use. S Basal Complete is a fluid that does not contain food but rather provides nutrients for the

worms' survival. For the control chips that did contain food, the S Basal Complete has trace metal solution in it that also ensured the survival of the HB101 food source.

Microfluidic Chip Control Group

The control group in this experiment is the fed condition. A population of approximately 600 JU775 gravid adult hermaphrodites were placed into different microfluidic chips that all contained egg-lay chambers. The chips were continually flushed with S. basal complete which included HB101. The fed worms were kept in the chip for a 24-hour duration. At the 24-hour mark, the egg-lay chamber was flushed, and eggs were collected for the next two hours. We then plated the progeny collected from the egg chamber of the microfluidic chip onto agar plates with OP50-1 food supply.

Microfluidic Chip Starvation

To generate a cohort of progeny whose mother had experienced starvation, we completed starvation in the microfluidic environment. A population of approximately 600 JU775 gravid adult hermaphrodites were placed into several microfluidic chips each with an egg-lay chamber. The gravid adults were loaded into a chip that does not contain food. The adults were then starved for 24 hours within the chip. Since gravid adults lay eggs about every 2.5 hours, the adults would have laid about 300 eggs each within the 24 hour starvation period. Therefore, at 24 hours the chip was flushed with S. basal complete (buffer without food) and the eggs were collected and were not used in the experiment. I did not use these eggs in the experiment because they might have not experienced the full 24-hour maternal starvation. I then waited two hours to allow the adults ample time to lay their offspring and to ensure the eggs had experienced the full starvation through their mother. Following the 2 hours, all the chips were flushed again,

and all the eggs were collected as one starved cohort for use in the experiment. We then plated the progeny collected from the egg chamber of the microfluidic chip onto agar plates with OP50-1 food supply. Overview of the microfluidic starvation protocol is depicted in figure 3 (below). It is important to note that many of the gravid adults that were loaded into the chip eventually shrunk due to starvation and were lost through the chips outflow. Therefore, there was typically a smaller cohort of starved progeny that was collected when compared to the control. Additionally, due to starvation, some of the adult worms experienced the phenotype of matricidal hatching where offspring hatch inside the uterus and causes maternal death. Pickett et al, (2013) found that a disruption in insulin/IGF-1 signaling (a results of starvation) increases the instance of matricidal hatching. This also contributed to the smaller cohort of starved progeny collected.

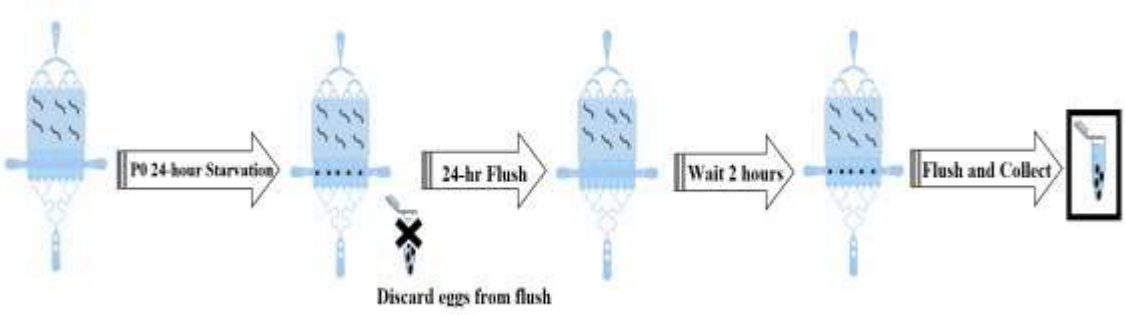

Figure 4: Experimental protocol for microfluidic starvation.

Lifespan Assay

The eggs collected from the microfluidic chip were placed on multiple agar plates with food (OP50-1). Once the eggs from the chip hatched and were Day 1 adults, I placed 40 gravid adults per **replicate** from each condition onto separate FUDR plates.

Each of these plates contained FUDR, which is a compound which ensures the worms are not able to produce viable offspring. Therefore, these plates contained the same 40 individuals for the duration of the experiment. Two subsequent transfers were completed onto additional FUDR plates to rid plates of eggs. The G1 for each condition were then placed on scanner plates and onto the automated lifespan machine (ALM). The automated lifespan machine (ALM) measured lifespan by calculating the age at death based on worm movement (Stroustrup et al., 2013). Overview of experimental protocol for G1 is depicted in figure 4.

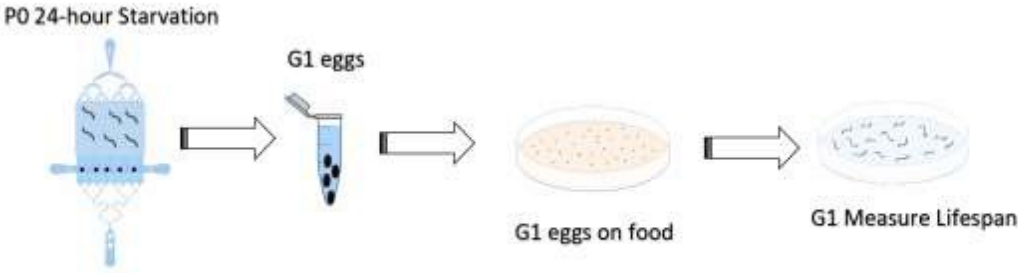

Figure 5: Experimental protocol for measuring G1 lifespan. Following 24-hour starvation in microfluidic chip, eggs will be collected and placed on agar plates with food. Once the eggs hatch, 40 gravid adults will be placed on scanner plates to measure lifespan.

The remaining G1 worms on plates were maintained to grow generation two (G2) and generation three (G3) cohorts. In order to produce generational cohorts, the individuals were age synchronized by completing egg-lays. **Egg-lays** involve placing 25 gravid adults on a plate and giving them ample time to lay eggs. After the gravid adults lay their eggs, the adults are flamed off the plate and only the eggs remain. Therefore, I took some remaining G1 adults and completed an egg lay to collect G2 eggs. Once the eggs from the egg lay hatched and developed, they were G2 gravid adults. Forty G2 gravid adults per replicate for each condition then underwent FUDR transfers, were

placed on scanner plates, and eventually onto the ALM. Additional G2 adults were used to complete another egg-lay to collect G3 eggs. Once the G3 eggs from the egg lay hatched and developed, they were G3 gravid adults. Forty G2 gravid adults per replicate for each condition then underwent FUDR transfers, were placed on scanner plates, and eventually onto the ALM. Overview of measuring lifespan for G2 and G3 is depicted in Figure 5.

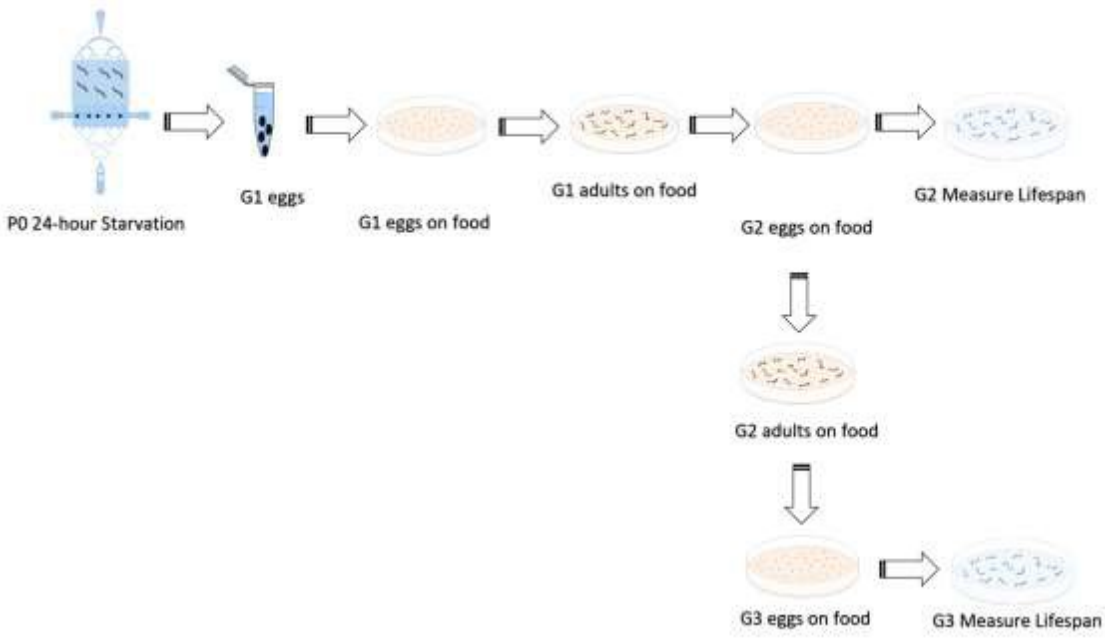

Figure 6: Experimental protocol for measuring G2 and G3 lifespan.

Bacterial Strains Used

We used HB101 E coli bacteria as a food source for worm maintenance prior to experimentation, and within the chip (for the fed condition). Specifically, we choose to use HB101 because this bacterial line has been used in microfluidic chips for starvation (Hibshman et al., 2016). After the worms were removed from the chip, they were placed on plates with OP50 bacteria. We switched food sources because the subsequent FUDR and scanner plates that are used for the lifespan assay have OP50 bacteria on them.

Additionally, HB101 grows a thicker lawn and would make it difficult for the ALM to detect worm movement. In making this decision we looked at the literature which compared the two bacterial strains. The biggest difference between them is that HB101 has a greater carbohydrate concentration. However, Brooks *et al*. found that despite the difference in composition of the bacterial strains, there was no significant difference in lifespan between worms maintained on the different bacterial sources. Therefore, we decided that changing bacterial strains should not confound our results.

Automated Lifespan Machine (ALM) Storyboarding

Once the ALM scanner run was completed the software was used to generate storyboards. Each storyboard contained several images of what the machine detected as a worm death. I would go through each picture and verify worm death. If the machine had detected an object on the plate that was not a worm, I would exclude that image from the storyboard. Next, for the images that correctly identified worms, I would analyze each frame of the storyboard and verify that the correct time of death was marked. Specifically, I marked time of death once the worms ceased movement and when the worm began to extrude its contents outside of its body. Extrusion is visualized when a small circle forms in the middle of the worm's body and its contents are ejected. Once the circle was fully formed, worm death was marked at that time point. If the worm did not extrude, I would look for the time when the worm ceased movement and began expanding. I marked time of death at that time point because worms tend to swell after death. To ensure experimenters who completed storyboarding where marking time of death in the same manner, and thereby controlling for any associated bias, I created a

storyboarding procedure that detailed the above factors that were considered in determining worm death.

Brood Size and Viability Assay

The same microfluidic starvation protocol was followed to obtain fed and starved cohorts. The eggs collected from the microfluidic chip were then placed on a plate with OP50 bacteria. I waited two days until the worms were staged at L4. I then took 25 fed and 25 starved L4 worms and placed each one on a plate by itself. The initial step was completed in the evening, so the next step could be completed 36 hours after initial picking. I specifically choose 36 hours because it takes 12 hours for L4s to become day one adults thus leaving 24 hours of time for the day 1 adults to lay their progeny. Thirty-six hours after, I counted how many eggs and L1s were on the Day 1 plates. I then transferred the fed and starved worms and placed each one on a plate by itself labeled Day 2. Every 24 hours, the worms were transferred and the eggs/L1s were counted. Transfers stopped on Day 4 because the worms were laying little to no progeny. Data was then compiled into an excel spreadsheet for further analysis. To assess the viability of the progeny, I counted the adults present on all the plates (plates labeled days 1-4) and divided that number by the total number of eggs present for that day.

Results

Lifespan Assay

Lifespan was investigated to determine if there was a transgenerational effect seen in progeny after maternal starvation. Average lifespan values in days are shown in the table and graph below:

Generation	Replicate Number	Fed Average Lifespan (Days)	Standard Error	Starved Average Lifespan (Days)	Standard Error	P-value Between Conditions
G1	Rep 1	14.67	0.69	12.35	0.52	0.0354*
G2	Rep 1	11.90	0.25	10.2	0.30	<0.0001*
G2	Rep 2	19.27	0.29	19.00	0.41	0.6653
G2	Rep 3	20.33	0.40	20.77	0.27	<0.0001*
G2	Rep 4	13.61	0.70	20.14	0.45	0.7905
G3	Rep 1	14.64	0.31	12.38	0.33	<0.0001*
G3	Rep 2	14.32	0.31	16.08	0.34	0.0002*

Table 1: Average lifespan in days for each generation, replicate, and condition (fed vs starved). P-value was calculated using Log-rank test and any value below 0.05 was considered significant. * = a significant p-value between conditions of fed and starved

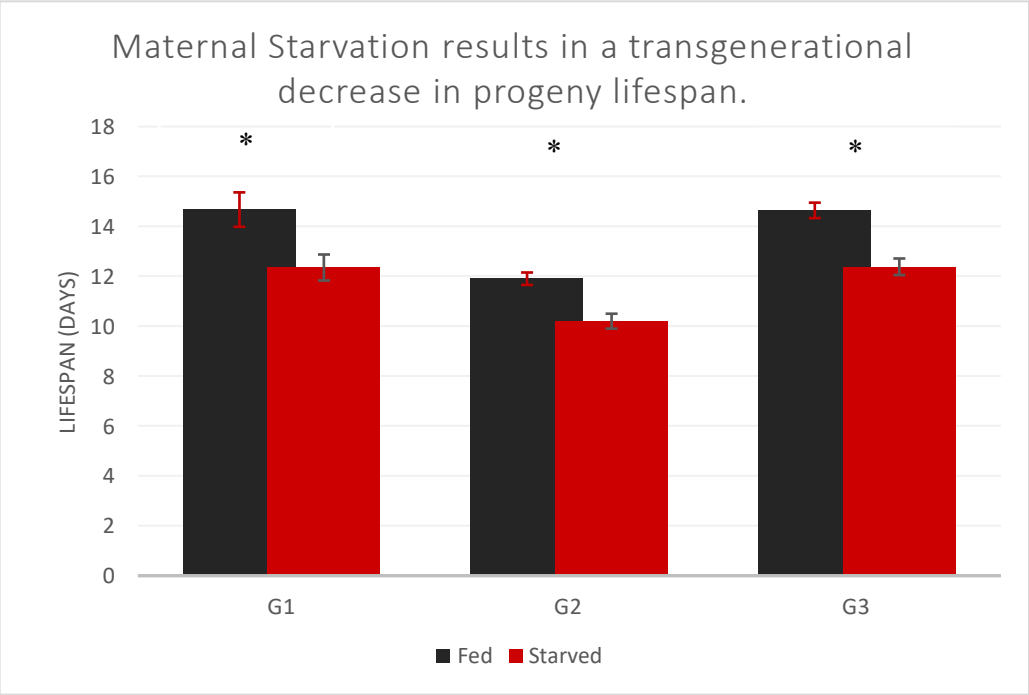

Figure 7: Transgenerational effects on lifespan across a population. Includes data for generation one replicate one, generation two replicate one, and generation three replicate one. * = a significant p-value between conditions of fed and starved

Data from table one and figure seven show G1 Rep 1, G2 Rep 1, and G3 Rep 1 all had a significant decrease in lifespan of starved progeny when compared to control. Since the decrease in lifespan was observed over all three generations, there is evidence to support a transgenerational effect.

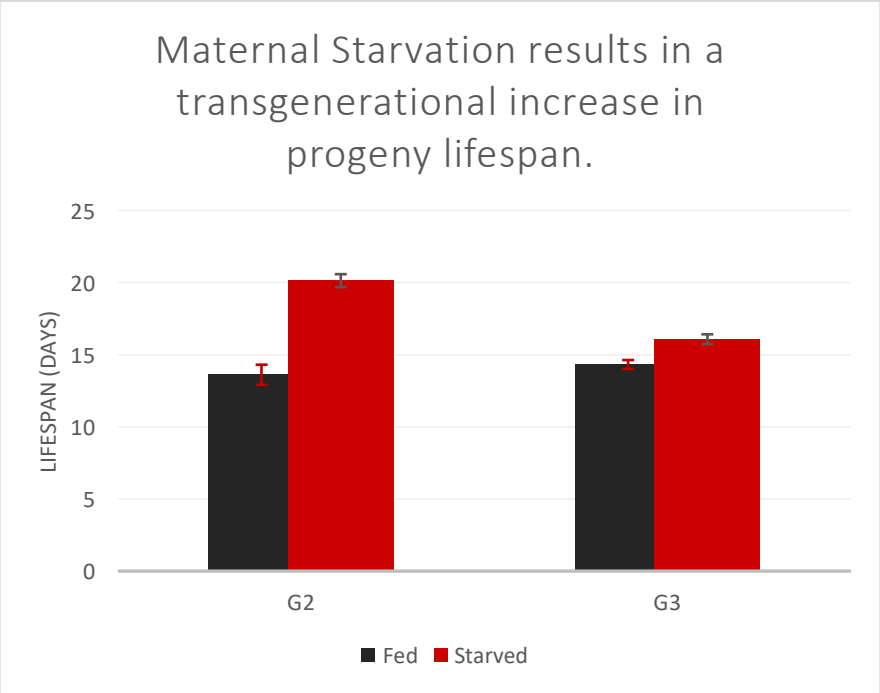

Figure 8: Transgenerational effects on lifespan within a population. Includes data for generation two replicate four and generation three replicate two. * = a significant p-value between conditions of fed and starved

As shown in table one and in figure eight, G2 Rep 4 and G3 Rep 2 indicated a significant increase in lifespan of starved progeny when compared to control. The offspring of the G2 Rep 4 was G3 Rep 2 and therefore, they are direct descendants of the same starved maternal cohort. This result indicates a transgenerational effect and an increase in lifespan of starved progeny within a population. However, this increase in lifespan opposes the effect seen with the other replicates that indicated a decrease in lifespan.

Reproduction Assay

Reproduction of generation one starved progeny was collected to assess whether starvation of maternal adults can induce a direct effect in progeny brood size and viability. Data collected on the average number of eggs per day and average total brood size is displayed in table 2 below:

Average Number of Day 1 Eggs Laid		Average Number of Day 2 Eggs Laid		Average Number of Day 3 Eggs Laid		Average Number of Day 4 Eggs Laid		Average Total Brood Size	
Fed	Starved	Fed	Starved	Fed	Starved	Fed	Starved	Fed	Starved
231	236	67.1	59.6	23.0	21.8	5.55*	23.8*	326	341

Table 2: Average number of eggs per day for each condition and average total brood size for each condition. Significance is denoted with a * and was determined by having a p-value less than 0.05 between fed and starved conditions.

Data collected on generation one brood size indicated a significant difference in the amount of eggs laid on Day 4. Data shows that the fed progeny were laying an average of 5.55 eggs on day 4 while starved progeny were laying an average of 23.8 eggs. Significance was determined using a two-tailed, paired t-test which indicated a p-value of 0.002. Qualitative notes taken during the experiment indicate that day 4 eggs appeared inviable in both fed and starved conditions. Nevertheless, it appears that the starved progeny continued to lay inviable eggs into day 4 while the fed progeny were in the process of halting the production of eggs. However, there was no significant difference in the amount of progeny laid between fed and starved conditions on days 1 through 3. Additionally. there was no significant difference in the viability of the progeny between fed (76.90% viability) and starved (76.12% viability) conditions.

Discussion

We completed lifespan and reproductive assays to address a hypothesis that adult starvation could cause transgenerational effects in lifespan and reproduction. Through the analysis of phenotypes of lifespan, brood size, and viability we discovered unexpected results. While results did support our hypothesis that starvation could cause transgenerational effects, the results were inconsistent. Thus, a definitive conclusion to whether starvation causes a transgenerational effect cannot be drawn. However, of great interest is the bimodal trend in lifespan discussed below that was independent of starvation or generation number.

Transgenerational Effects

The results indicated a transgenerational effect due to starvation, when looking at generation one, two, and three. However, the data was inconsistent to whether starvation caused an increase or a decrease in lifespan of starved progeny. An increase in lifespan could have resulted from a decrease in insulin-like growth factors (IGF-1). Insulin-like peptides within the worm (which can also bind and activate human insulin receptors) bind the Daf-2 receptor (Hua et al., 2003). This leads to an activation of AGE-1 (Hua et al., 2003). Downstream, this results in the phosphorylation of DAF-16/FoxO transcription factors (Hua et al., 2003). The role of DAF-16/FoxO is to promote survival when worms go into L1 arrest in response to the environmental stressor of starvation (Hua et al., 2003). Therefore, these transcription factors have been a target of research for investigating longevity. Additionally, a decrease in glucose causes an increase in AMP which in turn activates AMPK (Greer et al., 2007). AMPK upregulates oxidative phosphorylation resulting in an increase in reactive oxygen

species (ROS) (Greer et al., 2007). An increase in reactive oxygen species will promote the upregulation of antioxidant enzymes like SOD and catalase in order to destroy the ROS (Greer et al., 2007). This will in turn increase lifespan as the organism is able to build an arsenal of antioxidant enzymes that can decrease oxidative stress.

The lifespan assay results also indicated a decrease in lifespan of starved progeny in generation two and three. The decrease in lifespan in the starved progeny could have been a result of too much stress on the maternal organism. The stress could have impacted the germline epigenetically through some unknown pathway. Perhaps, the mother who experienced starvation, was not able to allocate all the necessary resources to yolk sac and egg.

Overall, given the results of the lifespan assay showed variable results, I do not feel a causal relationship can be drawn between starvation and a transgenerational effect in lifespan.

The Search for a more Reasonable Conclusion

In search of a more reasonable conclusion to draw from the data resulted, I graphed all the generations and replicates on a single graph to visualize the results. After graphing the results of each replicate on the same graph, it became apparent that there was an interesting result seen below in figure 9.

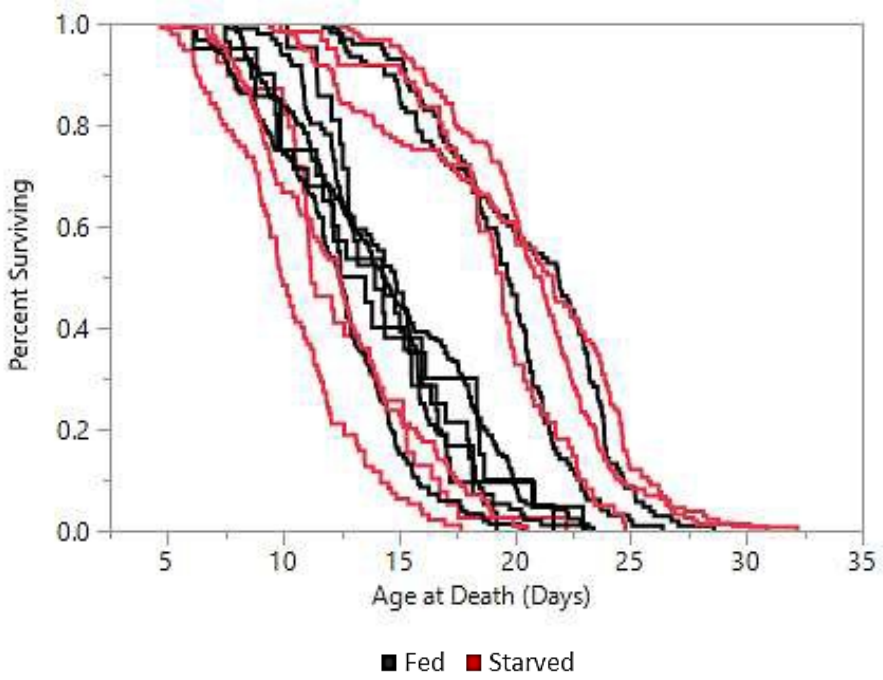

Figure 9: Lifespan curve for generation one (G1), generation two (G2), and generation three (G3) replicates for the fed and starved conditions.

When looking at figure 9 (above), it appears that some of the starved progeny are either living a shorter lifespan or a longer lifespan. When looking at the control group, the same effect is evident. Therefore, the graph does not indicate a direct change in lifespan due to the conditions of starvation. Instead, it indicates some worms exhibit a long lifespan and some exhibit a short life span independent of starvation. Figure 10, below, shows the same data as figure 9, but is color coded to demonstrate long-living and short-living phenotypes.

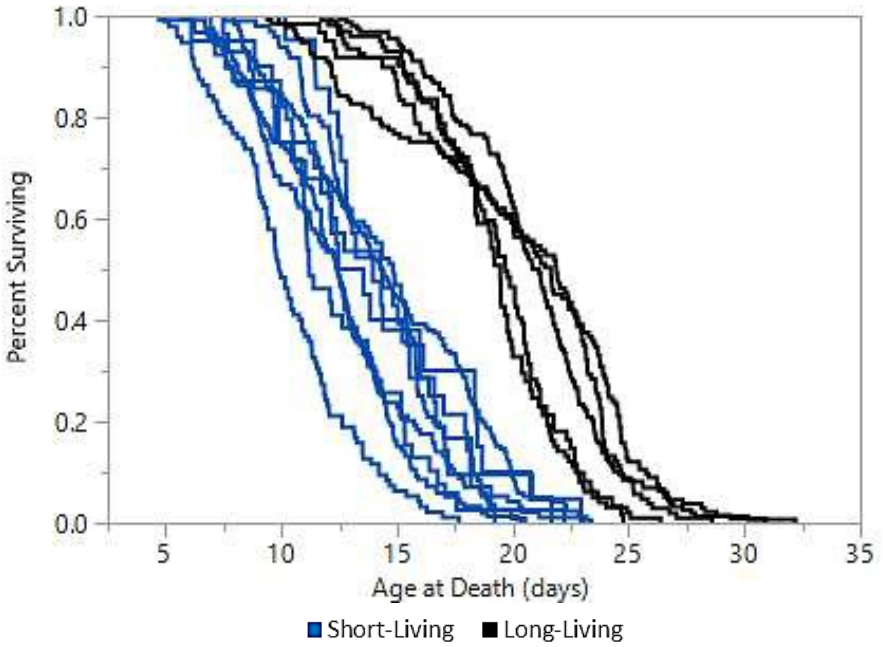

Figure 10: Lifespan curve for generation one (G1), generation two (G2), and generation three (G3) replicates for the fed and starved conditions. This is the same graph as depicted in figure 9 but is color coded differently to show long-living and short living worm types.

To ensure the short-living and long-living worm effect was also seen on each individual scanner plates of progeny, I graphed each individual plate for all generations in figures 11 A and B, below.

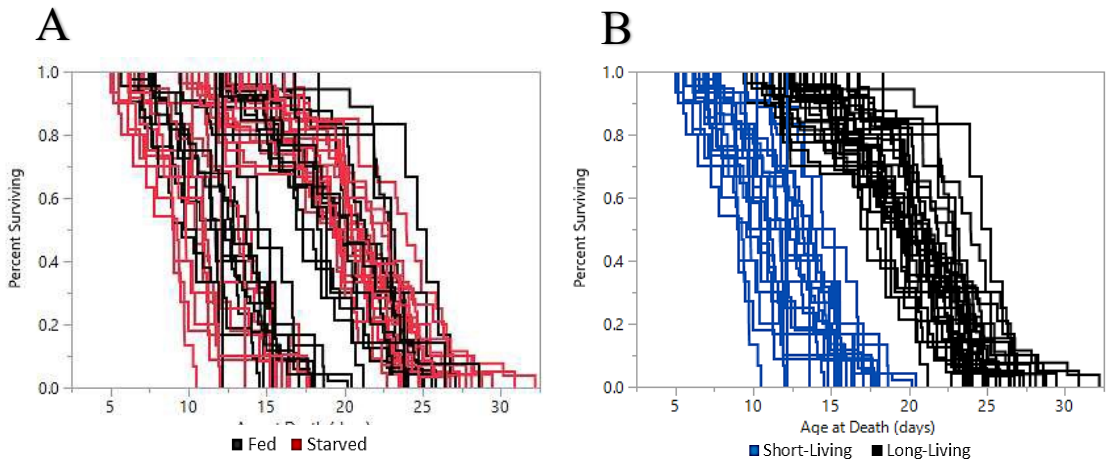

Figure 11 A and B: Lifespan curve for generation one (G1), generation two (G2), and

generation three (G3) by individual plate for the fed and starved conditions. **B.** This is the same graph as A but is color coded differently to show long-living and short living worm types.

Figure 11 A and B provide more evidence for a bimodal trend. Independent of starvation and the generation number, some worms have a short lifespan and others have a long lifespan. Lastly, I wanted to ensure that the bimodal trend was seen within just one generation. Therefore, I can ensure the trend was not a result of one generation exhibiting just one length of lifespan. Figure 12a-d (below) show only generation 2 results.

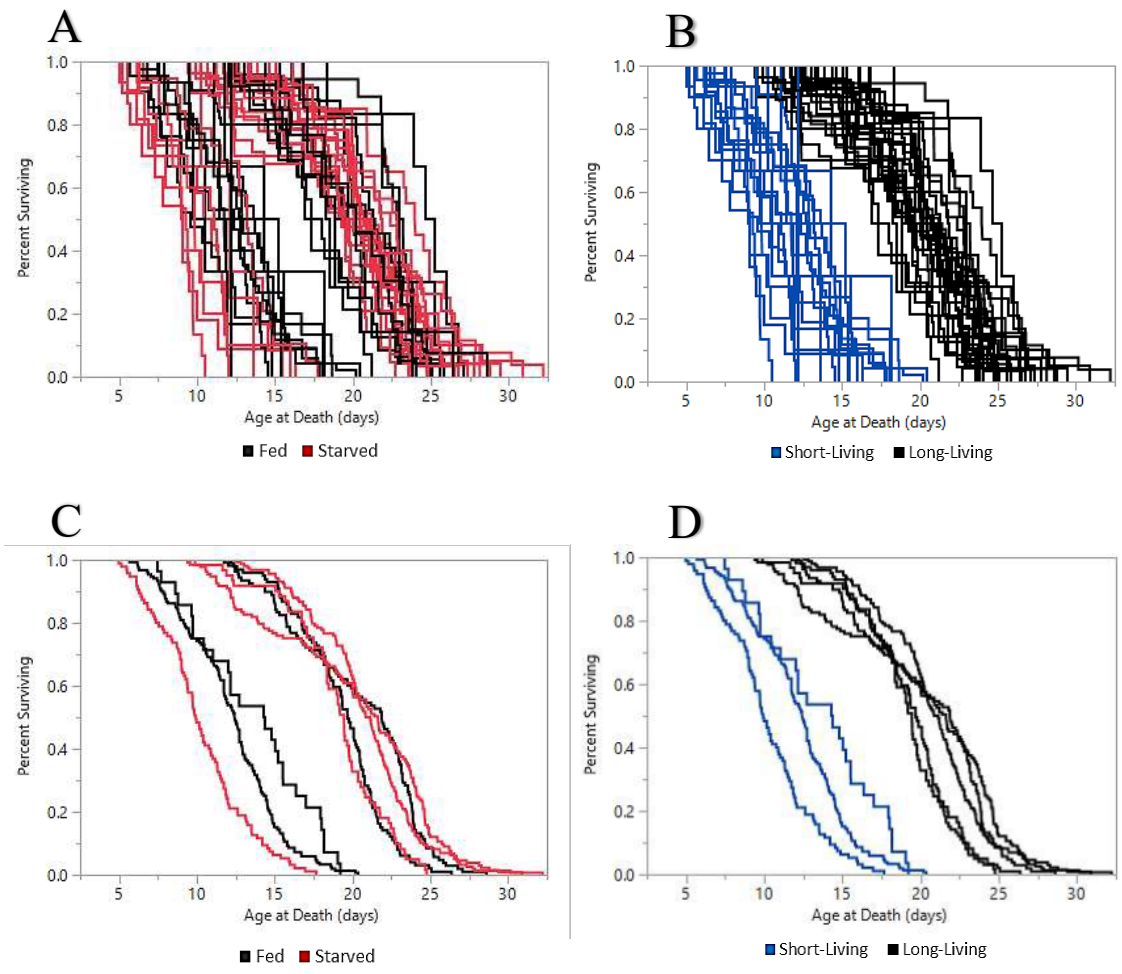

Figure 12 A-D: A. Lifespan curve for generation two (G2) by individual plate for fed and starved conditions. **B.** This is the same graph as A but is color coded differently to show long-living and short living worm types. **C.** Lifespan curve for generation two (G2) by replicate on each scanner for fed and starved conditions. **D.** This is the same graph as C but is color coded differently to show long-living and short living worm types.

Figure 12 A-D clearly show that the same bimodal trend was seen within generation two, independent of starvation. The trend is shown by individual plates on each scanner (figures 12A and 12B) and by each replicate (figures 12C and 12D).

Bimodal Lifespan Result

Further analysis of lifespan data indicated that some cohorts showed high early life mortality while others exhibited long lifespans. The bimodal trend was observed across different generations, within a single generation, and on individual plates as well as whole scanner replicates. Given there was no observed intermediate lifespan between the long living and short living worms, it is possible that these worms are in different physiological states that affect their lifespan. Also, it is possible that the worms could be shifting between physiological states due to some unknown environmental factor. The same result was observed in *C. briggsae*, another nematode that is similar to C. *elegans* (Lucanic, 2017). However, to this date, the literature does not provide an answer to why this effect is observed.

Exploring the Mortal Germline Effect as a Possible Explanation

An interesting phenomenon in the field of C. *elegans* research that has observed a bimodal trend in lifespan, is the mortal germline effect. It is the idea that some worm mutants' germlines can reproduce viable offspring for several generations but eventually the germline becomes sterile (Smelick & Ahmed, 2005). Smelick et al.

postulate that the sterility is due to accumulation of cellular damage and replicative telomere shortening. Telomeres are extra repeat sequences of nitrogenous bases at the ends of chromosomes. They shorten with each DNA replication and eventually become so short that the DNA appears broken (Smelick & Ahmed, 2005). Other cellular pathways are activated which can lead to cellular senescence which promotes oxidative damage. This pathophysiology occurs in the mortal germ cell phenotype. Additionally, it is shown that this phenotype is associated with a decrease in telomerase, an enzyme that can increase telomere length and mitigate the negative effects of telomere shortening (Smelick & Ahmed, 2005). Therefore, without telomerase, progressive telomere attrition occurs. After 10-15 generations of worms, very short telomeres can lead to genome instability and chromosomal fusion (Ahmed & Hodgkin, 2000). While some may think the germline mortality effect could be theoretically be the cause of the bimodal trends seen in this experiment, the worms used in this study were all retrieved from the same cohort. Specifically, this strain was acquired from a frozen population at the same time and the same cohort was maintained from there. Therefore, the worms should not be from vastly different generation times. At no point in the experiment were additional worms thawed from the frozen population. All in all, this does not seem like a realistic reason for this trend.

More Research is Required to Identify Cause of Bimodal Lifespan Trend

At this time, there is no explanation in the literature that explains the cause of the bimodal lifespan result. Therefore, this thesis cannot point to the cause of such a trend. However, the trend observed was very prominent and warrants additional research to identify the cause. Our tightly regulated experiment suggests that

investigator, maintenance of cohort, overall temperature or humidity, food availability are not factors that influenced the bimodal effect. Thus, the results of this experiment have brought about an unexpected result that requires additional mechanistic investigation.

Reproduction Assay

After viewing the inconsistent results seen with starvation and length of life, I decided to look at generation one reproduction. I choose to do this in order to investigate whether starvation was actually having a direct effect on the progeny, rather than the through indirect effects as hypothesized. Results showed that starved progeny laid more eggs on day 4 when compared to their fed counterparts. Qualitative notes indicated these eggs were inviable, often not having a full formed egg shape. Since *C. elegans* hermaphrodites are self-fertile and contain sperm and oocytes, the worms typically stop laying eggs when they run out of sperm needed to fertilize their eggs. However, with the starved progeny, it seems the worms continue to lay eggs regardless if they have run out of sperm. I do not believe that they have more sperm because the eggs looked unviable and repeat counts for adults indicated none of the eggs were viable. There could be an issue in the signaling pathway that leads worms to stop laying the eggs or in the recognition that the egg is fertilized or not. Further research would need to be done in order to determine the cause of these results.

Future Directions

A Note on Generation 1 Lifespan Assay Results

During each starvation, multiple microfluidic chips were used per condition (fed or starved) per run, and at the end, all progeny per group were pooled. On average the egg laying rate in microfluidic chips is slower than the egg laying rate on plates. Also, qualitative observations indicated the starved progeny lay fewer eggs than their fed controls. The timing of collection is set at a 2-hour interval to maintain the synchronization of the animals. However, the number of starved chips that could be set up at one time was not able to provide enough G1 progeny every time. The dexterity of the set-up and fine scale temporal resolution of progeny is compromised when too many microfluidic chips are set up. Currently, the maximum amount of starved microfluidic chips that can be set up is six chips. Future runs of this experiment would ensure the correct equipment was available to set up additional chips to be able to collect more G1 progeny.

Additionally, some of the chips would fail due to errors that could not be controlled. For instance, sometimes there were errors in the microfluidic chip making process that caused the chip to clog and stop the flow of pressure through the system. Other times a hair or particle got stuck in the chip making it difficult to visualize the arena. These chips ultimately failed. However, it is important to note that all the chips were hooked up independent of one another. In other words, if one chip failed, it did not affect the results of the other chips. Having said that, it is important to acknowledge that given we already had a less than optimal amount of chips that could be set up at one time, a chip failing made it even more difficult to retrieve G1 individuals to complete

the lifespan assay. Future runs of this experiment would ensure a greater number of microfluidic chips are set up to obtain enough G1 progeny to complete more replicates of the lifespan assay.

Bimodal Trend

Given the bimodal trend witnessed in this experiment, I believe first there needs to be more G1 and G3 replicates completed. There were four replicates of G2 data completed in this study. I would have liked to also have four replicates of G1 and G3 data. Further, I would like to test the hypothesis of whether epigenetic changes could be the cause of the trend. Some epigenetic mechanisms that have been identified in C. *elegans* include histone post-translational modifications, histone variant incorporation, non-coding RNAs, and nucleosome remodeling and exchange (Wenzel et al, 2011). In order to investigate if any of these changes are occurring, future research must look at genomic sequencing, siRNAs, and chromatin immunoprecipitation.

Reproductive Assay

To ensure the reproducibility of the results of the reproduction assay in this study, more replicates should be completed. Additionally, the count of eggs laid should be carried out to five days.

Conclusion

Stressors are an everyday part of every organisms' day to day life. Given all species endure environmental stressors, it is important to investigate how these stressors can impact future generations of offspring. By investigating these effects, we can ensure a better outcome for our children. We experimentally tested the effects of the stressor of starvation on lifespan and reproductive capabilities of C. *elegans*. This study did not produce enough evidence to definitely conclude that there is a causal relationship between starvation and a transgenerational effect on lifespan.

However, of great interest is the striking bimodal effect seen in lifespan, independent of starvation. Some worm cohorts showed high early life mortality while others exhibited long lifespans. To date, this effect has only been seen in one other species and the literature does not provide an explanation to why this trend has been observed. Our tightly regulated experiment suggests that investigator, maintenance of cohort, overall temperature or humidity, food availability are not factors that influenced the bimodal effect. Thus, the results of this experiment have brought about an unexpected result that requires additional mechanistic investigation.

Considering the bigger implications of this study, if we were able to isolate the epigenetic change causing the phenotype difference in lifespan, we could begin to understand how to induce the longer lifespan phenotype. Irrespective of what is causing the different phenotypes, it is clear that these worms exhibit different epigenetic factors. Because of the homology between C. *elegans* and humans, genetic discoveries that uncover stress response networks or epigenetic changes can greatly benefit medical research.

Specifically, the study and implementation of personalized pharmaceutical plans. If humans too have differences in epigenetic factors that can shorten or extend lifespan, we can pharmaceutically target these changes to secure a lengthened lifespan.

Glossary

Body mass index: a ratio which compares weight to height to serve as an indicator of being underweight or obese

Brood size: the amount of eggs or offspring the organism produces

Buffer: mixture of chemicals used to mitigate changes in pH

Egg-lay: Method used to synchronize age of a worm population; 20 gravid adults are put on a plate and given 2 hours to lay eggs, and after two hours they are flamed off of the plate

Embryological development: process by which an embryo develops

Environmental stressor: external stimuli found in organism's habitat and can create variances in fitness

Fetus: a mammal's unborn offspring

Fitness: an organism's ability to survive and reproduce in a given environment

Genome: the complete set of DNA in a cell or an organism

Gravid adult: an adult of reproductive age

Hermaphrodite: an individual that has both male and female reproductive organs and is able to reproduce without mating with another individual

L1 arrest: arrest in development during the first larval stage

Phenotype: an observable characteristic an organism has due to interaction of their genome with their environment

Progeny: offspring of an individual

Replicate: the repetition of the experimental protocol and condition

Stress: a physical, chemical or biological constraint that affects species fitness

Transgenerational effect: when the fitness of successive generations is affected by environmental stress that is not directly experienced by the progeny

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