PHARMACOLOGICAL STUDIES OF *C. ELEGANS* CULTURED IN AXENIC MEDIA: NICOTINE AND ITS EFFECTS ON GROWTH, BODY MORPHOLOGY, AND REPRODUCTION

by

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ABSTRACT

C. elegans is a soil nematode that has been used as a model organism for pharmaceutical and environmental adaptation studies for decades. The primary culture method for raising *C. elegans* is to use bacterial *Escherichia coli* media plates. Using bacterial media can be resource intensive, result in turbidity for microscopy based assays, and bacterial metabolism can affect any pharmaceuticals introduced to the system. Liquid axenic media is chemically synthetic, optically transparent, and can be utilized in automated microfluidic devices and closed systems. However, there has been no quantification of changes in morphology, development, reproductive fitness, or lifespan as a result of adaptation to liquid axenic media. The WormSlide microfluidic device was developed and used with high temporal resolution automated microscopy to quantify the morphological changes, growth rates, reproductive behavior, and lifespan in liquid axenic *C. elegans* Habitation and Reproduction (CeHR) media. The media was also used to quantify certain phenotypic effects brought about by teratogenic nicotine exposure at various concentrations. This was used as a proof of concept that axenic media can be used as an alternative for pharmaceutical assays on *C. elegans* and that the baseline data acquired for CeHR will enable more laboratories to make effective use of the media.

Chapter 1

INTRODUCTION

1.1 Caenorhabditis elegans as a Model Organism

The nematode *Caenorhabditis elegans* has been utilized as the model organism of choice for research groups over the past four decades. First isolated in Bristol, England by Sydney Brenner in 1974, the one millimeter long transparent roundworm has been a popular choice for molecular biologists, biochemists, and geneticists around the world. *C. elegans* belongs to the pseudo-coelomate family of worms which exhibit a symmetrical body plan. The internal organs, consisting of the digestive tract, gonadal arms, and specialized reproductive structures are contained within the body cavity. Commonly found in soil and compost samples in terrestrial ecosystems, these animals are easily maintained in a laboratory environment. *C. elegans* feed through a pharyngeal pumping mechanism, in which the pump draws in suspended particles and expels liquids, allowing only solid nutrients to be consumed by the worm. The primary food source for *C. elegans* is bacteria, and in laboratories they are commonly fed *Escherichia coli.* The *E. coli* is commonly grown as a lawn on nematode growth media (NGM) plates [1]. This is the standard growth media for *C. elegans* laboratory strains, and the majority of experiments are conducted in these conditions. The transparency of the worms' cuticle is advantageous for microscopists. This transparency allows high quality differential interference contrast (DIC) imaging of individual animals. The result is the ability to acquire high resolution images of internal organs and examine organogenesis processes over periods of time. These

animals have an invariant number of somatic cells, and the cataloguing of the complete cell lineage has been achieved. The ease of creating transgenic nematodes has also resulted in a variety of fluorescent reporter strains.

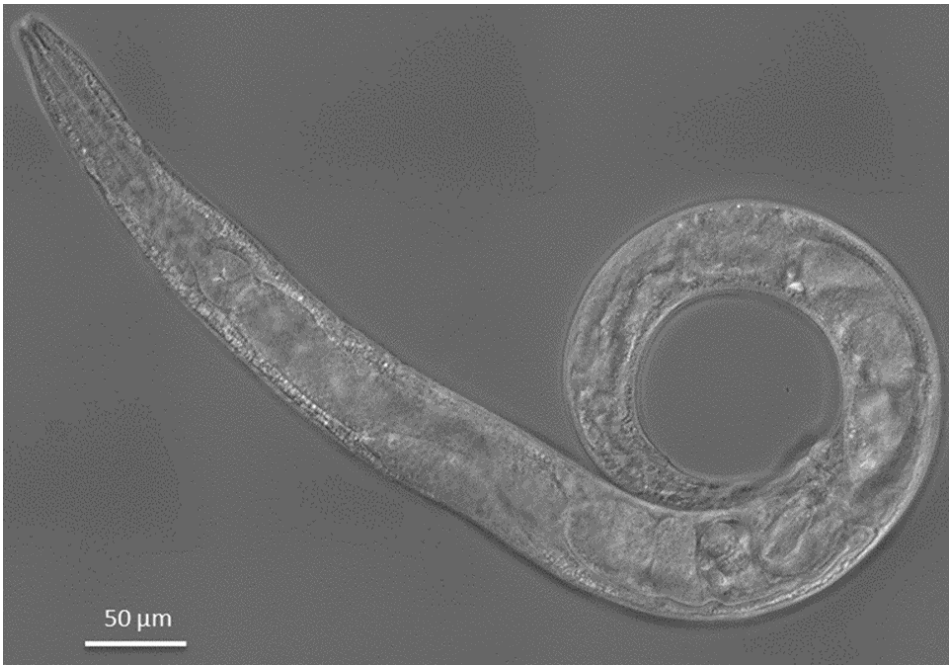

Figure 1.1: An image of a gravid adult *C. elegans* taken using differential interference contrast (DIC) imaging. This technique used to visualize many morphological and interior features.

The majority of these animal are naturally hermaphrodites, and males exist as less than 0.2% of a population of lab raised wild-type (N2) animals [1]. Self-fertilization results in nearly 300 progeny per parent, with each subsequent filial generation having the identical genetic background of the preceding parental generation. This isogenic background means that *C. elegans* can function as a platform for studying clonal discordance and phenotypic variation. *C. elegans* are desirable

model systems for geneticists due to this low level of molecular diversity. *C. elegans* have a close degree of genetic homology to humans, which makes them an ideal organism for pharmacogenetic experiments and experiments for modeling genetic changes resulting from extreme environments such as outer space. An estimated 40% of protein coding genes in *C. elegans* have orthologs in humans, while an estimated 60-70% of human genes share an orthologs in these nematodes [2]. Many bioinformatics tools and databases have taken advantage of the homology present in *C. elegans*. Many of these tools are also well established partly because these worms were the first multicellular organisms to have their genomes completely sequenced [1].

The reproductive life cycle of *C. elegans* is very rapid in comparison to other model organisms. A single embryo will proceed through four larval phases and become a self-fertilizing reproductive adult at approximately 50 hours post hatch, at standard laboratory temperatures of 22 °C [3]. This rapid generational time scale combined with the high number of offspring produced per parent means that researchers can grow up very large quantities of animals relatively quickly and conduct generationally dependent surveys in a short amount of time.

C. elegans continues to be an emerging model organism for a growing repertoire of biological functions. A simple anatomy, rapid reproduction rate, offspring of an identical genetic background, and ease of cultivation, have allowed for this worm to become a robust system to study human diseases, pharmacogenomics, adaptation to environmental changes, learned behavior and memory, and as a platform to explore the biological implications of long-term space travel.

1.1.1 Anatomy

Understanding the anatomical characteristics of *C. elegans* is important to be able to interpret phenotypic changes in response to a change in environmental conditions. The worm is sheathed in a cuticle, which functions as a barrier and also is responsible for body morphology and shape [4]. This cuticle is constructed through cellular secretions from the hypodermis, which also serves as the main body of the animal and the primary barrier to the diffusion of external molecules. The cuticle is composed of a matrix of collagen, and mutations or changes in expression of these collagen coding genes result in morphological changes. Molting of *C. elegans* occurs four times in the animal's lifecycle, and involves the shedding of the previous cuticle layer. Immediately prior to molting the animal goes through a period of greatly reduced activity known as lethargus, followed by the dissociation of the cuticle, termed ecdysis [5]. The period of lethargus during the molting stage is approximately two hours. During periods of overcrowding, limited food, or other environmental stressor, the late L1 stage may go into arrest, and enter the dauer stage. This dauer larva is nonmotile, and activities such as feeding and pharyngeal pumping cease [6]. Dauer recovery occurs once the food supply becomes abundant or the environment returns to a favorable condition. The L1 worm proceeds into the L2, L3, and L4 stages, becoming a young adult after the L4 molt. *C. elegans* of the N2 wild-type strain live on average for 12-18 days. Although animals from the same brood share the same genetic background, phenotypic variation does occur. Whether this is due to mutations in a single gene, multiple genes, or changes in epigenetic regulation is not yet known, and is likely dependent on the degree and type of variation observed.

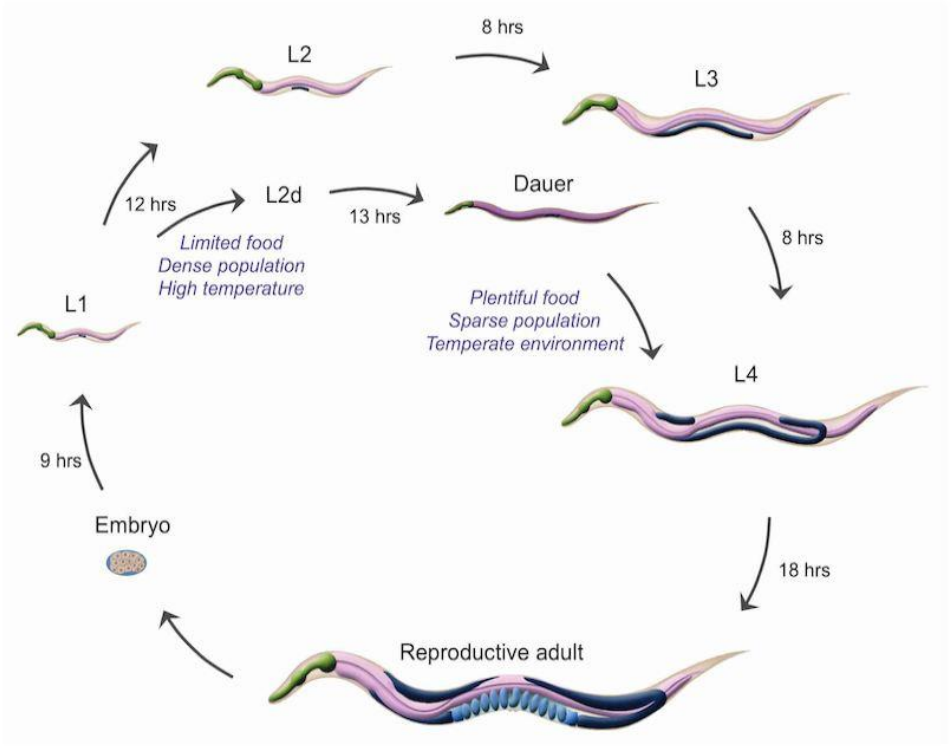

Figure 1.2: Graphical representation of *C. elegans* life cycle at 20 °C. Figure courtesy of [8], Figure 6.

Feeding is accomplished through the pumping of the pharynx, which is controlled by a collection of specialized neurons. After the liquid suspension is expelled, solid food particles are ground up and proceed through the intestinal tract of the animal. Defecation is a behavior that is present in *C. elegans*, and has been studied in detail. There are several such behavioral activities associated with feeding. *C. elegans* will actively exhibit foraging behavior in search of food. Additionally, animals will undergo a period of quiescence if they are properly satiated by food of a high quality or an abundance of nutrients [7]. Feeding, defecation, locomotion, and

other muscle movements are all controlled through the nervous system, which contains 302 neurons [1].

1.1.2 Reproduction

The reproductive mechanism of *C. elegans* hermaphrodites is of primary interest to many researchers. A somatic gonad and the germline are organized in one contiguous U-shaped tube, of which there are two in the animal. These tubes lay across the longitudinal axis of the worm, and both terminate in a shared egg laying apparatus. Beginning at the proximal end of the germline is the distal tip cell (DTC). The DTC assists in the regulation of germ cell activity and in the development of the gonadal arm prior to adulthood. Germ cells are located near the DTC in the mitotic zone, and as they move through the germ line in the distal direction, they enter the meiotic transition zone, which consists of the majority of the germ line. The germ line is covered in sheath cells, which also assist in the engulfing of germ cells undergoing apoptosis in the pachytene loop. The developing germ cells mature into oocytes, and proceed to the spermatheca [8]. The spermatheca contains the male gametes, and each gonadal arm contains one of these chambers and approximately 150 sperm cells [9]. The female gametes pass through this structure, where they are fertilized.

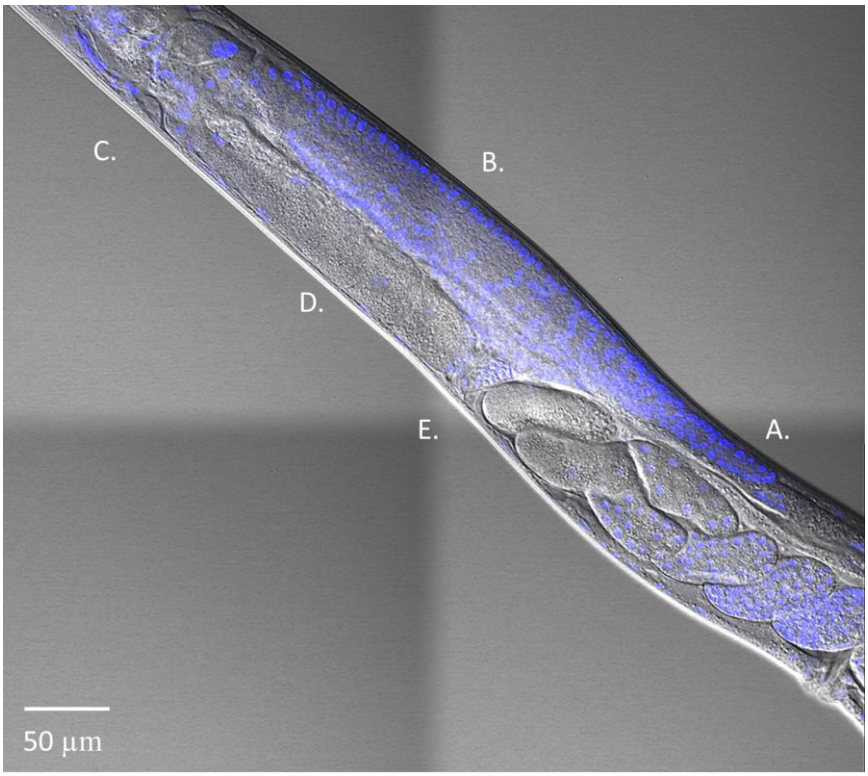

Figure 1.3: Example of the C. elegans germline stained with DAPI. A.) The distal tip cell represents the proximal end of the germline. B.) The meiotic transition zone is where germ cells begin development into oocytes. C.) The pachytene loop is where a number of germ cells undergo apoptosis. D.) Immature oocytes pass through to the spermatheca E.), where they are now fertilized oocytes contained within the uterus.

The mature fertilized oocyte passes into the uterus, which links both of the germlines present in the animal. The fertilized egg will develop internally in the uterus for approximately 150 minutes, although this is temperature dependent. Once development to the 100 cell stage has occurred, the egg will be expelled via the vulva, which is positioned on the ventral side of the animal [8]. The egg laying rate of a gravid adult hermaphrodite can vary from between four to ten eggs an hour, and further influences, such as sensory cues and chemical stimulants, influence the

stochastic nature of this function. After expulsion from the vulva, the *C. elegans* embryo will undergo an *ex utero* development period outside of the parent for nine hours before becoming motile as an L1 stage larva.

1.1.3 *C. elegans* as Models for Pharmaceutical Study

New pharmaceutical products go through vigorous rounds of testing, from laboratory synthesis to clinical trials, before being approved for human usage by a governmental regulatory body. This can lead to potentially life-saving drugs being in the experimental phase for decades. In addition, downstream effects of these drugs to subsequent filial generations is not always known. *C. elegans* are able to produce multiple generations very rapidly, meaning that these potential teratogenic effects of these drugs can be ascertained on this model organism. The constant feeding behavior of *C. elegans* enables pharmaceuticals to be applied using basic techniques. Animal handlers are not required to regularly dose organisms, nor are considerations such as the weight of an individual animal needed to be accounted for in the experimental design process. Exposing *C. elegans* to pharmaceuticals first involves either introducing the drug into the bacterial growth media while still in liquid form. Once the media plates solidify, they can be seeded with bacteria, and worms inhabiting the plate will uptake the drug as they feed on the exposed microbes. The recent advancement in automation technology has enhanced the applications of drug-screening in *C. elegans*. The COPAS BIOSORT system, which can sort thousands of animals based on size and life stage is one such machine [10]. Identification and screening of individual animals by size and stage remains an important consideration for pharmaceutical studies in nematodes, and is technically demanding and laborious if done by hand, as this requires picking single worms and transferring them to fresh

drug plates. Phenotypes are also manually scored by eye. However, many automated and computerized sorting systems are expensive and impractical for many research labs.

C. elegans is a widely used platform for psychoactive drug studies. The most consumed social drugs in the world are nicotine, ethanol and caffeine. Although these drugs have been consumed recreationally for thousands of years, there are still unanswered questions about molecular mechanisms, downstream generational effects, and the mechanics of addiction in response to these drugs. Nicotine in particular has been implicated in the largest number of premature deaths in the world since the 1990's, and with the advent of liquid nicotine vapor pens and e-cigarettes as alternatives to smoking, the need exists for further research into the teratogenic, morphological, and developmental effects of nicotine.

Nicotine is classified as a stimulant and agonist of cholinergic receptors [11]. The molecular targets in *C. elegans* are nicotinic acetylcholine receptors. To date, all molecular interactions of nicotine in *C. elegans* that have been observed take place with the levamisole receptor subtype. Acute and chronic exposure to nicotine is responsible for many physiological and behavioral effects in *C. elegans*. Acute exposure can result in an increase in egg-laying rate, both hypercontraction and paralytic muscle responses that are dose dependent, and changes in body bending endpoints [12]. Chronic nicotine exposure can lead to increased tolerance, downregulation of certain nicotinic receptors, long-term egg-laying behavior changes, and changes in developmental time points. Of particular interest is transgenerational effects of nicotine in nematodes. While few of these studies have been conducted, there is a biological impact on the F1 generations of nicotine using organisms, whether

through transgenerational inheritance, or in the case of humans, through environmental mechanisms like second-hand smoke. It has been observed that nicotine associated changes in locomotion, body bending angles, and speed of travel in *C. elegans* can be inherited several generations downstream in filial generations that were not exposed to nicotine [13]. Research has also expanded to include studies on microRNA's, so that expression profiles may be used to identify possible molecular targets for the transgenerational effects of nicotine [14].

There are a number of limitations and challenges that must continue to be surmounted to improve upon drug studies in *C. elegans*. One such limitation is the need for quantitative software to analyze behaviors such as egg-laying, lethargus, and long-term locomotion. While many such software suites have been developed, and freeware worm trackers are available from several laboratories, many of these tools are designed with specific observation setups in mind. Certain optical tools are not always financially viable for a nematode laboratory, and oftentimes large numbers of replicate animals are needed, making these experiments difficult for labs with limited manpower. Additionally, the temporal resolution of many nematode specific behaviors are either stochastic or variable, or require visualization and analysis over the long-term. The lack of experimental standardization in the field also adds a degree of complexity to experimental design. Dosage time points, dosage concentrations, and method of pharmaceutical application are all factors that remain inconsistent across studies, even those using the same drug. Many studies utilize full grown adults, which does not lead to an understanding of the effects of drugs in developing animals. Therefore, efforts are needed to implement more comprehensive guidelines for *in vivo* dosing and treatment options [15]. Irrespective of these drawbacks, the evolution of *C.*

elegans drug-based studies to incorporate liquid-based assays, computer automation, and higher-throughput screening samples will result in significant progress, as further prototypes and proof-of-concept methods are developed [16].

1.1.4 *C. elegans* as Models for Adaptation to Space-flight Conditions

Space travel is considered the final frontier of human technological limitations. Experiments on humans in a microgravity environment are expensive to undertake, owing to safety, training, and engineering factors. These nematodes are the perfect organisms to use for space travel and microgravity experiments. Rapid generational times allow the effects of space travel on subsequent generations of potential colonists to be researched, and the animals require very little in the way of consumable resources or maintenance, as opposed to the feeding necessities when using animals such as drosophila or mice. Due to the strict aseptic environment required aboard spacecraft and the International Space Station liquid, bacterial free media is preferred for *C. elegans* experiments. The culturing techniques used must also be automated, due to the lack of available manpower onboard the space station [17]. The small size of the animal also allows large numbers required for high-throughput genomics studies or studies requiring a large number of replicates to be easily transported for use in onboard microgravity environments. The animals also display a hardiness to environmental perturbations, as demonstrated when the experimental group of worms aboard the space shuttle *Columbia* survived the craft's catastrophic breakup during reentry in 2003 [18].

There have been a number of space flight experiments undertaken with *C. elegans* in recent years. It has been theorized that a microgravity environment can lead to increased lifespan and delayed senescence in organisms, and gene-regulation

studies of *C. elegans* in spaceflight have identified genes involved in longevity, which suggests that space environmental cues delay the aging process in *C. elegans* [19]. As with any other organism used for spaceflight experiments, the primary challenge remains that of life-support mechanics and engineering. An automated system utilizing axenic media would operate within NASA limitations for sterility and crew interactions, and present a method for conducting long-term *C. elegans* experiments in low Earth orbit.

1.1.5 Experimental Considerations

The technique of maintaining stocks of *C. elegans* for experimental purposes in the laboratory has not changed since the mid-1970's. *C. elegans* are cultured on nematode growth media (NGM) petri dishes containing a base of agar and salts, and are fed from *E. coli* lawns that are cultivated on the plates. The *OP50* strain of *E. coli* is the most common, although others such as *NA22* can be substituted for a variety of reasons, such as the desire for a faster growing bacterial lawn, or better quality of bacteria.

The process of casting an NGM plate and seeding it with viable bacteria to culture *C. elegans* can take approximately 36 hours. Plates are non-reusable, as once the bacterial food source has been consumed, the plate can no longer be reseeded with new bacteria or used for animal habitation. *C. elegans* that are on starved plates are transferred individually to fresh food sources through a device known as a worm picker, or en masse via the chunking technique. If specific time points are needed to be examined during the animals life time, or drug additions must be introduced at a specific life stage, these animals must be individually picked and separated. Mixed populations of worms can all be synchronized to the same generation if desired. The

ability to synchronize a population by age is another extremely useful characteristic of these worms. Gravid adults can be exposed to a caustic bleaching buffer, which kills all living worms and eliminates any residual bacteria. All that remains of the solution is the embryos that were previously laid or maturing inside the parent, protected from the bleaching agent by a chitinous shell. These embryos can either be transferred directly to fresh plates, or allowed to hatch in M9 buffer, where they will remain at an arrested L1 stage of development until introduction to a food source. *C. elegans* can also be cultured in monoxenic liquid media. S-basal media is seeded with bacterial cells, which are usually heat-killed to prevent the accumulation of deadly toxins that prevents *C. elegans* from reproducing past the first generation.

Although the principle methods of *C. elegans* maintenance have not changed, there are a number of drawbacks and considerations for evaluation by the field. The lack of time needed to grow up large amounts of animals enables a large amount of tissue to be harvested, and greatly simplifies the logistical workflow of such experiments as RNA-Seq or ChIP-Seq [20]. In accomplishing this however, the raising of large number of animals necessary to generate the amount of tissue needed for high-throughput genomic experiments can be resource-intensive. Investments in time are required to manufacture new plates, and the process of continually transferring and feeding animals requires significant labor and technical skill. As the ever growing number of animals consume food at a faster rate, the number of plates and amount of media reagents and bacterial stocks needed to maintain the population exponentially increase. For behavioral, locomotor, or mechanical experiments, isolating the required number of animals for statistical purposes is a labor intensive and time-consuming process. Microscopes needed for visual scoring of certain assays may not be readily

available at all institutions, and imaging many individual worms at a variety of time points for quantifiable scoring may not always be practical. In monoxenic liquid media, bacterial particles result in a very turbid environment, making microscopy techniques difficult to use. Animal swimming behavior and motion in all three dimensions adds a challenge to focusing on a single animal for a prolonged period of time. Additionally, the particulate nature of the media precludes the use of taking advantage of advanced microfluidic techniques and automated technology applications. Tubing can become fouled with bacterial debris and detritus, halting media flow. Drugs introduced into NGM plates as a method of deliverance run the risk of undergoing molecular or structural changes by the heat needed to cast NGM plates.

There are also biological factors and influences to consider. When introducing pharmaceuticals to a population of *C. elegans* for long-term study, such as assaying the effects of chronic drug dosing or looking at epigenomic or transcriptomic changes, the confounding variable of *E. coli* metabolism is often overlooked. The *E. coli* will metabolize the drug, potentially changing not only concentration of the compound that is being ingested by the *C. elegans*, but also exposing the animals to the metabolic byproducts of the bacterial metabolism [21]. This effect is undesirable for studies looking to minimize outside perturbations in order to determine the effects of a certain pharmaceutical on *C. elegans*. Because axenic media is chemically synthetic and contains no organism as a food source, media replenishment is easy, and optical transparency also results in characteristics desirable for observation and microscopy-based assays.

1.2 Axenic Media Culture

Raising *C. elegans* in liquid monoxenic culture involves S-basal media and *E. coli* cells. These liquid cultures are often turbid and contain masses of bacterial cells, which clog the fine tubing used in many microfluidic devices. Additionally, using live *E. coli* in liquid cultures results in the buildup of toxic byproducts, preventing worms from reproducing past the F1 generation. The alternative is to use heat or UV-killed bacteria, but the problem of fouled tubing by biomass still remains. These setups also lack the transparency needed to undertake microscopy applications without first removing the worms from their native environment. Current liquid cultures utilizing bacteria still suffer from the same resource intensive requirements as NGM plates and have limited applications. As a prerequisite for spaceflight experiments on board the International Space Station (ISS), only limited forms of bacterial cultures may be used for experimental purposes [17]. The use of *C. elegans* as a model organism to study the effects of spaceflight in a true microgravity environment has consistently used liquid media. Using bacterial media on ISS missions would require a dedicated mission specialist to maintain the animals on these cultures.

An optically transparent, axenic liquid media would be ideal for drug studies, spaceflight experimentation, microfluidic applications, and long term culturing to generate tissue for high-throughput genomic assays. The first research into developing an axenic media alternative was by Lu and Goetsch in 1993, who modified *C. briggsae* Maintenance Medium with glucose [22]. The result was *C. elegans* Maintenance Media (CeMM). CeMM was further modified by the Department of Defense, and has seen use on NASA spaceflight experiments. CeMM has been used because of its synthetic nature, which fulfills the desired lack of bacteria for spaceflight experiments [21]. Further development of CeMM by Clegg et al. resulted

in another version of axenic media, *C. elegans* Habitation and Reproduction media. Further modified by Hamza et al., CeHR has been used to examine the effects of heme concentration in nematodes [23].

However, synthetic media is still not a widespread technique amongst the *C. elegans* community, and there is much information that is unknown about how these animals proliferate and survive in this environment. Basic parameters needed to establish the baseline morphological and developmental effects of this media, such as growth rate, fecundity, and lifespan have not been determined.

The standard technique of culturing *C. elegans* en masse on bacterial growth media, has a number of drawbacks that limit its applications and experimental flexibility. Bacterial metabolism of drug products can confound pharmacogenetic assays. The solid matrix and bacterial content of the media prevents it from being used in spaceflight experiments, and the turbidity of bacterial liquid cultures prevent the use of microscopy techniques. Consequently, the nature of culturing large numbers of worms in this media also precludes the opportunity to easily study or analyze individual animals for the purpose of categorizing phenotypic variation. The depletion and need for replenishment of the bacterial food source also makes tracking animals for their entire lives difficult and impractical. An axenic, optically transparent chemically synthetic media, specifically CeHR, exists to provide alternatives to these challenges. Currently, no baseline developmental, morphological, or reproductive data on *C. elegans* cultured in CeHR exists. The common culture method is to either grow CeHR and other axenic media animals in FEP bags, T75 flasks, or in scintillation vials, methods which do not allow for easy observation or scoring of behavior and morphological traits. These volumes are large, and animals are intermixed in large

populations. A technique to segregate these animals and visualize them in a simple system is crucial to establishing the baseline effects of CeHR on adapting worms.

1.3 The Current State of Microfluidics

Microfluidics is the use of microscale, submillimeter volumes in a system, either through flow based or static-drop liquid handling systems. The field first became popularized in the late 1990's with the advent of the lab-on-a-chip (LOC) system. The LOC concept is focused around the construction of extremely small micro devices utilizing fluids for a variety of applications, including PCR plates and precise measurements of hydrodynamic forces. The use of small volumes of liquid is efficient in terms of reagent consumption, and the small surface area of these devices makes it ideal for scanning microscopy systems. The most common material for the construction of microfluidic chips is polydimethylsiloxane (PDMS).

PDMS is a silicon based polymer with a number of characteristics that make it ideal for microfluidics geared towards biological applications. PDMS kits consists of an elastomer that does not solidify until the addition of a curing agent, which makes it ideal to fit any number of molded surfaces. The ratio of curing agent to elastomer can be controlled, resulting in a polymer with varying tensile and structural strength. PDMS is non-toxic, chemically inert and optically transparent, and remains the microfluidic material of choice for many bioengineering devices [24]. PDMS is capable of gas transfer, ensuring that closed systems do not suffocate any organisms contained within. Sealing of PDMS-based microfluidic devices can be accomplished by using several PDMS slabs of varying structural intensity, or covalently bonding a glass microscope slide to an open face of the PDMS device. This latter option is possible due to the silanol groups contained within PDMS. Exposure of a PDMS

surface to oxygen plasma results in these hydrophilic silanol groups migrating to the surface. These groups can then be covalently bonded with likewise exposed groups on a glass surface. Bonding is near instantaneous, and results in significant tensile strength. There are fluid dynamic considerations to take into account when using liquid in PDMS devices. PDMS is hydrophobic, and has a low surface wettability. When using aqueous solutions, the PDMS must be made more hydrophilic. This is usually accomplished by surface modification treatments with non-ionic detergents, or by immediately introducing aqueous fluids directly after oxygen plasma exposure [25].

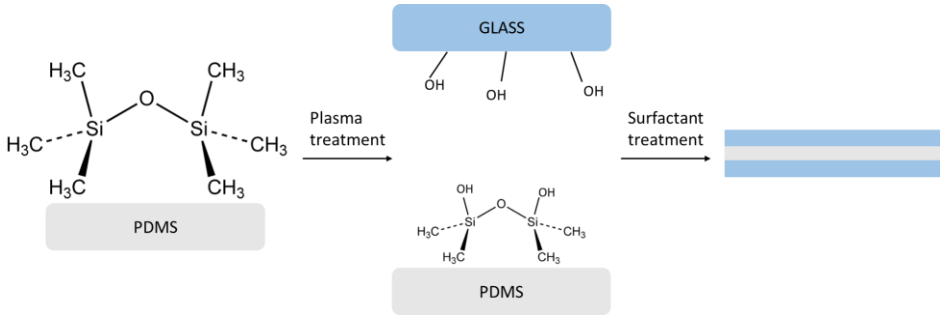

Figure 1.4: Example of the basic workflow to construct a sealed PDMS device. The hydrophilic silanol groups are exposed on the PDMS immediately after plasma treatment. After bonding, surfactants can be applied to further enhance hydrophilicity.

C. elegans experiments that are done on a per-worm basis can be laborious and time-consuming. The use of microfluidic devices in nematode laboratories can drastically reduce much of the manual handling and immobilization techniques required when attempting to observe single animals [26]. Current devices constructed for *C. elegans* use range in applications from high-throughput imaging techniques to

immobilization for gonadal dissections. Oftentimes, animals are introduced into one-time use devices as young adults for a specific temporal assay, and then discarded. The inexpensive construction costs of PDMS usually make these devices a disposable asset, although they can be reused. Standard microfluidics techniques and devices used for *C. elegans* assays do have limitations. Animals are often assayed for short periods of time, and transferred between different media substrates if the same animal is needed for multiple time point experiments [27]. In devices that image animals for long-term periods, immobilization is often used to procure high-quality images. Immobilization can incur developmental side effects, and is also not representative of a natural environment without constraints [28]. Close replicates to the natural *C. elegans* laboratory environment have been replicated in a microfluidic device. In 2016, the van Zon group at the Foundation for Fundamental Research on Matter Institute for Atomic and Molecular Physics in the Netherlands constructed a device capable of time lapse microscopy in order to better understand post-embryonic development [29]. The microchip was limited to utilizing a bacterial food source, and studies of fecundity were not undertaken. Additionally, the food source was not replenished, and would have precluded the use of any pharmaceutical compounds. The physical walls of the device also prevented natural movement of the animal in containment. Additionally, the average length of fully gravid adults was sub one-millimeter, slightly shorter than what is usually observed in wild-type animals. This containment may have triggered adaptational changes in the overall body length (Figure 1.5).

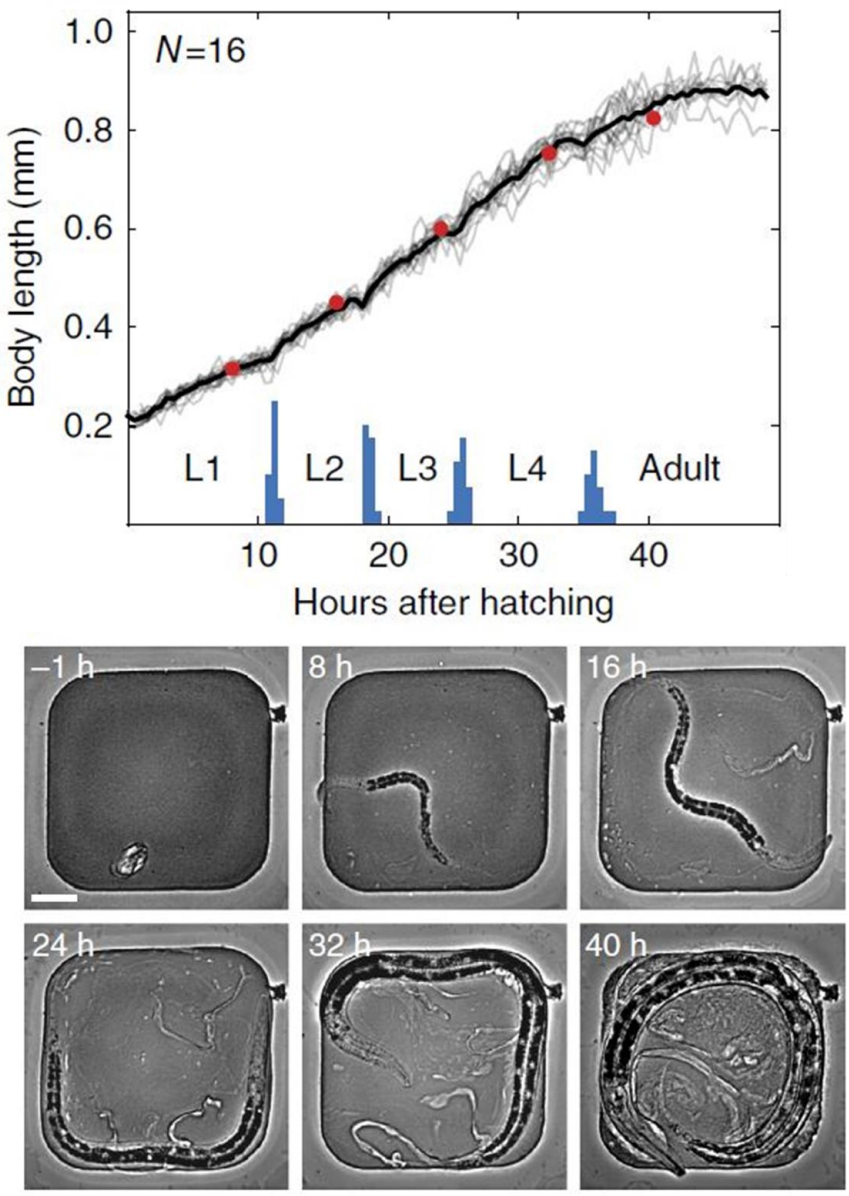

Figure 1.5: *C. elegans* growth over time in a bacterial based microchip observation system. Animals were constrained in a limited mobility environment and observed over 48 hours. Figure from [29], Figure 1.

The small liquid volumes needed to make these devices effective, as well as the ability to manufacture transparent, glass compatible observation devices makes

microfluidic LOC devices the ideal choice to conduct experiments in liquid axenic media. However, to date, the long-term examination of *C. elegans* from the post-embryonic L1 stage to reproduction in a sustainable, liquid environment has not been studied.

1.4 Project Hypothesis and Aims

C. elegans have been cultivated on bacterial plates for several decades, and remains the primary method of growing these animals. Limitations to bacterial food sources include resource and labor intensity, bacterial metabolism of pharmaceutical compounds, difficulties in automation workflows, the inability to be used in spaceflight experiments, and the preclusion of the use of microfluidics. To that end, axenic media forms have been developed. The most recent development in the field of liquid axenic media is *C. elegans* Habitation and Reproduction media. To date, no quantification of the animal fecundity, morphology, or developmental time frame of animals raised in this media has been undertaken. Additionally, no long-term observations of animals in a microfluidic device to reproductive age has been made, nor have pharmaceutical studies been attempted utilizing CeHR media.

The central focus of this project is the hypothesis that axenic media, specifically CeHR, can be utilized to examine the developmental and morphological effects of common psychoactive drugs, specifically nicotine, on *C. elegans*. To this end, the primary engineering task focused on the development of a single-animal microfluidic containment system capable of housing and culturing individual animals. Complementation of the microfluidic device with an automated microscopy and imaging set up allowed for a high frequency of data collection of individual animals. The implementation of this system in concert with the benefits of axenic CeHR media

allowed for the previously unknown characterization of *C. elegans* morphology, life cycle, life span, fecundity, behavior and development in CeHR, and for the proof-of-concept demonstration of CeHR as an alternative media to be used for pharmacological studies.

Chapter 2

MATERIALS AND METHODS

2.1 *C. elegans* Culturing Methods

All standard culturing methods are standardized according to previous protocol [30]. Unless otherwise noted, *C. elegans* were cultured at 20 °C on nematode growth media (NGM) plates with *Escherichia coli* as a food source. N2 (wild-type) and all transgenic strains (DZ365 and MD701) were acquired from the Caenorhabditis Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440**)**.

Nematode growth media (NGM) plates were made in 1 liter batches using the following reagents: 3 g NaCl, 2.5 g peptone, and 17 g of agar. Flasks were then filled with distilled water to 1 liter. Flasks were autoclaved using a media cycle, and then allowed to cool to 40 °C in a water bath for approximately 45 minutes. Swirling the media to prevent solidification, 25 ml of 1 M KPO_4, 1 ml of 1 M $MgSO_4$, and 1 ml of 1 M $CaCl_2$ was added to the flask containing 1 liter of NGM. As a final step, 1 ml of 5 mg/ml cholesterol in ethanol was added, before pouring 35 ml of NGM into 100 mm plates. After overnight solidification, plates were kept in storage at 4 °C.

2.1.1 Feeding

After solidification, plates were seeded with 200 µl of *OP50 Escheria coli* bacteria. Using aseptic technique, bacteria was spread using a sterile plate spreader. Plates were sealed with parafilm and stored inverted at laboratory temperature. *C.*

elegans were transferred from starved plates to fresh plates either by using a platinum wire and worm picker tool, or synchronized L1 stage animals were pipetted directly onto the plates in M9 buffer in volumes of 100 µl.

M9 buffer was made by combining 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, and 1ml of 1 M $MgSO_4$ with deionized H_20 for a final volume of 1 liter. The M9 buffer was then aliquoted in 100 ml amounts in Wheaton bottles, and autoclaved for 60 minutes using the liquid cycle. Sterilized M9 buffer was then stored at room temperature.

2.1.2 Population Synchronization

Bleaching buffer was prepared immediately prior to synchronization. Buffer was made using 5 N NaOH, 5% Chlorox bleach, and 0.1 M NaCl in a 1:2:6 ratio. Contaminated plates, or plates with gravid worms were washed down with 10 ml of M9 buffer. The wash was transferred into 15 ml Falcon tubes. Worms were centrifuged for 1 minute at 1,000 × g. The supernatant was aspirated, and two more M9 washes were used to clear as much bacteria as possible from the culture. After the final wash, 10 ml of bleaching buffer was added to the worm pellet. Worms were vortexed every 30 seconds for nine minutes, and progress of the bleaching reaction checked using a brightfield stereoscope at 40× magnification. After bleaching for nine minutes and checking for the presence of any worm carcasses, the bleaching mixture was centrifuged for 1 minute at 1,000 × g. The supernatant was aspirated and the pellet washed with sterile, autoclaved deionized water underneath a laminar flow hood. This washing procedure was repeated three more times. Pellets were then resuspended in either M9 buffer and embryos allowed to hatch overnight, or the pellet was resuspended in CeHR for axenic media experiments and culturing.

2.2 CeHR Media Production

CeHR media (without pasteurized skim milk) was prepared in batches of 400 ml and not stored for longer than six months. Media preparation was done in a sterile laminar flow hood using scrupulous aseptic technique. Aliquots of vitamin mix, nucleic acid mix, and milk were stored at -20 °C prior to making the media. These components were thawed 24 hours in advance to media preparation. The following reagents were added to a 0.22 µm filter unit, in specific orders and amounts: 12.5 ml of 2 mM hemin chloride, 67.5 ml of H_2O, 5 ml of vitamin mix, 5 ml of 2.4 mM inositol, 5 ml of 2 mM choline, 50 ml of H_20, 10 ml of nucleic acid mix, 50 ml of mineral mix, 10 ml of lactalbumin, 10 ml of essential amino acids, 5 ml of non-essential amino acids, 10 ml of 450 mM KH_2PO_4, 25 ml of 1.45 M d-glucose, 15 ml of 1 M HEPES sodium salt at pH 7.0, and 115 ml of deionized H_20. The filtration unit was then removed, and 0.5 ml of 5 mg/ml cholesterol in 99% ethanol was added. The CeHR was aliquoted out into 50 ml Falcon tubes filled to 40 ml each, and stored at -20 °C. Immediately prior to use, 10 ml of pasteurized skim milk was added to the tube for a final concentration of 20% milk. CeHR with milk retained its optimum nutrient properties for approximately two weeks at 4 °C storage. CeHR was protected from light at all times.

Table 2.1: *C. elegans* Habitation and Reproduction Media Reagents

Vitamin Growth Factor Mix	**Per 100 ml:**
N-acetyl-α-D-glucosamine	0.15 g
DL-alanine	0.15 g
Nicotinamide	0.075 g
D-pantethine	0.0375 g

Table 2.1 continued

Vitamin Growth Factor Mix	**Per 100 ml:**
DL-pantothenic acid, hemi calcium salt	0.075 g
Folic acid	0.075 g
Pyridoxamine 2HCl	0.0375 g
Pyridoxine HCl	0.075 g
Flavin mononucleotide, sodium salt	0.075 g
Thiamine hydrochloride	0.075 g
1 N KOH	5 ml
p-aminobenzoic acid	0.075 g
Cyanocobalamin (B12)	0.0375 g
Folinic acid, calcium salt	0.0375 g
Nicotinic acid	0.075 g
Pyridoxal 5-phosphate	0.0375 g
α-L-lipoic acid	0.0375 g
Nucleic Acid Mix	**Per 100 ml:**
Adenosine 5' -monophosphate, sodium salt	1.74 g
Cytidine 5' -phosphate	1.84 g
Guanosine 5' -phosphate	1.82 g
Uridine 5' -phosphate, disodium salt	2.04 g
Thymine	0.63 g
Mineral Mix	**Per 500 ml:**
$MgCl_2 \cdot 6H_2O$	2.05 g
Sodium citrate	1.45 g
Potassium citrate monohydrate	2.45 g
$CuCl_2 \cdot 2H_2O$	0.035 g
$MnCl_2 \cdot 4H_2O$	0.1 g
ZnCl2	0.05 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.3 g
$CaCl_2 \cdot 2H_2O$	0.1 g
Other Reagents	**Per 500 ml:**
Choline diacid citrate	5 ml
myo-inositol	5 ml
Hemin chloride	12.5 ml
Deionized water	117.5 ml
Lactalbumin hydrolysate	10 ml

Table 2.1 continued

Vitamin Growth Factor Mix	**Per 100 ml**
Essential amino acid mix	10 ml
Nonessential amino acid mix	5 ml
450 mM KH_2PO_4	10 ml
D-glucose	25 ml
Cholesterol	0.5 ml
Pasteurized skim milk	100 ml

2.2.1 CeHR Media Culturing

Gravid *C. elegans* were washed off 100 mm NGM petri dishes with M9. Worms were centrifuged at 1000 × g, washed and pelleted twice. The bleaching buffer was then added to the pellet. The pellet was vortexed every 30 seconds for 9 minutes, at which point worms were then pelleted down a third time at 1000 x g. Worms were then washed four times with sterile deionized water before the embryo pellet was transferred to 1 ml of CeHR contained in a scintillation vial. Additionally, a final concentration of 250 μg/ml of lysozyme in Tris-HCl buffer at pH 8.0 was also added to prevent microbial contamination during the initial handling step. CeHR was replaced every seven days. For 1 ml stock cultures, this involves discarding 900 μl of CeHR and replacing with the same volume of fresh media.

2.3 Microfluidic Mold Construction

WormSlide molds were constructed by designing topographical features using the Autodesk Fusion 360 software. These features were then 3D-printed using the FormLabs 2 photo polymerizing desktop 3D printer. Stereolithograph files generated from Autodesk were imported into the FormLabs Preform software. No supports were used in generating the models prior to printing. Feature models were then processed

using 91 % isopropyl alcohol according to manufacturing specifications, involving washing features for 10 minutes in pre-rinse step, and then for another 10 minutes in a post-wash step. The features were then dried and allowed to go through final curing under UV light for one hour at 50 °C.

After processing, the features and walls were then joined to four mm thick acrylic sheets via chloroform. Chloroform was applied sparingly to the juncture between feature and acrylic through Pasteur pipette in a dropwise manner. The components were then subject to pressure from a steel block to enhance integrity and binding of the structures. Bonding of the finished mold was allowed to occur under this pressure for 24 hours to ensure structural integrity.

2.3.1 Microfluidic Mold Assembly

The body of the WormSlide was constructed out of polydimethylsiloxane (PDMS) in a 10:1 curing agent ratio. For the construction of a single WormSlide body, 1 ml of curing agent was added to a 50 ml Falcon tube, followed by the addition of 9 ml of elastomer. The two components were manually stirred via a glass rod for several minutes, until the mixture resembled a froth of bubbles. The Falcon tube was then placed under vacuum for a one hour degassing. The PDMS was then poured onto the molds manually, and an acrylic sheet larger than the mold dimensions was used to cover the open side. Binding clips were used to hold the sheet in place, and molds were placed upright to allow the flow of air bubbles away from the well features. The WormSlide molds containing PDMS were heated to 60 °C for 12 hours. After curing, the molds were allowed to cool, and then the WormSlide bodies were carefully removed from the molds using a blunt ended micropipette tip. WormSlides were then cut to the size of a Type 1.5 microscope cover glass using a razor. Any remaining

PDMS on the open sides of the wells were removed using tweezers, with uncured oligomer and fragments being removed by double-sided tape. The models were then submerged and stored until use in 100% ethanol to remove any uncured oligomers and oils.

2.3.2 Animal Insertion

Immediately prior to use, WormSlides were allowed to dry for 3 hours from the ethanol treatment. WormSlide faces were then subject to the use of 3M tape to remove any residual oils or dust particles from surfaces to be subject to bonding. WormSlides were then autoclaved using the solid setting for 50 minutes and allowed to cool in a sterile environment. A microscope coverslip and a cover slide were exposed to 5 minutes of ozone treatment at 50 °C to ensure sterility. WormSlides were subjected to 30 seconds of plasma treatment immediately following 5 minutes of vacuum. Both faces of the device were exposed to the plasma during the same treatment by inserting a sterile acrylic block underneath the open face of the well to lift the WormSlide. Additionally, the microscope slide and cover glass that were previously sterilized were also subject to plasma treatment, with the sterilized side also being the treatment face. The microscope cover glass was then covalently bonded using tweezers to the bottom of the WormSlide.

Embryos were recovered from bleaching, and 100 µl of embryo suspension in CeHR was placed on the lid of a 30 mm petri dish. Additionally, this suspension was further diluted with another 200 µl of CeHR. The embryos in this suspension were then subject to manual withdrawal with a P20 pipette and gel-loading tips, in volumes of 6 µl. These embryos and the accompanying CeHR media were used to individually load all 45 wells of the bottom sealed WormSlide

The plasma treated microscope slide was then placed over the WormSlide, and allowed to covalently bond under a steel weight for 30 minutes. Approximately 700 µl of CeHR was then manually introduced into the WormSlide by using a 25 GA hypodermic needle. A second needle without the syringe was placed opposite of the insertion point to act as pressure release point. Once the WormSlide was fully filled with media and no bubbles were present in the device, both needles were removed. Bubble traps at either end of the device prevented bubbles from obscuring imaging and creating a lens effect.

2.3.2.1 Pharmaceutical Assays

Nicotine stock solution in DI H_20 of 1 M concentration was prepared in volumes of 1 ml and stored at 4 °C. Using 99% nicotine, 160 µl of this stock was added to 840 µl of water to generate the 1 M concentration of nicotine. The necessary concentration of stock was diluted with CeHR to reach the desired concentrations for downstream assays, and synchronized embryos were washed in the mixture. Loading and filling of the WormSlide were conducted as previously described. No media exchange occurred during the assays.

2.4 Pharyngeal Pumping Assay

Synchronized embryos were allowed to grow in scintillation vials with CeHR. Once animals reached 96 hours of age, adults were transferred to WormSlides. Pharyngeal pumping scoring was conducted on worms once they reached 96 hours of age. Pumps per minute (PPM) were scored visually, counting pumps for each worm scored three times across 10 minutes to account for variability. Fifteen worms were selected, and these same worms were assayed for pharyngeal pumping every 24 hours.

Wells that contained more than one animal were discounted, and pump counts were measured only when thrashing activity of the animal ceased. Pumping was imaged using a 10× objective lens. No depressants or mechanical input was used to prevent the animals from freely moving.

2.5 Lifespan Scoring

A population of gravid N2 animals were grown on NGM media and bleached using standard synchronization methods. Embryos were then allowed to develop in CeHR conditions in scintillation vials for 72 hours. Floxuridine (FUdR) was made in a 10 mM concentration using DI H_20, followed by filter sterilization and aliquots were made of 1 ml each. FUdR was stored at -20 °C. FUdR was added to the CeHR to the desired final concentration, and animals were allowed to incubate for 24 hours. Media was then exchanged, and single animals were then inserted into individual wells of the WormSlide, and scored as alive or dead under a stereoscope every 24 hours. Media was not replenished in the WormSlide for the duration of the lifespan assays.

For scoring the lifespan of worms grown on OP50 plates, worms at the L4 stage were transferred to seeded NGM plates containing 120 µM of FUdR. These plates were made by adding 6 ml of 10 mM FUdR to the NGM mix during the cooling step, prior to plating. The plates were then seeded with *E. coli* using the standard technique.

2.6 Big Brother Assay

WormSlide wells were imaged using an inverted microscope (Nikon TE200) and utilizing a Nikon Plan Fluor 4×/0.13 objective for automated imaging and a Nikon

Plan Fluor 10×/0.30 objective was used for pharyngeal pumping and phenotype identification. The WormSlide was maneuvered into position using an XY-axis motorized stage, while an autofocuser was used to focus on individual wells. The system was controlled via a microcontroller and a custom program written in LabVIEW. A USB 3.0 Point Grey Grasshopper3 camera was used for photomicrography. Images were taken at two frames per second for 20 seconds for 168 hours unless otherwise noted. This resulted in each WormSlide being fully imaged every 33 minutes.

2.6.1 Image Processing

Images were processed in FIJI prior to further analysis [31]. Processing consisted of cropping the image stack so only the periphery and interior of the well remained in the frame. The image stack was then saved as an .AVI file. The video file was then exported to WormLab 4.1 (MBF Bioscience, Williston, VT) where data was processed using the custom settings available in the program. Statistical analysis and figure generation was done using Microsoft Excel and Origin (OriginLab, Northampton, MA) software programs.

2.7 Microscopy Sample Preparation

Fixation and storage of animals at -20 °C prior to DIC imaging was undertaken several days prior. Cultures of synchronized embryos were prepared in CeHR, and 1000 µl was aliquoted into a 1.5 ml tube. Worms were spun at 400 × g for 2 minutes. Supernatant was aspirated and the pellet washed twice in M9 buffer. On the final wash, worms were heat killed by placing the tube in a 70 °C water bath for 30 seconds to ensure thermic relaxation and an elongated posture. A final aspiration was done to

remove all but 50 µl of solution. This was followed by 1 ml of fixative solution. The fixative solution was made by mixing 16% paraformaldehyde in 0.1M phosphate buffered saline Tween 20 (PBST) buffer, for a final concentration of 4% paraformaldehyde. PBST was made by dissolving the following in 200 ml distilled H20: 2 g of NaCl, 0.05 g of KCl, 0.36 g of Na_2HPO_4, 0.06 g of KH_2PO_4, and 500 µl of Tween-20. The pH was adjusted dropwise to 7.2 via HCl titration, and the volume was adjusted to 250 ml with water. Sterilization was done via filtration with a 0.22 µm filter apparatus. Batches of toxic fixative were made in aliquots of 4 ml and stored at 4 °C. After the addition of the fixative buffer, the Eppendorf tube was placed in a Styrofoam box containing dry-ice for 3 minutes. The tube was then thawed out in a 40 °C water bath. This freeze cracking procedure was conducted once more. The tube was then placed on a shaker at 4 °C for 8 hours. After fixing for 8 hours, the tube was spun down at 1,000 × g for one minute, and the fixative was removed and replaced with 1 ml of PBST. This washing procedure was done twice more, and the fixed worms in a final volume of 1 ml PBST were stored indefinitely at -20 °C.

Slide preparation was done by placing a few drops of molten 3% agar on a cover slide. This cover slide was sandwiched between two spacer slides that had labeling tape once over lengthwise, and a second cover slide was placed down to sandwich and flatten the agar. After approximately 2-3 minutes, the top cover was removed via perpendicular motion, and 3 µl of buffer containing fixed worms was dropped onto the agar pad.

2.8 Imaging Fixed Samples

Imaging of fixed samples for DIC imaging was completed using the Nikon TE 200 inverted scope used for the Big Brother assays. Objectives used were Nikon Plan Fluor 10×/0.3 and Nikon Plan Fluor 40×/0.6 objectives.

2.9 Imaging of Fluorescent Strains

Fluorescent strains were imaged using techniques described previously. Agar pads were prepared as previously described. A suspension of 3 µl of heat killed animals were mounted on the pads. Imaging was done using a Zeiss LSM 710 confocal microscope, utilizing a Zeiss 20×/0.8 Plan Apochromat objective for imaging of green fluorescent protein and a Zeiss Plan Neofluar 10×/0.3 objective for DIC images.

2.10 Statistical Tests

All statistical tests were completed in Origin. One-Way ANOVA's were used for comparison of means along with Tukey Kramer post-hoc tests, and Kaplan Meier survival curves were completed using the log-rank test to determine the significance of lifespan curves. All *P*-values were set to 0.05 for significance.

Chapter 3

RESULTS

3.1 Using the WormSlide for Big Brother Assays

Segregation of individual animals into wells allows for a reduction in the noise that could result from animal interactions. The WormSlide was used to segregate individual embryos into 45 wells that measured 1.2 millimeters square and held a working volume of 6 µl. An individual WormSlide was secured onto a 3D printed microscope stage, and was imaged over a period of seven days. Each pass of the WormSlide in order to take 20 frames of images for each well took 33 minutes as a result of scanning speed and the time required for the autofocus to function. This technique of constant surveillance of individual animals was termed, the Big Brother assay.

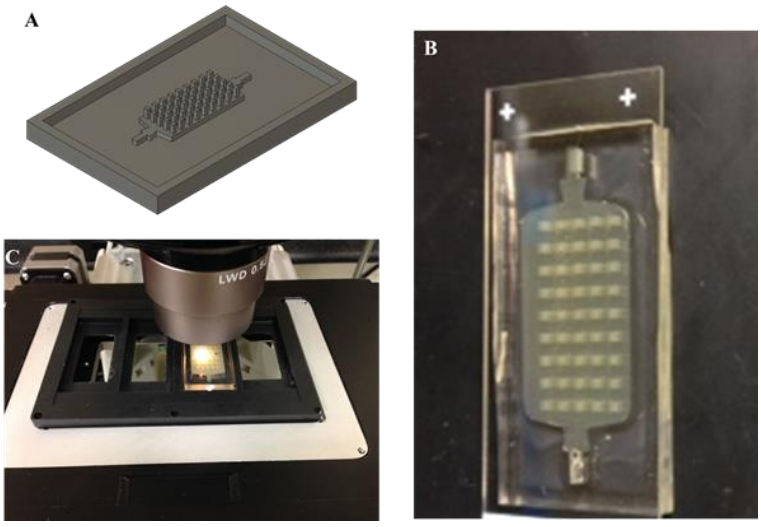

Figure 3.1: Representation of the components of the WormSlide and Big Brother assay system. (A) The WormSlide is produced from a 3D printed mold, which is then cast in PDMS. (B) The WormSlide is sealed between a glass coverslip and a glass coverslide, and has 45 individual wells and has a working volume of approximately 800 µl. (C) The WormSlide is scanned using an automated translator, and imaging takes place on an inverted microscope.

The manual loading of the wells was conducted under a stereoscope so that the correct number of embryos can be inserted as often as possible. There was a time limit to this process of thirty minutes, as after this time limit loading of the WormSlide with liquid becomes more difficult. This increased difficulty is due to the reversion of hydrophilic silanol groups within the PDMS, which returns the material to its hydrophobic, low wettability state. The majority of the wells were able to be accurately loaded within this time frame.

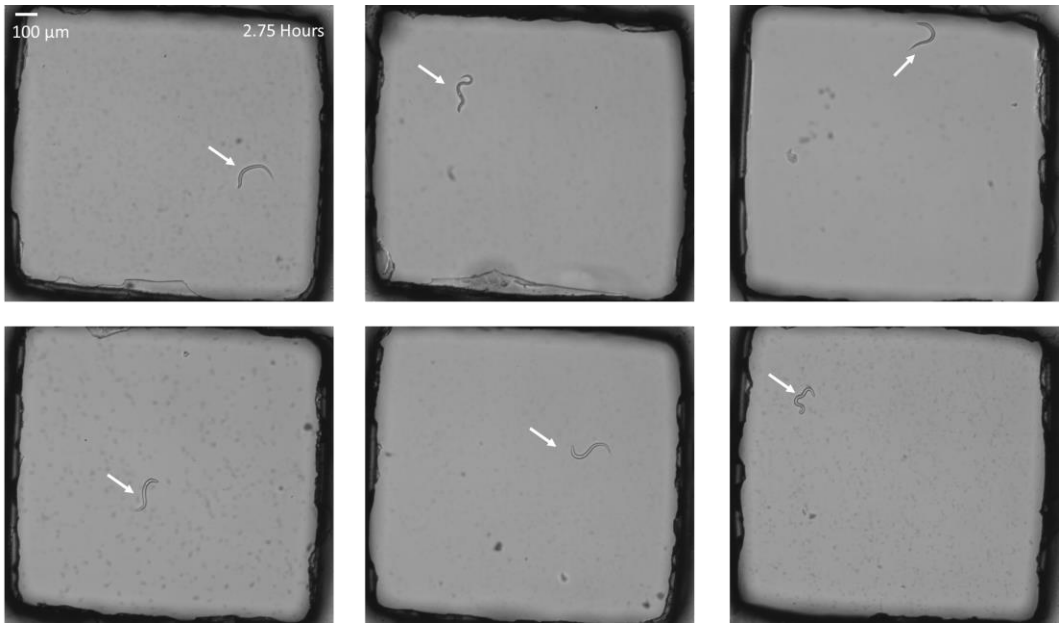

Figure 3.2: WormSlide wells each contain a single animal. Forty-five wells are loaded with individual embryos from a bleached, synchronized sample via micropipette.

After the assay is complete, the images taken during the seven day long observation period are compiled into a video, and analysis completed via the WormLab software. The WormLab software is capable of tracking worms over any period of time, and has a number of parameters that can be used to track worms for as long as desired. There are a number of worm behaviors that add a degree of complexity to using the WormLab software with animals in liquid media. The recognition is based on thresholding of the image, and animals that interact too closely with the well wall are unable to be recognized as individuals. Out of focus animals that are exploring in the Z-axis direction are also discounted by the software. Worm shapes are carefully determined by user settings, and monitoring of the analysis step ensures that no particles or PDMS fragments are recognized as worms.

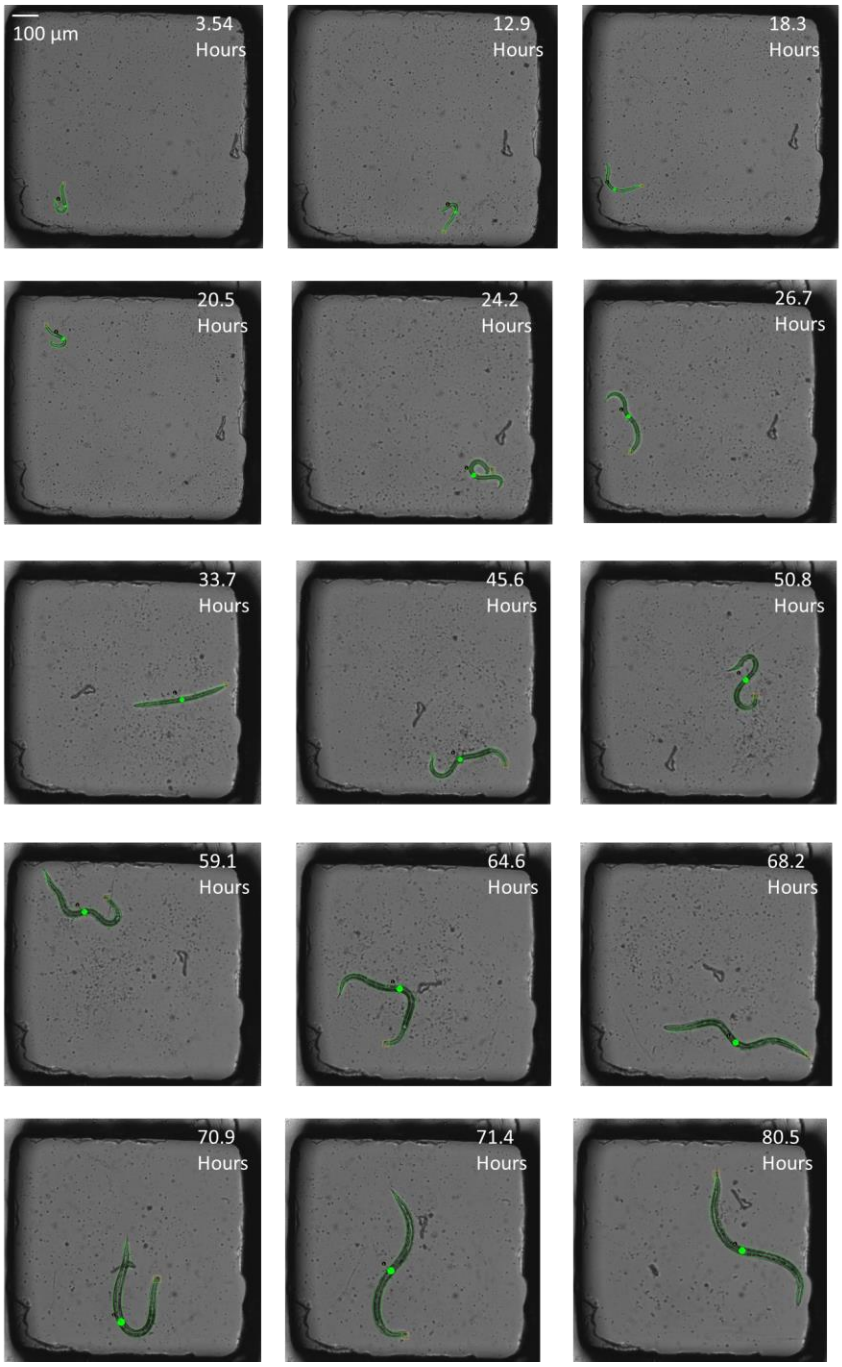

Figure 3.3 Example of WormLab skeletal recognition. WormLab recognizes *C. elegans* shapes based on the centroid of the elongated shape. Animals that remain clear of the well walls are easily recognized by the software. The majority of interactions with well walls, other worms, and Z-axis exploration and out-of-focus animals are ignored by the software.

The measurements that were made using the WormLab analysis software included: length, width, speed, and positioning. These four measurements were limited to, at maximum, 100 hours of metrics. This is a result of the confined nature of the well once reproduction occurs. As the number of individual animals in a well increases past approximately three to four animals, separating individual worms from each other becomes difficult, as the unique shape and centroid positioning that WormLab uses to identify worms becomes distorted by activity (Figure 3.4).

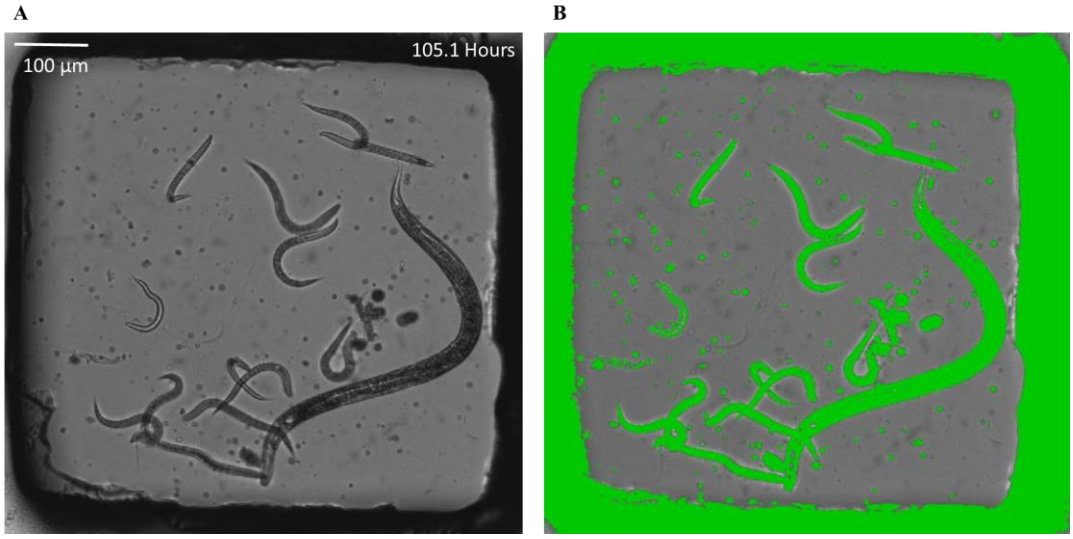

Figure 3.4: Example of worm identification after reproduction. After reproduction, the number of worms in any particular well increases, compounding the difficulty that WormLab has in terms of identifying both F1 worms and the P0 parent as they begin to interact with each other.

3.2 Growth, Morphology, and Reproduction in CeHR

Phenotypic changes are observed in animals that grow in CeHR, compared to a bacterial food source. These changes necessitate the establishment of the baseline effects of CeHR in terms of growth, morphology, and reproduction. Changes in the

body morphology of P0 generation worms raised in CeHR were observed and calculated over a period of 100 hours. This was the maximum time allowable in which the WormLab software was able to discern individual animals. The changes in body length and body width in CeHR raised animals were also compared to animals grown on OP50 *E. coli* NGM plates, and animals grown in milk-free CeHR. Worms in CeHR and CeHR without milk were measured via WormLab for 20 seconds every 33 minutes until 100 hours had passed. The length and widths measured over this 20 second period were then averaged, and charted over time (Figure 3.5). Samples of animals fed on a diet of OP50 were fixed every eight hours with DIC images being collected and measured.

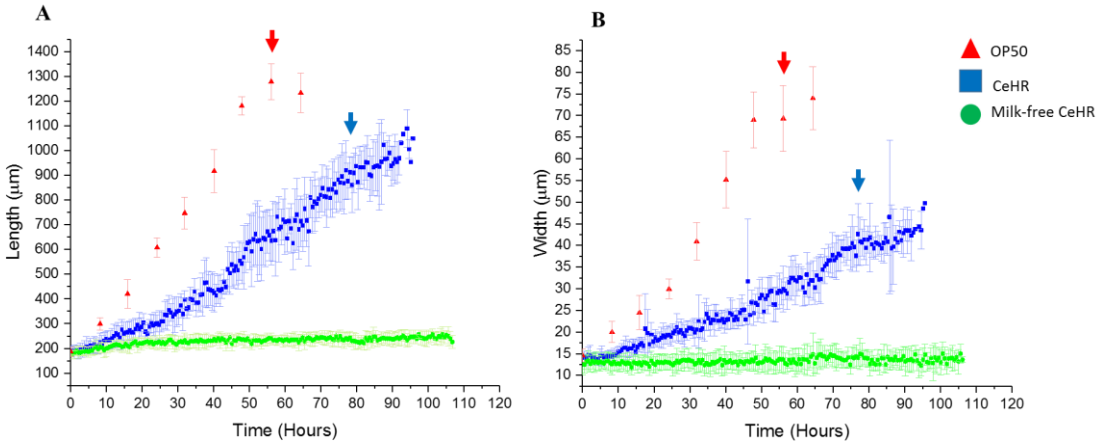

Figure 3.5: Changes in morphology occur over time. *C. elegans* display different rates of change and morphologies that is dependent on the media source. A.) Mean lengths and widths (±S.D.; CeHR n=24, Milk-free CeHR n=23) for CeHR and milk-free CeHR animals were taken every 33 minutes. B.) Mean lengths and widths (±S.D; n=10) for OP50 raised worms were taken every 8 hours. Arrows indicate the average hour of egg-laying behavior.

Worms grown fed on a bacterial diet reach their maximum length and width nearly 24 hours before their CeHR counterparts. These OP50 fed worms were on average 15 % longer and 37 % wider than the smaller CeHR cultured animals. Animals grown in milk-free CeHR do not grow at all, and maintain constant lengths and widths, despite exhibiting movement and activity that indicate that the animals were healthy. A large amount of variation can be seen at certain time points, especially for animals grown in CeHR. This variation, as shown by the standard deviation bars, is partially a result of animal interactions with other objects. Extremities of the worm, the head or tail, can be elongated in the software if they make contact with the wall of the well, and width can be artificially increased if the centroid position of the worm is joined with masses of laid eggs. Final morphology distributions of animal length and width of worms taken 24 hours after first egg-laying behavior was observed show that OP50 worms (Figure 3.6B) are longer and wider than adult CeHR animals (Figure 3.6A). Measurements of CeHR cultured adults grown in scintillation vials rather than constrained wells show the same relationship, indicating that the smaller length of CeHR animals is not due to environmental factors (data not shown).

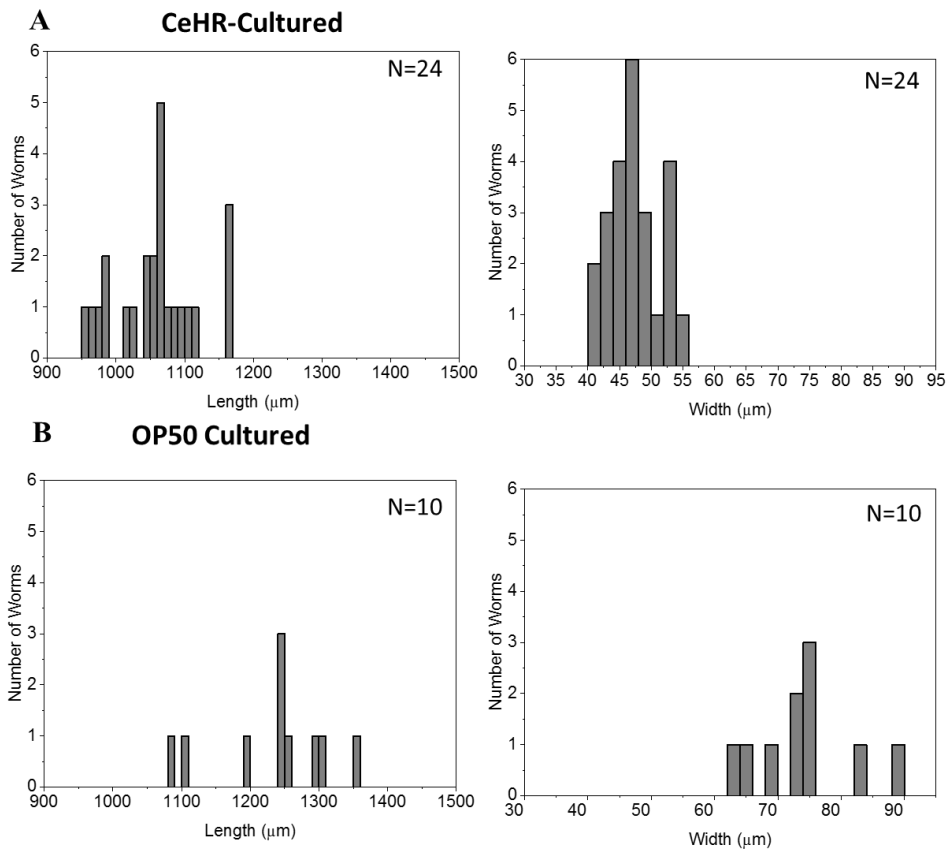

Figure 3.6: Length and width distribution of post-24 hour gravid adults. A.) *C. elegans* grown in CeHR axenic media (n=24, length $\bar{x}$ = 1059 µm ± 58.87 µm, width $\bar{x}$ = 48 µm ± 4.99 µm) exhibit a smaller, more streamlined body sized than their counterparts B.) grown on OP50 media plates (n=10, length $\bar{x}$ = 1232.59 µm ± 80.19 µm, width $\bar{x}$ = 73.97 µm ± 7.3 µm).

Determining the approximate transition points between the different larval stages is necessary for future developmental experiments. The lethargus periods that signify the transition from one larval stage to another were identified for individual animals. The distribution of these periods show that they last approximately the same two hour duration as animals grown on OP50 media (Figure 3.8), but occur at

significantly delayed time points (Figure 3.7). The brief plateau in length changes that occur throughout development can also be correlated to each specific life stage. Variability is present among all four lethargus stage transitions.

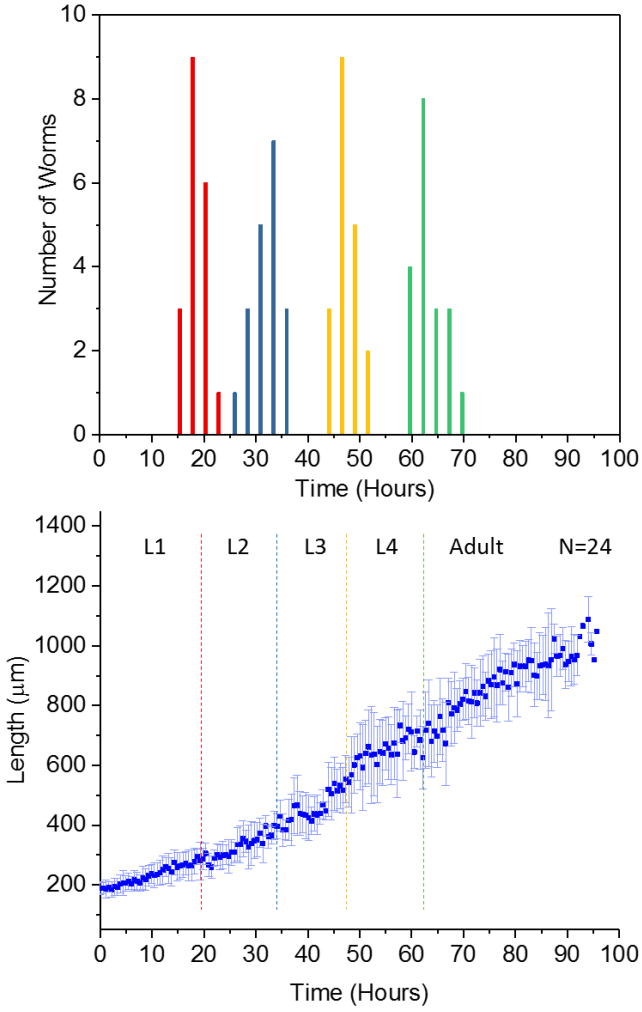

Figure 3.7: Changes in length over time overlaid with a distribution of the occurrence of lethargus periods (n=24). Not all animals undergo each succeeding period at the same time, as evidenced by the variation in distribution, particularly in the transition from L4 to the adult stage.

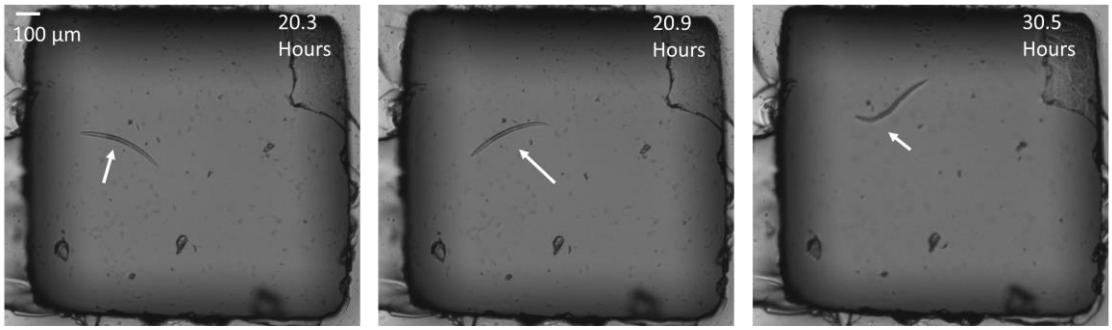

Figure 3.8: *C. elegans* undergoing lethargus from the L1 to the L2 stage. The duration of the lethargus stage of animals grown in CeHR remains the same as that observed in OP 50-fed animals. The lethargus period is approximately 2 hours in length. The animal exhibits very little locomotor activity, and maintains a rigid body form.

The differences between each larval stage and the adult stage of *C. elegans* grown on agar plates versus liquid CeHR media are not only present in size and width, but are also dependent on the time of transition (Figure 3.9).

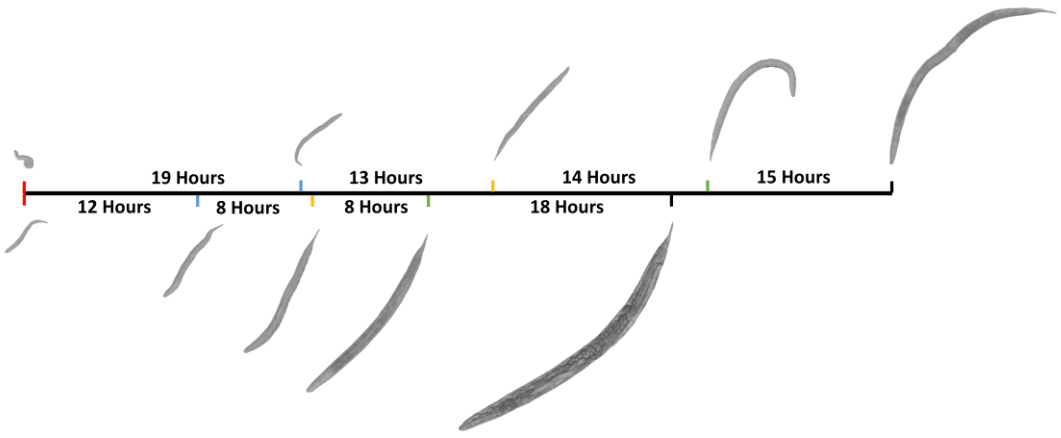

Figure 3.9: The life cycle of the average CeHR-cultured *C. elegans* (top) as compared to agar grown, bacterial-fed animals (bottom). The life cycle of CeHR grown P0 animals was approximated by observing the occurrence of lethargus periods, as well as certain morphological features such as vulval development. The time labels signify the length of time before the animal proceeds to the next growth phase, beginning with the L1 stage.

In addition to observing size changes and lethargus periods as a means of identifying the life stage of a particular animal, the size and shape of the vulva can also be used as determinant of life stage. The vulva undergoes development during the L4 stage, and can be used to determine whether the animal is early, mid, or late-stage L4. The fully developed vulva of the adult is also a distinctive feature that can be used to identify fully grown worms (Figure 3.10). The vulva of adult CeHR worms protrudes more than their OP50 counterparts. The germlines of animals grown in the differing media conditions also exhibit differences, with CeHR raised animals having a significantly reduced number of offspring in the gonadal region than animals raised on agar, and the orientation of developing embryos in CeHR animals are in the

horizontal position, rather than the vertical position that is usually observed (Figure 3.11).

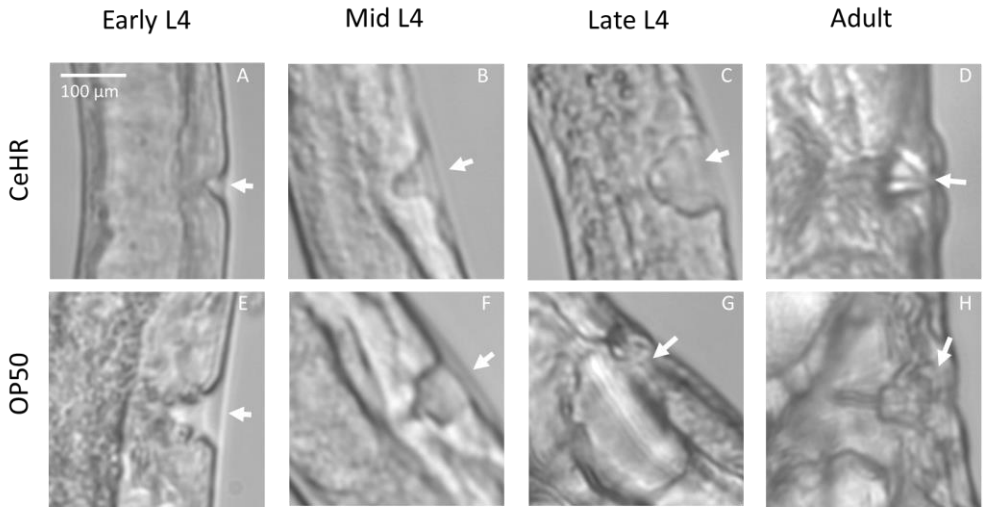

Figure 3.10: Comparison of CeHR and OP50 vulvas. A.) Early L4 vulval development in CeHR animals, followed by B.) mid-stage and C.) late-stage, before finally reaching the D.) adult stage vulva. Figures E-H show the vulval development of OP50 worms.

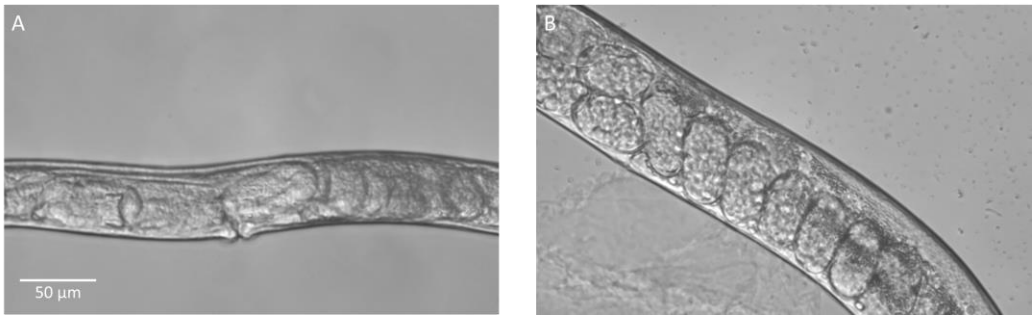

Figure 3.11: CeHR-raised animals have a greatly reduced brood size and germline morphology compared to OP50-fed animals. A.) The germline of animals grown in CeHR does not contain the number of oocytes as compared to those of B.) OP50 fed animals. Additionally, the orientation of CeHR embryos is horizontal, as opposed to vertical, as found in OP50 fed worms.

Changes in internal reproductive organs in response to adaptation to CeHR were visualized through fluorescence confocal microscopy. The germline structure was truncated in CeHR worms in comparison to the well-defined germline found in OP50 raised animals. Apoptosis is a naturally occurring process in the germline of *C. elegans*, and this process was observed to be occurring in animals grown in both conditions (Figures 3.12 and 3.13). Differences in the structure of the spermatheca were also visualized. The spermatheca in OP50 wild-type animals is a thick organ that covers the width of the animal, while in CeHR adults the spermatheca is observed to be a longer, tube-like structure than was seen in agar grown animals (Figure 3.14).

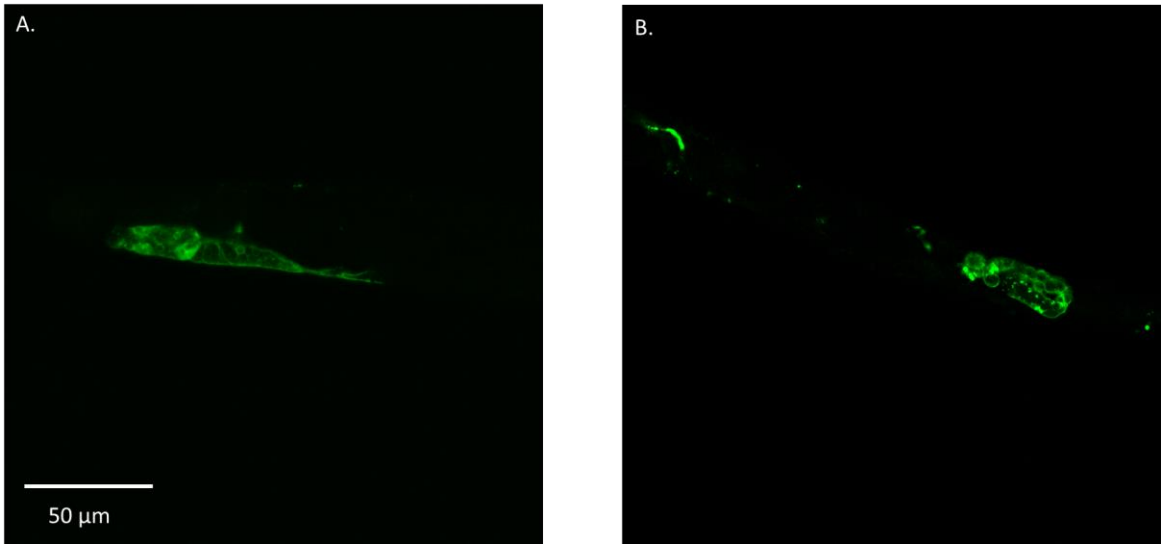

Figure 3.12: Confocal images taken at 20× of *C. elegans* germlines. A.) The germline of OP50 worms begins as a tube, and the loops around to join the uterus. Apoptosis can be observed occurring within the pachytene loop, visualized as circles. B.) By contrast, the germline of worms grown in CeHR appears to be truncated and malformed, and it too is undergoing apoptosis.

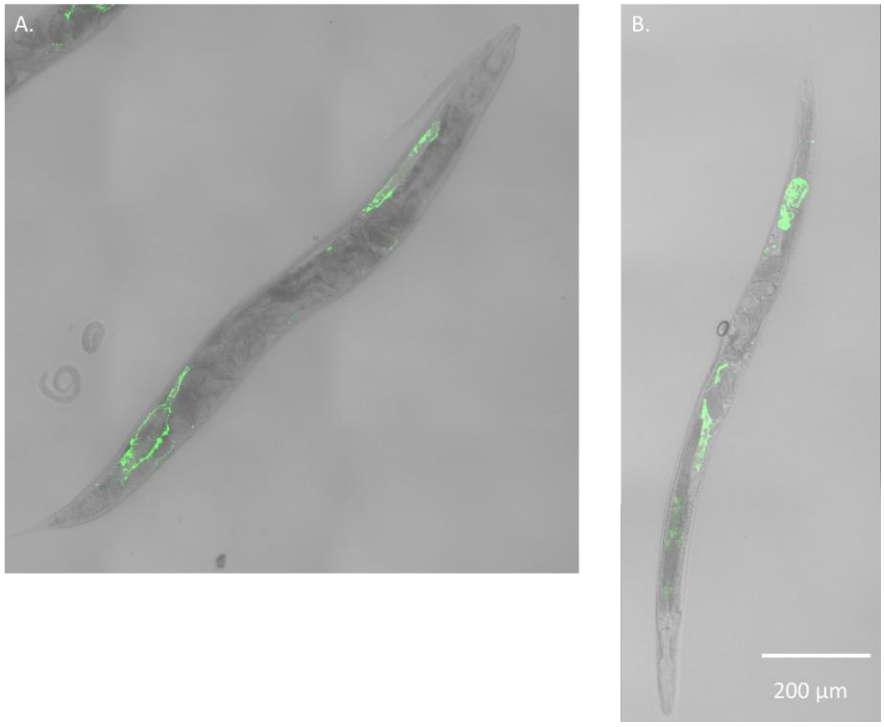

Figure 3.13: DIC images of the *C. elegans* germline in both OP50 animals and CeHR grown animals. A.)The posterior germline can be visualized, as can a number of neatly ordered oocytes in OP50 animals. B.) By contrast, this animal in CeHR exhibits the truncated germline seen in CeHR animals, and additionally a reduced number of oocytes can also be observed.

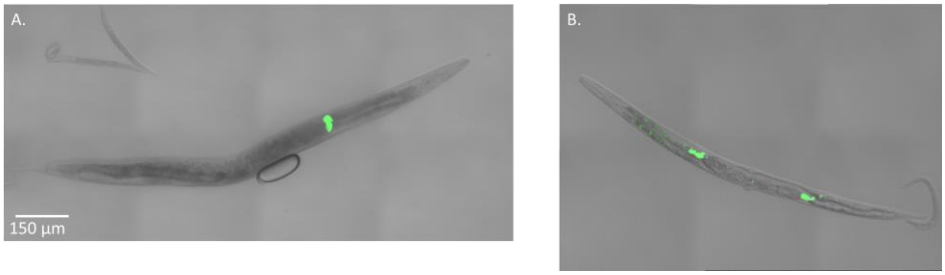

Figure 3.14: GFP images of the *C. elegans* spermatheca. A.) OP50 animals have two spermatheca with 150 cells each. The orientation of this gravid OP50 raised adult is obscuring the second spermatheca. B.) CeHR cultured worms also have two spermatheca, although the orientation is dramatically different. The longitudinal layout of the spermatheca in comparison to the wider confirmation of the OP50 worm's spermatheca can be seen.

Pharyngeal pumping rates in CeHR animals was also measured. The average pumping rate for 24 hour old adults was measured on the first day of containment as 214 pumps per minute, and then generally declined with each subsequent day, although there was a large degree of variation present among individual animals (Figure 3.15). Some animals also exhibited no pumping behavior during the duration of the recording on later days. A decrease in pumping is generally observed with increasing senescence, however a complete cessation of pumping activity in wild type animals has not been reported in the literature thus far.

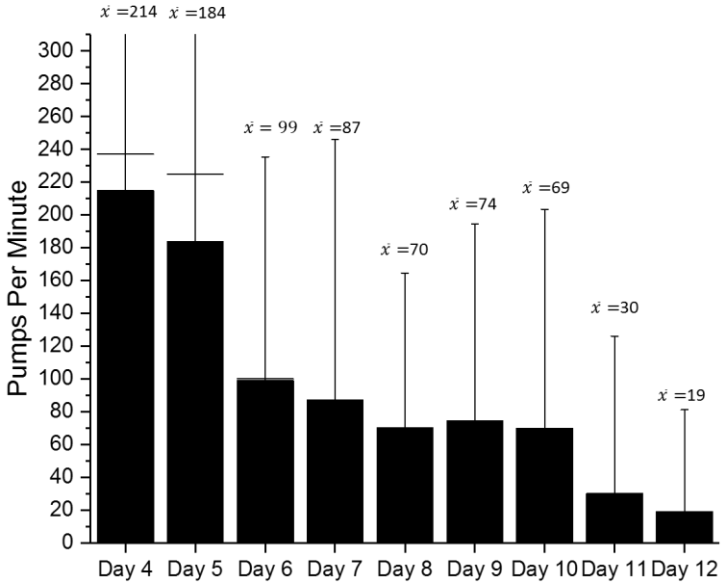

Figure 3.15: Pharyngeal pumping rate of adult worms (n=15) was measured over 8 days in the WormSlide.

The lifespan of CeHR animals was measured by treating with FUdR to prevent the production of offspring, and scoring the animals daily as alive or dead. FUdR concentration does have an effect on lifespan at higher concentrations. At a concentration of 50 μM FUdR shortens lifespan in CeHR control animals by an average of 3 days, a statistically significant difference. There was no such difference between 40 μM FUdR and 30 μM FUdR concentrations. The average lifespan for 50 μM FUdR conditions was 21.8 days, 25.1 days for 40 μM FUdR, 25.6 days for 30 μM FUdR, 13.9 days for OP50 NGM, and 20.9 days for animals grown in milk-free CeHR.

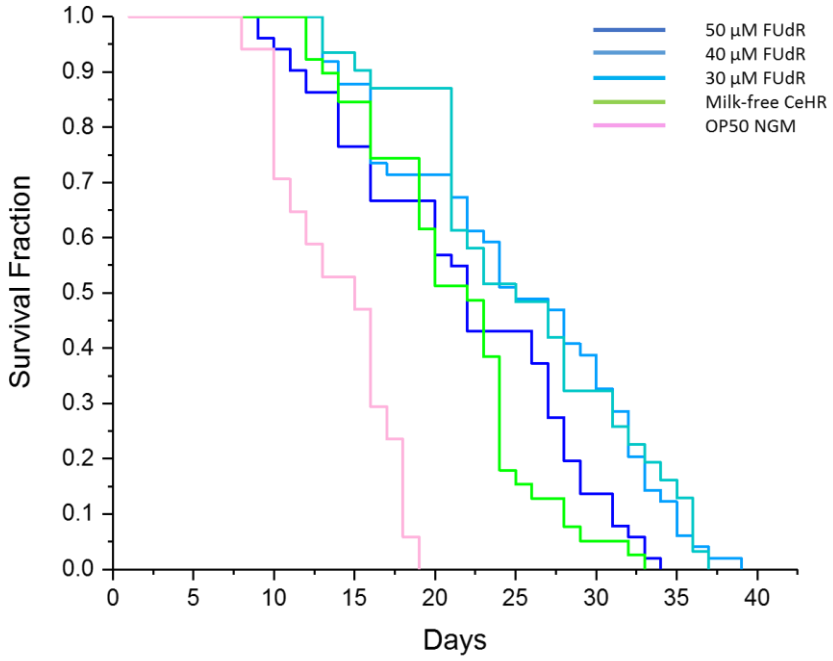

Figure 3.16: Survival curves of CeHR grown *C. elegans*. Worm conditions were exposure to different concentrations of FUdR, grown in CeHR in the absence of milk, and comparison to worms grown on OP50 bacterial plates. Worms at 50 µM FUdR (n=51), 40 µM FUdR (n=49), 30 µM FUdR (n=31), on OP50 agar plates (n=17) and lack of milk in CeHR (n=39) were compared using the Kaplan-Meier log-rank test. Results of statistical tests are $P<0.05$ for: 50 µM FUdR and 40 µM FUdR, 50 µM FUdR and 30 µM FUdR , 40 µM FUdR and milk-free CeHR, 30 µM FUdR and milk-free CeHR, and all conditions compared to OP50 fed animals. No statistical difference was found between 50 µM FUdR and milk-free CeHR, or 40 µM FUdR and 30 µM FUdR.

3.3 Changes in Development and Morphology as a Result of Nicotine Exposure

CeHR has the potential to be utilized as an alternative form of media for pharmaceutical assays. *C. elegans* embryos were exposed to varying concentrations of nicotine in CeHR and grown in the WormSlide. Length measurements were taken over

time from individual animals and averaged to establish growth curves. A concentration of 1 mM nicotine does not significantly delay egg laying time, but a slight decrease in overall length of approximately 100 μm is seen. Concentrations above 5 mM prevent reproduction, and animals exposed to 5 mM lay their eggs beginning at 87 hours. None of these animals exceeded 800 μm in length, and variability was high, with several animals maintaining an overall length of less than 500 μm (Figure 3.17).

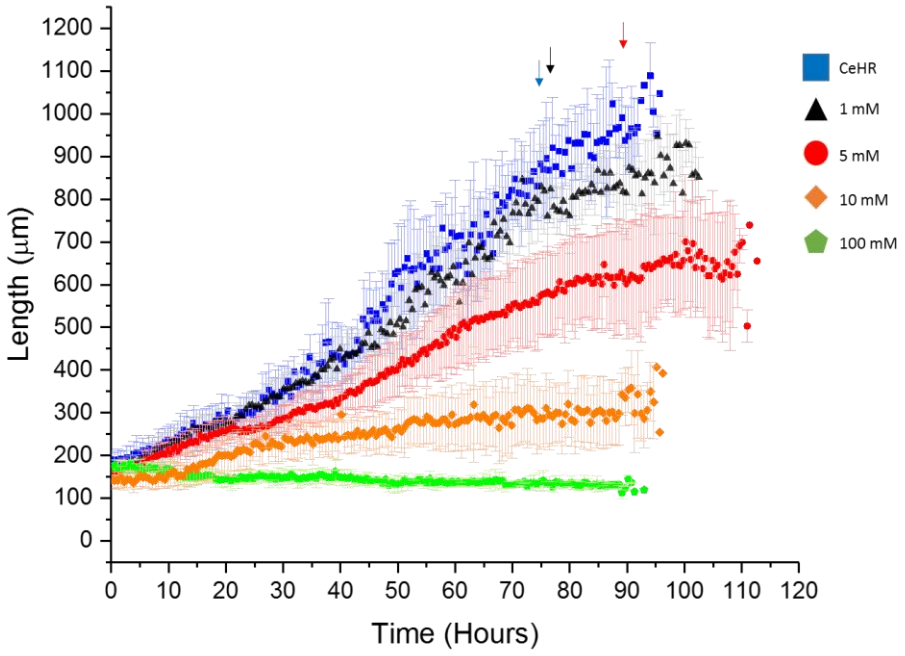

Figure 3.17: *C. elegans* average length change over time after embryonic exposure to nicotine concentrations. Delays in growth and development are present after embryonic exposure to nicotine. The retardation of development is dependent upon the concentration of nicotine that the embryos are initially exposed to. Mean length and widths are shown, with measurements being taken every 33 minutes for 100 hours or until animal death (±S.D.; CeHR n=24, 1 mM nicotine n=26, 5 mM nicotine n=39, 10 mM nicotine n=26 , 100 mM nicotine n=30) . Egg lay times are represented with the colored arrows.

Animals exposed to concentrations of 10 mM did not advance to reproductive age, and hatch at a shorter length than worms exposed to decreased concentrations. The amount of 100 mM was toxic to the animals, with no animal surviving past eight days. The decrease in length seen (Green plot, Figure 3.17) is a result of the shriveling conformation the worm body takes when it is sick or dead. These worms then move to

the side of the well, where they are obscured and the full body length cannot be measured by the WormLab software.

Delays in development were also observed in response to nicotine exposure. The developmental time points between the various L-stages of larva are equivalent between control animals in CeHR and those exposed to 1 mM nicotine. This is evidenced by the overlap of time for both conditions seen in the distribution of when lethargus occurs prior to each stage transition (Figure 3.18). Exposure to 5 mM of nicotine results in the earliest observations of lethargus in worms occurring at the same time points as the later developing animals in the control and 1 mM conditions. The most severe delay appears to be during the L2-L3 transition and the L4 transition into adulthood (C. and D., Figure 3.18). Animals exposed to concentrations higher than 5 mM generally did not undergo the lethargus stage seen prior to growth, although a small proportion of animals grown in 10 mM nicotine did undergo lethargus activity around 35 hours after hatching and experienced minimal growth, accounting for the first plateau in the change in length over time.

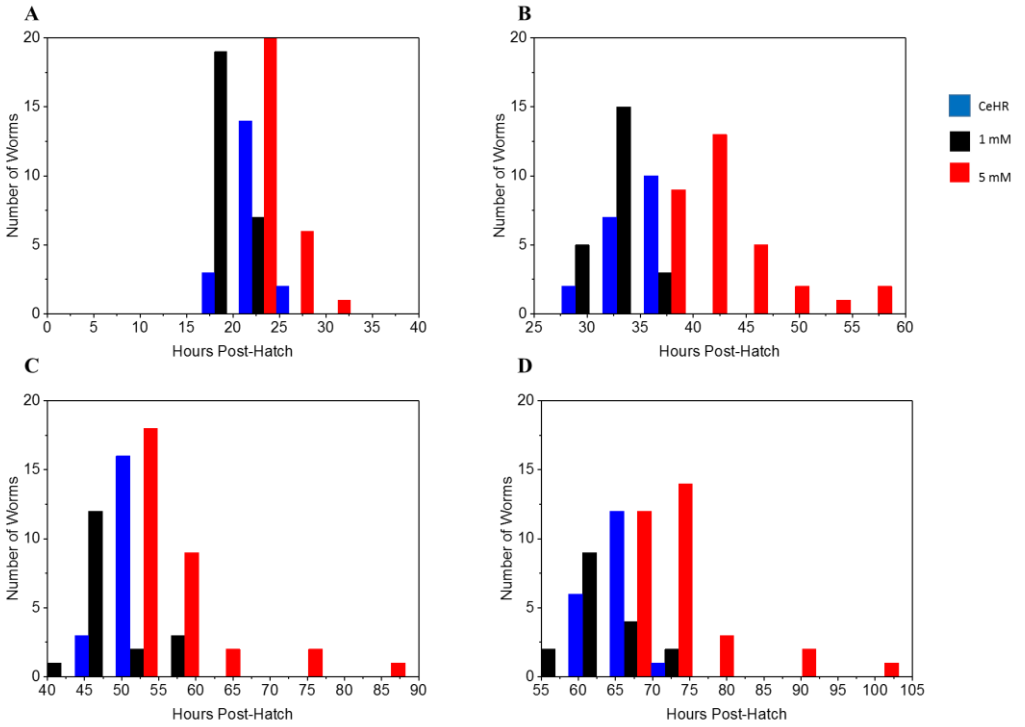

Figure 3.18: Distribution of lethargus time points for animals after nicotine exposure. Only animals exposed to 1 mM nicotine and 5 mM nicotine undergo the 4 lethargus stages and subsequent reproduction. Animals exposed to 10 mM nicotine have only been observed undergoing the transition from L1 to L2, and higher doses of nicotine result in complete absence of growth and L-stage transition. A.) Shows transition times for L1-L2 stage, B.) displays those for L2-L3, C.) shows L3-L4 stage transition times, and D.) shows the transition times from L4 to adulthood.

Lifespan in response to embryonic nicotine exposure is reduced. A concentration of 100 mM nicotine is toxic and animals do not live past eight days. A concentration of 10 mM nicotine is more related to the survival curves seen in wild-type animals grown on agar. Control animals remain the longest lived out of any of the conditions tested. The log-rank test was used to determine the statistical significance between each group. Animals grown in 30 μM FUdR had a mean lifespan of 25.6

days, compared to a mean lifespan of 18.9 days in 1 mM nicotine, 11.6 days in 5 mM nicotine, 11.9 days in 10 mM nicotine, and 5.3 days in 100 mM nicotine.

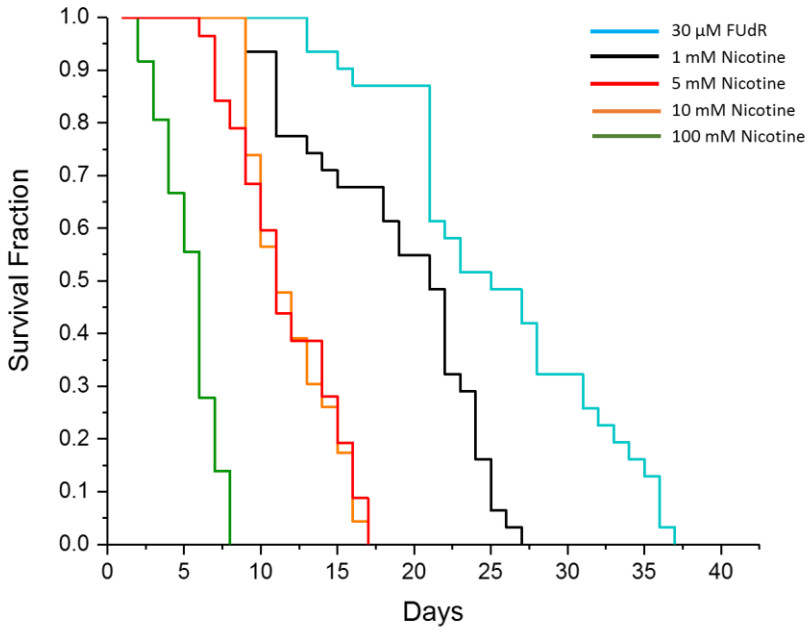

Figure 3.19: Survival curves of nicotine-exposed worms. Worms conditions were exposure to different concentrations of nicotine at 50 µM FUdR. Comparison is to 30 µM FUdR concentration CeHR cultured worms. Worms at 30 µM FUdR (n=31), 1 mM nicotine (n=31), 5 mM nicotine (n=57), 10 mM nicotine (n=23), and 100 mM nicotine (n=36) were compared using the Kaplan-Meier log-rank test. Results of statistical tests are $P<0.05$ for all conditions in combination with each other, with the exception of 5 mM nicotine compared to 10 mM nicotine.

3.4 Reduced Fecundity in the Presence of Nicotine

Fecundity is a measure of reproductive fitness. Nicotine is known to have effects on the reproduction of organisms, including effecting development times of

offspring, the onset of reproductive maturity, and reducing the number of offspring. This reduction in offspring does not occur in *C. elegans*. Animals grown in control CeHR conditions have on average 25 offspring per parent (Figure 3.20). This is a ten-fold reduction in the amount of offspring seen in wild type animals grown on agar NGM plates [23]. The mean number of offspring for animals that were exposed to 1 mM of nicotine as embryos in CeHR is 26, which is not a statistically significant difference between control animals in CeHR. Worms from the 5 mM nicotine condition group had a statistically significant increase in the number of offspring as related to the other two conditions, as determined through the One Way ANOVA statistical test. The reason for this increase in offspring is not known, although it has been found in other studies that the number of offspring does increase in response to low doses of nicotine exposure.

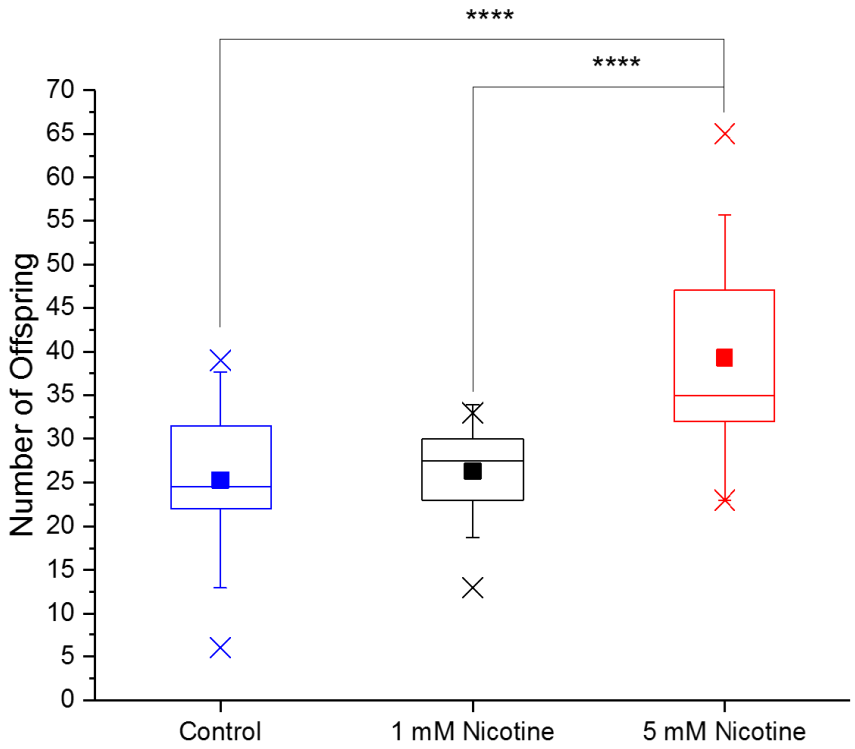

Figure 3.20: Differences in the number of offspring between different nicotine conditions. The average number of offspring for control (25) and 1 mM nicotine (26) adults are not significantly significant. However both of these numbers are significantly less than the average number of offspring (39) observed in the 5 mM test condition. Squares indicate the mean, X's indicate outliers. (n=24 worms for control, n=26 for 1 mM nicotine, n=23 for 5 mM nicotine. Gravid adults that prematurely burst or experienced internal hatching were omitted from fecundity counts. ****$P \leq 0.0001$).

Egg laying times are also changed in gravid adults as a result of chronic exposure to nicotine. The mean egg lay time observed for control worms was 73 hours, and for 1 mM nicotine worms it was observed to be 76 hours. As a further example of developmental delay as a result of embryonic exposure to nicotine, the

worms in the 5 mM condition group had a mean egg lay time of 85 hours (Figure 3.21).

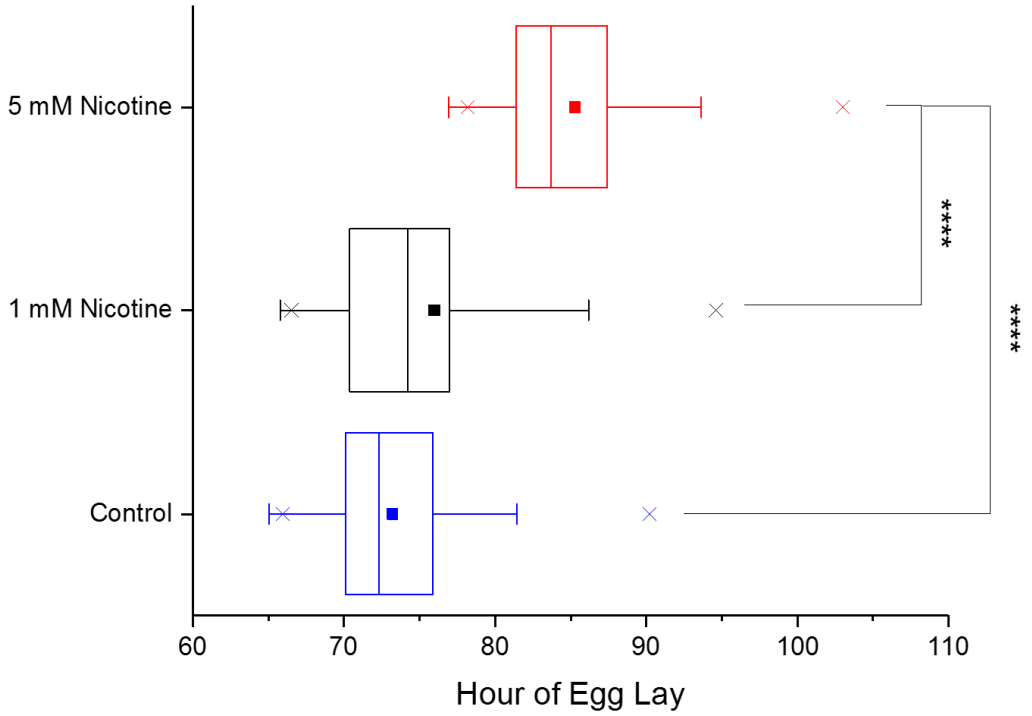

Figure 3.21: Egg laying times in response to teratogenic nicotine exposure. The mean lay times for control and 1 mM nicotine conditions are similar to each other, and no difference in significance was found. The lay time for the 5 mM nicotine condition was however significantly delayed. Squares indicate the mean, X's indicate outliers. (n=24 worms for control, n=26 for 1 mM nicotine, n=23 for 5 mM nicotine. Gravid adults that prematurely burst or experienced internal hatching were omitted from fecundity counts. ****$P \leq 0.0001$).

Downstream effects of embryonic nicotine exposure in the P0 generation may also carry down to the F1 generation. *C. elegans* undergo approximately nine hours of *ex utero* development time after being expelled from the vulva of the parent when grown on agar plates. This *ex utero* time fluctuates widely in axenic CeHR media, and even more so among F1 worms from parents that were subjected to embryonic nicotine exposure. The mean *ex utero* development time for F1 control animals was 7 hours, which is relatively similar to that seen in agar raised animals. However, there is a standard deviation of 2.4 hours, and outliers of animals taking either an extended 12 hours of development before hatching, or those that hatch into L1 larva within 2 hours of being laid by the parent. Offspring from parents exposed to 1 mM nicotine had a mean *ex utero* development time of 8.2 hours, and those offspring spawned from the 5 mM nicotine group had a mean development time of over 13 hours (Figure 3.22). All of these development times were significantly different from each other, which indicates that even there is no statistical difference between control and 1 mM nicotine animal fecundity, there is a trend that suggests downstream effects significantly affect the F1 offspring.

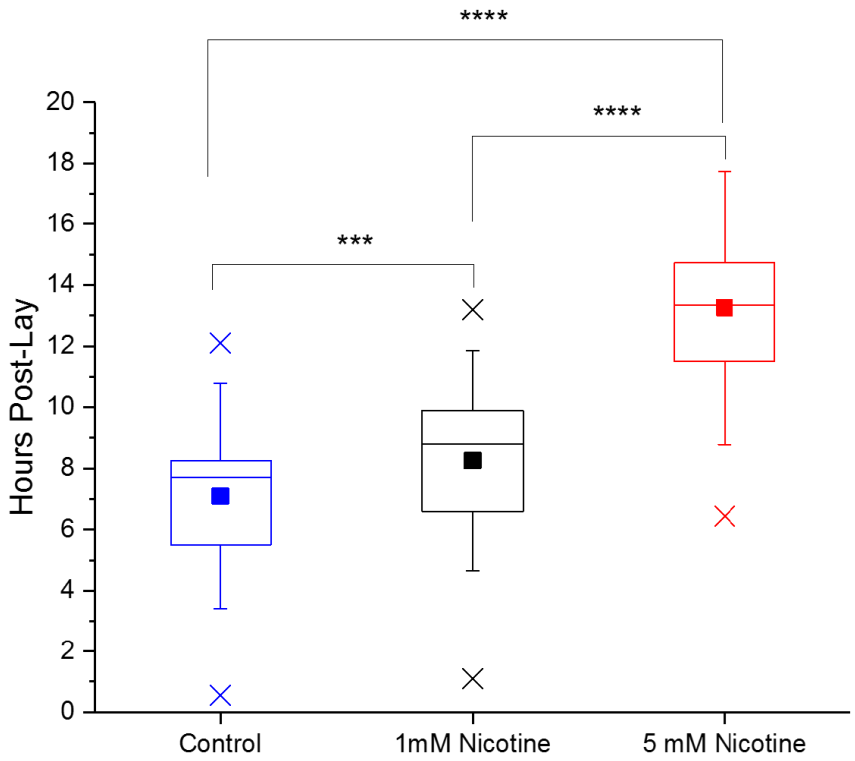

Figure 3.22: *Ex utero* development time is significantly different than control animals grown on OP50 media. The mean development times for F1 larva were significantly different among parents across all three conditions. Squares indicate the mean, X's indicate outliers. (n=118 F1 worms for control, n=122 for 1 mM nicotine, n=147 for 5 mM nicotine. *** P≤0.001, ****$P \leq 0.0001$)

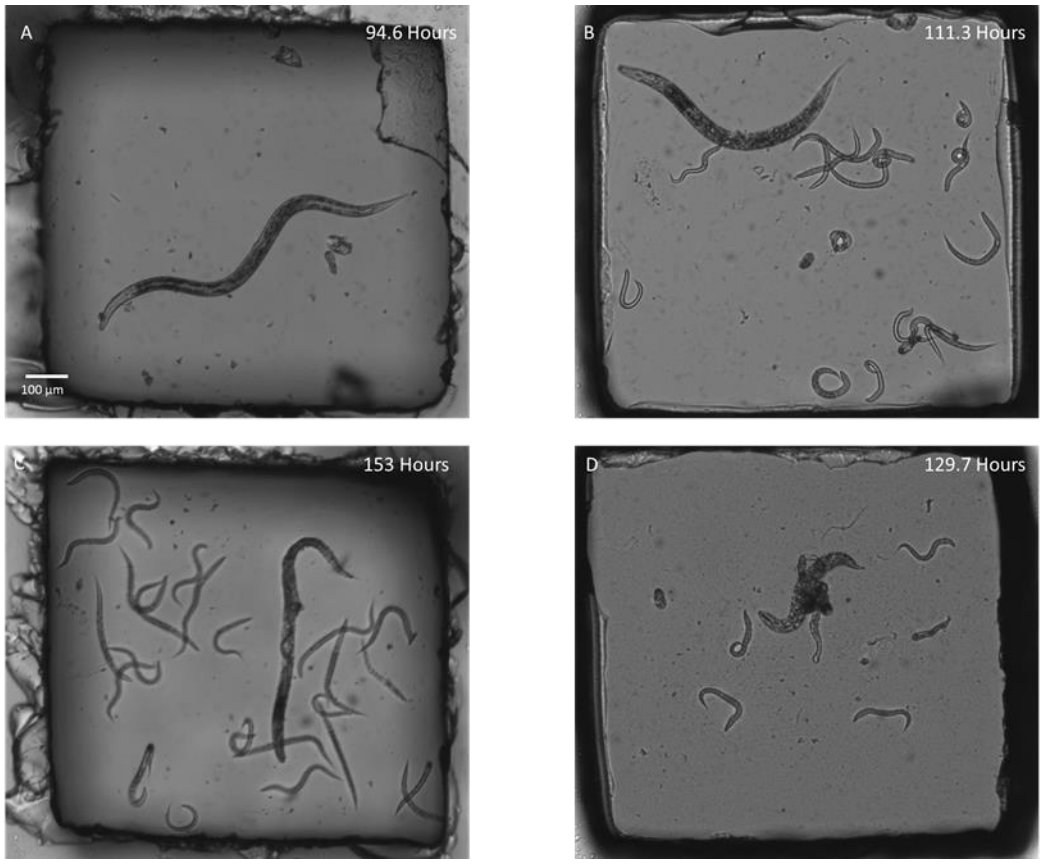

Figure 3.23: A.) The majority of CeHR control worms produce progeny with no difficulty. B.) A small number of the population was observed to experience internal hatching of embryos, which resulted in the death of the parent. C.) For worms grown in 5 mM nicotine, the majority of egg laying events were uninterrupted. D.) Several animals in 5 mM nicotine conditions ruptured during the egg laying process.

3.5 Locomotive Behavior Observations of Nicotine Affected Animals

Other analytical tools can be used to study the *C. elegans* in liquid media. Analysis of the position of the centroid can be used to plot position over time. A representation of the activity and spacial preferences of individual worms can then be generated (Figure 3.24). Of particular note is the decrease in activity levels seen in animals. Worms in the 10 mM nicotine condition group tend to stay in one location for

a longer duration, as shown by the lack of position markers over the area of the entire well. Those worms in the 100 mM nicotine group do not show exploratory behavior, and drift towards the right side of the well. This is most likely a result of the paralysis of movement, and the shifting of the WormSlide as it moves around platform.

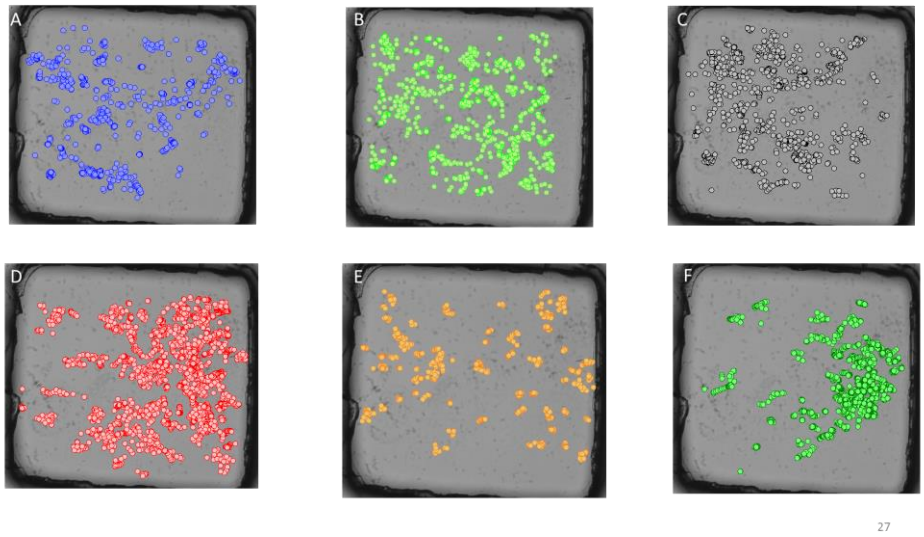

Figure 3.24: Locational changes within individual wells. The positions of individual animals over 100 hours are represented. Individual worms in the conditions of A.) control CeHR, B.) milk-free CeHR, C.) 1 mM nicotine, D.) 5 mM nicotine, E.) 10 mM nicotine and F.) 100 mM nicotine are represented.

Speed is another indicator of locomotive ability. While speed widely fluctuates across an animal's lifetime, dependent on such events such as lethargus and ecdysis, a distribution of the speed range for each condition was compiled (Figure 3.25). All conditions at a concentration of 10 mM of nicotine or less exhibit a normal distribution of speed ranges. The toxicity of 100 mM concentrations of nicotine are

evident in that the speed distribution are between 0 and 25 μm/S, while the average speed for every other condition is nearly double that, with only no individuals showing a complete lack of motion.

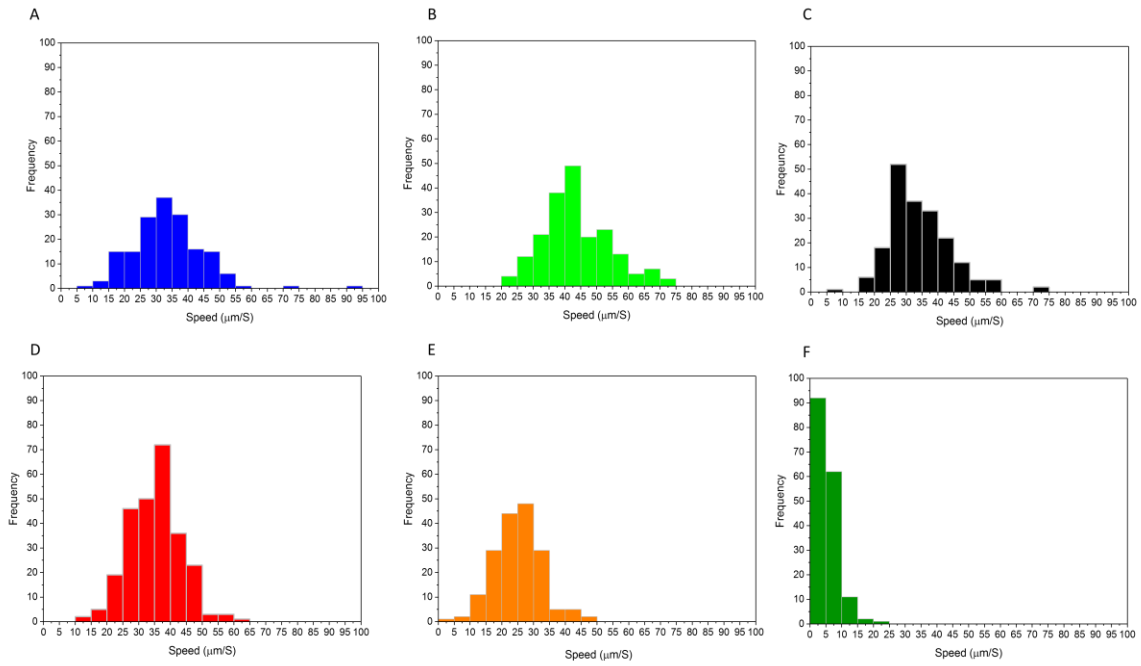

Figure 3.25: Histogram of average lifetime speed ranges. The lifetime speed range of the population of each condition was measured, in order to show the distribution of speeds in response to different drug dosages. The conditions of A.) control CeHR (n=24), B.) milk-free CeHR (n=23), C.) 1 mM nicotine (n=26), D.) 5 mM nicotine (n=39), E.) 10 mM nicotine (n=26), and F.) 100 mM nicotine (n=30) are represented.

Chapter 4

DISCUSSION

4.1 The Advantages of WormSlide Implementation for CeHR Assays

Microfluidic enclosures to study living organisms offer several advantages over traditional culture techniques. Small amounts of fluids limit the consumption of expensive reagents, allow the end-user to exercise careful control over the final concentrations of chemicals introduced into the system, and result in a smaller field of view to be examined for microscopy purposes. The WormSlide is an advantageous method for culturing and observations of *C. elegans* in liquid, as compared to other bulk fluid methods. Containers such as sterile bags or scintillation vials necessitate the use of larger amounts of axenic media to culture worm populations. Additionally, the large surface area and three dimensional nature of current liquid media enclosures do not allow for simple observations and screening of mutant or variable phenotypes to be easily undertaken.

The WormSlide eliminates these issues in *C. elegans* liquid culturing methods. The WormSlide is a completely self-sealed closed system, minimizing the risk of bacterial or fungal contamination from laboratory surfaces and the outside environment. The WormSlide has a working volume of approximately 800 µl, resulting in minimal reagent consumption. PDMS enables gaseous exchange between the device and the outside environment, ensuring that the nematodes contained within are not oxygen deprived, and glass coverings on both faces of the device enable it to be used for microscopy assays. Forty five individual wells set within the chip are

capable of holding a single *C. elegans*, which is sustained from the embryonic stage through to propagation and early growth of the F1 generation.

The segregation of individual animals within the device allows for higher quality metrics to be gathered, limiting influential noise from interactions stemming from multiple worms. Fecundity counts and precision measurements of the lethargus and ecdysis period timescales are difficult in systems containing multiple animals. The Big Brother assay concept is built around measurements that are taken every 33 minutes per individual well, ensuring that behaviors that occur with regular frequency, such as lethargus are not missed, and that the likelihood of imaging stochastic events in worm behavior is increased. The time required to image all 45 wells can be decreased, at the expense of fidelity of autofocusing. Autofocusing is an integral part of the system, as healthy larval stage animals exhibit exploratory behaviors and investigate their environments in the Z-axis. This vertical exploration and movement out of the well enclosure is another variable in *C. elegans* behavior and locomotion that can be scored, an analysis that cannot be conducted on agar plates.

Investigation of phenotypic variation and screening for mutant phenotypes is an important feature of the WormSlide device and Big Brother assay. For example, certain animals in 10 mM nicotine experienced the first lethargus stage of growth, while others failed to. Additionally, some animals in 5 mM nicotine were capable of reproduction, while others were not and did not even grow to adulthood. These phenotypes, whether the result of stochastic changes in gene expression or mutations displaying incomplete penetrance, would be difficult to observe in bulk liquid systems, or systems dependent on manual imaging by the end user. Difficulties in measuring phenotypic variation stem from challenges in acquiring large sample sizes and

eschewing traditional techniques, such as examining population aggregates [32]. High content imaging is one of way of circumventing this problem, and the WormSlide and the Big Brother assay system represent a move in this direction for *C. elegans* studies.

There are challenges in working with CeHR media. CeHR is incredibly rich, which results in rapid growth of contaminating microbes, so precise ascetic technique is required when working with the media. Additionally, while the time and reagents required to manufacture CeHR are a positive investment, not all laboratories will have the necessary time or materials to produce CeHR in bulk. In regards to microfluidics, differences in PDMS batches and in material handling techniques need to be minimized in order to consistently reproduce the experimental environment.

The WormSlide relies on the use of liquid axenic media. Axenic media is optically transparent, compatible with PDMS constructed micro devices, and can function as a solvent for various pharmaceutical compounds. *C. elegans* cultured in axenic media exhibit morphological and developmental adaptations to a chemically synthetic liquid environment. Body plan sizes, fecundity, and development time points of N2 laboratory strain worms differ from worms fed on a standard bacterial diet of OP50 strain *E. coli*. Environmental perturbations and chemical changes must both play a role in the changes observed in CeHR cultured *C. elegans*. Characterizing these various phenotypic changes are important to establishing baseline data for future experiments examining morphological changes in CeHR, as well as functioning as a baseline for future drug studies utilizing CeHR media.

4.2 *C. elegans* Adaptations to CeHR Media

4.2.1 Changes in Body Plan Morphology

C. elegans raised on OP50 bacteria grow to be slightly over one millimeter long at adulthood. Animals cultured in CeHR from the embryonic stage grow slightly shorter and thinner. This altered body plan may provide better mobility for animals growing in a liquid environment. Animals raised in S-basal bacterial media exhibit a thrashing motion in their locomotive behavior, and animals raised in CeHR exhibit similar movement characteristics, in which the more streamlined body plan would allow the animal to propel itself along greater distances with less effort [33]. Comparisons in the Reynolds number between CeHR-adapted and naïve swimming animals would further add to understanding the mechanics behind *C. elegans* swimming behavior [34]. This change towards a more linear body plan is analogous to adaptations seen in spaceflight conditions, in which astronauts will exhibit muscle and cartilage wasting in microgravity. Previous studies have shown that fluid dynamics alter *C. elegans* body length. In liquid OP50 media, animals grown in liquid exhibited significantly longer body length, as well has higher contraction levels. This altered physique was a result of an upregulation via TGF-β/DBL-1 signaling in liquid [35]. In TGF and *dbl* mutants, a decrease in length when animals were grown in liquid bacteria compared to wild-type N2 worms was seen. DBL-1 controls the expression of certain collagens, and research into the downregulation in collagen and cuticle genes in CeHR is currently being conducted in the Sabanayagam lab. These collagens are needed for animal growth and increased length. The implications of these prior results combined with the length of CeHR raised animals is that although the necessary fluid dynamics of CeHR are present for changes in TGF and myosin expression upregulation, the

nutritional requirements compared to liquid bacteria are significantly different as to lower expression of these genes needed for increased length in a liquid environment.

Animals cultured in CeHR are significantly thinner than those grown on OP50. Animals grown in other axenic media cultures have demonstrated reduced volume and mass, and based on the dimensions of CeHR grown animals, these animals also likely have a reduced volume and mass [36]. There are several possible pathways involved in this outcome, and they are perhaps interconnected. Axenic media has been found to upregulate metabolic activities in *C. elegans*, possibly as a result of dietary restriction. An increased metabolic rate would result in a body plan better suited to stressful environments, and result in changes including reduced fat stores, and the inability to support large numbers of progeny. Additionally, because CeHR is not as nutrient rich as an *E. coli* based food source and is likely lacking several molecules synthesized by biological entities, *C. elegans* that are raised in CeHR may have to synthesize a greater number of their own biomolecules. Fat storage in nematodes depends on the particular strain of bacteria used. It is known that carbohydrate levels in the food source of nematodes inversely correlates with fat storage in these animal, but the relationship between these two macromolecules is still unknown [37]. Because there is a high concentration of glucose in CeHR, it is possible that carbohydrate concentration and uptake may play a role in regulation of fat storage. Changes in the concentration of glucose in CeHR media, followed by labeling of lipid storages would lead to further understanding of this supposed mechanism.

4.2.2 Adaptations in Feeding Behavior

C. elegans feeding is dependent on the activity of the pharynx. The pharynx, as previously described, intakes solid food and particles where they are transported to the

intestine. Any liquid is expelled from the suspended particles. In an all liquid environment such as CeHR, pharyngeal pumping would not be beneficial in any form of liquid uptake. Nevertheless, pharyngeal pumping is still a behavior that is observed in CeHR raised P0 generation animals. In the presence of bacterial food sources, animals will pump on average 200-300 times per minute [7]. In CeHR media, this pumping average is decreased, and becomes less frequent as the animal ages. Some animals, cultured in CeHR as P0 embryos, exhibited a complete cessation of pharyngeal pumping after 168 hours of age. This threshold of no pharyngeal pumping activity is usually only seen in *eat* defective mutants or dead animals. The *eat-2* gene is a nicotinic acetylcholine receptor that is necessary for pharyngeal pumping [38]. The animals assayed were wild-type, and no defections in the *eat-2* gene were known to have occurred. Additionally, there is a degree of variability in pumping behavior of CeHR grown animals. It is known that the marginal cell (MC) neuron is partially responsible for regular pumping of the pharynx [38]. MC-independent pumping possibly occurs in the absence of food, and the irregular pumping rates seen in CeHR raised animals may be a result of MC-independent pumping, as the media lacks a solid food source. Solid particles are still present in the media, resulting from shed cuticles as the animals' age, detritus from the bleaching procedure and possibly defecation behavior. The excretory orifice of the animal is seen on the rear side, but observation of defecation has not occurred. These solid particles do seem to induce pumping behavior in animals on contact with the mouth region. Since subsequent generations will not need to intake food through pharyngeal pumping, it is possible that expression of the genes necessary to maintain proper and regular pumping and particle intake will be downregulated, resulting in offspring of later generations that do not exhibit

pharyngeal beating behavior. A four day pharyngeal pumping study with F9 generation adults did show a decline in pumping activity over time, but cessation of pumping activity was not observed widespread (data not shown).

Pharyngeal pumping and feeding behaviors are also indicators of food quality and satiety. Quiescence is defined as a sleep-like behavior, in which animals stop exploring their environments and enter into a sleeplike state. When *C. elegans* are satiated based on the quality of their food source, they cease food intake, indicated by a lack of rapid pumping, and become quiescence [39]. Animals in CeHR do exhibit a cessation of these activities, possibly as a result of satiety quiescence. Further experimentation with varying qualities of CeHR is necessary to properly examine the implications and frequency of satiety quiescence in *C. elegans* grown in axenic media. Since many assays based on food quality are dependent on starving animals, these experiments remain challenging to conduct with CeHR in a closed system microfluidic environment. Because the dauer larval stage that is indicative of starvation conditions have not yet been observed in CeHR, determining a temporal or nutrition dependent threshold for starvation remains elusive.

4.2.3 CeHR Media Leads to Delays in Developmental Progress

The developmental checkpoints of N2 *C. elegans* grown on OP50 bacteria is well known. In the presence of food, progression through the different molting stages occurs at regular intervals. This process can be halted in response to changing environmental conditions, including the presence of stressors or absence of food. *C. elegans* gown in CeHR progress through all four larval stages, although at later time points than what is observed in agar grown animals. The reason for this delayed development is unknown, and it is likely the result of the nutrient value of CeHR

being sufficiently different from bacterial food sources as to change the expression of micro RNA switches believed to play key roles in these cycle checkpoints [40]. These miRNA switches primarily include the *lin* family of micro RNA's, specifically *lin-4*, in which loss of function mutants display developmental retardation. *Lin-4* targets expression of the protein LIN-14, which is a heterochronic gene involved in timing of cell division, although the wider mechanism of developmental effects is not known [41]. A period of lethargus, which is defined as another sleep-like state analogous to quiescence, yet with reduced responsiveness to mechanical stimulation [42]. While the time between each L-stage and the time required for P0 animals grown in CeHR are delayed, the actual length of the lethargus period prior to molting, approximately two hours, remains the same as animals fed on a bacterial diet. Worms undergoing lethargus become rigid, and are less responsive to stimuli in individual wells, which primarily stem from fluid disturbance by nearby swimming worms or physical contact with another animal. Testing the effects of interruption of this lethargus state on development and growth is challenging with the current WormSlide system, as no mechanism is currently implemented to induce mechanosensation during the automated imaging process.

C. elegans behavior post-lethargus in CeHR remains similar to that observed in plate grown animals. This behavior consists of a process called ecdiysis, where the old cuticle is shed following lethargus. This process is characterized by the rapid longitudinal rotation of the worm along its central axis. This motion separates the cuticle from the animal, and this rotating behavior is observed in CeHR grown animals immediately following the two hour lethargus period. Multiple cuticles can be observed in individual wells, evidence of animals undergoing the molting stages.

Animals grown in CeHR with the absence of skim milk do not undergo these developmental changes, regardless of whether they are grown in glass scintillation vials or PDMS WormSlides. In fact, animals grown in CeHR without milk remain at the L1 stage for over seven days without exhibiting any signs of lethargus or ecdysis. These animals do appear to undergo quiescence states of shorter duration than lethargus, such as those associated with satiety, but there is no forward development. This would be indicative of a lack of crucial nutrients in CeHR that is only present with the addition of milk. This lack of growth means that milk, or the molecules contained therein, must remain a primary reagent in CeHR and any downstream successors of CeHR in order to facilitate rapid development and reproduction.

4.2.4 Reductions in Brood Size and Reproductive Fitness

C. elegans brood size is dictated by the amount of available sperm cells contained in the spermatheca available for self-fertilization of oocytes. In wild-type N2 worms grown on bacterial plates, brood size is approximately 300 animals, which are laid as eggs over a period of 48 hours, with four to ten eggs being laid per hour [1]. In CeHR-cultured animals this total is markedly reduced by ten-fold. *C. elegans* in CeHR give rise to an average of 25 offspring over the same 48 hour period observed in NGM grown animals. Reduced fecundity of animals grown in axenic media has been reported in the past, but never quantified or studied in-depth. The number of offspring per parent is also stochastic. Although uncommon, animals grown in CeHR culture also exhibit internal hatching of offspring, killing the parent animal. This severe phenotype may be the result of defects in either vulval muscle neurons resulting in failure to expel the embryos, or a physical defect or blockage of the vulval structure itself. Animals in CeMM also exhibit an increased likelihood to die from internal

hatching or a defective vulva, which is to be expected due to the similarities in chemical composition of the two media [43]. CeMM differs from CeHR with the absence of hemin chloride, milk, lactalbumin, and cholesterol. Experiments in CeMM have shown a rate of internal hatching in approximately 8% of adult animals. It has also been hypothesized that brood size may be directly related to the cost of reproduction. A reduction in brood size would be a strategy necessary to devote more energy into ensuring a smaller, but more robust filial generation would be capable of surviving a foreign, stressful environment. The structural changes in CeHR raised *C. elegans* that are indicative of a reduction in offspring quality, namely an elongated spermatheca and deformed germline, may be a result in a shift from a r-type selection model to a fecundity more associated with K-selection. K-selection organisms have fewer offspring and invest more energy and parental nurturing to increase the chances of offspring success, while r-selection organisms have large amounts of offspring and very little nurturing occurs on the part of the parent [44]. While *C. elegans* have always been classified as r-selection organisms, this change in number of offspring is a shift from that model.

4.2.5 CeHR Cultured Worms Undergo an Increase in Lifespan

Senescence in *C. elegans* remains an important research subject, especially because of its variable nature even in organisms of identical genetic backgrounds in identical environments [45]. The WormSlide's function of segregating animals in individual wells allows for simpler, more accurate and reproducible measurements to be taken of animals in their life or death state. The self-contained nature of the device also prevents the need to forcibly transfer animals across plates to maintain a food source. Since the WormSlide in its current iteration does not support separation of the

P0 from subsequent filial generations, floxuridine (FUdR) is used to induce sterility in hermaphrodites. The somatic cells in *C. elegans* post-adulthood do not undergo cell division, and multiple studies show that FUdR concentrations do not have any impact on the physiology or lifespan on wild-type N2 worms grown on NGM plates [44]. CeHR animals subjected to FUdR treatment are longer lived and exhibit a longer median lifespan than NGM raised worms, and lifespan in axenic media is also independent of lower FUdR concentration for N2 animals. A concentration of 50 µM FUdR, half of what is normally used to stop reproduction in agar worms, significantly reduces the lifespan of CeHR animals compared to those who were exposed to 40 or 30 µM FUdR. This could be indicative of the threshold for toxicity to floxuridine.

There are a number of possible mechanisms that result in the longer lifespan of axenically cultured worms. One theory of aging and senescence that is universal across living organisms is the free radical theory of aging. This theory states that the production and accumulation of reactive oxygen species (ROS) are responsible for aging. These reactive oxygen species are chemically very reactive and can damage cells and their structures [47]. There is evidence that overexpression of certain oxidative stress resistance genes, such as *gst-10*, significantly extend lifespan in wild-type worms. Antioxidant additives used to supplement media sources have also been considered as a strategy to extend lifespan. In monoxenic cultures, the generation of free radicals through bacterial metabolism could possibly be a source of the oxidative stress that effects *C. elegans* as they age [48]. As CeHR is a form of axenic media, the increase in ROS from microbial propagation and growth would not be present. It is possible that the overall oxidative stress experienced by *C. elegans* in axenic CeHR media is less than that experienced by worms grown on monoxenic cultures. *C.*

elegans placed in CeHR media as P0 embryos must exhibit an organism wide adaptation response to liquid axenic media. Although the osmotic stress response in *C. elegans* is poorly understood, it has been shown that osmotic stress adaptation can result in lifespan extension in the presence of FUdR. Changes in osmolarity were conducted by placing animals in varying concentrations of NaCl for 24 hours. The postulated mechanism is that FUdR activates the base-excision repair pathway in somatic cells which increases acute stress resistance and lifespan [49].

This increase in lifespan may also be a result of dietary restriction. Previous versions of axenic media, such as *C. elegans* Maintenance Medium (CeMM) has resulted in lifespan, development rate, and fecundity rates analogous to animals grown on a reduced bacterial diet to the point of malnutrition [21]. However, this may not be indicative of a reduction in health. Adaptations to different environmental conditions manifest themselves in different ways, and this may simply be the result of adaptations to a reduced gravity environment stemming from the buoyancy forces in liquid, or an environment lacking a certain metabolic component stemming from microbes. The length of developmental periods and the length in which adults in CeHR remain gravid are very similar to those raised on bacterial media, and are indicative of healthy animals.

However, dietary restriction has also been challenged as the reason for the differences observed in lifespans between NGM and axenically raised animals. AXM is a growth media made of soy peptone and yeast extract that can be formed into solid plates, analogous to NGM without an *E. coli* lawn. Based on studies with AXM compared to live microbial food sources, it has been postulated that there is a requirement for metabolic activity and a metabolic byproduct in living bacteria that

accounts for phenotypic differences seen in *C. elegans* grown in this way. The study found that AXM nutrient content is 6-fold higher than *E. coli* based plates, so that this particular axenic media does not qualify for dietary restriction [50]. Animals grown on the AXM regardless showed signs of malnutrition and physical phenotypes consistent with being raised on an axenic diet. Whether a metabolic byproduct or non-diffusible protein in *E. coli* is responsible for rescuing the effects of axenic media is currently unknown.

Studies on *C. elegans* lifespan with CeHR confirm that animals raised in axenic media do exhibit longer lifespans. This increase in lifespan is likely FUdR independent, but the mechanism of how CeHR induces a longer period of life til senescence is unknown. Factors such as reduced reactive oxygen species, the onset of an osmotic stress response, and the effects of dietary restriction, or a combination of all three may be responsible for this increase in lifespan. Observations have been made of incidental bacterial contamination in the WormSlide rescuing the effect of reduced fecundity and malformed germline in P0 CeHR worms, so the possibility remains that bacterial contamination of CeHR would shorten *C. elegans* lifespan. CeHR and the WormSlide microfluidic device provide a unique opportunity for continued study of lifespan factors and pathways that may affect lifespan in *C. elegans*.

4.3 Teratogenic Effects of Nicotine in CeHR Cultured *C. elegans*

Tobacco consumption in the United States results in the death of over half a million individuals every year and hundreds of billions of dollars spent on prevention and treatment [51]. A growing number of Americans are turning to alternative sources of nicotine, including patches, gum, e-cigarettes, and vape pens. These devices all utilize nicotine in its liquid form. Nicotine, as the primary psychoactive component of

tobacco, is a known teratogen. The exposure of developing organisms to nicotine is known to cause developmental and cognitive changes, including exposure at the embryonic stage [52]. Several studies have been conducted in order to characterize the effects of nicotine on *C. elegans*, utilizing both acute and chronic exposure time points. Unlike ethanol studies, there is no standardized molar concentration of nicotine that has been decided upon by the worm community, nor is the half-life of nicotine or metabolic kinetics of nicotine known in *C. elegans*. Additionally, all of the published nicotine studies to date utilize bacterial plates, which introduces the confounding variable of microbial metabolism of the nicotine product. Definitions of acute and chronic exposure also vary amongst individual studies. As an example, two studies published within two years of each other differ significantly in time periods and concentrations used. One paper uses 24 hours as its definition of chronic exposure, the other grows animals until the L4 stage. The thresholds for low and high concentrations of nicotine are, respectively, 50 µM and 500 µM [53]. The second study utilizes amounts of 20 µM and 20 mM when comparing phenotypes and gene expression in comparison to concentration differences [13]. This range of amounts can give radically different results. Recently, studies have shown that *C. elegans* can be a viable model to study the effects of liquid nicotine on development. Nematodes were exposed to liquid nicotine from cartridges found in e-cigarettes. This study was again conducted on NGM plates with a bacterial food source, which has the potential to metabolize the nicotine and change the dynamics of its effect on the animals [54]. Accounting for all of these variables and experimental considerations, the WormSlide with CeHR axenic media was used to examine *C. elegans* embryos grown in nicotine supplemented CeHR at various concentrations. Although the potential chemical

interactions of nicotine with the reagents found in CeHR are currently unknown, growing embryos in a nicotine environment without replenishment or separate dosing points allows for a standard baseline to be established and the utility of the WormSlide and CeHR for pharmaceutical studies to be demonstrated.

4.3.1 Scoring Morphological Deformations

The severity of morphological deformations is related to the nicotine concentration to which embryos are exposed. Embryos exposed to concentrations of 100 mM and greater do not show any growth. These animals die within one week of exposure, indicating that this level of nicotine is the threshold of toxicity for teratogenic nicotine studies in *C. elegans*. Lower concentrations, 10 mM and 5 mM specifically, result in more thorough development, but there are also physical deformations present. Worms exposed to 10 mM nicotine are stunted in their physical growth compared to lower concentrations. These worms do undergo the lethargus phase transitions and do show evidence of ecidysis of the cuticle, albeit at delayed time points. The duration of the lethargus period is however still the same as that measured in the control animals. This is possibly as a result of downregulation of genes involved in the signaling pathways necessary to activate the transition into succeeding growth checkpoints, or differential expression of the microRNA's mentioned earlier, that serve as regulators of developmental time points.

4.3.2 Nicotine Induces a Severe Decrease in Reproductive Fitness

Nicotine is known to have a number of downstream effects on reproduction and fecundity in a number of species. Nematodes are no exception, and nicotine exposure is known to cause downstream effects such as changes in egg-laying time,

duration, and number of eggs laid in a given time period. Nicotine exposure experiments on OP50 plates have shown that egg laying frequency increases in the first 24 hours of egg laying behavior, and this behavior manifests itself earlier than in nicotine-free worms [55]. Animals grown in concentrations greater than 5 mM of nicotine do not reach gravid adulthood and hence do not reproduce. In fact, animals exposed to 5 mM nicotine have a significantly increased number of progeny compared to wild-type animals grown in CeHR. The reason for this is as of yet unknown, however a recent study conducted by Smith et al. showed that chronically low doses causes a significantly increase in egg-laying behavior and brood size [55]. This study however did not study brood sizes, so it is not known if this increase in egg laying is simply a temporal phenomenon, or a result of a greater number of progeny. The onset of egg laying behavior in wild-type and 1 mM raised animals differed by an average of two hours, which while a slight delay, was statistically significant. In comparison, the onset of egg laying was significantly delayed by 10 hours in 5 mM nicotine. Nicotine at 1 mM concentration did not have any of the reproductive effects on gravid adult animals seen in those exposed to 5 mM nicotine. Those raised in 5 mM nicotine begin their egg laying behavior later than animals raised in standard CeHR conditions, on average 12 hours later post-hatch. Ex utero development times are also effected depending on nicotine concentration. The average development time for embryos after expulsion from the parent increases as nicotine concentration increases. Additionally, the *ex utero* development time of control animals is very brief in some instances, less than two hours in several cases observed. This implies that there is a defect in the timing mechanism of the vulval nerves, resulting in embryos being held internally for a longer gestation period than is normally observed in bacterial media. Some control

CeHR animals also exhibit internally hatching larva which kill the parent, indicative of these timing defects. Structural defects or blockage of the vulva itself does not appear to be responsible for these wild-type events, as there is no rupturing of this region of the body, and a number of eggs are laid normally throughout this stage. Prolapsed or otherwise compromised vulvas are present in animals grown in 5 mM of nicotine (Figure 3.23). These events are characterized by a burst of internal organs from the cuticle of the adult worms, followed shortly thereafter by death. The frequency of these bursting events were observed in 15% of the sample population. These bursting animals were not counted in the scoring of ex utero development time, egg lay time, or total brood size.

There is evidence for defective vulvas based on the variability of *ex utero* development times observed. The shortened development time of less than two hours of certain outlier animals could be a result of defects in the vulval timing machinery, resulting in gravid parents holding onto their eggs for a longer duration than those seen in agar grown worms. This holding of the eggs may be the source of the internal hatching observed in a small proportion of wild-type CeHR animals.

4.4 Teratogenic Nicotine Results in Shortened Lifespan

Nicotine has been previously established to shorten the lifespan of *C. elegans*, and this trend is seen in animals grown in CeHR. While the lifespans of 5 mM and 10 mM exposed worms are not significantly different, all of the experimental conditions had significantly reduced lifespan compared to control animals in 30 µM FUdR. The mechanism behind the reduction in lifespan seen in humans who consume tobacco is poorly understood, as there are over one thousand additionally compounds in cigarettes in addition to nicotine. What is known is that nicotine exposure in *C.*

elegans can increase the expression of certain kinases and phosphatases that are traditionally associated with cancer [51]. It is likely that the reduction in lifespan seen in response to teratogenic nicotine exposure is the result of changes in multiple signaling cascades, brought about by interactions of nicotine with the various nicotinic acetylcholine receptors in these nematodes.

4.5 Conclusions

The nematode *C. elegans* has been utilized by biologists as a model organism for the better part of four decades, and methods used to culture and observe these animals have remained similar. Bacterial food sources are still widely used, which are incapable of being supported by automated systems, require animals to be consistently transferred to fresh media plates to avoid starvation, and can alter the structure of pharmaceuticals added as a result of bacterial metabolism. Liquid axenic media alternatives exist and have been utilized for space biology and gene expression assays. CeHR is the most recently developed of these media types, yet no baseline data exists on the effects of the media on animal lifespan, growth, morphology, or fecundity. Additionally, the applications of CeHR in conjunction with a microfluidic system or as an alternative for pharmaceutical experiments have not been explored.

The development of the PDMS based WormSlide is not only able to be used to segregate individual animals in liquid media, but also is used to demonstrate the utility of CeHR as a medium to conduct pharmaceutical assays. Using the Big Brother assay technique, worms are able to be recorded in a natural, physically unconstrained state for 20 seconds every 33 minutes. By utilizing the flexibility of automated microfluidics, it was found that CeHR media results in phenotypic changes in P0 animals, including a shorter and slimmer body plan, delayed growth and

developmental time points, reduced brood size, and reduced rates of pharyngeal pumping. The WormSlide also showed that chronic nicotine exposure at the embryonic stage severely amplifies several of the phenotypes naturally seen in CeHR, such as reduced body size and reduced fecundity, and also reflects other behaviors and phenotypes seen in nicotine tests carried out on bacterial media, such as delayed onset of egg-laying and paralysis at higher concentrations.

The current technique of Big Brother assays that the WormSlide is used for could be expanded to accommodate a greater number of WormSlides. Each slide contains 45 individual wells, and a greater number of devices would enable a greater number of replicates or multiple conditions to be examined in technical run. A flow system would also be beneficial, as the effect of drugs introduced at specific time points could be examined, as well as pulse chase experiments to simulate withdrawal symptoms. Future experiments should focus on establishing the dynamics that various pharmaceuticals may have with CeHR reagents, specific time point experiments related to drug introduction, and conducting gene expression experiments, so that a transcriptome can be established from which RNAi and other molecular assays can be conducted. In terms of pharmaceutical implementation, other types of pharmaceuticals could be utilized in conjunction with the WormSlide and CeHR media. Specifically relating to nicotine, specific time point and dosage experiments, taking advantage of the nature of nicotine as a stimulant in certain concentrations and as a depressant in others, could further improve upon the knowledge of how nicotine effects mechanosensation and locomotion. Further experiments could also incorporate the downstream effects of nicotine on F1 and F2 generations of offspring. The use of CeHR liquid media opens new avenues for how biologists can take advantage of

microfluidics and computer automation to expand their knowledge of how organisms adapt to different environments and how pharmaceuticals can affect an organism such as *C. elegans*, without needing to account for certain confounding variables, such as bacterial metabolism, resource and manpower constraints, and difficulties in live-imaging in other types of liquid containment vessels.

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