MECHANISMS OF MATING-BEHAVIOR DETERIORATION IN EARLY AGING MALE *C. ELEGANS*

A Dissertation

by

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ABSTRACT

Aging has been a subject of interest throughout history. Scientific studies have focused on lifespan regulation, but ignored many other aspects of aging such as behavioral decline. Research using the model organism *C. elegans* has contributed significantly to the aging field. In this dissertation, I used *C. elegans* males to determine the molecular mechanisms of behavioral deterioration during aging. Through mating potency assays, I found that the mating behavior of *C. elegans* declines at early adulthood, as the mating potency of 3-day-old wild-type males is significantly lower than 1-day-old males. Meanwhile, using both pharmacological tests and calcium imaging, I showed that the excitability of the mating circuit increased during early adulthood. This is consistent with the observation that old males exhibit reduced control over their ability to mate.

Caloric restriction is an efficient non-genetic intervention to increase lifespan. I demonstrated here that it also improves mating behavior in 3-day-old males, possibly through reducing the excitability of the mating circuitry by up-regulation of potassium channels and additional metabolic enzymes.

To explore the relationship between metabolic status and behavioral deterioration, I characterized the dynamics of male mating deterioration in males containing a deletion in the metabolism-regulator *sir-2.1*. *sir-2.1* encodes a NAD^+ dependent histone deacetylase, which might be involved in regulating aging. I discovered that *sir-2.1(0)* males have a premature decline in mating potency and an accelerated increase in the

excitability of the mating circuitry. Direct mating behavior observations indicated that a significant proportion of 2-day-old *sir-2.1(0)* males cannot transfer their sperm into their hermaphrodite mates. Through Ca^{2+} imaging, I found that the genital sex muscles are hyper-contracted during sperm transfer. This hyper-contraction blocks the vas deferens and obstructs sperm release. Furthermore, through qPCR, measurements of metabolites, and diet supplementation, I found that the potentially enhanced catabolism in 1-day-old *sir-2.1(0)* and 2-day-old wild-type males generates excess reactive oxygen species (ROS). ROS increases the excitability of the mating circuitry and leads to the mating potency decline in subsequent days. Meanwhile, anabolic processes such as gluconeogenesis/glyceroneogenesis are also elevated. These processes shunt pyruvate from oxidative processes to lipid synthesis, and serve as a potential compensatory mechanism to reduce energy and ROS production.

In conclusion, I demonstrated that a complex behavior in *C. elegans* deteriorated during early aging due to the physiological state change, which is possibly caused by ROS induced by both metabolic and stress-response alteration.

DEDICATION

This work is dedicated to all the worms killed by me, and all the plants and fish I tried to keep alive in the past 6 years.

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NOMENCLATURE

ACh	Acetylcholine
AChR	Acetylcholine receptor
ARE	Arecoline
CFX	Cycloheximide
EC_{50}	Effective concentration to cause response in 50% of the population
ETC	Electron Transport Chain
FRTA	Free Radical Theory of Aging
IIS	Insulin/IGF-1 signaling
LEV	Levamisole
mAChR	Muscarinic acetylcholine receptor
nAChR	Nicotinic acetylcholine receptor
NAC	N-acetyl-cysteine
NAD^+	Nicotinamide adenine dinucleotide
Nam	Nicotinamide
OXPHOS	Oxidative phosphorylation
p.c.s.	Postcloacal sensilla
PEPCK	Phosphoenolpyruvate carboxykinase
prc	Spontaneous spicule protraction
ROS	Reactive oxygen species
SOD	Superoxide dismutase

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CHAPTER I

INTRODUCTION

Two major evolutionary theories of aging

Aging is a complex and universal phenomenon, which is associated with progressive deterioration of physiological function, decline of fitness, and increased possibility of age-related diseases and eventually mortality (Rose 1991). Thus, aging is usually associated with lifespan. In nature, different species have different lifespans; the mechanism underlying this phenomenon is a mystery. Also, from an evolutionary point of view, why does natural selection favor survival and reproduction rather than stopping aging in the first place? In fact, aging may not be stopped. Even though lifespan can be significantly prolonged, there is no report of immortal organisms.

These questions promote multiple theories to explain the relationship between aging and evolution/natural selection. From the 1940's, evolutionary biologists argued that aging occurs because the force of natural selection becomes inefficient as organisms age, especially when they are in post-reproduction (Ljubuncic and Reznick 2009). This idea is later well supported by mathematical modeling and experiments (Williams 1957, Hamilton 1966, Charlesworth 2000). It is generally accepted that during evolution, extrinsic factors such as predators, food resources, parasites, and infectious bacteria/virus cause the death of organisms even before they had a chance to reach the limits of their natural lifespans. Therefore, reproduction is the best way to promote

species' survival and would be the target of natural selection. Post-reproduction, the force of natural selection would decline over the aging process, unless such selection is related to the survival of the offspring. For instance, the parents should live long enough to take care of progeny, however, their death after maturation of progeny might benefit the species by saving resources (Fabian 2011). In addition, reproduction provides a mechanism for diversifying organisms to be more adaptive to the changes of the nature (Griffiths A 1999). Thus, the natural selection would mainly function at the relatively early stages in life to select those who reproduce.

Based on the notion that the force of natural selection starts to decline during aging, Medawar developed the first complete written formulation and graphical model to explain the evolution of aging, known as the mutation accumulation hypothesis (Medawar 1952). According to this theory, deleterious mutations, such as those seen in many genes related to neurodegenerative disease, are hardly under selection during evolution, since they would only become a problem in the unlikely event that the organism lived for an extended amount of time.

Since reproductive fitness is preferentially selected by natural selection during evolution, George C. William pushed the mutation accumulation theory a step further and developed the antagonistic pleiotropy hypothesis of aging (Williams 1957). In his theory, gene mutations selected for the benefit of reproduction during early age may display deleterious effects on fitness at advanced age. In other words, the deleterious genes are not selected against, instead they are selected for due to their beneficial contribution to reproduction. These contributions include but are not limited to

development of the reproductory system, and to the relocation of energy to maintain the germline cells. Thus, enrichment of fitness factors for reproduction might be a genetic trade-off for fitness at an older age.

Both the mutation-accumulation and antagonistic-pleiotropy hypothesis of aging help to explain how aging evolves and why there are so many detrimental aspects of aging. However, with the advance of human civilization, the extrinsic factors that affect human aging are diminishing and human lifespan has extended tremendously. Unfortunately, this extended lifespan is associated with health problems arising from aging. These health problems not only cause personal pain but also are also social burdens. Thus, understanding the molecular mechanism of aging would definitely provide strategies to promote the span during which humans remain healthy and potentially cure or at least minimize the diseases related to aging.

Molecular theories of aging

Is the aging process simply a wearing-out process analogous to the breaking down of a car or even a piece of paper? From all aging research conducted so far, and the work I will present, the answer is not straightforward. However, that wearing-out contributes to aging seems commonsense. The scientific questions to ask then are: (i) what cause the wearing-out process, (ii) do any mechanisms exist to counteract the wearing-out process, and if so, (iii) what are they? Additionally, would minimizing wearing-out factors or amplifying detoxification decrease the chances of diseases, or

enhance fitness at an advanced age? To answer these questions, some important theories have emerged.

Free radical theory of aging (FRTA)/Oxidative stress theory

Free radicals are defined as molecules containing one or more unpaired electrons in their atomic orbitals. The unpaired electrons dramatically increase the reactivity of the molecule. In nature, strong radiation will generate a large amount of radicals, which are almost lethally detrimental to living organisms through inducing mutations, cancer and aging. Radiation's devastating effects were unfortunately verified by irradiation of living organisms after nuclear explosions and rare nuclear accidents (Hempelmann and Hoffman 1953).

In living organisms, spontaneous free radicals were first recorded in 1954 (Commoner, Townsend et al. 1954). Through a newly developed technique called paramagnetic resonance absorption, free radicals were measured in lyophilized tissues such as leaf, liver and egg. Most intriguingly, the amount of free radicals are relatively higher in the metabolically active tissues such as leaf or liver than those metabolically non-active tissues, indicating that free radicals might be the intermediate products of metabolism processes and positively associated with the metabolism rate.

Based on the chemistry of radiation and the fact that living organisms generate free radicals, in 1956, Dr. Harman proposed that aging is caused by the deleterious

attacks to the cellular components including nucleotides, proteins, and lipids by the free radicals that are spontaneously generated in the living cell (Harman 1956).

Free radicals are generated during the cellular respiration process. For an organism to function, survive and reproduce successfully, efficient energy production is a necessity. During evolution, oxidative phosphorylation (OXPHOS) in the mitochondrion replaced anaerobic fermentation and served as a more efficient way to generate ATP, a uniform energy currency for all living organisms. During this oxidation-reduction process, electrons donated from NADH and succinate travel through the electron transport chains (ETC) complexes I to IV to oxygen and the energy released from the series of redox reactions promote the generation of the proton gradient. ATP synthase then utilizes the energy released from the influx of protons back to the mitochondrial matrix to generate ATP via oxidative phosphorylation, a process known as chemiosmotic coupling (Mitchell 2011). However, the OXPHOS that occurs in the mitochondrion is not perfectly efficient and actually serves as a major contributor of endogenous oxidative stress. Up to 3% of the electrons can leak from respiratory complexes I and III under normal conditions and be transferred to oxygen prematurely. The addition of electrons to the oxygen forms the superoxide anion $\bullet O_2^-$, a primary form of reactive oxygen species (ROS). Additional oxygen-derived radicals as well as reactive molecules that are not radicals will be generated. For instance, hydrogen peroxide, is not a non-radical but is a highly and strong oxidant. In addition, through the Fenton reaction, hydrogen peroxide can produce hydroxyl radicals (HO•) (Stohs and Bagchi 1995), which can react with many biomolecules and change their functional properties. Oxidization of

lipid, a process referred as lipid peroxidation, generates fatty acid radicals. These radicals covalently modify proteins (Arguelles, Cano et al. 2011). Thus, oxygen originated radicals and reactive molecules are major contributors for oxidative stress, and the free radical theory is after referred to as the oxidative stress theory.

To cope with both exogenous and endogenous reactive stress, organisms must optimize their metabolism to generate sufficient energy for reproductive success at the first place. Additionally, they must minimize the generation of detrimental ROS through developing an efficient antioxidant system by expressing a series of scavenger genes such as superoxide dismutase (SOD), peroxidase and catalases (McCord and Fridovich 1969, Greenwald 1990). Meanwhile, organisms also need to evolve strategies to remedy the oxidative damage that occurs. Those machineries include the DNA repair system and protein quality control machinery (Sancar, Lindsey-Boltz et al. 2004, Buchberger, Bukau et al. 2010).

Thus, by combining both evolutionary and molecular theories, aging can be viewed as an overall product of balance between maintaining efficient reproduction success and damaging effects of deleterious products. The presence of oxidative phosphorylation as a major source of energy is essential for survival and reproduction in the animals, leads to the production of the deleterious byproducts such as ROS. In addition, living organisms unavoidably face environmental toxic molecules such as oxygen as an oxidant itself. To combat against both endogenous and exogenous stresses, they developed anti-stress systems.

Mitochondrial theory of aging

While the free radical theory of aging (FRTA) explains harmful factors potentially causing aging, it does not specify the mechanism of deterioration that the free radicals are responsible for. As an extension of the FRTA, the mitochondrial theory of aging specifies that mitochondria might be the primary target of ROS. Firstly, ROS are generated simultaneously along the electron transport chain during the production of ATP. They will preferentially attack biomolecules localized nearest to their sites of production, including mitochondrial DNA (mtDNA), proteins and phospholipids. Secondly, the mitochondrion does not have sophisticated machineries compared to the cytosol of the cell to repair damaged mtDNA nor for protein quality control. As a result, damage to mtDNA and direct modification of OXPHOS complex proteins by ROS, would affect the function of mitochondria. Eventually the mitochondria become less efficient and produce even more ROS. Thus a vicious cycle could be formed to amplify the damaging effects of ROS. With aging, mitochondrial function declines, which can no longer provide sufficient "power" for other essential cellular events, therefore eventually leading to senescence.

However, the mitochondrial theory of aging needs more direct experiments to be supported or dismissed (Jacobs 2003). In addition, during aging, biomolecules in other compartments are also unavoidably modulated by ROS. Even though these damage/modifications may not affect lifespan per se, they have the potential to pathologically contribute to many age-related diseases. In other words, ROS-mediated

modification of biomolecules beyond mitochondria may affect health-span if not lifespan.

Mitohormesis theory

Caloric restriction has been reported to efficiently extend lifespan in a remarkable range of organisms from yeast, worms and flies to rodents, although it is still controversial in primates (Heilbronn and Ravussin 2003, Colman, Anderson et al. 2009, Mattison, Roth et al. 2012). The mechanism underling caloric restriction is still a subject of debate (Koubova and Guarente 2003). According to the free radical theory of aging, one major effect of caloric restriction is through repressed mitochondrial respiration to reduce the generation of ROS and extend lifespan. However, recent studies showed that under caloric restriction, especially glucose restriction, mitochondrion respiration is enhanced due to utilization of lipid through fatty acid β-oxidation. As a result, the organism has an increased production of ROS, which is necessary for the lifespan extension, because eliminating ROS with antioxidants abolishes the lifespan extension (Schulz, Zarse et al. 2007, Ristow and Zarse 2010). The concept of adaptive response induced by the detrimental molecules is called hormesis, and because this specific phenotype is related to mitochondrial respiration, it is referred as mitohormesis (Yun and Finkel 2014).

Superficially, it seems that the mitohormesis theory is contradictory to the free radical theory of aging, which states that the free radicals attack the biomolecules and

cause aging. However, the mitohormesis phenotype may actually still serve as evidence that free radicals are eventually detrimental to the organism, because anti-stress (including oxidative stress) responses provoked by ROS finally benefits the biological system (Liochev 2013). Therefore, the mitohormesis phenotype might still be supporting the free radical theory, although it adds one more dimension of the function of ROS due to the complexity of the biological system. It also raises the question of how to properly use antioxidants as nutrient supplements.

In this regard, ROS can function not only as damage inducers but also as physiological signaling molecules that are involved in the physiological regulation. These physiological processes include oxygen sensing, regulation of vascular tone, enhancement of several important signal transduction pathways such as EGF and insulin signaling, and immunological function to defend against pathogens (Droge 2002). ROS involved in the physiological regulation are majorly generated through NAD(P)H oxidase located on the membrane. More importantly, treatment of organisms with moderate amount of oxidant such as hydrogen peroxide promotes the protective responses against oxidative stress (Yang and Hekimi 2010).

C. elegans **as a model organism to study aging**

The nematode *Caenorhabditis elegans* (*C. elegans*) emerged as an experimental model organism after two publications by Sidney Brenner on its remarkable basic genetic features (Brenner 1974, Sulston and Brenner 1974). Due to its relatively small

genome size, transparency, sensitivity to mutagenesis/RNAi, mapped cell lineage and neuromuscular connections, and self-reproducing properties (hermaphrodite), studies using *C. elegans* have contributed significantly to understand variety of basic biological processes such as RNA interference, apoptosis, developmental and behavioral mechanisms.

Additionally, due to its relatively short lifecycle and lifespan, *C. elegans* is an ideal organism to study these processes. Long-lived or short-lived mutants can be identified either through mutagenesis or RNAi to reveal the mechanisms of the aging process. Staring from fertilized embryo, it takes about two days for a worm to go through four larval stages and reach adulthood. The adult worm can live about 10 to 20 days under standard laboratory conditions. During development, dauer, an alternative of larval stage three, can be formed due to a harsh environment such as lack of food (Cassada and Russell 1975).

Mutagenesis of worms helps to identify genes involved in aging regulation. During the early 1980s, scientists used a mutagenesis screen to look for long-lived worms and recovered several candidate strains. However, those strains also displayed eating defects, suggesting that caloric restriction, rather than more specific genetic factors, were solely responsible for lifespan regulation. Later, through several rounds of outcrosses, *age-1(hx546)* was obtained without a feeding defect. These mutants had a longer lifespan but lower fertility, due to an additional mutation in the strain (Friedman and Johnson 1988) (also see review in (Kenyon 2011)).

It was not until 1993, when Cynthia Kenyon published in *Nature* that *daf-2* mutants can live twice as long as wild type (Kenyon, Chang et al. 1993), that studies on the molecular pathways regulating aging started to grow exponentially. *daf-2(1370)* is a dauer-constitutive mutation, which promotes dauer formation independent of food availability or population density at a non-permissive temperature. Dauer, as an alternative third larval stage, can survive almost 120 days, which is 10 times longer than wild type. After bypassing the critical period to form dauer, the *daf-2(e1370)* mutant will develop into adult, and live a significantly longer lifespan (Kenyon, Chang et al. 1993). This discovery opened up the door for identification of pathways and mechanisms involved in lifespan regulation. Analogous to the pathway that regulates dauer formation, *daf-16* is required for *daf-2(e1370)* lifespan extension (Kenyon, Chang et al. 1993, Lin, Dorman et al. 1997). Later, scientists found that the extension of lifespan in *age-1* mutant also requires *daf-16* (Dorman, Albinder et al. 1995).

The molecular cloning of *age-1*, *daf-2* and *daf-16* was very informative and indicated that manipulating a conserved nutrient sensing pathway, the insulin/IGF-1 pathway, could delay aging at least in term of lifespan. AGE-1 is a phosphatidylinositol 3- kinase, a known down stream target of insulin/IGF pathway (Morris, Tissenbaum et al. 1996). *daf-2* encodes a insulin/IGF-1 receptor (Kimura, Tissenbaum et al. 1997) and DAF-16 is a FOXO transcriptional factor (Lin, Dorman et al. 1997, Ogg, Paradis et al. 1997). Both genetic screens and molecular cloning connect all three molecules to a conserved linear phosphorylation cascade pathway, insulin/IGF-1 signaling. The details

of this signaling pathway and its potential mechanisms in lifespan regulation are discussed in the following sections.

Signaling pathways involved in aging regulation

Extensive studies on aging since 1993 indicate that nutrient sensing signaling pathways, which ultimately alter metabolism and stress responses, potentially regulate longevity. Caloric restriction promotes longevity through modulation of the nutrient sensing signaling pathways. Although the metabolic shifts are complicated, it seems that extension of lifespan is usually correlated with enhanced responses to stresses including oxidative damage, thermo-stress and microbial stresses. In the following sections, I briefly review several known molecular pathways that regulate aging (Figure 1).

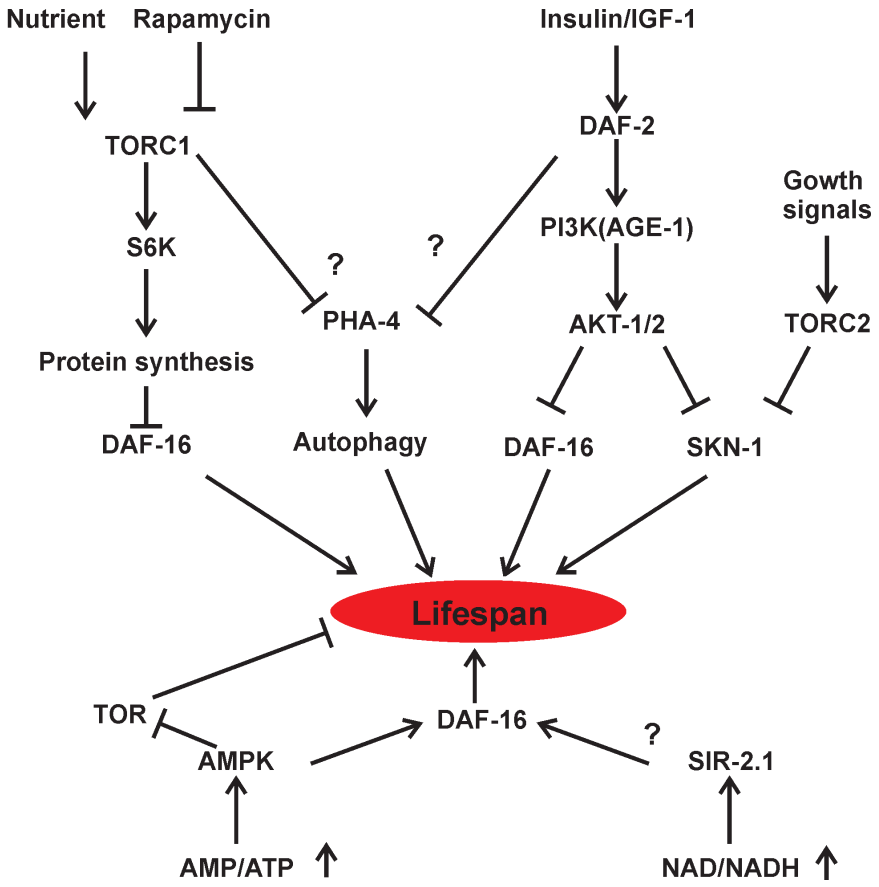

Figure 1 Molecular signaling pathways involved in lifespan regulation and potential interactions between them.

Reducing Insulin/IGF-1 signaling extends lifespan in animals ranging from worms to mammals

Since the discovery that reducing insulin/IGF-1 signaling (IIS) extends the lifespan in *C. elegans*, studies have determined the mechanisms involved. Sequencing results indicated that DAF-2 is the only insulin/IGF-1 receptors in *C. elegans*. DAF-2 displays more than 30% similarity to the human insulin receptor, IGF-1receptor, and

insulin-receptor related receptor (Kimura, Tissenbaum et al. 1997, Pinkston-Gosse and Kenyon 2007). Upon activation by insulin/IGF-1, DAF-2 initiates a phosphorylation cascade mediated by PI3K (AGE-1) and AKT-1/AKT-2, which eventually phosphorylates DAF-16 and inhibits its translocation into nuclei (Kenyon 2005). However, in *daf-2* mutants, hypophosphorylated DAF-16 can enter the nuclei to function as a transcription factor, which initiates the transcriptional program for dauer formation and lifespan extension.

Determining the transcriptional targets of DAF-16 is critical to decipher the mechanism of lifespan extension in worms with reduced IIS. Through comparing the mRNA levels in *daf-2(-)* and *daf-16(-)*, Murphy and colleagues found that two sets of genes (about 100 genes in total) are potentially regulated by this signaling pathway (Murphy, McCarroll et al. 2003). Genes up-regulated in *daf-2(-)* but repressed in *daf-16(-)* might contribute to the extension of the lifespan (Category I). Conversely, those genes with the opposite profile might shorten the lifespan (Category II). It turns out that many stress-response genes such as mitochondria superoxide dismutase (*sod-3*), *mtl-1*, catalase (*ctl-1* and) and small heat shock proteins fall into the category I set of genes, potential candidates for the organism to defeat the free radicals and extend lifespan. Additionally, in *daf-2(-)* animals, other potential lifespan effectors such as antimicrobial genes are also up-regulated in a *daf-16*-dependent manner. Many insulin-like genes belong to category II, indicating a positive feedback loop to amplify IIS.

One prominent metabolic enzyme up-regulated in *daf-2(-)* is isocitrate lyase/malate synthase (GEI-7), which is a critical enzyme involved in the glyoxylation

cycle. Metabolic Shift from the TCA cycle towards the glyoxylation cycle, an anabolic pathway, is necessary for the lifespan extension in *daf-2(-)*. This may occur through reducing the ROS generation from oxidative phosphorylation (Shen, Song et al. 2014).

Similar to DAF-16, another transcriptional factor, SKN-1, is also under the direct regulation of IIS signaling. SKN-1 functions in parallel to DAF-16 to extend lifespan, although DAF-16 is the prominent effector of lifespan extension in *daf-2(-)*.

The subset of target genes regulated by SKN-1 includes many genes involved in the detoxification process such as glutathione-S-transferase (Tullet, Hertweck et al. 2008). In contrast to DAF-16, SKN-1 is not required for dauer formation in the IIS reduced worms.

In summary, the insulin/IGF signaling pathway regulates the expression of both metabolisc and stress-response genes. Thus this signaling pathway potentially affects aging through regulation of oxidative stress. This regulation occurs through the modulation of metabolism to reduce the generation of ROS as well as through the reduction of ROS via scavenger enzymes.

TOR pathway regulates aging

Another well-studied pathway that regulates aging is the TOR pathway. This conserved nutrient-sensor TOR (Target of Rapamycin) kinase integrates nutrient availability with regulation of growth in two protein complexes: TORC1 and TORC2. Under rich nutrient conditions, activated TORC1 promotes protein synthesis and inhibits

autophagy, while TOCR2 is activated through growth signals and activates kinases including AKT and SGK, which are also under the regulation of IIS (Loewith 2011). Both genetic and pharmacological inhibition of TOR activity after critical development periods promotes lifespan extension in yeast, nematodes, fruit flies and rodents (Kapahi, Chen et al. 2010). It is possible that the effect of lifespan extension by inhibition of TOR signaling is mediated through both inhibition of protein synthesis and promotion of autophagy. Inhibition of mRNA translation extends lifespan in a DAF-16 and SKN-dependent manner, which is similar to reducing TORC1 activity (Pan, Palter et al. 2007). However, extension of lifespan by reducing TORC2 requires SKN-1 but not DAF-16. Although both TOR and IIS converge on the transcriptional factors SKN-1 and DAF-16, the molecular mechanisms might differ (Robida-Stubbs, Glover-Cutter et al. 2012).

Autophagy is a process that recycles biomolecules and organelles. It is reported that dietary restriction and reduced IIS or TOR signaling promote autophagy, which is necessary but not sufficient to promote lifespan extension (Hansen, Chandra et al. 2008). DAF-16 is dispensable for the occurrence of autophagy under both dietary and reduced IIS situations. However, the FOXA transcription factor PHA-4 is required to promote autophagy during dietary restriction but not in response to reduced IIS (Hansen, Chandra et al. 2008). PHA-4 also up-regulates genes that encode ROS scavengers including *sod-1*, *sod-2*, *sod-4*, and *sod-5*, but not *sod-3*, which is a well-established target of DAF-16 (Panowski, Wolff et al. 2007). In addition, similar to dietary restriction, both reduction the function of TOR and its target S6 kinase promote lifespan mediated by the PHA-4 (Sheaffer, Updike et al. 2008). These results suggest that nutrients might activate TOR

signaling and its downstream effector S6 kinase to antagonize the activity of PHA-4, which is essential to promote stress response and autophagy. The mechanism of PHA-4 regulation requires further study.

AMPK increases lifespan

Another energy sensor, AMPK, is activated by a high AMP/ATP ratio, which is usually the consequence of dietary restriction. Indeed, AMPK is the mediator of lifespan extension in one dietary restriction regime, in which the dietary restriction is initiated at the fourth day of *C. elegans* adulthood. In this situation, both AMPK and DAF-16 are required to promote lifespan extension, although enhanced DAF-16 nuclear localization is not observed. This resembles the effect induced by inhibition of TORC1, raising the possibility that AMPK regulates TORC1. If this is true, it would be consistent with mammalian studies, in which AMPK inhibits the function of mTOR via direct phosphorylation (Bolster, Crozier et al. 2002). Additionally, *in vitro* experiments showed that AMPK directly phosphorylates DAF-16 at non-AKT sites, potentially enhancing the transcriptional function of DAF-16 (Greer, Dowlatshahi et al. 2007).

Sirtuin proteins and aging

Sirtuin proteins, a class of histone deacetylase, are involved in metabolic regulation, stress responses and potentially in aging-related diseases (Houtkooper,

Pirinen et al. 2012). The first sirtuin discovered was silent information regulator 2 (SIR2) in yeast. Overexpression of SIR2 extends replicative lifespan possibly through its function as a regulator of transcriptional silencing of mating-type loci, telomeres and ribosomal DNA (rDNA). SIR2 prevents the homologous recombination that occurs during rDNA replication and thus reduces the formation of extrachromosomal rDNA circles. Such circles promote replicative aging (Kaeberlein, McVey et al. 1999). Later, Sir2 was identified as a NAD^+-dependent histone deacetylase, consistent with its role in silencing the heterochromatin through deacetylating specific sites in histones (H3K9 and H4K16) and thus compacting the chromosome to limit the access of the transcriptional factors (Imai, Armstrong et al. 2000).

Although whether overexpression of the invertebrate sir2 ortholog extends lifespan is still under intensive debate (Burnett et al., 2011; Viswanathan and Guarente, 2011), sirtuin family proteins have been shown to regulate glucose and fat metabolism (Houtkooper et al. 2012). In addition to histones, many metabolism regulators such as FOXO, PGC-1 α and PPAR-α are the substrates of sirtuin proteins (Morris 2013). Thus, sirtuin can potentially integrate metabolic status through NAD^+/NADH with the global regulation of chromosome structure with adaptive metabolism alteration through modulation of FOXO, PGC-1 α and PPAR-α.

During fasting, an increased NAD^+ level activates SIRT1, the mammalian ortholog of Sir2. Activation of SIRT1 promotes PCG-alpha mediated gluconeogenesis gene expression to increase the secretion of glucose from the liver to maintain the glucose level in the blood stream. Meanwhile, SIRT1 inhibits glycolysis through PCG

and promotes fatty acid-β oxidation by activating PPAR-alpha. However, the role of SIRT1 in gluconeogenesis is controversial and requires further investigation (Liu, Dentin et al. 2008, Wang, Kim et al. 2011).

In addition to the regulation of metabolism, sirtuin proteins mediate an oxidative stress response by regulating antioxidant gene expression through transcription factors such as FOXO in a 14-3-3 (a scaffold protein)-dependent manner (Berdichevsky et al., 2006; Merksamer et al., 2013; Webster et al., 2012). Overall, sirtuin proteins could be involved in age-related diseases, such as type II diabetes and neurodegenerative diseases (Houtkooper et al., 2012; Satoh et al., 2011).

Although several signaling pathways are well characterized for regulation of lifespan, precise mechanisms of aging are unknown. Characterization the behavior decline and its underling mechanism will help to understand how organisms age.

Locomotion deterioration during aging

The decline of neuromuscular function that accompanies aging contributes to declines of performance in typical behaviors (Herndon, Schmeissner et al. 2002, Huang, Xiong et al. 2004). A relatively well-studied model for behavioral decline is *C. elegans* locomotion behavior (Huang, Xiong et al. 2004, Murakami, Bessinger et al. 2008, Iwasa, Yu et al. 2010). On a solid culture surface, *C. elegans* exhibits sinusoidal body movement by alternating the contraction and relaxation of the dorsal and ventral body wall muscles. The pattern of body wall muscle contraction is produced by interactions

between cholinergic excitatory and GABAergic inhibitory motor neurons (McIntire, Jorgensen et al. 1993). A young adult animal moves continuously and exhibits a well-coordinated, sinusoidal pattern. As it ages, the body movements become progressively less continuous and coordinated (Herndon, Schmeissner et al. 2002, Huang, Xiong et al. 2004). Together with acetylcholine (Ach) and GABA, the neurotransmitters dopamine and serotonin regulate basal slowing of non-starved animals in response to food and enhanced slowing when the animal encounters food after starvation, respectively (Sawin, Ranganathan et al. 2000, Chase, Pepper et al. 2004). During aging, the basal slowing response is increased so that the difference between the two slowing responses is diminished (Murakami, Bessinger et al. 2008). In a liquid environment, *C. elegans* displays an alternative thrashing/swimming locomotory pattern (Pierce-Shimomura, Chen et al. 2008). As the animals age, the frequency of body wall muscle contractions during swimming is also decreased (Schreiber, Pierce-Shimomura et al. 2010).

Reduced rate of motor decline during aging is used as a proxy in *C. elegans* to identify molecular pathways that extend lifespan. Interventions that extend lifespan usually delay the behavior deterioration that occurs during aging. For instance, reducing insulin-like signaling by limiting the activity of the insulin-like receptor, *daf-2*, can prominently extend lifespan and delay locomotory deterioration (Huang, Xiong et al. 2004). By performing RNAi screens for aged animals with improved locomotory vigor in liquid, researchers have identified novel molecules that modify the rate of behavioral aging. Down-regulation of EGF signaling, through mutation of the two negative regulators, HPA-1 and HPA-2, or knocking down the level of RAS-related Rag GTPase

RAGA-1, can delay the age-related deterioration of swimming ability (Iwasa, Yu et al. 2010, Schreiber, Pierce-Shimomura et al. 2010). These studies identified molecules that attenuate age-related locomotory decline. However, much less is known about the cellular mechanisms that result in this decline in the first place.

Research has shown that locomotory decline in *C. elegans* is associated with sarcopenia in body wall muscles, characterized by deformed nuclei and muscle fiber loss and disorganization (Glenn, Chow et al. 2004). This might explain the decline of behavior at an advanced age. However, the behavior begins to decline at an early phase of aging, when there is no significant loss of muscle fibers (Glenn, Chow et al. 2004, Murakami, Bessinger et al. 2008) or significant neuronal morphology change (Herndon, Schmeissner et al. 2002, Tank, Rodgers et al. 2011). Manipulation of serotonin signaling suppresses the increased basal slowing response at adult day 4 prior to the timing of sarcopenia onset (Glenn, Chow et al. 2004, Murakami, Bessinger et al. 2008). This raises the possibility that the physiological state, which determines the timing and coordination of muscle contraction, might be suboptimal prior to the onset of significant muscle contractile apparatus damage. However, locomotion behavior might be not sensitive enough to subtle physiological changes that occur prior to the deterioration of muscle structure, such that it won't help to uncover these changes contributing to behavioral decline.

The neuromuscular basis of male mating behavior in *C. elegans*

Relative to other general *C. elegans* behaviors such as pharyngeal pumping and locomotion behavior, the complexity of male mating might render it less robust as the male ages and the physiology of neuromuscular circuits change. The cellular and molecular mechanisms that regulate the spicule insertion step of mating are well described, allowing one to monitor the physiology and functional changes in the circuit as the male proceeds through adulthood (Gruninger, Gualberto et al. 2006, LeBoeuf, Gruninger et al. 2007, Gruninger, Gualberto et al. 2008, LeBoeuf, Guo et al. 2011, Liu, LeBeouf et al. 2011). *C. elegans* has two sexes, hermaphrodite and male. Male worms display a complex but stereotypical courtship behavior. *C. elegans* male mating requires precise coordination between the male genital muscles, in conjunction with the locomotion neuromuscular circuit, to be executed successfully.

Mating behavior can be divided into the following steps: response, backing, turning, vulva location, spicule insertion and sperm transfer (Figure 2A) (Liu and Sternberg 1995). The backing and vulva location behaviors are initiated and executed by tail-located sensory neurons (ray neurons in Figure 2B). When the male's fan-shaped tail contacts the cuticle of the hermaphrodite, he uses the locomotory neuromuscular circuit to move backwards, scanning along the hermaphrodite cuticle for the vulva. If the male fails to locate the vulva on one side, he will make a turn at the end of the hermaphrodite and continue backwards locomotion. Once he encounters the vulva, the sensory neurons including HOA, HOB, PCA, PCB, PCC and ray neurons are activated, the male will then

contract sex-specific muscles located in his tail, in order to press against the vulval lips. He then repetitively prods the vulva with his spicules, in an attempt to breach the tight vulval slit. This is achieved through rapid contractions of the protractor muscles, which are attached to his spicules (Figure 2B). Once his spicules partially penetrate the vulva, the protractor muscles fully contract allowing complete spicule insertion. Sperm transfer occurs following full spicule penetration (Barr and Garcia 2006, Liu, LeBeouf et al. 2011).

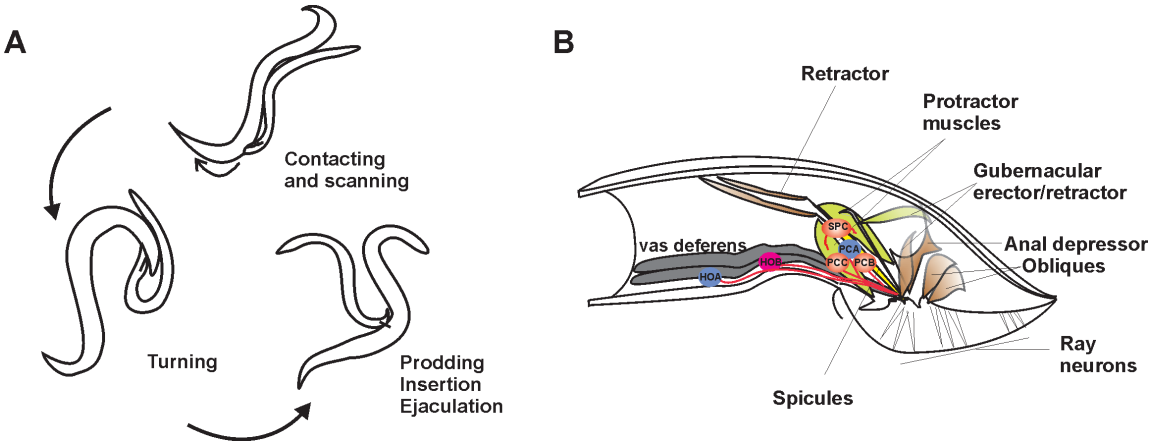

Figure 2 Male mating behavior and its neuromuscular basis.
(A) A cartoon diagram of stereotypical steps during *C. elegans* male mating; (B) A simplified neuromuscular basis for the male mating behavior.

Not only is the cellular basis of the mating behavior well described, but also the molecular basis has been explored in our lab. Acetylcholine (ACh) is the main excitatory neurotransmitter for spicule protraction. Cholinergic neurons PCB and PCC sense the vulva and cause contraction of the oblique muscles. As a result, the male can maintain his tail on the vulva, at the same time, the oblique muscles communicate with spicule

protractors via gap junction to promote spicule prodding. (Figure 2B). The prodding behavior requires a sarcoplasmic reticulum calcium channel during twitching of the spicule protractor muscles (ryanodine receptor, UNC-68). ACh released from cholinergic SPC neurons activates acetylcholine receptors (AChR) expressed on the membrane of the protractor muscles to promote their full contraction and spicule protraction. Different from twitching of the protractor muscles, the tonic contraction of these muscles requires a L-voltage gated calcium channel/EGL-19 (Garcia, Mehta et al. 2001). We also characterized an ERG-like voltage-gated potassium channel /UNC-103 that plays a role in the regulation of the excitability of the mating circuitry. Loss of function of UNC-103 leads to the spontaneous contraction of the protractor muscles and permanent protraction of spicules (referred to as prc phenotype) in 30% of the male population even in the absence of hermaphrodite. Food deprivation suppresses the prc phenotype by up-regulation of EAG-potassium channel (EGL-2) in a CAMKII/UNC-43 dependent manner (LeBoeuf, Gruninger et al. 2007, LeBoeuf, Guo et al. 2011, LeBoeuf and Garcia 2012). Utilizing prior knowledge we obtained previously, I will explore the effects of aging on the mating circuitry and behavior as described in the Dissertation objectives.

Dissertation objectives

Most studies in the aging field focus on the regulation of lifespan but ignore the physiological alterations that occur prior to drastic morphology deterioration during aging. *C. elegans* male mating behavior, a complex but stereotyped behavior, might be

sensitive to such physiological alterations and provide an efficient assay for fertility failure. In this dissertation, I used *C. elegans* male mating behavior as my model to characterize the molecular and cellular mechanism(s) underlying the behavioral deterioration that occurs during early aging.

Chapter II includes the detailed materials and methods I used throughout this research. In Chapter III, I first characterized the dynamics of the deterioration rate of wild type male mating behavior using a mating potency assay. Surprisingly, this complex behavior starts to decline in early adulthood, when no obvious morphology changes such as muscle disorganization or sperm dysfunction are observed. Direct observation of mating behavior suggested that the decline of mating potency is due to behavioral defects including ectopic prodding at non-vulva regions and abnormal turning. Through pharmacological tests and calcium imaging, I determined that during aging, the excitability of the mating circuitry is increased. It is possible that the hyper-excitability in the mating circuitry potentially causes the mating defects during aging.

To further illustrate the correlated relationship between the hyper-excitability and mating behavior decline, in Chapter IV, I addressed the question of reducing the excitability to delay mating behavior deterioration. Using a quadruple heterozygous mutant stain of ACh receptors genes, which mediate the sensitivity to mating cues, improves mating at the third day of adulthood, when significant deterioration in mating ability is first observed. Another intervention I used is caloric deprivation during the L4 stage that immediately proceeds adulthood. Caloric restriction has been shown to suppress the *unc-103* and *slo-1* loss of function induced prc phenotype. Indeed, transient

food deprivation for about 20 hrs as the male matures into an adult also significantly increases the mating ability of 3-day-old males. These males display a reduced neuromuscular excitability but not a prolonged lifespan. I further determined that the long-term effects of transient starvation on mating potency require protein synthesis during early adulthood, and the proteins synthesized that are potentially important for this are potassium channels.

To determine the underlying mechanism(s) of increased excitability in the mating circuitry during aging, in Chapter V, I studied the function of a metabolism regulator SIR-2.1 in maintaining male mating behavior during aging. *sir-2.1(0)* males display a premature mating behavior decline compared to wild type males. At day 2 of adulthood, *sir-2.1(0)* males' mating ability drops significantly due to an ejaculation deficiency but not a short lifespan, muscle disorganization, or sperm dysfunction. I further demonstrated using calcium imaging and pharmacological drug tests that the ejaculation might be the consequence of the hyper-excitability and hypertonic contraction of the dorsal sex muscles, which pinch closed the vas deferens.

Inspired by the free radical theory of aging and complicated roles of SIR-2.1 in metabolism regulation and anti-oxidative stress, I tested whether ROS plays a role in altering the excitability of the mating circuitry. First, I confirmed that *sir-2.1(0)* males are more sensitive to oxidative stress induced by a ROS-generator, paraquat. Then I showed that manipulation of the ROS level does affect mating efficiency and the excitability of the mating circuitry. Boosting ROS by paraquat significantly reduces mating in even 1-day-old wild type males in a dosage-dependent manner, accompanied

by an increase in the excitability of the mating circuitry. Meanwhile, reducing ROS levels by feeding worms with an antioxidant (N-acetyl-cystein) can promote mating ability.

In the last part of Chapter V, using qPCR and metabolic measurements, I demonstrate that both catabolism and anabolism processes in *sir-2.1(0)* are possibly up-regulated. However, the anti-oxidative stress system is compromised. Combining all these data, I propose that enhanced catabolism promotes the generation of ROS, and with a compromised anti-oxidative stress system, *sir-2.1(0)* males suffer more oxidative stress, which increases the excitability of the mating circuitry and reduces mating ability. Interestingly, the anabolic pathway which is responsible for the synthesis of fat is also increased in *sir-2.1(0)* males. This increased anabolic level serves as a sub-optimal compensation mechanism to shunt pyruvate to fat synthesis and away from the oxidation through TCA and OXPHOS.

In chapter VI, I summarized all the experiments and conclusions from Chapters III-V, and also discussed our results, significance of the discoveries, implications and potential future directions.

CHAPTER II

EXPERIMENTAL PROCEDURES

Strains and medium

Worms were grown at 20°C on nematode growth media (NGM) plates seeded with *E. coli* strain OP50, except for the *pha-1(e2123)* strain, which was maintained at 15°C (Schnabel and Schnabel 1990). The alleles used in this work include: *lite-1(ce314)* (Edwards, Charlie et al. 2008) on LGX; *unc-29(e193)*, *pck-2(ok2586)* on LGI (Lewis, Wu et al. 1980); *pck-1(ok2098)*, *pha-1(e2123)* (Schnabel and Schnabel 1990), *unc-64(e240)* (Brenner 1974) and *unc-103(n1213)* (Park and Horvitz 1986) on LGIII; *sir-2.1(ok434)* on LGIV; *him-5(e1490)* (Hodgkin, Horvitz et al. 1979), *acr-18(ok1258)*, *gar-3(gk305)* (Liu, LeBoeuf et al. 2007), *acr-16(ok789* and *egl-2(rg4)* (LeBoeuf, Gruninger et al. 2007) on LG V. Males containing only the *him-5(e1490)* mutation are referred to as wild type; *him-5(e1490)* males have been shown to mate efficiently as wild type (Hodgkin 1983). All the *ok* alleles were generated by the *C. elegans* Gene Knockout Consortium (Oklahoma). *sir-2.1(ok434)*, *pck-1(ok2098)* and *pck-2(ok2586)* animals were out-crossed 4 times with the *him-5(e1490)* strain. The deletions in those three mutants were detected through PCR using primers listed in the appendix A.

Modified media used here included NGM medium containing glucose (2%), paraquat, N-acetyl-cystine (NAC) (Sigma, MO), and nicotinamide (Nam) (Sigma, MO) respectively. The latter three were added at the indicated concentration when the

temperature of the medium cooled to about 60°C. *E. coli* OP50, used for the special medium containing glucose and NAC, was UV-killed and concentrated to make sure the worms were not food deprived. To assay the effects of translational inhibition on male behavior, cycloheximide (Sigma, St. Louis, MO), freshly dissolved in water at a concentration of 20 mg/ml, was added to the surface of NGM plates with or without *E. coli*. The drug was allowed to soak into the plate overnight. The final concentration of cycloheximide in the plates was 250 μg/ml. This concentration was either lethal to larvae or stalled larval growth. If the CHX treatment was required in an experiment, I waited until the males molted from their L4 cuticle, and then immediately placed them on the CHX-containing plates (LeBoeuf, Guo et al. 2011). I assume that the animals ingested these compounds as they feed on *E. coli* or absorbed them through their cuticle.

Mating potency assay

Males were isolated from non-crowded plates, either at the late L4 stage (when cells in the male tail spike have completely migrated anteriorly) or for the cycloheximide assays (see below), after they newly crawled out of their L4 molt. They were kept individually or in groups of 20-30, on 1-2 cm diameter lawns of bacteria. ~30% of males that contain the *unc-103(n1213)* deletion (referred to as *unc-103(0)*) and ~70% of males that contain both the *unc-103(n1213)* and *egl-2(rg4)* deletions (the *egl-2(rg4)* deletion is referred to as *egl-2(0)*), respectively, display the constitutive protracted spicule phenotype (LeBoeuf, Gruninger et al. 2007, LeBoeuf, Guo et al. 2011). Exceptional

males, which did not display the abnormal behavior, were used for the mating potency assays. L4 *pha-1(e2123)* hermaphrodites were isolated and grown at 20°C, one day before the mating potency assays were conducted. A 1-day-old *pha-1* hermaphrodite and an adult male were picked to 5 mm diameter lawns (10 μl of *E. coli*, grown overnight in LB media at 37°C without aeration, was spotted onto an 3.5 mm NGM agar plate to make a 5 mm diameter lawn) to increase the chance of mating. I scored the male as sexually potent if 2-3 days later the plate contained cross-progeny. The 0- or 1-day-old males' mating potency under well-fed or starved conditions was set as 100%, and the normalized percentages of potent males were calculated as follows: normalized percentage of potent males = (non-normalized percent of potent (n)-day-old males/ non-normalized percent of potent 1-day-old males) X 100%.

If males were required to be starved, they were serially transferred to *E. coli*-less NGM agar plates, using a mouth pipette and water as a vehicle. To inhibit bacterial growth, the plates contained streptomycin at a final concentration of 30 μg/ml. An 8 M glycerol ring was applied to the edge of the agar to discourage males from crawling and desiccating on the sides of the plates. After the starvation period, males were transferred, using a worm pick, to NGM plates containing *E. coli*.

Lifespan and stress resistance assays

L4 males were isolated from non-crowed plates and raised 20-30 per plate. The males that can respond to gentle touch with a platinum wire were counted and transferred to new plates every day. Males that dried on the wall of the Petri plate were censored from the assay on the day they died. Log-rank (Mantel-Cox) test was used to analyze the lifespan curves

To assess males' sensitivity to paraquat, L4 males were transferred to plates containing 10 mM paraquat and scored at 24 and 48 hrs.

Sex muscle fiber observation

To visualize the structure of the sex muscles during aging, I made transgenic *C. elegans* expressing actin tagged at the N-terminus with YFP. The plasmid was constructed as follows: I PCR-amplified the 1.2kb genomic sequence of *act-1*, using primers that contained homologous sequences around the insertion position (next to the YFP gene) of the vector pGW322YFP. The primers that were used to linearize the plasmid pGW322YFP removed the stop codon of the YFP gene. The primers used were included in the Appendix A. The linearized pGW322YFP vector was then recombined with *act-1* DNA, using the Clontech in-fusion enzyme kit (Clontech, Mountain View, CA), to produce the plasmid pXG30. pXG30 additionally contains the gateway cassette in front of the YFP: actin gene fusion. To express YFP:actin in the sex muscles, I

replaced the gateway cassette with the *unc-103*E promoter (Reiner, Weinshenker et al. 2006) in pXG30, using LR clonase (Invitrogen, Carlsbad, CA), to construct the plasmid pXG31. 50 ng/µl of pXG31 and 150ng/µl pUC18 were injected into *him-5(e1490)* males. The transgenic L4 males were picked and visualized at different ages using fluorescence microscopy.

Staining sperm with SYTO-17

The red fluorescence dye SYTO-17 (Invitrogen, Eugene, OR) was diluted in a solution of 100 mM NaCl, 50 mM KH_2PO_4, 3 mM $CaCl_2$, 3 mM $MgSO_4$ to a final concentration of 70 µM. Males were incubated in 1 ml of the dye for three hours in the dark at 20°C. They were then removed from the dye and paired with hermaphrodites. The next day, sperm in the hermaphrodite's spermatheca were visualized by fluorescence microscopy.

***In vitro* sperm activation assay**

Sperm activation assays were accomplished as described (L'Hernault and Roberts 1995, Smith and Stanfield 2011). Briefly, three 2-day-old males, isolated at L4 stage, were cut at the posterior portion with a needle in 20µL sperm media (50mM Hepes, pH7.0, 45mM NaCl, 25 mM KCl, 1 mM $MgSO_4$, and 5 mM $CaCl_2$) on a slide. This media is freshly supplemented with polyvinylprolidone (PVP) 40,000 molecular weight

(Sigma, MO) and the activator pronase (Roche) at the final concentration of 10 mg/mL and 500 μg/mL. A coverslip with a thin layer of Vaseline applied around the edge was put on the top of the sperm media to form a chamber over the sperm. After 5 min incubation, activated sperm with pseudopods and inactive ones were counted using a compound microscope fitted with a 100X objective. ~50-60 sperm cells were counted in each sample section.

Drug-induced spicule protraction

To assay agonist-induced spicule protraction, I dissolved the acetylcholine agonists levamisole (ICN Biomedicals, Aurora, OH) and arecoline in water to make a stock solution of 1 mM and 100 mM respectively. I then serially diluted the stock solution in water as needed. 1 ml of the drug was added to a three well round-bottom Pyrex titer dish. Five to ten males were then transferred to the drug bath. The males were observed for five minutes at 20°C with a stereomicroscope; they were considered responsive to the drug if their spicules remained protracted for ≥ 5 seconds. Drug baths were changed after 30 males were observed.

Assessment of mating behaviors

Mating behavior was observed from two to five minutes for each male using a dissecting stereomicroscope. Ten 2-day-old *unc-64(e246)* adult hermaphrodites were

placed on a 5 mm diameter bacterial lawn. Using a mouth pipette and water as a vehicle, a male was transferred in the center of the hermaphrodite group. A hand-held timer or a lab-written Microsoft Excel macro was used to record certain aspects of mating behavior (Liu, LeBoeuf et al. 2007). During mating, the following parameters were recorded: when and how many times a male contacted a mate after he was introduced to the bacteria; how long he required to turn successfully at the end of a mate after he initiates the mating sequence; when and how many times a male contacted the vulva; when and how long the male spent attempting to insert his spicules. A different population of males was used to obtain data for each behavioral metric. The efficiency of spicule insertion, E_{SI}, was calculated from recordings made during the first 120 seconds of contact between the male and the hermaphrodite. If the male successfully inserted his spicules before the 120 seconds were over, then the observation was stopped. E_{SI} = (time (sec) spent at spicule insertion attempts / total time (sec) in contact with hermaphrodite, up to 120 sec) X (1/time (sec) in contact with the hermaphrodite, such as backing or turning, but not attempting insertion) X (1+ (0 if no penetration, otherwise time (sec) remaining after a successful penetration / 120 sec)). A hypothetical E_{SI} of 1.99 would mean that the male located the vulva and inserted his spicules approximately 1 sec after contact with the hermaphrodite; whereas a hypothetical E_{SI} of 0.0 meant that the male spent his first 120 seconds contact but not attempting spicule insertion.

The ability of males to sense the vulva was calculated by counting the number of times he stopped at the vulva divided by the total number of times he stopped and/or passed by the vulva. The turning quality was calculated as: the number of smooth turns

(defined as the male tail keeping contact with hermaphrodite and turning without hesitation) divided by the total number of turns. Ejaculation assays were conducted in two ways. I directly observed sperm transfer after spicule insertion, and determined if sperm drained into the *unc-64(e240)* hermaphrodite's uterus. Additionally, cross-progeny were counted 1-2 days after spicules insertion.

Ca^{2+} imaging

The genetically encoded calcium indicator G-CaMP1.3 was used to visualize calcium transients in the male sex muscles. The Gateway reading frame cassette B (Invitrogen) was blunt-end cloned into the XbaI site of plasmid pTG29, a previously described vector containing the GFP-based calcium indicator G-CaMP1.3 (Gruninger, Gualberto et al. 2008). The resulting G-CaMP Gateway destination vector, pTG30 was then recombined using LR clonase (Invitrogen) with the Gateway entry vector pLR22, which contains the *lev-11* promoter (LeBoeuf, Gruninger et al. 2007), to generate the plasmid pTG32. To visualize red fluorescence in the same cells as G-CaMP1.3, the gateway destination vector pGW322DsRed was recombined with pLR22 to generate the plasmid pLR132. pGW322DsRed was generated by cloning an AgeI-SplI fragment containing the mDSred gene from the plasmid pDC68 (a gift from Dr. Daniel Chase, University of Massachusetts) into the AgeI-SplI site of plasmid pGW322YFP (Garcia and Sternberg 2003), replacing the YFP gene with the monomeric DsRed gene.

Transgenic animals were generated using standard protocols (Mello, Kramer et al. 1991). To generate animals that contain G-CaMP expressed in all sex and body wall muscles, an injection mixture containing 10 ng/µl of pTG32, 10 ng/µl of pLR132, 50 ng/µl of pBX1 and 130 ng/µl of pUC18 was injected into *pha-1(e2123);him-5(e1490);lite-1(ce314)* (Granato, Schnabel et al. 1994). A stable transgenic line, which expressed green and red fluorescence at approximately the same intensity, *pha-1(e2123); him-5(e1490); lite-1(ce314); rg*Ex430 [P*lev-11*: G-CaMP; P*lev-11*: mDsRed], was kept for analysis.

Construction of animals that contain G-CaMP expressed in all male sex muscles was previously described (Gruninger, Gualberto et al. 2008). In short, a mixture containing 12 ng/µl of pLR136 (a plasmid containing both G-CaMP expressed from the *unc-103*E promoter and pha-1(+)), 2 ng/µl of pLR132 (a plasmid containing DsRed expressed from the *unc-103*E promoter),50 ng/µl pBX1 and 136 ng/µl of pUC18 was injected into *pha-1(e2123);him-5(e1490);lite-1(ce314)* to generate the line *pha-1(e2123);him-5(e1490); lite-1(ce314; rg*Ex197[P*unc-103*E:G-CaMP; P*unc-103*E:mDsRed].

Transgenic males were separated from hermaphrodites at mid or late L4 stage (a stage when the tail spike hypodermal cells have finished their anterior retraction). At day 1 and day 3, the males were either directly visualized during copulation (without a microscope coverslip), or they were immobilized between a microscope coverslip and 10% (for 1-day-old males) and 8% (for 3-day-old males) Noble agar pads containing Polybead polystyrene 0.1µm microspheres (Polysciences, Inc., WA). Mating and

immobilized animals were visualized on an epifluorescence-equipped Olympus BX51 microscope. Using a 40X long working distance objective, immobilized worms were imaged for approximately 1 minute and mating worms were imaged for about 5 minutes. The G-CaMP and DsRed fluorescence signals at the male tail were recorded, simultaneously, using a Dual View Simultaneous Image splitter (Photometrics, Tucson, AZ) and a Hamamatsu ImagEM Electron multiplier (EM) CCD camera, at the speed of approximately 30 frames per second.

Ca^{2+} data were analyzed using the Hamamatsu Simple PCI (version 6.6.0.0) software and Microsoft Excel, as described previously (LeBoeuf, Guo et al. 2011, Liu, LeBeouf et al. 2011). Four region-of-interests (ROIs), of equal area, were generated in the Simple PCI software. Two of the ROIs were used to measure the background signal in the green and red channels, and two ROIs were used to measure the fluorescence of the male anal depressor muscle and the male spicule protractor muscles in both channels. The mean pixel intensity (MPI) was measured for every ROI in every frame, in each recording. The data were then transferred from Simple PCI to Microsoft Excel. For each frame of the recordings, the values of the background ROIs were then subtracted from their respective ROIs that quantified the muscle fluorescence.

The red channel was used as a reference to analyze the green channel. The fluorescence measured in the red channel, in theory, should not change during the course of the recording. However, flickering of the mercury arc lamp, movement/contraction artifacts and photobleaching will cause fluorescence changes from frame to frame independent of fluorescence changes due to calcium transients. To correct for these

artifacts, in each frame, the background subtracted MPI in the red channel was plotted with respect to time and a one-phase decay curve (to correct for photobleaching) was fitted over the data points using GraphPad Prism (version 4.03). The fitted curve serves as an arbitrary reference to quantify the magnitude of non-interesting fluorescence. For each frame, the background subtracted red channel MPI was divided by the interpolated value to give a correction value. The inverse of that correction value, for each frame, was then multiplied by the subtracted green channel MPI of the respective frame. This corrects the values from the green channel, so that the fluorescence changes reflect calcium transients rather than experimental artifacts. The values for each frame of the recording was then calculated as $\Delta F/F0$ = [(corrected MPI (frame n)-corrected MPI (frame 0(initial frame)))/corrected MPI (frame 0)] X 100. The values were then plotted with respect to time. Generally, photobleaching did not occur in the green channel, but when it was obvious, a one-phase decay curve was fitted over the data points using GraphPad Prism. The inverse proportion of decay, for each frame, was then multiplied to the respective experimental data point to re-adjust the plots.

To compare the data between experiments, the mean $\Delta F/F0$ and the standard deviation from the mean were calculated using Microsoft Excel and GraphPad Prism; the standard deviation was then plotted and compared using the Mann-Whitney non-parametric statistical test. The standard deviation was used as a simple measure to reflect how much spontaneous activity occurred in the muscles during the recordings.

Transgenic constructs for *sir-2.1(0)* rescue

DNA primers are listed in the Appendix A. The *sir-2.1* genomic sequence, plus 2kb upstream of its ATG, was PCR-amplified from N2 DNA. The PCR product was digested and ligated between the *SphI* and *SalI* sites of pSX322YFP to obtain the plasmid pXG5. To obtain promoters for driving *sir-2.1* expression, the *sir-2.1* endogenous promoter was removed via PCR-mutagenesis from pXG5 to construct pXG6. The Gateway ATTR cassette frame A was inserted in front of the *sir-2.1* genomic sequence to make the destination clone pXG7. Plasmids (pXG8, pXG9 and pXG11) that promote neuronal, muscular and intestinal expression of *sir-2.1* were obtained through Gateway LR reactions between pXG7 and pLR35 (*Paex-3*) (LeBoeuf, Gruninger et al. 2007), pLR22 (*Plev-11*) (Gruninger, Gualberto et al. 2008) and pBL50 (*Pges-1*) (Urano, Calfon et al. 2002), respectively. pXG5 (25 ng/µL), pXG8 (10 ng/µL), pXG9 (1 ng/µL) or pXG11 (50 ng/µL) were injected to *sir-2.1(0)* hermaphrodites and transgenic animals were selected via YFP fluorescence. pXG5 (50 ng/µL) was injected into wild type hermaphrodites to obtains strains with overexpression of *sir-2.1* (referred as *sir-2.1(OE)*).

Real-time PCR

300 day 1 and day 2 adult males were frozen and accumulated over a period of time. RNA was extracted by Trizol, and cDNA was synthesized by SuperScript II (Life

technology, NY) using around 2 µg total RNA, as described in (LeBoeuf and Garcia 2012). The RT-qPCR reactions were performed using BIO-RAD CFX96 real-time system and SsoFast EvaGreen supermix. 11 candidate reference genes were tested to see whether their expression changed from day 1 and day 2 in both wild-type and *sir-2.1* males (Hoogewijs, Houthoofd et al. 2008); from our analyses, *act-1* and *gpd-2* were selected as the reference to normalize the expression of the metabolic genes. Many of the primers used to detect the expression of metabolic enzymes are described in (Castelein, Hoogewijs et al. 2008). Other primers for additional metabolic and antioxidant stress genes are listed in the Appendix A. Three replicates were conducted on the same RNA samples. I used the t-test to determine which mRNA transcripts in *sir-2.1*(0) males were significantly different from their cognate wild type transcripts.

ATP, glucose, glycogen and lipid measurements

To measure ATP and glucose, 100 males were collected at different ages, frozen and thawed 3 times. The worms were homogenized, and the supernatant was collected and measured using an ATP determination Kit (Life technology, NY) and the Glucose Oxidase Assay Kit (Life technology, NY). The ATP and glucose were normalized to the amount of dsDNA quantified by picoGreen (Life technology, NY).

To stain glycogen, 1-day-old *sir-2.1(0)* and wild-type virgin males were transferred to 2% agar pads. The pads containing both genotypes were then placed over a bottle of iodine crystals for 30 seconds (Frazier and Roth 2009). The pictures were taken

by a Leica compound miroscope mounted with OLYMPUS DP70 camera. The RGB images were then converted to 16-bit gray scale, and the mean gray levels of the isthmus regions were measured using the SimplePCI image quantification software (Hamamatsu). The mean gray level was reversely correlated with the red signal.

Oil Red O staining was done according to (O'Rourke, Soukas et al. 2009). Briefly, males were collected and washed with PBS, and then fixed with Modified Ruvkuns witches brew (MRWB) buffer containing 1% paraformaldehyde (PFA) for 1hr. Worms were then washed with PBS and suspended in 60% isopropanol for 15 minutes at room temperature. The 60% isopropanol was removed and worms were bathed in 60% Red Oil O staining solution overnight. The RGB images were taken by a Leica compound mircoscope mounted with OLYMPUS DP70 camera. The images were quantified by ImageJ according to (Mehlem, Hagberg et al. 2013).

CHAPTER III

CHARACTERIZATION OF MATING BEHAVIOR DETERIORATION IN WILD TYPE *C. ELEGANS* DURING AGING[*]

The mating potency of *C. elegans* males significantly drops by the third day of adulthood

To measure how *C. elegans* male mating behavior changes during adulthood, I performed a male mating potency assay. Briefly, in this assay, an individual adult male of different ages was paired with a single 1-day-old adult hermaphrodite containing the *pha-1(e2123)* allele at 20°C. The *e2123* temperature sensitive mutation in *pha-1* allows one to score the presence of adult cross progeny as a rapid indicator of a successful mating event. Any self progeny that are homozygous for the recessive temperature sensitive *e2123* mutation cannot undergo embryonic and early larval development at 20°C. From the mating potency assays, I found that for N2 males (Most *C. elegans* labs use N2 as their wild-type reference), 90%, 68%, 37%, 37% and 36% (n=20 for each) of males were potent at adult day 1, 2, 3, 4 and 5, respectively (Figure 3A), relative to males that were paired with a hermaphrodite soon after adult molt (denoted as day 0). Statistical analysis indicates that at adult age day 3, the N2 males mating potency

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declines significantly (p=0.0031 for 3-day-old males versus 1-day-old males, and p=0.0001 for 3-day-old males versus 0-day-old males, Fisher's exact test).

To obtain sufficient numbers of males for the experiment, N2 males must be maintained through continual large-scale crossings with hermaphrodites. For ease of further experiments, I tested if *him-5(e1490)* males have the same kinetics of mating behavioral decline as N2 males. The *e1490* mutation in *him-5* increases the incidence of spontaneous males from 0.1% to 30%, by increasing X-chromosome non-disjunction events during meiosis (Hodgkin, Horvitz et al. 1979, Goldstein 1986). From assaying the mating potency of *him-5* males, I found that relative to 0-day-old males, 90%, 77%, 54%, 24% and 10% (n=40 for each) of the males were potent at day 1, 2, 3, 4 and 5, respectively (Figure 3B). Similar to the behavior of N2 males, there was a significant drop of male mating potency in 3-day-old *him-5* males compared either to 0-day-old males (p<0.0001) or 1-day-old males (p=0.0012). Because there was little difference between N2 males and *him-5* males in the kinetics of mating potency, I used *him-5* males in the subsequent assays, and refer to them as the wild type for ease of comparison.

The percentage of males that are potent could vary due to the population density of the culture plate, where males originated, or the fluctuations of humidity and temperature of when the assay was conducted. Therefore, I asked at what age (day 2 or day 3 relative to day 1) the mating potency drops with consistent measureable statistical significance. I conducted four mating potency trials on separate days and found that consistent among the trials, male mating potency at day 3 significantly dropped relative

to day 1 or day 2, although the percentage of potent males varied ranging from approximately 30% to 60% (Figure 3C). The quantitative variation indicates that data obtained from experiments should be analyzed and compared within a self-contained experimental setting and not between different settings. Additionally, since the difference of mating potency between 1-day-old and 3-day-old males was robust, these two ages were used for further analysis.

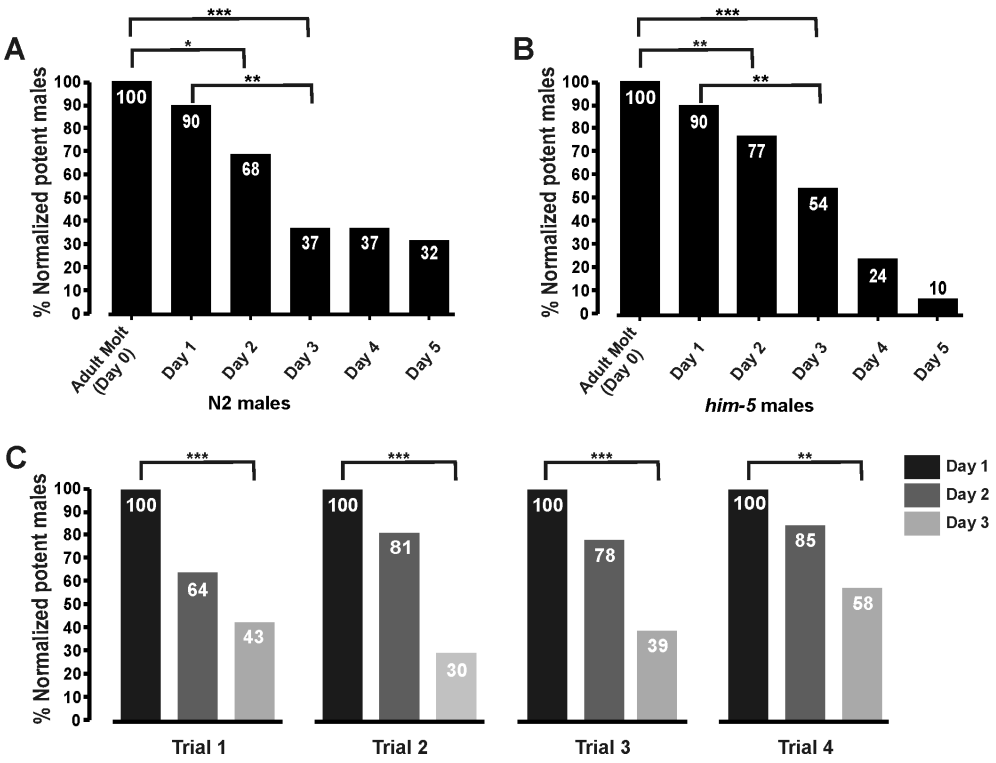

Figure 3 *C. elegans* mating potency declines significantly by the third day of adulthood.
(A) Relative mating potency of N2 males normalized to 0-day-old males (n=20 for each day). The normalized percentage for each day is listed on the top of each bar. Fisher's exact test was used to compare the mating potency between different aged males. Asterisks *, ** and *** indicate the $p<0.05$, 0.01 and 0.0001 respectively. (B) Relative mating potency of normalized *him-5* males (n=40 for each day). (C) Four independent trials of mating potency assays in *him-5* males (n=30 for each column).

Gems and Riddle reported that males raised together have shorter lifespan than males raised in isolation (Gems and Riddle 2000). This phenotype was confirmed (Figure 4A). It is possible that rearing males in groups might affect male mating potency. In the assays described above, 20-30 L4 males were reared together and allowed to develop to adults prior to using them in the potency assay; those adult males generally attempted to mate continuously with each other by crawling together and scanning each other, which could damage the males in some unknown aspects. To determine if males raised in a group artificially reduced the male mating potency, I conducted the mating potency assay using males reared in isolation and compared them with the males that were raised in groups of 20. I found that males raised in isolation or in groups have the same mating potency trend at day 1 and day 3 (Figure 4B). At day 5, the solitary-reared males have a slightly, but not significantly higher mating potency. These results indicated that the male mating potency decline at day 3 is not correlated to rearing density.

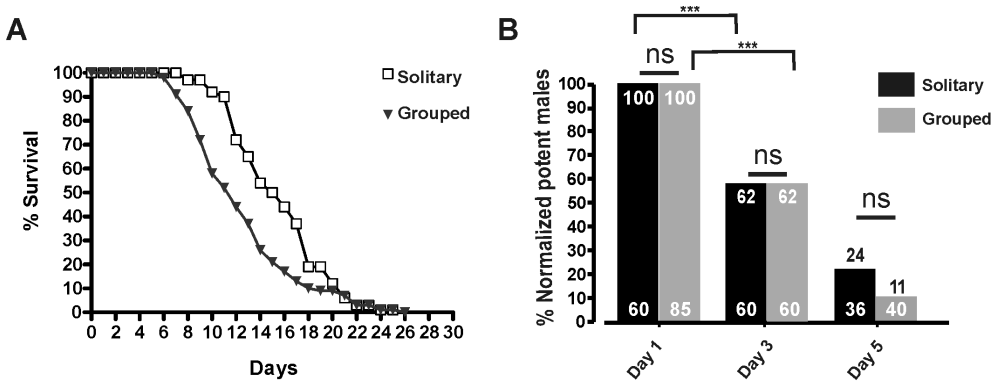

Figure 4 Male mating behavior decline is not dependent on culture conditions. (A) Adult lifespan of males maintained in isolation (triangles) or in groups of 20 per plate (squares). (n=97 for solitary males; n=205 for grouped males); *** $p<0.0001$, Log-rank (Mantel-Cox) Test (B) Normalized mating potency of *him-5* males reared in groups of 20 or in isolation. The black bar represents males kept in isolation, whereas the light gray bar represents males kept in groups. The number of males assayed for each day is listed on the bottom of each bar.

The mating behavior deterioration is not due to structural dysfunction

Advanced aging is accompanied with severe structural deformities such as sarcapenia and even cell death. To determine whether the mating behavior deterioration I observed at the third day of the adulthood is due to structural dysfunction, I construct a plasmid with the actin gene fused to *yfp* to observe the microfilament organization in the sex muscles of males at different ages. As 3-day-old males, they have similar microfilament orientation and mass compared to 1-day-old males (Figure 5A). However, 8-day-old males display significant shrinkage phenotype and also disorganization of microfilament arrangement. Although I did not inspect the morphology of the neurons in 3-day-old males, there is report that neurons in aging *C. elegans* can keep their integrity throughout their lifespan (Herndon, Schmeissner et al. 2002).

To exclude the possibility that the mating behavior deterioration is due to sperm dysfunction, I did two experiments: first, if the decline of a male's ability to sire progeny is due to dysfunctional sperm, mating potency should drop even if hermaphrodites that are easier to mate with are used. The 1-day-old *pha-1* hermaphrodites present a challenge to the males, as they are actively moving and their vulvas have not yet been stretched from repeated egg-laying. To make mating easier for the males, I conducted the mating potency assay using 4-day-old *pha-1* hermaphrodites. These hermaphrodites are less active, have a more dilated vulval opening from extended egg-laying and are depleted of self-sperm. I found that the percentage of 3-day-old potent males was significantly increased from 37% to 70% (p=0.02) when I used older *pha-1(e2123)* hermaphrodite as mates (Figure 5B). Second, if the sperm are dysfunctional, for those males that displayed successful ejaculation, they might not produce viable progeny. To address this, sperm DNA in the male's testis were stained with the red fluorescent dye SYTO-17 and tested if hermaphrodites that contained fluorescent sperm from 3-day-old adult males produced cross progeny (Hill and L'Hernault 2001). From overnight matings between stained 1-day-old males and *pha-1* hermaphrodites, 27/30 hermaphrodites contained stained male sperm in their spermatheca; all 27 hermaphrodites produced cross progeny. From matings between stained 3-day-old males and hermaphrodites, 6/30 hermaphrodites contained stained male sperm in their spermatheca; all 6 hermaphrodites produced cross progeny. These observations indicate that the sperm of 3-day-old males are still functional. This left the possibility that the reduction in mating potency could be due to a decline in some aspect of behavioral execution.

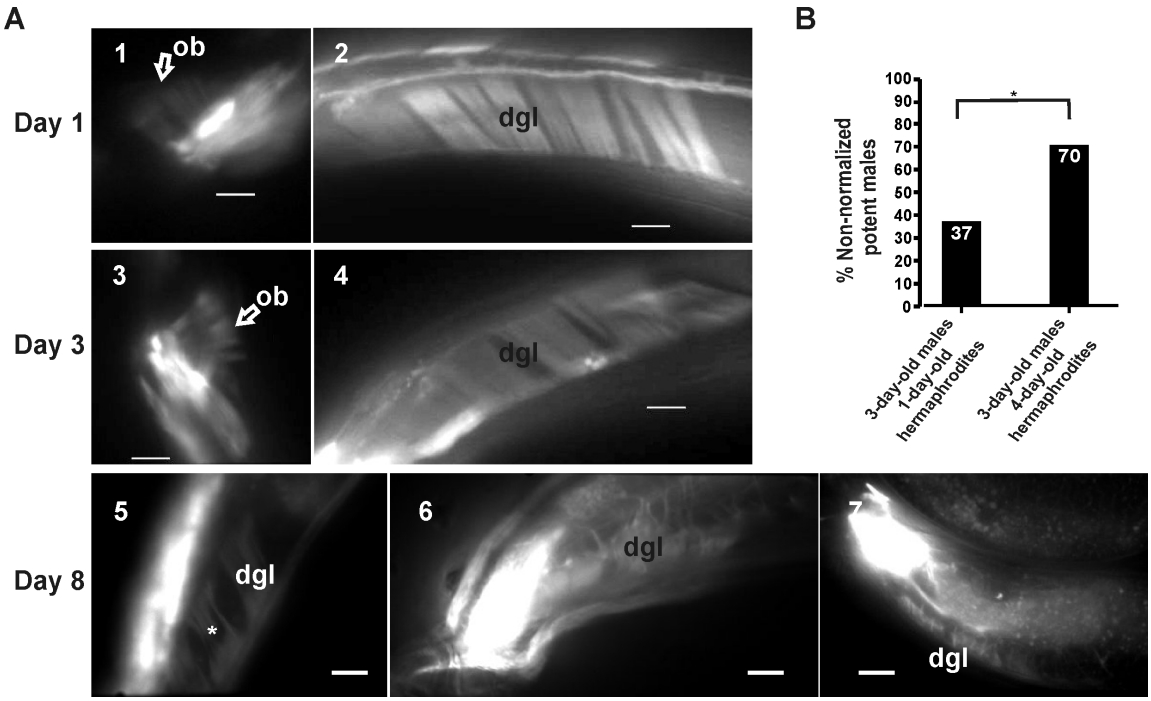

Figure 5 Mating potency decline is not due to sex muscle disorganization or sperm dysfunction.
(A) Negative grey scale fluorescent images of the adult male tail muscles. The overall structure of male-specific sex muscles indicated by YFP:ACTIN in 3-day-old male shows no significant difference as compared to that of 1-day-old male. (1) and (3) show the male tail structure with noticeable striated oblique muscles of 1-day-old and 3-day-old males respectively. (2) and (4) show diagonal muscles of 1-day-old and 3-day-old males respectively. Scale bar is 10 μm. (5), (6) and (7) show disorganized diagonal muscles of 8-day-old males. I observed hole-like structure in the diagonal muscles indicated by *. (ob) oblique muscles; (dgl) diagonal muscles. (B) Mating potency of 3-day-old *him-5* males using 1-day-old and 4-day-old *pha-1* hermaphrodites as mates. Fisher's exact test Asterisks * indicates the $p<0.05$ (N=40 for each case).

Aberrant behavior displayed during mating of 3-day-old males

Since it is unlikely that structural dysfunction is the primary cause of the deterioration of mating behavior during early aging, the altered physiological state in the

3-day-old of males likely plays an important role in the decline of mating potency. Determining which aspect of mating behavior is altered would provide a clue about how the physiology of the mating circuitry is changed. To address this, I observed the mating behavior of 1- and 3-day-old males, which were paired with 2-day-old paralyzed *unc-64(e240)* hermaphrodites, to compare behavioral parameters more uniformly between copulation events. For a male to initiate the mating behavior, he has to rely on the functionality of his chemosensory cilia, which sense the chemical and mechanical cues from the hermaphrodites (Barr and Sternberg 1999, Barr and Garcia 2006). The male crawls backwards along the hermaphrodite after the ray sensory neurons, located in his tail, contact his mate (Koo, Bian et al. 2011). When 3-day-old males were introduced to the mating lawn, they took twice as long as 1-day-old males to contact a hermaphrodite (99 ± 52 secs for 1-day-old male, n=20; 182 ± 146 secs for 3-day-old males; mean ± SD, p=0.02, unpaired t-test) (Figure 6A). 3-day-old males did not initially explore the new lawn, but after 30 seconds they would initiate exploration and locate a mate within six minutes. Once a male's tail contacted a hermaphrodite, his behavior was observed for up to 5 minutes and recorded how well he executed each step of mating behavior. 3-day-old males immediately initiated mating with the same efficiency as young males; the mean number of contacts before backing behavior was two for both young and old males (n=20 for each) (Figure 6B). Thus sufficient numbers of ray sensory neurons located in male's tail are functional to mediate the contact response step of mating. During backward locomotion, if the male reaches the end of hermaphrodite, he will make a turn to continue scanning along the hermaphrodite cuticle (Loer and Kenyon 1993). After the

3-day-old males made contact, they took slightly longer than younger males to make a successful turn at the ends of the hermaphrodite (7 ± 3 secs for 1-day-old male, n=20; 15 ± 15 secs for 3-day-old males; mean ± SD, p=0.03, unpaired t-test) (Figure 6C). Instead of turning, the older males would rub their tails back and forth at the ends of the hermaphrodites or consecutively attempt, abort and reattempt a turn for a few seconds. They would make a successful turn with their tail, but then they would not carry through the turn with continued backward locomotion. Instead, they would move forwards and then backwards again to reattempt the turn. Interestingly, when the aged males were hesitant in their turns, the curvature of their body resembled that of a male trying to insert his spicules into the vulva (Figure 6D). More frequently, the 3-day-old males would stop their locomotion at non-vulva regions, which also accounts for why they took longer to make one turn after contact.

Sensing the vulva is regulated by the hook and post-cloacal sensilla. After a successful turn, 3-day-old males had no trouble locating the vulva, as compared to young males (Figure 6E), indicating that these neurons still function at day 3. However, they were not as efficient as younger animals at maintaining their position over the vulva during spicule insertion attempts or breaching the vulva lips. After the postcloacal sensilla (p.c.s.) neurons and hook neurons sense the vulva, the male stops at the vulva and simultaneously initiates spicules prodding via rhythmic contractions of the protractor muscles (Garcia, Mehta et al. 2001). Once the spicules partially penetrate the vulva, the SPC neurons will stimulate the protractor muscles to fully contract. Consequently, the spicules will fully insert (Liu, LeBeouf et al. 2011). During the first

two minutes of mating, only 15% of 3-day-old males (n=20) were able to insert their spicule completely, whereas 50% of 1-day-old males (n=20) were able to penetrate their mates (Figure 6F). The efficiency of spicule insertion (E_{SI}), a metric that combines how fast they initiate spicule insertion attempts, how tenacious they sustain reattempts and how fast they completely insert their spicules within two minutes (see Materials and methods), was higher for 1-day-old males compared to 3-day-old males (the average E_{SI} was 0.04 for 1-day-old males, n=20 and 0.004 for 3-day-old males, n=20) (Figure 6F). In conclusion, while older males are capable of performing the specific steps of mating behavior, they do so at a lower efficiency than younger males. Therefore, the inefficient behavioral execution exhibited by 3-day-old males during mating with paralyzed hermaphrodites would magnify during copulations with moving hermaphrodites, which would be a likely cause for reduced mating potency.

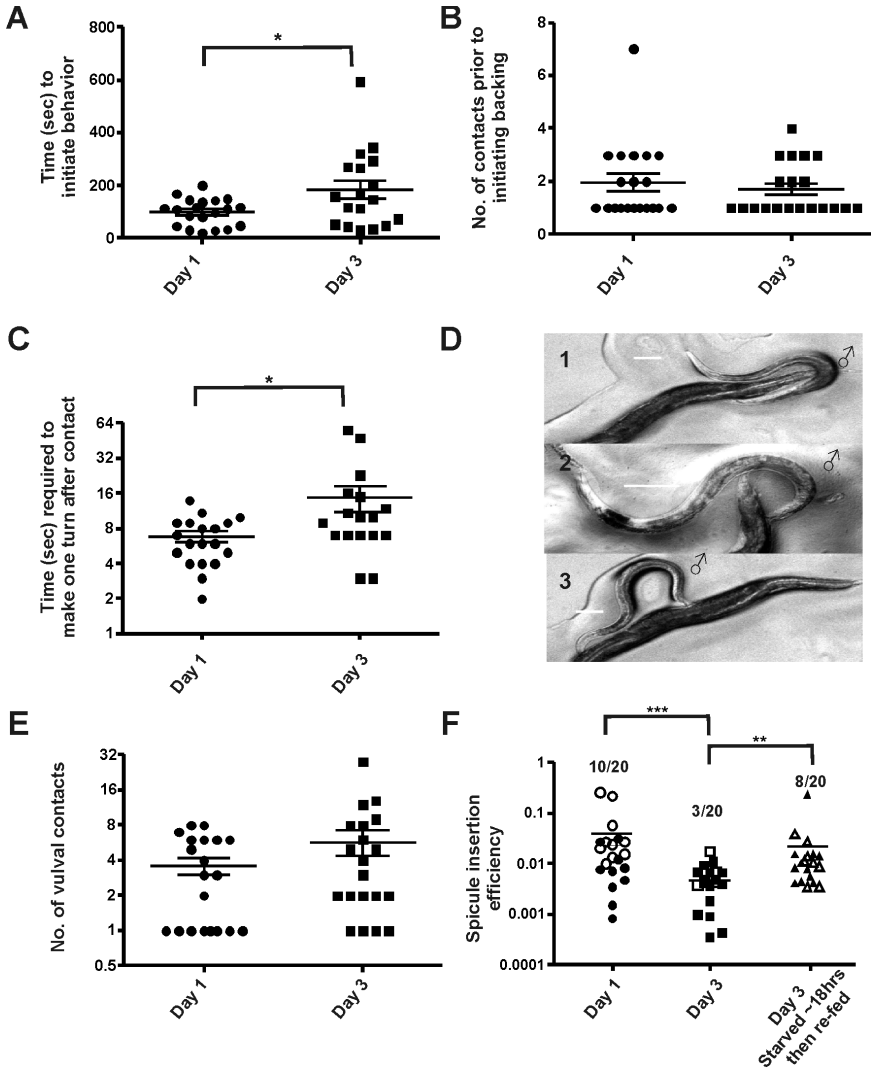

Figure 6 Specific steps in mating behavior are affected as the male ages.
(A) The time required for the males to initiate mating behavior. Each dot represents the metric of a single male observed. * $p<0.05$; unpaired t-test. (B) The number of contacts the males made prior to initiating backward locomotion along the hermaphrodites cuticle. (C) The time between the male initiating mating behavior and completing the first turn at the end of the hermaphrodite. * $p<0.05$; unpaired t-test. (D) The top panel (1) depicts a 1-day-old male moving backwards along the hermaphrodite and turning at the end of his mate. The posture of a 3-day-old male (2) during backward locomotion resembles a male attempting to insert his spicules (3). Scale bar is 100 µm. (E) The number of vulval contacts prior to successful insertion of spicules. (F) Spicule insertion efficiency (E_{SI}) during 2-minutes of observation. Open symbols indicate that the males successfully inserted their spicules. The number of success insertion is listed on the top of each column. * $p<0.05$; ** $p<0.001$; *** $p<0.0001$; Mann-Whitney non-parametric test.

Spicule muscle excitability increases during adulthood

During the behavioral observations, I noticed that 3-day-old males spent a significant amount of time at areas outside the vulva region and assumed a posture that was reminiscent of spicule insertion attempts (Figure 6D). I asked if this increase in apparent spicule insertion attempts outside the vulva region was correlated with increased sex muscle excitability. To address this question, I used G-CaMP, a GFP-derived fluorescent calcium sensor to monitor the Ca^{2+} transients in the sex muscles during mating (Nakai, Ohkura et al. 2001). I observed Ca^{2+} transients in the sex muscles (Figure 7) of 3-day-old males, when they randomly stopped at non-vulval regions.

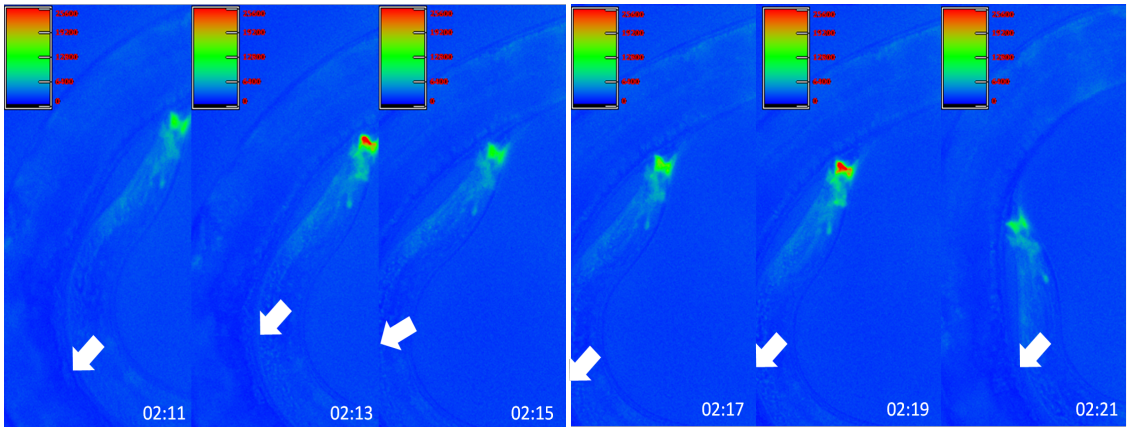

Figure 7 An old male displays ectopic spicule insertion behavior at non-vulva regions.
The arrows point to the hermaphrodite vulva and sex muscles of the male tail express G-CaMP. The green indicates the background fluorescence of G-CaMP. Once Ca^{2+} is released into the muscle cytoplasm, the G-CaMP fluorescence becomes more intense, indicated by the yellow and red false colors.

These observations led me to hypothesize that components of the mating circuit might be hyper-excited, resulting in the ectopic spicule insertion behavior. If this was the case, mating relevant components such as the spicule muscles of older males might display more spontaneous Ca^{2+} transients, even when the males are not mating. To test this idea, I imaged the Ca^{2+} transients in the spicule-associated muscles from immobilized 1- and 3-day-old males (Figure 8A). Compared to 1-day-old males (Figure 8B), 3-day-old males displayed more Ca^{2+} transients (Figures 8C, D, E).

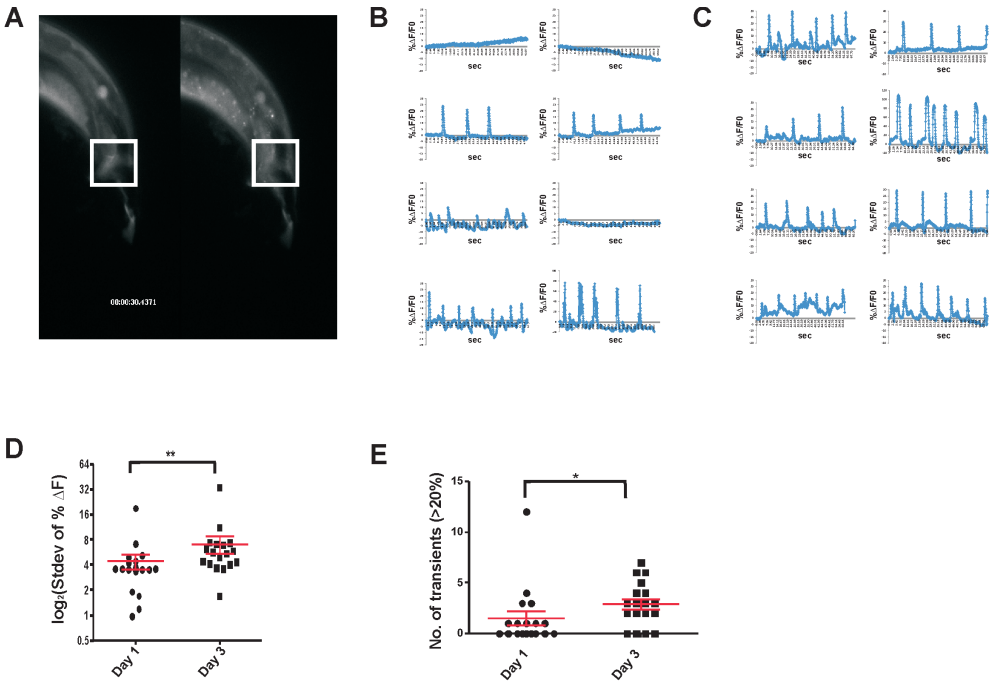

Figure 8 Immobilized older males display increased Ca^{2+} transients in the sex muscles.
(A) A male was immobilized on an agar pad and recorded in the green (left) and red (right) fluorescence channels simultaneously. The mean gray pixel intensity was measured in the rectangular region of interest. (B) Plots of Ca^{2+} transients in 8 representative 1-day-old males. (C) Plots of Ca^{2+} transients in 8 representative 3-day-old males. (D) Standard deviation from the mean ΔF/F0 for each male. N=18 for each group. ** p<0.01; Mann-Whitney non-parametric Test. (E) No. of transient peaks with amplitude larger than 20% (p <0.05) change; Mann-Whitney non-parametric Test**.**

The increased incidence of Ca^{2+} transients in 3-day-old male sex muscles suggested the non-stimulated excitability level of the mating circuit increases as the male ages. I hypothesized that the increased excitability might result in hypersensitivity of the spicule-associated muscles to agonist stimulation. The cholinergic SPC motor neurons make direct synapses with the spicule muscles. To respond to secretions from these neurons, the muscles express levamisole (LEV)-sensitive ionotropic acetylcholine receptors (AChRs) made up of subunits encoded by the *unc-38*, *unc-63*, *lev-8*, *lev-1* and *unc-29* genes. To test the hypothesis that increased calcium transients might be related to hypersensitivity to cholinergic stimulation, I used the acetylcholine agonist LEV to stimulate protractor muscle contraction. Low concentrations of LEV can increase the acetylcholine receptors' closed to open probability, causing excitable cells to fire. To ascertain the sensitivity of males to the drug, I bathed 1- and 3-day-old males for five minutes in various concentrations of LEV, and quantified how many males protracted their spicules for greater than 5 seconds. I found that the LEV sensitivity of the spicule muscles for 3-day-old males was approximately seven times greater than compared to younger males (LEV EC_{50}: 884 μM for 1-day-old males, 100 nM for 3-day-old males) (Figure 9). This result is consistent with the mating behavior observations and increased calcium transients, indicating that the overall excitability of the male mating circuit increases as the male ages.

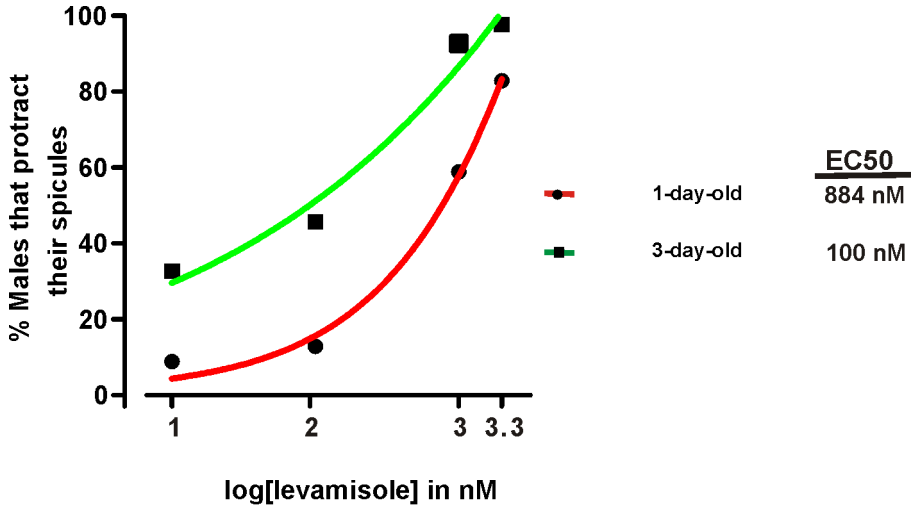

Figure 9 3-day-old wild type males are more sensitive to levamisole.
≥30 males were assayed for each concentration. The EC_{50} for each treatment or strain was calculated and listed on the right of each corresponding line.

Chapter summary

In chapter III, I characterized how wild type *C. elegans* male mating behavior deteriorates during aging. First, I utilized a very simple mating potency assay using *pha-1(e2123ts)* hermaphrodite as mates, which allowed quick scoring of successful mating events through existence of viable cross progeny. Via the mating potency assay, I demonstrated that *C. elegans* males, either from the N2 or *him-5(e1490)* strains, display mating behavior deterioration at the the third day of the adulthood, after which they can still live about 7-13 days. Also, the decline of mating potency is independent of the way I raised them. When the males were raised individually, although they can live significantly longer than males cultured in a group, isolated males still have a dramatic

drop of mating at the third day of adulthood. Thus, the lifespan extension is not necessarily correlated with a delay of behavior deterioration.

To determine the underlying mechanism of mating-behavior decline that occur during early aging, I observed the sex muscles structure of different aged males. 3-day-old males have well-organized muscle filament arrangement, excluding the possibility of sarcopenia-caused mating behavior deterioration. Sperm dysfunction could also result in decreased mating success. However, this is unlikely, since I observed that 100% of 3-day-old males who successfully transferred sperm produced viable progeny. Also, another group studying on *C. elegans* males reported that the sperm activity could be preserved at least up to day 5 of adulthood (Chatterjee, Ibanez-Ventoso et al. 2013). Therefore, I concluded that the deterioration of mating behavior at such an early age is not due to structural dysfunction but actually neuromuscular circuit disregulation.

Through direct observation of mating steps in 3-day-old and 1-day-old males, I found that although 3-day-old males required slightly more time to initiate mating, most of them could sense the hermaphrodite and initiate mating eventually. Considering that during a mating potency test, males remain with hermaphrodites for an unlimited amount of time, the time required to initiate mating may not be the limiting factor contributing to mating failure. After males start to scan for the vulva, 3-day-old males displayed ectopic spicule prodding behavior more often with more calcium transients observed during this ectopic behavior. Also, they turn with a posture that mimics that of insertion, indicating that the mating circuitry might be hyper-excitable. 3-day-old males can sense the vulva as efficiently as 1-day-old males indicating a full function of their sensory neurons.

Taken all together, it might be that the ectopic prodding and aberrant turning behavior contribute to the lower spicule insertion efficiency and thus lower mating potency. The hyper-excitability of the mating circuitry in the 3-day-old males is confirmed by the pharmacological drug tests.

CHAPTER IV

REDUCING THE EXCITABILITY OF THE MATING CIRCUITRY PROLONGS THE MATING BEHAVIOR*

Reducing the expression of ACh Receptor genes increases the mating potency in 3-day-old males

My pharmacological assays and Ca^{2+} imaging experiments indicated that during aging, the excitability of male mating circuit components such as the male sex muscles is increased. Therefore, I asked if the hypersensitivity of the cholinergic mating circuit components was related to the mating potency decrease in 3-day-old males. To answer this, I reduced the expression levels of several acetylcholine receptor (AChR) genes and asked if the manipulation can increase the mating potency of older males.

The male sex muscles express the GAR-3, ACR-18, ACR-16 and UNC-29 AChRs (Liu, LeBeouf et al. 2011). I assayed the mating potency of males that contain a single homozygous mutation in these AChR genes. Relative to 1-day-old males, the mating potency of 3-day-old *gar-3(gk305)* and *acr-18(ok1258)* males dropped significantly, and was similar in kinetics to wild-type males (Figure 10A). In this study, I found that for *acr-16(ok789)* males, there was also a significant drop at day 3 compared

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to 1-day-old males. *unc-29* is expressed in the body wall muscles and the lack of functional UNC-29 makes the animals severely defective in locomotion, which obviously eliminates the ability of the mutant males to mate. To circumvent this problem, locomotion was restored to the mutant males by driving the expression of an *unc-29* cDNA, using the *acr-8* promoter that expresses in the body wall muscles, but not the male sex muscles (Liu, LeBeouf et al. 2011). Similar to *acr-16* males, the body wall restored *unc-29* male mating potency dropped significantly at day 3 relative to 1-day-old males (Figure 10A). Taken together, these results suggested that reducing the function of single AChR genes does not decrease the rate of mating potency decline by day 3.

I then asked if reducing the expression of all four AChR genes could reduce the rate of mating potency decline. Males that are homozygous for mutations in all 4 genes cannot mate; therefore, males that are heterozygous mutant for all of these AChR genes were used to conduct the mating potency assays. I reasoned that one functional genomic copy of these four AChR genes would be sufficient to allow the males to mate. But one copy of these genes might also reduce the sex muscle excitability in 3-day-old males and possibly increase mating potency.

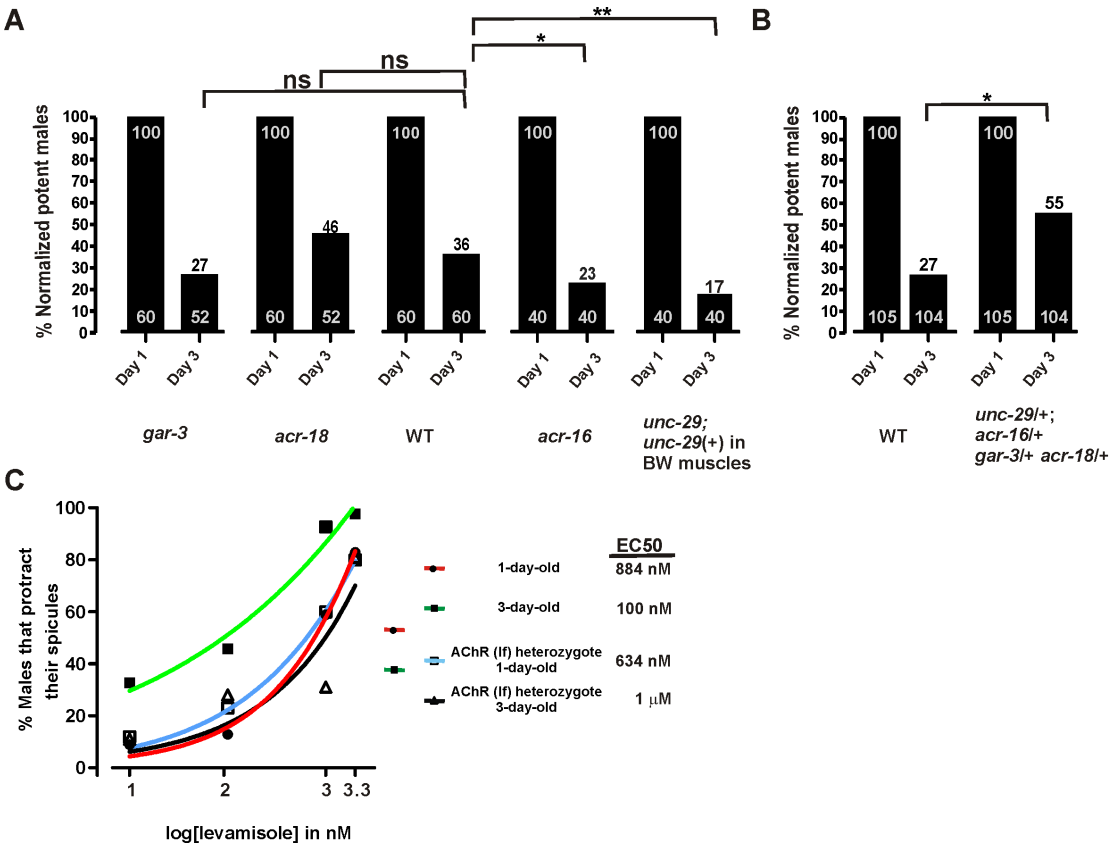

Figure 10 Manipulation of AChR gene dosage can improve the mating potency of 3-day-old males.
(A) Relative mating potency in single AChR mutant homozygous males. (B) Relative mating potency in AChRs heterozygous mutants. The number of males assayed for each strain is listed within the bottom of each bar and the normalized percentages are listed within the top of each bar. *p<0.5; ** p<0.01; Fisher's exact test. (C) AChRs heterozygous mutants have a lower sensitivity to levamisole at the third day of adulthood. ≥30 males were assayed for each concentration. The EC_{50} for each treatment or strain was calculated and listed on the right of each corresponding line.

To test this hypothesis, I assayed the mating potency of the heterozygous mutant males at day 1 and 3. As expected, I observed a significant improvement of mating potency at day 3, 55% for heterozygous males compared to 27% for wild-type males (Figure 10B). I then assayed the 1- and 3-day old heterozygous AChR males' response

to LEV and found that the LEV sensitivity of the spicule muscles for 3-day-old heterozygous males was significantly lower than that of similarly aged wild-type males (LEV EC_{50}: 1 μM for 3-day-old heterozygous males, 100 nM for 3-day-old *him-5* males) (Figure 10C). Thus, reducing one functional genomic copy of each AChR gene was sufficient to reduce the excitability of males mating circuit components on day 3. This observation suggests a correlation between the changes in the excitability of male mating circuit components and the efficiency of copulation behavior during aging.

Transient starvation in 1-day-old males can reduce sex muscle excitability and increase mating potency in 3-day-old males

Although genetic manipulation can reduce the excitability of sex muscles and increase mating potency in 3-day-old males, I asked if there is another less-invasive strategy that can be applied to wild-type males, in order to achieve the same outcome. Previous work has showed that caloric deprivation can reduce male sex muscle excitability. 3-18 hrs of transient starvation during early adulthood can suppress fictive spontaneous mating behavior and sex muscle spasms caused by deletion of the *unc-103* encoded *ether-a-go-go* related gene (ERG)-like K^+ channel (Reiner, Weinshenker et al. 2006, LeBoeuf, Gruninger et al. 2007, Gruninger, Gualberto et al. 2008). The suppressive effect can last for days after re-feeding, and is partly facilitated by the compensatory increased expression and function of the *ether-a-go-go* (EAG) K^+ channel encoded by the gene *egl-2* (LeBoeuf, Guo et al. 2011).

Since transient starvation can reduce the excitability of components of the mating circuit in young adult males, I asked if it has a similar effect on 3-day-old males. To address this question, I used LEV to stimulate the male spicule protraction circuit in 3-day-old males that were transiently starved for the first 18~20 hr of adulthood. As expected, I found that the sensitivity to LEV of these transiently starved males was significantly lower than that of well-fed 3-day-old males (LEV EC_{50}: 251nM for transiently starved, 3-day-old males; 100nM for 3-day-old males) (Figure 11A).

I then asked if transient starvation could also positively affect the mating behavior of 3-day-old males and found that male mating behavior can be improved by transient starvation during early adulthood. At day 1, 83% (n=69, 100% after normalization) of males after transient starvation can produce the cross progeny versus 93% (n=60, 100% after normalization) of well-fed males. At day 3, a significant improvement in mating potency was observed in males that experienced transient starvation. 71% (n=70) of starved males can sire progeny; while only 41% (n=60) of well fed males can mate (Figure 11B). In agreement with the mating potency assay, during mating behavior, the efficiency of spicule insertion (E_{SI}) of 3-day-old males that were transiently starved during early adulthood is significantly higher than their continuously fed cohorts. During 2 minutes of observation, 40% of the transiently starved 3-day-old males inserted their spicules, and the average E_{SI} was 0.02, similar to 18-24 hr males and significantly different from aged well-fed males (p=0.005, Mann-Whitney U test) (Figure 6F).

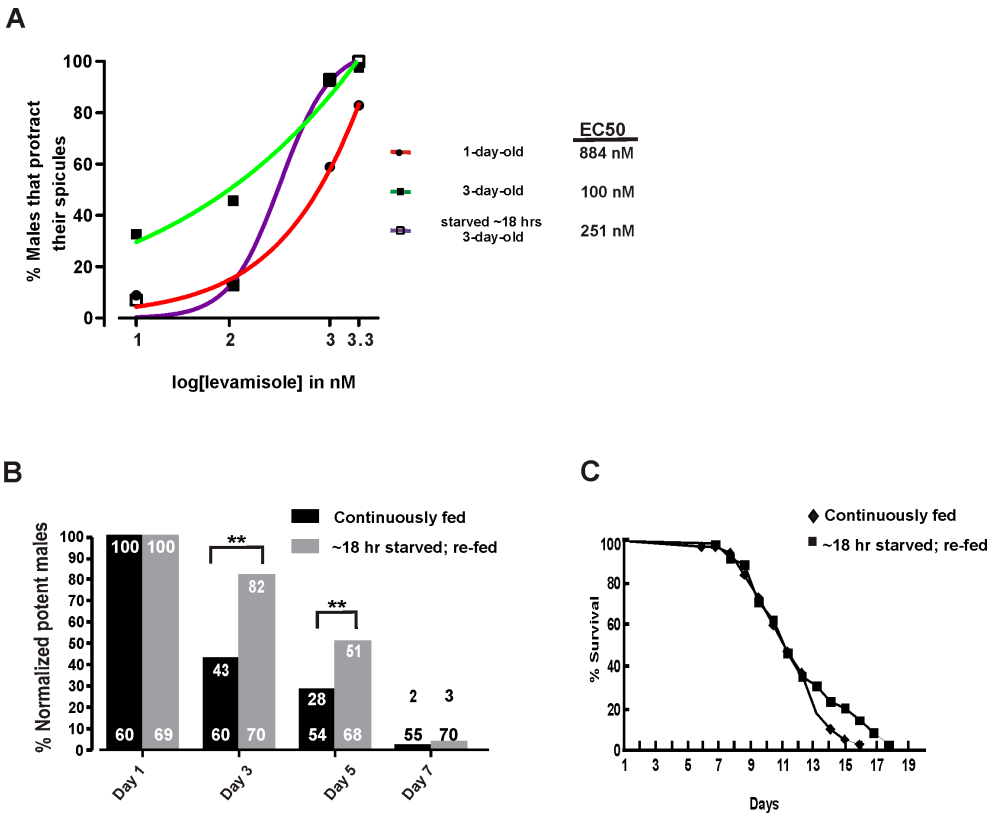

Figure 11 Transient starvation can improve the mating behavior without extending lifespan.
(A) Transient starvation reduces the sensitivity of 3-day-old males to levamisole. (B) Changes in mating potency of transiently starved and well fed aged males. ** $p<0.01$; Fisher's exact test. (C) Adult lifespan of transiently starved and fed wild-type males (n=78). $p=0.2$ ns; Log-rank (Mantel-Cox) test.

Since caloric restriction has been shown to extend lifespan in *C. elegans* (Klass 1977, Hosono, Nishimoto et al. 1989), it is possible that the increased mating potency of 3-day-old males by transient starvation is a consequence of lifespan extension rather than the modulation of cell excitability. Therefore, I conducted a lifespan assay for both well-fed males and males transiently starved for ~18hrs and then re-fed and found that the duration of transient starvation that can increase mating potency in 3-day-old males

is not sufficient to extend lifespan (Figure 11C). This result is consistent with my hypothesis that increase of the mating potency by transient starvation during early adulthood is correlated with the reduction of cell excitability in 3-day-old males.

The long-term effect of transient starvation on mating potency requires protein synthesis during early adulthood

To address how transient starvation prolongs male mating potency, I first asked if food deprivation slows down the natural, yet undefined progressive changes in the physiology of the mating circuit, so that 3-day-old males can mate better. If so, transiently starving the males anytime from adult molt to day 3 should increase the mating potency of 3-day-old males. Since ~20 hrs starvation of males starting from late L4 or early adulthood was sufficient to improve male mating potency at day 3, I asked if ~20 hrs of starvation at day 2 can also prolong male mating potency. Males were fed for the first 24 hrs of their adulthood, then starved for ~20 hrs, then re-fed for an additional 24 hrs before they were assayed for their mating potency. I found that transiently starving the males at day 2 of adulthood cannot prolong mating potency (Figure 12), indicating that starvation needs to occur early in a males' development to have lasting effects on mating potency.

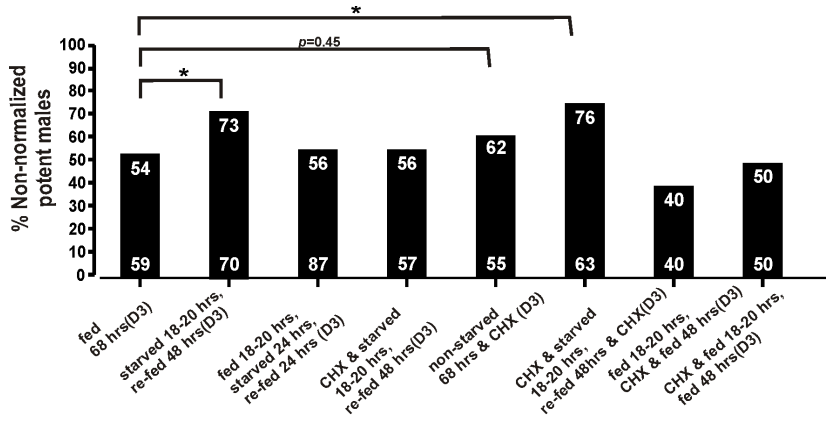

Figure 12 The effect of a protein synthesis blocker, cycloheximide, on male mating potency.

I then asked if protein synthesis during starvation is required to increase the mating potency at the third day of adulthood. To address this, I starved the males (immediately after adult molt) in the presence of cycloheximide (CHX), a drug that reduces translation by blocking the ribosomal translocation step (Ernest 1982). After 18-20 hrs on plates with CHX but lacking food, the males were removed from the drug and transferred to plates containing food. I then scored the 3-day-old male mating potency and found that the percentage of potent males was reduced from 73% (n=70) to 56% (n=57) (p<0.05) (Figure 12). This suggests that under normal conditions, protein synthesis occurs in young starving males that later improves mating potency in older males. One consequence of those newly synthesized proteins might be to offset or reprogram the physiological changes that occur in the mating circuit during aging.

The protein synthesized or the protein synthesis process itself during early adulthood might play a negative role in maintaining mating potency during aging. In contrast to the negative effect of CHX on starved males, continual exposure to CHX

during one day of starvation and two days of re-feeding can restore the positive effect of transient starvation; the percentage of 3-day-old males that can sire progeny increased from 56% to 76% (Figure 12). This indicates that under normal well-fed conditions there might be molecules synthesized during adulthood that are detrimental to the physiological state of the mating circuit. I then asked if perturbing protein synthesis in continuous feeding conditions also positively affects the mating potency of 3-day-old males. I found that continuously blocking protein synthesis slightly, although not significantly, affects 3-day-old males (54% compare to 62% of 3-day-old males can sire progeny p=0.45), whereas exposure to CHX only at day 1 (54% compared to 50% of 3-day-old males can sire progeny) or only at day 2 (54% compared to 40% of 3-day-old males can sire progeny) has no effect, suggesting that reducing protein synthesis in general does not increase the mating potency. Taken together, these results suggest that early adulthood is a sensitive period when new proteins, synthesized in response to transient starvation, can offset the deteriorative effect of protein synthesis during re-feeding, to prolong the mating potency.

UNC-103 and EGL-2 potassium channels mediate the effect of transient starvation on mating potency

Previous works from our lab demonstrated that 3 to 18 hrs of transient starvation could attenuate the excitability of the mating circuit for an extended period of time, through the activity of EAG family K^+ channels. Deletion of the *unc-103*-encoded ERG-

like K^+ channel (*unc-103(0)*) leads to an unregulated increase in the excitability of the male mating circuit, causing a percentage of males to display spontaneous spicule-muscle spasms. As a consequence, the attached spicules are protracted constitutively from the cloacal opening (Garcia and Sternberg 2003, Gruninger, Gualberto et al. 2006). The *unc-103(0)* spontaneous spicule protraction phenotype can be suppressed by starvation, partially through the up-regulation of *unc-103's* paralog, the *egl-2*-encoded EAG K^+ channel. In wild-type males, robust expression of *egl-2* in the sex muscles occurs approximately two days after adulthood; however, its temporal expression can be expedited by starvation, and the effects on attenuating cell excitability can last for days (LeBoeuf, Gruninger et al. 2007, LeBoeuf, Guo et al. 2011). This led us to ask if the EGL-2 and UNC-103 K^+ channels could be molecules that respond to short-term starvation, attenuate cell excitability, and consequently reduce the rate of male mating decline.

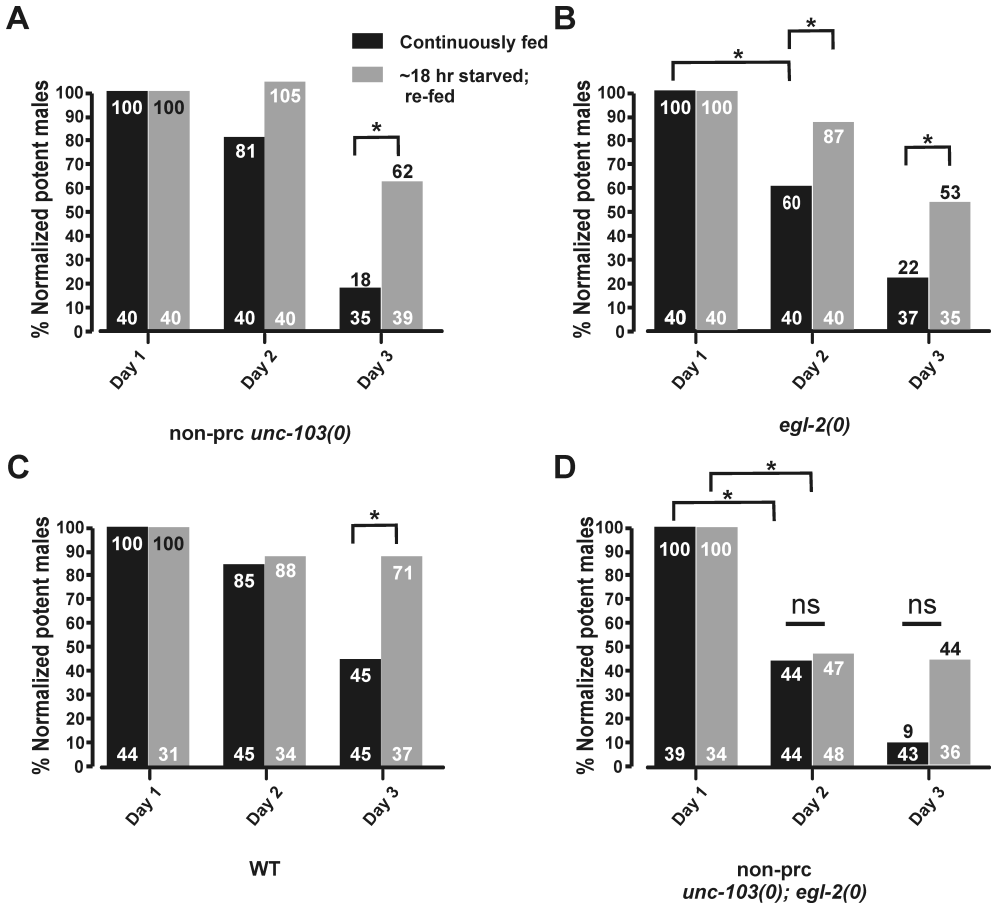

Figure 13 Potassium channels are required to mediate the effect of transient starvation on mating potency.
Numbers at the top of the bars are normalized percentages. Below are the numbers of males assayed. The *unc-103* mutation causes a certain percentage of males to protract their spicules constitutively, the Prc phenotype. Males used in the mating potency assay did not display the abnormal phenotype, and are referred to as non-Prc males. (A) Changes in mating potency of transiently starved and fed aged *unc-103(0)* males. ** $p<0.001$; Fisher's exact test. (B) Changes in mating potency of transiently starved and well fed aged *egl-2(0)* males. * $p<0.05$; Fisher's exact test. (C) Changes in mating potency of transiently starved and well fed aged wild-type males. (D) Changes in mating potency of transiently starved and well fed aged *unc-103(0); egl-2(0)* males. * $p<0.05$; Fisher's exact test.

To address the hypothesis, I used *unc-103(0)* and *egl-2(0)* single- and *unc-103(0); egl-2(0)* double-mutant males to test if transient starvation's effect on mating decline is perturbed by mutations in these EAG family K^+ channel genes. In wild-type males, *egl-2* expression in the male sex muscles is robust at day 2 of adulthood, suggesting that the K^+ channel might be important during that period (LeBoeuf, Guo et al. 2011). Therefore, I assayed the mating potency of wild type, *unc-103(0)* and *egl-2(0)* single and double mutant males on day 1, 2 and 3. The mating potency kinetics for *unc-103(0)* single mutant males resembled wild type. Mating behavior did not significantly decline until day 3, and transient starvation improved the mating potency (from 18% to 62%, $p<0.05$ n=40) (Figure 13A). Unlike wild type or *unc-103(0)* males, the mating potency of *egl-2(0)* males measurably declined by day 2. However, after starvation, 87% (n=40) of mutant males were potent, which was significantly higher than their well fed cohorts (60% n=40) (Figure 13B). Therefore, single K^+ channel mutations do not obviously interfere with processes that occur during and after transient starvation.

Mutations in both K^+ channels did affect the male's mating potency after starvation. Similar to *egl-2(0)* males, the mating potency of *unc-103(0); egl-2(0)* animals significantly drops at day 2 (from 100% to 44%, n=39 and 34, respectively, $p=0.02$). However, unlike in *egl-2(0)* males, transient starvation did not improve the mating potency of the double mutants (45% for well-fed males vs. 47% for starved males, n=44 and 48, respectively) (Figure 13D). I did observe a slight, but not statistically significant difference (probably due to the limited sample size) in mating potency of *unc-103(0); egl-2(0)* males at day 3 between well-fed and starved conditions. Therefore, I cannot rule

out that there are additional molecules that might have a minor involvement in the transient starvation response. I concluded that increase in K^+ channels function during transient starvation might result in slowing the decline of mating potency during aging.

Chapter summary

In this chapter, I explored two ways to improve the mating behavior of 3-day-old males. The first one is through manipulation of ACh receptors. Individual homozygous mutants of ACh receptors either do not affect mating at third day of adulthood, or they make males mate even worse. However, quadruple heterozygous mutations improved mating of 3-day-old males and reduce the excitability of the mating circuits. This suggests that the hyper-excitability might cause behavioral deterioration, which occurs during early aging.

The second way to extend the mating behavior is through transient food deprivation from late L4 to early adulthood for about 20 hrs. The time window for the starvation is critical, as transient starvation initiated at day 2 cannot prolong mating. By using a protein synthesis blocker, cycloheximide, I found that protein synthesis during the starvation at L4 to early adulthood is required to improve mating in the long term. However, continuing to supply worms with CFX can counteract the effects of blocked protein synthesis during food deprivation, suggesting that stage-dependent synthesis of protein played opposite roles in maintaining male mating ability. As important regulators

of excitability in the mating circuitry, potassium channels including UNC-103 and EGL-2 are at least partially required to mediate the effects of transient starvation.

CHAPTER V

SIR-2.1 REGULATES MATING BEHAVIOR THROUGH REGULATION OF METABOLIC HOMEOSTASIS AND STRESS RESPONSE*

SIR-2.1 maintains male mating during early aging

C. elegans male mating behavior deteriorates during early aging. N2 and *him-5(e1490) C. elegans* males' mating capability begins to decline at day 3 of their adulthood, although their median lifespan is 11 to 12 days (Guo, Navetta et al. 2012). I demonstrated that transient starvation of young males can extend their mating span, partially through up-regulation of *ether-a-go-go* K^+ channels (LeBoeuf, Guo et al. 2011); however, our data also suggested that transient starvation can improve mating through additional mechanisms (Guo, Navetta et al. 2012). Considering that metabolism is altered in food deprived males (Tan, Luo et al. 2011), I tested whether perturbing the histone deacetylase metabolic regulator, *sir-2.1*, affects the functional span of copulation behavior in fed and transiently starved/re-fed males.

In adult hermaphrodites, animals that lack *sir-2.1* have increased intestinal lipids (Walker, Yang et al. 2010), a phenotype opposite of starved animals. Likewise, I found that 1-day-old *sir-2.1(ok434)* null *(0)* males also contain more lipids than wild type (Figure 14). In addition, I observed that 2-day-old wild-type males have more fat (Figure

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14). Thus, I asked if *sir-2.1(0)* males might have altered mating due to metabolic dysregulation. Allowing the males to mate for five hours, I found that well-fed aging *sir-2.1(0)* males' mating ability drops prematurely, compared to wild-type males (Figure 15A). Even under unlimited mating conditions, the mating potency of 2-day-old *sir-2.1(0)* drops to 42% ($p < 0.0001$, $n = 47$) (Figure 15B).

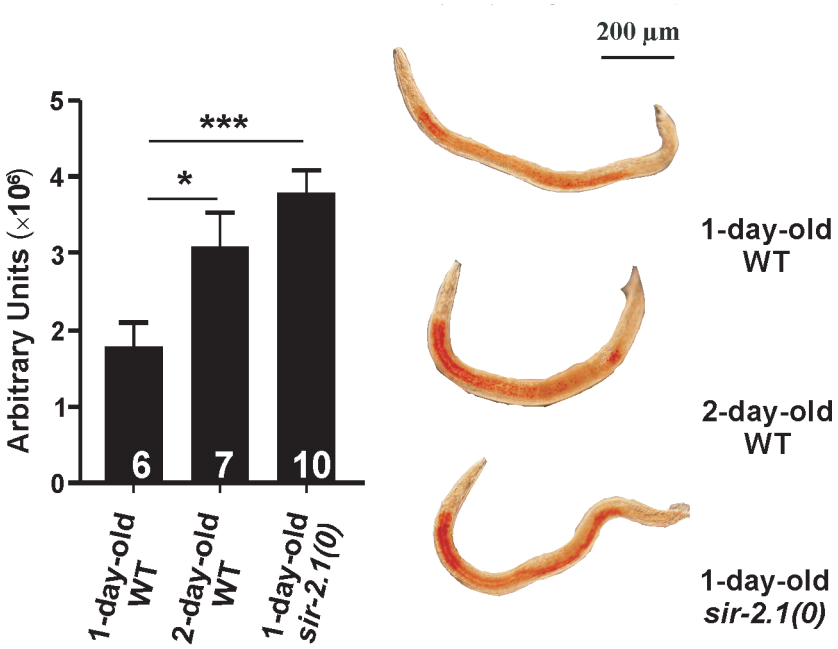

Figure 14 *sir-2.1(0)* and older wild type males have more lipid content.

I then asked if transient starvation can suppress the mating defect in *sir-2.1(0)*. To do so, I starved *sir-2.1(0)* males for ~20 hours from L4, and conducted a 5 hours mating potency assay. Transient starvation improved mating of 2-day-old *sir-2.1(0)* males from 13% to 75% ($p<0.0001$, Figure 15C), but at day 3, the mating potency of transiently starved *sir-2.1(0)* males was still lower than wild type. Thus, similar to wild

type, the metabolic alteration and/or up-regulated EAG K+ channel functions caused by starvation alleviate some of the dysfunction caused by the *sir-2.1* deletion. However, the mutant's reduced mating potencies between day 1 and 3 under both conditions indicate that SIR-2.1 contributes to the functionality of the mating circuits during this period.

To confirm that premature mating deterioration in *sir-2.1(0)* males is caused by the *ok434* allele, I introduced into *sir-2.1(0)* animals a rescuing transgene containing the *sir-2.1* endogenous promoter driving the *sir-2.1* genomic sequence fused to *yfp*. The extrachromosomal expression of *sir-2.1* significantly improved the mating potency of 2-day-old *sir-2.1(0)* males from 26% to 75% ($p<0.0001$, Figure 15D). *sir-2.*1 is expressed broadly in *C. elegans* (Bamps, Wirtz et al. 2009), thus I further conducted tissue specific rescue assays. However, I found that none of the tissue specific promoters driving the expression of *sir-2.1*, including neuronal, muscle and intestinal promoters can rescue the premature mating decline (Figure 15E). This suggests that *sir-2.1* is required in multiple tissues to maintain male mating.

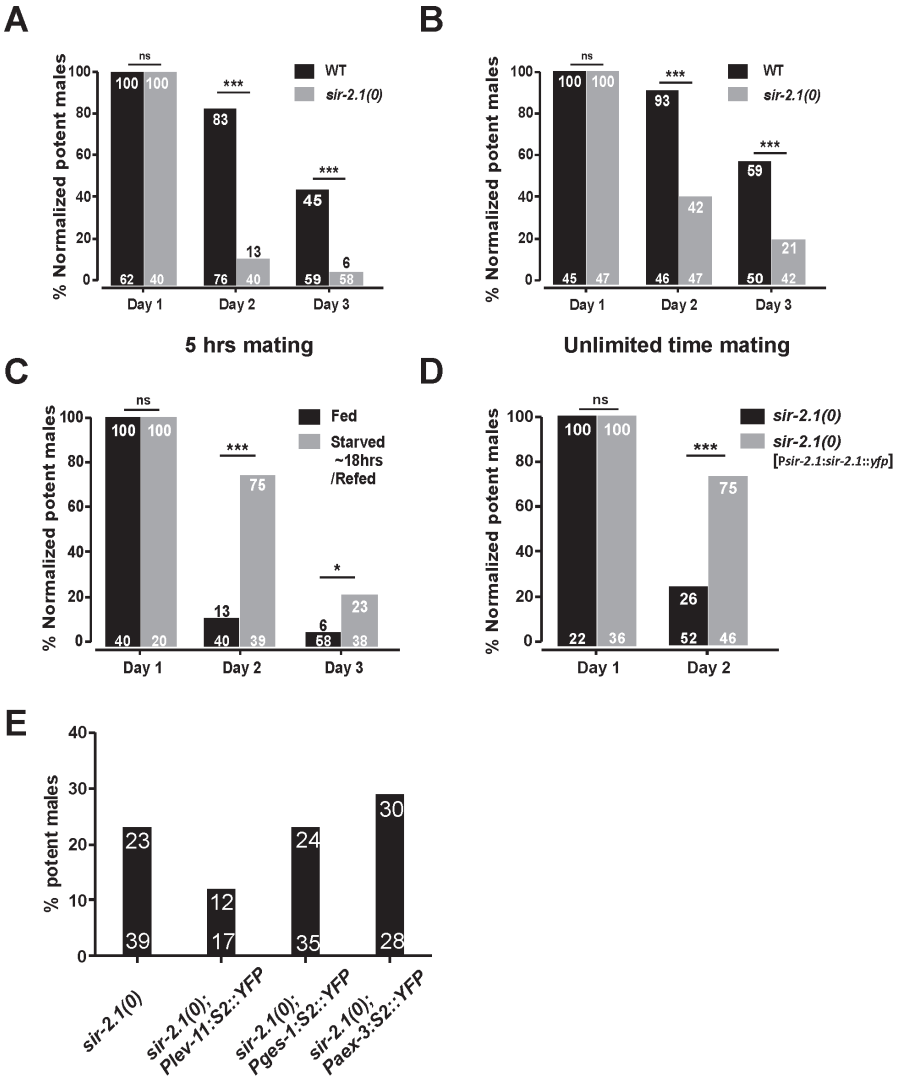

Figure 15 *sir-2.1(0)* mating ability deteriorates prematurely.
Mating potency of wild-type and *sir-2.1(0)* males. Copulations were allowed to occur for 5 hours (A) or for an unlimited time (B). The number of males in each assay is listed at the bottom of each bar. The numerical percentage of wild-type males that mated on day 1 was normalized to 100%. The normalization factor was then applied to the other experimental conditions. The normalized percentages for each day are listed on the top. Fisher's exact test was used to compare the mating potency prior to normalization. (C) Transient starvation reduces *sir-2.1(0)* mating deficiency. (D) Mating potency of *sir-2.1(0)* and rescued strain *sir-2.1(0); rg*EX*399 [Psir-2.1:sir-2.1::yfp]*. ns, not significant. Asterisks *, ** and *** indicate the $p < 0.05$, 0.01 and 0.0001, respectively. (E) Tissue specific expression of *sir-2.1* does not rescue the reduced mating potency of *sir-2.1(0)* males at day 2. The *lev-11* promoter expresses *sir-2.1* in all body wall and sex muscles. The *ges-1* promoter expresses *sir-2.1* in the intestine. The *aex-3* promoter expresses *sir-2.1* in all neurons.

sir-2.1(0) males mating deterioration is not due to structural dysfunction

To exclude the possibility that *sir-2.1(0)* mating deficiency at day 2 is due to a shorter lifespan, I conducted a lifespan assay and found that *sir-2.1(0)* males lived as long as wild type (Figure 16A). Another possibility for the mating deterioration is morphological deformities of the sex musculature. However, in 2-day-old *sir-2.1(0)* males expressing a functional yfp:actin transgene (Figure16B), I did not observe any obvious muscle fiber disorganization, which normally occurs in 8-day-old wild-type males (Guo, Navetta et al. 2012). Although I did not inspect neural morphology, published studies showed that neural morphology does not change in *C. elegans* during aging (Herndon, Schmeissner et al. 2002).

Another potential explanation for the lower mating efficiency is that sperm activation in 2-day-old *sir-2.1(0)* males is defective. *C. elegans* male sperm are stored in the seminal vesicle as a non-activated form and become activated after transfer into a hermaphrodite. Regulated by proteases, individual sperm goes through a morphological change to form a pseudopod. This pseudopod provides mobility for the sperm to fertilize the hermaphrodite oocyte (Smith and Stanfield 2011). To test whether the low mating potency of 2-day-old *sir-2.1(0)* males is due to failure in sperm activation, I did an *in vitro* sperm activation assay and found that similar to wild type, 92.0 ± 4.3% of sperms from 2-day-old *sir-2.1(0)* can be artificially activated by pronase (Figure 16C).Taken together, I speculate that the premature mating decline in *sir-2.1(0)* is due to

physiological changes, rather than the structural degeneration of either neuromuscular circuits or sperm.

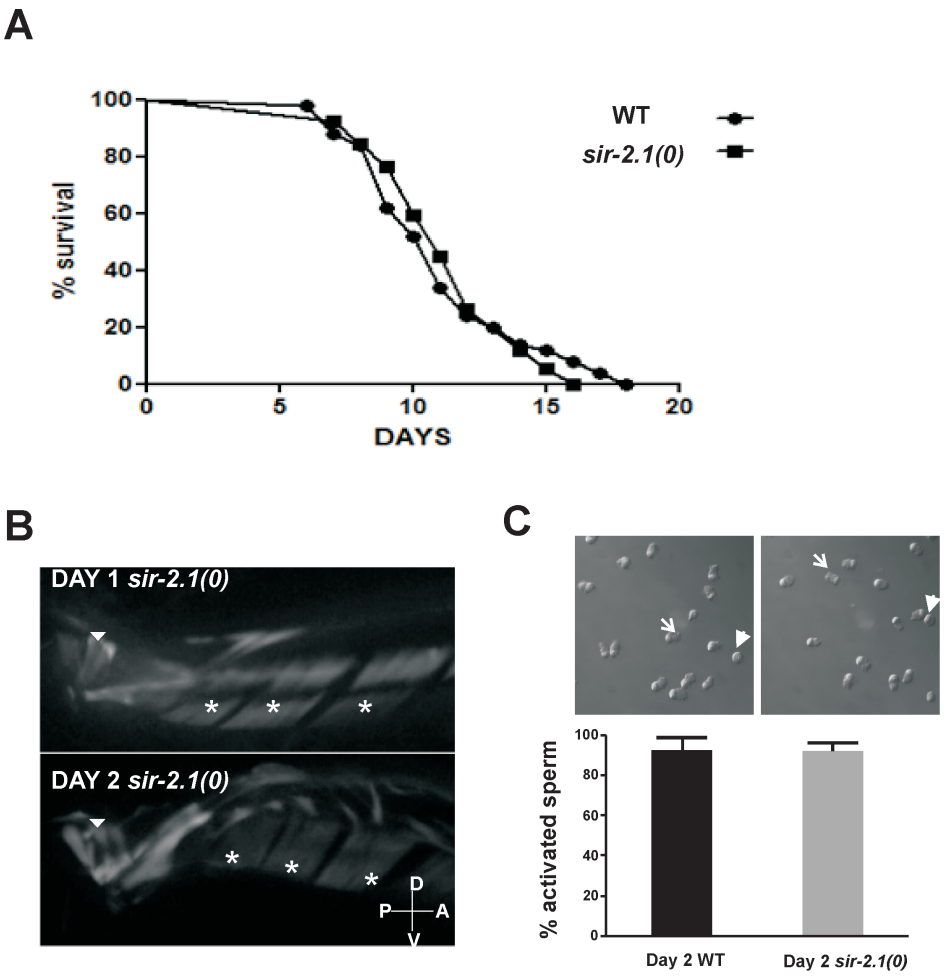

Figure 16 *sir-2.1(0)* males mating deterioration is not due to structural dysfunction. (A) Adult lifespan of wild-type (circles) and *sir-2.1(0)* (squares) males (n=50 for wild-type males; n=76 for *sir-2.1(0)* males) (Log-rank (Mantel-Cox) Test). (B) Muscle fiber organization in the genital muscles of 1-day-old and 2-day-old *sir-2.1(0)* males. Asterisks indicate the diagonal muscles, arrow head indicates the oblique muscles. (C) *In vitro* sperm activation assays of 2-day-old wild-type and *sir-2.1(0)* males. Representative images are shown on the top of percentage bars. Arrow indicates the activated sperm with pseudopod, and solid arrowhead indicates the inactivated sperm. No significant differences were observed between wild type and *sir-2.1(0)* males (unpaired t-test, N=5 trials). In each trial, 50-60 sperm cells were analyzed.

sir-2.1(0) males mating circuit becomes more excitable

I showed that wild-type mating deterioration at day 3 is correlated with an increased excitability in the mating circuitry (Chapter III, Figure 8 and 9)(Guo, Navetta et al. 2012). Hence, I hypothesized that *sir-2.1(0)* mating decline might also be due to a premature increase in cellular excitability. To test this, I used two acetylcholine (ACh) agonists, levemisole (LEV) and arecoline (ARE) to determine the response of wild-type and *sir-2.1(0)* males at multiple ages. In the male spicule intromission circuit, LEV binds to ionotropic ACh receptors, whereas ARE is a nonselective ACh agonist (Liu, LeBoeuf et al. 2007, Correa, LeBoeuf et al. 2012). Activation of ACh receptors depolarizes the male's neurons and muscles, and ultimately causes sex muscle contractions; as a result, males protrude their copulatory spicules. I found that at day 1, *sir-2.1(0)* and wild-type males had similar response to a sub-threshold effective concentration of ARE (50μM) (Figure 17A(i)). However, 2-day-old *sir-2.1(0)* males were more sensitive to agonist stimulation and required significantly less time to respond (Figure 17A (ii)).

Additionally, 2-day-old *sir-2.1(0)* males were more sensitive to sub-threshold LEV stimulation. 58% *sir-2.1(0)* compared to 35% of wild type protracted their spicules in 500 nM LEV ($P<0.05$, $n>30$) (Figure 17B (ii)). These results indicate that loss of *sir-2.1* in males alters the spicule intromission circuit's excitability during early aging.

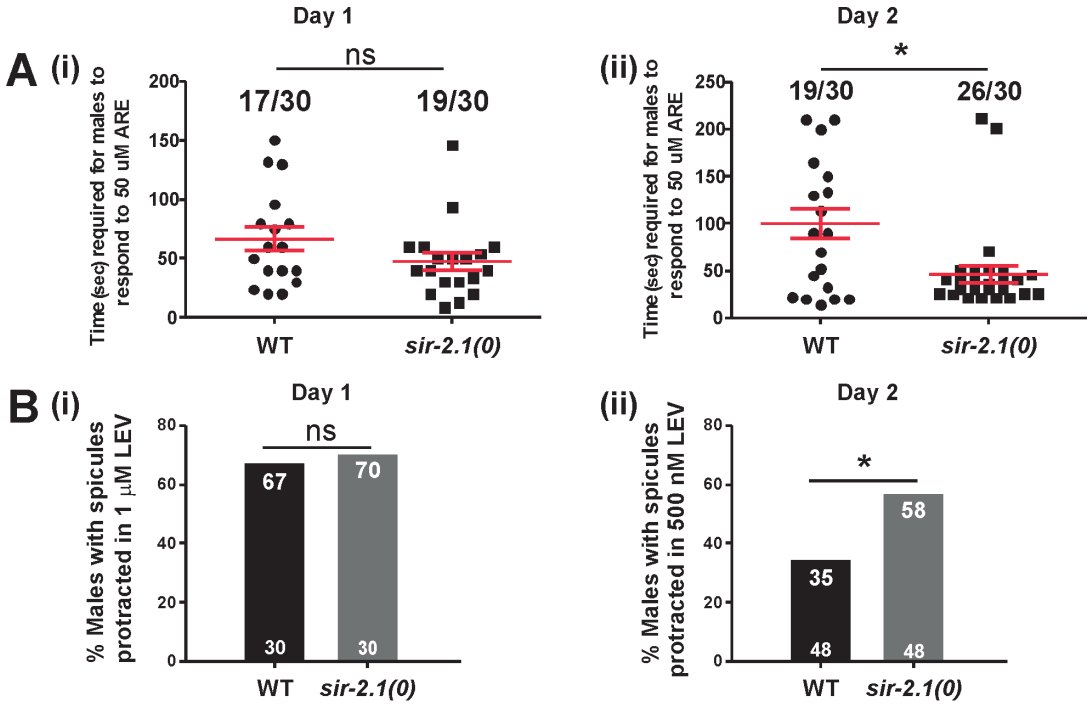

Figure 17 *sir-2.1(0)* males' sex circuitry becomes more excitable during aging. (A) 1-day-old wild-type and *sir-2.1(0)* males (n=30) have similar response to the ACh agonist arecoline (ARE). The time required for those males to protrude their spicules out in 50 µM ARE solution are not significantly different (i) (unpaired t-test), whereas 2-day-old *sir-2.1(0)* males require significantly less time to respond to ARE (ii) (unpaired t-test). Mean and SEM are indicated. (B) 1-day-old wild-type and *sir-2.1(0)* males (n=30) have similar response to the ACh agonist levamisole (LEV) (i). However, 2-day-old *sir-2.1(0)* males are more sensitive to LEV (ii). (Fisher's exact test).

Hyper-excitability leads to an ejaculation defect

To address how hyper-excitability disrupts copulation, I observed the mating behavior of 2-day-old *sir-2.1(0)* and wild-type males and found that 2-day-old *sir-2.1(0)* males performed most of the mating steps similarly to the wild-type control (Figure 18A, B, and C). Although 2-day-old *sir-2.1(0)* males can effectively insert their spicules, a significant number of them failed to transfer sperm into the hermaphrodite (Figure 18D).

Upon spicule insertion, sperm moved out from the seminal vesicle and traveled through the vas deferens; however, they remained stuck in the vas deferens and did not drain through the cloacal opening. Even the exceptional *sir-2.1(0)* males that successfully ejaculated, transferred less sperm and produced fewer progeny (Figure 18E).

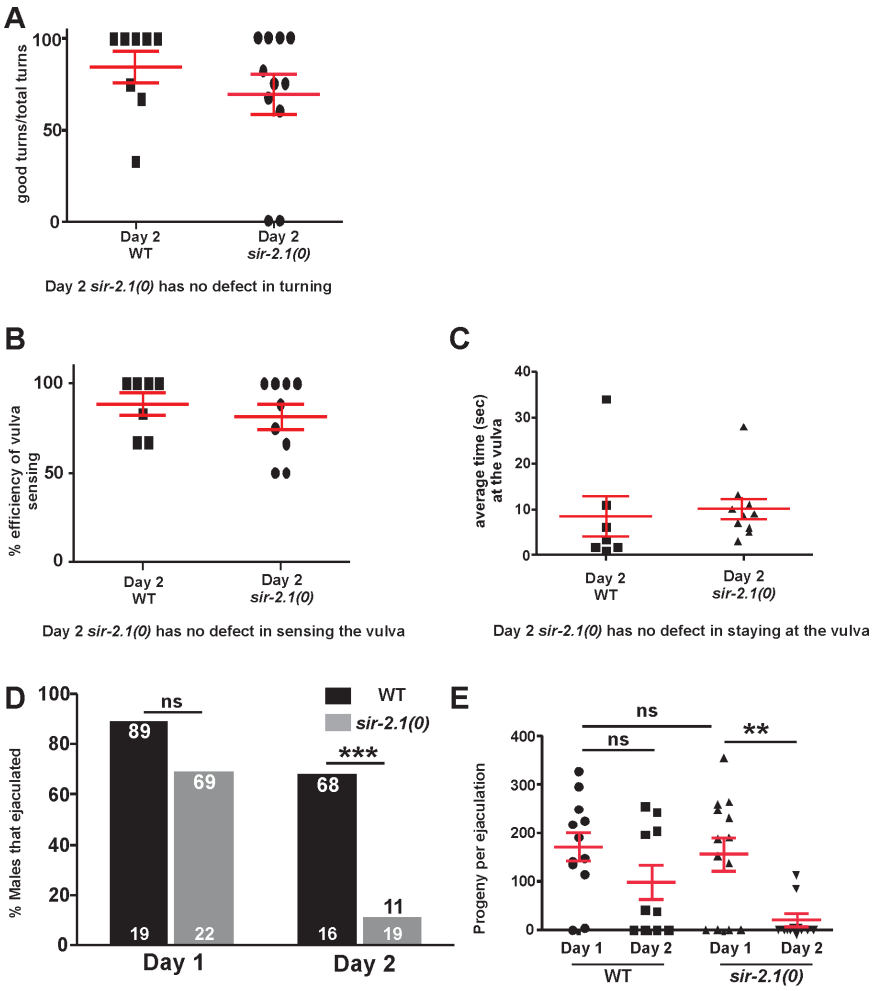

Figure 18 2-day-old *sir-2.1(0)* males display ejaculation defects.
(A) 2-day-old *sir-2.1(0)* males have no defect in turning behavior, (B) sensing the vulva of the hermaphrodite and (C) staying at the vulva. (D) The percentages of 2-day-old wild-type and *sir-2.1(0)* males that ejaculated during copulation. (Fisher's exact test). (E) The numbers of cross progeny produced by individual 2-day-old wild-type and *sir-2.1(0)* with *unc-64(e240)* hermaphrodites. Mean and SEM are indicated (unpaired t-test).

The male copulatory spicules are attached to 3 sets of sex muscles: the retractor, the protractor and the anal depressor muscles (Figure 2B). Contraction of the protractor muscles leads to spicules insertion into the vulva (Garcia, Mehta et al. 2001). During the normal ejaculation step of mating behavior, after spicule penetration, the posterior gubernaculum erector and retractor muscles contract, presumably to pull the proctodeum posteriorly, so that sperm can drain from the vas deferens (Figure 2B) (Liu, LeBoeuf et al. 2007). In *sir-2.1(0)* males, I speculated that after spicule insertion, the abnormal increased cell excitability causes the spicule protractor and anal depressor muscles to hyper-contract, which during sperm transfer, would pinch close the vas deferens opening. To test this, I imaged the Ca^{2+} in the spicule-associated dorsal protractor and anal depressor muscles (region-of-interest, ROI, indicated in figures 3A and 3B), by expressing G-CaMP3 in these sex muscles of both *sir-2.1(0)* and wild-type males (Tian, Hires et al. 2009, Guo, Navetta et al. 2012). During the mating behavior of 2-day-old wild-type males, the G-CaMP3 ΔF/F0 increased to 129.0 ± 32.5 % (n=5) at the time of spicule insertion, and the Ca^{2+} signal started to decline to 86.7 ± 30.8 % during the 10 sec period after spicule insertion (Figure 19). This indicates that the spicule protractor muscles partially relax after spicule insertion. However, in 2-day-old *sir-2.1(0)* males, ΔF/F0 increased up to 204.3 ± 97.5 % (n=5) upon spicule insertion. Unlike wild type, Ca^{2+} transients did not decrease as much, and the ΔF/F0 fluctuated at about 129.8 ± 33.0 % (Figure 19B). The sustained higher Ca^{2+} levels in 2-day-old *sir-2.1(0)* males suggest

that spicule protractor and anal depressor muscles are hyper-contracted and pinch the vas deferens opening, thus blocking sperm release.

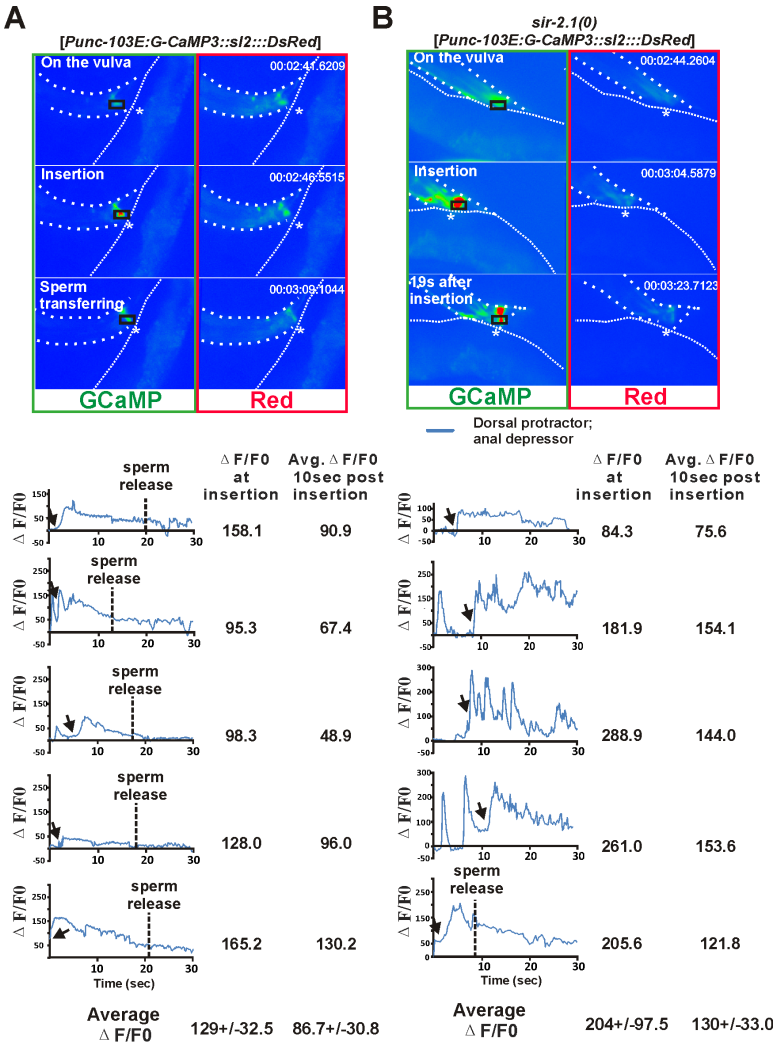

Figure 19 Ca^{2+} imaging of spicule-associated muscles in mating males.
Pseudo-colored images of Ca^{2+} in the spicule muscles of 2-day-old wild-type and *sir-2.1(0)* males during mating (A) and (B) are representative frames to show the Ca^{2+} levels of the spicule-associated muscles during spicule insertion attempts, penetration and the start of sperm transfer (~10 sec after insertion for wild type) or 19 seconds after insertion (for *sir-2.1(0)* males). The asterisks indicate the hermaphrodite vulva. Below the images, the Ca^{2+} transients in the protractor and anal depressor muscles (indicated by the black rectangle in A and B) are plotted for 5 individual wild type (A) and *sir-2.1(0)* males (B), respectively.

Reactive oxygen species (ROS) leads to the mating deterioration

C. elegans hermaphrodite studies showed that SIR-2.1 promotes the antioxidant genes expression through its association with the FOXO/DAF-16 transcription factor (Berdichevsky, Viswanathan et al. 2006). *sir-2.1* null hermaphrodites are more sensitive to stresses such as reactive oxygen species (ROS) (Rizki, Iwata et al. 2011). Therefore, I asked if ROS-induced damage contributes to the premature mating deterioration. I confirmed that similar to hermaphrodites, *sir-2.1(0)* males are also more sensitive to paraquat, a ROS-generator. Mutant males are less viable in 10 mM paraquat after 24 hrs; 89% of *sir-2.1(0)* males survived, compared to 99% of wild type ($p<0.01$, $n>100$) (Figure 4A). When exposure time reached 48 hours, the difference between two strains became more obvious, 39% of wild-type males survived, compared to 4% of *sir-2.1(0)* ($p<0.001$) (Figure 20A).

Since *sir-2.1(0)* males are more sensitive to oxidative stress, I hypothesized that during aging, accumulated ROS from metabolism might contribute to the decreased mating efficiency and to the increased excitability of the spicule intromission circuit. To test this, I grew wild-type males on plates containing 1 mM paraquat from late L4 to adult and assayed their mating ability and genital muscle excitability. After exposure to paraquat for 24hrs, males showed significant decline in mating potency (Figure 20B). Additionally, these males also displayed increased genital muscle sensitivity to the day 1 EC_{50} concentration (1μM) of LEV. 56% of wild type protracted their spicules; however,

83% males exposed to paraquat responded to the ACh agonist (p<0.05, n=54) (Figure 20C).

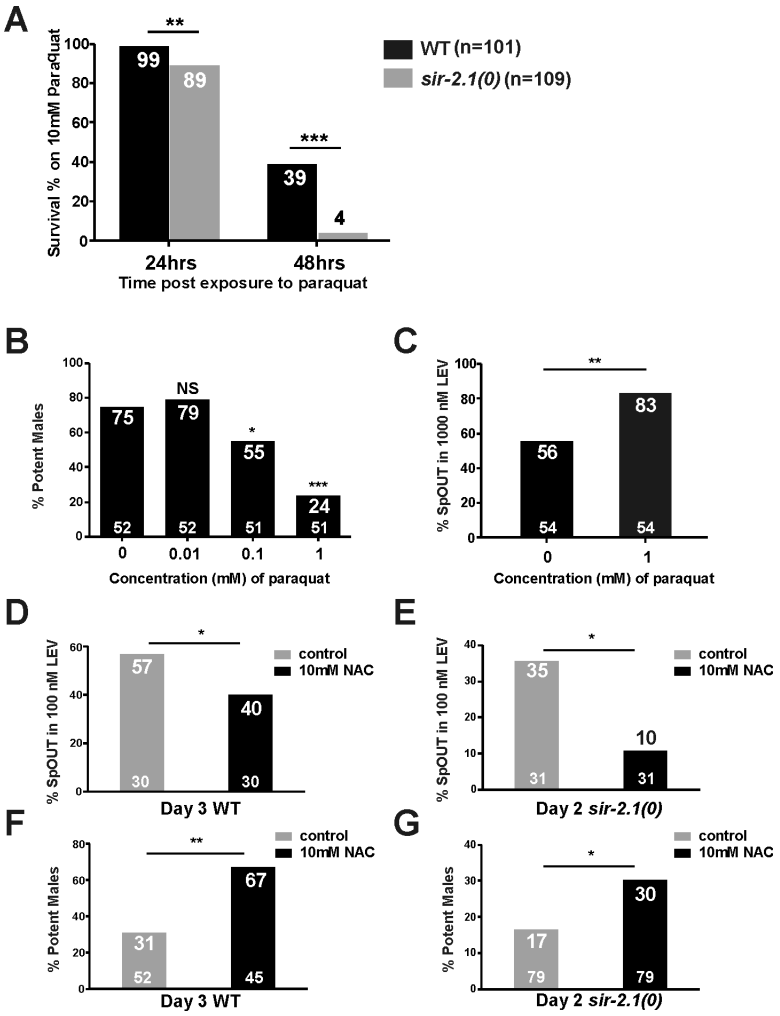

Figure 20 ROS contributes to the mating deterioration.
(A) Survival rates of wild-type and *sir-2.1(0)* males on NGM containing 10 mM paraquat at 24hrs and 48hrs post paraquat exposure. (B) Mating potency of 1-day-old wild-type males exposed to 0.01, 0.1 and 1 mM paraquat. (C) The percentages of males with their spicules protruding out (SpOUT) in response to 1µM levamisole (LEV) after treatment with 1mM paraquat. (D-G) Exposing males to N-acetyl-cystine (NAC) improves mating. The percentages of 3-day-old wild-type (D) and 2-day-old *sir-2.1(0)* (E) males that protrude their spicules out in response to 100 nM LEV after NAC exposure. Mating potency of 3-day-old wild-type (F) and 2-day-old *sir-2.1(0)* (G) males after NAC exposure (Fisher's exact test).

To further test if ROS contributes to the copulation decline, I supplemented the males' media with the antioxidant N-acetyl-cysteine (NAC) (Schulz, Zarse et al. 2007), and asked if NAC can delay genital muscle excitability changes and improve fertility. Indeed, when I exposed wild-type and *sir-2.1(0)* males to NAC from L4 to adulthood day 3 and adulthood day 2, respectively, the antioxidant decreased the males' sensitivity to 100 nM LEV (the EC_{50} concentration for older males (Guo, Navetta et al. 2012)) (Figures 20D and 20E) and increased their mating potency (Figures 20F and 20G). These results are consistent with the idea that ROS contributes to the behavioral decline.

sir-2.1(0) males might have altered metabolism and reduced ROS scavenge capability

Next, I asked why 2-day-old *sir-2.1(0)* males are more sensitive to ROS. Metabolism as a major source of endogenous ROS stress might contribute to behavioral decline. SIR-2.1's role in regulating metabolic processes has not been well described in *C. elegans* hermaphrodites, and scarcely in males. Therefore, I compared the expression levels of 55 genes involved in multiple metabolic processes including: glycolysis, gluconeogenesis/glyceroneogenesis, citrate acid cycle, glyoxylate cycle, fatty acid metabolism and electron transport chain (ETC)/oxidative phosphorylation (OXPHOS) (Castelein, Hoogewijs et al. 2008) between age-matched *sir-2.1(0)* and wild-type males.

Out of the 55 genes I surveyed, 17 showed statistically significant changes (Figure 21); the information for all the genes is tabulated in the Appendix B.

Through real-time PCR, I found that mRNAs encoding key enzymes involved in the initiation of glycolysis (hexokinase (F14B4.2) and glucose-6-phosphate isomerase (Y87G2A.8)) and fatty acid oxidation (fatty acid acyl-CoA synthetase (C46F4.2)) were up-regulated in 1-day-old *sir-2.1(0)* and 2-day-old wild-type males, relative to 1-day-old wild type (Figures 21A and 21B). This is consistent with the published observation that hexokinase is also up-regulated in the whole body of conditional *sirt1* knock-out mice (Gomes, Price et al. 2013). Most enzymes involved in the TCA cycle did not change in their levels (Appendix B). In contrast, expression of ETC/OXPHOS components (*cco-1*, W09C5.8) was reduced in *sir-2.1(0)* (Figure 21F). Other genes that were significantly up-regulated include key anabolic enzymes like fatty acid desaturase (*fat-5,6,7*), pyruvate carboxylase (PC) (*pyc-1*) and phosphoenolpyruvate carboxykinase (PEPCK) (*pck-1* and *pck-2*), isocytrate lysase (*icl-1*) and aconitase-cytosol (*aco-1*), which are important for fatty acid biosynthesis, gluconeogenesis, glyceroneogensis and glyoxylate cycle (Figures 21D and 21E) (Yang, Kalhan et al. 2009). Fatty acid desaturase plays a critical role in lipid/triglyceride biosynthesis (Van Gilst, Hadjivassiliou et al. 2005, Flowers and Ntambi 2008). PC catalyzes the carboxylation of pyruvate to oxaloacetate (OAA), the first step that shunts pyruvate from glycolysis to gluconeogenesis/glyceroneogenesis. Alternatively, OAA, an intermediate of TCA, can be converted to phosphoenolpyruvate (PEP) by PEPCK directly inside the mitochondrion or transported and converted to PEP by PEPCK in cytosol (Figure 22A).

The up-regulation of fatty acid desaturase is consistent with the increased lipid staining in *sir-2.1(0)* males (Figure 14) (Figure 22).

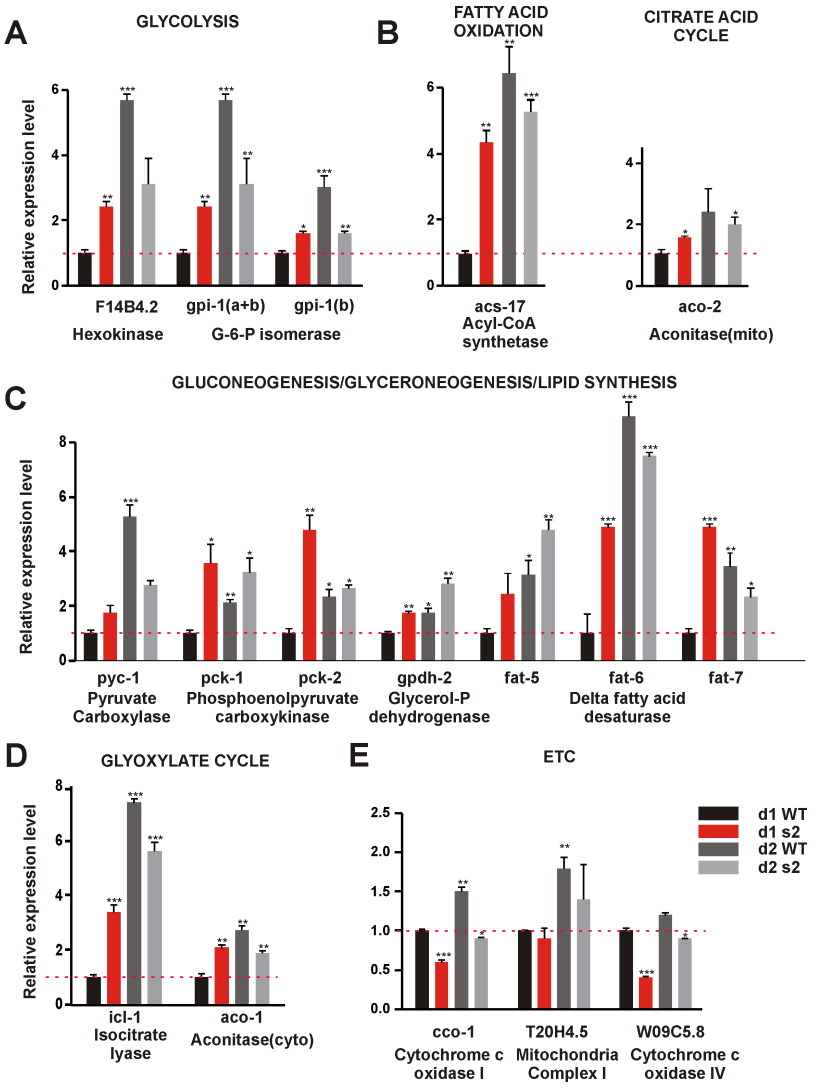

Figure 21 *sir-2.1(0)* males have altered expression of metabolic genes.
Relative mRNA expression level of genes involved in metabolic processes such as glycolysis (A), TCA cycle (B), fatty acid oxidation(C), Gluconeogenesis/glyceroneogenesis/lipid synthesis (D), Glyoxylate cycle (E) and ETC (F) in 2-day-old wild type, 1-day-old and 2-day-old sir-2.1(0) males relative to 1-day-old wild type. d1 WT refers to day1 wild type; d2 WT refers to day 2 wild type; d1 s2 refers to day1 *sir-2.1(0)*; d2 s2 refers to day 2 *sir-2.1(0)* (unpaired t-test compared to 1-day-old wild type).

To confirm if the changes in mRNA levels of those metabolic enzymes reflect functional alterations in the metabolic processes, I also measured ATP, glucose and glycogen accumulation in wild-type and *sir-2.1(0)* males. Consistent with increased expression levels of glycolysis and fatty acid oxidation genes, *sir-2.1(0)* males produced significantly more ATP at day 1. At day 2, wild type ATP levels increased to match the level of *sir-2.1(0)* males. But at day 3, *sir-2.1(0)* males again accumulated more ATP (Figure 22B). This data suggests that *sir-2.1(0)* and older wild-type males might have an enhanced catabolism, consistent with the potential to generate more ROS.

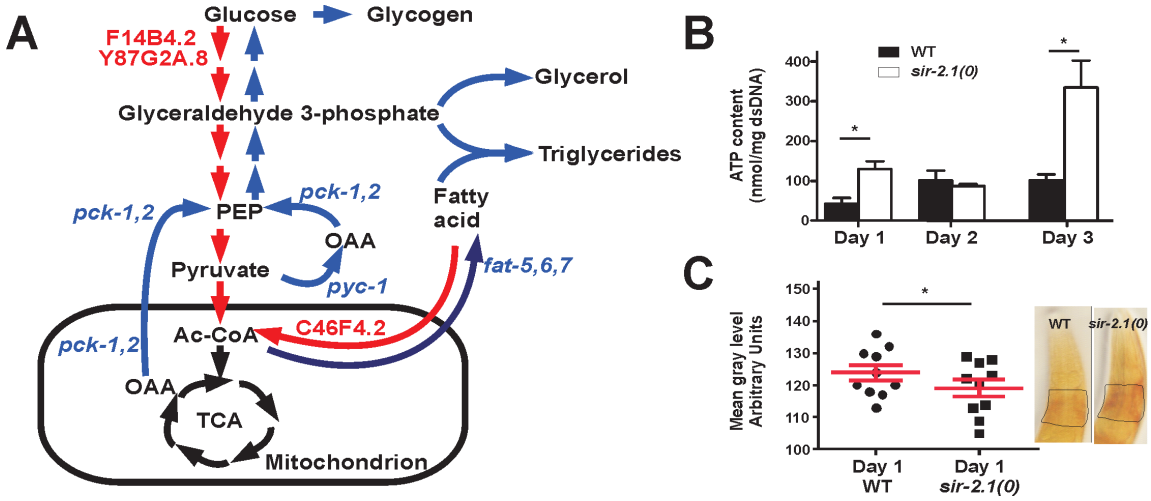

Figure 22 *sir-2.1(0)* males might have enhanced metabolism.
(A) Schematic illustration of main metabolic enzymes which have altered expression in *sir-2.1(0)* males. Red arrows indicate catabolic pathways. Blue arrows indicate anabolic pathways. (B) ATP content measured in 1, 2 and 3-day-old wild-type and *sir-2.1(0)* males. (C) Glycogen staining in 1-day-old wild type and *sir-2.1(0)*. The glycogen staining level is quantified by measuring the mean gray level of the ROI indicated on the top right corner. The mean gray level is inversely correlated with the iodine stain.

Based on metabolic roles of PEPCK (Yang, Kalhan et al. 2009), up-regulation of *pck* genes could lead to excess glucose/glycogen in *sir-2.1(0)* males. Although similar amounts of glucose were detected in *sir-2.1(0)* and wild-type males (data not shown), more glycogen was synthesized in the mutant (Figure 22C). In addition to gluconeogenesis, PEPCK catalysis of OAA to PEP is also a key step for the synthesis of glycerol-3-phosphate, which is used in triglyceride biosynthesis (Figure 22A) (Nye, Hanson et al. 2008). Indeed, males lacking functional *pck-2,* but not *pck-1* have less lipid content (Figure 23A). Additionally, *sir-2.1(0)* males that lack *pck-2,* but not *pck-1*, showed reduced lipid staining (Figure 23A), indicating that *pck-2* is necessary for the up-regulation of fat synthesis in *sir-2.1(0)*. Taking together the real-time PCR results, accumulation of metabolic products and hypersensitivity to paraquat, I propose that in *sir-2.1(0)* males, glycolysis and fatty acid oxidation are up-regulated to provide excessive NADH to the electron transport chain. Since I also measured reduced expression of ETC components cytochrome c oxidase, more ROS might be generated via electron leak (Lee, Hwang et al. 2010). However, I hypothesized that the enhanced expression of enzymes involved in anabolic processes might be a suboptimal self-compensatory mechanism to shunt excess pyruvate from being oxidized in the TCA cycle.

To test if the up-regulation of *pck* genes *in sir-2.1(0)* is a compensatory response, I assayed the mating potency of *pck-1(0)* and *pck-2(0)* single mutants and *sir-2.1(0); pck-1(0)* and *pck-2(0); sir-2.1(0)* double mutants. At day 1, *sir-2.1(0)* and *pck-2(0)* males mated comparable to wild type (Figure 23B). However at day 2, the potency of *pck-2(0)*

males started to decline similarly to *sir-2.1(0)*. In contrast, for males containing both *sir-2.1(0)* and *pck-2(0)*, their mating potency dropped at day 1 (Figure 23B). This indicates that without *pck-2*, males that contain or lack *sir-2.1* display accelerated behavioral decline. Similar to the requirement for functional *pck-2*, males that lack *sir-2.1* also needed *pck-1* to maintain their mating potency at day 1; however, *pck-1* was not required for *sir-2.1(+)* males to mate efficiently at day 2 (Figure 23C).

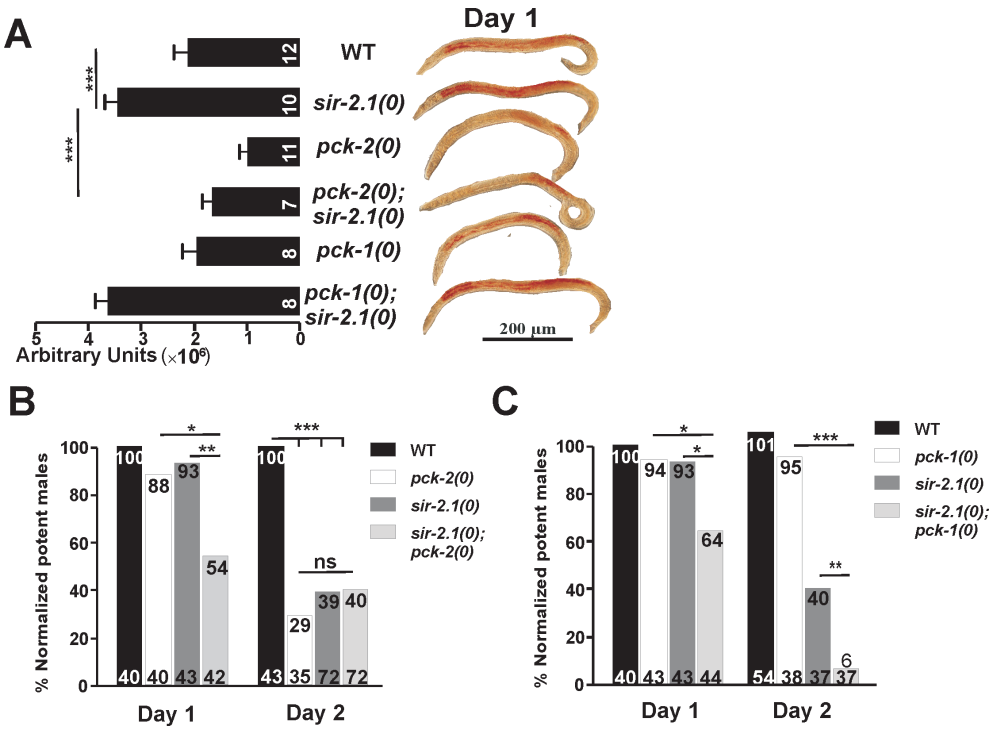

Figure 23 Potentially enhanced anabolism as a compensatory mechanism.
(A) Oil Red O staining of wild type and mutant *C. elegans* males. (B) *sir-2.1(+)* and *sir-2.1(0)* need *pck-2* to maintain their mating at day 2 and day 1 respectively. All percentages of mating potency are normalized to that of 1-day-old wild-type male. (C) *sir-2.1(0)* requires *pck-1* to maintain their mating at day 1 and day 2, while *sir-2.1(+)* males do not need *pck-2* to maintain their mating at either day 1 or day 2.

Next, I reasoned that if excessive glycolysis contributes to the behavioral deterioration, artificially adding extra glucose to the males' media could accelerate their mating decline. To test this, I grew males on UV-killed-OP50 NGM plates supplemented with 2% glucose, from hatched larvae up to the adult age prior to behavioral decline, which is day 1 or day 2, for *sir 2.1(0)* and wild-type males, respectively. I found that the glucose reduced mating potency of 1-day-old *sir-2.1(0),* but not 2-day-old wild type (Figure 24), indicating that wild type can cope with the extra glucose better than *sir-2.1(0)*.

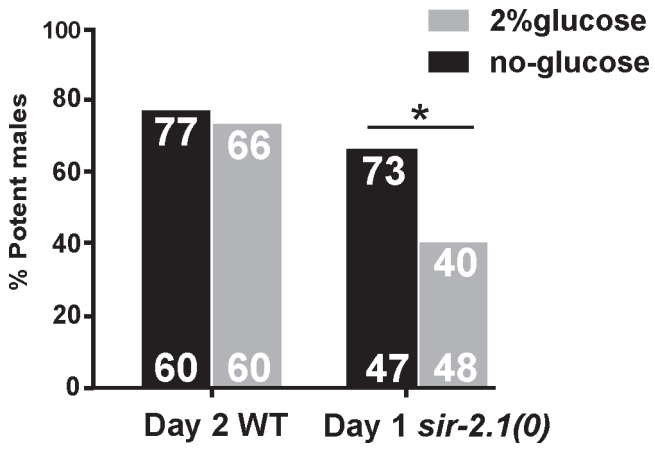

Figure 24 Glucose reduces 2-day-old *sir-2.1(0)* mating.
2% glucose reduces 1-day-old *sir-2.1(0)* mating potency, but not 2-day-old wild-type males (Fisher's exact test).

I hypothesized that unlike wild type, *sir-2.1(0)* males cannot efficiently respond to the oxidative stress generated by the enhanced catabolism. To test this, I used qRT-PCR to measure the mRNA levels of antioxidant genes: superoxide dismutase (*sod-1, 2*,

3, *4* and *5*), catalase (*ctl-1*, *2*) and glutathione transferase (*gst-10* and *gsto-1*) relative to 1-day-old wild-type males (Figure 25). As expected, the expression of *sod-1*, *sod-5*, *gst-10* and *gsto-1* was reduced in 1-day-old *sir-2.1(0)* and 2-day-old wild type (Figure 25). For *sod-2*, day 1 expression was also reduced in *sir-2.1(0)*, but this gene's expression increased in both wild type and *sir-2.1(0)* at day 2, possibly a stress response. For *sod-3* and *ctl-2*, their day 1 expression was similar in both strains; however at day 2, *sod-3* expression became higher and *ctl-2* expression became lower in mutants. Finally, *sir-2.1(0)* males displayed an increased *ctl-1* expression at day 1, which is also reported in antioxidant-compromised *daf-16(0)* mutant. The enhanced expression of *ctl-1* is considered as an adaptive response (Yanase, Yasuda et al. 2002). These results indicate that in addition to a potentially altered metabolism, which could generate excessive ROS, *sir-2.1(0)* males might also have a comprised antioxidant response, which is consistent with their hypersensitivity to excessive glucose intake (Figure 23) and to the ROS generator (Figure 20).

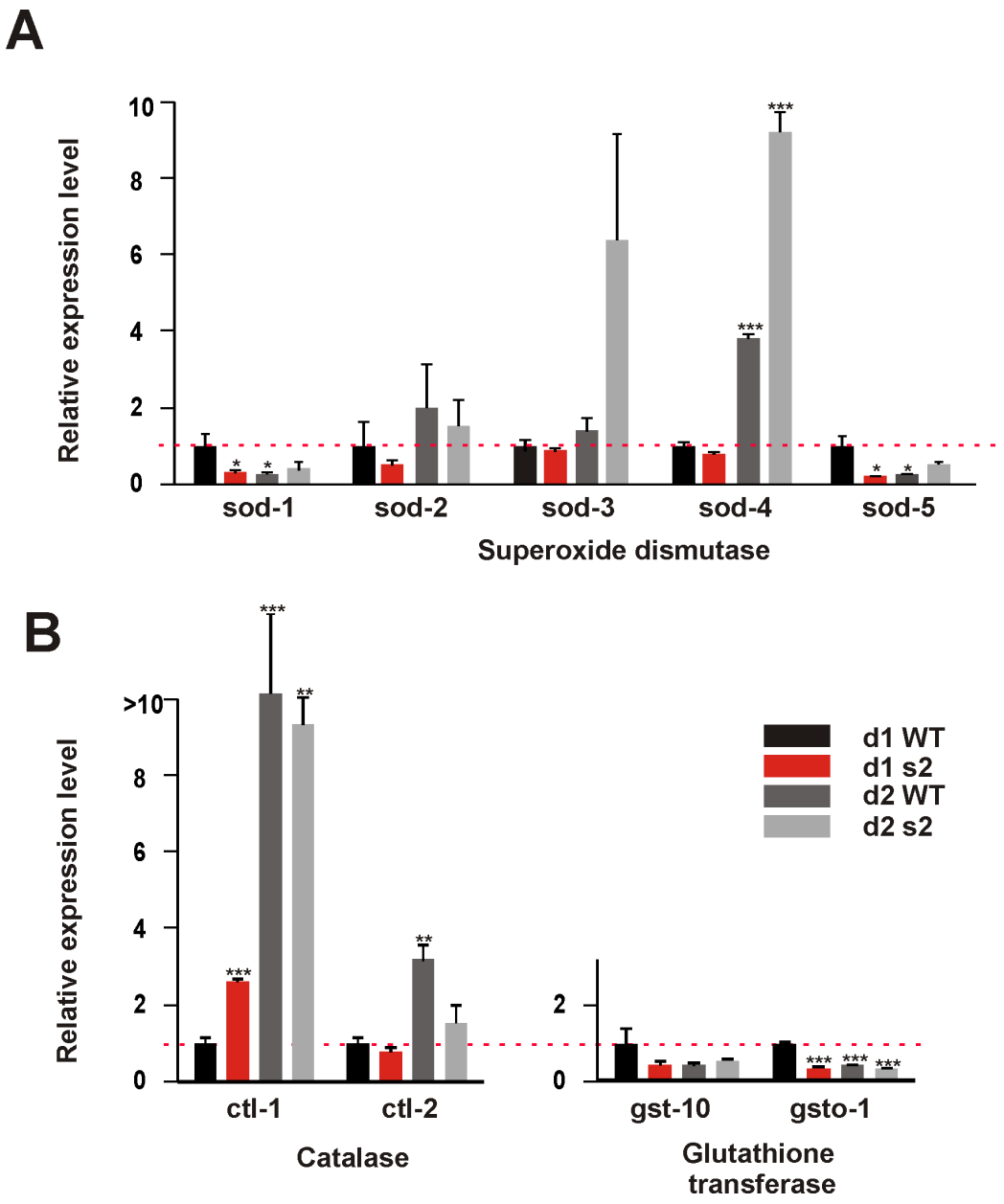

Figure 25 *sir-2.1(0)* males have compromised expression of anti-oxidant genes. Relative mRNA expression level of anti-oxidant genes superoxide dismutase (A), catalase (B) and glutathione transferase (C) in 1, 2-day-old wild type and *sir-2.1(0)* males (unpaired t-test).

Nicotinamide delays the deterioration of male mating behavior

Based on the above results, one could hypothesize that increasing SIR-2.1 expression or activity might delay mating deterioration during aging. However, I found that transgenic overexpression of *sir-2.1* does not improve the mating potency of 3-day-old wild type (Figure 26A). It is unlikely that the fusion with YFP disrupts SIR-2.1 function, because the same transgene can rescue the *sir-2.1(0)* phenotype. Thus, I speculate that up to a point, the expression level of *sir-2.1* is not rate limiting for SIR-2.1 activity during early aging. However, one could also speculate that the normal endogenous levels of NAD^+ limit the function of SIR-2.1. To test this, I grew males in the presence of the NAD^+ precursor nicotinamide (Nam) at 200 μM concentration (Houtkooper, Canto et al. 2010), and then conducted the mating potency. Indeed, Nam exposure significantly improves 3-day-old wild-type mating potency, but not 2-day-old *sir-2.1(0)* males (Figure 26B and 26C). This result is consistent with the idea that excess NAD^+ might stimulate SIR-2.1 activity. But additionally, excess NAD^+ might also reduce ROS production by relaxing the demand of oxidizing NADH back to NAD^+; as a corollary to this possibility, the lack of excess Nam to positively affect the *sir-2.1(0)* male's behavior might be aggravated by the abnormally high expression of catabolic enzymes in the mutant males. To further test if overexpressing SIR-2.1 activity can promote mating behavior in older males, I exposed 3-day-old transgenic SIR-2.1 over-expressed males with exogenous Nam, but found that excess SIR-2.1 does not amplify the positive effect of Nam (Figure 26D). Thus I cannot exclude the possibility that Nam

or possibly NAD^+ additionally promotes behavioral extension through mechanisms parallel to SIR-2.1 activity.

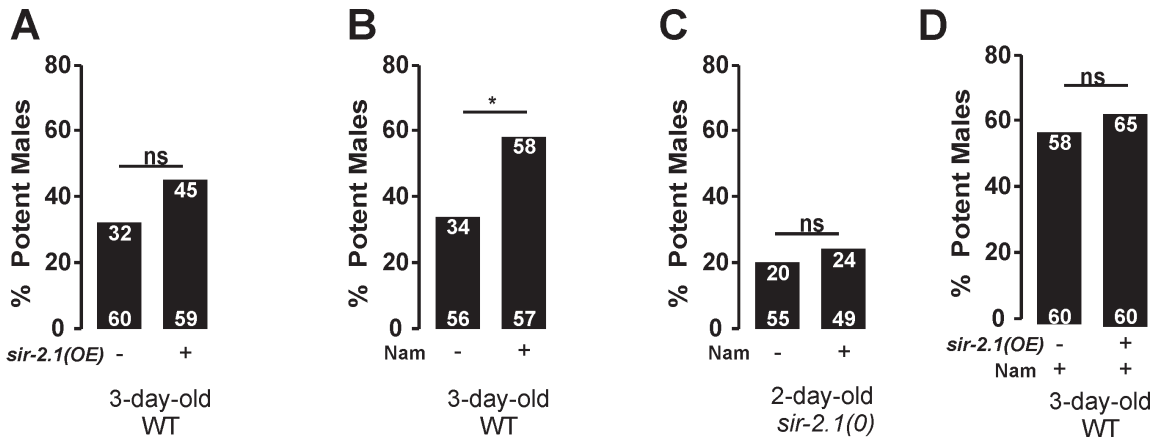

Figure 26 Exogenous nicotinamide improves mating during aging.
sir-2.1 overexpression cannot increase mating potency of 3-day-old wild type (A). However, feeding with a NAD^+ precursor nicotinamide (Nam) significantly improve the mating potency of 3-day-old wild type (B) but not 2-day-old *sir-2.1(0)* males (C). Overexpression of *sir-2.1* cannot further promote the effect of exogenous Nam (D).

Chapter summary

In chapter V, I determined the role of SIR-2.1 in maintaining male mating behavior during aging. Unlike wild-type males, *sir-2.1(0)* males display a premature decline of mating behavior. At day 2 of adulthood, only around 20 -40% of *sir-2.1(0)* males could mate successfully. Similar to wild type, 2-day-old *sir-2.1(0)* males have well-organized sex muscles structure and functional sperm. In addition, *sir-2.1(0)* males live as long as wild type, excluding the possibility that *sir-2.1(0)* premature behavioral decline is due to shorter lifespan.

sir-2.1(0) males display mating behavior deterioration due to behavior deficits. Through direct behavior observation, it seems that a significant proportion of 2-day-old *sir-2.1(0)* males fail to ejaculate. This phenotype can be explained by the hyper-excitability of the mating circuitry. Pharmacological drug tests implicated that although 1-day-old *sir-2.1(0)* males have similar sensitivity to both ACh agonists levamisole and arecoline, 2-day-old *sir-2.1(0)* males become more sensitive. By monitoring the sex muscle activity through calcium imaging, I found that the sex muscles, including the protractor and anal depressor, contract to insert the spicules into hermaphrodite vulva; in *sir-2.1(0)* males, the calcium increases significantly higher compared to wild-type males. After spicule insertion, in wild type, calcium in those sex muscles starts to decline followed by sperm transferring; however, in *sir-2.1(0)*, the calcium signal sustains a higher level and the muscles stay contracted, blocking the transfer of sperm.

In the last part of chapter V, I determined that ROS might be a potential cause for the hyper-excitability in the mating circuitry during early aging. Supplementing the worms' food with the ROS generator paraquat increases the excitability and reduces the mating potency, however, feeding worms with an antioxidant has the opposite effect. *sir-2.1(0)* males display a premature decline in mating behavior due to the fact that *sir-2.1(0)* males are under significantly more oxidative stress. I then determined that the excess ROS might be caused by altered metabolism status and compromised anti-oxidative stress response. Combining both qPCR data and quantitative measurements of the end-products of metabolism pathways, I speculated that at least during early aging, the glycolysis process is enhanced, which promotes oxidative phosphorylation to produce

more than enough ATP and ROS. However, the anti-stress system is not keeping up. In *sir-2.1(0)* this alteration occurs even sooner, which leads to the mating behavior deterioration at the second day of the adulthood. Intriguingly, I discovered that in addition to enhanced catabolism, anabolism processes are also up regulated, especially leading to lipid genesis, consistent with the Red Oil O staining of fat. It turns out that enhancement of anabolism is a sub-optimal compensation mechanism. When it is abolished, *sir-2.1(0)* males can not even mate at the first day of adulthood. In summary, SIR-2.1 is required to maintain male mating ability during early aging through optimize the metabolism processes and stress response to reduce oxidative stress, which are responsible for the hyper-excitability in the mating circuitry.

CHAPTER VI

SUMMARY OF EXPERIMENTS AND DISCUSSION*

Summary of experimental results

To study the potential mechanisms underlying behavioral deterioration during aging, I used a simple mating potency assay to characterize the dynamics of mating behavior decline. Interestingly, the *C. elegans* males mating behavior displayed a dramatic deterioration at the third day of adulthood, prior to any drastic morphological alterations, such as muscle disorganization or sperm dysfunction. Observation of mating behavior, pharmacological drug tests and calcium imaging of the sex muscles during both non-mating and mating states suggest that the hyper-excitability of the sex muscles disrupts behavioral coordination and leads to reproduction failure.

Lowering the neuromuscular excitability of the mating circuit through genetic and non-genetic manipulations increases mating potency. Males that carried heterozygous mutations in four acetylcholine receptor (AChR) genes, which mediate the response of the sex muscles to the mating cues, displayed extended mating vigor.

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* Reprinted with permission from Xiaoyan Guo, Andrew Navetta, Daisy G. Gualberto, and L. René García (2012) Behavioral decay in aging male C. elegans correlates with increased cell excitability. Neurobiol Aging. 33(7): 1483.e5–1483.23. (Copyright 2012 Elsevier) and Xiaoyan Guo, L. René García (2014) SIR-2.1 integrates metabolic homeostasis with the reproductive neuromuscular excitability in early aging male *C. elegans*. *eLife* :e01730 (Copyright 2014 elife Science Publication)

Transient starvation during the late L4 stage had a long-term effect on mating. This effect may be mediated by reprograming the expression of potassium channels to regulate the excitability of the mating circuitry.

Through studying males defective for the metabolic regulation via the mutation of histone deacetylase/SIR-2.1, I found that mating behavior was vulnerable to reactive oxygen species (ROS). Oxidative stress increased the excitability of the mating circuitry and reduced the mating efficiency. *sir-2.1(0)* males displayed a defect in ejaculation, which causes the premature decline in mating vigor. I observed that in 2-day-old *sir-2.1(0)* males, calcium transients increased more in the dorsal sex muscles and remained higher after spicule insertion, which caused sex muscle hyper-tonic contraction. The hyper-contracted sex muscles blocked the vas deferens opening, inhibiting sperm transfer from the male into the hermaphrodite.

SIR-2.1 is required to maintain male mating ability during aging, through regulation of both metabolism and the anti-stress response. qPCR data and quantification of the metabolic end-products indicate that in *sir-2.1(0)* males, the enhanced catabolism occurs one day earlier than wild type, accompanied by a comprised anti-oxidative stress response. As a result, *sir-2.1(0)* males suffer significantly more oxidative stress, which increases the excitability of the muscle and reduces the mating behavior. Meanwhile, the anabolism pathway, which ultimately synthesizes fat, is also up regulated, serving as a sub-optimal compensation mechanism to dampen the detrimental effects caused by the by-products generated from enhanced catabolism.

Using male mating behavior to discover the mechanism of behavioral decline prior to the drastic morphological changes that occur at an advanced age

Aging is associated with progressive behavioral decline that is relatively easier to investigate at an advanced age. In *C. elegans*, age-related behavioral decline is manifested through slower movement and decreased feeding, similar to other well-studied invertebrate and vertebrate model organisms (Forster, Dubey et al. 1996, Huang, Xiong et al. 2004, Simon, Liang et al. 2006). The decline in those general behaviors is usually correlated with the loss of cell integrity, such as cytoskeletal disorganization in the worm's body wall and pharyngeal muscle cells (Herndon, Schmeissner et al. 2002, Augustin and Partridge 2009). However, early indicators of behavioral decline can occur prior to the onset of gross morphological and structural changes to the cells. For example, people start losing memory capability in their late 20's, when there are no obvious pathological changes to their brain structure (Salthouse 2003). Therefore, the study of decline at an advanced age may not allow the identification of mechanisms responsible for early-phase behavioral decline. It would be advantageous to identify and explore a behavioral model that is sensitive to early phase changes in the neuromuscular circuitry during aging.

The complex mating behavior performed by *C. elegans* males offers an ideal model to study the early effects of aging on behavior. Male mating requires accuracy and coordination between the different groups of sex muscles; studying this behavior might uncover subtle physiological changes in the mating circuitry that reduce the efficiency of

copulation (Garcia, Mehta et al. 2001, Liu, LeBeouf et al. 2011). In contrast, a general behavior, such as locomotion on solid media, has been shown to be robust and require environmental manipulation, such as crawling into a food source or swimming in liquid media, to study the subtle or measurable changes that occur during early aging (Murakami, Bessinger et al. 2008, Hsu, Feng et al. 2009). Unlike general behaviors, the *C. elegans* male mating behavior starts to decline significantly at a very early stage of adulthood, when there is no obvious muscle structure disorganization or sperm dysfunction. Hence, I can use mating behavior, as a compliment to other successful behavioral models, to identify early manipulable factors that promote behavioral decline and, thus, provide potential therapeutic targets to delay the onset of structure dysfunction in neuromuscular cellular components.

Loss of precise regulation of neuromuscular excitability during aging affects the execution of behavior

To determine the physiological changes that occur in the *C. elegans* male mating circuit during aging, I carefully observed the sub-steps of mating in 1 and 3-day-old males. I found that ectopic spicule insertion behaviors, at non-vulval regions, were displayed in the older males. This suggests that the excitability state of the mating circuit might be increasing during early aging. I further confirmed that the excitability of the mating circuit increases during early aging, by showing that spicule muscles of non-mating 3-day-old males displayed more spontaneous Ca^{2+} transients and are much more

sensitive to the ACh agonist LEV stimulation, relative to 1-day-old males. Therefore, it is possible that the increased excitability in the mating circuitry, during early phase aging, contributes to the decline of copulation success.

The excitability of the neuromuscular circuitry is critical to control the execution of behavior. Changes in the excitability of neurons and muscles are associated with behavioral decline during aging among many organisms; reduced cell excitation would lead to the failure of initiating or maintaining behaviors, whereas increased excitability may result in exaggerated, promiscuous or uncoordinated behaviors. Similar to *C. elegans* males, other invertebrates, such as the mollusc *Lymnaea stagnalis,* display an increased sensitivity of excitable cell membranes to acetylcholine stimulation with age (Frolkis, Stupina et al. 1984). In contrast, many reports in vertebrate systems have demonstrated that the general excitability, in either neurons or muscles, is reduced during aging (De Luca, Mambrini et al. 1990, Karakelides and Nair 2005, Lopes, Smaili et al. 2007). For instance, the hippocampus-dependent learning ability decreases during aging, which is correlated with a decreased excitability in hippocampal neurons (Wu, Oh et al. 2002). Similarly, Ca^{2+} released from the sarcoplasmic reticulum is impaired during muscle contraction of old animals, resulting in feeble force generation (Jimenez-Moreno, Wang et al. 2008). However, similar mechanisms might be responsible for the differences in excitability states during aging between invertebrates and vertebrates. In the mammalian brain, calcium-imaging studies have demonstrated a significant increase of intracellular Ca^{2+} in response to a prolonged synaptic stimulation in hippocampal neurons of aged animals (Thibault, Hadley et al. 2001). Likewise, in the skeletal muscles

of aged animals, spontaneous intracellular Ca^{2+} leakage from oxidized sarcoplasmic reticulum ranodine receptor calcium channels contributes to the ultimate degradation of muscle activity (Andersson, Betzenhauser et al. 2011). This is consistent with our finding that there are increased Ca^{2+} transients in the male mating circuit. However, the Ca^{2+} current in mammals plays a signaling role, and the increase of Ca^{2+} activates calcium dependent potassium channels, which in turn hyperpolarizes the cell membrane to attenuate excitability (Murchison and Griffith 1995, Murchison and Griffith 1996, Thompson, Moyer et al. 1996). Whereas, in the muscles of invertebrates, such as *C. elegans*, the influx of Ca^{2+} through voltage-gated calcium channels (VGCCs) propagates depolarizing action potentials, and promotes excitation contraction coupling (Hagiwara and Byerly 1981, Maryon, Saari et al. 1998). Although the idiosyncratic cellular output may differ between aging cells of invertebrates and vertebrates, *C. elegans* male mating behavior can be used as a model to investigate the mechanism of increased Ca^{2+} transients, which appears to be conserved among species during aging.

Manipulation of cell excitability benefits behavior during aging

Manipulation of cell excitability can be used as a strategy to delay the deterioration of behavior. Excitability can be affected by changes to neurotransmitter pathways or the ion channels that maintain appropriate polarization. In the *C. elegans* male mating circuitry, acetylcholine is used to promote sex muscle contractions. Through halving the gene dosage of AChR genes, I found that the excitability of the

mating circuitry decreased and, concurrently, mating behavior was significantly improved at the third day of adulthood. The promiscuous spicule insertion behavior displayed by 3-day-old males mirrors the previously reported observation that 4-day-old *C. elegans* hermaphrodites display an exaggerated locomotor behavioral response when they enter an area of solid media containing food (Murakami, Bessinger et al. 2008). When young starved hermaphrodites crawl into an area containing their food, *E. coli*, they greatly reduce their velocity; this behavior is termed, the enhanced slowing response, and is regulated by the neurotransmitter serotonin (Sawin, Ranganathan et al. 2000). Well-fed 4-day-old *C. elegans* hermaphrodites inappropriately display this behavior, and reducing serotonin signaling, similar to our experiments when I reduce ACh signaling, attenuates the basal slowing response (Murakami, Bessinger et al. 2008). In addition to neurotransmitter signaling, many channels, such as sodium, potassium and calcium channels, also contribute to establishing and maintaining the membrane excitability. During aging, one potential reason for the decreased excitability in hippocampal neurons is increased intracellular Ca^{2+}, possibly through modification of Ca^{2+} channels. The increased Ca^{2+} then activates the calcium dependent potassium channels, such as the voltage- and calcium-dependent BK channel, which hyperpolarizes the cell membrane (Murphy, Fedorov et al. 2004). Therefore, decreasing the function of potassium channels is a potential strategy to increase cell excitability and promote learning. Indeed, in aged mammals, increasing neuronal excitability, through targeted deletion of a potassium channel subunit, Kvβ 1.1, in learning-associated pyramidal neurons in the hippocampal area CA1, enhanced learning and memory functions

(Murphy, Fedorov et al. 2004). Taken collectively, the available research suggests that genetic manipulation of neurotransmitter pathways or cell membrane excitability components is a feasible method to delay the deterioration of behavior.

In addition to genetic manipulation, our results demonstrate that transient food deprivation can reduce the excitability of the mating circuit and prolong copulation behavior after re-feeding. During starvation, the excitability of mating circuits is suppressed, probably to focus the animal's attention towards searching for food (Gruninger, Gualberto et al. 2008). Once feeding is resumed, the male's mating ability can be recovered within 1 hr (unpublished results) and, subsequently, mating ability is improved at the third day of adulthood. Caloric restriction has a positive effect on organisms ranging from yeast, worms and flies to mammals by extending lifespan. However, unlike in our experiments, prolonged dietary restriction has to be used to extend lifespan (Houthoofd and Vanfleteren 2006, Houthoofd, Gems et al. 2007, Greer and Brunet 2009). I was able to produce a positive, lasting effect on mating ability using about 20 hrs of food deprivation that does not affect males' lifespan. Thus, the increased mating ability in aged males is not due to lifespan extension, but could be a result of permanent changes to the mating circuitry produced by transient starvation.

A previous study suggests that the specific onset of food deprivation during *C. elegans* hermaphrodite adulthood is not critical to increase lifespan (Kaeberlein, Smith et al. 2006). Food deprivation either starting from the second, fourth or post-reproduction tenth day of adulthood would significantly increase the animal's longevity. In contrast, I found that only newly molted adult males are responsive to short-term starvation, since

starving males later in adulthood does not confer any beneficial effects. Taken together, these results suggest that the mechanisms of improving male mating potency by food deprivation differ from that of increased lifespan by caloric restriction.

Caloric restriction may extend lifespan through physiological changes such as increased autophagy, increased resistance to environmental stresses, decreased metabolic oxidative damage and overall decreased translation levels (Masoro 2005, Hansen, Chandra et al. 2008). However, protein synthesis is required during starvation to improve mating behavior. Young adulthood could be a critical developmental period that can be modified by transient starvation, to produce molecules, including potassium channels, which will offset the increased excitability. This hypothesis is consistent with our results that UNC-103 and EGL-2 potassium channels mediate the effect of transient starvation. Blocking protein synthesis during starvation and during re-feeding can restore the positive effect of starvation. These results suggest that during aging, signaling molecules might accumulate, which increase cell excitability. Combining the *sir-2.1(0)* study, it is possible that blocking protein synthesis might contribute to inhibiting the enhanced catabolism occurred during aging, thus reducing the generation of ROS.

SIR-2.1 is required to maintain male mating behavior during early aging

SIR-2.1 is a modulator of behavior and is required to maintain mating in aging males. 1-day-old *sir-2.1(0)* males can mate similarly to wild type, suggesting that *sir-2.1* is not essential for mating. However, unlike wild-type males, the mating ability of *sir-*

2.1(0) prematurely drops at day 2. This is due to hyper-excitability of the reproductive circuitry that coordinates spicule intromission and ejaculation. The hyper-excitability of the spicule muscles causes the male proctodeum to block the connection between the vas deferens and the cloacal opening, which indirectly obstructs the transfer of sperm. The mutant phenotype resembles the behavioral, physiological and pharmacological changes that occur in older wild-type males. This indicates that in wild type, SIR-2.1 maintains the functional excitability of the intromission and ejaculation circuit, possibly by slowing down the deteriorative events that accumulate during aging. I suggest that as the male ages, SIR-2.1 regulates the amounts of catabolic, anabolic and free radical scavenging enzymes to balance the energy demands needed for rapid reproductive motor responses, with the generation of damaging metabolic by-products, such as ROS.

My work indicates that the male's cellular physiology is correlated with his ability to mate. Perturbation of the male's physiology through genetic mutations or through dietary alterations during early adulthood will affect his ability to mate later in life. The physiology of wild-type males is likely changing from day 1 to day 2, as determined by the level of mRNAs encoding metabolic enzymes and the amount of terminal metabolic products. These physiological changes can ultimately lead to excessive carbon flow into the TCA cycle, and consequently, more NADH to be oxidized by ETC complexes (Figure 27). This promotes ROS generation via electron leak (Federico, Cardaioli et al. 2012), which can be reflected by behavioral decay at day 3. Analysis of *sir-2.1(0)* males allowed us to extrapolate how this protein deacetylase regulates male physiology. Lack of SIR-2.1 will induce these deleterious changes to

occur sooner, and degenerative behavioral responses in the mutant males are measured at day 2. Under standard laboratory conditions, wild-type males are raised constitutively on abundant *E. coli* until senescence. I speculate that since SIR-2.1 uses NAD^+ as a cofactor to deacetylate proteins, the physiological changes that occur after two days of constitutive feeding in wild type adults might be due to reduction in SIR-2.1 function, via the lower ratio of NAD^+ to NADH in wild type. The phenomenon of altering NAD^+ to NADH levels in vertebrate cells is shown to reduce the activity of SIRT1 (Braidy, Guillemin et al. 2011). Decreased activity of SIR-2.1 will not only aggravate a bias towards catabolism, but will also decrease the levels of ROS scavengers. This is consistent with a hermaphrodite study, which showed that *sir-2.1* overexpression can protect the organism from ROS, possibly via HCF-1 and FOXO/DAF-16, to regulate the expression of stress response genes (Rizki, Iwata et al. 2011).

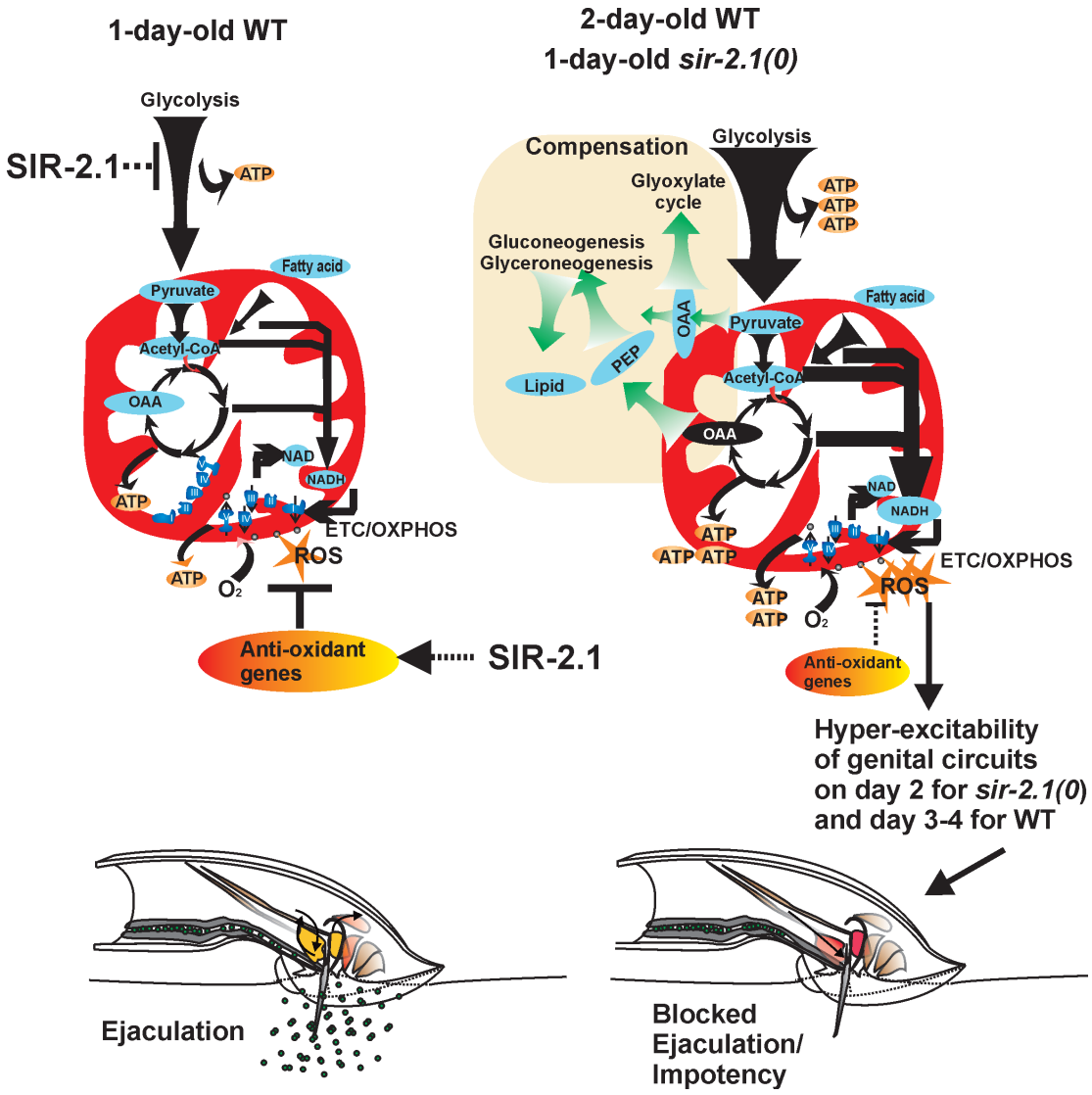

Figure 27 A cartoon of the metabolism and behavior that occurs in wild-type and *sir-2.1(0)* males during early aging.
For successful reproductive behavior, SIR-2.1 is required to maintain proper carbon flow to meet the male's energy demands and balance the generation of ROS. In 1-day-old old *sir-2.1(0)* males, catabolism such as glycolysis and fatty acid oxidation is enhanced, and consequently, oxidative phosphorylation and generation of ROS are also increased. Without SIR-2.1, ROS accumulation by day 2 of adulthood can lead to hyper-excitability of the male's genital neuromuscular circuitry. This results in blocked ejaculation and impotency. It is possible that in 2-3 day-old wild-type males, the NAD^+-dependent SIR-2.1 activity declines due to a lower ratio of NAD to NADH; thus older wild-type males might have a similar physiology as 1-day-old *sir-2.1(0)* males.

Enhanced anabolism serves as a compensation mechanism

Although our qPCR analyses suggest that enhanced catabolism might be occurring in 1-day-old of *sir-2.1(0)* and 2-day-old wild-type males, their mating ability could be facilitated via anabolic compensatory mechanisms. In addition to enhanced expression of catabolic genes, mRNAs encoding enzymes such as pyruvate carboxylase and phosphoenolpyruvate carboxykinase (PEPCK) are also up-regulated. This could be a likely reason for why the males contain more measurable lipids and glycogen. I propose that the up-regulation of anabolic processes is a self-compensatory mechanism to divert carbon from the TCA cycle (Figure 27). *sir-2.1(0)* males that are mutant for PEPCK genes, lose their ability to generate fat and fail to mate efficiently on day 1. Likewise *sir-2.1 (+)* males with a mutation in PEPCK genes also display premature mating decline. I hypothesize that anabolic pathways could act as a homeostatic mechanism to reduce ROS production. This idea raises the possibility that obesity, as a phenotype might be a compensatory mechanism to alleviate the effects of other underlying metabolic dysfunctions. A recent study showed that non-obese diabetic patients have higher mortality than overweight ones (Carnethon 2012). Another study showed that a lifestyle intervention focusing on weight loss did not reduce the rate of cardiovascular events in obese adults with type II diabetes (Wing, Bolin et al. 2013), challenging the traditional viewpoint that obesity is a major contributor to metabolic disorders.

Future directions

Does reproduction regulate the metabolic status?

Most studies in the aging field did not characterize alterations that occur during early aging, but instead focused on comparing differences between young and very old animals. In this work, I characterized changes that occur during the first three days of adulthood in worms. One obvious physiological change that occurs during this short period is an increase in ATP production, potentially indicating an enhanced catabolism. Additionally, males lacking the histone deacetylase/SIR-2.1 produce even more ATP. However, ATP production is reduced at an advanced age (Gruber, Ng et al. 2011). This is possibly due to the damaged mitochondria, which ultimately accelerates aging (mitochondrion theory of aging). Thus, perturbation of ATP production is one sign of aging in animals. In spite of this, ATP production cannot be used as a prediction for lifespan. Some lifespan extension mutants such as *daf-2(e1370)* have the ability to generate more ATP at both young and older ages. However, the anti-stress responses are also significantly elevated in these mutants (Murphy, McCarroll et al. 2003). Mild mutations in mitochondrion proteins extend lifespan by increasing the ROS companied with less ATP generation. In these mutants, the increased ROS serves as a stress signal to amplify the stress response. As a consequence, this promotes lifespan (Ristow and Zarse 2010, Hwang and Lee 2011). The *C. elegans* genome doesn't contain the gene encoding the mitochondrion uncoupling protein (UCP), which uncouples protons

gradient with oxidative phosphorylation (OXPHOS) and thus generates heat via proton leakage. Expression of exogenous *upc* genes from zebrafish in worms extends lifespan, potentially by reducing the generation of ATP and ROS at the same time (Sagi and Kim 2012). Thus, under different contexts, including genetic backgrounds, the production of ATP is not positively linked to the lifespan extension. A more important issue regulating aging is the sum of ROS generated and ability to the combat them.

What is the mechanism of ATP increase during early aging? Under normal conditions, I propose that consistent with the antagonistic pleiotropy hypothesis of aging (Williams 1957), during early ages of adulthood, the increase of ATP production might be beneficial for reproduction, because maintaining reproduction and sexual behavior is a energy-expensive process. However, ROS, the byproduct generated during ATP production, will affect the cellular function, which eventually damages the mitochondrion function. Thus, at an older age, the cell cannot produce more ATP. Though, at this age, less ATP does not mean less ROS, because the inefficient electron transport chain might leak more electrons compared to younger animals.

Prior mating experience may also impact ATP production and thus metabolic status. All *C. elegans* males I used in this study were virgins. It is possible that the lack of reproduction causes the males to generate more energy, so that they can anticipate the opportunity for reproduction. It is also possible that during early adulthood, the males are always generating more ATP until the mitochondria function is damaged. To test these possibilities, I will compare ATP levels between virgin and non-virgin males at different ages. More ATP produced in 2-day-old virgin males compared to non-virgin

males would suggest a communication between mating success and energy production. Similar amount of ATP would suggest that reproduction status is not involved in energy regulation. Additionally, I will perform the mating potency assay with non-virgins males at the third day of adulthood to determine whether prior reproduction success has a positive effect on mating behavior at a later stage. All these experiments will help me to explore the communication between the reproduction system and the metabolism of the whole system.

Reproductive success might regulate the metabolic status via SIRT-2.1. *sir-2.1(0)* virgin males, at least during early aging, produce more ATP than wild-type males. The reproduction system of a virgin male might send signals to other somatic tissues to generate ATP through inhibition of the function of SIR-2.1. To test this, I would compare ATP amount in virgin and non-virgin males lacking SIR-2.1. Additionally, I would like to find a way to develop an in vivo assay to monitor the activity of SIR-2.1 in virgin and non-virgin males.

How are the metabolism status and anti-stress responses regulated by SIR-2.1?

The coupling of metabolism and ROS production might explain why SIR-2.1 has a dual function in regulating both metabolism and stress responses. As a NAD^+/NADH dependent metabolism regulator, it is possible that SIR-2.1 regulates the redox state. When OXPHOS is enhanced, the increased ratio of NAD^+/NADH activates SIR-2.1 to fine-tune the activity of glycolysis to reduce OXPHOS. At the same time, SIR-2.1 up

regulates the expression of stress genes to minimize the detrimental effects of ROS generated during OXPHOS. Therefore, without *sir-2.1,* the system lost the ability to optimize the metabolism and oxidative stress.

SIR-2.1 regulates metabolism and stress response through gene expression regulation. In *sir-2.1(0)* males, the expression of many metabolic genes are altered. However, whether SIR-2.1 directly regulates the expression of these gene-functions has yet to be determined. One possibility is that SIR-2.1, as a histone deacetylase, globally inhibits gene expression through compacting the chromosome. If this were the case, removing *sir-2.1* would cause global up-regulation of gene expression. However, the data indicates that this possibility is unlikely. Of all genes I detected in *sir-2.1(0)*, only a small portion of them were significantly up-regulated compared to wild type. Some scavenger genes are down regulated, indicating a more specific mechanism involved in SIR-2.1 regulation of gene expression. Thus it might be worthwhile to do Chromatin Immunoprecipitation Sequencing (ChIP-seq) to determine the direct and indirect targets of the SIR-2.1.

Key enzymes of both catabolism and anabolism processes in *sir-2.1(0)* are up regulated. It is possible that enhanced catabolism is directly regulated by *sir-2.1(0),* as a primary response, while anabolism up-regulation might be an adaptive response, serving as a compensatory mechanism. Phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme of gluconeogenesis, is significantly up regulated in *sir-2.1(0)*. There are two forms of PEPCK, cytosol and mitochondrial forms. *pck-1* is predicted to be the cytosol form, while *pck-2* might be the mitochondrial form. Although the cytosol form of

PEPCK is well studied, the function of mitochondrion form is not. My work here suggests that PCK-1 and PCK-2 may function differently.

Deletion of either *pck* genes significantly reduces the mating of *sir-2.1(0)* at day 1, however, they may play different roles because of the following two observations. First, unlike *pck-2*, lack of *pck-1* does not affect day 2 mating of wild type. Second, *pck-2* is required to maintain the lipid genesis in both wild type and *sir-2.1(0)*, while *pck-1* is not. It is possible that in *pck-1(0)*, *pck-2* can be up-regulated/compensated in a SIR-2.1 dependent manner. In *pck-2(0)* animals, however, *pck-1* can not be up-regulated or up-regulation of *pck-1* fails to compensate for the loss of *pck-2*. Thus, I hypothesize that *pck-2* might be under a complicated regulation: in the *pck-1(0)* context, SIR-2.1 up regulates PCK-2 function directly; while in *sir-2.1(0)* context, PCK-2 is also regulated possibly through other unknown regulation due to the altered catabolism.

Understanding the interaction of *pck* genes and how they are regulated would help to determine the mechanism organisms use to cope with altered metabolism under different situations. As I discussed above, the up-regulation of *pck-2* serves as an adaptive response, thus, determining how *pck-2* is regulated might be a chance to discover how metabolic pathways can be changed to compensate for damage that arises in other metabolic pathways. To address this question, I generated a knock-in strain with the endogenous *pck-2* tagged with *yfp* through the CRISPR-CAS9 system (Friedland, Tzur et al. 2013). Using this powerful tool, I can test the above hypothesis by comparing *pck-2* expression level in *pck-1(0)*, *sir-2.1(0); pck-1(0)* and wild-type males. Secondly, I can perform an EMS mutagenesis on the knock-in strain to screen for the mutants, in

which background *pck-2* expression is significantly altered. The mutation can be mapped by combining traditional two-factors mapping, SNP mapping and whole genome sequencing. Genes identified from this mutation screen can help me to understand how anabolism pathways are regulated.

How does oxidative stress contribute to mating behavior decline?

The free radical theory of aging states that aging is the result of free radicals-induced molecular damages (Harman 1956). Recently, this notion has been challenged by the hormesis theory, which posits that moderate amounts of physiological or environmental insults can reinforce cellular processes that reduce stress-induced damage (Schulz, Zarse et al. 2007, Afanas'ev 2010). However, in support of the free radicals theory of aging, artificially applied ROS is reported to change the excitability of cultured neurons and muscles through chemically damaging ion channels (Danson and Paterson 2006). Therefore, aspects of this theory of aging might still apply to physiological changes in neural muscular systems and behavioral decay. Consistent with this, I showed here that genetic and environmental conditions, which can lead to oxidative stress, caused increased excitability of the male mating circuitry and behavioral decay during early aging. The question is then how does ROS affect cell excitability and eventually behavioral output?

There is not a strict correlation between oxidative stress and changes in the electrical properties of neurons and muscles. Oxidation of different types of ion channels

changes their conductance and alters the cell's excitability (Annunziato, Parmaccione et al. 2002). One mammalian study showed that oxidative stress hyperpolarizes the resting potential, but extends the duration of the action potential in cardiac ganglion (Whyte, Hogg et al. 2009). Mitochondria ROS has been shown to trigger Ca^{2+} increases in the pulmonary arterial myocytes (Waypa, Marks et al. 2002, Waypa, Guzy et al. 2006). In a study using glia, L-type voltage-gated Ca^{2+} channels (L-VGCC) were found to be a target of ROS. After modification by ROS, their conductance of Ca^{2+} was increased (Bond and Greenfield 2007). Different from vertebrate skeletal muscles, L-VGCC in *C. elegans* propagates action potentials, and the entry of external Ca^{2+} directly promotes excitation-contraction coupling (Lee, Lobel et al. 1997, Maryon, Saari et al. 1998). Previous work showed that the pore forming subunit of L-VGCC, EGL-19, in *C. elegans* is required for sustained tonic contraction of the copulatory spicule muscles (Garcia, Mehta et al. 2001). Therefore, oxidation of L-VGCC in *C. elegans* might contribute to the increased excitability of mating circuits.

Other major targets of ROS are voltage-gated K^+ channels. One *C. elegans* study indicates that oxidative stress reduces cell excitability by increasing the conductance of K^+ channels (Sesti, Liu et al. 2010). In another *C. elegans* study, oxidation of voltage-dependent potassium channel/KVS-1 slows down its inactivation, leading to hyperpolarization and sensory function loss (Cai and Sesti 2009). The ERG-like K^+ channel UNC-103 is a major excitability regulator of the sex circuit (Reiner, Weinshenker et al. 2006). Although there is no report of oxidative modification on UNC-103, human encoded H-ERG channels can be activated by oxidative stress, so that

cells become hyperpolarized and less excitable (Cui and Zhang 2013). Considering that ROS increases the excitability of the mating circuit, it is possible that L-VGCC is more prone to be oxidized than K^+ channels in male reproductive cells.

To determine whether enhanced L-VGCC's function due to the oxidative stress leads to mating failure, first I want to test if reducing the function of L-VGCC can improve mating behavior. I propose to treat worms with a L-VGCC blocker Nemadipine-A to mildly reduce the activity of the *C. elegans* L-VGCC, EGL-19, and ask whether this supplementation can improve mating in aged males (Kwok, Ricker et al. 2006). However, the dosages of the drug and time window of the treatment still need to be determined, as complete blocking EGL-19 will abolish mating behavior. Alternatively, I can use a genetic way to theoretically reduce EGL-19 function by testing the mating of males that carry one wild-type copy of *egl-19* and one copy of the partial loss function of *egl-19* allele *n582*. However, if improvement in mating behavior is observed, this might be due to the reduced excitability that occurs from lowering calcium influx.

To further test how oxidative stress regulates the physiological properties of those channels, it might be necessary to clone the *C. elegans* versions of L-VGCC/EGL-19 and ERG-like potassium channel/UNC-103 into *Xenopus* oocytes. This would allow me to to address if these channels are subjected to the regulation of oxidative modification and which amino acids are modified by the oxidative stress. I will address the later by through either injecting RNA transcribed from randomly mutagenized PCR products or site-directed mutation of cysteine, the potential target of oxidation

modification. I do need to test whether expression of *egl-19* alone is sufficient to function autonomously without co-expression of other subunits, although there is a report that expression of the alpha subunit can form functional voltage-gated calcium channel (Catterall 1991). Knowing which amino acids are more sensitive to the oxidative modification would allow us to modify the gene accordingly and in vivo test whether reducing the sensitivity of the protein to oxidative stress benefits behavior.

Conclusion

C. elegans is a powerful model organism to study molecular mechanisms regulating behavior decline as the organism ages. Due to its quick lifecycle, short lifespan, and easily amendable and conserved genome, extensive studies have focused on identifying signaling pathways that control lifespan. However, less research has focused on the functional decline that occurs during aging. In this dissertation, I took advantage of the accumulated knowledge on the regulation of a complex behavior displayed by male *C. elegans*. I characterized dynamics of male mating behavior deterioration as well as the physiological alterations, which potentially contribute to the behavioral decline. Prior to dramatic structural damage, physiological changes including altered metabolism, stress response, and hyper-excitability of the neuromuscular circuitry impact the males' ability to sir progeny when they are only 3 days old. In conclusion, I have identified previous unknown mechanism that impact behavioral

decline as an organism ages. These mechanisms may offer conserved targets for alleviation of age-related behavioral dysfunction in human.

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APPENDIX A

PRIMERS USED IN THIS STUDY

Primers used to construct actin:YFP:	
Actin-infbackbone(YFP) F	CATTCGTAGAATTCCAACTGAGCGC
Actin-infbackbone(YFP) R	TTTGTATAGTTCATCCATGCCATGTGTAATC
Actin-pGW322YFP inf F	GGCATGGATGAACTATACAAAATGTGTGACGAC GAGGTTGCCGC
Actin-pGW322YFP inf R	CAGTTGGAATTCTACGAATGTTAGAAGCACTTG CGGTGAACGATGG
Primers used to detect sir-2.1 deletion:	
Sir-2.1reverse	GAAAATCCTGCTCCGTTCTACAATTTTGCGAGAG
Sir-2.1forward	CTAGATCAAATGAGCATTCGGCTCCAGGAAAGAC
Primers used to detect *pck-2* deletion:	
pck-2delF	TGGGTTAGGAGTTGGTGGAG
pck-2delinsideR	TCTGGGTTGATAGCGTAGAG
pck-2delR	CAAACAAGTGTGTGAGGAGC
Primers used to detect *pck-1* deletion:	
W05G11.6-delF	CCATGACCAGAATGGGAACC
W05G11.6-delR	GCGTGAACTCTCTTCTCAAG
Primers used to PCR genomic *sir-2.1* and ligate to pSX422YFP to generate pXG5:	
Sir-2.1Sphlforward	CAGAGCATGCCCAATTCAAGTTCAGCAACCCGAG AAAGTGCAGAATGATG
Sir-2.1SalIreverse	ATGAGTCGACGATACGCATTTCTTCACACAAATG CGAGAATGTTTCAGGATCAATC
Primers used to remove sir-2.1 promoter from pXG5:	
Cassette sir-2.1F	ACGATGTCACGTGATAGTGGCAACGATTC
Cassette sir-2.1R	GCAAGCTTATCGATGATAAGCTGTCAAACATGAG
Primers used to sequence sir-2.1 in pXG5:	
sir-2.1F1	CCGTTGACGCAGTTGGTTC
sir-2.1F2	GACTTCTTGATGATGGTGCC
sir-2.1F3	GAAAATCCTGCTCCGTTCTAC
sir-2.1F4	CCAGGACAGTTCGTACCATC
sir-2.1F5	GCTCGGAAATTGTGATGACATC
sir-2.1F6	CTGCTCATCAAACCGTCTTTC

sir-2.1R1	CCGTATGTTGCATCACCTTC
sir-2.1R2	CCTCGTCAGATAGTACAAAGTC
sir-2.1R3	TCGTCAGCCACCGACATTC
sir-2.1R4	TCCTCACGAATCTCATTCCC
sir-2.1R5	TTCCGATATTCTGGCCCGC
sir-2.1R6	TAAGTACCGCGTTCTCTGAC
Primers not mentioned in the reference:	
qPCRsod-1F	CATGGTGGACCAAAATCCGAG
qPCRsod-1R	TCCGGCATGAACAACCATAG
qPCRsod-2F	TCACCGCAATTAAGAGCGAC
qPCRsod-2R	GTTGCCTCAAGTGGATCCTG
qPCRsod-3F	GGCTGTTTCGAAAGGGAATC
qPCRsod-3R	GGTTCTCCACCATCCTTAG
qPCRsod-4F	ATATTGAGTCACCGGCTTCC
qPCRsod-4R	GCGTCCCAAGTCATCAGTTT
qPCRsod-5F	CATGGAGGAAGAGATTCCGT
qPCRsod-5R	ACGTCCGATAACAGTGTTCG
qPCRgst-10F	TGGGAAGAGTTCATGGCTTG
qPCRgst-10R	TTGTTGACACAATCCTCGCG
qPCRctl-1F	GTGATGACATTCGAACAAGCTG
qPCRctl-1R	CTTGTTCGACCTCAGCGAAA
qPCRctl-2F	GAACTACTTCGCTGAGGTTG
qPCRctl-R	GGATGTAGTTTGGTCCAAGG
qPCRgsto-1F	GACAGGTTAACTGCGGTAGC
qPCRgsto-1R	TAACCTGGTTGAGATCCAGC
qPCRT20H4.5F	GTTTCGGAGTGATGCTTGGT
qPCRT20H4.5R	GAAGAGCGTGTTCTCCTCTG
qPCRW09C5.8F	TCGTCTCGACTACTGGTATC
qPCRW09C5.8R	AAGCTGTAGCGGTACAAGAG
qPCRcco-1F	CTTGCTGGAGATGATCGTTAC
qPCRcco-1R	CGGAATCTTGCTCACACATG
F1qPCR-W05G11.6	CTCGCCGAGCACATGTTGATCAT
qPCR-W05G11.6R1	TTCATCCAGGCGATGTCGTCTCC
FqPCR-R11A5.4	TGCTCATCCAAACTCGCGTTTCG
qPCR-R11A5.4R	AAATGAGTGGTACTCCCTGTGG

APPENDIX B

SUPPLEMENTARY QPCR DATA

In the following tables, d1 and d2 refer to day 1 and day 2 respectively; wt and s2 refer to wild-type control and *sir-2.1(0)*.

Expression of genes involved in the glycolysis process.						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Hexokinase	Y77E11A.1	d1wt	1	0.1064	30.38	0.11994
		d1s2	1.00096	0.09779	29.25	0.12784
		d2wt	1.77357	0.1901	28.34	0.14846
		d2s2	1.06502	0.10453	29.25	0.13744
	F14B4.2	d1wt	1	0.1092	29.87	0.12506
		d1s2	2.37567	0.17308	27.49	0.07589
		d2wt	5.66306	0.18025	26.16	0.0154
		d2s2	3.0559	0.17532	27.22	0.07543
	H25P06.1	d1wt	1	0.07682	29.3	0.07851
		d1s2	1.03449	0.0552	28.12	0.049
		d2wt	1.33519	0.07229	27.67	0.06967
		d2s2	0.93007	0.04654	28.37	0.06662
Glucose-6-phosphate isomerase	Y87G2A.8 (a+b)	d1wt	1	0.06089	26.99	0.03998
		d1s2	1.60147	0.07027	25.17	0.02196
		d2wt	3.04774	0.37286	24.17	0.17293
		d2s2	1.55307	0.06019	25.31	0.0485
	Y87G2A.8 (b)	d1wt	1	0.10034	30.77	0.1218
		d1s2	1.72797	0.1772	28.85	0.13551
		d2wt	5.5517	0.17828	27.08	0.02998
		d2s2	3.62102	0.30323	27.87	0.11757

Expression of genes involved in the glycolysis process. (Continued)						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Fructose-1,6-bisphosphate aldolase	F01F1.12 (a)	d1wt	1	0.07908	26.57	0.10628
		d1s2	0.33052	0.04283	27.06	0.174
		d2wt	0.49573	0.03912	26.02	0.09411
		d2s2	0.1619	0.0726	28.25	0.64688
	T05D4.1	d1wt	1	0.05522	26.35	0.06802
		d1s2	1.21215	0.06225	24.96	0.02843
		d2wt	0.9874	0.11207	24.8	0.15068
		d2s2	1.2802	0.08237	25.04	0.09259
Triosephosphate isomerase	Y17G7B.7	d1wt	1	0.17981	38.01	0.25607
		d1s2	1.20467	0.20831	36.63	0.23991
		d2wt	1.2318	0.33723	36.14	0.38974
		d2s2	0.82106	0.10723	37.34	0.18829
	C33D9.9	d1wt	1	0.36286	37.33	0.52103
		d1s2	0.34533	0.23638	37.75	0.98517
		d2wt	1.24927	0.23692	35.43	0.26599
		d2s2	1.26672	0.51877	36.03	0.5908
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	T09F3.3	d1wt	1	0.05407	26.1	0.06607
		d1s2	0.61891	0.03508	25.68	0.04481
		d2wt	1.25235	0.05681	24.2	0.01335
		d2s2	1.27157	0.0691	24.8	0.07812
	K10B3.8	d1wt	1	0.10255	21.56	0.13909
		d1s2	1.02461	0.09314	20.26	0.02697
		d2wt	0.88932	0.04181	20.18	0.03537
		d2s2	0.88156	0.0594	20.25	0.07722

Expression of genes involved in the glycolysis process. (Continued)						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Enolase	T21B10.2 (a)	d1wt	1	0.03624	23.39	0.01374
		d1s2	0.92983	0.0838	22.23	0.02081
		d2wt	1.38641	0.12321	21.37	0.11441
		d2s2	0.92294	0.11591	22.02	0.17129
	T21B10.2 (a+b)	d1wt	1	0.098	22.59	0.13208
		d1s2	1.23891	0.11034	21.02	0.00619
		d2wt	1.74328	0.08379	20.24	0.0382
		d2s2	1.56025	0.09918	20.46	0.07016
Phosphofructokinase	Y71H10A.1	d1wt	1	0.16373	30.16	0.22288
		d1s2	0.97783	0.09276	29.06	0.1233
		d2wt	1.27875	0.03963	28.59	0.02741
		d2s2	0.55169	0.05507	29.98	0.14131
	C50F4.2	d1wt	1	0.23574	31.69	0.33756
		d1s2	1.23372	0.07486	30.28	0.05462
		d2wt	3.78108	0.38765	28.2	0.13331
		d2s2	3.33461	0.11416	29	0.04894

Expression of genes in the gluconeogenesis pathway.						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Pyruvate kinase	F25WT.3	d1wt	1	0.04235	23.25	0.03448
		d1s2	0.99869	0.10241	21.99	0.07359
		d2wt	1.63074	0.08157	20.99	0.0136
		d2s2	1.35219	0.13152	21.33	0.1273
	ZK593.1	d1wt	1	0.03681	28.04	0.03934
		d1s2	1.21151	0.27977	26.5	0.29374
		d2wt	1.65971	0.20095	25.76	0.15965
		d2s2	1.02116	0.10231	26.53	0.12515
Pyruvate carboxylase	D2023.2	d1wt	1	0.1063	28.39	0.14915
		d1s2	1.6859	0.29039	26.37	0.19247
		d2wt	5.151	0.40664	24.47	0.08915
		d2s2	2.72635	0.34155	25.45	0.16564

Expression of genes in the gluconeogenesis pathway. (Contiuned)						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
phosphoenol pyruvate carboxykinase	R11A5.4(*pck-2*)	d1wt	1	0.13312	24.78	0.18063
		d1s2	1.70055	0.06513	22.97	0.04305
		d2wt	2.48293	0.33331	22.23	0.18777
		d2s2	1.57492	0.07683	23.22	0.04714
	pck-2	d1wt	1	0.14636	26.5	0.13719
		d1s2	4.7221	0.53461	22.89	0.01764
		d2wt	2.28587	0.2627	24.02	0.00603
		d2s2	2.55634	0.08994	24.04	0.01345
	pck-1	d1wt	1	0.12249	26.84	0.05208
		d1s2	3.45599	0.70984	23.35	0.29409
		d2wt	2.115	0.1175	24.46	0.04725
		d2s2	3.17199	0.52016	23.99	0.21079
F-1,6-biphosphatase	K07A3.1	d1wt	1	0.04266	24.32	0.03086
		d1s2	1.13446	0.0494	23.09	0.05241
		d2wt	1.43954	0.10222	22.55	0.09079
		d2s2	0.55338	0.09766	24.26	0.24918
G-6-phosphphate translocase	F47B8.10	d1wt	N/A	N/A	N/A	N/A
		d1s2	N/A	N/A	N/A	N/A
		d2wt	N/A	0.04342	39.29	0.13621
		d2s2	N/A	N/A	N/A	N/A
Pyruvate dehydrogenase E1α	T05H10.6	d1wt	1	0.03834	26.09	0.01497
		d1s2	0.78968	0.03435	25.38	0.05234
		d2wt	0.60241	0.19084	25.58	0.45456
		d2s2	0.27711	0.10825	27.03	0.55994

Expression of genes in citrate acid cycle.						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Pyruvate dehydrogenase E1β	C04C3.3	d1wt	1	0.07035	26.58	0.07776
		d1s2	1.26375	0.23267	25.19	0.26335
		d2wt	3.72248	0.43859	23.44	0.15974
		d2s2	0.00365	0.00735	33.77	2.90576
Citrate synthase	T20G5.2	d1wt	1	0.10134	22.23	0.13616
		d1s2	1.31588	0.03924	20.79	0.02551
		d2wt	2.06941	0.08433	19.94	0.03471
		d2s2	1.31784	0.10372	20.93	0.10081
Aconitase, mitochondrial	F54H12.1	d1wt	1	0.13449	27.77	0.16436
		d1s2	1.53767	0.05131	25.21	0.03618
		d2wt	2.30131	0.71774	24.46	0.44261
		d2s2	1.90564	0.24451	24.98	0.18396
Aconitase, cytosolic	ZK455.1	d1wt	1	0.12927	29.69	0.16641
		d1s2	2.05196	0.07236	26.72	0.03974
		d2wt	2.72634	0.17997	26.14	0.05017
		d2s2	1.89931	0.08	26.91	0.05718
Isocitrate dehydrogenase (NAD)	F43G9.1	d1wt	1	0.06922	23.73	0.05369
		d1s2	0.85378	0.01904	22.03	0.00514
		d2wt	1.05185	0.06683	21.56	0.04299
		d2s2	1.19415	0.02359	21.62	0.01973
Isocitrate dehydrogenase (NADP)	F59B8.2	d1wt	1	0.12679	28.09	0.16239
		d1s2	0.87492	0.06233	26.35	0.09774
		d2wt	1.02765	0.11557	25.95	0.14061
		d2s2	1.01594	0.10399	26.22	0.14624

Expression of genes in citrate acid cycle. (Continued)						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Ketoglutarate dehydrogenase	T22B11.5	d1wt	1	0.0852	27.06	0.08955
		d1s2	0.92173	0.03518	25.25	0.04499
		d2wt	2.21564	0.15223	23.81	0.05721
		d2s2	1.1497	0.02662	25.01	0.02631
Succinyl CoA synthetase	C05G5.4	d1wt	1	0.06868	28.91	0.05225
		d1s2	0.57666	0.07992	27.77	0.1974
		d2wt	0.58982	0.10811	27.57	0.25174
		d2s2	0.46495	0.05514	28.16	0.16987
Succinate dehydrogenase (iron-sulfur protein)	F42A8.3	d1wt	1	0.04333	30.83	0.01041
		d1s2	1.14911	0.10651	28.95	0.07013
		d2wt	0.67775	0.04777	29.66	0.06432
		d2s2	1.2025	0.19453	29.23	0.22579
Succunate dehydrogenase (cytochrome b)	T07C4.7	d1wt	1	0.16323	34.83	0.22728
		d1s2	0.87279	0.09869	33.35	0.13406
		d2wt	0.32814	0.05253	34.71	0.21708
		d2s2	0.82808	0.10891	33.78	0.18031
Fumarase	H14A12.2	d1wt	1	0.13212	35.9	0.18385
		d1s2	1.36871	0.25682	33.77	0.25424
		d2wt	0.95472	0.0582	34.24	0.03914
		d2s2	1.7979	0.26381	33.73	0.20328
Melate dehydrogenase (organelle)	F20H11.3	d1wt	1	0.06461	23.99	0.07847
		d1s2	0.94151	0.06889	22.4	0.05003
		d2wt	0.987	0.06123	22.28	0.04249
		d2s2	1.21334	0.06002	22.38	0.04004
Melate dehydrogenase (cytosolic)	F46E10.10	d1wt	1	0.11714	24.37	0.16133
		d1s2	0.71597	0.06565	23.17	0.09411
		d2wt	0.55651	0.08463	23.49	0.20477
		d2s2	0.6839	0.11868	23.59	0.24329

Expression of genes in the lipid hydrolysis process.						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Lipase	F28H7.3	d1wt	1	0.04352	25.49	0.03754
		d1s2	0.81159	0.05886	24.12	0.04801
		d2wt	0.77005	0.04925	24.14	0.04806
		d2s2	0.70344	0.11261	24.67	0.22327
Glycerol kinase	R11F4.1	d1wt	1	0.17583	28.3	0.24236
		d1s2	1.1224	0.21894	26.92	0.27667
		d2wt	2.46814	0.31071	25.36	0.1728
		d2s2	0.9673	0.13902	27.22	0.20304
Glycerol-3-phosphate dehydrogenase	K11H3.1	d1wt	1	0.42698	24.96	0.61143
		d1s2	1.7284	0.07835	22.96	0.04039
		d2wt	1.70531	0.17698	22.55	0.1389
		d2s2	2.81157	0.22471	22.34	0.10737
	F47G4.3	d1wt	1	0.14307	29.92	0.19233
		d1s2	1.4549	0.07836	28.17	0.05824
		d2wt	1.485	0.08642	27.71	0.06264
		d2s2	1.39296	0.16464	28.32	0.16525
3-hydorxyacyl-CoA dehydrogenase	F54D5.7	d1wt	1	0.06274	28.11	0.05079
		d1s2	0.88408	0.03712	27.07	0.03201
		d2wt	1.10091	0.08902	26.33	0.10239
		d2s2	1.16959	0.10188	26.75	0.11842

Expression of genes involved in the fatty acid oxidation process						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Enoyl-CoA hydroatase	F38H4.8	d1wt	1	0.09907	34.37	0.12172
		d1s2	0.66258	0.10765	33.75	0.22867
		d2wt	1.16806	0.16251	32.51	0.19277
		d2s2	0.79939	0.26406	33.57	0.4747
Acyl-CoA Synthase	C46F4.2	d1wt	1	0.09066	32.49	0.10721
		d1s2	4.53407	0.4092	29.1	0.11961
		d2wt	6.76741	0.82569	28.09	0.16691
		d2s2	5.5156	0.35384	28.9	0.08245

Expression of genes in the glyoxylate cycle.						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Isocitrate lyase/malate synthase	C05E4.9	d1wt	1	0.07561	26.81	0.09335
		d1s2	3.36432	0.22589	23.8	0.02909
		d2wt	7.41247	1.14013	22.61	0.20872
		d2s2	5.58599	0.36158	23.25	0.07998
Malic enzyme	Y48B6A.12	d1wt	1	0.06434	26.52	0.0737
		d1s2	1.67237	0.11601	24.52	0.03847
		d2wt	4.06895	0.24001	23.18	0.03954
		d2s2	2.33602	0.38009	24.21	0.22974

Expression of genes in anaerobic pathways.						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Cytosolic SDH/Fumarate reductase	F48E8.3	d1wt	1	0.20048	35.2	0.28367
		d1s2	1.90932	0.12751	33.01	0.02731
		d2wt	3.57631	0.25556	32.05	0.07036
		d2s2	1.43598	0.43968	33.6	0.4391
Lactate dehydrogenase	F13D12.2	d1wt	1	0.04483	27.56	0.03158
		d1s2	0.82503	0.06495	26.58	0.06605
		d2wt	0.70204	0.08217	26.76	0.15111
		d2s2	0.41437	0.09562	27.75	0.32943
Alcohol dehydrogenase	K12G11.3	d1wt	1	0.07593	26.97	0.09389
		d1s2	0.66419	0.10877	26.3	0.21745
		d2wt	1.99339	0.19172	24.66	0.11651
		d2s2	0.46046	0.01733	27.01	0.02496
6-phosphogulconolactonase	Y57G11C.3	d1wt	1	0.07334	28.8	0.0895
		d1s2	0.50309	0.03407	28.53	0.03179
		d2wt	1.2089	0.12347	27.21	0.12662
		d2s2	0.58388	0.03114	28.49	0.05998

Expression of genes in the pentose phosphate shunt pathway						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Ri(bul)ose-5-phophate isomerase	B0280.3	d1wt	1	0.29004	37.88	0.39933
		d1s2	0.98072	0.15028	36.31	0.21248
		d2wt	1.39019	0.18323	35.88	0.17428
		d2s2	1.435	0.1554	36.15	0.12427
Transketolase	F01G10.1	d1wt	1	0.07753	29.1	0.04574
		d1s2	1.38374	0.09912	27.03	0.0834
		d2wt	2.9183	0.14019	26.03	0.03079
		d2s2	3.00048	0.21047	26.31	0.03571
Transaldolase	Y24D9A.8 (a+b)	d1wt	1	0.07997	26.09	0.05376
		d1s2	0.80874	0.07255	24.8	0.11414
		d2wt	1.12486	0.05587	24.4	0.03578
		d2s2	1.57824	0.10407	24.23	0.00915
	Y24D9A.8 (a)	d1wt	1	0.07972	32.08	0.053
		d1s2	1.47371	0.08766	29.92	0.06034
		d2wt	2.05191	0.46138	29.52	0.3184
		d2s2	3.05124	0.37824	29.26	0.15172

Expression of genes in the electron transport chain.						
Enzymes	**Genes**		**Expression**	**SEM**	**Mean Ct**	**SEM**
Mitochondria complex I	T20H4.5	d1wt	1	0.11492	25.03	0.116
		d1s2	1.08133	0.07415	23.34	0.01035
		d2wt	1.78884	0.12983	22.35	0.10434
		d2s2	1.12046	0.01721	24.1	0.01899
Cytochrome C oxidase I	cco-1	d1wt	1	0.01801	21.46	0.01243
		d1s2	0.58967	0.0349	20.86	0.02543
		d2wt	1.49174	0.05977	19.97	0.03664
		d2s2	0.87353	0.02486	20.43	0.04069
Cytochrome C oxidase I	W09C5.8	d1wt	1	0.02702	21.65	0.03438
		d1s2	0.43065	0.0191	21.5	0.02114
		d2wt	1.24058	0.32254	20.43	0.37241