UNIVERSITY OF CALIFORNIA,
IRVINE

# Reproductive senescence and control of germline stem cells in the *Caenorhabditis elegans* hermaphrodite germline

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Adrian Paz

Dissertation Committee:
Assistant Professor Olivier Cinquin, Chair
Professor Kavita Arora
Professor Thomas Schilling
Assistant Professor Susanne Rafelski

2016

## DEDICATION

I would like to dedicate my dissertation to my family. To my parents, Aurelia and Lucio. To my siblings Miguel, Guillermina, Rigoberto, and Richard.

To my family in laws, Mr. Anthony Kim, Mrs. Theresa Kim, Tony, Vivian, and Samson. To my wife, Juliet, and son, Matthew who bring inspiration and joy with every smile and laugh.

## TABLE OF CONTENTS


| | | PAGE |
|---|---|---|
| LIST OF FIGURES | | iv |
| ACKNOWLEDGEMENTS | | v |
| CURRICULUM VITAE | | vi |
| ABSTRACT OF THE DISSERTATION | | viii |
| CHAPTER 1 : | **Introduction** | 1 |
| CHAPTER 2 : | **Intermittent fasting can delay reproductive cessation without reducing brood size** | 12 |
| | Introduction | 13 |
| | Methods | 15 |
| | Results | 18 |
| | Discussion | 29 |
| CHAPTER 3 : | **Characterization of the *C. elegans* reproductive response to increased demand** | 32 |
| | Introduction | 34 |
| | Methods | 36 |
| | Results | 38 |
| | Discussion | 49 |
| CHAPTER 4 : | **Live imaging reveals active infiltration of mitotic zone by its stem cell niche** | 52 |
| | Introduction | 53 |
| | Methods | 57 |
| | Results | 59 |
| | Discussion | 69 |
| CHAPTER 5 : | **Conclusion** | 71 |
| REFERENCES | | 80 |

## LIST OF FIGURES


| | | Page |
|---|---|---|
| **Figure 1.1** | Life cycle of *Caenorhabditis elegans.* | 4 |
| **Figure 1.2** | Gonadal arm of adult, hermaphrodite *C. elegans.* | 5 |
| **Figure 2.1** | Food regimen control and embryo laying detection in real-time. | 20 |
| **Figure 2.2** | Microfluidic chip conditions optimized for *C. elegans* culture. | 23 |
| **Figure 2.3** | Brood sizes under different conditions relevant to culture on microfluidic chips. | 24 |
| **Figure 2.4** | Daily and total brood size of mated hermaphrodites under IF treatments. | 26 |
| **Figure 2.5** | Reproductive activity and brood size of hermaphrodites under IF. | 27 |
| **Figure 2.6** | Embryo size of hermaphrodites under 6h-1h IF. | 28 |
| **Figure 3.1** | Germ cell cycle length in mated and unmated individuals. | 40 |
| **Figure 3.2** | Germline compartment organization in mated and virgin hermaphrodites. | 42 |
| **Figure 3.3** | Daily and total brood size of hermaphrodites mated with sterile males. | 45 |
| **Figure 3.4** | Reproductive activity of hermaphrodites mated with sterile males prior to mating with fertile males. | 46 |
| **Figure 3.5** | Length of the longest DTC process along the distal-proximal axis of the germline. | 48 |
| **Figure 4.1** | The *C. elegans* gonad forms a tube that is closed at the distal end, where stem cells are located. | 56 |
| **Figure 4.2** | Worm immobilization device. | 61 |
| **Figure 4.3** | Device fabrication process. | 63 |
| **Figure 4.4** | Gonadal arms extruded, fixed, and stained with antibodies and DAPI show indistinguishable morphology whether they were repeatedly immobilized and imaged live. | 64 |
| **Figure 4.5** | DTC process extension is not detectably affected by repeated immobilization in the device. | 65 |
| **Figure 4.6** | Time lapse imaging of the DTC at 12h or 1h intervals reveals growth of individual processes. | 66 |
| **Figure 4.7** | Time lapse imaging of DTC processes shows an active growth and infiltration of the mitotic zone. | 68 |

**ACKNOWLEDGEMENTS**

My development as a scientist and the completion of my dissertation could not have been possible without Professor Olivier Cinquin. His mentorship and guidance allowed me to progress and learn to develop the necessary skills. His enthusiasm was contagious and provided additional motivation to my project. I am truly grateful for his time and patience towards matters that pertained to my research, as well as, personal life. I would also like to thank the members of the Cinquin lab for creating a great research environment by sharing advice, suggestions, and ideas. I would especially like to thank Amanda Cinquin for taking time out of her busy day to teach me experimental procedures and offer valuable discussion and ideas that influenced my project. I would like to thank Michael Chiang for his help in the data analysis of the germ cell cycle experiments. I would like to thank Evan Lister for his assistance with the irradiator and constructive comments that helped improve my presentations.

I would like to thank professor Elliot Hui for the great collaborative relationship and for guidance in the development of the worm microfluidic chips. I would like to thank Brandon Wong for the design and manufacturing of the worm immobilization chip utilized to do time lapse imaging of the DTC. My project would not have progressed without the undergraduate students who have helped with experiments and data analysis. I would also like to thank Jafar for taking the responsibility of carrying on the intermittent fasting project while I was on leave and for his continued commitment. Other undergraduates who helped with the data analysis, Brian, Abanob, Josh, and Mark. I would also like to thank Sarun for his contributions to the DTC project.

I would like to thank my committee members for all of the comments and advice that helped shape my project. I would like to thank the Center for Complex Biological Systems (CCBS) for their support and funding through the opportunity award. I would like to thank the minority science program (MSP), especially Dr. Marlene de la Cruz and Dr. Luis Mota-Bravo, for welcoming me to UCI and providing several resources that have helped me get through the first two years of graduate school. I would like to thank professor Mike Mulligan for providing financial assistance that allowed me to focus on research.

**CURRICULUM VITAE**

**Adrian Paz**

| | | |
|---|---|---|
| EDUCATION | University of California, Irvine<br>Ph.D. Biological sciences | 2008-2016 |
| | California state university, Northridge<br>B. S. Biology: Biotechnology | 2006-2008 |
| RESEARCH EXPERIENCE | Principal Investigator: Olivier Cinquin Ph.D.<br>*University of California, Irvine*<br>A microfluidic approach to investigate the effects of dietary restriction on reproductive aging in *Caenorhabditis elegans*. | *2009-2016* |
| | Principal Investigator: Beverley Fermor Ph.D.<br>*Duke University Medical Center*<br>Effects of oxygen tension on reactive species induced DNA damage and its repair in articular cartilage. | *Summer 2007* |
| | Principal Investigator: Michael L. Summers Ph.D.<br>*University of California, Northridge*<br>Regulation of heat shock gene expression in differentiated cyanobacterium *Nostoc punctiforme*. | 2006-2008 |
| | Principal Investigator: Stan T. Metzenberg Ph.D.<br>*University of California, Northridge*<br>Investigation of proteins involved in exogastrulation. | *Summer 2005* |
| HONORS AWARDS | CCBS Opportunity award (Center for Complex Biological Systems) | *2010* |
| | UCI MSP (Minority Science Program) | *2008-2009* |
| | Duke SROP (Summer Research Opportunity Program) | *2007* |
| | MBRS RISE (Research Initiative for Scientific Enhancement) | *2006-2008* |
| | Summer TRAILS (Team Research Approach in Laboratory Science) | *Summer 2006* |

PUBLICATIONS

**A. Paz,** P. Thomas, J. A. Souz E. E. Hui, and O. Cinquin, "Intermittent fasting delays reproductive cessation without sacrificing brood size," (Manuscript in preparation, 2015).

M. Chiang, S. Hallman, A. Cinquin, N. R. de Mochel, **A. Paz**, S. Kawauchi, A. L. Calof, K. W. Cho, C. C. Fowlkes, and O. Cinquin, "Analysis of in vivo single cell behavior by high throughput, human-in-the-loop segmentation of three-dimensional images," *BMC Bioinformatics*, vol. 16, no. 1, p. 544, Nov. 2015.

A. Cinquin, L. Zheng, P. H. Taylor, **A. Paz**, L. Zhang, M. Chiang, J. J. Snow, Q. Nie, and O. Cinquin, "Semi-permeable Diffusion Barriers Enhance Patterning Robustness in the C. elegans Germline," *Dev Cell*, vol. 35, no. 4, pp. 405–417, Nov. 2015.

M. Chiang, A. Cinquin, **A. Paz**, E. Meeds, C. A. Price, M. Welling, and O. Cinquin, "Control of Caenorhabditis elegans germ-line stem-cell cycling speed meets requirements of design to minimize mutation accumulation," *BMC Biol.*, vol. 13, no. 1, p. 51, Jul. 2015.

**A. Paz*,** B. G. Wong*****, M. A. Corrado, B. R. Ramos, A. Cinquin, O. Cinquin, and E. E. Hui, "Live imaging reveals active infiltration of mitotic zone by its stem cell niche," *Integr. Biol.*, 2013.

*** denotes equal contribution**

**ABSTRACT OF THE DISSERTATION**

Reproductive senescence and control of germline stem cells in the *Caenorhabditis elegans* hermaphrodite germline.

By

Adrian Paz

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2015

Professor Olivier Cinquin, Chair

Menopause is one of the earliest age related declines in humans. Uncovering factors that influence reproductive aging is of interest with regard to human reproductive health, and is also an important first step in understanding the overall aging process. The *Caenorhabditis elegans* hermaphrodite germline has been established as a highly-suitable model system, but many questions remain regarding the factors that modulate its aging.

Although IF is well known to extend lifespan in a variety of species including *C. elegans*, less is known about its effect on reproductive aging. I show that IF can delay worm reproductive cessation without reducing brood size. As a first step in establishing means to understand the ways in which IF exerts its effects on reproductive aging, I characterize germline changes that occur as reproductive activity is modulated; I chose this approach because reproductive aging is likely to be linked to reproductive activity and to germline stem cell (GSC) activity. Specifically, I find that GSCs of individuals with low reproduction rates randomly occupy a dormant state in which they do not cycle. I also show that increasing reproduction rate alters germline compartment makeup, which occurs

independently of oocyte fertilization. A strong candidate for regulation of at least some of these responses is the stem cell niche provided by the distal tip cell (DTC). Indeed, niche architecture has been hypothesized to play an important role in stem cell regulation. The DTC extends processes whose functional significance is unknown. Using time-lapse imaging, I show this growth to be an active and highly-dynamic process. This suggests that process growth may be an important component of the regulation of GSC behavior.

Overall, I report striking, novel behaviors of the *C. elegans* reproductive system as a whole, and of GSCs in particular. I also report a new intervention by which reproductive cessation can be delayed without sacrificing brood size. This lays the foundation for future studies to identify the underlying causes of reproductive aging, and to identify ways in which this aging is intrinsically controlled and can be further manipulated by external intervention.

# Chapter 1

## Introduction

Aging is defined as the progressive decline of organ and tissue function and the increase in disease and mortality. Advances in medicine have extended the average human life span in both men and women. With people living longer there has been increased focus on extending health span, the length of time an individual remains healthy. One of the earliest age related declines experienced by women is menopause, female reproductive cessation, between the ages of 48 and 55 (Hill et al. 1991; Austad 1994). Because of the age related decline in oocyte quality, female reproductive capacity begins to diminish dramatically after 35 years of age. Women who become pregnant at an age of 35 or older have increased risk of birth defects and miscarriages (Packer,et al. 1998; Broekmans et al. 2007; Hansen et al. 2008). Reproductive aging has become a medical issue of increasing importance because more women today tend to delay childbearing.

Since the main purpose of animal life is to reproduce it is intriguing that reproductive cessation takes place well before the end of life. While much work has been done to understand the aging process, little is currently known about the regulation of reproductive aging. A better understanding of reproductive aging will provide insight into maternal age-related infertility and child birth defects. In this thesis I utilize the *C. elegans* hermaphrodite germline as a model system to investigate how food availability and germline behavior impact reproductive aging.

The *C. elegans* hermaphrodite germline as a model system for aging studies

The relatively short life span of *C. elegans*, 2-3 weeks (Figure 1.1), makes it possible to conduct rapid analysis of survival and reproductive aging phenotypes. *C. elegans* has two sexes, hermaphrodite and male. Hermaphrodite worms can self-fertilize or mate with male worms, which allows for manipulation of reproductive output. These attributes along with a large genetic toolset make the *C. elegans* worm a powerful model system for reproductive aging studies. Additional attributes such as the transparency of tissues and the spatiotemporal layout of its reproductive system facilitate the study of reproduction, germline stem cell biology, the transition from mitosis to meiosis, and niche morphology (Henderson et al. 1994; Hubbard and Greenstein 2000; Kimble and Crittenden 2007).

The *C. elegans* hermaphrodite germline is a relatively simple self-renewing tissue that is composed of two symmetric arms that form a syncytial, tube-shaped structure (Figure 1.2). Each gonadal arm is capped at the distal end by a single somatic cell, the distal tip cell (DTC), that maintains stem cells by providing a ligand for the Notch receptor expressed by the germ cells (Henderson et al. 1994; Gao and Kimble 1995). The DTC is essential for maintenance of the mitotic zone (MZ) as a whole: removal of Notch signaling results in germ cell entry into meiosis (Austin and Kimble 1987; Lambie and Kimble 1991). Hyperactive Notch signaling produces ectopic proliferation throughout the germline (Berry, et al. 1997; Anita S-R Pepper 2003). The DTC is also important for gonad development, specifically promoting germline elongation and patterning. The distal most germ cells are putative germline stem cells and give rise to the rest of the proliferating germ cells in the MZ. As germ cells in the MZ divide they are displaced in the proximal direction, and progress through differentiation (Kimble and Crittenden 2005; Crittenden et al. 2006).

Germ cells exit the MZ, initiate meiosis in the transition zone (TZ) and progress through the pachytene stage more proximally. As germ cells migrate proximally they reach the bend region, which is the end of the pachytene stage and where germ cell apoptosis takes place. Surviving syncytial germ cells begin to cellularize and grow larger; after completing the diplotene stage they become arrested at diakinesis. Oocytes are then activated by major sperm protein (MSP), complete meiotic maturation, and are ovulated into the spermatheca, were they are fertilized by sperm. The fertilized embryo is pushed into the uterus and laid through the vulva.

In the presence of food newly hatched *C. elegans* larvae undergo four molts until reaching adulthood in approximately 48 hours (Byerly et al. 1976). Hermaphrodites self fertilize to produce approximately 300 progeny within the first five days of adult hood. Total number of progeny produced, however, is limited by the finite number of self sperm that are produced during their late larval stages of hermaphrodites. By mating with male worms hermaphrodites receive additional sperm, so that sperm is no longer a limiting factor, which increases total reproductive output by over two fold and extends reproductive span from ~5 to ~9 days. In spite of the increase in reproductive activity after mating, hermaphrodites still experience a relatively large post reproductive time period.

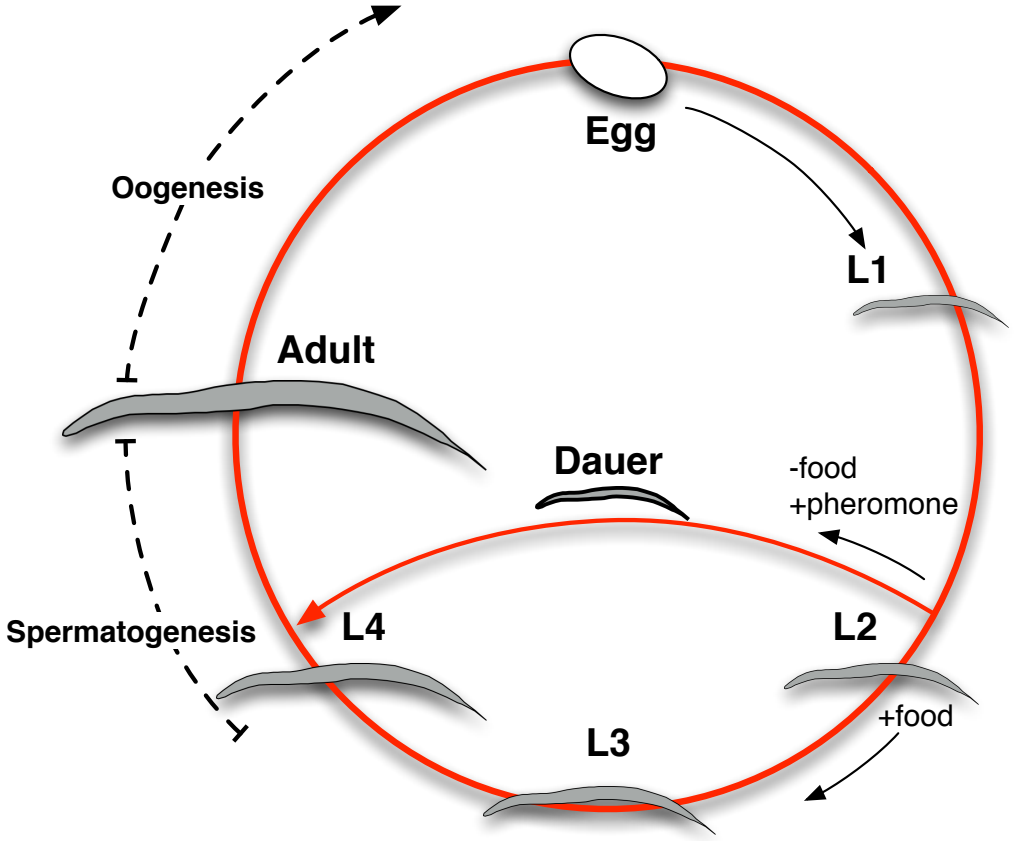



**Figure 1.1.** Life cycle of *Caenorhabditis elegans*. Under well fed conditions newly hatched larvae undergo four larval stages before reaching reproductive adulthood within ~48 h of hatching. Spermatogenesis is restricted to the late larval stages and produces approximately 300 sperm. Upon reaching adulthood the germline switches from spermatogenesis to oogenesis. Life span under standard laboratory culturing conditions is 2-3 weeks (Byerly et al. 1976).

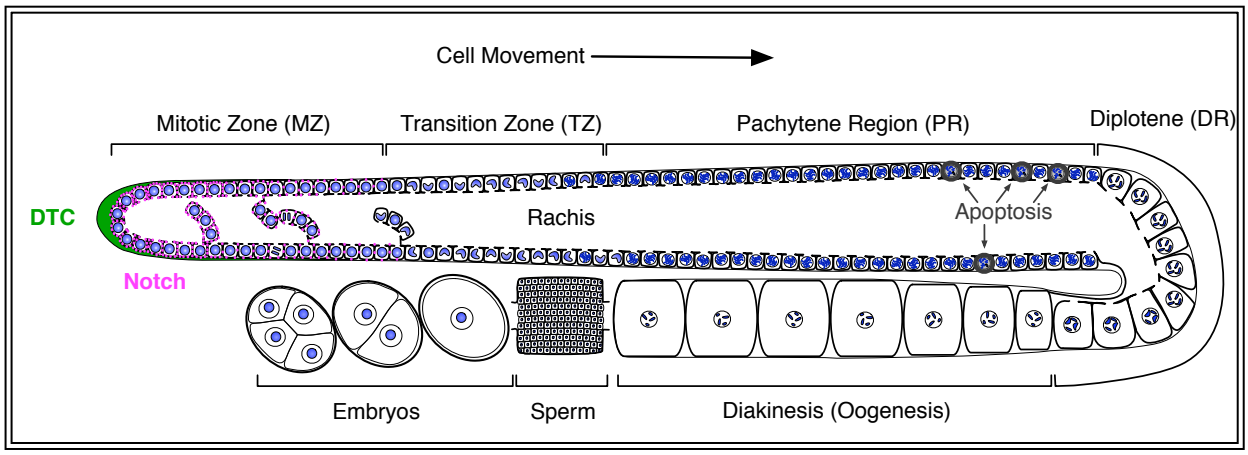



**Figure 1.2.** Gonadal arm of adult, hermaphrodite *C. elegans*
. Stem cells reside at the distal end within the MZ, and cells progressively differentiate as they are displaced from that end. Stem cells are maintained by the distal tip cell, which acts as a stem cell niche by presenting Notch ligands to germ cells.

Reproductive aging in the *C. elegans* hermaphrodite

Prior to my thesis work other studies used the *C. elegans* hermaphrodite as a model system for investigation of reproductive aging (Klass 1977; Hughes et al. 2007; Andux and Ellis 2008). One of the first major findings was that caloric restriction (CR) extends life span and delays reproductive cessation in *C. elegans* hermaphrodites. This has been shown by reducing food availability and by genetic manipulation. The ion channel *eat-2* controls pharyngeal pumping. Mutations in *eat-2* limit food intake, providing a genetic mimic of CR; *eat-2* mutants were initially identified because of their drastic increase in life span, up to 50%, and were later found to also have delayed reproductive aging. Another hallmark of reduced food intake is reduced brood size. While CR studies have greatly increased our understanding of aging, how reproductive aging is regulated remains to be determined. In particular the relationship between food availability, reproductive life span, and brood size requires further investigation.

Hughes et al (2007) suggested that reproductive aging is controlled by a time-dependent mechanism rather than a usage-dependent mechanism. By controlling sperm availability they were able to manipulate reproduction in wild-type and mutant hermaphrodites deficient in sperm production. They found that increasing reproductive output in wild-type hermaphrodites by mating with male worms did not alter late progeny production. In a second experiment they utilized hermaphrodites carrying a mutation in *fog-2,* which encodes a protein required for the formation of self-sperm, to control early progeny production. They demonstrated that reducing early progeny production by mating *fog-2* hermaphrodites with wild-type males at progressively later days did not alter late progeny production. Decreasing reproductive output early in life does not delay reproductive decline; thus reproductive aging may be independent of the number of early progeny production with time being instead the major factor. But questions remain as to whether the reproductive system retains some forms of activity such as germ cell cycling even when worms are not reproducing, and as to how that activity is regulated and may contribute to reproductive aging.

Caloric restriction and longevity

The effects of caloric restriction on mammalian life span was first documented over 80 years ago in the rat *Rattus norvegicus* (McCay et al. 1935). Since then CR has been shown to consistently and effectively delay aging in several model organisms, including the fruit fly *Drosophila melanogaster,* mice *Mus musculus,* and *C. elegans*. More recently a CR study on adult rhesus macaques has been shown to delay the onset of age related diseases and extend life span (Colman et al. 2009). Some of the hallmarks of CR are elevated resistance to

stress, delay in the onset of age related diseases, delay of reproductive senescence, and decrease in brood size. Typically there is an increase in life span as food intake is reduced, but a point is reached when further reduction in food intake results in reduced life span due to starvation. Klass (1977), using *C. elegans*, demonstrated that reducing food availability increased the average life span but also reduced brood size. Hermaphrodites cultured under high food availability have high brood size and a median life span of sixteen days (Gems and Riddle 1996). In contrast hermaphrodites cultured at lower food availability showed reduced brood size and an average life span of twenty six days (Klass 1977). The general conclusion is that CR is correlated with increased life span, increased reproductive span, and decreased brood size.

CR studies have also revealed pathways, common amongst most model organisms, that are involved in life span extension. In *C. elegans* Insulin/IGF-1 signaling (IIS) plays a major role in growth and development as well as CR-induced longevity (Dillin et al. 2002; Antebi 2007; Wang et al. 2014). CR can dramatically increase life span in *C. elegans,* which was shown to be mediated in part by *daf-16*, a *C. elegans* FOXO transcription factor (Greer et al. 2007; Antebi 2007) that is regulated by *aak-2*, a *C. elegans* orthologue of AMPK (Greer et al. 2007). The fact that a broad range of species display a similar response to CR suggest that there is a conserved mechanism controlling longevity that could extend to humans (although there is currently no evidence that reducing food intake extends life span in humans, studies have recently begun to address this question; Holloszy and Fontana 2007).

In addition to CR, intermittent fasting (IF) has received much interest due to its ability to modulate life span (Martin et al. 2006; Honjoh et al. 2009; Uno et al. 2013; Anton and Leeuwenburgh 2013). IF regimens consist of alternating periods of feeding and fasting.

What is interesting about IF is that it can extend life span and in some instances improve health benefits of many species including mammals (Goodrick et al. 1990; Mattson and Wan 2005; Fontana and Klein 2007; Anderson et al. 2009). A recent study applied an IF regimen, alternating between feeding and fasting states every 24 or 48 hours, to adult *C. elegans* and found a 40 and 56% increase in life span, respectively (Honjoh et al. 2009). RHEB-1 was found to mediate life span extension under IF in part by the insulin/insulin growth factor (IGF)-like signaling effector DAF-16 through suppression of DAF-2. This is in contrast to CR life span extension which is regulated by DAF-16 through *aak-2* but not *daf-2*. This shows that IF life span extension works through pathways that only partially overlap with those elicited by CR (Honjoh et al. 2009). Overall, there is strong evidence that it is not just the number of calories but also the temporal patterns of feeding that control longevity. Very little is known however about the impact of IF on reproductive aging. In chapter two of this thesis I investigate changes in the time of reproductive cessation in response to IF.

Mechanisms of reproductive senescence

Stem cells are essential in tissue maintenance and regeneration, thus changes in their capacity to generate fresh cells may greatly impact tissue senescence (Geiger et al. 2014; Saffrey 2014). Tissue aging is thought to occur, in part, because stem cells undergo age related decline of replicative ability (Rando 2006; Sharpless and DePinho 2007; Boyle et al. 2007). In aging *Drosophila* females and males for example GSCs proliferate at a slower rate compared to younger individuals (Boyle et al. 2007). Similarly the activity of

hematopoietic stem cells in mice and neural stem cells in rats become compromised with age (Geiger and Van Zant 2002; Hattiangady and Shetty 2008).

A possible mechanism by which IF delays reproductive cessation is through modulation of specific subcomponents of the *C. elegans* reproductive system . The MZ in *C. elegans* hermaphrodites germline contains stem cells whose descendants differentiate as they leave the MZ. As germlines age, however, the number of germ cells in the MZ decrease suggesting a lowering of germ cell activity (Luo et al. 2010; Crittenden et al. 2006). Proliferation in the MZ has also been shown to be modulated through insulin signaling in response to environmental stimuli (Michaelson et al. 2010; Dalfó et al. 2012). However, prior to my studies only the cell cycle rates of young unmated adult *C. elegans* were reported. Thus it is not known whether stem cell cycling decreases in older age individuals, and whether it increases in response to increased reproductive output. It would be expected that the cell cycle rate adjusts to reproductive demand. In *C. elegans* hermaphrodites germ cell usage can be drastically increased by mating with male worms (Hodgkin and Barnes 1991). Continuous mating can increase reproductive output from 300 to 1000 progeny and extend reproductive activity from six days to twelve compared to unmated hermaphrodites (Hodgkin and Barnes 1991; Hughes et al. 2007). I thus predict that this level of increased progeny production would also cause an increase in germ cell cycling. There is a large gap in the literature with respect to changes in *C. elegans* compartments with age or increased reproductive production.

The first step to determine whether stem cell cycling influences reproductive senescence is to investigate whether cell cycle rates change with progeny production. In chapter three I investigate whether increased reproductive activity increases germ cell

divisions by measuring cell cycle progression in individuals with different levels of reproductive activity. My results provide evidence that hermaphrodite germlines enter a dormant state in which germ cells are not actively cycling. This dormant state is present at higher frequency in unmated hermaphrodites that have depleted the majority of their sperm, suggesting a link between germ cell cycling behavior and reproductive activity. In addition I find that mating induces changes in other germline compartments and germline stem cell niche morphology. These results suggest germ cell behavior is linked to reproductive activity and may play a role in reproductive senescence.

Regulation of germline stem cells

A strong candidate for regulation of germline stem cell behavior is the stem cell niche. The concept of the stem cell niche, a specialized microenvironment that controls stem cell activity, was first proposed over 3 decades ago by Schofield (Schofield 1978). Advances in genetics and imaging techniques have allowed identification of stem cell niches in various systems, which vary in morphology and mechanism (Li and Xie 2005; Morrison and Spradling 2008; Drummond-Barbosa 2008). Niches do not simply provide molecules, they physically contact stem cells to create a unique environment (Hall 1999; Li and Xie 2005). Niches provide spatial and mechanical cues that influence the fate of stem cell daughters. The *Drosophila* fly testis niche is located at the tip of the testis and forms the apical hub. The number of hub cells decreases with age which correlates with the slowing of the GSCs division rate (Wallenfang et al. 2006). The changes in hub cell number not only point to the dynamics of the niche but also suggest that niche architecture contributes to stem cell behavior. Similarly the intestinal crypt niche is composed of Paneth cells The

number of Paneth cells are crucial in defining the niche (Sato et al. 2010). A decrease in the number of Paneth cells results in a decrease in the number of stem cells and the remaining stem cells crowd around the remaining Paneth cells (Sato et al. 2010). Stem cells are believed to compete for Paneth cell surface which suggests that niche architecture plays an important role in stem cell maintenance. These studies have increased the basic understanding of cellular and molecular functions of niches, but how they regulate self-renewal remains largely known.

In the *C. elegans* germline the DTC provides the germline stem cell niche. The DTC main body, the cap, maintains extensive contact with the distal most germ cells (Crittenden et al. 2006; Byrd et al. 2014). A central and intriguing feature of the DTC is its thin tentacle-like processes that extend proximally and contact germ cells further from the cap. DTC processes begin to extend proximally as worms enter adulthood and continue rapid growth in the first three days, some reaching the MZ boundary (Crittenden et al. 2006). Nothing is known about the functional significance of the DTC processes but it has been speculated that they control germ cell behavior. Evidence has provided support for niche architecture as an important component in stem cell regulation.

In chapter four I investigate DTC process growth by first developing a worm immobilization device that allows long term imaging of the DTC with minimal perturbation to germline function. I then show dynamic DTC process growth in both distal and proximal directions that have not been reported previously.

## Chapter 2

## Intermittent fasting can delay reproductive cessation without reducing brood size


Abstract

One of the first aging phenotypes experienced by humans is reproductive decline. Model organisms such as *C. elegans* have been valuable in identifying factors that regulate longevity. Caloric restriction (CR) has been consistently shown to increase longevity in a number of organisms including *C. elegans*. One of the hallmarks of CR is that it delays reproductive cessation but at the expense of brood size. There is growing interest in intermittent fasting (IF). Although studies have shown IF to extend life span, further exploration is needed to determine whether IF can delay reproductive cessation. Here we develop a microfluidic chip with which we can quantify the number of progeny from individual worms to investigate the impact of IF on reproductive cessation. Our results demonstrate that IF delays reproductive cessation in a fasting frequency dependent manner. We also identify a specific IF regimen that extends reproductive span without sacrificing brood size. Our findings suggest that the trade off between reproductive longevity and brood size can be circumvented by IF.

# Introduction

CR is one of the most effective interventions to increase life span and has been observed in a wide range of organisms including worms (Klass 1977; Lee et al. 2006) and more recently in primates (Colman et al. 2009). CR delay of reproductive cessation results in a dramatic decrease in total brood size (Klass 1977; Reznick et al. 2004; Hughes et al. 2007). Similarly IF has been shown to extend life span and health span. Interestingly, IF was recently shown to extend life span in *C. elegans* through signaling pathways that only partially overlap with those elicited through CR (Honjoh et al. 2009). Aging studies have largely focused on somatic aging with much less attention being paid to reproductive aging let alone the impact of IF on reproductive cessation. Since reproductive success is essential for organismal fitness, dissection of reproductive cessation will provide valuable insights into the aging process. Here I characterize the effects of IF on reproductive cessation and determine whether IF can extend reproductive activity without sacrificing brood size.

*C. elegans* longevity can be modulated by CR and IF, making it an ideal model system to investigate the impact food availability on reproductive aging. A major limitation lies in the inability to provide dynamic food patterns. Current worm culture methods control food availability on solid or liquid medium, or use genetic manipulations to limit food intake. Solid medium can be utilized to enforce fasting periods, eliminating the bacterial food source (Kaeberlein et al. 2006), or to enforce CR using serially diluted bacterial lawns (Greer et al. 2007). Initial CR studies in *C. elegans* utilized liquid medium with food availability restricted by dilution of bacteria (Klass 1977). More recently axenic medium and chemically defined liquid medium have been developed and shown to induce a CR-like

phenotype in *C. elegans* (Houthoofd et al. 2002; Szewczyk et al. 2006). The worm mutant *eat-2* has reduced pharyngeal pumping rate and decreased food intake making it a genetic model for CR studies (Avery 1993).

The present culturing methods enable caloric restriction in a static fashion, by providing a constant food concentration throughout a worm's life span. In some instances studies have alternated between two feeding states, well fed and starvation, but on the scale of days. It is feasible to manually switch individuals between fasting and feeding states, on the scale of hours or minutes, within a short time frame (Kopito and Levine 2013; Uno et al. 2013) but impractical to carry out for the duration of the worm reproductive life span, thus making life long IF experiments technically difficult. To overcome these culturing difficulties we developed a microfluidic chip that allows programmed control of dynamic food patterns delivered to individual worms using a dual syringe pump system that alternates between rich and poor food media. To track embryo laying events in real time, I first designed in collaboration with Philip Thomas (Hui laboratory) a microfluidic setup including a worm habitat that provides for immediate removal of laid embryos, and channels that provide for live video capture of these embryos as they exit the device. To ensure that the reproductive cessation I measured was not simply due to sperm depletion, I used young adult hermaphrodites that are mated prior to loading into the microfluidic device. Using this experimental setup, I derived evidence that reproductive activity is sensitive to IF patterns and that reproductive cessation can be delayed without a reduction in brood size.

# Methods

## Design rationale

We designed worm habitat dimensions that would allow 1) free movement of worms in any direction, 2) retention of the parent worm under a continuous media flow, and 3) fast removal of embryos from the habitat. The similarity between adult hermaphrodite width, which can reach up to 80μm, and the dimensions of the embryos, ~50 μm long with a diameter of 30 μm, required careful tuning of device dimensions. The worm habitat and upstream areas are 100 microns high since a lower value leads to constant compression of adults and shortens their life span. The loading channels have cross-sectional area of 80 x100 microns to avoid loading damage to adult worms. The habitat splits into four outlets with cross section 35x50 microns. The sharp drop in cross-sectional area downstream of the habitat prevents adult worms from escaping, and the multiple outlets help to clear the eggs which are frequently laid in groups of two to three. The device contains a single media inlet that splits into 20 individual channels and evenly distributes media into each worm habitat. The channels upstream of the habitats are large enough to facilitate passage of young adult hermaphrodites with minimal constriction.

## Device fabrication

The microfluidic device was made using PDMS soft lithography. In brief, SU-8 3000 (MicroChem) was spun to create the 50 micron layer and exposed through a transparency photomask(CAD/Art) on a Karl Suss MA-56 mask aligner. After the post-exposure bake, the second 50 micron layer was added and exposed using its own photomask. The rest of the

steps in the SU-8 3000 series protocol were followed as normal. These master molds were passivated overnight in a vacuum chamber using 1,1,2,2-tetrahydrooctyl-1-triethoxysilane. 3mm high devices were cast from Sylgard 184 (Dow Corning) and 23 gauge holes were cored using a biopsy punch (Syneo). These devices were cleaned and plasma bonded to standard microscope slides using an air plasma (Harrick). From here straight stainless steel pins (New England Small Tube) were used to connect the tubing to the device ports.

Worm loading and media preparation

*C. elegans* N2 hermaphrodites were cultured on NGM agar as previously described (Brenner 1974), staged for mating 4 hours after L4, and mated with young adult males (L4 + 1 day) for 8 hours. The resulting mated hermaphrodites were loaded into the device inlet port and individually drawn into habitats. To load worms individually, the corresponding outlet tubing to a habitat was lowered to create a gentle suction that would draw a worm straight from the inlet into a habitat. JW1025-1 or HB101 *E. coli* strains were grown overnight to OD 5.0 in Luria-Bertrani medium with kanamycin or streptomycin, respectively. Bacterial pellets were spun down and resuspended in S-medium, pre-filtered through a 5.0 micron filter, and brought to 1e10 cells/mL (OD 10.0). Depending on the type of experiment, one or more syringes filled with different concentrations of *E. coli* and a PTFE stir bar were utilized. Syringes were placed on syringe pumps and in the vicinity of a stir plate so that the media could be constantly stirred. Each syringe then connected to an inline bubble trap through a 2.0 micron syringe filter (IDEX), and from there connected to its own device inlet port using gas permeable silicone tubing (Cole-Parmer).

Long term imaging

Since *C. elegans* development and feeding is sensitive to temperature and blue light (Klass 1977; Ward et al. 2008), the microfluidic chip was imaged on a dissection scope camera with fiber optic red light illumination, and all components were placed inside a 20°C incubator. Syringe flow rates were set to 400 μL per hour (Harvard Apparatus), and switching between different syringes was controlled through a custom script. Media syringes were changed once a day, replacing the syringe, media, 2.0 micron filter, bubble trap, and inlet tubing. At least once per experiment waste was collected from each of the outlet ports, transferred onto agar plates, and checked after 48 hours for the presence of male L4 offspring to verify if a particular worm was mated successfully.

Statistics

Error bars were computed using 83% confidence intervals and stars indicate significance of p-value computed using the Wilcoxon rank sum test through the R project.

## Results

### Device design

Microfluidic devices have been a powerful tool for *in vivo* studies, permitting short term immobilization of *C.elegans* for imaging and physiological analysis (Zeng et al. 2008; Gilleland et al. 2010; Wong et al. 2013). Microfluidic devices have also been utilized for long term culture to track entire life span (Wen et al. 2014; Hulme et al. 2010). Until recently microfluidic devices lacked the ability to assay reproductive capacity of worms. One study introduced a microfluidic chip that confined embryos; however hatching events could only be quantified for only the early portion of the reproductive life span (Kopito and Levine, 2013). Another microfluidic tracking system was able to quantify embryo hatching events for the entirety of the reproductive life span (Li et al. 2014). Here we report a microfluidic device designed to accommodate twenty worms; unlike previously-published devices, it allows tracking of embryo laying events in real-time while applying dynamic food patterns. We designed a habitat with a restriction exit large enough to allow embryo passage but small enough to restrict the parent worm. Every worm habitat was designed with four exit ports that link to a single exit channel that permits progeny collection on a worm by worm basis.

### Automated food delivery and embryo detection in real-time

A major limitation of present worm culture methods is the inability to present dynamic food patterns to worms. Microfluidic chips can provide a platform for automated delivery of IF patterns that would otherwise be impractical (Figure 2.1i). To achieve precise control and delivery of food medium on the scale of minutes we utilized a programmable

dual syringe pump system. The first syringe contains bacterial food source at 1e10 cells $mL^{-1}$ (Figure 2.1ii) while the second syringe contains only S medium (Figure 2.1iii). Each syringe connects to a bubble trap (Figure 2.1iv) which is then connected to the microfluidic chip inlet. Syringe pumps are programmed to alternate between on and off states and dispense media so that each worm habitat experiences a constant flow rate of 20 μL/h throughout the experiment, which efficiently flushes out embryos while allowing parent worms to move freely. Lower flow rates tested, 10 μL/h and 15 μL/h, allowed worm movement but were not sufficient to immediately flush out the embryos. For the control experiment worms were presented with food continuously, using a single syringe containing the desired food concentration.

Analysis of embryo laying events in real-time relies on continuous video acquisition of habitat exit channels to capture embryos as they are flushed out (Figure 2.1vi). Continuous video capture over the reproductive life span results in large video files that would be difficult to analyze in a timely fashion. To minimize the video footage we first restricted video capture to an embryo observation zone (Figure 2.1vii) close to channel exits. We further filtered video frames lacking embryos using an algorithm that detects movement in real time (Figure 2.1viii). Each individual channel in the resulting video frames containing embryo movement was then cropped. Embryo “candidates” were first identified by an automated analysis that also provided a measurement of their area (Figure 2.1x). To ensure high accuracy, embryo candidates were then manually curated (Figure 2.1ix).

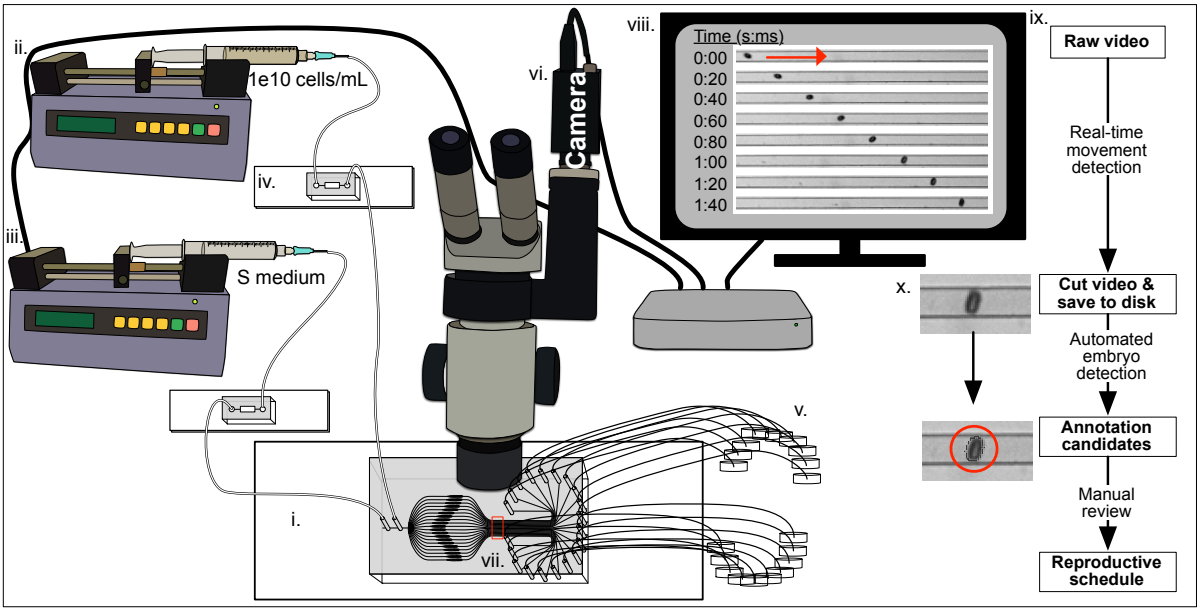



**Figure 2.1**. Food regimen control and embryo laying detection in real-time. (i-viii) Schematic of experimental set-up. (i) Up to 20 mated hermaphrodites are loaded into the microfluidic chip, 1 worm per habitat. (ii-iii) Food delivery to microfluidic chip is controlled using two syringe pumps. The first pump delivers medium with food (1e10 *E. coli* cells/mL in S medium), while the second pump delivers S medium only. Syringe pumps are programmed to alternate between periods of on and off states at desired time intervals. (iv) Silicone tubing connects the syringe to an air-bubble trap which is then connected to the worm microfluidic chip. (v) Media flows continuously, flushing embryos out which are then collected and plated for each channel. (vi-viii) Continuous video recording captures embryos as they travel through observation zone in real-time. (ix-x) Video processing pipeline. Raw footage is processed using a movement detection algorithm; footage containing movement is stored to disk. An automated embryo detection algorithm is applied to the embryo footage; embryo "candidates" undergo manual review to determine the reproductive schedule for each worm.

Optimization of device culture conditions

I determined microfluidic device conditions that would allow reproductive output comparable to worms cultured on solid medium. *C. elegans* reproductive output as well as life span is sensitive to bacterial concentration in liquid medium (Klass 1977). I first set out to determine proper liquid medium food concentration that would allow worms to produce brood size comparable to solid culture. I tested two food concentrations, 1e9 cells $mL^{-1}$ and 1e10 cells $mL^{-1}$, and assayed reproductive activity. Worms cultured with a food concentration of 1e10 cells $mL^{-1}$ produced a brood size comparable to that of worms kept on solid medium and thus fed "*ad libitum"* (Figure 2.2A). HB101 has been established as a high quality food source for worms (Shtonda and Avery 2006), and was initially tested under our microfluidic conditions. Trials with HB101, however, displayed a high prevalence of biofouling within our chip, disrupting media flow and preventing adequate food delivery to worms (Figure 2.2B). I next tested *E. coli* strain JW1025-1, which lacks curli, adhesive fimbrial structures, and is well characterized for reduced biofilm formation (Saldaña et al. 2009). I cultured young mated adult worms in the microfluidic device with a JW1025-1 concentration of 1e10 cells $mL^{-1}$ in S medium and did not detect any biofouling (Figure 2.2B). I did however notice a high incidence of embryo accumulation in several of the worm habitats. A small subset of habitats did not have embryo accumulation which suggested that the habitat exits were large enough to allow embryo passage. I thus hypothesized that embryo stickiness (noted by Cox and Hardin, 2004; see also Figure 2.2C) was the most likely cause of the problem. To test if embryo accumulation was due to embryo sticking I incorporated Tween-80, which has been proven to be non-toxic in various species (Thackaberry et al. 2010), to minimize that stickiness. I tested 0.1% and 0.5% Tween-80,

and found that concentration 0.5% was sufficient to flush out embryos from the worm habitat (Figure 2.2C). I also noticed that the natural exploration behavior of hermaphrodites (Qin and Wheeler 2007) caused their anterior or posterior ends to temporary clog the habitat exit, resulting in significant reduction or complete stoppage of media flow (Figure 2.2D). To remedy worm clogging of the habitat exit we designed a habitat with multiple exit ports. Incorporating four exit ports provides an open passage to maintain media flow in the event that the parent worm temporarily clogs one of the ports.

Since *C. elegans* has been shown to be sensitive to different bacterial food sources with respect to development and reproductive schedule, I next set out to determine if JW1025-1 negatively affected reproductive schedule. To test this I cultured worms on solid medium with either JW1025-1 or HB101 bacteria for at least 3 generations and then tracked the reproductive activity of selfed and mated hermaphrodites (Figure 2.3A-B). The reproductive schedule of selfed hermaphrodites feeding on JW1025-1 was virtually identical to that on HB101. Mated hermaphrodites fed JW1025-1 had an increase in daily brood size later in reproductive life and increased in total brood size. This shows that JW1025-1 does not negatively affect brood size of worms and is a suitable food source.

I next asked whether the presence of Tween-80 in our microfluidic culture significantly affected brood size. Previous findings demonstrated that worm viability is not significantly reduced by Tween concentrations up to 2% (Katiki et al. 2011). To test whether 0.5% Tween-80 has any negative effects on reproductive output I loaded young mated adult hermaphrodites in the microfluidic chip and cultured with or without 0.5% Tween-80 for the entire reproductive life span (Figure 2.3C-D). Total brood size with 0.5% Tween-80 was not significantly different to that without Tween-80.

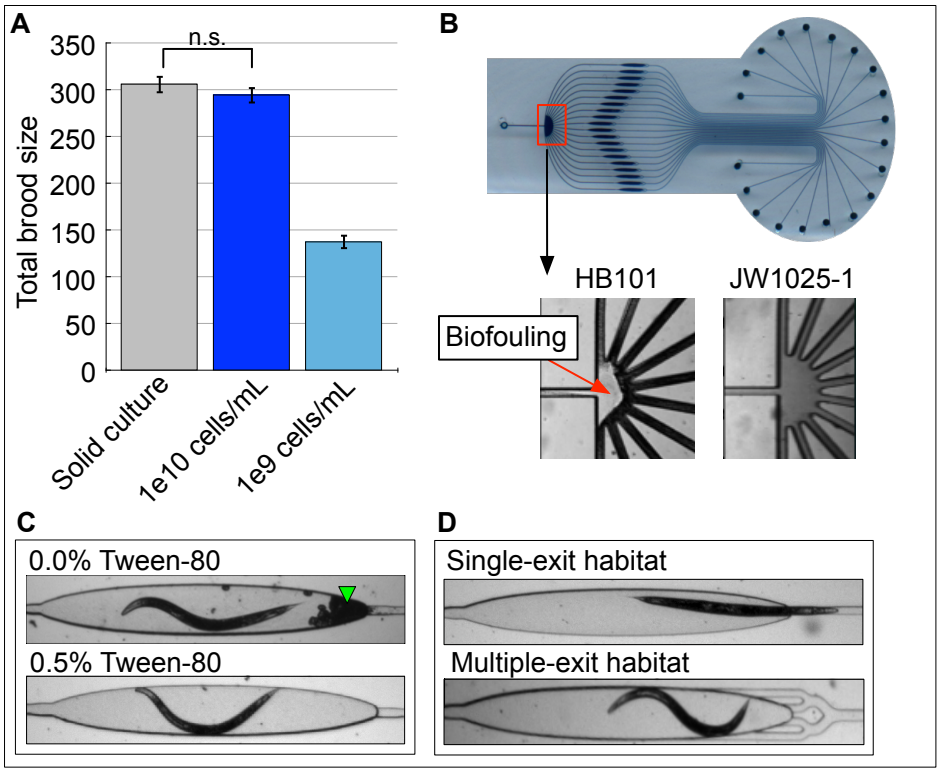



**Figure 2.2**. Microfluidic chip conditions optimized for *C. elegans* culture. (A) Worms cultured in 1e10 cells/mL of food source have a mean total brood size that is comparable to that on solid medium. (B) HB101 in liquid culture produced biofouling in the chip and prevented proper food delivery to worms. (C) Bacterial strain JW1025-1 does not produce biofouling, but a high frequency of embryo clumping in worm habitats (green arrow head) prevented measurement of real-time embryo laying events. Supplementing media with 0.5% Tween-80 eliminates embryo clumping in worm habitats. (D) Natural worm movement periodically plugs the single exit habitat, significantly disrupting media flow. Multiple habitat exit ports prevent plugging of exit channel and allow uninterrupted media flow.

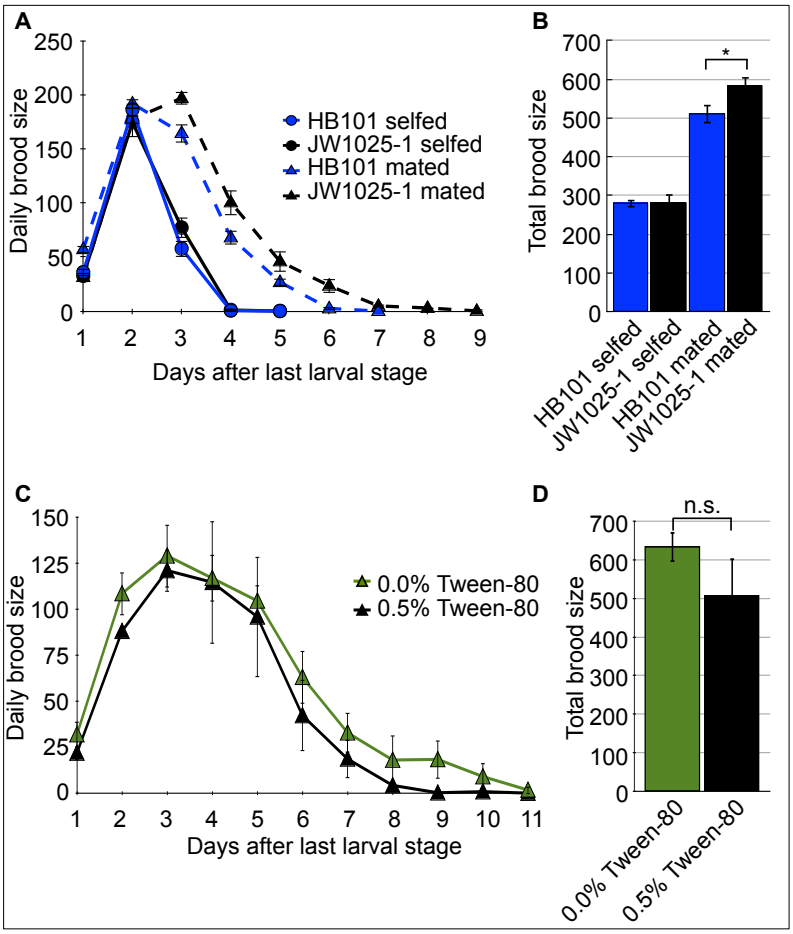



**Figure 2.3**. Brood sizes under different conditions relevant to culture on microfluidic chips. (A-B) Daily and total brood sizes of selfed and mated hermaphrodites are not strongly affected by feeding HB101 or JW1025-1. (C-D) Tween-80 at a concentration of 0.5% does not significantly affect daily and total brood sizes.

IF delays reproductive cessation without decreasing brood size

I next set out to apply an IF regimen to worms in the microfluidic device and track reproductive activity in real-time. Trial experiments with HB101 revealed that IF extends reproductive span to various degrees, in a way that depends on fasting frequency. Interestingly one IF regimen, alternating between six hours of full *E. coli* feeding and one hour of starvation, gave a modest extension of mean reproductive span and produced a brood size that did not significantly differ from control (Figure 2.4A-B). Applying a one hour IF interval significantly increased mean reproductive span by 45% compared to the continuously fed control (Figure 2.4D). This one hour IF interval however significantly reduced total brood size, by 32%. Regimens with fasting every six hours or every ten minutes did not significantly extend reproductive span (Figure 2.4D). I repeated the 6h-1h IF regimen utilizing bacterial strain JW1025-1, which yielded comparable results to those obtained with HB101 (25% extension of reproductive activity and no significant drop in total brood size; Figure 2.5B-C). In addition, analysis of embryo size revealed that IF individuals have a 9% reduction in embryo area compared to the control group (Figure 2.6).

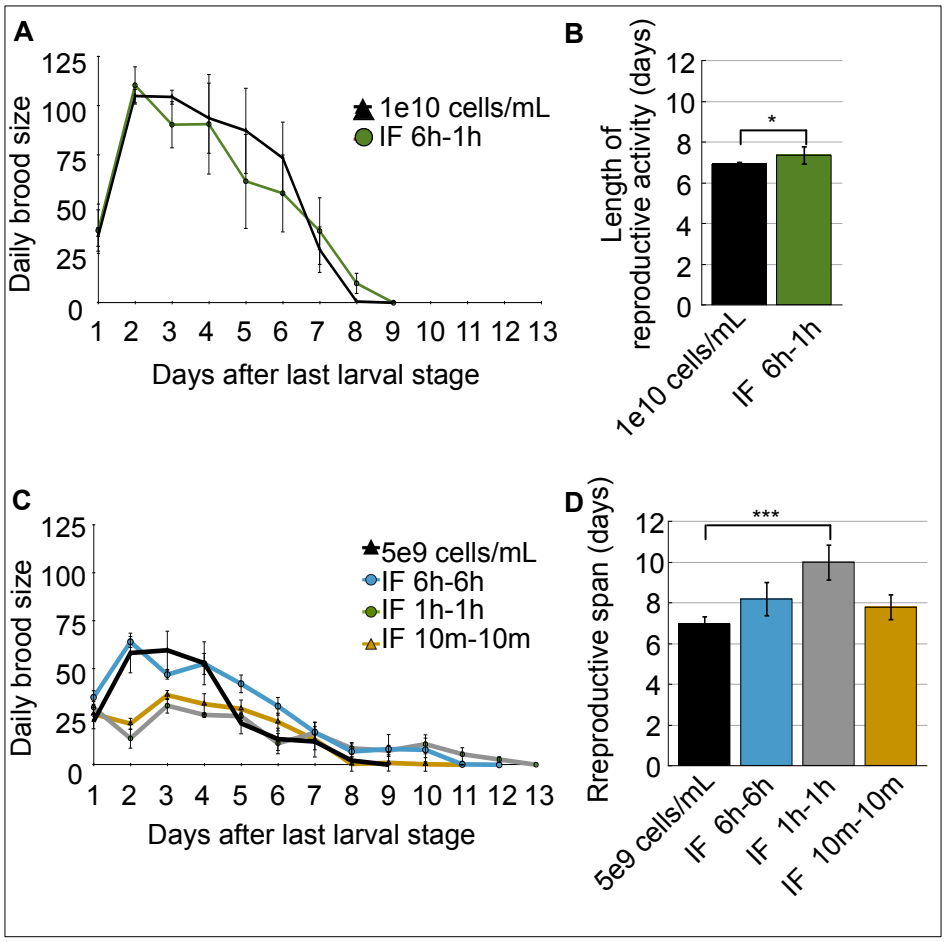



**Figure 2.4**. Daily and total brood sizes of mated hermaphrodites under IF treatments. (A-D) Young adult hermaphrodites were mated and transferred to a chip and either fed continuously (1e10 or 5e9 cells/mL HB101) or given an IF regimen (alternating between 1e10 cells/mL and fasting periods). (B) 6h-1h IF regimen extends reproductive span with no significant change in brood size compared to continuously fed individuals. (C-D) 6h-6h or 10min-10min IF regimens do not significantly increase reproductive span. Interestingly, 1h-1h IF does significantly increase reproductive span.

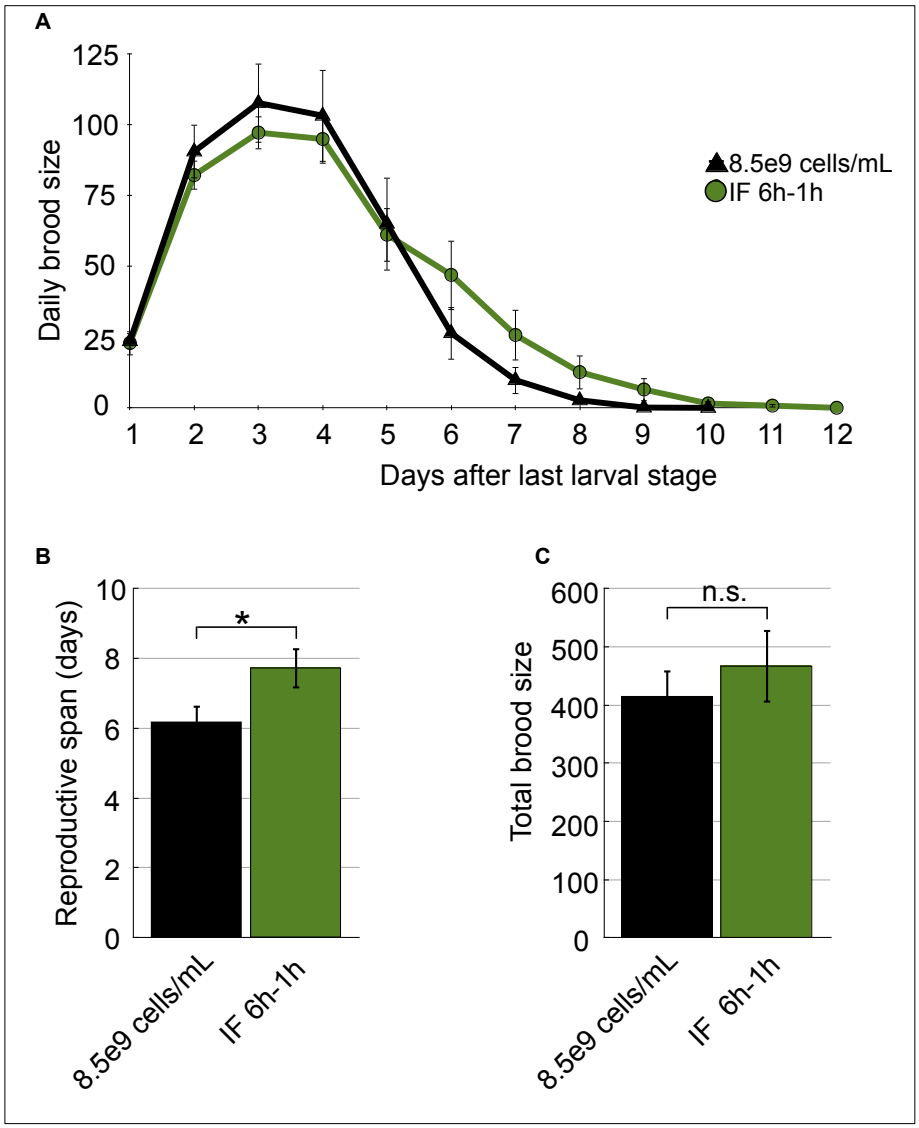



**Figure 2.5**. Reproductive span and brood sizes of hermaphrodites under IF. (A-C) Young adult hermaphrodites were mated and transferred into a chip and were given either an IF regimen (alternating between 6hrs of 1e10 cells/mL JW1025-1 and 1hr of fasting) or a continuous food regimen (8.57e9 cells/mL JW1025-1). (B) Length of reproductive activity is significantly extended, by 25%, when IF regimen is applied. (A-C) Interestingly there is no decrease in daily or total brood size in the IF treatment when compared to the control.

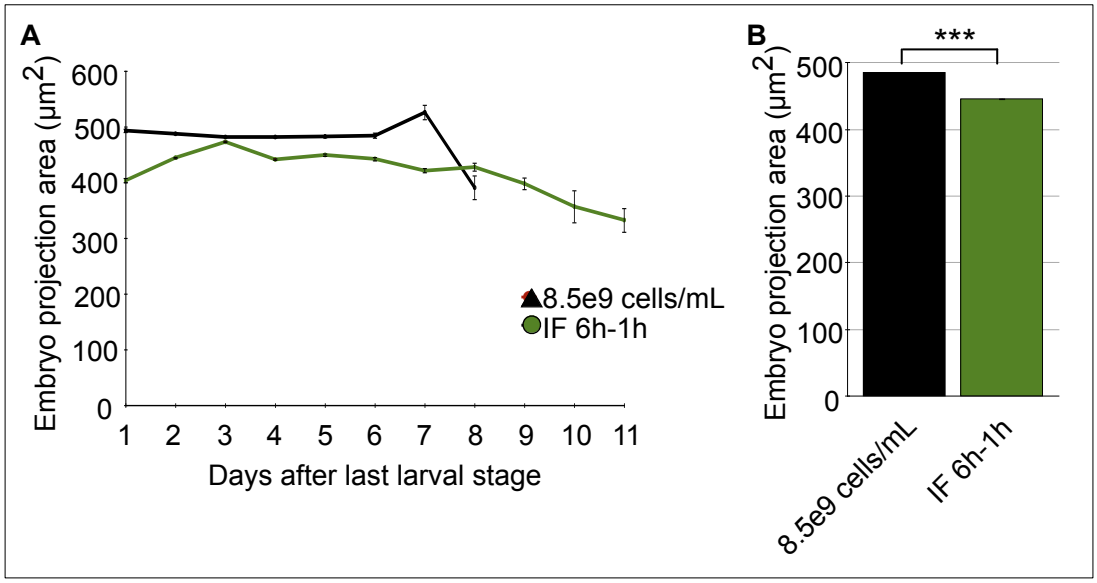



**Figure 2.6**. Embryo size of hermaphrodites under 6h-1h IF. (A-B) Mean embryo size of hermaphrodites given an IF regimen alternating between 6hrs of 1e10 cells/mL JW1025-1and 1hr of fasting is significantly reduced compared to hermaphrodites continuously fed the same average food concentration (8.57e9 cells/mL JW1025-1).

## Discussion

Previous studies have utilized a static food source to modulate reproductive span (Klass 1977; Johnson et al. 1984; Lee et al. 2006). Although it is well understood that reduced food availability delays reproductive aging, it was not known whether IF provides any reproductive life span benefits. Here I introduced a microfluidic platform capable of delivering IF throughout a worm's life span and document reproductive activity in real time. I first established microfluidic chip conditions adequate for long term worm culture and reproductive activity comparable to solid culture conditions. Previously reported microfluidic chips have significantly lower brood size compared to solid food culture conditions (Hulme et al. 2010; Li et al. 2014). A common feature of previous microfluidic devices was that they delivered a solution with food concentration of 1e9 cells $mL^{-1}$. The most recent example being a microfluidic chip which tracked reproductive output of unmated hermaphrodites and reported a lower average brood size, 200 progeny (Li et al. 2014), compared to 280 progeny with our 1e10 cells $mL^{-1}$ culture (Figure 2.2A). A possible reason for the low reproductive output of previously reported microfluidic culture systems is inadequate food concentration: I determined that, under the experimental conditions I used, the optimal food concentration was higher than others have used. This suggests that previous studies are reporting data on worms experience CR, which should be borne in mind when interpreting their findings.

Reducing caloric intake is known to extend reproductive activity of several organisms, but that is generally associated with a drastic decrease in total brood size (Klass 1977; Chippindale et al. 1993; Bishop and Guarente 2007). An important aspect of aging

research is not only to simply extend life span but to maintain normal tissue function. Here I report reproductive benefits afforded by a specific IF regimen. My results show that different IF frequencies can vary reproductive schedule and total reproductive output. By providing IF at one hour intervals we significantly extended reproductive span of mated hermaphrodites, but as in CR it also drastically decreases total brood size. By contrast, a 6h-6h IF regimen did not significantly extend reproductive span nor did it decrease total brood size. This shows that reproductive span can be modulated through not only the total number of calories but the food pattern presented. Most interesting is the result that by applying a one hour fasting period every six hours I was able to delay reproductive cessation without decreasing total brood size of mated hermaphrodites. This is in contrast to previous studies that consistently demonstrate a significant decrease in brood size after CR (Friedman and Johnson 1988; Austad 1989; Reznick et al. 2004; Burger et al. 2007). Extension of reproductive activity without reducing brood size had only been observed in *C. elegans* by genetic manipulation, in a *daf-16; daf-2* background (Hughes et al. 2007), by exposure to ethosuximide (an anticonvulsant that affects neural activity), or by culturing worms at a cold temperature (Evason et al. 2005; Hughes et al. 2007). Similar results have been derived in *Drosophila. D. melanogaster* females carrying a heterozygous loss of function in the gene *Indy* are long lived and produce more offspring over a longer period of time (Rogina et al. 2000). The *C. elegans* homolog of *Indy*, NaDC2, was also shown to extend life span of worms however no reports of extended progeny production (Fei et al. 2003). Flies carrying an EcR mutation also have increased life span, fecundity and fertility (Simon et al. 2003). My results, however, are the first to show extended reproductive span without reduction in brood size in response to food availability. It will be highly interesting to

identify genetic pathways and cellular mechanisms through which IF acts to extend reproductive span.

Reproductive aging in *C. elegans* is also marked by decline in oocyte quality (Andux et al. 2008; Luo et al. 2011). Studies have demonstrated a correlation between oocyte size and oocyte quality: the smaller the egg the higher the increase in embryonic mortality (Andux et al. 2008). My findings show a decrease in embryo size from the IF treatment. Future experiments aim to recover embryos from the chip and determine if there is a decrease in embryo viability. This will aid in characterizing the trade offs between embryo quality and reproductive activity.

My results are of interest with respect to evolutionary theories of aging. The disposable soma theory proposes a metabolic tradeoff between reproduction and aging (Kirkwood 1977). Similarly the antagonistic pleiotropy theory proposes a trade off between early and late reproduction (Williams 1957). My results indicate that the apparent trade off between reproductive aging and brood size may be avoided, which challenges leading aging theories. This apparent paradox may be resolved by considering maternal investment in embryos, which is perhaps reflected in embryo size. Overall, future experiments using the system that I established will enable a number of future studies of *C. elegans* reproductive aging.

## Chapter 3

## Characterization of *C. elegans* germline compartments and reproductive activity.

Abstract

Little is known about the regulation of the germ cell cycling in the mitotic zone (MZ) or of the number of germ cells in different compartments of the *C. elegans* germline. These compartments are defined as the MZ and various zones corresponding to different stages of meiosis: the transition zone (TZ), the pachytene region (PR), and the diplotene region (DR). The MZ contain mitotically cycling germ cells that become displaced proximally into the TZ. Germ cells displaced into the TZ begin differentiation and enter meiotic prophase I, and progress through meiosis until they reach the DR. Hermaphrodites have been shown to experience a decrease in MZ germ cell number with age, which could be due in part to a decrease in germ cell cycling. Because changes in germline stem cell activity may be related to reproductive aging, I first decided to ask how germ cell cycling responds to increased reproductive activity. Specifically, I analyzed germ cell cycle rates in mated hermaphrodites and in unmated, self-fertilizing hermaphrodites at the beginning and middle of their reproductive life span. Increasing reproductive activity by mating hermaphrodites did not significantly increase the cell cycle rate of cells in actively cycling MZ. Instead I found that MZ from mated individuals stay in an active, constantly cycling state, unlike those from unmated individuals that switch back and forth between an actively-cycling state and a dormant state. Since increasing oocyte fertilization alters cycling I then asked if it also affects cell numbers in the different germline compartments. I found that mated individuals displayed reorganization of germline compartments and changes in germ cell niche morphology that are independent of progeny production, and that may be consistent with

anticipation of increased reproductive activity. Overall, I demonstrate the existence of intriguing new regulatory links between reproductive activity and germ cell cycling and differentiation.

## Introduction

To further our understanding of mechanisms by which IF can delay reproductive cessation, I investigated changes in stem cell cycling and in the makeup of compartments of the *C. elegans* reproductive system in response to modulation of reproductive activity. Such changes may be in part responsible for the delay of reproductive cessation observed under some IF conditions. The germline stem cells for example have been shown to influence aging of the soma in *C. elegans* hermaphrodites (Arantes-Oliveira et al. 2001). Consistent with these studies, decreased cycling in older mammals and *Drosophila* implicate a link between stem cell cycling and aging (Rando 2006; Sharpless and DePinho 2007; Boyle et al. 2007). In *C. elegans,* size of the MZ can be modulated through insulin signaling and germline shrinkage can be induced by starvation (Angelo et al. 2009; Seidel and Kimble 2011; Dalfó et al. 2012). However there is a lack of knowledge regarding the changes in the germline makeup and activity with age or in response to environmental changes. It is also unknown how these germline changes are regulated, but the stem cell niche is a likely candidate since it maintains the MZ (Kimble and Crittenden 2007; Dalfó et al. 2012). To investigate the relationship between germ cell cycling and reproductive activity I relied on the fact that reproductive activity can be modulated by mating. *C. elegans* has two naturally-occurring sexes: hermaphrodite and male. Hermaphrodite worms can self-fertilize with the ~300 self sperm produced during the last larval stages and are also able to receive additional sperm by mating with male worms. Mating provides a method to greatly extend hermaphrodite reproductive activity (up to two-fold; Hodgkin and Barnes 1991). The germ cell cycle rate of young adult hermaphrodites has already been determined (Crittenden et

al. 2006; Fox et al. 2011; Chiang et al, 2015), but it is not known whether this rate changes with germline age or reproductive activity (Crittenden et al. 2006; Fox et al. 2011). Aging hermaphrodite germlines show a decrease in the size of their MZ and an increase in the number of apoptotic germ cells, which does suggest significant changes in germ cell behavior (Gumienny et al. 1999; Crittenden et al. 2006).

Here I report that the *C. elegans* MZ can enter a dormant state that is predominant in individuals with low reproductive activity. These results show a relationship between reproductive activity and MZ cell cycling. In addition I find that mating alters the size of germline compartments; specifically, the number of germ cells in the PR is increased while the number of germ cells in the TZ and DR is decreased. This change in germline compartment makeup is independent of oocyte fertilization. I also demonstrate that DTC length increases in response to mating, independently of oocyte fertilization. This germline mating response suggests that there could perhaps be an anticipation mechanism for increased progeny production.

## Methods

### Worm strains and maintenance

Strains used were Bristol N2, *glp-1(q224 ts) III; him-5(e1490) V* (obtained by cross of JK1107, Austin and Kimble, 1987, and CB4088, Hodgkin, et al, 1979), AV125: *spe-8(hc40) I; dpy-4(e1166) IV* (Stanfield and Villeneuve 2006), BA17: *fem-1(hc17) IV* (Ward et al. 1986), JK2868: *qIs56*[*lag-2::gfp*] *V* (Blelloch et al. 1999), CNQ11: *nos-3 gld-3*; qIs56[*lag-2::gfp*] *V*, CNQ12: *gld-2*(*q497*) *gld-1*(q485) I /[hT2[qIs48](*I*;*III*)]; *qIs56*[*lag-2::gfp*] *V*, and CNQ:16 *unc-36*, *glp-1/unc-32,glp-1.* Strains were maintained as previously described (Brenner 1974) using *E. coli* HB101 as a food source. Worms were staged at the mid-L4 stage by visual inspection of the vulva. *glp-1(q224); him-5* males were cultured at the permissive temperature 15$^{o}$C. Hermaphrodite mating was preformed on 35mm diameter plates (CC7672-3340, USA Scientific, Ocala, FL), at a density of 1:3 hermaphrodite to male ratio.

### Immunohistochemistry and imaging of extruded gonads

To label S-phase germ cells we utilized the thymidine analogue 5-Ethynyl-2'-deoxyuridine (EdU; C10337, Life Technologies, Grand Island, NY). To prepare labelled *E. coli*, strain MG1693 was grown in minimal medium (Brenner 1974) supplemented with glucose (Ito and McGhee 1987) and 75mM EdU. Immediately following seeding, plates were stored at 4$^{o}$C. Plates were warmed to 20$^{o}$C prior to use. Young adult worms, 24 hours after the last larval stage (L4), were fed EdU-labeled *E. coli* for 30 minutes in the dark, pulsed, and returned to non-labeled bacteria for experiments that required a chase. Germlines were extruded and fixed and processed as described (Cinquin et al. 2010) using 0.1 µg/ml DAPI to label DNA and 1:200 anti-PH3 antibody (9706, Cell Signaling, Beverly, MA) to label

M-phase cells, and imaged at ~0.3 μm z intervals with LSM 710 or 780 confocal microscopes (Carl Zeiss MicroImaging, Oberkochen, Germany), using a 63x objective.

Whole gonad imaging and scoring germline compartments

Extruded germlines were fixed and stained with 0.1 μg/ml DAPI, 0.16 μM Alexa 594-conjugated Phalloidin (A12381, Life Technologies) and 1:200 anti-PH3 antibody. They were imaged at 0.6 μm z intervals with LSM 710 or 780 confocal microscopes using a 63x objective. Several panels were imaged for each gonadal arm there were subsequently stitched (Preibisch et al. 2009). Germline compartment boundaries were scored along the distal-proximal axis and were based on nuclei morphology from DAPI fluorescent confocal stacks.

Cell cycle analysis

A spatial cytometry pipeline was used to process images of C. *elegans* gonadal arms as described (Chiang et al, 2015a; 2015b).

Statistics

Error bars were computed using 83% confidence intervals and stars indicate significance of p-value computed using the Wilcoxon rank sum test through the R project.

## Results

### Mating increases the average germ cell cycling rate by reducing time spent by MZs in a dormant state.

I first asked whether increasing reproductive activity would induce an increase in germ cell cycling. To measure germ cell cycle length of mated and unmated individuals I conducted an 5-Ethynl-2'-deoxyuridine (EdU) pulse chase experiment. At day one of adulthood mated germlines that labeled with EdU had an average cell cycle length of 4.9 hours, close to the value of 5.49 hours for unmated germlines (Figure 3.1A). I next assayed cell cycle length at day three of adulthood, when mated gonads have significantly higher reproductive activity than unmated gonads, and when I thus expected a substantial difference in cell cycle length between mated and unmated gonads. For gonads that labeled with EdU, the average cell cycle length at day 3 of adulthood was 4.8 hours for mated germlines and 5.8 hours for unmated germlines (Figure 3.1B). The small difference in cell cycle length was not significant, suggesting that the germ cell cycle rate in actively-cycling gonads is independent of elevated reproductive activity.

Surprisingly, at day three ~40% of unmated germlines lacked any EdU incorporation within 1 h of labeling, but all germlines did label after 4 h of continuous EdU labeling (Figure 3.1C). Two potential explanations for the lack of EdU incorporation are that worms are not ingesting the EdU labeled bacteria, or that at any given time germlines are not actively cycling and are instead in a dormant state. An argument against lack of food ingestion is that both mated and unmated hermaphrodites display similar pharynx pumping rates for the first 5 days of adulthood (Pickett et al. 2013). Analysis of cell cycle progression on a germline by germline basis revealed desynchronization in the unmated

population, which was not observed in the mated germline population (Figure 3.1D). These results show that increasing reproductive activity does not significantly increase germ cell cycling rate of active germlines but instead reduces the number of dormant germlines in the population.

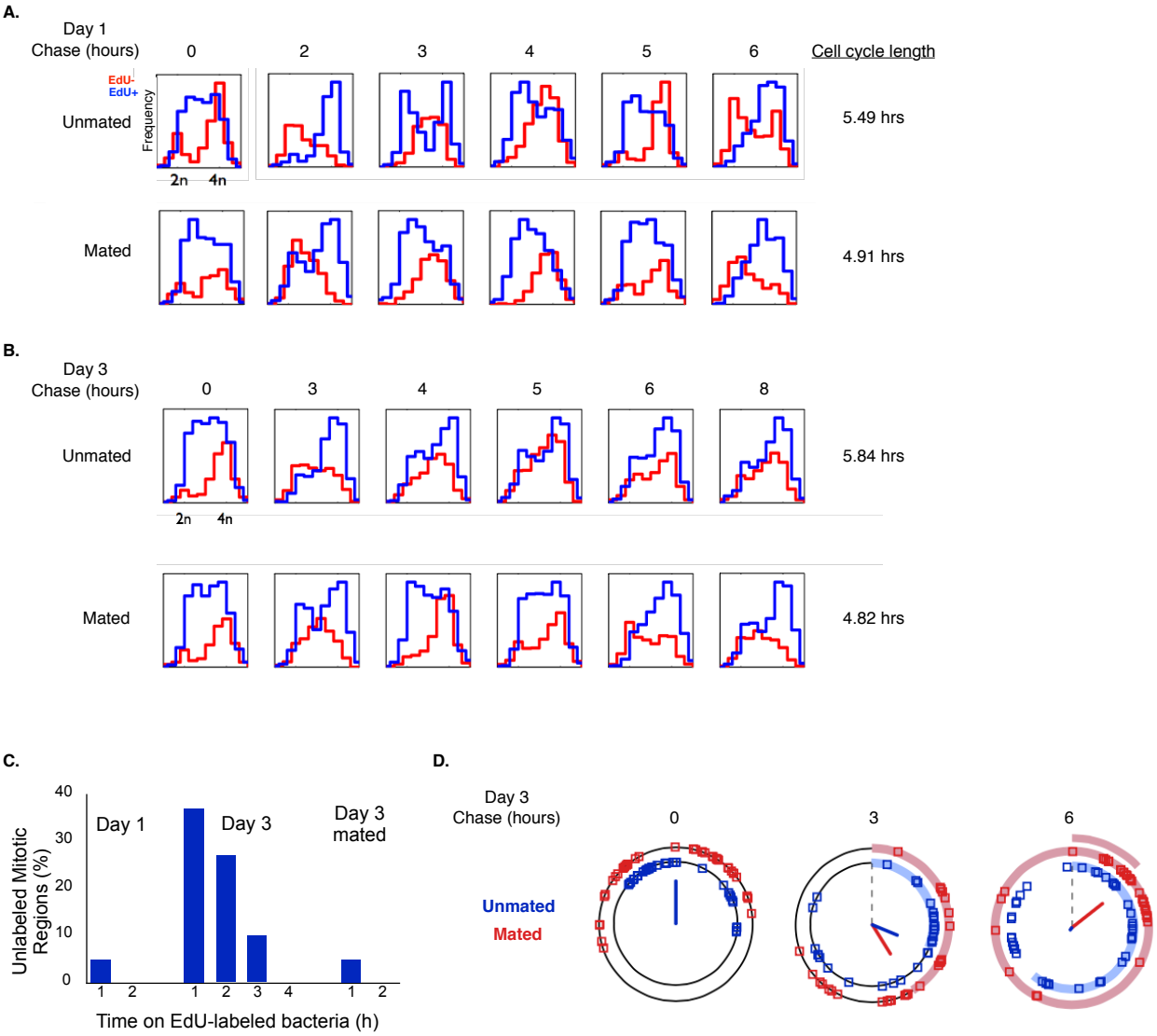



**Figure 3.1.** Germ cell cycle length in mated and unmated gonads. (A-B) I first assayed cycle progression of individual MZs of unmated and mated gonads at day one of adulthood with a 30 minute EdU pulse followed by a range of chase times represented by each column. Each set of histograms shows DNA content of EdU-positive cells (blue) and EdU-negative cells (red). DNA content histograms show cell cycle progression. The original histogram is reconstituted at approximately 5 hours for both unmated and mated populations. (C) Continuous EdU labeling time course for selfed and mated gonads. (D) Day 3 cell cycle progression after EdU pulse-chase is depicted by the position on the circle. The inner circle represents unmated cell cycle progression (blue square) and the outer circle represents mated (red squares). The center line in each circle is the resultant computed as the vector sum of individual gonad positions, which shows average cycle progression (angle of vector) and amount of dispersion between MZs (length of line diminishes with increasing dispersion). MZs from day 3 unmated hermaphrodites lose synchrony, as shown by small magnitude of resultant at later chase times.

Mating alters cell numbers in various germline compartments.

I next set out to determine if increasing reproductive activity altered germline compartment makeup. I scored the total number of germ cells in each gonad compartment for the first four days following the mating period. The total number of germ cells did not significantly differ between mated and unmated hermaphrodites on the first two days, while on the third day there was a marginal increase in the total number of germ cells of in mated hermaphrodites (Figure 3.1A).

Analysis of the germline compartments showed that mating alters the distribution of germ cells more than it does total germ cell number. The number of germ cells in the MZ of mated individuals does not significantly change between days one and four of adulthood in response to mating. In contrast, unmated individuals experience a 37% decrease in the number of MZ germ cells over the first four days of adulthood (Figure 3.1B), which is consistent with previous findings (Crittenden et al. 2006). The distribution of proximal germ cells in mated individuals is significantly altered: the transition zone (TZ) and diplotene region (DR) decrease in cell number while the pachytene region (PR) shows an increase (Figure 3.1C-E). This redistribution of germ cells is observed shortly after mating and is maintained for the first four days of adulthood (Figure 3.1F-J). Interestingly, unmated individuals display a similar germ cell redistribution at day two (Figure 3.1H-J).

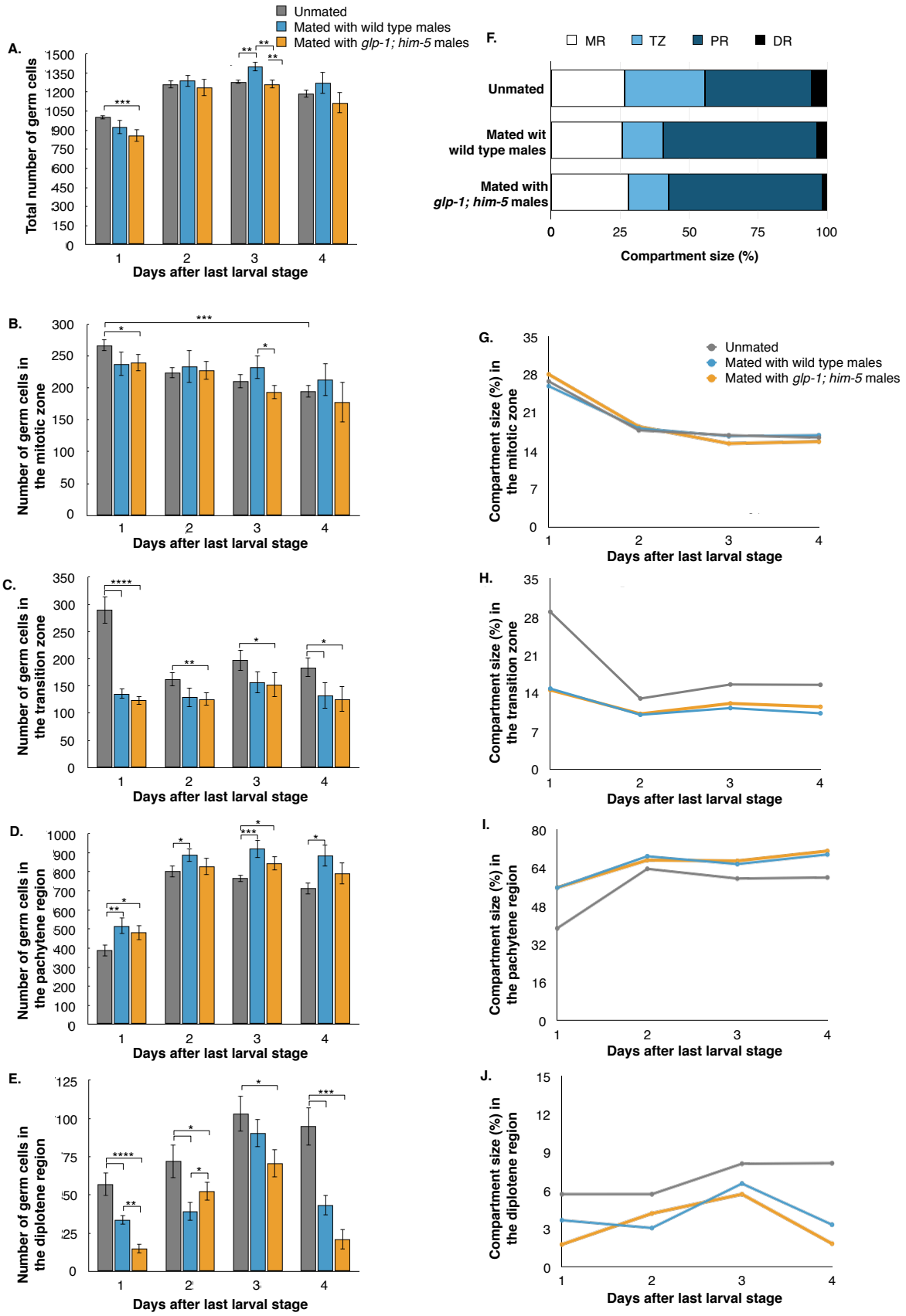

A.
Unmated
Mated with wild type males
Mated with glp-1; him-5 males
Total number of germ cells
Days after last larval stage
B.
Number of germ cells in the mitotic zone
Days after last larval stage
C.
Number of germ cells in the transition zone
Days after last larval stage
D.
Number of germ cells in the pachytene region
Days after last larval stage
E.
Number of germ cells in the diplotene region
Days after last larval stage
F.
MR
TZ
PR
DR
Unmated
Mated wit wild type males
Mated with glp-1; him-5 males
Compartment size (%)
G.
Unmated
Mated with wild type males
Mated with glp-1; him-5 males
Compartment size (%) in the mitotic zone
Days after last larval stage
H.
Compartment size (%) in the transition zone
Days after last larval stage
I.
Compartment size (%) in the pachytene region
Days after last larval stage
J.
Compartment size (%) in the diplotene region
Days after last larval stage

**Figure 3.2.** Germline compartment organization in mated and virgin hermaphrodites. To assay the germline response to mating, hermaphrodite worms were mated with either wild-type or *glp-1(q224); him-5* male worms during the first day of adulthood. Compartment boundaries were discerned based on DNA morphology. (A) On the third day of adulthood, mated hermaphrodites have significantly more total germ cells than unmated hermaphrodites. (B) The number of cells in the MZ of hermaphrodites mated with wild-type males remains constant throughout the first four days; this is in contrast to selfed hermaphrodites, which show a significant decrease by day 4. (C) Mating substantially decreases the number of TZ cells at day one of adulthood. (D) Mating increases the number of PR cells. (E) DR cell number in mated worms is lower across all time points and never reaches selfed levels. (F-J) Mating with wild-type or with *glp-1(q224); him-5* sperm-less males has virtually identical effects on the size of germline compartments.

Mating with sterile males leads to germline compartment reorganization

I next aimed to determine whether germline compartment reorganization observed in mated hermaphrodites is a result of an increase in oocyte fertilization or a response to male presence. To differentiate between increased oocyte fertilization and male presence I utilized *glp-1(q224); him-5* sterile males. The *q224* allele provides a temperature sensitive mutation in Notch; males shifted during development to the restrictive temperature produce only 4-8 sperm (Austin and Kimble 1987). To confirm sterility of *glp-1(q224); him-5* males I utilized *spe-8; dpy-4* hermaphrodites, which have a dumpy phenotype and are unable to activate their own sperm. The use of this strain allows discrimination of embryo production as a result of fertilization by male sperm from embryo production as a result of fertilization by hermaphrodite self-sperm activated by transferred male seminal fluid. Mating of *spe-8; dpy-4* hermaphrodites with *glp-1(q224); him-5* males produced progeny with dumpy phenotype, indicating that functional seminal fluid and not sperm is transferred by *glp-1(q224); him-5* males.

To test wether germ cell redistribution is dependent on increased oocyte fertilization I mated hermaphrodites with *glp-1(q224); him-5* males and assayed germline compartment

makeup immediately after the mating period and subsequently every 24 h for 3 days. The germline compartment reorganization observed in hermaphrodites mated with wild-type males was also detected in hermaphrodites mated with *glp-1(q224); him-5* sterile males (Figure 3.2). For example, there was a significant decrease in MZ germ cells in germlines mated with *glp-1(q224); him-5* sterile males compared to unmated germlines, which is virtually identical to the decrease seen after mating with wild-type males (Figure 3.2B). Similarly there was a decrease in the number of TZ and DR germ cells and an increase in PR germ cells (Figure 3.2C-E). The germ cell distribution between compartments in gonads of hermaphrodites mated with *glp-1* males followed a profile that was distinct to unmated gonads through the first four days (Figure 3.2G-J). These results suggest that germline compartment reorganization is not dependent on increased oocyte fertilization.

Since redistribution of germ cells between compartments is observed at day one of adulthood in mated individuals, before reproductive activity differs between mated and unmated hermaphrodites, I hypothesized that germlines may be able to anticipate and prime germ cells for increased usage. I thus set out to determine whether anticipation of increased reproductive activity would be advantageous by increasing total brood size. To test whether mating anticipation affects reproductive activity I mated hermaphrodites with either *glp-1(q224); him-5* sterile males or *fem-1(hc17ts)* sterile males and tracked reproductive output. *fem-1(hc17ts)* males are temperature sensitive mutants and fail to develop a vas deferens when raised at the non-permissive temperature, preventing transfer of any sperm or fluid (Nelson, et al. 1978). *fem-1(hc17ts)* males have been reported to display rudimentary mating behavior (Hodgkin and Doniach 1997). Through observations of mating encounters I determined that *fem-1(hc17ts)* males probe hermaphrodites but fail

to maintain position over the vulva for successful copulation. I first mated hermaphrodites with sterile males at day one of adulthood. I then removed hermaphrodites from sterile males and transferred them to a fresh plate, from which point I scored daily reproductive activity. Mating with sterile males does not provide additional sperm, thus I did not expect an increase in brood size. However, I observed a significant decrease at day two in reproduction of hermaphrodites mated with sterile males (Figure 3.3).

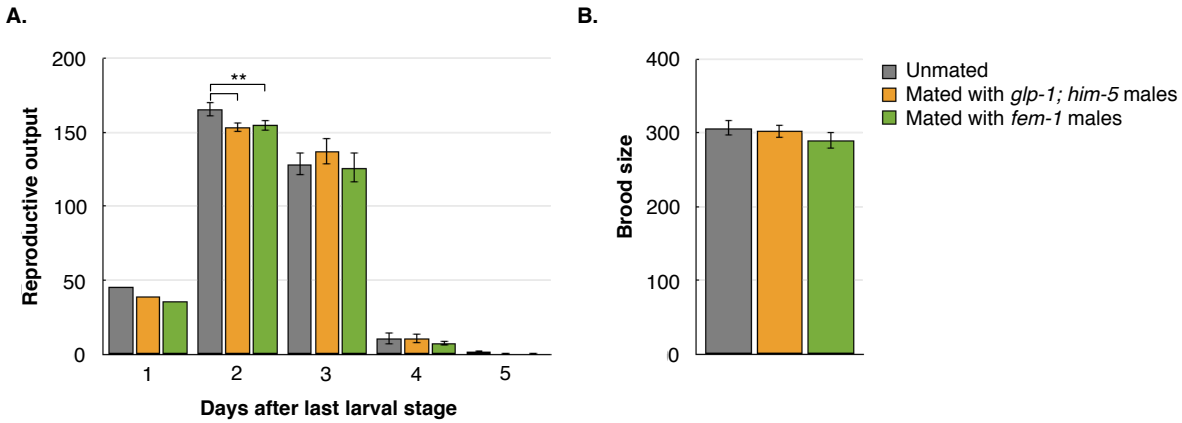
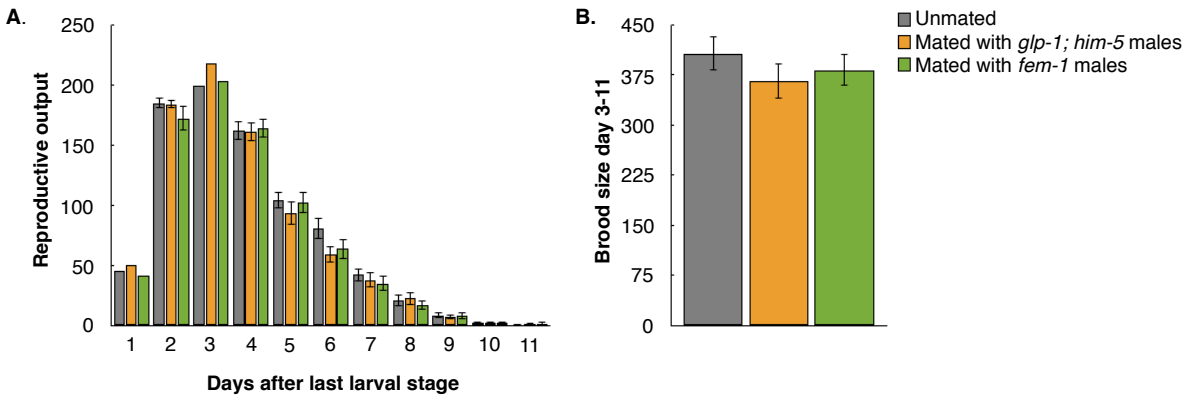



**Figure 3.3.** Daily and total brood size of hermaphrodites mated with sterile males. Embryo laying was lower at day two of adulthood, but total brood size was not significantly different, suggesting that mating with sterile males may slightly delay reproduction.

I next tested wether pre-mating with sterile males could maximize reproductive output of hermaphrodites mated late in reproductive life with fertile males. I pre-mated hermaphrodites with sterile males at day one of adulthood and with wild-type males at day three. Hermaphrodites not exposed to sterile males but mated with wild-type males at day three served as the control group. I tracked reproductive output daily until the end of reproduction. Pre-mating with sterile males made no detectable difference (Figure 3.4).




DTC process extension is sensitive to mating.

The DTC develops several processes that extend proximally, and whose length increases with age (Crittenden et al. 2006) and is a likely regulator of germ cell behavior. Because mating alters germline behavior I next asked whether the length of the longest DTC process is also altered by mating. I utilized hermaphrodites expressing *lag-2::gfp*, which labels DTC processes, and mated them with either wild-type males or sterile males at day one of adulthood and tracked DTC process growth over a four day time course. Hermaphrodites mated with wild-type males or *glp-1; him-5* sterile males did not display significantly altered length of the longest DTC process compared to unmated hermaphrodites (Figure 3.5A). Hermaphrodites mated with *fem-1(hc17ts)* sterile males however showed increased length (Figure 3.5A). Unlike *glp-1; him-5* males, *fem-1(hc17ts)* males do not transfer seminal fluid. Thus DTC process growth is extended independently of increased oocyte fertilization and seminal fluid.

To further investigate how mating influences DTC process length, and to complement my results derived using sterile males, I utilized hermaphrodites whose germlines do not produce gametes and thus do not increase reproductive activity in

response to mating. Hermaphrodites carrying *gld-2 gld-1* or *gld-3 nos-3* pairs of mutations exhibit ectopic germ cell proliferation and do not produce gametes (Francis et al. 1995; Kadyk and Kimble 1998; Kraemer et al. 1999; Eckmann et al. 2002; Eckmann et al. 2004). Similarly *glp-1(gf)*, a gain of function Notch mutant, causes ectopic germ cell proliferation and little to no germ cell differentiation (Berry et al. 1997). I first measured DTC process length of each mutant strain at days one and two of adulthood, which is when the greatest process growth is expected. DTC process length in *gld-2 gld-1* and *gld-3 nos-3* individuals was substantially lower compared to wild-type (Figure 3.5. B). From days one to three however, *gld-3 nos-3* showed a modest process extension, while *gld-2 gld-1* process length appeared to plateau after day one. Surprisingly, DTC process length of *glp-1(gf)* tumors was not significantly different from wild-type (Figure 3.5. B).

I then tested whether DTC process length of *gld-2 gld-1* double mutants is extended by mating with wild-type males during the first day of adulthood. Mating did lead to an increase the length of the longest process (Figure 3.5C), similar to the DTC process length extension seen in wild-type hermaphrodites mated with *fem-1(hc17ts)* males (Figure 3.5A). These results suggest that length of the longest process does not depend on reproductive activity but is responsive to external stimulus. Both *gld-2 gld-1* and *glp-1(gf)* germlines contain a higher number of distal M-phase germ cells compared to *gld-3 nos-3,* suggesting differences in germ cell cycling. The fact that the three different tumorous germlines have different DTC process profiles also suggest that germ cell behavior plays a role in determining DTC process length.

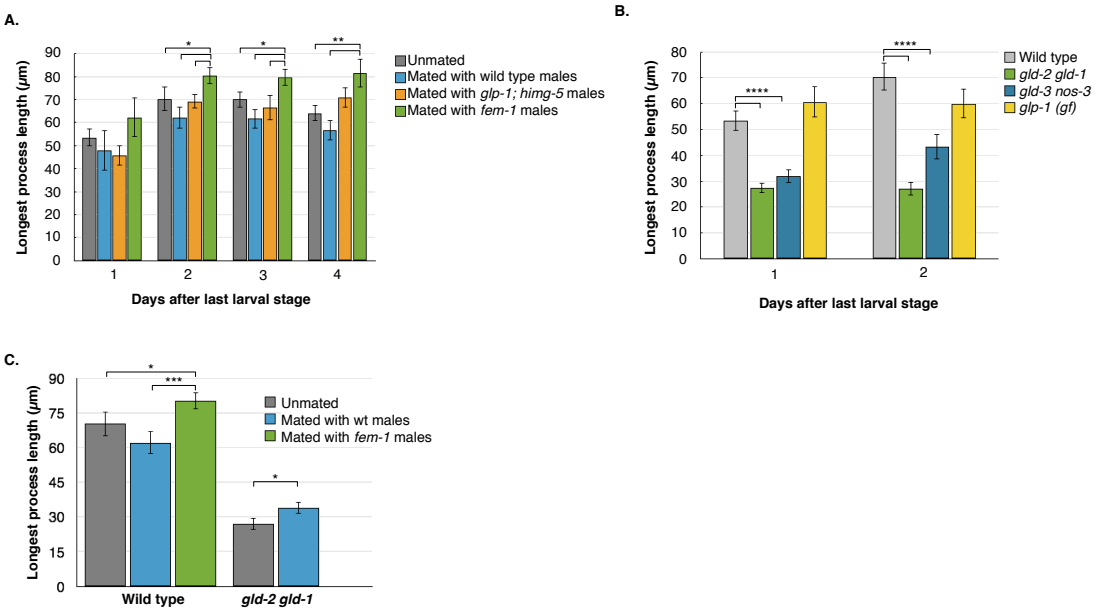



**Figure 3.5.** Length of the longest DTC process along the distal-proximal axis of the germline. The physical DTC process length was measured along the distal proximal axis of the germline. To test whether mating influenced DTC morphology, hermaphrodites were mated with male worms during the first day of adulthood. (A) DTC process length in wild-type hermaphrodites that were selfed or mated with wild-type or mutant males. (B) DTC process length in tumorous mutants. (C) DTC process length response to mating in wild-type or tumorous backgrounds.

## Discussion

My findings add to the understanding of the *C. elegans* germline cycling and compartment regulation. First I demonstrate that actively cycling germlines of mated or unmated individuals do not differ in cycling rate. Instead what I find is that germlines stochastically enter a dormant state whose occupancy is linked to reproductive activity. Populations with low reproductive activity (unmated hermaphrodites at day 3 of adulthood) have a high percentage of dormant germlines. Populations with high reproductive activity (unmated hermaphrodites at day 1 and mated hermaphrodites at day 3) have a low percentage of dormant germlines. These results suggest a link between germ cell cycling activity and reproductive activity which may play a role in reproductive senescence.

I found that hermaphrodite germlines undergo a compartment reorganization after mating with male worms. This germline compartment reorganization results in a higher allocation of germ cells to the pachytene region. Compartment reorganization can also be induced by mating hermaphrodites with *glp-1(q224); him-5* sterile males, which shows that it is independent of male sperm, and is accompanied by a reduction in the reproductive schedule at day two of adulthood. Together these results suggest a response mechanism that may prime the germline for increased fertilization.

Hermaphrodites can detect and respond to male presence through physical contact (Morsci et al. 2011). Since *glp-1(q224); him-5* sterile males transfer seminal fluid the next logical question to ask is whether germline compartment reorganization is dependent on seminal fluid or whether male presence is sufficient to elicit compartment reorganization.

This could be tested in future work, by mating hermaphrodites with *fem-1(hc17ts)* sterile males and asking if there is a change in germline makeup.

Pachytene is where germ cells spend the majority of their time and where over half of the germ cells undergo apoptosis. It is known that the primary source of oocyte growth is the cytoplasmic streaming that originates from pachytene-stage germ cells that are believed to function as nurse cells (Wolke et al. 2007; Nadarajan et al. 2009). One potential explanation for an increase in pachytene cells in mated worms is to provide additional nurse cells to sustain production of a larger number of oocytes. Mechanistically, mated gonad compartment reorganization could be a result of germ cells progressing through pachytene at a slower rate and thus exiting the PR at a low frequency. One argument against delayed progression through pachytene is that mated individuals have comparable embryo laying rate to unmated individuals in the first two days; thus differentiated germ cells are exiting the germline at similar rates. In addition previous studies demonstrate that sperm promote ovulation and oocyte maturation (McCarter et al. 1999; Miller et al. 2001; Nadarajan et al. 2009) and progression of germ cells through pachytene has been shown to be regulated by the presence of sperm (Jaramillo-Lambert et al. 2007). These results make it unlikely that mated gonads, which are supplemented with a large amount of sperm, delay germ cell progression through pachytene. Another possibility is that levels of apoptosis decrease, leading to an increase in the total number of germ cells in the germline, specifically in pachytene. One would predict that apoptosis levels would either remain constant or increase since there is strong evidence that apoptosis is important for maintaining oocyte quality in *C. elegans* and other species as well (Cavaliere et al. 1998;

Andux and Ellis 2008). This could be tested in future work using standard assays for apoptosis.

The DTC has been shown to play a role in regulating germ cell proliferation in response to external stimuli (Dalfó et al. 2012). Here I show that the extent over which the DTC makes contact with germ cells is sensitive to mating. Hermaphrodites display enhanced DTC process growth if mated with sterile males. Tumorous germlines lacking a combination of cell differentiation factors have reduced DTC process length but the growth can be enhanced if mated by male worms. One possibility is that the male presence alters the state of the germline which then increases DTC growth; alternatively, mating may alter DTC growth through somatic signaling. In any case, these results show that DTC morphology is sensitive to the state of the germline and thus suggest feedback loop from germ cells to its niche. Further investigation is required to determine the mechanisms through with DTC processes are extended, which will provide insight into their functional significance.

# Chapter 4

## Active infiltration of mitotic zone by its stem cell niche


Abstract

Stem cells niches are increasingly recognized as dynamic environments that play a key role in transducing signals that allow an organism to exert control on its stem cells. Live imaging of stem cell niches in their in vivo setting is thus of high interest to dissect stem cell controls. Here we report a new microfluidic design that is highly amenable to dissemination in biology laboratories that have no microfluidics expertise. This design has allowed us to perform the first time lapse imaging of the *C. elegans* germline stem cell niche. Our results show that this niche is strikingly dynamic, and that morphological changes that take place during development are the result of a highly active process. These results lay the foundation for future studies to dissect molecular mechanisms by which stem cell niche morphology is modulated, and by which niche morphology controls stem cell behavior.

## Introduction

Regulation of fundamental stem cell behaviors—cycling and self-renewal—is a topic of high interest for understanding development and for making therapeutic use of stem cells. Stem cells are found in niches, whose makeup varies between organs and species (Losick et al. 2011). The crucial role of these niches in stem cell maintenance is well established (Kimble 1981), but the sophistication of stem cell regulation goes well beyond controlling their numbers by delimiting a fixed volume in which they can exist. Numerous lines of evidence suggest stem cell niches to be dynamic environments that allow for control not only of stem cell number (Toledano et al. 2012), but also of the behavior of stem cells and their descendants (Raaijmakers et al. 2010)—perhaps in a way that allows for tight coordination between various stem cell types (Lander et al. 2012), and that allows for control by the nervous system (Méndez-Ferrer et al. 2008). Characterizing niche dynamics will thus be a key step in dissecting in vivo extrinsic controls of stem cells and their descendants.

The *C. elegans* germline stem cell niche is a powerful model system to study niche dynamics. Indeed, this niche is well defined and can be imaged live in intact animals. It is made of a single somatic cell, the distal tip cell (DTC), which acts as a niche at least in part by presenting germline stem cells with Notch ligands. The DTC is located at the distal end of the gonadal arm, which essentially forms a tube of germ cells that are displaced in a distal to proximal direction as they proceed through differentiation (Figure 4.1). Notch signaling in germ cells is required for maintenance of stem cells and the mitotic zone, as shown by premature germ cell differentiation when the DTC is ablated (Kimble 1981) or when the Notch receptor is genetically inactivated (Austin et al. 1989). As hermaphroditic larvae go

through the last larval stage L4 and enter adulthood, the DTC extends striking long filopodia-like structures called ‘‘processes’’ that carry at least one Notch ligand (Fitzgerald and Greenwald 1995; Hall 1999; Crittenden et al. 2006). The precise role of these processes remains unaddressed, but their similarity with structures found in other species and other organs (Cinquin 2009) strongly suggests that they are of functional significance.

The *C. elegans* germline stem cell niche is furthermore a relevant model system to study interactions between niches and their host organism. Indeed, expression of Notch ligand by the DTC has been shown to be regulated by extrinsic TGF-beta signaling, possibly under neuronal control (Park et al. 2010; Dalfó et al. 2012). DTC morphology is also altered during adult reproductive diapause (Angelo and Van Gilst 2009), suggesting a potential mechanism by which a small pool of stem cells is kept in a quiescent state for future regeneration. Control of Notch ligand levels on DTC processes and control of DTC process morphology could thus provide for coordination between germline stem cells and the rest of the organism.

But despite the high relevance of the DTC as a model dynamic stem cell niche and despite its striking morphology, little is known about the dynamics of its morphology. Although it is known that the processes start extending when hermaphrodites reach adulthood and continue doing so over the course of at least 3 days (Crittenden et al. 2006), it is not known whether this extension is the result of active DTC growth, or merely the result of DTC membrane being passively dragged by proliferating germ cells as they are being displaced in a distal to proximal direction. Furthermore nothing is known about its control of the MZ size or feedback of germ cells controls DTC morphology. Arguing for passive growth is the adhesion often observed between stem cells and their niches

(Marthiens et al. 2010), the close association of the DTC with the distal-most germ cells (Hall 1999; Crittenden et al. 2006), and the apparent internalization of DTC content by germ cells (Henderson et al. 1994). Arguing for active growth is that the extension of DTC processes takes place in hermaphrodites but not in males (Morgan et al. 2010), at a specific stage of development. Resolving this question would be an important first step in addressing the role of DTC morphology as a control of germline stem cells.

The lack of current data on DTC morphology results in large part from limitations in tools to perform minimally-disruptive live imaging of *C. elegans*. Imaging of fixed samples only makes it possible to study population-level behavior; stochasticity and variability between individuals can mask dynamics, especially on a short time scale. Live imaging can be performed with permanent immobilization of worms, but such immobilization results in strong defects in germ cell proliferation (our unpublished observations). While methods exist to reversibly immobilize worms, they carry biological or technical drawbacks. Biological drawbacks include non-immediate recovery or other perturbation of animal physiology (as for anesthesia, reversible cooling Chung et al. 2008), or exposure to $CO_2$ (Chokshi et al. 2009), or repetitive transfers of worms that can lead to damage (as for agarose bead immobilization). Technical drawbacks exist for methods that rely on worm confinement based on deformable membranes, suction channels or tapered channels (Rohde et al. 2007; Hulme et al. 2007; Allen et al. 2008), or are based on temperature-controlled transition of pluronic acid between liquid and gel phases (Krajniak and Lu 2010). These devices are based on complex, multi-layered microfluidic designs that require specialized equipment and expertise to build, and/or external pneumatic, hydraulic, or temperature control systems that also require specialized expertise to use. We thus

designed a simple device, the manufacture and use of which are accessible to biology labs with no ad hoc technical expertise. This device allowed us to reversibly immobilize worms under nearly-physiological conditions, and to characterize the dynamics of DTC processes over short time intervals in individual worms.

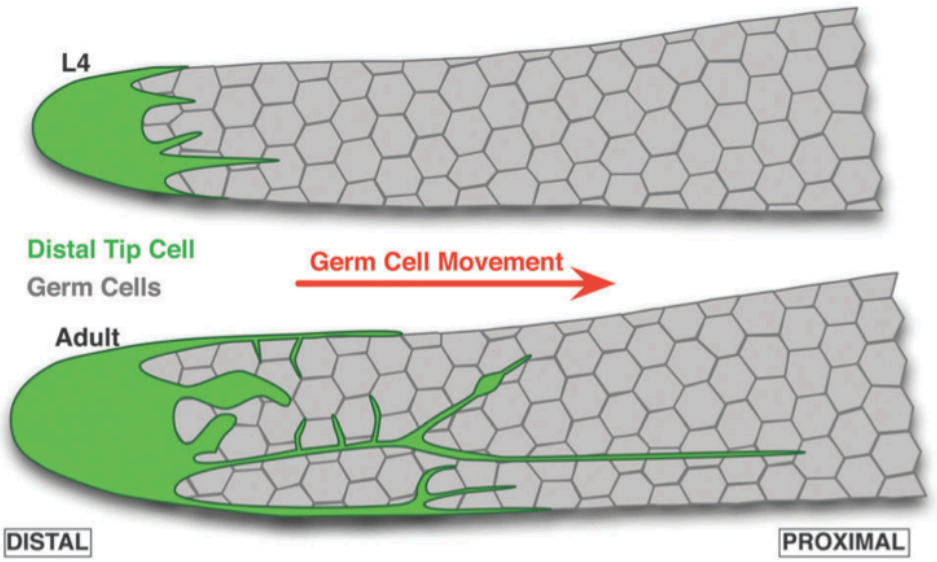



**Figure 4.1.** The *C. elegans* gonad forms a tube that is closed at the distal end, where stem cells are located, and open at the proximal end (not shown) from which differentiated gametes exit. The Distal Tip Cell (DTC) acts as a stem niche, at least in part by expressing Notch ligands. The DTC extends processes that start growing from the last larval stage (L4). These processes tightly intercalate between germ cells, especially in the distal region where stem cells are located, and in mature adults extend past the mitotic zone in which cells are proliferating.

# Methods

## Worm handling

The strain used was JK2868 (qIs56[*lag-2::gfp*] V) which was maintained at 20C outside of the microfluidic device as described (Brenner 1974) using E. coli HB101 as a food source. Loosely synchronized populations were obtained by isolating young adults from unsynchronized populations; devices were loaded with descendants that were at the L4 stage (as identified by visual inspection of vulval shape) at the desired time. The microfluidic device was operated with S medium supplemented with E. coli HB101 concentrated to $10^9$–$10^{10}$ mL; constant gravity flow of the medium through the device guarded against local depletion of bacteria. During time course imaging, worms were immobilized for a maximum of 2 minutes at each time point. In between time points, the channels were expanded by releasing compression, allowing the worms to move freely. Both pharynx pumping and head foraging movements were observed in released worms. We did not assay whether worms actively feed during the short periods of immobilization, but normal reproductive output, mitotic index, and DTC growth suggest that potential nutrient deprivation during immobilization is not detrimental.

Gonad extrusion and staining was performed as described (Crittenden and Kimble 2014). Mitotic index was computed as the ratio of pH3-positive cells to the total number of cells counted in the mitotic zone. Live imaging of permanently-immobilized worms reported in Figure 4.5 was performed using agarose and polystyrene beads.

Progeny counts

Hermaphrodites were passaged every day to fresh plates, until the end of reproductive activity. Plates on which embryos had been laid were incubated for B2 days to let progeny hatch and grow in size; progeny were counted before they reached the adult stage. Mothers that crawled off agar (n = 3) were censored from the analysis.

Imaging

Images were acquired on a Zeiss Cell Observer SD spinning disc confocal microscope, and on a Yokagawa CSUX M1-N Spinning Disk Confocal (Solamere Technology Group) mounted on a Nikon TI-E motorized inverted microscope and equipped with a Hamamatsu 9100c-13 EM-BT EMCCD camera. Residual movement in confocal stacks was corrected using the StackReg ImageJ plugin (Thévenaz et al. 1998). Ray tracing rendering was performed using Volocity (PerkinElmer, Waltham, MA).

Statistics

Calculations were performed by using R (http://r-project.org) or Microsoft Excel. Unless otherwise stated, the asymptotic Wilcoxon rank sum test was used for pairwise comparisons (two-sided tests).

## Results

### Device design and fabrication

Construction of microfluidic devices typically requires specialized equipment and training to perform photolithographic patterning and lamination of channels and actuated structures. Devices for *C. elegans* manipulation can require even greater complexity than is typical. For example, a nonstandard photoresist that is thick and capable of thermal reflow is required to form rounded channels that are both compatible with pneumatic valves and large enough to fit worms (Rohde et al. 2007). This resist is very costly in comparison to standard photoresists. Actuation of on-chip components also requires a complex network of pneumatic tubing and computer-controlled solenoid valves, and in some cases, the piping of hot and cool liquids onto the chip (Krajniak and Lu 2010). Here, we achieve large rounded channels simply by molding off of pencil leads in a very low-cost process. Further, device actuation is achieved simply by pressing on the structure.

The core of the immobilization device is a microfluidic channel that can be tuned in diameter. The channel cross section is roughly triangular and is formed by sealing an elastomeric stamp with cylindrical ridges against a No. 2 glass coverslip (Figure 4.2A). Downward compression on the elastomer collapses the cylindrical ridges against the glass, thus narrowing the channel. The dimensions of the channel were designed such that a young adult worm could move freely in an uncompressed channel and be immobilized in a compressed channel (Figure 4.2B and C). The channel was designed to be narrower towards the inlet and outlet so that by partially compressing the device, a non-immobilized worm could be confined to the center of the device in order to keep it from drifting out of the microscope field of view. Individual worms were loaded through access ports punched

at either end of the channel, and vacuum aspiration was used to draw the worm into the channel. Cut pipette tips were inserted into the access ports to serve as liquid reservoirs, and continuous flow could be maintained in the channel by asymmetric loading of the two reservoirs. Controlled compression of the device was accomplished by using a clamping structure machined in poly(methyl methacrylate) (Figure 4.2D–F). The bottom of the clamping structure tapers to a small oval hole to allow high-resolution microscope objectives to approach the sample while minimizing bending of the coverslip during compression. The structure was held together by three sets of nuts and screws, and compression was adjusted by turning the nuts while observing worm mobility under a microscope.

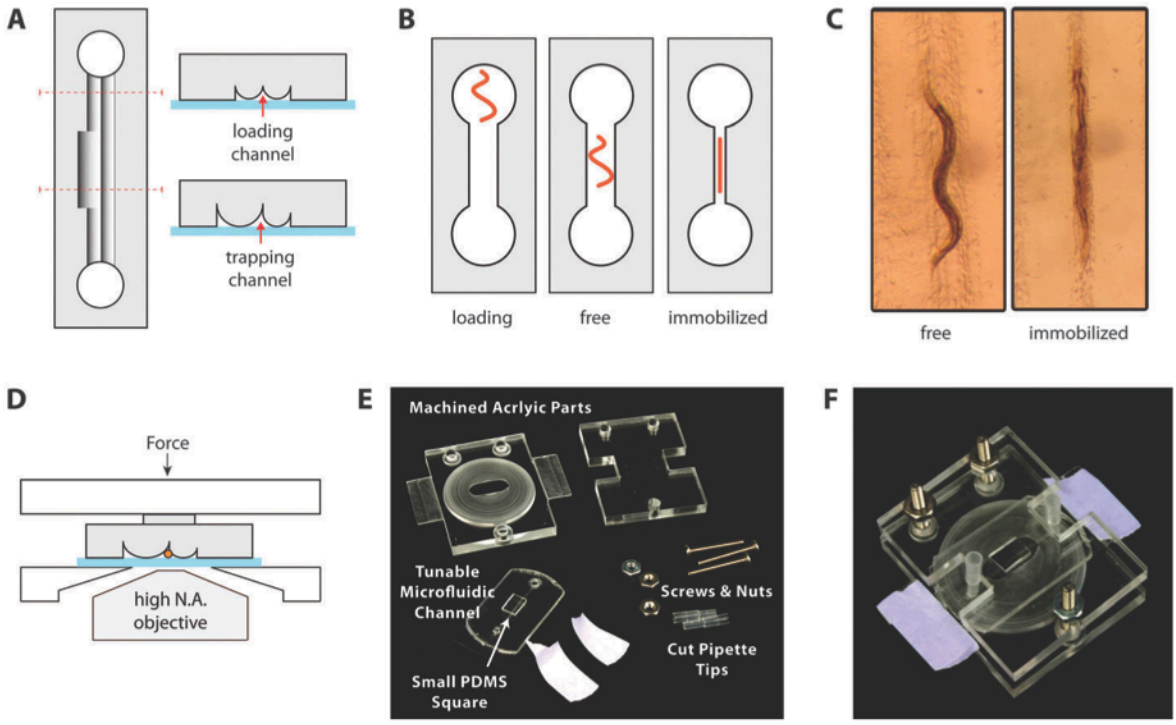



**Figure 4.2.** Worm immobilization device. (A) Cross sections. A tunable-diameter microfluidic channel is formed by sealing an elastomer with cylindrical ridges onto a glass coverslip. The loading channels are narrower than the worm-trapping region to confine the worm in the middle of the device. (B) A worm is placed into one of the access ports and loaded by aspiration. The device is compressed to constrict the channel and immobilize the worm. (C) Microscope images of a worm in the device. An adult worm can be cultured for hours in an uncompressed channel and reversibly immobilized for imaging at specific time points. (D) An acrylic clamping rig maintains compression of the elastomer against the glass, while allowing microscope access for imaging through the coverslip. (E) The clamping rig includes slats to facilitate mounting on a microscope stage. A PDMS square (6 x 3 x 2 $mm^3$) is placed over the microchannels to focus the compression force. (F) Assembled device.

Fabrication of the elastomeric microchannels was accomplished without the need for any specialized equipment. As detailed in Figure 4.3, a mold was created from sculpting clay (Original Sculpey Clay, Polyform Products, Elk Grove Village, IL) and 0.3 mm and 0.5 mm leads (Pentel, Torrance, CA) typically used in mechanical pencils. The leads were embedded into the clay in pairs and pressed down under a glass slide until the tops of the leads were flush with the surface of the clay. This structure was baked at 100 1C for 10 min and then used as a mold to cast a second piece of clay. The surface of the mold was wet with

a small amount of water prior to casting in order to prevent sticking. The second piece of clay, the inverse of the first mold, was also baked at 100 1C for 10 min and then used as a master to cast polydimethylsiloxane (PDMS) stamps. PDMS casting was accomplished by thoroughly mixing prepolymer and linker (Sylgard 184, Dow Corning, Midland, MI) at 15 : 1 by weight, pouring this mixture over the clay mold, degassing in a vacuum chamber, and baking at 80 1C for 40 minutes. Access holes were created in the molded PDMS stamps by using a 3 mm biopsy punch, and the stamps were then plasma bonded (2 min, ambient gas) to glass coverslips to create tunable microchannels. Plasma bonding was helpful to eliminate the possibility for worms to slide into parallel channels. However, bonding was not absolutely essential as long as the PDMS stamp remained pressed against the glass.

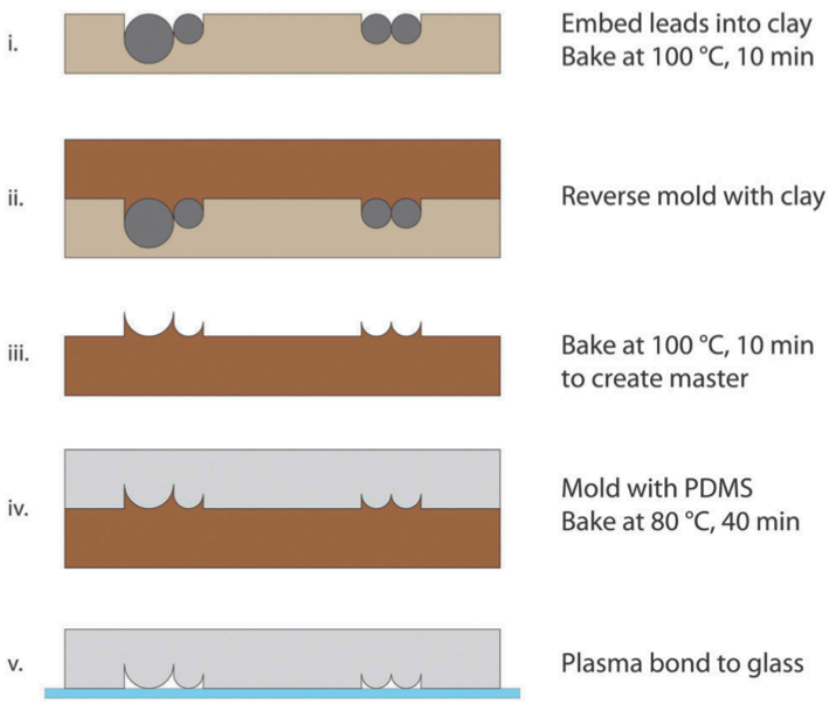



**Figure 4.3.** Device fabrication process. (i) 0.5 mm and 0.3 mm mechanical pencil leads are pressed into molding clay until the tops of the cylinders are flush with the clay surface, and the entire structure is baked. The use of a wider lead results in a larger channel diameter in the completed device. (ii) Additional sculpting clay is pressed into the baked structure. (iii) The reverse-molded clay is baked to form the master mold. (iv) The master is used to cast a PDMS stamp. (v) Access holes are created in the PDMS by using a 3 mm biopsy punch, and the PDMS stamp is attached to a glass coverslip by plasma bonding.

Device validation

We first assayed the impact of worm immobilization and imaging on germ cell cycling and on reproductive activity. We immobilized and acquired 0.2 mm-interval confocal stacks of young adult hermaphrodites 9 times at one-hour intervals; each individual was imaged in less than 2 minutes. Controls were incubated in culture medium identical to that of imaged worms, but were kept in 24-well plates rather than in our device and were not immobilized or imaged. After 9 hours, imaged and control worms were each split into two groups. One group had gonads stained for DNA and phosphorylated histone

H3 to assay mitotic index, and another group was returned to agar plates to score reproductive output.

Overall gonad morphology of imaged and control worms was indistinguishable (Figure 4.4). Average mitotic index was 3.0 for imaged worms (n = 11), and 3.5 for control worms (n = 11). This 15% difference was not significant ($p > 0.33$) and both values fall within the range of previous reports (e.g. ref. 10 and 26), which suggests that germ cell cycling is not appreciably perturbed by immobilization or imaging.

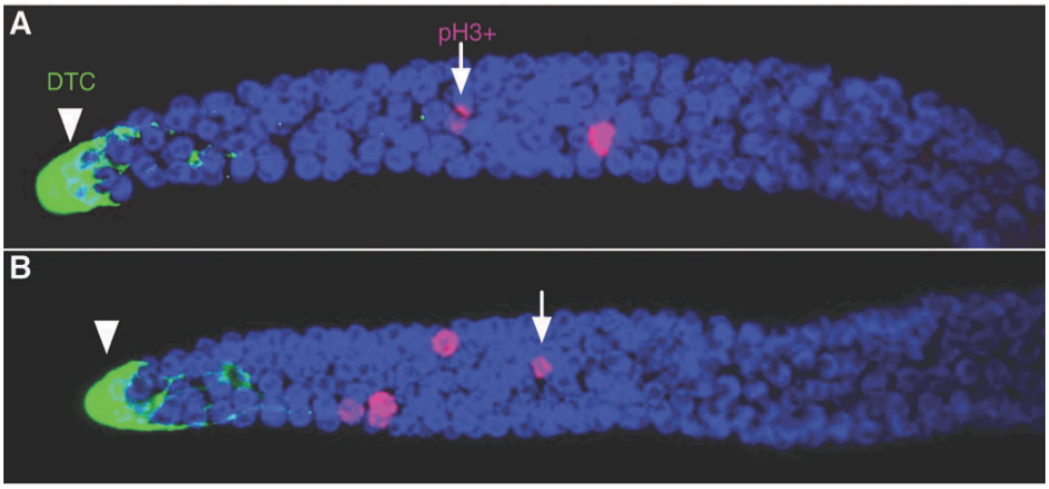



**Figure 4.4.** Gonadal arms extruded, fixed, and stained with antibodies and DAPI show indistinguishable morphology whether they were repeatedly immobilized and imaged live (A) or kept in identical liquid culture medium without immobilization or live imaging (B). The DTC is shown with an arrowhead, and examples of pH3-positive cells with arrows. Images shown are standard deviation projections of confocal stacks.

Average number of progeny was 170 for imaged worms (n = 9), and 248 for control worms (n = 21). The difference—30%—is modest and not statistically significant ($p > 0.11$). Furthermore, this difference stems specifically from 3 imaged specimens that died within 3 days of imaging; when excluding specimens that died within the first 3 days after imaging, average progeny number is 247 (n = 6) and is indistinguishable from the control

average of 259 progeny (n = 20; p > 0.6; 1/21 control died within 6 days). We thus conclude that immobilization and imaging in our device do not appreciably affect reproductive output.

We next asked whether the dynamics of DTC process extension were affected by hermaphrodite immobilization and imaging. We compared DTC growth in single worms that were immobilized and imaged at multiple time points, against average DTC growth in worms maintained in standard liquid culture and sacrificed for imaging at a single time point (Figure 4.5). Worms in the device and control worms were all in S medium supplemented with E. coli. No significant difference was observed in the length of the longest DTC process over 4 rounds of immobilization spanning 36 hours (p > 0.05 with t-test; n = 7).

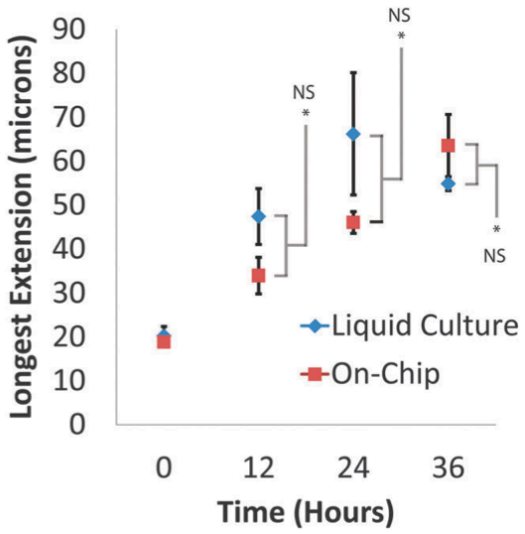



**Figure 4.5.** DTC process extension is not detectably affected by repeated immobilization in the device. Liquid culture: hermaphrodites were cultured for the specified amount of time in S-medium supplemented with E. coli, and permanently immobilized with polystyrene beads for imaging at the time indicated. On chip: worms were kept in the device and temporarily immobilized for live imaging of processes at each indicated time point.

Device reveals dynamics that are obscured in population averages

To ask whether the dynamics of DTC growth are uniform across time and individuals, we imaged the DTC at 1 hour intervals from L4. While the population average of length of the longest DTC process progressed linearly, striking variability was apparent between individuals. Some experienced slow growth, while others experienced rapid growth (Figure 4.6). The difference in growth rates could be attributable to slight differences in developmental stages of the individuals in the population. In any case, imaging individuals through time reveals that the population average is not representative of individual behavior. Furthermore, spurts of growth observed in fast-growing DTCs suggested the possibility of an active growth mechanism rather than passive DTC stretching over time.

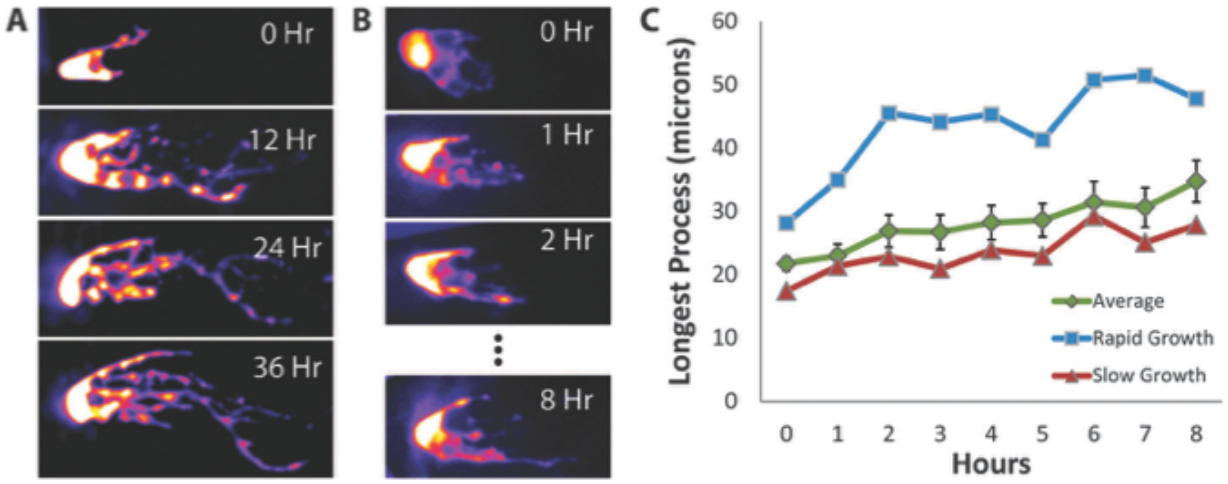



**Figure 4.6.** Time lapse imaging of the DTC at 12 h (A) or 1 h (B) intervals reveals growth of individual processes. (C) Dynamics of growth differ between individuals. While average growth is close to linear (green curve), some DTCs experience apparent spurts of growth (blue curve) while others experience more even, slow growth (red curve).

Local remodeling, backwards growth, and branch points

We next asked what local shape changes underlie the overall lengthening of the DTC. We tracked individual DTCs through time at 1 hour intervals. We made three striking observations. First, process elongation can take place following two distinct steps: a process first forms a ring, which subsequently regains a linear topology (Figure 4.7A). Second, growing processes can form branch points that give rise to multiple small nascent extensions (Figure 4.7C); after a period of time, a single extension appears to grow further while others retreat, suggesting potential competition between nascent extensions (Figure 4.7C to E). Third, process elongation can take place not only along the direction of overall DTC growth (from distal to proximal), but also in the reverse direction (Figure 4.7D). Branch points with multiple nascent extensions or backward growth of processes were observed in 5 of 10 samples.

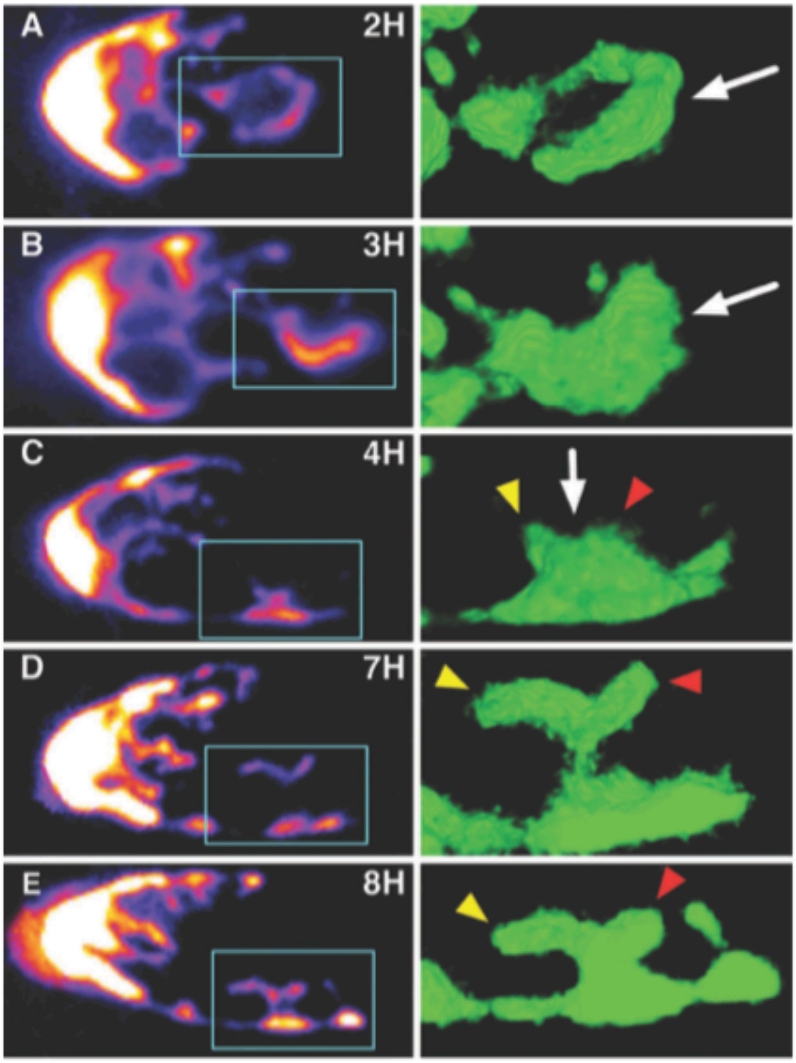



**Figure 4.7.** Time lapse imaging of DTC processes shows an active growth and infiltration of the mitotic zone (left: confocal section; right: ray tracing rendering of region boxed on left). Two important features emerge. First, some processes extend proximally by forming a transient ring-like structure (arrow on row A) that subsequently regains a linear topology (arrow on row B). Second, some processes form a bulb-like structure (arrow on row C) that changes shape over time and results in distal and thus ''backward'' growth of an extension (yellow arrow heads in C–E). Such extensions appear to tightly intercalate between germ cells. Another extension shows more limited growth (red arrowheads).

## Discussion

Our observations of DTC processes over time strongly suggest that their growth is an active process. The transient ring structure that we observe during process elongation shows that processes are not simply stretched by a pulling force generated by germ cell migration along the distal to proximal axis. A ring structure similar to what we observed has been reported by electron microscopy (Hall 1999), lending evidence that our ring is not an artifact (due for example to a very thin central layer that does not contain a detectable amount of cytoplasmic GFP).

The branch points with nascent extensions suggest that the DTC could be actively exploring the environment formed by germ cells. The fact that in some instances only one of these extensions appears to persist and grow suggests possible competition between processes. Biases in the outcome of that competition could result from local signaling between DTC processes and germ cells, perhaps transduced by changes in membrane tension (Heiman and Shaham 2010; Houk et al. 2012).

Our results suggest that the *C. elegans* germline stem cell niche is a dynamic entity that actively seeks contact with a subset of proliferating germ cells. Active movement of the stem cell niche has been observed in the Drosophila ovary (Morris and Spradling 2011) (this movement does not result in net migration); human hematopoietic stem cells also have a dynamic, elaborate contact surface with osteoblasts that plays a role in polarization (Gillette et al. 2009). Dynamic control of the contact surface between stem cells and their niche could thus be a general feature of stem cell systems. The *C. elegans* germline, when imaged live with a device such as the one we report here, provides a powerful model

system to address this dynamic control: the niche can be imaged in its native environment, and the relationship between niche and stem cells dissected with genetic tools.

An important question to address in the future will be the functional significance of the spatial pattern of contact between cycling cells and the DTC, which is established by the infiltration process reported here. A role of the DTC in controlling the length of the mitotic zone has long been suspected (Crittenden et al. 1994; Fitzgerald and Greenwald 1995; Hall 1999), but the shortening of the mitotic zone in aging worms despite concomitant DTC process lengthening could be at odds with this idea (Crittenden et al. 2006). One possibility is that DTC spatial patterns contribute to the control of transitions between differentiation states identified more recently within the mitotic zone (Cinquin et al. 2010).

# Chapter 5

# Conclusion

The ability to reproduce declines with age, which impacts quality of life. Both female humans and *C. elegans* hermaphrodites experience an age related decline of the reproductive system and have a long post-reproductive life span. I utilized the *C. elegans* hermaphrodite as a model to understand this reproductive aging. Specifically, my thesis work aimed to study how food availability influences reproductive aging and, since reproductive aging may be linked to reproductive activity, to investigate the germline response to modulation of the rate of reproduction. In this chapter I review findings from my thesis work, implications and future directions.

## Modulation of reproductive aging

Both CR and IF are well known for their ability to extend organism life span and to delay age related phenotypes. Interestingly, it has been shown in *C. elegans* that CR and IF modulate life span through pathways that only partially overlap. Despite the common impact of CR and IF on somatic aging, studies of *C. elegans* reproductive aging have only focused on the benefits of CR: to my knowledge, there are no reports on the impact of IF on reproductive cessation. In the case of *C. elegans,* this is likely because precise control of food availability throughout the duration of the animals' reproductive life span is a major technical challenge. Application of high-frequency IF regimens throughout a worm's reproductive span requires automation, which we were able to achieve though the use of a microfluidic platform. Microfluidic chips have been used as a tool for a variety of studies. These include temporary immobilization of worms to conduct physiological analysis, drug

screening (e.g. Gilleland et al. 2010; Lockery et al. 2012), and to perform life long analysis of worm development and aging studies (Krajniak and Lu 2010; Hulme et al. 2010). Until recently none of the microfluidic chips have been used to study worm reproductive activity (Kopito and Levine 2013; Li et al. 2014). To properly assay reproductive aging we developed a microfluidic platform which allows application of an IF regimen throughout the entire reproductive life span of mated hermaphrodites. A unique feature of our chip is the ability to flush out laid embryos immediately after being laid without flushing out the parent worm. Thus we are able to track reproductive activity in real time.

Results show that not only the duration but also the frequency of the fasting period can impact reproductive output. Specifically, my studies show that short fasting periods at a low frequency can extend reproductive span without reducing brood size (Chapter 2). This is in sharp contrast to CR studies which show delayed reproductive aging but diminished reproductive output. These findings show that IF is a viable intervention for increasing reproductive span and perhaps "reproductive health span". These findings are also useful to query aging theories such as the "disposable soma" theory (Kirkwood 1977) or the antagonistic pleiotropy theory that puts forth the notion of trade-offs between early and late reproduction (Williams 1957). It has indeed been noted that the fact that reducing early progeny production does not increase late progeny production in *C. elegans* hermaphrodites is perhaps in conflict with current theories of aging (Hughes et al. 2007). My results along with previous findings suggest that current aging theories may require modifying to reconcile these contradictions, or that other tradeoffs need to be considered. For example, I have not assayed fitness of the progeny produced under IF conditions. It may

be that this fitness is impacted, which could be tested using the current chip design by collecting laid embryos and measuring their own reproductive schedule outside of the chip.

Germ cell behavior and reproductive activity

As *C. elegans* worms age, not only is there a decline in oocyte fertilizability but there is also a deterioration of the reproductive tissue, as assayed by morphology (Garigan et al. 2002; Luo et al. 2010). Along with this tissue degeneration, there is a decrease in the number of MZ nuclei (Luo et al. 2010). There is however a gap in the literature in the characterization of other germline compartments of aged hermaphrodite worms or of worms with increased progeny production. I identified the presence of a germline dormant state that was found to be predominant in individuals with low reproductive activity (Chapter 3). Populations with higher progeny production rates had a lower number of dormant germlines. These results suggest that the state of the germline, actively cycling or dormant, is regulated by reproductive activity and may play a role in reproductive cessation.

I further demonstrated that mating alters germline makeup, a response that I found to be independent of reproductive activity or male sperm. I presented evidence that mating not only alters germline compartment makeup but DTC morphology as well. Mating hermaphrodites with sterile males produced similar results as mating with wild-type males: it increased the number of germ cells in PR while decreasing the number of germ cells in TZ and DR. The most likely explanation for the increase in germ cells in the pachytene region is that germ cells are entering the pachytene region at a higher rate as opposed to exiting more slowly. This compartment reorganization may be a germline

priming mechanism for increased fertilization. In addition, I found that mating altered DTC morphology by increasing proximal process extension. It may be that changes in niche morphology are a result of the changes in germ cell activity, which would suggest a feedback loop going from germ cells to its niche. Another, non mutually-exclusive possibility is that mating directly triggers DTC processes extension which in turn modulates germ cell activity. Further experiments will be required to distinguish between these possibilities.

Regulation of germline stem cells

The DTC plays a central role in maintaining the MR and is a prime candidate for regulation of germline stem cell behaviors such as occupancy of the dormant state reported in chapter 3. Indeed, niche architecture has been implicated in regulation of stem cell behavior in *Drosophila* testis and mouse intestinal crypt (Wallenfang et al. 2006; Sato et al. 2010). Although I did not have a chance to address specifically the relationship between germline stem cell cycle control and DTC processes, the tools that I have developed will provide a solid basis for such future studies.

The tools that I developed did allow me, as a first application, to address one important aspect of the DTC. The DTC extends intricate processes proximally whose functional significance remains mysterious. One theory is that DTC processes are simply dragged along by germ cells that are migrating in the proximal direction. This is unlikely because germ cells migrate proximally at a much faster rate than DTC process growth, and because typically only a single process reaches the MZ boundary while the remaining processes only grow a fraction of the distance. If DTC processes were passively dragged by germ cells, we would expect a much faster extension rate and all processes to be of similar

length, which is not what we observe. As a first step in better understanding the regulation of germline stem cells by their DTC, I characterized the fine dynamics of DTC process growth. This had not been achieved prior to my work, most likely because of the difficulty in conducting time lapse imaging of *C. elegans:* there were no methods we identified as suitable to strongly immobilize a worm without inflicting injury.

Using a new microfluidic chip that allowed me to perform time-lapse imaging I showed that the growth rate of processes varies across individuals of the same age. This helps to explain the varying process lengths that are observed. One possibility strongly suggested by my data is that DTC processes alternate between periods of slow and rapid growth as opposed to continuous steady growth until reaching their final length. Such growth spurts would be more compatible with a dynamic, rather than a passive growth process. Further analysis of DTC morphology revealed local active growth of processes. If processes were dragged along by germ cells as they move proximally we would not expect growth in the distal direction. My data, however, shows that processes do extend in the distal direction, which is evidence that processes are actively infiltrating the germline. It is known that at least two germ cell populations exist within the MZ (Cinquin et al. 2010), a distal stem cell pool and a transit amplifying pool. A recent study utilized a *lag-2* promoter driven myristoylated GFP, which localizes to DTC membrane, and showed results consistent with our observations of high density process infiltration in the distal end (Byrd et al. 2014). It would be interesting to determine whether DTC process infiltration and formation of ring structures function to localize Notch signaling. This would help address the functional role of DTC processes in germline stem cell regulation.

Implications for reproductive aging

Like human females, the *C. elegans* hermaphrodites have a long post-reproductive life span (Hughes et al. 2007; Cant and Johnstone 2008; Luo et al. 2009). In addition, both humans and worms display a decline in oocyte quality with age. Food availability regulates longevity in worms and mammals, and have been implicated in the regulation of mammalian reproductive aging (Castrillon et al. 2003; Selesniemi et al. 2008; Tilly and Sinclair 2013). An important goal in human reproductive aging is not only to extend reproductive capacity but to also reduce the maternal age-associated birth defects. My IF results (chapter 2) suggest that reproductive cessation can be delayed without reducing brood size. Understanding the mechanistic underpinning of this delay in reproductive aging may eventually suggest potential therapeutic interventions to delay the reproductive decline in humans.

My results presented in Chapter 3 suggest that germline stem cell behavior is closely related to reproductive activity and may be involved in reproductive cessation. This is in line with other studies which implicate changes in stem cell behavior, senescence and reduction of self-renewal, in aging and in the development of age-related diseases in mice (Campisi 2000; Zhang and Herman 2002). Understanding the relationship between stem cell behavior and reproductive cessation in worms may provide insights into how that cessation can be delayed. In chapter 4 I investigated the DTC, whose control of germline stem cells may be in part responsible for age-related changes in their behavior. Results show sophisticated morphological changes that occur in the niche micro-environment as individuals age; these changes may play a role in stem cell regulation that extends beyond the well-established role in maintenance of an undifferentiated state. This is compatible

with studies which implicate niche environment in age related decline of stem cell self-renewal (Ryu et al. 2006; Jones et al. 2008). Since control of stem cell behavior is in large part exerted by their niche, understanding this relationship will provide insight that will allow better manipulation of stem cells.

Future directions

My thesis work along with previous studies have characterized different aspects of reproductive aging in *C. elegans*, but many questions remain. To further our understanding of reproductive aging we must first address whether trade-offs play a role in the aging process. One possibility is that there are trade offs between brood size and worm life span, as it has been well documented that life span and reproductive activity are negatively correlated. This would mean that IF individuals would have a decrease in life span compared to continuously fed individuals. However, common aging phenotypes of worms, such as decrease in movement and size, was not noticeably different between our control and IF fed individuals. It will be important for future studies to determine whether there are changes in life span under these IF regimens. This can be achieved using our current microfluidic chip design.

Another possibility is that IF negatively impacts offspring fitness. It is well documented that perturbations of maternal environment can result in transgenerational effects (Frost et al. 2009; Carone et al. 2010; Dunn and Bale 2011). Further studies can focus on the effects of IF with respect to life span and progeny fitness to determine whether trade offs can be avoided or redirected, which again can be achieved using our current microfluidic chip design.

This study has identified a dormant state occupied by germline stem cells under conditions of reduced reproductive activity. It may be that stem cell dormancy helps delay reproductive cessation, which should be addressed in future studies. This dormancy may be part of a broader set of responses that occur in responses to changes in reproductive activity; for example, I also identified changes in hermaphrodite germline compartment makeup that occur in response to the presence of males. Most interestingly, these changes occur independently of increased oocyte fertilization. This suggests the existence of previously-unsuspected regulatory mechanisms that may integrate sensing of environmental cues with regulation of germline stem cell cycling and of progression through meiosis, which are likely strongly linked to the progression of reproductive aging.

Finally, my findings raise intriguing questions for future study with respect to the worm germline stem cell niche. I showed that DTC processes extension is sensitive to mating and their growth is active and dynamic. One important question to resolve will be how this extension is regulated; at this point, nothing is known about molecular mechanisms that control DTC process length. Another important question to address will be the impact of DTC on the spatial distribution of Notch signaling. The Notch receptor is concentrated in the distal region of the germline mitotic zone (Crittenden et al. 1994), but there are currently no direct readouts of Notch signaling (an antibody specific to the cleaved, activated form of Notch does not exist in worms). Development of markers for Notch activity will be critical to determine how DTC processes shape the profile of Notch signaling, and how that profile impacts germline stem cell behavior such as progression through the cell cycle. An alternative method to test the role of processes would be to laser ablate processes and determine germ cells responses in terms of e.g. mitotic zone size and

cell cycle speed. Overall, a better understanding of the control exerted over stem cells by their niche will likely be instrumental in understanding how IF delays reproductive cessation, which will in turn aid in understanding the reproductive aging process both in *C. elegans* and in other species.

## References


Allen, Peter B, Allyson E Sgro, Daniel L Chao, Byron E Doepker, J Scott Edgar, Kang Shen, and Daniel T Chiu. 2008. "Single-Synapse Ablation and Long-Term Imaging in Live *C. elegans*." *Journal of Neuroscience Methods* 173 (1). NIH Public Access: 20–26. doi: 10.1016/j.jneumeth.2008.05.007.

Anderson, Rozalyn M, Dhanansayan Shanmuganayagam, and Richard Weindruch. 2009. "Caloric Restriction and Aging: Studies in Mice and Monkeys." *Toxicologic Pathology* 37 (1): 47–51. doi:10.1177/0192623308329476.

Andux, Sara, and Ronald E Ellis. 2008. "Apoptosis Maintains Oocyte Quality in Aging Caenorhabditis elegans Females." *PLoS Genetics* 4 (12). Public Library of Science: e1000295. doi:10.1371/journal.pgen.1000295.

Angelo, Giana, and Marc R Van Gilst. 2009. "Starvation Protects Germline Stem Cells and Extends Reproductive Longevity in *C. elegans*." *Science (New York, NY)* 326 (5955): 954–58. doi:10.1126/science.1178343.

Anita S-R Pepper, Darrell J Killian E Jane Albert Hubbard. 2003. "Genetic Analysis of Caenorhabditis elegans Glp-1 Mutants Suggests Receptor Interaction or Competition." *Genetics* 163 (1). Genetics Society of America: 115.

Antebi, Adam. 2007. "Genetics of Aging in Caenorhabditis elegans." *PLoS Genetics* 3 (9). Public Library of Science: e129. doi:10.1371/journal.pgen.0030129.

Anton, Stephen, and Christiaan Leeuwenburgh. 2013. "Fasting or Caloric Restriction for Healthy Aging." *Experimental Gerontology* 48 (10): 1003–5. doi:10.1016/j.exger. 2013.04.011.

Austad, Steven N. 1989. "Life Extension by Dietary Restriction in the Bowl and Doily Spider, Frontinella Pyramitela." *Experimental Gerontology* 24 (1): 83–92.

Austin, Judith, and Judith Kimble. 1987. "Glp-1 Is Required in the Germ Line for Regulation of the Decision Between Mitosis and Meiosis in *C. elegans*." *Cell* 51 (4): 589–99.

Austin, Judith, Eleanor M Maine, and Judith Kimble. 1989. "Genetics of Intercellular Signalling in *C. elegans*." *Development (Cambridge, England)* 107 (Supplement). The Company of Biologists Limited: 53–57.

Avery, Leon. 1993. "The Genetics of Feeding in Caenorhabditis elegans." *Genetics* 133 (4): 897–917.

Berry, Laura W, B Westlund, and T Schedl. 1997. "Germ-Line Tumor Formation Caused by Activation of Glp-1, a Caenorhabditis elegans Member of the Notch Family of

Receptors." *Development (Cambridge, England)* 124 (4): 925–36. http://dev.biologists.org/content/124/4/925.full.pdf.

Blelloch, Robert, S S Anna-Arriola, D Gao, Y Li, J Hodgkin, and Judith Kimble. 1999. "The Gon-1 Gene Is Required for Gonadal Morphogenesis in Caenorhabditis elegans." *Developmental Biology* 216 (1): 382–93. doi:10.1006/dbio.1999.9491.

Boyle, Monica, Chihunt Wong, Michael Rocha, and D Leanne Jones. 2007. "Decline in Self-Renewal Factors Contributes to Aging of the Stem Cell Niche in the Drosophila Testis." *Cell Stem Cell* 1 (4): 470–78. doi:10.1016/j.stem.2007.08.002.

Brenner, Sydney. 1974. "The Genetics of Caenorhabditis elegans." *Genetics* 77 (1): 71–94.

Broekmans, Frank J, Erik A H Knauff, Egbert R te Velde, Nick S Macklon, and Bart C Fauser. 2007. "Female Reproductive Ageing: Current Knowledge and Future Trends." *Trends in Endocrinology & Metabolism* 18 (2): 58–65. doi:10.1016/j.tem.2007.01.004.

Burger, Joep M S, Dae Sung Hwangbo, Vanessa Corby-Harris, and Daniel E L Promislow. 2007. "The Functional Costs and Benefits of Dietary Restriction in Drosophila." *Aging Cell* 6 (1): 63–71. doi:10.1111/j.1474-9726.2006.00261.x.

Byerly, L, R C Cassada, and R L Russell. 1976. "The Life Cycle of the Nematode Caenorhabditis elegans. I. Wild-Type Growth and Reproduction." *Developmental Biology* 51 (1): 23–33.

Byrd, Dana T, Karla Knobel, Katharyn Affeldt, Sarah L Crittenden, and Judith Kimble. 2014. "A DTC Niche Plexus Surrounds the Germline Stem Cell Pool in Caenorhabditis elegans." *PLoS ONE* 9 (2). Public Library of Science: e88372. doi:10.1371/journal.pone.0088372.

Campisi, Judith. 2003. "Cellular Senescence and Apoptosis: How Cellular Responses Might Influence Aging Phenotypes." *Experimental Gerontology*. 38(2003): 5-11.

Cavaliere, Valeria, Carlo Taddei, and G Gargiulo. 1998. "Apoptosis of Nurse Cells at the Late Stages of Oogenesis of Drosophila Melanogaster." *Development Genes and Evolution* 208 (2): 106–12. doi:10.1007/s004270050160.

Carone, Benjamin R, Lucas, Fauquier, Naomi, Habib, Jeremy M, Shea, and Caroline E, Hart. 2010. "Paternally Induced Transgenerational Environmental Reprogramming of Metabolic Gene Expression in Mammals." *Cell* 143: 1084-1096.

Castrillon, Diego H, Lili, Miao, Ramya, Kollipara, and James W Horner. 2003. "Suppression of Ovarian Follicle Activation in Mice by the Transcription Factor Foxo3a." *Science* 301: 215-218. doi:10.1126/science.1086336.

Chiang, Michael, Amanda Cinquin, Adrian Paz, Edward Meeds, Christopher A Price, Max Welling, and Olivier Cinquin. 2015a. "Control of Caenorhabditis elegans Germ-Line Stem-Cell Cycling Speed Meets Requirements of Design to Minimize Mutation Accumulation." *BMC Biology* 13 (1). BioMed Central Ltd: 51. doi:10.1186/s12915-015-0148-y.

Chiang, Michael, Sam Hallman, Amanda Cinquin, Nabora Reyes de Mochel, Adrian Paz, Shimako Kawauchi, Anne L Calof, Ken W Cho, Charless C Fowlkes, and Olivier Cinquin. 2015b. "Analysis of in Vivo Single Cell Behavior by High Throughput, Human-in-the-Loop Segmentation of Three-Dimensional Images." *BMC Bioinformatics* 16 (1). BioMed Central Ltd: 544. doi:10.1186/s12859-015-0814-7.

Chokshi, Trushal Vijaykumar, Adela Ben-Yakar, and Nikos Chronis. 2009. "CO2 and Compressive Immobilization of *C. elegans* on-Chip." *Lab on a Chip* 9 (1): 151–57. doi:10.1039/b807345g.

Chung, Kwanghun, Matthew M Crane, and Hang Lu. 2008. "Automated on-Chip Rapid Microscopy, Phenotyping and Sorting of *C. elegans*." *Nature Methods* 5 (7): 637–43. doi:10.1038/nmeth.1227.

Cinquin, Olivier. 2009. "Purpose and Regulation of Stem Cells: a Systems-Biology View From the Caenorhabditis elegans Germ Line." *The Journal of Pathology* 217 (2): 186–98. doi:10.1002/path.2481.

Cinquin, Olivier, Sarah L Crittenden, Dyan E Morgan, and Judith Kimble. 2010. "Progression From a Stem Cell-Like State to Early Differentiation in the *C. elegans* Germ Line." *Proceedings of the National Academy of Sciences* 107 (5): 2048–53. doi:10.1073/pnas.0912704107.

Colman, Ricki J, R M Anderson, S C Johnson, E K Kastman, K J Kosmatka, T M Beasley, D B Allison, et al. 2009. "Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys." *Science (New York, NY)* 325 (5937): 201–4. doi:10.1126/science.1173635.

Cox, Elisabeth A, and Jeff Hardin. 2004. "Sticky Worms: Adhesion Complexes in *C. elegans*." *Journal of Cell Science* 117 (Pt 10): 1885–97. doi:10.1242/jcs.01176.

Crittenden, Sarah L, E R Troemel, T C Evans, and Judith Kimble. 1994. "GLP-1 Is Localized to the Mitotic Region of the *C. elegans* Germ Line." *Development (Cambridge, England)* 120 (10): 2901–11.

Crittenden, Sarah L, and Judith Kimble. 2014. "Confocal Methods for Caenorhabditis elegans." *StemBook*. doi:10.3824/stembook.1.95.1.

Crittenden, Sarah L, Kimberly A Leonhard, Dana T Byrd, and Judith Kimble. 2006. "Cellular Analyses of the Mitotic Region in the Caenorhabditis elegans Adult Germ Line." *Molecular Biology of the Cell* 17 (7): 3051–61. doi:10.1091/mbc.E06-03-0170.

Dalfó, Diana, David Michaelson, and E Jane Albert Hubbard. 2012. "Sensory Regulation of the *C. elegans* Germline Through TGF-Β-Dependent Signaling in the Niche." *Current Biology : CB*, April. Elsevier Ltd, 1–8. doi:10.1016/j.cub.2012.02.064.

Dillin, Andrew, Douglas K Crawford, and Cynthia Kenyon. 2002. "Timing Requirements for Insulin/IGF-1 Signaling in *C. elegans*." *Science (New York, NY)* 298 (5594): 830–34. doi:10.1126/science.1074240.

Drummond-Barbosa, Daniela. 2008. "Stem Cells, Their Niches and the Systemic Environment: an Aging Network." *Genetics* 180 (4): 1787–97. doi:10.1534/genetics.108.098244.

Dunn, Gregory A, and Tracy L Bale. 2011. "Maternal High-Fat Diet Effects on Third-Generation Female Body Size via the Paternal Lineage." *Endocrinology* 152 (6). Endocrine Society Chevy Chase, MD : 2228–36. doi:10.1210/en.2010-1461.

Eckmann, Christian R, Brian Kraemer, Marvin Wickens, and Judith Kimble. 2002. "GLD-3, a Bicaudal-C Homolog That Inhibits FBF to Control Germline Sex Determination in *C. elegans*." *Developmental Cell* 3 (5): 697–710. doi:10.1016/S1534-5807(02)00322-2.

Eckmann, Christian R, Sarah L Crittenden, Nayoung Suh, and Judith Kimble. 2004. "GLD-3 and Control of the Mitosis/Meiosis Decision in the Germline of Caenorhabditis elegans." *Genetics* 168 (1): 147–60. doi:10.1534/genetics.104.029264.

Evason, Kimberley, Cheng Huang, Idella Yamben, Douglas F Covey, and Kerry Kornfeld. 2005. "Anticonvulsant Medications Extend Worm Life-Span." *Science (New York, NY)* 307 (5707): 258–62. doi:10.1126/science.1105299.

Fitzgerald, Kevin, and Greenwald, Iva. 1995. "Interchangeability of Caenorhabditis elegans DSL Proteins and Intrinsic Signalling Activity of Their Extracellular Domains in Vivo." *Development (Cambridge, England)* 121 (12): 4275–82.

Fontana, Luigi, and Samuel Klein. 2007. "Aging, Adiposity, and Calorie Restriction." *Jama* 297 (9): 986–94. doi:10.1001/jama.297.9.986.

Fox, P M, V E Vought, M Hanazawa, M H Lee, E M Maine, and T Schedl. 2011. "Cyclin E and CDK-2 Regulate Proliferative Cell Fate and Cell Cycle Progression in the *C. elegans* Germline." *Development (Cambridge, England)* 138 (11): 2223–34. doi:10.1242/dev.059535.

Francis, Ross, M K Barton, Judith Kimble, and T Schedl. 1995. “Gld-1, a Tumor Suppressor Gene Required for Oocyte Development in Caenorhabditis elegans.” *Genetics* 139 (2): 579–606.

Friedman, David B, and T E Johnson. 1988. “A Mutation in the Age-1 Gene in Caenorhabditis elegans Lengthens Life and Reduces Hermaphrodite Fertility.” *Genetics* 118 (1): 75–86.

Frost, Paul C, Dieter Ebert, James H Larson, Michelle A Marcus, and Nicole D Wagner. 2010. “Transgenerational Effects of Poor Elemental Food Quality on Daphnia Magna.” *Oecologia* 162: 865-872. doi:10.1007/s00442-009-1517-4

Gao, Dali, and Judith Kimble. 1995. “APX-1 Can Substitute for Its Homolog LAG-2 to Direct Cell Interactions Throughout Caenorhabditis elegans Development.” *Proceedings of the National Academy of Sciences of the United States of America* 92 (21): 9839–42.

Geiger, Hartmut, and Gary Van Zant. 2002. “The Aging of Lympho-Hematopoietic Stem Cells.” *Nature Immunology* 3 (4). Nature Publishing Group: 329–33. doi:10.1038/ni0402-329.

Gems, David, and D L Riddle. 1996. “Longevity in Caenorhabditis elegans Reduced by Mating but Not Gamete Production.” *Nature* 379 (6567): 723–25. doi:10.1038/379723a0.

Gilleland, Cody L, Christopher B Rohde, Fei Zeng, and Mehmet Fatih Yanik. 2010. “Microfluidic Immobilization of Physiologically Active Caenorhabditis elegans.” *Nature Protocols* 5 (12): 1888–1902. doi:10.1038/nprot.2010.143.

Gillette, Jennifer M, Andre Larochelle, Cynthia E Dunbar, and Jennifer Lippincott-Schwartz. 2009. “Intercellular Transfer to Signalling Endosomes Regulates an Ex Vivo Bone Marrow Niche.” *Nature Cell Biology* 11 (3). NIH Public Access: 303–11. doi:10.1038/ncb1838.

Goodrick, C L, D K Ingram, M A Reynolds, J R Freeman, and N Cider. 1990. “Effects of Intermittent Feeding Upon Body Weight and Life Span in Inbred Mice: Interaction of Genotype and Age.” *Mechanisms of Ageing and Development* 55 (1): 69–87. doi:10.1016/0047-6374(90)90107-Q.

Greer, Eric L, Dara Dowlatshahi, Max R Banko, Judit Villen, Kimmi Hoang, Daniel Blanchard, Steven P Gygi, and Anne Brunet. 2007. “An AMPK-FOXO Pathway Mediates Longevity Induced by a Novel Method of Dietary Restriction in *C. elegans*.” *Current Biology* 17 (19). NIH Public Access: 1646–56. doi:10.1016/j.cub.2007.08.047.

Gumienny, Tina L, E Lambie, E Hartwieg, H R Horvitz, and M O Hengartner. 1999. "Genetic Control of Programmed Cell Death in the Caenorhabditis elegans Hermaphrodite Germline." *Development (Cambridge, England)* 126 (5): 1011–22.

Hall, David. 1999. "Ultrastructural Features of the Adult Hermaphrodite Gonad of Caenorhabditis elegans: Relations Between the Germ Line and Soma." *Developmental Biology* 212 (1): 101–23. doi:10.1006/dbio.1999.9356.

Hansen, Karl R, N S Knowlton, A C Thyer, and J S Charleston. 2008. "A New Model of Reproductive Aging: the Decline in Ovarian Non-Growing Follicle Number From Birth to Menopause." *Human ….*

Hattiangady, Bharathi, and Ashok K Shetty. 2008. "Aging Does Not Alter the Number or Phenotype of Putative Stem/Progenitor Cells in the Neurogenic Region of the Hippocampus." *Neurobiology of Aging* 29 (1): 129–47. doi:10.1016/j.neurobiolaging. 2006.09.015.

Heiman, Maxwell G, and Shai Shaham. 2010. "Twigs Into Branches: How a Filopodium Becomes a Dendrite." *Current Opinion in Neurobiology* 20 (1): 86–91. doi:10.1016/ j.conb.2009.10.016.

Henderson, Samuel T, D Gao, E J Lambie, and Judith Kimble. 1994. "Lag-2 May Encode a Signaling Ligand for the GLP-1 and LIN-12 Receptors of *C. elegans*." *Development (Cambridge, England)* 120 (10): 2913–24.

Hodgkin, Jonathan, and T Doniach. 1997. "Natural Variation and Copulatory Plug Formation in Caenorhabditis elegans." *Genetics* 146 (1): 149–64.

Hodgkin, Jonathan, and T M Barnes. 1991. "More Is Not Better: Brood Size and Population Growth in a Self-Fertilizing Nematode." *Proceedings. Biological Sciences / the Royal Society* 246 (1315): 19–24. doi:10.1098/rspb.1991.0119.

Hodgkin, Jonathan, H R Horvitz, and S Brenner. 1979. "Nondisjunction Mutants of the Nematode CAENORHABDITIS ELEGANS." *Genetics* 91 (1): 67–94.

Holloszy, John O, and Luigi Fontana. 2007. "Caloric Restriction in Humans." *Experimental Gerontology* 42 (8): 709–12. doi:10.1016/j.exger.2007.03.009.

Honjoh, Sakiko, Takuya Yamamoto, Masaharu Uno, and Eisuke Nishida. 2009. "Signalling Through RHEB-1 Mediates Intermittent Fasting-Induced Longevity in *C. elegans*." *Nature* 457 (7230): 726–30. doi:10.1038/nature07583.

Houk, Andrew R, Alexandra Jilkine, Cecile O Mejean, Rostislav Boltyanskiy, Eric R Dufresne, Sigurd B Angenent, Steven J Altschuler, Lani F Wu, and Orion D Weiner. 2012. "Membrane Tension Maintains Cell Polarity by Confining Signals to the Leading Edge During Neutrophil Migration." *Cell* 148 (1-2): 175–88. doi:10.1016/j.cell.2011.10.050.

Houthoofd, Koen, Bart P Braeckman, Isabelle Lenaerts, Kristel Brys, Annemie De Vreese, Sylvie Van Eygen, and Jacques R Vanfleteren. 2002. "Axenic Growth Up-Regulates Mass-Specific Metabolic Rate, Stress Resistance, and Extends Life Span in Caenorhabditis elegans." *Experimental Gerontology* 37 (12): 1371–78. doi:10.1016/S0531-5565(02)00173-0.

Hubbard, E J, and D Greenstein. 2000. "The Caenorhabditis elegans Gonad: a Test Tube for Cell and Developmental Biology." *Developmental Dynamics : an Official Publication of the American Association of Anatomists* 218 (1): 2–22. doi:10.1002/(SICI)1097-0177(200005)218:1<2::AID-DVDY2>3.0.CO;2-W.

Hughes, Stacie E, Kimberley Evason, Chengjie Xiong, and Kerry Kornfeld. 2007. "Genetic and Pharmacological Factors That Influence Reproductive Aging in Nematodes." *PLoS Genetics* 3 (2): e25. doi:10.1371/journal.pgen.0030025.

Hulme, S Elizabeth, Sergey S Shevkoplyas, Alison P McGuigan, Javier Apfeld, Walter Fontana, and George M Whitesides. 2010. "Life Span-on-a-Chip: Microfluidic Chambers for Performing Lifelong Observation of *C. elegans*." *Lab on a Chip* 10 (5). The Royal Society of Chemistry: 589–97. doi:10.1039/b919265d.

Hulme, S Elizabeth, Sergey S Shevkoplyas, Javier Apfeld, Walter Fontana, and George M Whitesides. 2007. "A Microfabricated Array of Clamps for Immobilizing and Imaging *C. elegans*." *Lab on a Chip* 7 (11): 1515. doi:10.1039/b707861g.

Ito, Kenichi, and J D McGhee. 1987. "Parental DNA Strands Segregate Randomly During Embryonic Development of Caenorhabditis elegans." *Cell* 49 (3): 329–36.

Jaramillo-Lambert, Aimee, Marina Ellefson, Anne M Villeneuve, and JoAnne Engebrecht. 2007. "Differential Timing of S Phases, X Chromosome Replication, and Meiotic Prophase in the *C. elegans* Germ Line." *Developmental Biology* 308 (1): 206–21. doi:10.1016/j.ydbio.2007.05.019.

Jones, D Leanne, and Amy J Wagers. 2008. "No Place Like Home: Anatomy and Function of the Stem Cell Niche." *Nature Reviews Molecular Cell Biology* 9 (1): 11–21. doi:10.1038/nrm2319.

Kadyk, Lisa C, and Judith Kimble. 1998. "Genetic Regulation of Entry Into Meiosis in Caenorhabditis elegans." *Development (Cambridge, England)* 125 (10): 1803–13.

Kaeberlein, Tammi L, Erica D Smith, Mitsuhiro Tsuchiya, K Linnea Welton, James H Thomas, Stanley Fields, Brian K Kennedy, and Matt Kaeberlein. 2006. "Life Span Extension in Caenorhabditis elegans by Complete Removal of Food." *Aging Cell* 5 (6): 487–94. doi: 10.1111/j.1474-9726.2006.00238.x.

Katiki, Luciana M, Jorge F S Ferreira, Anne M Zajac, Carol Masler, David S Lindsay, Ana Carolina S Chagas, and Alessandro F T Amarante. 2011. "Caenorhabditis elegans as a Model to Screen Plant Extracts and Compounds as Natural Anthelmintics for Veterinary Use." *Veterinary Parasitology* 182 (2-4): 264–68. doi:10.1016/j.vetpar. 2011.05.020.

Kimble, Judith. 1981. "Alterations in Cell Lineage Following Laser Ablation of Cells in the Somatic Gonad of Caenorhabditis elegans." *Developmental Biology* 87 (2): 286–300.

Kimble, Judith, and Sarah L Crittenden. 2005. "Germline Proliferation and Its Control." *WormBook*, 1–14. doi:10.1895/wormbook.1.13.1.

Kimble, Judith, and Sarah L Crittenden. 2007. "Controls of Germline Stem Cells, Entry Into Meiosis, and the Sperm/Oocyte Decision in Caenorhabditis elegans." *Annual Review of Cell and Developmental Biology* 23: 405–33. doi:10.1146/annurev.cellbio. 23.090506.123326.

Kirkwood, T B L. 1977. "Evolution of Ageing." *Nature* 270 (5635): 301–4. doi: 10.1038/270301a0.

Klass, Michael R. 1977. "Aging in the Nematode Caenorhabditis elegans: Major Biological and Environmental Factors Influencing Life Span." *Mechanisms of Ageing and Development* 6 (6): 413–29.

Kleemann, Gunnar A, and A.L. Basolo. 2007. "Facultative Decrease in Mating Resistance in Hermaphroditic Caenorhabditis elegans with Self-Sperm Depletion." *Animal Behaviour* 74 (5). Elsevier: 1339–47.

Kopito, Ronen, and Erel Levine. 2013. "Durable Spatiotemporal Surveillance of Caenorhabditis elegans Response to Environmental Cues." *Lab on a Chip*. doi: 10.1039/c3lc51061a.

Kraemer, Brain, Sarah Crittenden, Maria Gallegos, Gary Moulder, Robert Barstead, Judith Kimble, and Marvin Wickens. 1999. "NANOS-3 and FBF Proteins Physically Interact to Control the Sperm–Oocyte Switch in Caenorhabditis elegans." *Current Biology* 9 (18): 1009–18. doi:10.1016/S0960-9822(99)80449-7.

Krajniak, Jan, and Hang Lu. 2010. "Long-Term High-Resolution Imaging and Culture of *C. elegans* in Chip-Gel Hybrid Microfluidic Device for Developmental Studies." *Lab on a Chip* 10 (14): 1862–68. doi:10.1039/c001986k.

Lambie, Eric J, and Judith Kimble. 1991. “Two Homologous Regulatory Genes, Lin-12 and Glp-1, Have Overlapping Functions.” *Development (Cambridge, England)* 112 (1): 231–40.

Lander, Arthur D, Judith Kimble, Hans Clevers, Elaine Fuchs, Didier Montarras, Margaret Buckingham, Anne L Calof, Andreas Trumpp, and Thordur Oskarsson. 2012. “What Does the Concept of the Stem Cell Niche Really Mean Today?.” *BMC Biology* 10: 19. doi:10.1186/1741-7007-10-19.

Lee, Garrick D, Mark A Wilson, Min Zhu, Catherine A Wolkow, Rafael de Cabo, Donald K Ingram, and Sige Zou. 2006. “Dietary Deprivation Extends Life Span in Caenorhabditis elegans.” *Aging Cell* 5 (6): 515–24. doi:10.1111/j.1474-9726.2006.00241.x.

Li, Linheng, and Ting Xie. 2005. “STEM CELL NICHE: Structure and Function.” *Annual Review of Cell and Developmental Biology* 21 (1): 605–31. doi:10.1146/annurev.cellbio.21.012704.131525.

Li, Siran, Howard A Stone, and Coleen Tara Murphy. 2014. “A Microfluidic Device and Automatic Counting System for the Study of *C. elegans* Reproductive Aging.” *Lab on a Chip* 15 (2). The Royal Society of Chemistry: 524–31. doi:10.1039/C4LC01028K.

Lockery, Shawn R, S Elizabeth Hulme, William M Roberts, Kristin J Robinson, Anna Laromaine, Theodore H Lindsay, George M Whitesides, and Janis C Weeks. 2012. “A Microfluidic Device for Whole-Animal Drug Screening Using Electrophysiological Measures in the Nematode *C. elegans*.” *Lab on a Chip* 12 (12): 2211–20. doi:10.1039/c2lc00001f.

Losick, Vicki P, Lucy X Morris, Donald T Fox, and Allan Spradling. 2011. “Drosophila Stem Cell Niches: a Decade of Discovery Suggests a Unified View of Stem Cell Regulation.” *Developmental Cell* 21 (1): 159–71. doi:10.1016/j.devcel.2011.06.018.

Marthiens, Veronique, I Kazanis, L Moss, K Long, and C ffrench-Constant. 2010. “Adhesion Molecules in the Stem Cell Niche - More Than Just Staying in Shape?.” *Journal of Cell Science* 123 (10): 1613–22. doi:10.1242/jcs.054312.

Martin, Bronwen, Mark P Mattson, and Stuart Maudsley. 2006. “Caloric Restriction and Intermittent Fasting: Two Potential Diets for Successful Brain Aging.” *Ageing Research Reviews* 5 (3): 332–53. doi:10.1016/j.arr.2006.04.002.

Mattson, Mark P, and Wan, Ruiqian. 2005. “Beneficial Effects of Intermittent Fasting and Caloric Restriction on the Cardiovascular and Cerebrovascular Systems.” *The Journal of Nutritional Biochemistry* 16 (2005): 129-137. doi: 10.1016/J.jnutbio.2004.12.007.

McCarter, James, Bart Bartlett, Thanh Dang, and Tim Schedl. 1999. “On the Control of Oocyte Meiotic Maturation and Ovulation in Caenorhabditis elegans.” *Developmental Biology* 205 (1): 111–28. doi:10.1006/dbio.1998.9109.

McCay, C M, M F Crowell, and L A Maynard. 1935. “The Effect of Retarded Growth Upon the Length of Life Span and Upon the Ultimate Body Size.” *The Journal of Nutrition* 1 (1935): 63-79.

Méndez-Ferrer, Simón, Daniel Lucas, Michela Battista, and Paul S Frenette. 2008. “Haematopoietic Stem Cell Release Is Regulated by Circadian Oscillations.” *Nature* 452 (7186): 442–47. doi:10.1038/nature06685.

Miller, Michael A, V Q Nguyen, M H Lee, M Kosinski, T Schedl, R M Caprioli, and D Greenstein. 2001. “A Sperm Cytoskeletal Protein That Signals Oocyte Meiotic Maturation and Ovulation.” *Science (New York, NY)* 291 (5511): 2144–47. doi: 10.1126/science.1057586.

Morgan, Dyan E, Sarah L Crittenden, and Judith Kimble. 2010. “The *C. elegans* Adult Male Germline: Stem Cells and Sexual Dimorphism.” *Developmental Biology*, July. doi: 10.1016/j.ydbio.2010.07.022.

Morris, Lucy X, and A C Spradling. 2011. “Long-Term Live Imaging Provides New Insight Into Stem Cell Regulation and Germline-Soma Coordination in the Drosophila Ovary.” *Development (Cambridge, England)* 138 (11): 2207–15. doi:10.1242/dev.065508.

Morrison, Sean J, and Allan C Spradling. 2008. “Stem Cells and Niches: Mechanisms That Promote Stem Cell Maintenance Throughout Life.” *Cell* 132 (4): 598–611. doi: 10.1016/j.cell.2008.01.038.

Morsci, Natalia S, Leonard A Haas, and Maureen M Barr. 2011. “Sperm Status Regulates Sexual Attraction in Caenorhabditis elegans.” *Genetics*, October. doi:10.1534/ genetics.111.133603.

Nadarajan, Saravanapriah, J Amaranath Govindan, Marie McGovern, E Jane Albert Hubbard, and David Greenstein. 2009. “MSP and GLP-1/Notch Signaling Coordinately Regulate Actomyosin-Dependent Cytoplasmic Streaming and Oocyte Growth in *C. elegans*.” *Development (Cambridge, England)* 136 (13): 2223–34. doi:10.1242/dev.034603.

Nelson, Gregory A, K K Lew, and S Ward. 1978. “Intersex, a Temperature-Sensitive Mutant of the Nematode Caenorhabditis elegans.” *Developmental Biology* 66 (2): 386–409.

Packer, Craig, Marc Tatar, and Anthony Collins. 1998. “Reproductive Cessation in Female Mammals.” *Nature* 392 (6678). Nature Publishing Group: 807–11. doi: 10.1038/33910.

Park, Donha, Annette Estevez, and Donald L Riddle. 2010. “Antagonistic Smad Transcription Factors Control the Dauer/Non-Dauer Switch in *C. elegans*.” *Development (Cambridge, England)* 137 (3): 477–85. doi:10.1242/dev.043752.

Pickett, Christopher L, Nicholas Dietrich, Junfang Chen, Chengjie Xiong, and Kerry Kornfeld. 2013. “Mated Progeny Production Is a Biomarker of Aging in Caenorhabditis elegans.” *G3 (Bethesda, Md.)* 3 (12): 2219–32. doi:10.1534/g3.113.008664.

Preibisch, Stephan, S Saalfeld, and P Tomancak. 2009. “Globally Optimal Stitching of Tiled 3D Microscopic Image Acquisitions.” *Bioinformatics* 25 (11): 1463–65. doi:10.1093/bioinformatics/btp184.

Qin, Jianhua, and Aaron R Wheeler. 2007. “Maze Exploration and Learning in *C. elegans*.” *Lab on a Chip* 7. Royal Society of Chemistry: 186–92. doi:10.1039/B613414A.

Raaijmakers, Marc H G P, Siddhartha Mukherjee, Shangqin Guo, Siyi Zhang, Tatsuya Kobayashi, Jesse A Schoonmaker, Benjamin L Ebert, et al. 2010. “Bone Progenitor Dysfunction Induces Myelodysplasia and Secondary Leukaemia.” *Nature* 464 (7290): 852–57. doi:10.1038/nature08851.

Rando, Thomas A. 2006. “Stem Cells, Ageing and the Quest for Immortality.” *Nature* 441 (7097). Nature Publishing Group: 1080–86. doi:10.1038/nature04958.

Reznick, David N, Michael J Bryant, Derek Roff, Cameron K Ghalambor, and Dionna E Ghalambor. 2004. “Effect of Extrinsic Mortality on the Evolution of Senescence in Guppies.” *Nature* 431 (7012): 1095–99. doi:10.1038/nature02936.

Rogina, Blanka, R A Reenan, S P Nilsen, and S L Helfand. 2000. “Extended Life-Span Conferred by Cotransporter Gene Mutations in Drosophila.” *Science (New York, NY)* 290 (5499): 2137–40. doi:10.1126/science.290.5499.2137.

Rohde, Christopher B, Fei Zeng, Ricardo Gonzalez-Rubio, Matthew Angel, and Mehmet Fatih Yanik. 2007. “Microfluidic System for on-Chip High-Throughput Whole-Animal Sorting and Screening at Subcellular Resolution.” *Proceedings of the National Academy of Sciences of the United States of America* 104 (35): 13891–95. doi:10.1073/pnas.0706513104.

Ryu, Buom-Yong, Kyle E Orwig, Jon M Oatley, Mary R Avarbock, and Ralph L Brinster. 2006. “Effects of Aging and Niche Microenvironment on Spermatogonial Stem Cell Self-Renewal.” *Stem Cells* 24 (6): 1505–11. doi:10.1634/stemcells.2005-0580.

Saffrey, M Jill. 2014. “Aging of the Mammalian Gastrointestinal Tract: a Complex Organ System.” *Age (Dordrecht, Netherlands)* 36 (3): 9603. doi:10.1007/s11357-013-9603-2.

Saldaña, Zeus, Juan Xicohtencatl-Cortes, Fabiola Avelino, Alan D Phillips, James B Kaper, José L Puente, and Jorge A Girón. 2009. "Synergistic Role of Curli and Cellulose in Cell Adherence and Biofilm Formation of Attaching and Effacing Escherichia Coli and Identification of Fis as a Negative Regulator of Curli." 11 (4): 992–1006. doi: 10.1111/j.1462-2920.2008.01824.x.

Sato, Toshiro, Johan H van Es, Hugo J Snippert, Daniel E Stange, Robert G Vries, Maaike van den Born, Nick Barker, Noah F Shroyer, Marc van de Wetering, and Hans Clevers. 2010. "Paneth Cells Constitute the Niche for Lgr5 Stem Cells in Intestinal Crypts." *Nature* 469 (7330). Nature Publishing Group: 415–18. doi:10.1038/nature09637.

Selesniemi, Kaisa, Ho-Joon Lee, and Jonathan L, Tilly. 2008. "Moderate Caloric Restriction Initiated in Rodents During Adulthood Sustains Function of the Female Reproductive Axis Into Advanced Chronological Age." *Aging Cell* (7): 622-629

Sharpless, Norman E, and Ronald A DePinho. 2007. "How Stem Cells Age and Why This Makes Us Grow Old." *Nature Reviews Molecular Cell Biology* 8 (9): 703–13. doi: 10.1038/nrm2241.

Shtonda, Boris Borisovich, and Leon Avery. 2006. "Dietary Choice Behavior in Caenorhabditis elegans." *The Journal of Experimental Biology* 209 (Pt 1): 89–102. doi:10.1242/jeb.01955.

Simon, Anne F, Cindy Shih, Antha Mack, and Seymour Benzer. 2003. "Steroid Control of Longevity in Drosophila Melanogaster." *Science (New York, NY)* 299 (5611): 1407–10. doi:10.1126/science.1080539.

Stanfield, Gillian M, and Anne M Villeneuve. 2006. "Regulation of Sperm Activation by SWM-1 Is Required for Reproductive Success of *C. elegans* Males." *Current Biology : CB* 16 (3): 252–63. doi:10.1016/j.cub.2005.12.041.

Szewczyk, Nathaniel J, I A Udranszky, E Kozak, J Sunga, S K Kim, L A Jacobson, and C A Conley. 2006. "Delayed Development and Life Span Extension as Features of Metabolic Lifestyle Alteration in *C. elegans* Under Dietary Restriction." *Journal of Experimental Biology* 209 (20): 4129–39. doi:10.1242/jeb.02492.

Thackaberry, Evan A, Stephen Kopytek, Phillip Sherratt, Kevin Trouba, and Barry McIntyre. 2010. "Comprehensive Investigation of Hydroxypropyl Methylcellulose, Propylene Glycol, Polysorbate 80, and Hydroxypropyl-Beta-Cyclodextrin for Use in General Toxicology Studies." *Toxicological Sciences : an Official Journal of the Society of Toxicology* 117 (2): 485–92. doi:10.1093/toxsci/kfq207.

Thévenaz, Philippe, U E Ruttimann, and M Unser. 1998. "A Pyramid Approach to Subpixel Registration Based on Intensity." *IEEE Transactions on Image Processing : a Publication of the IEEE Signal Processing Society* 7 (1): 27–41. doi: 10.1109/83.650848.

Tilly, Jonathan L, and David A Sinclair. 2013. “Germline Energetics, Aging, and Female Infertility.” *Cell Metabolism* 17 (6): 838–50. doi:10.1016/j.cmet.2013.05.007.

Toledano, Hila, Cecilia D’Alterio, Benjamin Czech, Erel Levine, and D Leanne Jones. 2012. “The Let-7–Imp Axis Regulates Ageing of the Drosophila Testis Stem-Cell Niche.” *Nature* 485 (7400). Nature Publishing Group: 605–10. doi:10.1038/nature11061.

Uno, Masaharu, Sakiko Honjoh, Mitsuhiro Matsuda, Haruka Hoshikawa, Saya Kishimoto, Tomohito Yamamoto, Miki Ebisuya, Takuya Yamamoto, Kunihiro Matsumoto, and Eisuke Nishida. 2013. “A Fasting-Responsive Signaling Pathway That Extends Life Span in *C. elegans*.” *Cell Reports* 3 (1): 79–91. doi:10.1016/j.celrep.2012.12.018.

Wallenfang, Matthew R, Renuka Nayak, and Stephen DiNardo. 2006. “Dynamics of the Male Germline Stem Cell Population During Aging of Drosophila Melanogaster.” *Aging Cell* 5 (4): 297–304. doi:10.1111/j.1474-9726.2006.00221.x.

Wang, Meng C, Holly D Oakley, Christopher E Carr, Jessica N Sowa, and Gary Ruvkun. 2014. “Gene Pathways That Delay Caenorhabditis elegans Reproductive Senescence.” *PLoS Genetics* 10 (12): e1004752. doi:10.1371/journal.pgen.1004752.

Ward, Alex, Liu, Jie, Feng, Zhaoyang, Xu, X, Z, Shawn. 2008. “Light-Sensitive Neurons and Channels Mediate Phototaxis in *C. elegans*.” *Nature Neuroscience* 11 (8). NIH Public Access: 916. doi:10.1038/nn.2215.

Ward, Samuel, Thomas M, Roberts, Susan Strome, Fredrick M, Pavalko, and Eileen Hogan. 1986. “Monoclonal Antibodies That Recognize a Polypeptide Antigenic Determinant Shared by Multiple Caenorhabditis elegans Sperm-Specific Proteins.” *The Journal of Cell Biology* 102 (5): 1778–86. doi:10.1083/jcb.102.5.1778.

Wen, Hui, Xinghua Gao, and Jianhua Qin. 2014. “Probing the Anti-Aging Role of Polydatin in Caenorhabditis elegans on a Chip.” *Integrative Biology* 6 (1): 35–43. doi:10.1039/c3ib40191j.

Williams, George C. 1957. “Pleiotropy, Natural Selection, and the Evolution of Senescence.” *Evolution* 11 (4): 398. doi:10.2307/2406060.

Wolke, Uta, Erin A Jezuit, and James R Priess. 2007. “Actin-Dependent Cytoplasmic Streaming in *C. elegans* Oogenesis.” *Development (Cambridge, England)* 134 (12): 2227–36. doi:10.1242/dev.004952.

Wong, Brandon G, Adrian Paz, Michael A Corrado, Brian R Ramos, Amanda Cinquin, Olivier Cinquin, and Elliot E Hui. 2013. “Live Imaging Reveals Active Infiltration of Mitotic Zone by Its Stem Cell Niche.” *Integrative Biology*. The Royal Society of Chemistry. doi:10.1039/c3ib20291g.

Zeng, Fei, Christopher B Rohde, and Mehmet Fatih Yanik. 2008. “Sub-Cellular Precision on-Chip Small-Animal Immobilization, Multi-Photon Imaging and Femtosecond-Laser Manipulation.” *Lab on a Chip* 8 (5): 653–56. doi:10.1039/b804808h.

Zhang, Yingpei, and Brian Herman. 2002. “Ageing and Apoptosis.” *Mechanisms of Ageing and Development* 123: 245-260.