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# Molecular links between nutrition, reproduction and aging

Manjunatha Thondamal

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# THÈSE

en vue de l'obtention du grade de

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**Laboratoire de Biologie Moléculaire de la Cellule**

École Doctorale : **Biologie Moléculaire Intégrative et Cellulaire**

présentée et soutenue publiquement le **18 Novembre 2014**

par

**Manjunatha Thondamal**

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Titre

**“Molecular links between nutrition, reproduction, and aging”**

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Directeur de thèse : **Dr. Hugo Aguilaniu**

Professor Adam ANTEBI, Rapporteur

Dr. Francois LEULIER, Examineur

Dr Herve GUILLOU, Rapporteur

Dr Hugo AGUILANIU, Directeur de these

Professor Jean-Marc LOBACCARO, Examineur

Professor Vincent LAUDET, Examineur

*to*  
*my family*

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## *Abstract*

Dietary restriction (DR) increases healthspan and longevity in many species, including primates, but it is often accompanied by impaired reproductive function. Whether signals associated with the reproductive system contribute to or are required for DR effects on lifespan has not been established. In my doctoral thesis presented here, we show that expression of DAF-9/CYP27A1 and production of the steroid hormone  $\Delta^7$ -dafachronic acid (DA) are increased in *C. elegans* subjected to DR. DA signaling through the non-canonical nuclear hormone receptor NHR-8/NHR and the nutrient-responsive kinase *let-363*/mTOR is essential for DR-mediated longevity. Steroid signaling also affects germline plasticity in response to nutrient deprivation and this is required to achieve lifespan extension. Results presented in my thesis demonstrate that steroid signaling is activated by nutrient scarcity and is required for DR effects on lifespan extension through TOR signaling. In the absence of proper steroid signaling, *let-363*/mTOR levels remain high during starvation and the number of germ cells within the proliferative zone of the germ line is no longer affected by nutrient availability. Interestingly, genetic reduction of germ cells alleviates the requirement for steroid signaling for DR-mediated lifespan extension. Genetically lowering the germ cell count mimics the response of the germ line to DR. These data demonstrate that steroid signaling links germline physiology to lifespan when nutrients are limited, and establish a central role for *let-363*/mTOR in integrating signals derived from nutrients and steroid hormones. We speculate that this induces a signal that is usually emitted when nutrients are scarce and the germ line becomes less active. Taken together, this thesis work suggests that, diet-induced lifespan extension is part of a coordinated response that involves reproductive phenotypes.

## *Résumé*

Une restriction alimentaire améliore la qualité du vieillissement et augmente la durée de vie chez de nombreuses espèces, y compris certains primates. Cependant, cette intervention s'accompagne souvent d'une baisse significative des capacités reproductives. Il est donc légitime de se demander si des signaux provenant du système reproductif contribuent aux effets positifs de la restriction alimentaire sur la longévité. Durant ma thèse, j'ai montré que l'expression de DAF-9/CYP27A1 et la production de l'hormone stéroïdienne D7- acide dafachronique (DA) sont augmentées chez *C. elegans* lorsque les vers sont soumis à une restriction alimentaire. De plus, la signalisation à l'acide dafachronique via le récepteur hormonal nucléaire NHR-8/NHR et la kinase *let-363/mTOR* est essentielle à l'augmentation de la durée de vie par restriction alimentaire. La signalisation stéroïde affecte également la plasticité de la lignée germinale en condition de jeûne. De plus, nous montrons que cette plasticité est nécessaire à l'augmentation de la longévité dans ce contexte de restriction. Les résultats présentés dans cette thèse démontrent que la signalisation des hormones stéroïdes est activée par le manque de nutriments et est requise pour l'augmentation de la longévité par la voie *mTOR*. En effet, chez un animal sauvage, le niveau d'expression de *let-363/mTOR* diminue en condition de jeûne. Ceci n'est pas observé lorsque les hormones stéroïdes sont absentes. De plus, le nombre de cellules de la lignée germinale au sein de la zone proliférative n'est plus affecté par le jeûne chez des animaux pour lesquels la synthèse d'hormones stéroïdes est inhibée. Une réduction artificielle du nombre de cellules germinales suffit à rétablir une réponse normale à la restriction alimentaire. Ceci suggère donc qu'il existe un lien étroit entre la lignée germinale et la longévité induite par une restriction alimentaire, et que ce lien repose en partie sur la signalisation des hormones stéroïdes. La kinase *let-363/mTOR* joue également un rôle central dans l'intégration de signaux nutritionnels et reproductifs. Nos données suggèrent également l'existence d'un signal entre lignée germinale et soma produit en condition de restriction alimentaire. L'augmentation de la durée de vie par restriction alimentaire implique donc une réponse systémique coordonnée qui implique l'appareil reproducteur.

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## Abbreviations

### **A**

A $\beta$  Amyloid- $\beta$  peptide  
AMPK AMP-activated protein kinase

### **B**

BD Bacterial deprivation

### **C**

*C. elegans* *Caenorhabditis elegans*  
CLS Chronological life span

### **D**

DR Dietary restriction  
dsRNA Double-stranded RNA  
DA Dafachronic acid  
*D. melanogaster* *Drosophila melanogaster*  
Daf Abnormal dauer formation

### **E**

*E. coli* *Escherichia coli*  
ER Endoplasmic reticulum  
ERC Extrachromosomal ribosomal DNA circle

### **F**

FU 5-fluorouridine

### **G**

GFP Green fluorescent protein  
GH Growth hormone  
GHR Growth hormone receptor

### **H**

HIF Hypoxia-inducible factor  
HSF Heat shock factor  
HSP Heat shock protein

### **I**

IGF1 Insulin-like growth factor 1  
IIS Insulin/IGF-1 signaling

### **L**

LH Luteinizing Hormone

## ***N***

NAD Nicotinamide Adenine Dinucleotide  
NGM Nematode growth medium

## ***P***

PI3-kinase Phosphatidylinositol-3OH kinase  
PIP2 Phosphatidylinositol 4,5-bisphosphate  
PIP3 Phosphatidylinositol 3,4,5-trisphosphate  
PKA Protein kinase A

## ***Q***

qRT-PCR Quantitative real time PCR

## ***R***

RLS Replicative life span  
RNAi RNA interference  
ROS Reactive oxygen species

## ***S***

S6K S6 kinase  
SCD Stearoyl-CoA-D9-Desaturase  
*S. cerevisiae* *Saccharomyces cerevisiae*  
siRNA Small interference RNA  
SOD Superoxide dismutase

## ***T***

TAG Triacylglycerol  
TOR Target of Rapamycin

## ***U***

UPR Unfolded protein response

Chapter I  
Introduction to  
Biogerontology

## **Chapter I Introduction to Biogerontology**

### **I-1 Aging**

Over the last decade, the field of biological gerontology has made exceptional progress in understanding the complex phenotype of aging, and this has been paralleled by an increased public interest in the scientific basis for this universal phenomenon. The advent of molecular techniques with improved precision together with rapid advances in the field of genetics has increased our ability to dissect the complex genetic and biochemical pathways that initiate and regulate aging. The increasing proportion of elderly persons in the populations of developed nations correlates with a dramatic increase in the incidence of age-related diseases and is pertinent to the burgeoning public interest in aging research. While there have been exciting discoveries in diverse areas of aging research, there are still enormous gaps in our understanding of the etiology of aging, and why the rate of aging, or senescence, varies significantly both between species and within individuals of the same species. The most profound and important contribution of modern biology to our understanding of aging is the discovery of conserved molecular mechanisms that can regulate senescence in vastly different organisms. Interestingly, many of these discoveries were based on theories and observations made long before the discovery of DNA and the subsequent study of individual genes. This thesis will summarize the evolution of aging theories, discuss the contributions of molecular biology to our understanding of the aging phenotype, and present novel experimental data on the genetic analysis of aging, providing a critical assessment of the current zeitgeist of the molecular links between nutrition, reproduction, and aging.

Aging is defined in an evolutionary biological context as “a persistent decline in the age-specific fitness components of an organism due to internal physiological deterioration” (Rose, 1994). This encompasses a decline in the efficiency of biological functions at the

molecular, cellular, and tissue levels. Typical phenotypic features of mammalian aging are decreases in skin elasticity, mobility, fertility, immunity, and stress resistance, accompanied by increases in the risk of many diseases, including cancer and cardiovascular and neurodegenerative diseases. Psychological changes accompanying aging include declines in reaction time and memory with concomitant increases in the likelihood of dementia. Although many theories have been proposed on the biological mechanisms of aging, the precise underlying molecular events remain unclear.

From an evolutionary standpoint, organismal aging is simple to rationalize. Once an organism has reached reproductive maturity and is capable of passing its genes to offspring, there is no longer selective pressure to combat the inexorable march toward biochemical equilibrium and death. In discussing the etiology of aging, it is useful to divide the life history of an organism into three discrete phases: development and growth, health and reproduction, and senescence and mortality. The first two phases, resulting in the reproductive fitness of an organism, are under intense selective pressure for survival, while the third senescent phase is evolutionarily invisible. During the development and growth phase, most organisms exhibit a decreasing rate of mortality as they approach sexual maturity and enter the health phase, at which point the rate of mortality is at its lowest. The reproductive phase is associated with a variable period of low age-associated mortality before transitioning to the senescent phase characterized by a marked increase in age-related mortality. Analyses of a number of organisms have shown that the rate of mortality changes with each life history phase; the lifespan of most organisms is highly plastic and can be either increased or decreased by environmental and genetic manipulations. These manipulations often affect changes in the duration of the health phase or in the observed rate of mortality during the senescent phase. Modern aging research seeks to characterize the phenotypic changes that occur during the transition from the health phase to the senescent



phase, and to elucidate the biochemical mechanism by which environmental and genetic manipulations affect the organism's lifespan. In this regard, observations on aging phenotypes and pathways that contribute to increased or decreased rates of senescence can be integrated into theories that seek to formulate a consensus etiology of aging applicable to all organisms.

On first inspection, aging might appear to be maladaptive because of its deleterious effects on fitness components. Nevertheless, an age-associated decline in fitness is almost universally observed in organisms that show a distinction between the germline and the soma (Bell, 1984; Charlesworth, 1994). One explanation for this apparent inconsistency hinges on the observation that the force of natural selection declines with increasing age (Medawar, 1952; Williams, 1957). This weakening is caused by the loss of individuals from younger age classes due to mortality hazards such as natural disasters, accidents, predation, and disease. Accordingly, if an allele has negative effects late in life, most of its carriers will have already died by the time those effects are expressed. Selection is therefore weaker against such an allele than against a similar allele expressed earlier in life when more carriers are alive.

## **I-2 Theories of aging**

The biological mechanisms that cause aging are still unclear. However, visible and quantifiable age-associated changes provide the basis for a number of theories on aging. In fact, there are more than 300 theories on mechanisms affecting aging and longevity (Medvedev, 1990). Although there is currently no consensus theory, the scientific community generally agrees that molecular damage and somatic maintenance influence aging and longevity, respectively. Influential theories of aging include the “rate of living theory of aging” of Max Rubner and Raymond Pearl (1908) and the “oxidative damage

theory of aging” of Denham Harman (1956).

Antagonistic pleiotropy and mutation accumulation are two non-mutually exclusive mechanisms generally thought to explain the evolution of aging in response to the decline in selective pressure. The antagonistic pleiotropy theory states that natural selection should actually favor genes that have deleterious effects late in life if these effects are outweighed by positive effects on fitness early in life (Williams, 1957; Hamilton, 1966). A special case of antagonistic pleiotropy is the disposable soma theory, in which the trade-off between early and late fitness components is specifically between reproduction and somatic maintenance (Kirkwood, 1977). There has been experimental support for genes with antagonistic effects, mainly from selection experiments (Rose and Charlesworth, 1980; Rose and Charlesworth, 1981b; Zwaan, 1995; Hunt, 2006), and there is some direct evidence for explicit antagonistic candidate genes (Gems, 1998), although that is not conclusive (Leroi, 2005).

### **I-2.1 Aging and life history theory**

Evolutionary theories of aging are part of life history theory, which seeks to describe and explain the evolution of age-specific schedules of survivorship and fecundity (Roff, 1992; Stearns, 1992; Partridge and Barton, 1996). Trade-offs between fitness components, such as those proposed by the antagonistic pleiotropy theory, are at the heart of the life history theory (Williams, 1957). One such trade-off between longevity and early fecundity is seen in the fruit fly *Drosophila melanogaster* (Zwaan, 1995) and males of the Australian black field cricket *Teleogryllus commodus* (Hunt, 2006), in which increased lifespan is artificially selected at the expense of early reproductive effort; that is, there is a genetic trade-off between lifespan and early reproduction. The same result is observed in other flies species, where increased lifespan is indirectly selected by allowing only older individuals to mate

(Partridge and Barton, 1993). This trade-off is thought to have evolved because the pool of resources is restricted by the acquisition of resources and has to be partitioned and allocated differentially with respect to life history components (Van Noordwijk and de Jong, 1986; Houle, 1991; Partridge and Barton, 1993; Kokko, 1998). The cost of reproduction is accordingly inherent in both life history theory and evolutionary theory of aging (Roff, 1992; Rose, 1994) and has been shown in a number of studies (reviewed in Reznick, 2000). The tight link between age-specific reproduction and survival, as the two most fundamental life history components, should be stressed here, because lifespan is measured per individual whereas survival probabilities or rates are traits of a population.

## **I-2.2 Damage theory of aging**

Many theories are based on the idea that aging is mainly caused by accumulated damage. These include the “wear and tear”, “waste accumulation”, “errors and repair”, “DNA damage”, “mitochondrial damage”, “disposable soma”, and “damage from metal ions” theories. Most of these theories are not pertinent to this thesis and are therefore not discussed further (for a review, see Medvedev, 1990). Although it is true that molecular and cellular insults accumulate over time, it is unclear whether they are a cause or a side effect of the aging process. For instance, errors in DNA repair contribute to DNA damage and mice deficient in DNA repair components have higher mutation rates; however, these mice do not exhibit accelerated aging (Narayanan, 1997). The effect of overexpression of DNA repair genes in mice is controversial, with one study showing delayed aging (Matheu, 2007) and another study seeing no effect (Zhou, 2001).

A prominent version of the damage theory of aging is the oxidative damage theory, which in turn evolved from the free radical theory proposed by Denham Harman in 1956.

The oxidative damage theory posits that aging results from molecular damage caused by reactive oxygen species (ROS), both free radical and otherwise (Beckman and Ames, 1998; Harman, 1956), which accumulate due to an imbalance between prooxidant and antioxidant defenses. All classes of biomolecules, including DNA, proteins, and lipids are affected. Various observations are consistent with this theory. For example, some long-lived animals have low levels of ROS production (Beckman and Ames, 1998; de Castro, 2004; Herrero and Barja, 1999; Ku, 1993; Sohal, 1993). The oxidative damage theory has been linked to the “rate of living theory”, in that an increased metabolic rate might cause a parallel increase in ROS levels, particularly during mitochondrial respiration, thereby accelerating damage and aging (Beckman and Ames, 1998; Finkel and Holbrook, 2000).

### **I-3 Model organisms in aging research**

Model organisms are critical in most fields of biology for generating and testing hypotheses and obtaining a deeper insight into biological processes that affect humans. The advantages of model organisms for aging research include the ease with which the genetic background, environment, and food supply can be standardized under controlled laboratory conditions. Additionally, genetic manipulations, such as alterations of single genes, can provide valuable information on which biological processes and pathways are involved in aging. Furthermore, the shorter lifespans of lower model organisms often used in aging research (e.g., the nematode *Caenorhabditis elegans*, the budding yeast *Saccharomyces cerevisiae*, and *Drosophila*) allow more rapid progress than is possible with higher organisms (e.g., *Mus musculus* and *Rattus* sp). In this thesis, I present data obtained using *C. elegans* as the model organism; therefore, the following discussions will focus on this invertebrate.

### **I-3.1 *C. elegans* as a model organism for studying aging**

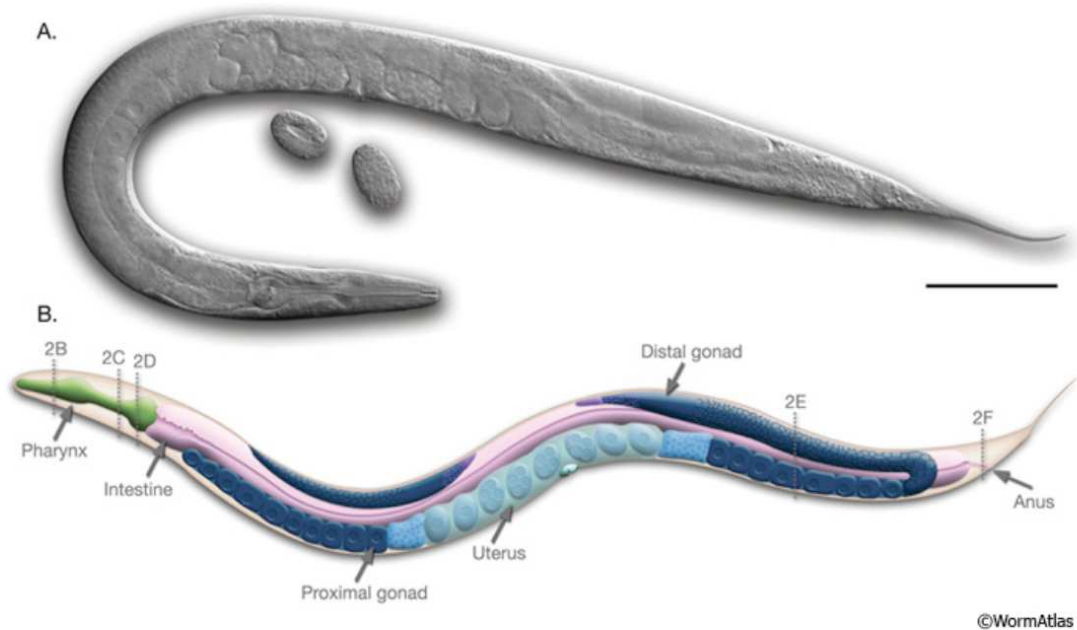
*C. elegans* is a small, free-living soil nematode that feeds on microbes, particularly those in compost. *C. elegans* was first proposed in 1948 as a multi-cellular model organism for the study of physiology (Dougherty and Calhoun, 1948). In 1974, Sydney Brenner, the godfather of *C. elegans* research, introduced this roundworm as a model organism for genetic studies (Brenner, 1974). *C. elegans* offers many advantages for this work, such as economy, ease of maintenance, and ease of observation and manipulation. Its small size (1 mm) makes it possible to grow large numbers of animals in mass culture, with the typical laboratory food source being *E. coli* strain OP50 (Brenner, 1974). Since *C. elegans* is largely transparent throughout its life span, phenotypic changes resulting from genetic manipulation are easy to observe. Manipulations of single genes are typically performed by chemical mutagenesis, gene knockdown by feeding with bacteria expressing gene-specific dsRNA (RNA interference; RNAi), or creating transgenic lines overexpressing the chosen gene.

The complete cell lineage of *C. elegans* has been described (Sulston, 1983) and its genome has been fully sequenced. Mutant strains are distributed via the *Caenorhabditis* Genetics Center, and the effects of transgenes or mutations in various mutant backgrounds can be tested easily by genetic crossing. Long-term storage is possible at -70°C, which is particularly convenient. Another major advantage of *C. elegans*, especially in biogerontology research, is their short lifespan; for wildtype animals this is 18–20 days at 20°C (Gems and Riddle, 1995). Typical of nematodes, *C. elegans* worms have a cylindrical body essentially consisting of an outer tube (body wall) and an inner tube. The body wall is made up of the cuticle, hypodermis, excretory system, neurons, and muscles. The inner tube consists of the pharynx, intestine, and gonad. The pharynx is located in the anterior part, the head region, which is responsible for food intake. This is directly connected to the intestine, which ends in the posterior part in the anus, where defecation occurs. The main difference

between hermaphrodites and males is the reproductive system (Figs. I-1 and I-2), which consists mainly of the gonad, where the gametes are produced.

### I-3.2 *C. elegans* hermaphrodites

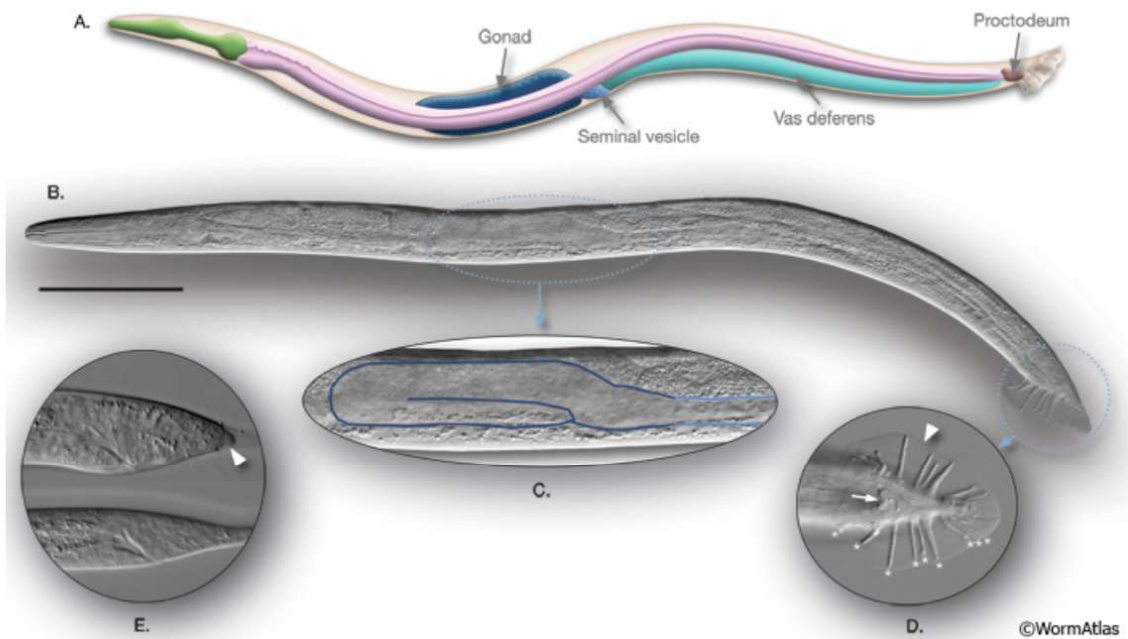
In hermaphrodites, the gonad consists of a somatic gonad, the germline, and the egg-laying apparatus (Fig. I-1). Two distal arms of the gonad (ovaries) converge onto a central uterus via two spermathecae. Oocytes are fertilized by the sperm as they pass through the spermathecae. The eggs pass along the uterus and are then released through the vulva, which penetrates the body wall in the ventral midsection (Brenner, 1973; Lewis and Fleming, 1995; Sulston, 1976).



**Figure I-1: *C. elegans* hermaphrodite anatomy:** (A) View using Nomarski microscopy. (B) Shows a schematic drawing of *C. elegans* (reproduced from WormAtlas.org et al.,). Scale bar shows 0.1 mm

### I-3.3 *C. elegans* males

Male and hermaphrodite worms show differences in body morphology, particularly at the posterior end. These changes begin in the L2 stage and are visible as a slight thickening of the tail (Fig. I-2) (Sulston, 1980; Sulston and Horvitz, 1977). Here, the sexual organ develops into a copulatory apparatus with nine sensory rays that help to transfer the sperm through the vulva of the hermaphrodites (Liu and Sternberg, 1995). The male reproductive system has only a single gonad arm, which includes a somatic gonad and germline. The reproductive system is connected to the proctodeum and anus through the seminal vesicle and vas deferens (Fig. I-2).



**Figure I-2: *C. elegans* male anatomy: (A) Schematic figure of male anatomy. (B) View using Nomarski microscopy. (C) Male gonad. (D) Male tail enlarged. (E) Male tail in L3 staged larva (Reproduced from WormAtlas.org et al.,). Scale bar shows 0.1 mm.**

### **I-3.4 *C. elegans* life cycle**

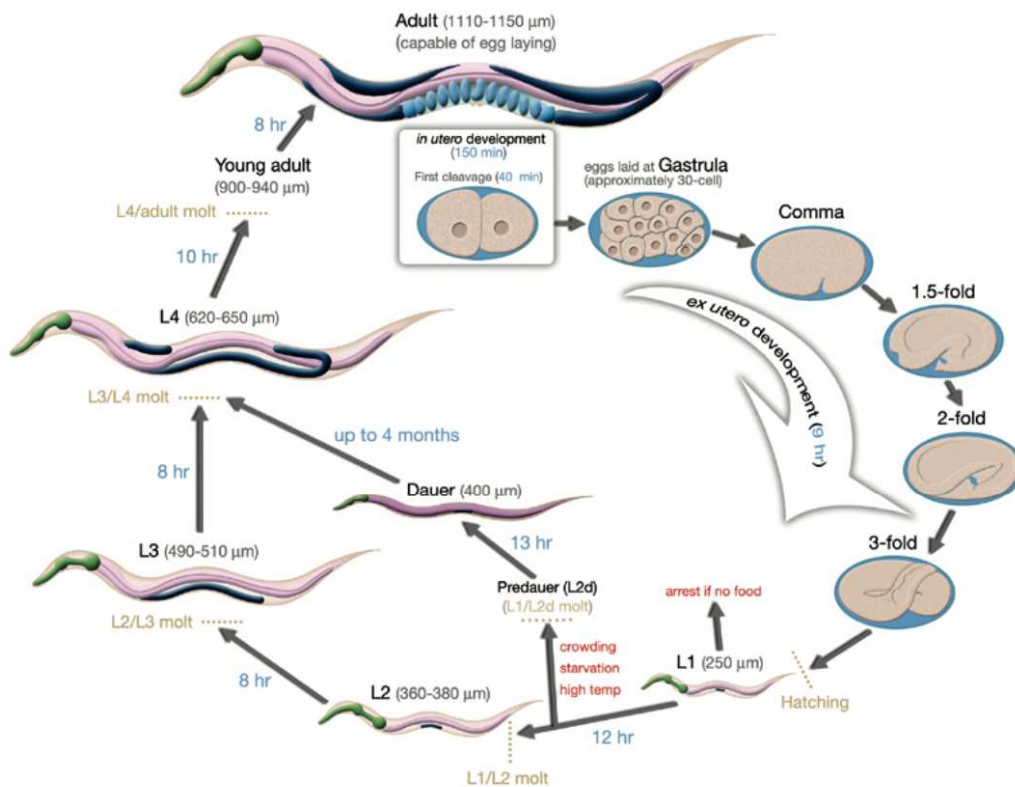
The development of *C. elegans* is temperature dependent (Byerly, 1976); under optimal conditions at 20°C, the reproductive cycle is around three days. Hermaphrodites are the predominant form (95.5%) and males are rare (0.05%). The hermaphrodites produce oocytes and sperm and can reproduce by self-fertilization. Males can fertilize hermaphrodites, and in this case, the male sperm is preferred to the hermaphrodite sperm. The brood size of a self-fertilized hermaphrodite can consist of up to 300 eggs. The typical hermaphrodite produces many more oocytes than sperm, so the size of the brood is limited by the number of sperm. Oocyte production is stimulated by mating with males, and in this case, a single mated hermaphrodite can produce more than 1000 progeny. The offspring hatch and develop through four different larval stages (L1–L4), with molts occurring between each stage (Fig. I-3). In addition to progressing sequentially through stages L1 to L4, *C. elegans* can take an alternative developmental route from L1/L2 to L4 known as the dauer diapause (Fig. I-3).

### **I-3.5 Dauer larvae**

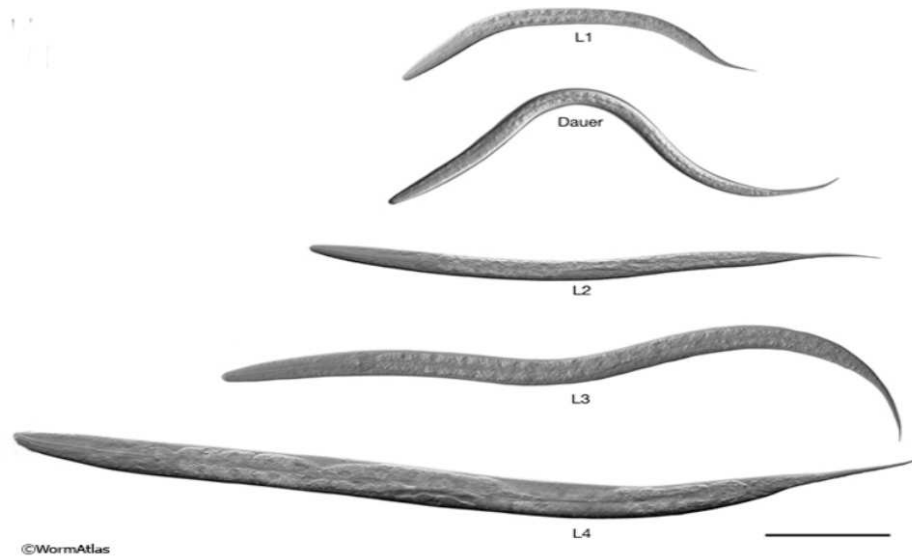
In the laboratory, *C. elegans* converts *E. coli* into biomass with an efficiency of nearly 50% (Lewis and Fleming, 1995). In the soil, *C. elegans* seeks to consume all available resources as quickly as possible as a means to outgrow competitors. During development, environmental cues such as limited food availability, high population density, high temperatures, and/or dauer pheromones (see below), can cause *C. elegans* to take an alternative developmental pathway at the L2 molt that results in an extended arrested stage, the so-called dauer larva diapause (L2d) (Fig. I-3, 4) (Golden and Riddle, 1984; Golden and Riddle, 1982). The dauer stage in *C. elegans* is facultative, which means that dauer larvae are formed only when the food supply is insufficient to support continued growth of the



population (Riddle, 1981, 1988, 1997). Preparation for the non-feeding dauer stage involves alteration of energy metabolism and accumulation of fat in intestinal and hypodermal cells; thus, dauer-specific behaviors are specialized to favor dispersal (moving to more favorable soil locations), which is key for evolutionary success. Dauer larvae can survive for many months, approximately ten times the normal life span, and then resume development when they re-encounter food. Thus, *C. elegans* may successfully migrate through sparse soil resources from one region of microbial bloom to another.



**Figure I-3: *C. elegans* life-cycle at 22°C.** 0 min is fertilization. The numbers in blue next to the arrows indicate the length of time the animal spends at a certain stage (Reproduced from Wormatlas.org et al.,).



**Figure I-4:** *C. elegans* larval stages. View with Nomarski microscopy. Scale bar shows 0.1mm (reproduced from WormAtlas.org et al.,).

Entry and exit from the dauer stage are developmental responses to specific chemosensory cues detected by amphid neurons located in the anterior of the worm. These cues inform the larva about the availability of food and its sufficiency to support reproduction. Worms arrested in the dauer stage are thinner and have thickened cuticles that confer a darkened appearance compared with developing worms (Fig. I-4). Dauer larvae do not eat or move, are more resistant to stress, and can survive for several months (Cassada and Russell, 1975).

### **I-3.6 Signaling pathways and molecular events that regulate dauer arrest**

Some key aspects of an organism's life history, such as mate selection and detection of population density, are regulated by pheromones secreted into the surrounding area (Karlson and Luscher, 1959; Gallo and Riddle, 2009). *C. elegans* pheromones were originally described as potent male attractants produced and secreted by hermaphrodites. Pheromones signaling a high population density and inducing dauer arrest were later identified and

characterized as small molecules containing both fatty acids and sugar moieties (Golden and Riddle, 1982; Jeong, 2005). Paik and colleagues first demonstrated that the ascaroside (-)-6-(3,5-dihydroxy-6-methyltetrahydropyran-2-yl)oxy heptanoic acid has dauer pheromone activity (Jeong, 2005). Although these pheromones have largely been characterized at the chemical level, the molecular mechanisms by which they promote dauer formation are still unknown. The small size and hydrophobic nature of such pheromones could allow them to enter cells; indeed, their binding targets do not have to be located on the cell surface. Whether such small molecules truly function as active “pheromone” signals or are simply passive cues secreted by individual worms also remains unclear. Systematic biochemical studies will be necessary to resolve these issues.

A number of dauer stage *C. elegans* mutants have been described and are classified according to their ability to promote or inhibit dauer arrest (Gerisch, 2001; Gottlieb and Ruvkun, 1994; Jia, 2002; Riddle, 1981; Thomas, 1993; Vowels and Thomas, 1992). As dauer larvae are phenotypically distinct and their formation is easy to observe, a large number of mutant *C. elegans* have been generated and screened. About 40 of these *daf* (dauer larvae formation) genes have been identified by selecting mutants that either do not produce dauer larvae even when starved (dauer-defective [Daf-d]) or do so even when food is abundant (dauer-constitutive [Daf-c]) (Riddle, 1981, Albert and Riddle, 1981). Many of these *daf* genes have been characterized, and for others, their potential biological functions have been inferred from comparisons to homologous protein products and then confirmed by biochemical studies (Table 1).

**Table 1. Genes implicated in regulation of the *C. elegans* dauer stage**

<i>daf</i> -genes	Assumed function
DAF-1	DAF-4 TGF-beta receptor
DAF-2	Insulin receptor
DAF-3, DAF-8, DAF 14	SMAD (cell signaling mediators)
DAF-5	Transcription coregulator (Ski homolog)
DAF-6	Patched-related protein
DAF-7	Development regulatory growth factor TGF- b
DAF-9	Steroid hydroxylase P450
DAF-10	Intraflagellar transport-particle
DAF-11	Guanylyl cyclase
DAF-12	Nuclear hormone receptor
DAF-16	FOXO transcription factor
DAF-18	Phosphatidylinositol-3,4,5-trisphosphate -3-phosphatase (PTEN homolog)

## **I-4 Pathways involved in determination of lifespan**

Biogerontology research aims to understand the biological basis of aging. One powerful approach to discovering the underlying mechanisms is first to identify interventions that slow and/or accelerate the aging process, and then to investigate the mechanisms of actions such interventions. In following sections, I briefly describe the various experimental manipulations by which aging can be modulated in *C. elegans* and other model organisms.

### **I-4.1 Dietary restriction**

Dietary restriction is the most potent experimental paradigm for increasing lifespan, and its effect is conserved in a wide variety of eukaryotic organisms. The discovery that caloric restriction increases lifespan was first reported by McCay and colleagues in 1935. They observed that rats maintained on a diet containing approximately 40% fewer calories than a standard diet had nearly 50% longer lifespans. Contemporary research has confirmed this phenomenon in a variety of mammals, including other rodents, dogs, and primates (Weindruch and Walford, 1988; Masoro, 2005; Kealy, 2002; Colman, 1998). Importantly,

the increased lifespan associated with dietary restriction (as it is now termed; DR) in rodents and primates is accompanied by a reduced incidence of many age-related diseases, including cancer, diabetes, and cardiovascular disease (Weindruch and Walford, 1988; Colman, 1998; Edwards, 2001; Fontana, 2004). The effect of food restriction on lifespan is also conserved in lower eukaryotic organisms. In this case, a reduction in specific dietary components may be more relevant than a reduction in total available calories *per se*, depending on the organism. For example, lifespan-extending interventions include: in *S. cerevisiae*, a reduction in glucose availability or mutation of genes involved in glucose metabolism; in *C. elegans*, partial removal of dietary bacteria or complete removal of all food sources (Lin, 2000; Kaeberlein, 2006; Lee, 2006); and in *Drosophila*, a reduction in methionine availability (Grandison, 2009) reduced amount of yeast or carbohydrates (Pletcher, 2005; Helfand, 2008). Regardless of the variations in the paradigm, the diversity of organisms that respond to DR by increasing their lifespan suggests that these phenotypes may have a common evolutionary origin and involve conserved genes and pathways.

The mechanisms by which DR confers extended longevity are a matter of considerable debate, with plausible explanations including a reduction in the onset of age-related disease, a more resilient and efficient metabolism, and an enhanced resistance to various forms of cellular stress and damage. The one clear conclusion from the study of the DR–lifespan connection is that in most organisms, lifespan is highly plastic and can be manipulated to promote both longevity and increased health. Because of the stringency of dietary conditions that result in extended longevity and improved health in mammals, the widespread adoption of DR by modern human populations is not likely to occur. There is therefore impetus to discover the genes and pathways that mediate the response to DR in order to generate mimetics that could be useful in extending the human healthspan and preventing the onset of terminal diseases. Model organisms with proven genetic tools for determining the ability of

specific genes and pathways to modulate observable phenotypes have been indispensable in characterizing the molecular basis of DR-mediated lifespan extension. Genes and pathways that are integral to the organismal response to DR and are conserved in model organisms include the target of rapamycin (TOR) nutrient-sensing and growth pathway, the insulin/IGF signaling (IIS) pathway, and *Sir2*, which encodes a member of the Sirtuin family of protein deacetylases.

#### **I-4.2 The target of rapamycin (TOR) pathway**

The TOR pathway is involved in regulating cell size and growth in response to a variety of internal and external environmental signals, including nutrient availability, growth factor concentrations, stress conditions, and cellular damage. TOR, a serine/threonine protein kinase, has been linked to various cellular responses to these cues through modulation of transcription, translation, nutrient partitioning, and autophagy (Reiling and Sabatini, 2006). In mammals, there are only circumstantial data to suggest that TOR is involved in mediating the DR phenotype, including a correlation between reduced insulin signaling and downregulation of TOR in cell culture models that results in a decrease of mitochondrial ROS production similar to that observed in DR models (Sarbasov, 2005; Kim, 2006). Long-lived Ames dwarf mice have reduced TOR activity, and this has been postulated to contribute to their extended lifespans (Sharp and Burtke, 2005). In a recent study, administration of the TOR inhibitor rapamycin to mice late in life increased the median and maximal lifespan in both males and females (Harrison, 2009). Downregulation of TOR signaling can also increase the lifespan of many lower eukaryotes, including yeast, *C. elegans*, and *Drosophila* (Kaeberlein, 2005; Jia, 2004; and Kapahi, 2004). In nematodes, loss-of-function mutation or RNAi knockdown of TOR pathway components extends lifespan; however, it is unclear whether this pathway is involved in the DR response (Vellai,

2003; Jia, 2004). Lifespan extension through DR and TOR signaling are correlated in *Drosophila*, as downregulation of TOR by either genetically, or by feeding with rapamycin increases lifespan but does not further extend DR-induced longevity (Kapahi, 2004). This observation, together with the fact that TOR inhibition in the fat body (the major metabolic organ in flies) is sufficient to extend lifespan, suggests that TOR activation is at least one element of the fly's response to DR.

### **I-4.3 The insulin/IGF-1 signaling (IIS) pathway**

Insulin and insulin-like growth factor (IGF-1) are major components of the IIS pathway in mammals, and have orthologs in *C. elegans* and *Drosophila*. The inability of cells to respond to insulin, termed insulin resistance, is a major cause of age-associated diabetes, and likely involves impaired mitochondrial function and both cellular metabolism (Wang and Wei 2010). Insulin resistance can be caused by chronically increased insulin secretion, which occurs under conditions of excess calories. It has been hypothesized that dietary restriction would ameliorate this effect by causing a decrease in insulin secretion and therefore increasing insulin sensitivity over the lifetime of an animal. Indeed, dietary restricted rodents show a reduction in serum insulin levels of up to 50%, and a reduction in IGF-1 of up to 22% (Masoro, 1992; Wetter, 1999; Breese, 1991; Sonntag, 1999). When the IGF-1 receptor is mutated in mice, heterozygous IGF-1R/+ mice display an increase in average lifespan of 25%, and a concomitant increase in oxidative stress resistance (Holzenberger, 2003). It is unclear if down-regulation of the insulin signaling axis results in effects on downstream targets that are consistent with dietary restricted animals; and thus whether or not insulin and IGF regulation is a major component of mammalian dietary restriction remains to be resolved. In nematodes, down regulation of insulin signaling is obtained by mutation of the *daf-2* gene (Kenyon, 1993), which is an insulin-like receptor, or

through mutation of the phosphoinositol-3 kinase or *age-1* (Friedman and Johnson, 1988) gene. These mutations result in a dramatic increase in lifespan which requires the forkhead transcription factor DAF-16 (Kenyon, 2005). When insulin signaling is down-regulated, DAF-16 is phosphorylated and migrates to the nucleus where it affects transcription of genes involved in stress response, somatic maintenance, and DNA repair. Lifespan extension through dietary restriction, however, does not require DAF-16, and long lived mutant nematodes that arise from insulin signaling mutation still experience an increase in lifespan when placed on dietary restriction paradigms (Lakowski and Hekimi 1998; Kaeberlein, 2006; Lee, 2006). This data suggests that CR and insulin signaling utilize different mechanism for lifespan extension, and insulin signaling is not a mediator of dietary restriction in these animals. In *Drosophila*, mutation of the insulin receptor substrate *chico* results in lifespan extension that appears to be mechanistically related to dietary restriction. However *chico* flies exhibit a shorter lifespan than controls at low food concentrations which cause a maximal lifespan through dietary restriction, (Clancey, 2002). This seems to indicate that mutations in *chico* have effects that reduce the ability of flies to respond to dietary restriction, and more data is needed to dissect whether or not dietary restricted flies experience changes in insulin signaling outputs.

Some studies imply that the effect of IIS on aging is tissue specific. For instance, mutations in the insulin receptor in adipose tissue (fat tissue) cause life span extension in mice, whereas in the liver it results in diabetes (Bluher, 2003; Michael, 2000). In invertebrates, specifically nematodes and fruit flies, the life span extending effect of IIS mutations is also associated with adipose tissue. The fat body in the fly and the intestine in the worm, both of which serve as adipose tissue, among other functions (Giannakou, 2004; Hwangbo, 2004; Libina, 2003). This link to fat tissue and longevity in model organisms is especially



interesting given that variation in genes responsible for fat transport in humans is also connected to longevity (Barzilai, 2003; Geesaman, 2003).

#### **I-4.4 Sirtuins**

Perhaps the best studied mediators of DR in lower eukaryotes, and possibly mammals, are the sirtuin protein deacetylase family member Sir2 and the mammalian ortholog SIRT1. Sirtuins are evolutionarily conserved nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases that regulate the expression of a variety of genes via interactions with transcription factors and histones (Imai and Guarente, 2010, Haigis and Sinclair, 2010). *Sir2* has been linked to several physiological processes relevant to senescence in eukaryotes, including metabolism, stress resistance, and apoptosis. Sir2 acts by deacetylation and modulation of key transcriptional regulators of these pathways, including PGC-1 $\alpha$ , FOXO, and p53 (Bauer and Helfand, 2009, Donmez and Guarente, 2010). *Sir2* expression has been linked to lifespan regulation and stress response in organisms ranging from yeast to humans, and in some organisms, Sir2 appears to be required for the response to DR. Although a direct link between lifespan extension and increased SIRT1 expression in mice has not yet been identified, it appears that SIRT1 is required for DR-mediated lifespan extension in some mouse strains (Bioley, 2008). Importantly, when SIRT1 is deleted from the mouse brain, there are no changes in several hallmarks of DR, including downregulation of the IGF-1 and growth hormone (GH) axis and increased physical activity (Chen, 2005, Cohen et. al., 2009). This shows *that at least some outputs that mediate* lifespan extension require a functional SIRT1. Overexpression of SIRT1 improves metabolic health in obese mice, including improved insulin sensitivity, glucose metabolism, protection from age-associated diabetes, and a reduction in lipid-induced inflammation (Banks, 2008, Pfluger, 2008).

Screening for small molecule activators of SIRT1 (STACs) resulted in the discovery of resveratrol, which activates SIRT1 *in vitro* (Howitz, 2003). Mice fed resveratrol are protected from the metabolic consequences of a high-fat diet, and the heart, skeletal muscle, and brain transcriptomes are highly similar to those of animals subjected to DR (Baur, 2006, Langouge, 2006, Barger, 2008). Recently, questions have been raised about the ability of resveratrol to activate SIRT1 and whether it has targets other than SIRT1 (Dasgupta and Milbrandt, 2007). Thus, further analyses will be required before a connection can be made between STACs, SIRT1, and the DR response.

Longevity in *S. cerevisiae* is typically measured in terms of the replicative lifespan, which is the number of mother cell divisions before senescence (Mortimer and Johnston, 1959). The buildup of extrachromosomal rDNA circles (ERCs) is thought to play a pivotal role in yeast aging since these structures are toxic to the yeast cell due to their impairment of replication and transcription factor activity (Sinclair and Guarente, 1997). Increased *Sir2* expression through the introduction of extra gene copies decreases ERC formation in yeast and concomitantly increases the lifespan. Conversely, when *Sir2* is mutated, ERC formation increases and the lifespan decreases (Kaeberlein et al., 1999). Thus, *Sir2* normally regulates the yeast lifespan by downregulating ERC formation. Because ERC formation is not a cause of aging in higher eukaryotes, the ability of *Sir2* to reduce ERCs is unremarkable when considering senescence in species other than yeast. However, ERC is not the only link between *Sir2* and yeast aging. *Sir2* also has a prominent role in the response of yeast to DR, which can be imposed by reducing glucose concentrations in the growth media or by mutating genes involved in glucose metabolism (Lin, 2000). In either paradigm, replicative lifespan is increased, and the effect on lifespan is dependent on the enzymatic activity of *Sir2* and concomitant reduction of ERCs (Lin, 2000). Despite the fact that ERCs are not

implicated in the lifespan of other organisms, this result raises the possibility that *Sir2* may be an evolutionarily conserved regulator of the response to DR.

The *C. elegans* genome encodes multiple orthologs of yeast *Sir2*, and the ortholog with the highest homology, *Sir2.1*, has a prominent role in lifespan regulation (Tissenbaum and Guarente, 2001). DR-induced lifespan extension in nematodes can be induced by dilution or complete removal of the bacterial food source, as well as by mutation of genes (*eat-2*) that control pharyngeal pumping and food intake (Klass, 1977; Kaeberlein, 2006; Lakowski and Hekimi, 1998). Mutation of *Sir2.1* suppresses the longevity enhancement conferred by the *eat-2* mutation (Wang and Tissenbaum, 2006) but not that by bacterial deprivation (Bishop and Guarente, 2007). This apparent discrepancy may result from the primary involvement of *Sir2.1* in the stress response rather than the DR response. Heat stress promotes the interaction of *Sir2.1* protein with DAF-16/FOXO, which initiates transcription of stress response genes (Berdichevsky, 2006, Wang and Tissenbaum, 2006). Interestingly, the DAF-16 gene product is also required for lifespan extension in IIS pathway mutants, but – as previously noted – it is not required for DR-mediated longevity. Transgenic *C. elegans* overexpressing *Sir2.1* show increases in lifespan of up to 50%, which is dependent on DAF-16 function (Wang and Tissenbaum, 2006). Mutation of *Sir2.1* reduces lifespan consistently only when nematodes are simultaneously under conditions of elevated stress (Viswanathan, 2005; Wang and Tissenbaum, 2006). Taken together, these findings indicate that the role of sirtuins in the nematode DR response remains ambiguous. They may be part of a broad stress response network that controls the response to nutrient deprivation as well as to other environmental and cellular stresses.

DR-induced lifespan extension in *Drosophila* is accompanied by reduced expression of

the class I histone deacetylase *Rpd3* (Rogina, 2002). In addition, heterozygous mutation of *rpd3* is capable of extending *Drosophila* lifespan and results in the increased expression of *Drosophila Sir2* (*dSir2*) mRNA. The model for DR mediated lifespan extension in flies is as follows: DR mediated repression of *Rpd3*, resulting in increased expression of *dSir2*, and lifespan extension affected through increased *dSir2* activity. Two experimental observations pertinent to this model are: (i) overexpression of *dSir2* either ubiquitously or specifically in the nervous system results in lifespan extension similar to that observed under DR, and (ii) DR does not extend the lifespan of *dSir2* mutants (Rogina and Helfand, 2004). The ability of *dSir2* to modulate the normal lifespan of *Drosophila* is affected by the genetic background, as homozygous mutation of *dSir2* has been shown to decrease lifespan or to have no significant effect on lifespan, depending on the fly strain and the mutated allele (Newman, 2002; Astrom, 2003; Pallos, 2008). Similarly, the sirtuin activator resveratrol has been shown to enhance lifespan in at least one genetic background but has no effect in other backgrounds under similar food and dosage conditions (Wood, 2004; Bass to Partridge 2007). Nevertheless, the idea that *Sir2* is an important regulator of the *Drosophila* response to DR is supported by the fact that extended lifespans were observed in independent laboratories using different *Sir2* overexpression systems, and by the observation that DR-induced lifespan extension and increased mobility do not occur in *dSir2* mutant animals (Rogina and Helfand, 2004; Parashar and Rogina, 2009; reviewed in Frankel, 2010).

#### **I-4.5 Germline signaling**

In *C. elegans*, removal of germline cells by laser ablation of germline precursor cells extends the lifespan by 60%, but this effect is nullified by removal of the somatic gonad. This finding suggests that there are opposing signaling pathways originating from the germline

and somatic gonad (Hsin and Kenyon, 1999). Many transcriptional regulators have been implicated in the regulation of lifespan by germline ablation. *daf-16/FOXO*, which is required for the long lifespan of *daf-2/InR* mutants, also regulates germline-mediated longevity. Several other components of the IIS pathway have been implicated in lifespan extension in germline-less animals, including DAF-18/PTEN (Berman and Kenyon, 2006), SMK-1/SMEK-1 (Wolf, 2006), and the transcription factor HSF-1 (Hansen, 2005). Factors that specifically regulate germline signaling have been identified and include the transcription elongation factor TCER-1/TCERG1 (Ghazi, 2009) and the ankyrin repeat-containing protein KRI-1/KRIT-1 (Berman and Kenyon, 2006).

A recent study from our own group showed that the nuclear hormone receptor NHR-80/HNF-4 regulates oleic acid synthesis via the fatty acid desaturases *fat-5*, *-6* and *-7*. Mutation of both *fat-6* and *fat-7* shortens the lifespan of germline-less animals, and this effect can be reversed by the addition of exogenous oleic acid to the culture (Goudeau, 2011). Flatt and colleagues have shown that misexpression of *bag of marbles (bam)* induces loss of germ cells in both male and female *Drosophila* and also increases their lifespans (Flatt, 2008). Although it is not yet clear whether the germline affects longevity in mammals, studies in mice have shown that prepubertally ovariectomized (11 months of age) females transplanted with ovaries from young mice have a life expectancy 40% longer than mice with intact ovaries (Cargill, 2003). These results suggest that signals from the reproductive tissues play an important role in regulating the lifespan in many species, including mammals.

## **I-5 Objective of this thesis**

The main objective of this work is to investigate the links between nutrition, reproduction, and longevity in *C. elegans*. Although studies are beginning to uncover the connections between reproduction and longevity, the signaling events that trigger reproductive slowdown (or shutdown) under nutritionally challenging conditions are not known.

In this thesis, I have asked whether known genetic mediators that are crucial during development, and later in regulating animal's lifespan, could also sense nutritional status of animal during adulthood. To test this, I have opted *C. elegans* as model organism to test the role of steroid signalling in mediating the DR response. Previous reports suggest that steroid signalling is required in deciding *C. elegans* developmental fate to enter dauer state or to reproductive development, and functional signalling is also crucial for mediating lifespan inducing signals from somatic tissue in germ-line less animals. Hence, we opted this signalling pathway to know whether the active steroid signalling couples reproductive to longevity in nutritionally challenged *C. elegans*.

Chapter II

Steroid Signaling is

Required for Dietary

Restriction Induced Lifespan

Extension in *C. elegans*

## **Chapter II Steroid Signaling is Required for Dietary Restriction Induced Lifespan Extension in *C. Elegans***

### **II-1 Introduction**

*C. elegans* continues to be an essential model organism for defining signaling pathways that regulate life history traits (Riddle and Albert, 1997). As discussed in Chapter I, when environmental conditions such as temperature and nutrition are optimal, *C. elegans* develops through four larval stages (L1–L4) to reach reproductive adulthood within approximately three days. However, under harsh environmental conditions such as limited food, high temperature, and high population density, *C. elegans* larvae may arrest development at the second molt to enter the dauer stage (Cassada and Russell, 1975). Food supply, temperature, and a constitutively secreted dauer-inducing pheromone play crucial roles in the decision to enter the dauer stage (Golden and Riddle, 1984a; Golden and Riddle, 1984b), and many additional signaling molecules have been shown to control dauer formation, including members of the TGF- $\beta$  family (Ren, 1996; Schackwitz, 1996), cyclic GMP (Birnbay, 2000), and components of the IIS pathway (Kimura, 1997; Pierce, 2001). Subsequent studies showed that these pathways converge on a nuclear hormone receptor, DAF-12 (Antebi, 2000), which was shown to be a key regulator of dauer versus non-dauer morphogenesis.

In *C. elegans*, *daf-7* encodes a TGF- $\beta$  family member (TGF- $\beta$  ligand) and is expressed in the ASI chemosensory neurons. Signals from DAF-7 are transduced through DAF-4 type II (Estevez, 1993) and DAF-1 type I (Georgi, 1990; Gunther, 2000) transmembrane receptor kinases. These receptors further control the activities of SMAD transcription factors encoded by *daf-8* (Estevez, 1997), *daf-14* (Inoue and Thomas, 2000), and *daf-3* (Patterson, 1997). Recently, it was shown that TGF- $\beta$  regulates germ cell proliferation in *C. elegans* through the action of *daf-5* (Dalfo, 2012). Another important signaling pathway in *C. elegans* is the



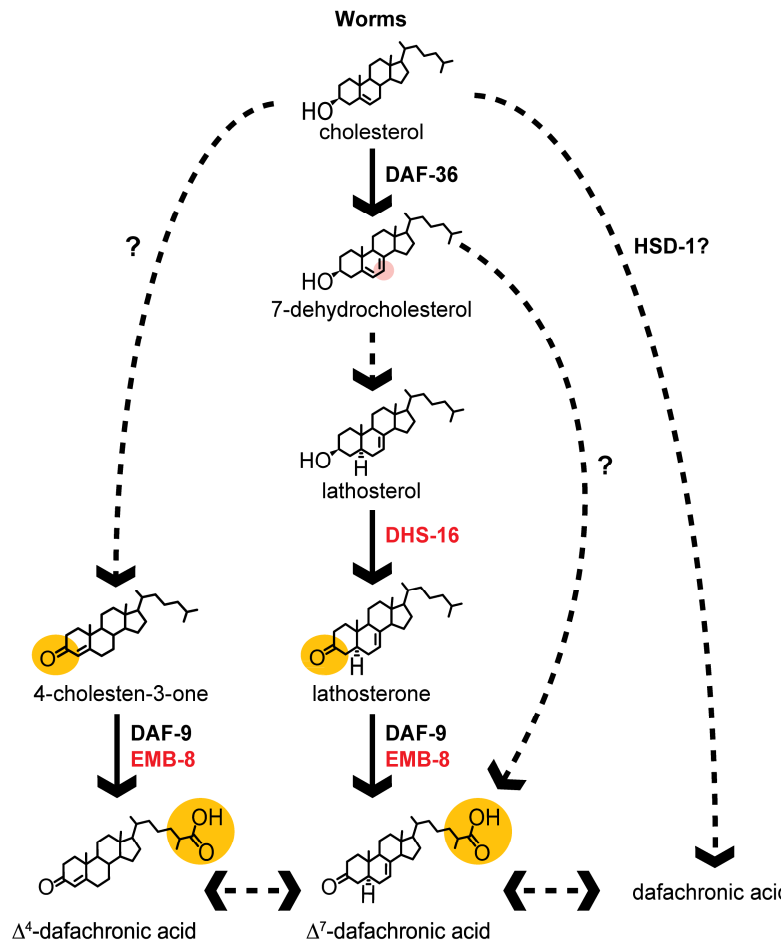
IIS pathway involving *daf-2*/InR, as discussed in Chapter I. DAF-2 is likely to signal via the PI3K catalytic subunit AGE-1 (Morris, 1996), Activation of PI3K inhibits the activity of the transcription factor DAF-16 (Lin, 1997; Ogg, 1997), which has been speculated to interact with DAF-3, DAF-8, and DAF-14 SMAD proteins to integrate DAF-7 and DAF-2 signals. The *daf-2*/InR signaling cascade also controls the lifespan of adult *C. elegans*. Temperature-sensitive *daf-2* mutants raised at a low temperature have an extended adult lifespan (Kenyon, 1993; Larsen, 1995; Gems, 1998). *daf-16* is also required for both dauer larva formation and the enhanced longevity of *daf-2* and *age-1* mutants.

Though much is known about the molecular regulators of these signaling pathways in controlling the development and/or adult lifespan of *C. elegans*, the mechanisms by which sensory transduction pathways are integrated to regulate appropriate organism-wide physiological responses are poorly understood. Studies on DAF-12, an NHR transcription factor related to the vertebrate vitamin D, pregnane X, and constitutive-androstane receptors (VDR, PXR, and CAR, respectively) (Antebi, 2000), have revealed the importance of nuclear receptors in transducing sensory signals to control animal physiology. DAF-12 is involved in both dauer formation and adult life span, and the *daf-12(m20)* mutation greatly enhances the extended lifespan of certain *daf-2* mutants (Larsen, 1995; Gems, 1998). Later work demonstrated that the bile acid-like steroid hormone dafachronic acid (DA) regulates *C. elegans* development and adult lifespan by acting on DAF-12 to direct the animal to enter the dauer stage or to proceed to reproductive development.

### **II-1.1 Dafachronic acid biosynthetic pathways**

$\Delta^4$ - and  $\Delta^7$ -DAs are synthesized from cholesterol in a multistep, branched pathway (Fig. II-

B). In the first step, the DAF-36/Rieske oxygenase converts cholesterol to 7-dehydrocholesterol. DHS-16 is a 3-hydroxysteroid dehydrogenase that converts the 3-alcohol to the 3-keto moiety.



**Figure II-A:** Dafachronic acid biosynthetic pathway from dietary cholesterol (Reproduced from Wollam, 2012).

Studies have revealed that *dhs-16* is required for lathosterone production (Rottiers, 2006). However, *dhs-16* mutant animals still produce low levels of  $\Delta^7$ -DA (Wollam, 2012), suggesting the existence of alternative pathways for  $\Delta^7$ -DA synthesis. *hsd-1* is not required for 4-cholesten-3-one and DA production but may be involved in producing alternative DAs (Wollam, 2012). Intermediate compounds and DAs may cross-regulate the synthesis of other molecules (Fig. II-A).

## II-1.2 The *daf-9* gene

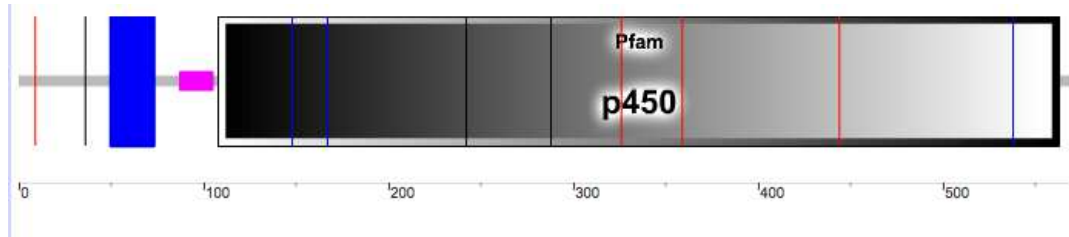
The *daf-9* gene encodes a conceptual protein of 557 amino acids with a predicted molecular weight of 64.7 kDa. BLAST searches indicate that it is a cytochrome P450 hydroxylase. The cytochrome P450 family consists of heme-containing mono-oxygenases that are involved in detoxification of xenobiotic compounds and the synthesis and degradation of physiologically important molecules such as steroid hormones. Phylogenetic analysis shows that DAF-9 is most similar to members of the CYP2 family (e.g., rabbit 2C3, a progesterone 16 $\alpha$ -hydroxylase) and to the steroid hydroxylases *Drosophila* CYP18 and human CYP17 and CYP21 (Nelson, 1998). The steroid hydroxylases contain three conserved functional domains: the oxygen-binding domain (Ono sequence), the steroid-binding domain (Ozols tridecapeptide), and the heme-binding domain (Tremblay, 1994). DAF-9 shares 22–35% identity with CYP18, CYP21, and CYP17 in the steroid-binding domain; 56%, 56%, and 38% identity, respectively, in the oxygen-binding domain; and 63–68% identity in the heme-binding domain. The highly conserved glycine and threonine in the oxygen-binding domain and the cysteine in the heme-binding domain are present in DAF-9 (Fig. II-B).

The *C. elegans* protein most closely related to DAF-9 is CYP23A1 (B0304.3), with an overall amino acid identity of 27%. The phylogenetic tree in Fig II-B (Nelson, 1998; Kialiang, 2002) shows the relationships between DAF-9 and its homologs. The homologous proteins CYP21 and CYP17 also play critical roles in the biosynthesis of mammalian steroid hormones. Decreasing the concentration of cholesterol, the precursor for steroids, enhanced the weak *daf-9(m540)* mutant phenotype, whereas providing steroids that bypass the requirement for DAF-9 function might reduce or eliminate the mutant phenotype. A simple

interpretation of these findings is that DA produced by DAF-9 acts on DAF-12 to bypass diapause, promote reproductive development and, perhaps, shorten lifespan (Fig. II-C).

The most obvious trait of *daf-9* mutants is *the unconditional* dauer-like arrest (Albert and Riddle, 1988). In four mutants with a severe phenotype (*e1406*, *m405*, *m641*, and *m642*), about 30%–40% of the arrested animals exit the dauer-like stage, molt, and become sterile (or nearly sterile) adults after several days. One homozygous viable mutant, *m540*, exhibits non-conditional dauer-like arrest but resumes development to fertile adulthood after 1–2 days.

Hedgecock et al., (1987) examined the reproductive capacity of *m540* hermaphrodites and reported a brood size of  $171 \pm 58$  ( $n = 10$ , mean  $\pm$  s.d.) at 20°C. The *daf-12* alleles *rh61*, *rh62*, *rh84*, and *rh50* (initially named *mig-8*) were isolated after ethylmethanesulfonate mutagenesis in F2 screens for mutants with distal-tip cell migration (Mig) phenotypes ( ). The recessive allele *rh50* was shown to have delayed expression of S4 gonadal programs but normal extragonadal development and dauer regulation. In *rh50* hermaphrodites, the compaction and divisions of gonadoblasts are normal through early L3, but thereafter, penetrant defects are evident in distal-tip cell migrations and herniation of the distal ovary (Antebi, 1998). The mean brood size of *rh50* mutants was reported to be 117 (range 40 to 146) (Antebi, 1998). Similarly, in *rh50* mutant males, linker cell migrations are essentially normal through early L3, but they then arrest without undergoing S4 movement.



**Oxygen-binding domain**

DAF-9	D	M	W	T	G	G	M	E	T	T	V	T	T	L	R	W
CeCYP23A1	D	L	F	F	A	G	M	E	T	T	V	T	T	L	K	W
hCYP17	D	I	F	G	A	G	V	E	T	T	T	S	V	V	K	W
hCYP21	D	L	L	I	G	G	T	E	T	T	A	N	T	L	S	W
DmCYP18	D	L	F	S	A	G	M	E	T	I	K	T	T	L	L	W

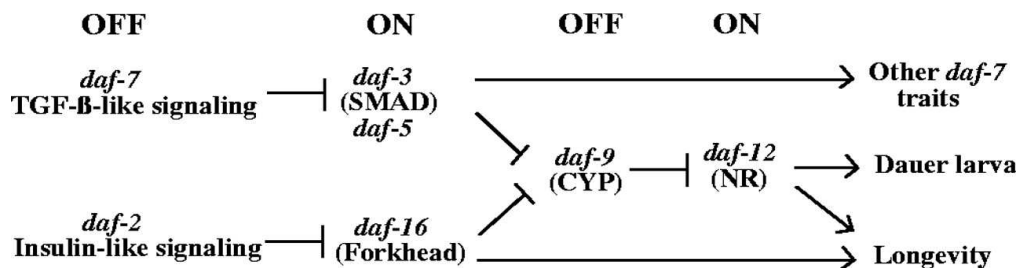
**Steroid-binding domain**

DAF-9	K	M	N	Q	T	P	Y	V	R	A	T	L	S	E	I	Q	R	L	A	N	V	L	P
CeCYP23A1	H	R	T	R	L	P	Y	V	Q	A	T	I	N	E	I	Q	R	I	A	N	I	L	P
hCYP17	D	R	N	R	L	L	L	L	E	A	T	I	R	E	V	L	R	L	R	P	V	A	P
hCYP21	D	R	A	R	L	P	L	L	N	A	T	I	A	E	V	L	R	L	R	P	V	V	P
DmCYP18	D	L	Q	Y	L	P	I	T	E	S	T	I	L	E	S	M	R	R	S	S	I	V	P

**Heme-binding domain**

DAF-9	P	F	G	L	G	P	R	V	C	L	G	E	R	I	A	R	T	E	L
CeCYP23A1	P	F	S	I	G	R	G	Q	C	L	G	E	S	L	A	R	A	E	L
hCYP17	P	F	G	A	G	P	R	S	C	I	G	E	I	L	A	R	Q	E	L
hCYP21	A	F	G	C	G	A	R	V	C	L	G	E	P	L	A	R	L	E	L
DmCYP18	P	F	G	V	G	R	R	M	C	L	G	D	V	L	A	R	M	E	L

**Figure II-B:** SMART homology search for DAF-9 revealed its homology to Cytochrome P450 (top). DAF-9 functional domain alignment with its closest homologs: *C. elegans* CYP23A1, human CYP17, human CYP21 and *Drosophila* CYP18 (bottom, Reproduced from Jia, 2002).



**Figure II-C:** Daur formation and longevity pathway (Reproduced from Jia, 2002).

Mutants carrying strong loss-of-function *daf-9* alleles have dark intestines due to transient excess fat storage and form dauer larvae constitutively (Daf-c). The larvae eventually develop into sterile adults that live ~25% longer than do wildtype *C. elegans*. Weak loss-of-function *daf-9* mutants have penetrant heterochronic delays in L3 gonadal leader cell migration (Mig), reduced fecundity, and modestly shortened lifespans. In contrast, *daf-12* null mutants have impenetrant heterochrony, pale intestines, defective dauer formation (Daf-d), and short lifespans (Antebi, 1998; Gerisch, 2001). *daf-9* phenotypes are *daf-12(+)* dependent, showing that *daf-12* acts downstream of *daf-9*. Moreover, *daf-9* mutants resemble *daf-12* ligand-binding domain (LBD) mutants, suggesting that loss of ligand production or binding specifies dauer formation. *daf-9* is expressed in potential endocrine tissues (two head cells, the hypodermis, and the hermaphrodite spermatheca) and appears to control developmental decisions for the entire organism.

Although much is known about the role of steroid signaling during the development of *C. elegans*, relatively little is known about its function in adult worms. Similarly, relationships between steroid signaling, development, and lifespan in germline-less animals have been analyzed, but not its role in regulating the adult lifespan under environmentally challenging conditions. In this chapter, I describe our initial studies on the role of steroid signaling in DR-induced lifespan extension in *C. elegans*.

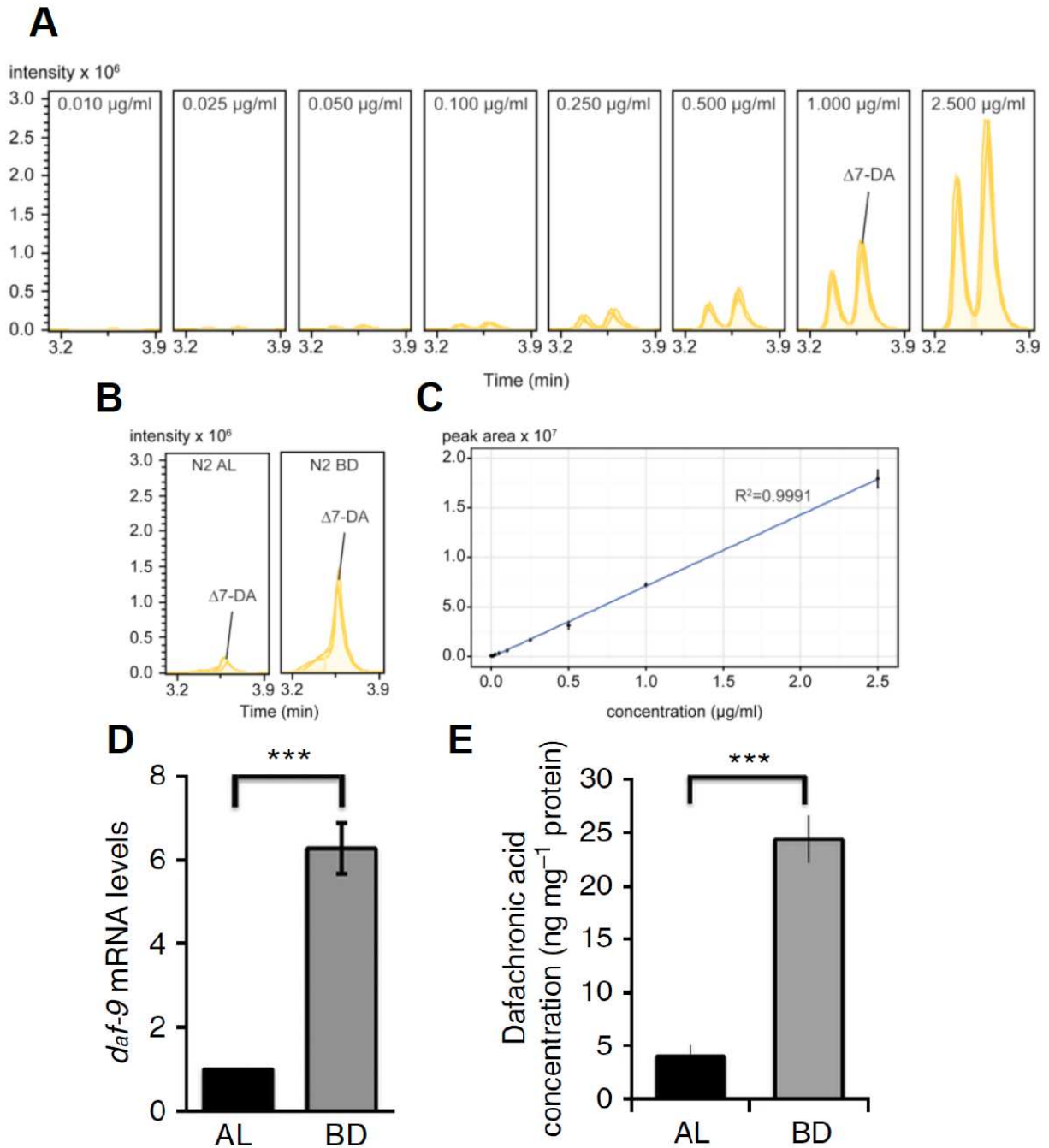
## II-2 Results

### II-2.1 Bacterial deprivation increases *daf-9* transcription and dafachronic acid production

DAs are known to be produced upon ablation of the germline, an intervention that extends lifespan in both worms and flies (Gerisch, 2007; Hsin, 1999; Flatt, 2008). We therefore asked whether *daf-9* transcript levels and DA production are altered in *C. elegans* subjected to bacterial deprivation (BD) compared with those fed *ad libitum* (AL). We found that transcription of *daf-9* and production of  $\Delta^7$ -DA were both increased approximately six-fold by BD (Fig. II-1A–D).

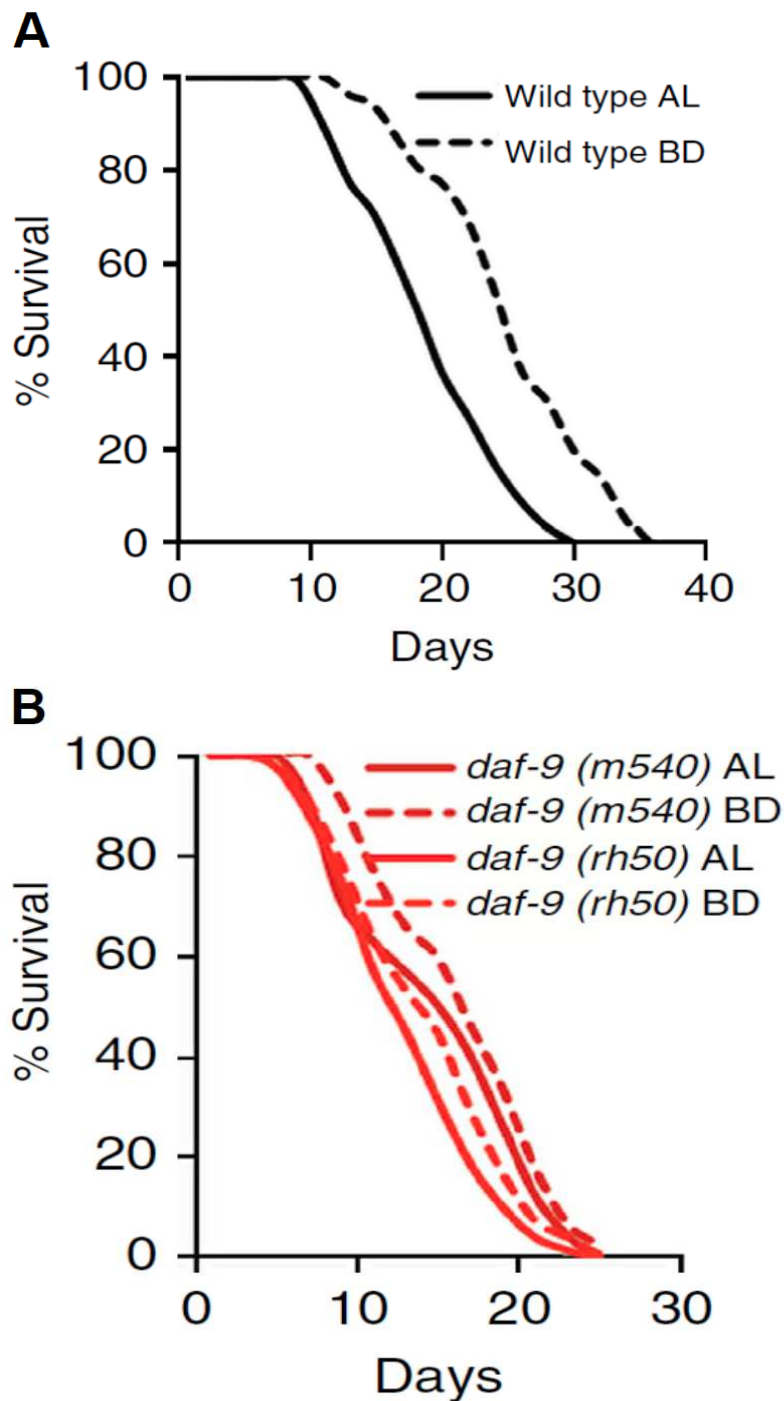
### II-2.2 Bacterial deprivation extends the lifespan of wildtype *C. elegans* but not of *daf-9* mutants

To determine whether DAs are required for the DR response, we examined the lifespans of wildtype and *daf-9* mutant animals. We found that complete removal of bacteria prolonged the lifespan of wildtype animals but failed to do so in two strains carrying different loss-of-function *daf-9* alleles (*rh50* and *m540*; Fig. II-2 and Table 2). To exclude the possibility that *daf-9* mutants are constitutively nutrient deprived, we measured the pharyngeal pumping rate and found it to be similar to that of wildtype animals under both AL and BD conditions (Fig. II-3B). Thus, *daf-9* activity is required for lifespan extension of *C. elegans* through BD.



**Figure II-1: *daf-9* transcript levels and DA quantities were induced in worms subjected to Bacterial Deprivation (BD).** (A) High resolution extracted ion chromatograms of  $m/z$   $413.3061 \pm 0.005$  corresponding to different dafachronic acid concentrations used for calibration and subsequent quantification. The first peak represents  $\Delta 4\text{-DA}$  and the second  $\Delta 7\text{-DA}$ . (B) High resolution extracted ion chromatograms of  $m/z$   $413.3061 \pm 0.005$  corresponding to dafachronic acid from Wild type animals either fed ad libitum or subjected bacterial deprivation.  $\Delta 7\text{-DA}$  is the most abundant isomer. (C) Calibration curve for quantification of  $\Delta 7\text{-DA}$ . *daf-9* mRNA levels (D) and  $\Delta 7\text{-DA}$  levels (E) are increased on bacterial deprivation (BD) of wild-type *C. elegans* compared with animals fed ad libitum (AL; \*\*\* $P < 0.001$ ).  $n=3$  independent biological samples for  $\Delta 7\text{-DA}$  measurements and  $n=9$  independent biological samples for *daf-9* mRNA quantification. P values for QRT-PCR data were calculated using the two-tailed Student's t-test; Error bars represent s.d.





**Figure II-2: DAF-9 is required for dietary restriction-mediated lifespan extension.** (A) Nutrient deprivation extends the mean lifespan of wild-type animals by 41% (Mean LS,  $19.15 \pm 0.95$  days in AL vs.  $27.43 \pm 0.87$  days in BD;  $P < 0.0001$ ) but, (B) does not affect the mean lifespan of animals carrying the *m540* or *rh50* alleles of *daf-9* (Mean LS,  $15.81 \pm 0.830$  vs.  $17.14 \pm 0.69$  in BD;  $P = 0.2$  in *daf-9(m540)*; and  $15.81 \pm 0.830$  days vs.  $17.14 \pm 0.69$  days in *daf-9(rh50)*;  $P = 0.13$ ). *P* values for survival analysis were calculated using the log-rank (Mantel–Cox) test. Lifespan values indicate mean  $\pm$  95% CI.

### **II-2.3 *daf-9* is required for lifespan extension through mutation of *eat-2***

To determine whether *daf-9* is required for lifespan extension through other DR paradigms, the *daf-9(rh50)* allele was introgressed into *eat-2(ad1116)* mutant worms. *eat-2(ad1116)* mutants are impaired in pharyngeal pumping and ingest bacteria at a slower rate, thus providing a *bona fide* genetic surrogate for DR (Hekimi, 1998). *eat-2(ad1116);daf-9(rh50)* double mutants exhibited a lifespan comparable to that of *daf-9(rh50)* single mutants and shorter than that of *eat-2(ad1116)* single mutants (Fig. II-3 and Table 2). These data demonstrate that the requirement for *daf-9* activity in the response to DR is not restricted to the BD paradigm.

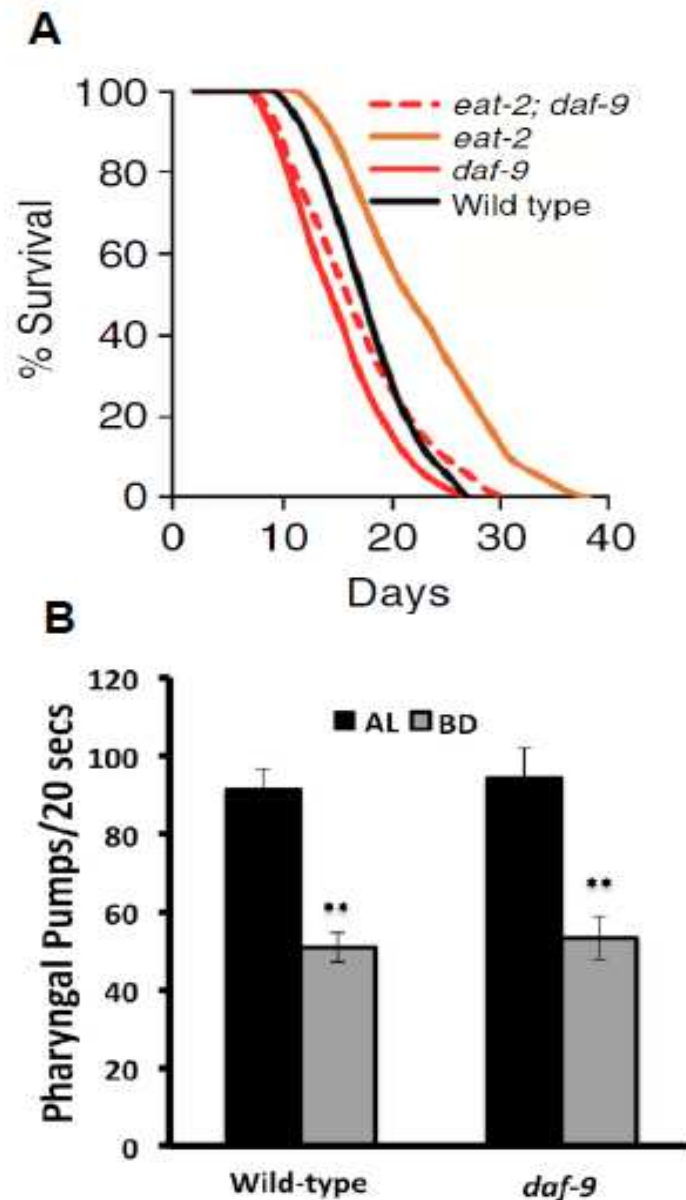
### **II-2.4 Lifespan extension induced by downregulation of insulin signaling is not affected by the *daf-9(rh50)* mutation**

One possible explanation for the inability of *daf-9(rh50)* mutants to respond to DR is that the mutation may compromise the animal's overall health and hinder their ability to extend the lifespan. To test this possibility, we examined the response of *daf-9(rh50)* mutants in which IIS was inhibited by RNAi-mediated silencing of *daf-2/InR*. Previous studies have shown that *daf-2* downregulation increases *C. elegans* longevity independently of DR. However, we saw no difference in the lifespans of wildtype animals or *daf-9(rh50)* mutants subjected to *daf-2* RNAi (Fig. II-4 and Table 2). These results therefore exclude the possibility that *daf-9(rh50)* mutant animals are constitutively unable to undergo lifespan extension.

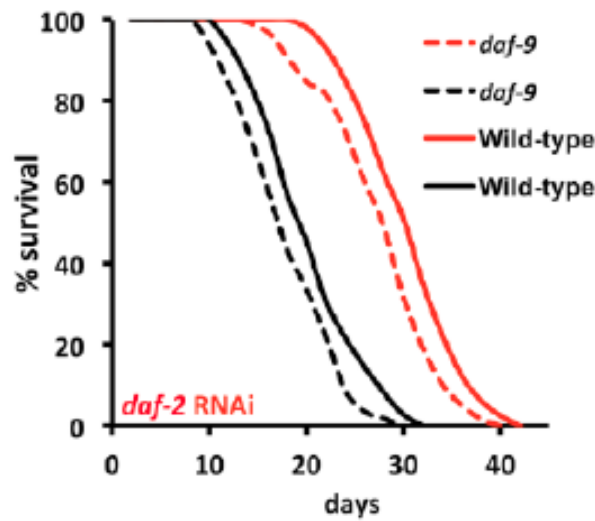
### **II-2.5 Induction of *daf-9* transcription is similar in insulin or TGF $\beta$ signaling mutants and wildtype animals**

A previous report has shown that DAs are produced in developing *C. elegans* under nutrient-favorable conditions (Gerisch, 2007). However, we found that DR also induces *daf-9*

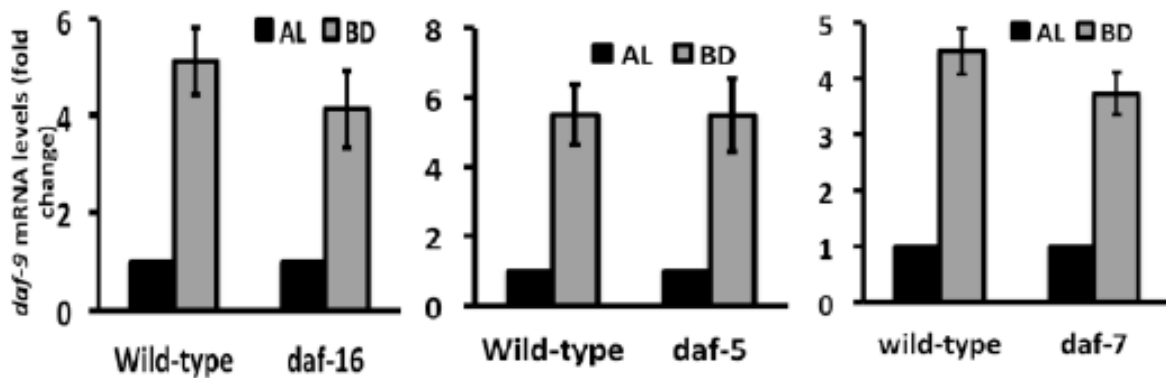
expression in adult mutants lacking functional insulin signaling (*daf-16*/FOXO mutant) or TGF $\beta$  signaling (*daf-5*/SKI and *daf-7*/TGF $\beta$  mutants; Fig. II-5). Thus, our observation that DAs are produced in gravid adult worms in response to starvation contrasts with the findings in worms during developmental stages.



**Figure II-3: *daf-9* is required for lifespan extension through *eat-2* mutation:** *eat-2(ad1116);daf-9(rh50)* double mutants and *daf-9(rh50)* single mutants exhibit similar mean lifespans (Mean LS of  $21.98 \pm 0.99$  days in *eat-2(ad1116)*,  $15.87 \pm 0.64$  days in *daf-9(rh50)*, and  $17.48 \pm 0.98$  days in *eat-2(ad1116);daf-9(rh50)*;  $P=0.1$ ).  $P$  values for survival analysis were calculated using the log-rank (Mantel-Cox) test. Lifespan values indicate mean $\pm$ 95% CI. Under BD conditions the pumping rate was significantly reduced in both wild-type (45%,  $P < 0.0027$ ,  $n = 10$ ) and *daf-9(rh50)* mutants (42%,  $P < 0.001$ ,  $n = 10$ ).  $P$  values were calculated using the two-tailed Student's t-test. Error bars, SEM.



**Figure II-4: *daf-2* RNAi extends the mean lifespan of wild-type and *daf-9(rh50)* mutant animals to the same extent:** *daf-2* RNAi extended the lifespan of *daf-9(rh50)* mutant and wild-type animals by 55% and 50%, respectively ( $P < 0.0001$  for both strains).  $P$  values were calculated using the log-rank (Mantel-Cox) test.



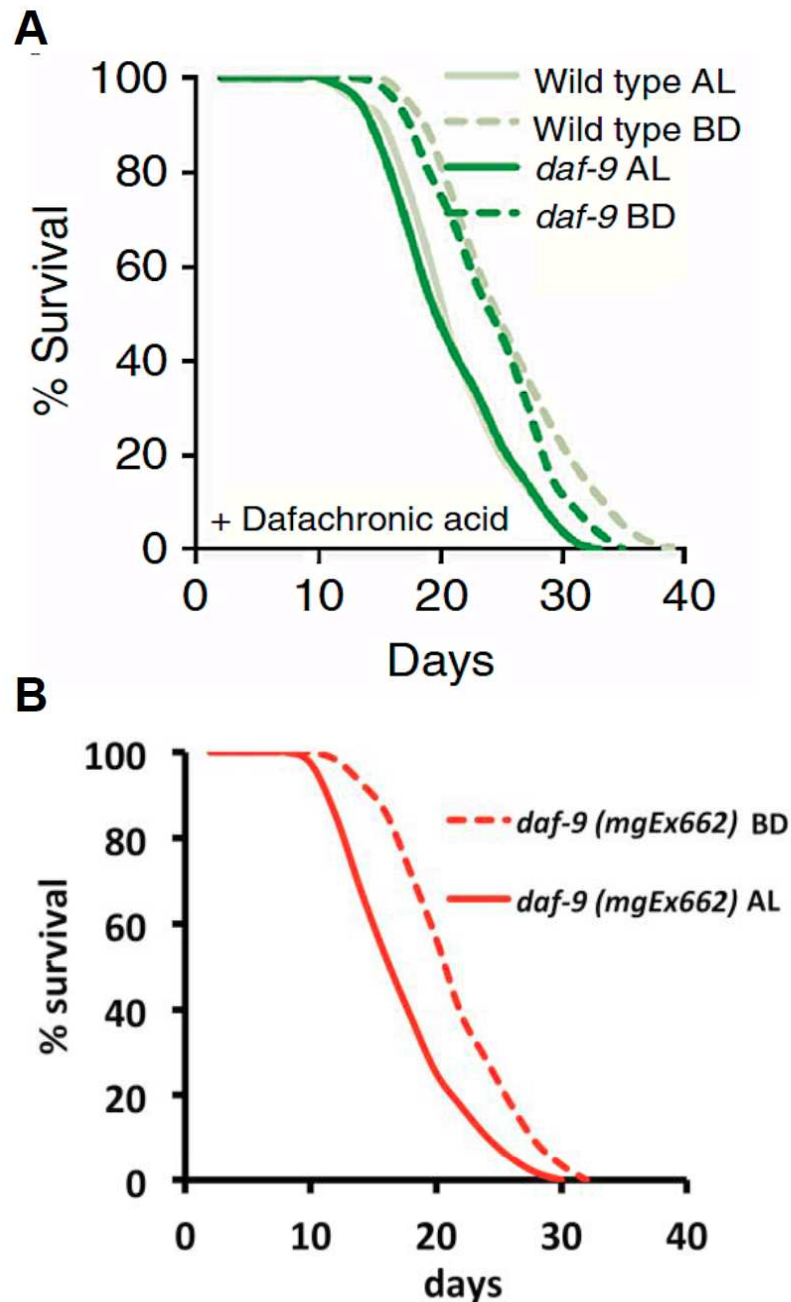
**Figure II-5: DR induces *daf-9* mRNA levels to similar extents in wild-type and *daf-16(mu86)*, *daf-5(e1386)* and *daf-7(e1372)* mutant animals:** Bacterial deprivation increased the transcript levels of the cytochrome P450 DAF-9 in wild-type animals (5.13-fold  $\pm$  0.71,  $P < 0.001$ ), *daf-16(mu86)* mutant (4.13-fold  $\pm$  0.8,  $P < 0.001$ ), *daf-5(e1386)* mutant (5.48-fold  $\pm$  1.05,  $P < 0.001$ ) and *daf-7(e1372)* mutant animals (3.73-fold  $\pm$  0.37,  $P < 0.001$ ).  $P$  values were calculated using the two-tailed Student's t-test. Error bars, SD.

## **II-2.6 Dafachronic acids are required, but not sufficient, for lifespan extension induced by bacterial deprivation**

To confirm that the requirement for *daf-9* in BD-induced lifespan extension was linked to the production of DAs, we examined the lifespans of *daf-9(rh50)* mutants with or without supplementation with 100 nM  $\Delta^7$ -DA (Adipogen<sup>®</sup>). We found that DA treatment rescued the DR longevity response of *daf-9(rh50)* mutant animals, and the lifespan extension was similar to that seen with wildtype animals (Fig. II-6A and Table 2). Thus, DAs are produced by *C. elegans* in response to food deprivation and are required for lifespan extension through DR. However, we found no significant differences in the lifespans of nutrient-deprived  $\Delta^7$ -DA-treated and non-treated wildtype animals (Fig. II-6A and Table 2). Next, we measured the lifespans of animals carrying an extrachromosomal array expressing the genomic *daf-9* gene (*daf-9(e1406);mgEx662[daf-9p::daf-9genomic::GFP]*). We found that the lifespan of these *daf-9*-overexpressing animals was not significantly different from that of wildtype animals (Fig. II-6B, Table 2). Together, these data suggest that DAs are necessary but not sufficient to increase the lifespan through BD.

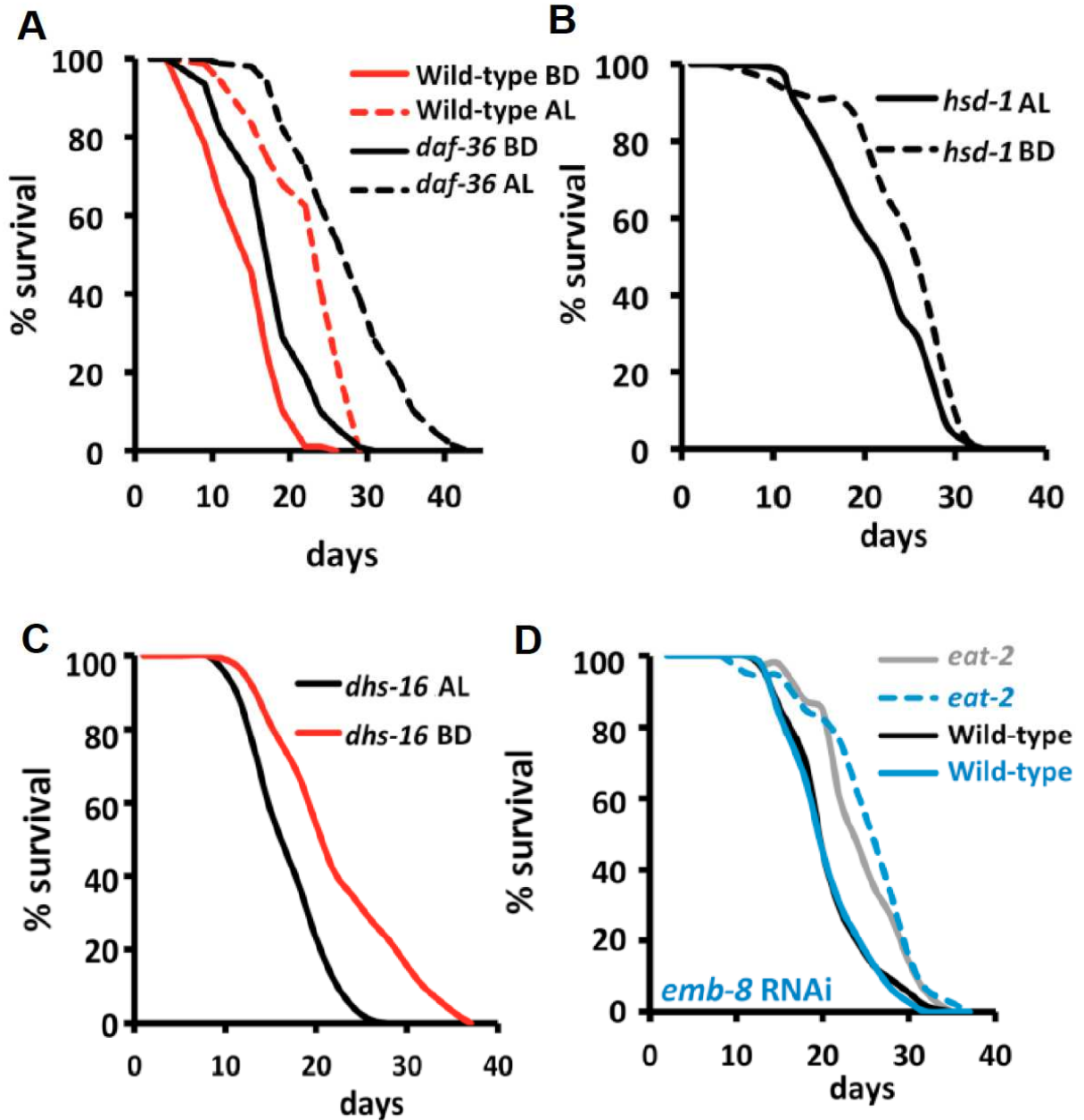
## **II-2.7 Additional steroidogenic genes are dispensable for DR-induced lifespan extension**

Other enzymes involved in the *C. elegans* steroid signaling pathway include *daf-36*, a Rieske-like oxygenase; *dhs-16*, a 3-hydroxysteroid dehydrogenase, *hsd-1*, a hydroxysteroid dehydrogenase homolog; and *emb-8*, an NADPH-cytochrome P450 reductase ortholog (Hsin and Kenyon, 1999; Jia, 2002; Rottiers, 2006; Motola, 2006; Wollam, 2012). We lifespanded the animals bearing mutations in genes involved in DA synthesis pathway. We found that these genes were dispensable for BD-induced lifespan extension (Fig. II-7 and Table 2), consistent with the notion proposed by others that DAs can be produced through an as yet uncharacterized alternative pathway (Wollam, 2012, Mahanti, 2014).



**Figure II-6: DAs are required, but not sufficient, for diet-mediated longevity enhancement :** (A) Addition of 100nM  $\Delta^7$ -DA does not affect the lifespan of wild-type animals under AL or BD conditions but restores the lifespan extension of *daf-9(rh50)* mutants (Mean LS: Wild-type,  $21.31 \pm 0.94$  days in AL+DA vs.  $26.27 \pm 1.02$  days in BD+DA,  $P < 0.0001$ ; *daf-9(rh50)*,  $18.54 \pm 0.94$  days in AL+DA vs.  $25 \pm 0.92$  days in BD+DA,  $P < 0.0001$ ). (B) *daf-9* overexpression did not enhance the DR response to bacterial deprivation. The mean lifespans of animals carrying a transgenic *daf-9* construct were  $18 \pm 1$  days in AL and  $22 \pm 1$  days in BD (22% increase,  $P < 0.0001$ ), P values for survival analysis were calculated using the log-rank (Mantel–Cox) test. Lifespan values indicate mean  $\pm$  95% CI.

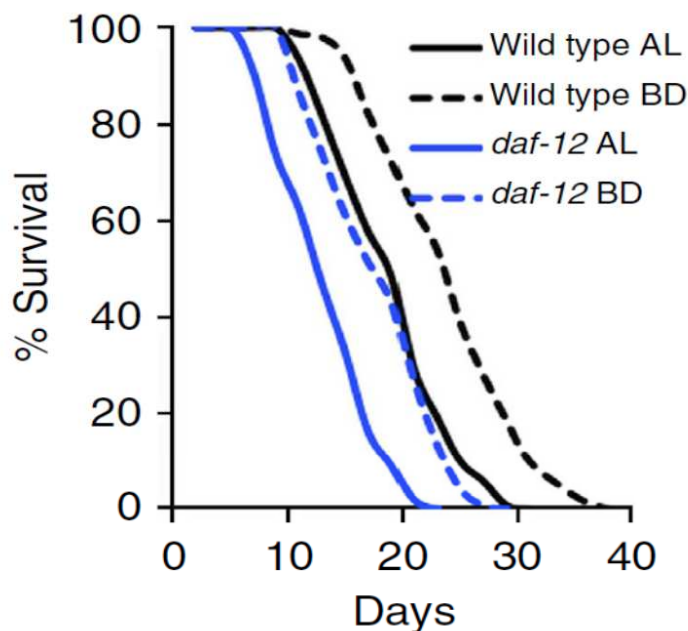




**Figure II-7: Inactivation of other steroidogenic genes does not affect the lifespan response to DR: BD extended the lifespan of *daf-36(k114)* (A; Mean LS, wild-type  $19.31 \pm 1.08$  days in AL vs.  $30.09 \pm 0.86$  in BD; *daf-36(k114)*  $14.95 \pm 0.93$  days in AL vs.  $22.69 \pm 1.34$  in BD), *hsd-1(mg433)* (B;  $22.15 \pm 1.1$  days in AL vs.  $25.3 \pm 1.15$  in BD,  $P < 0.0001$ ), and *dhs-16* (C;  $17.63 \pm 0.82$  in AL vs.  $22.83 \pm 1.03$  days in BD) mutant animals by 52% ( $P < 0.0001$ ), 20% ( $P < 0.001$ ) and 30% ( $P < 0.0001$ ), respectively. (D) Downregulating *emb-8* in *eat-2* mutant animals did not reduce the lifespan of these animals (empty vector  $27.18 \pm 1.02$  days vs. *emb-8 RNAi*  $25.84 \pm 0.96$  days  $P = 0.24$ ). P values for survival analysis were calculated using the log-rank (Mantel–Cox) test. Lifespan values indicate mean  $\pm$  95% CI.**

## II-2.8 *daf-12(rh61rh411)* mutants show a normal longevity response to bacterial deprivation

Next, we asked how DAs might mediate the effect of DR on lifespan. As mentioned earlier, the canonical steroid signaling pathway of *C. elegans* developing under optimal conditions involves the DAs produced by DAF-9 bind to the nuclear receptor, DAF-12. Therefore, we asked whether *daf-12* is involved in DR-mediated lifespan extension of adult *C. elegans*. We tested the class-3 *daf-12* allele, *daf-12(rh61rh411)*, which is a putative null allele and is *daf-d*. This allele carries two nonsense mutations affecting both the DNA binding domain (DBD) and the LBD. We found that the lifespans of wildtype animals and *daf-12(rh61rh411)* mutants were similarly extended by BD (Fig. II-8 and Table 2). This suggests that DAs can mediate the DR effect in the absence of DAF-12.



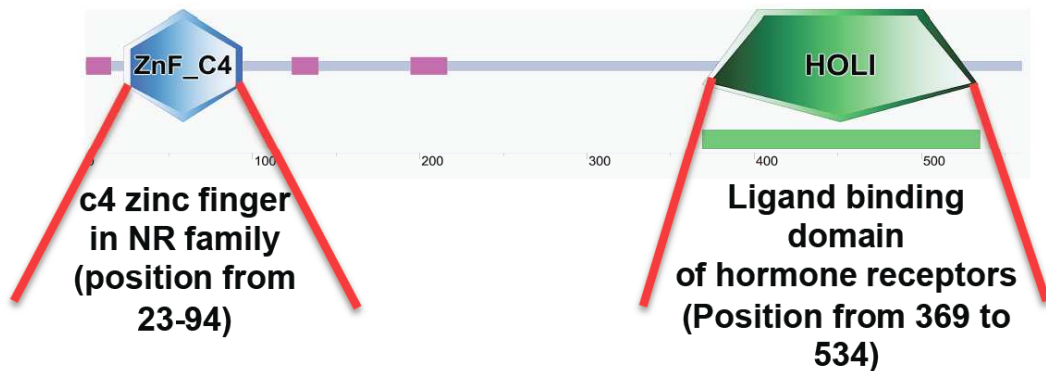
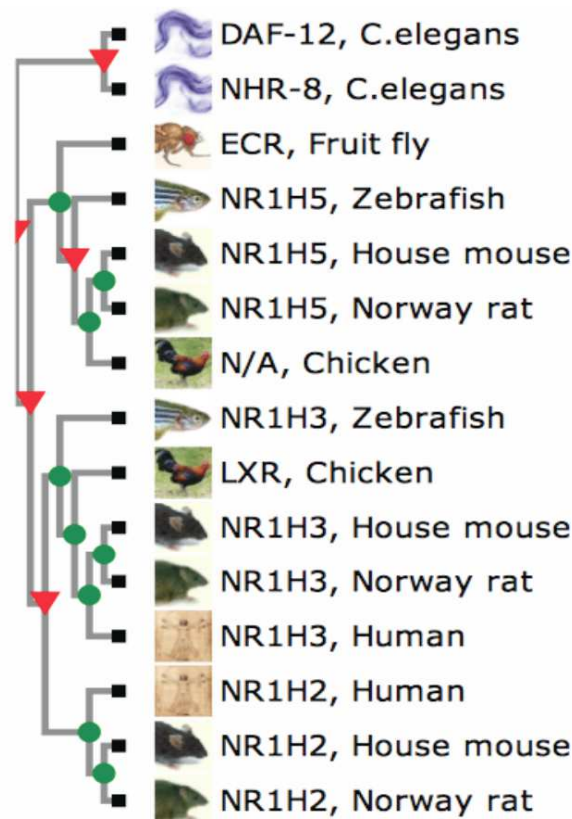
**Figure II-8: DAF-12 is not required for dietary restriction-mediated lifespan extension:** Bacterial deprivation extends the lifespan of *daf-12(rh61rh411)* mutant animals by 27% (Mean LS of  $13.69 \pm 0.76$  in AL vs.  $17.39 \pm 1.2$  days in BD,  $P < 0.0001$ ), similar to the effect on wild-type animals. P values for survival analysis were calculated using the log-rank (Mantel–Cox) test. Lifespan values indicate mean  $\pm$  95% CI.



## II-2.9 NHR-8 is required for lifespan extension mediated by dietary restriction

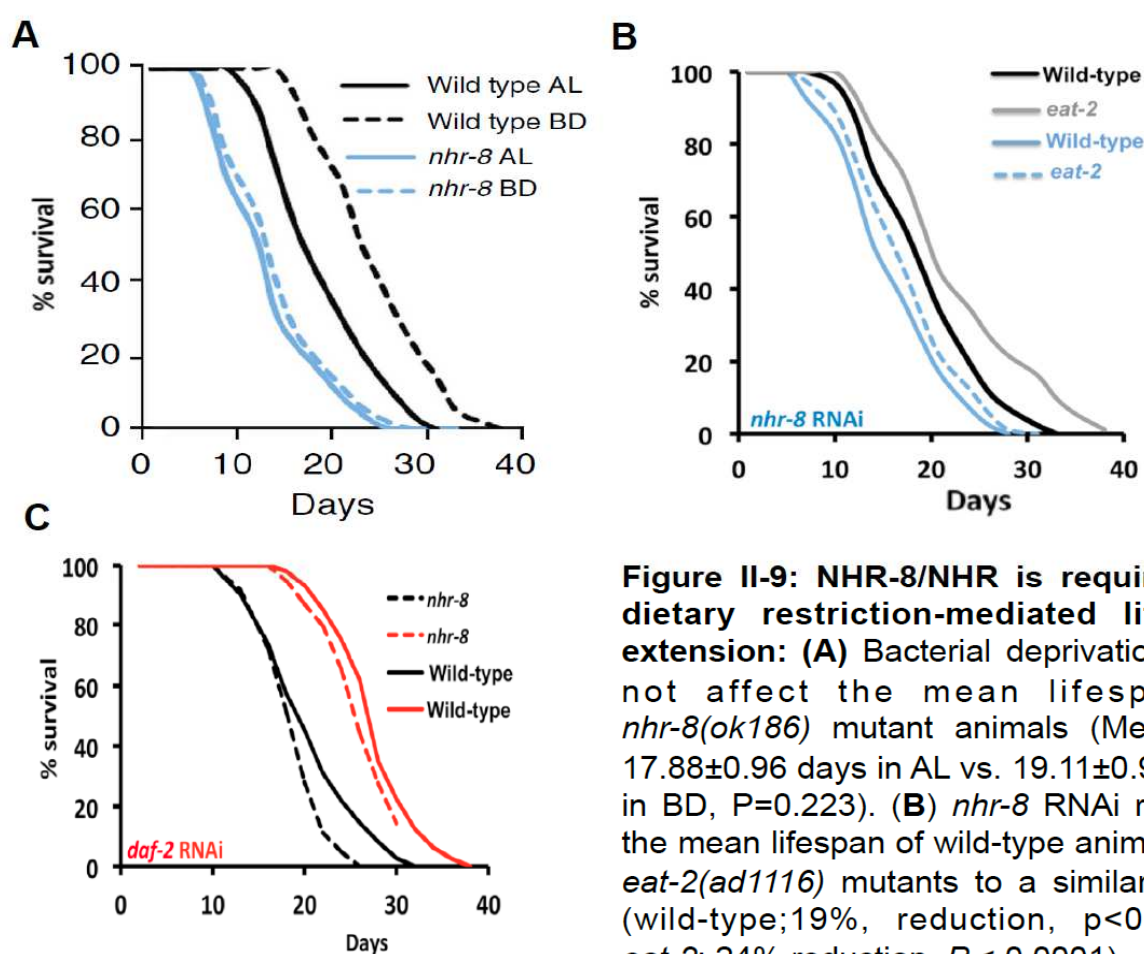
Our finding that *daf-12(rh61rh411)* mutant animals respond normally to DR prompted us to look for other NHRs that might mediate DA signaling. As described earlier, the *C. elegans* genome contains a second nuclear hormone receptor, NHR-8/NHR, which is closely related to *C. elegans* DAF-12, *Drosophila* HR96, and mammalian VDR, CAR, PXR, liver X receptor (LXR), and farnesoid X receptor (FXR) (Magner, 2013 and Fig. II-C). Recently, NHR-8/NHR has been shown to be involved in sterol homeostasis (Magner, 2013) and is thus a candidate receptor for mediating the effects of DAs on DR-mediated lifespan extension. Indeed, under cholesterol-deficient conditions, animals carrying an *nhr-8/NHR*-null mutation exhibit phenotypes reminiscent of DA deficiency; for example, facilitated dauer formation, defective gonadal migration, and shortened lifespans (Magner, 2013). Consistent with the notion that NHR-8 controls sterol production and homeostasis, these phenotypes could be rescued by supplementation with any sterol in the steroid signaling pathway.

We therefore asked whether NHR-8/NHR might be involved in the lifespan extension induced by DR. To bypass potentially confounding effects of *nhr-8/NHR* deficiency on development, we used the weak *ok186* allele of *nhr-8/NHR* (deletion within the LBD) and provided the animals with a standard concentration of cholesterol (5 mg/ml). Under these conditions, the *nhr-8(ok186)* mutants displayed no developmental defect or delay. When we measured the lifespans of these animals under AL feeding and BD conditions, we found that starvation did not extend their lifespan (Fig. II-9, Table 2). Consistent with our earlier data examining *eat-2(ad1116);daf-9(rh50)* double mutants, RNAi-mediated downregulation of *nhr-8* abrogated the long lifespan of *eat-2(ad1116)* mutants (Fig. II-9B, Table 2). These



**Figure II-C:** TreeFam for Nuclear receptor subfamily 1, group H, member 3 (NR1H3) which shows DAF-12 being the closest homolog of NHR-8 (top panel). Domains within *Caenorhabditis elegans* protein NHR-8. TreeFam data is reproduced from Wormbase and Protein domain predictions were from **SMART** (bottom panel, Simple Modular Architecture Research Tool, [http://smart.embl-heidelberg.de/help/smart\\_about.shtml](http://smart.embl-heidelberg.de/help/smart_about.shtml))

results confirm that *nhr-8*/NHR is required to respond to both DR paradigms. To ensure that our results did not simply reflect a general inability of *nhr-8(ok186)* mutants to undergo lifespan extension, we examined their response to *daf-2* RNAi. This treatment extended the lifespan of control animals and *nhr-8(ok186)* mutants to a similar extent (Fig. II-9C, Table 2).

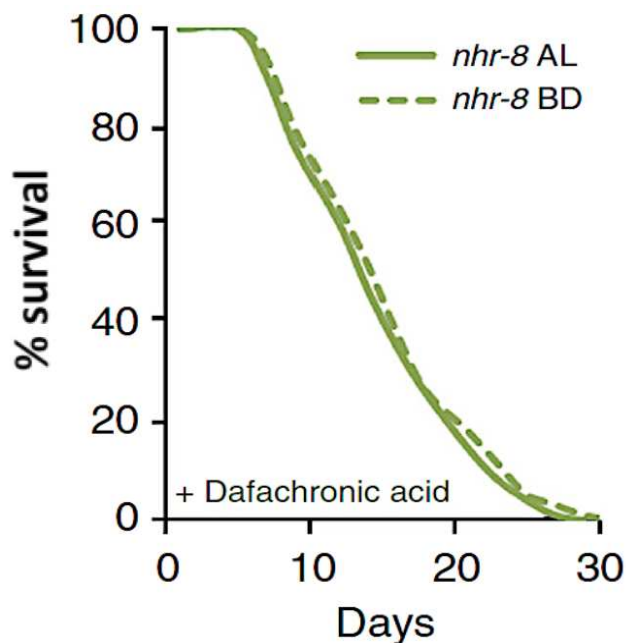


**Figure II-9: NHR-8/NHR is required for dietary restriction-mediated lifespan extension:** (A) Bacterial deprivation does not affect the mean lifespan of *nhr-8(ok186)* mutant animals (Mean LS,  $17.88 \pm 0.96$  days in AL vs.  $19.11 \pm 0.99$  days in BD,  $P=0.223$ ). (B) *nhr-8* RNAi reduced the mean lifespan of wild-type animals and *eat-2(ad1116)* mutants to a similar extent (wild-type; 19%, reduction,  $p < 0.0001$ ., *eat-2*; 24% reduction,  $P < 0.0001$ ).

(C) *daf-2* RNAi extended the lifespan of *nhr-8(ok186)* mutant and wild-type animals by 55% and 85%, respectively (Median LS; in wild-type animals, empty vector  $19.89 \pm 0.94$  vs. *daf-2* RNAi  $30.86 \pm 1.04$  days; *nhr-8(ok186)*, empty vector  $13.72 \pm 0.70$  vs. *daf-2* RNAi  $25.39 \pm 1.06$  ( $P < 0.0001$  for both strains).  $P$  values were calculated using the log-rank (Mantel-Cox) test. Lifespan values indicate mean  $\pm$  95% CI.

## II-2.10 Addition of $\Delta^7$ -DA does not increase the lifespan of *nhr-8(ok186)* mutants subjected to bacterial deprivation

We next asked whether supplementation with  $\Delta^7$ -DA could rescue the inability of *nhr-8(ok186)* mutants to respond to DR. We measured the lifespan of *nhr-8(ok186)* mutant animals under AL feeding and BD conditions with or without exogenous  $\Delta^7$ -DA (100 nM). Surprisingly,  $\Delta^7$ -DA supplementation had no effect on the lifespans of either fed or starved *nhr-8(ok186)* mutants (Fig. II-10, Table 2), suggesting that, in this context,  $\Delta^7$ -DA acts upstream of NHR-8.

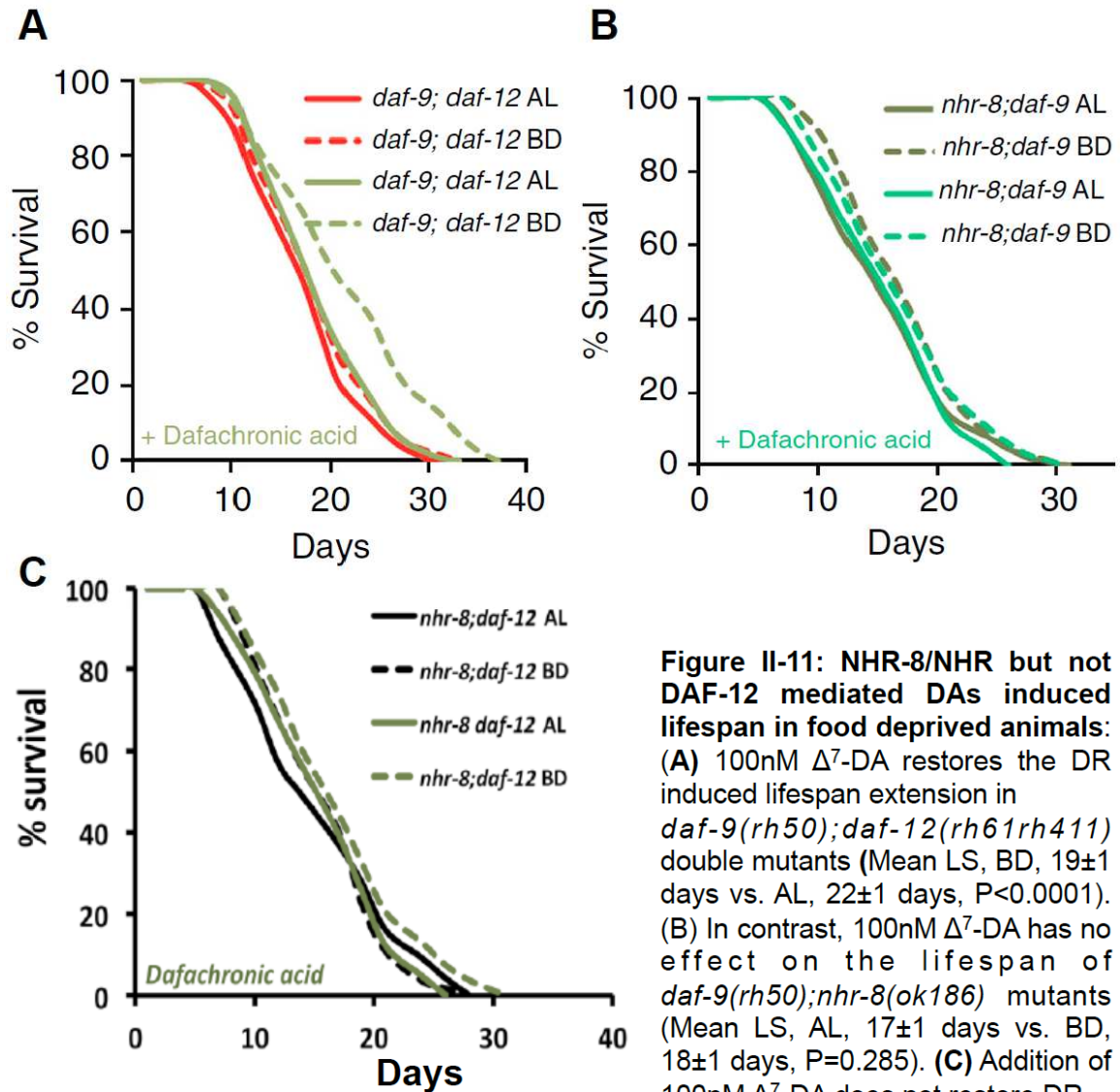


**Figure II-10:  $\Delta^7$ -DA does not increase lifespan in *nhr-8(ok186)* mutants:** Addition of 100nM  $\Delta^7$ -DA does not restore DR-induced lifespan extension of *nhr-8(ok186)* mutant animals (c); mean lifespans: BD,  $17 \pm 1$  days and AL,  $17 \pm 1$  days,  $P=0.642$ ).

## II-2.11 NHR-8, but not DAF-12, mediates the effects of dafachronic acids on the dietary restriction response

To further investigate the interaction between NHR-8 and  $\Delta^7$ -DA, we combined the *daf-12(rh61rh411)* and *nhr-8(ok186)* alleles with the *daf-9(rh50)* allele. We found that *daf-12(rh61rh411);daf-9(rh50)* double mutants could respond to DR only when  $\Delta^7$ -DA was provided (Fig. II-11A, Table 2), whereas *daf-9(rh50);nhr-8(ok186)* double mutants failed to

respond, even in the presence of  $\Delta^7$ -DA (Fig. II-11B, Table 2). Similarly, DR did not induce lifespan extension in *daf-12(rh61rh411);nhr-8(ok186)* double mutants whether or not  $\Delta^7$ -DA was added (Fig. II-11C, Table 2).



**Figure II-11: NHR-8/NHR but not DAF-12 mediated DAs induced lifespan in food deprived animals:** (A) 100nM  $\Delta^7$ -DA restores the DR induced lifespan extension in *daf-9(rh50);daf-12(rh61rh411)* double mutants (Mean LS, BD, 19±1 days vs. AL, 22±1 days, P<0.0001). (B) In contrast, 100nM  $\Delta^7$ -DA has no effect on the lifespan of *daf-9(rh50);nhr-8(ok186)* mutants (Mean LS, AL, 17±1 days vs. BD, 18±1 days, P=0.285). (C) Addition of 100nM  $\Delta^7$ -DA does not restore DR-

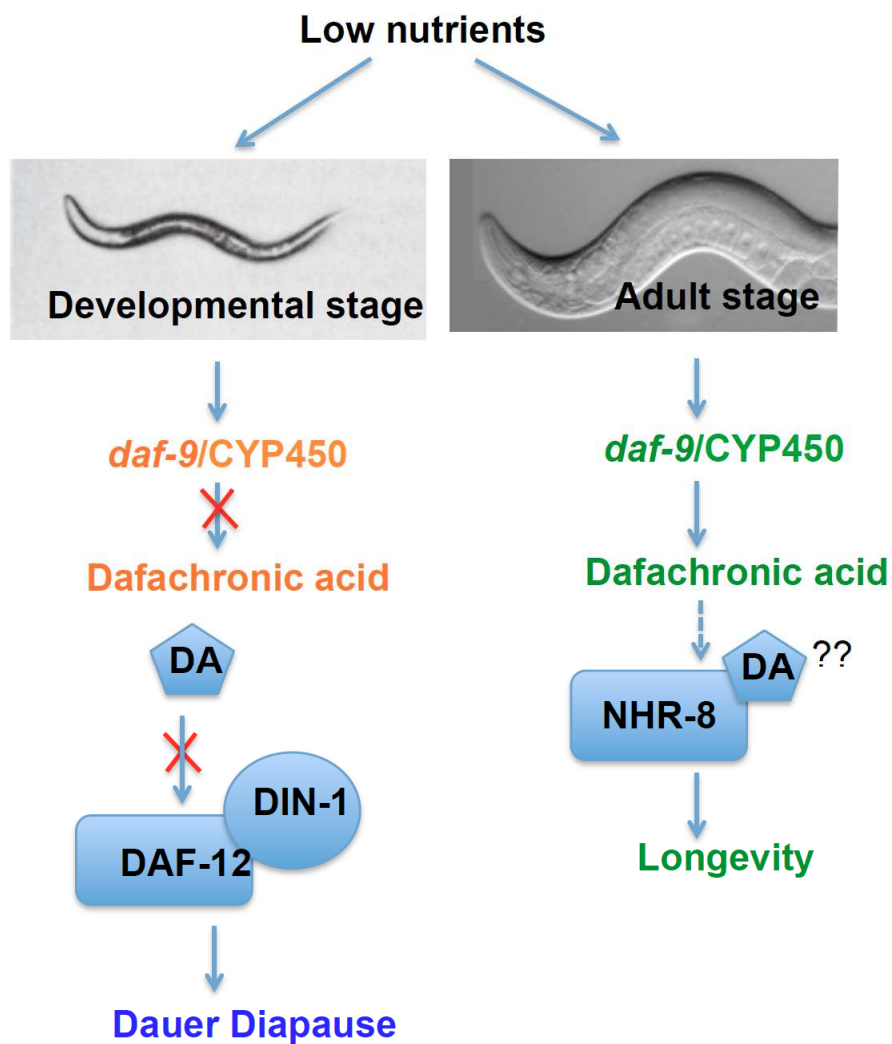
induced lifespan extension of *nhr-8(ok186)* mutant animals (c); mean lifespans: BD, 17±1 days and AL, 17±1 days, P=0.642). P values were calculated using the log-rank (Mantel–Cox) test. Lifespan values represents mean±95% CI.

## II-3 Conclusions and future perspectives

In this chapter, we asked whether steroid signaling plays a role in DR-mediated lifespan extension in *C. elegans*, and our results provide clear evidence that this is indeed the case. Our findings that *daf-9* transcription and DA production are induced in gravid adult worms in response to starvation contrasts with earlier findings in developing worms. However, one should take into consideration that the difference in the life history stages might affect the pattern of *daf-9* gene expression. In fact, there is evidence for this with other genes. Our results suggest that during development under favorable nutritional conditions, DAF-9 is activated to produce DAs, which triggers entry into reproductive development and hence leads to shorter lifespans. At the adult stage, however, the same gene is induced during conditions of nutrient scarcity, resulting in increased DA production and an extended lifespan. Although *daf-9(rh50)* is considered to be a weak allele of *daf-9*,  $\Delta^7$ -DA is sufficiently limiting in these animals to prevent their response to DR.

Our results additionally suggest that NHR-8, but not DAF-12, acts downstream of  $\Delta^7$ -DA to regulate the DR response. Thus, NHR-8/NHR has at least two independent functions in *C. elegans*. One is the regulation of larval development, which is observed in *nhr-8/NHR*-null mutants under cholesterol-deficient conditions and is rescued by any sterol, including  $\Delta^7$ -DA15. The second function is in DR-induced longevity, which is observed in animals carrying weak *nhr-8/ NHR* alleles and is not rescued by  $\Delta^7$ -DA (Fig. XX, Table 2). Our data show that lifespan extension induced by nutrient deprivation requires both NHR-8/NHR and  $\Delta^7$ -DA and that NHR-8 acts downstream of  $\Delta^7$ -DA. However, it is not yet clear whether  $\Delta^7$ -DA activates NHR-8 directly or indirectly. Indeed, in HEK293 cells, neither  $\Delta^4$ -DA nor  $\Delta^7$ -DA is able to transactivate NHR-8/NHR (Motola, 2006). Three scenarios are compatible with our observations. First, NHR-8 could be activated by another form of DA. This is

supported by a recent report that a variety of DAs can bind with different affinities and activate DAF-12 *in vivo* (13). Second, NHR-8 activation could require binding with co-activators that are present in the worm, but not in HEK293 cells. Finally, DA could activate NHR-8 through one or more unidentified factors. Further investigation will be necessary to determine how  $\Delta^7$ -DA activates NHR-8/NHR in *C. elegans*.



**Figure II-D: Steroid signalling at development and adult stage in *C. elegans* :** During development low nutrition condition blocks DAs being produced from steroid signaling pathways which involves *daf-9/CYP450*. In DAs absence, DAF-12 is constitutively repressed by DIN-1 (DAF-12 inhibitor), which then triggers animal to enter to dauer stage. In contrast, during reproductive adult stage, low nutrition induces *daf-9* expression and production of Dafachronic Acid. Unlike in developmental stage, NHR-8 but not DAF-12, mediates the effects of DA or its metabolites, to increase the lifespan in *C. elegans*.

## Chapter III

# TOR Signaling is Controlled by Steroid Signaling



## Chapter III TOR Signaling is Controlled by Steroid Signaling

### III-1 Introduction

DR is remarkable for its ability to extend both lifespan and healthspan. Various experimental DR regimens have been used in studies of species ranging from yeast to mammals. In *C. elegans*, longevity induced by different DR regimens is mediated by distinct genetic pathways (Greer and Brunnet, 2009). Previous studies have shown that the low-energy sensing AMP-activated protein kinase *aak-2*/AMPK and *daf-16*/FOXO are necessary for longevity induced by a solid dietary restriction (sDR), with AMPK acting upstream of *daf-16*/FOXO, perhaps via direct phosphorylation (Greer, 2007). Subsequent studies from the same group showed that AMPK and DAF-16 are also necessary to induce lifespan in response to a peptone dilution DR regimen, whereas the response to a DR mimetic, resveratrol, requires AMPK but not DAF-16 (Greer and Brunnet, 2009).

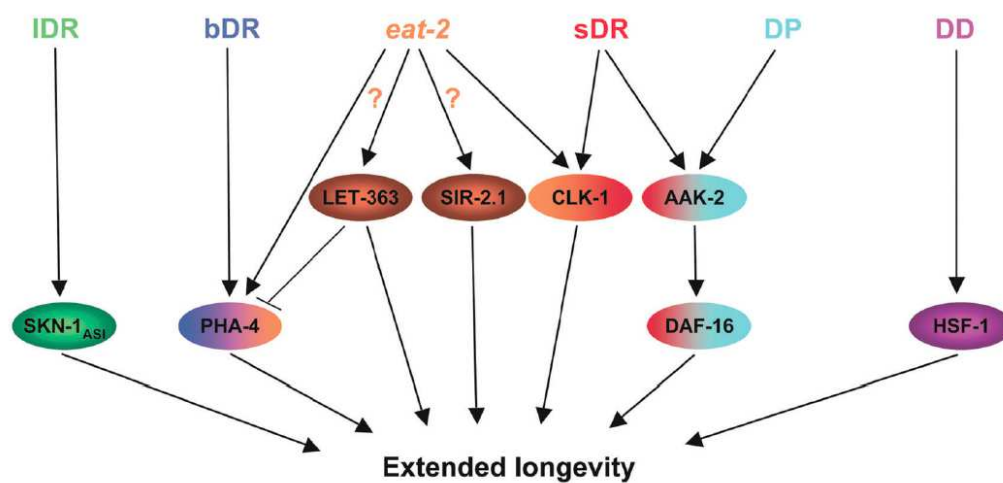
The sirtuin family of NAD-dependent protein deacetylases has also been proposed to mediate the effects of DR on lifespan (Guarente, 2000). One gene, *sir-2.1*, was shown to mediate lifespan extension by resveratrol in some studies (Wood, 2004 and Viswanathan, 2005) but not in others (Bass, 2007). However, the studies by Greer and Brunnet (2009) suggest that *sir-2.1* is not necessary for lifespan extension by sDR.

In 2007, Panowski et al., showed that another forkhead transcription factor, *pha-4*/FOXA, is required for lifespan extension through at least two DR paradigms; dilution of bacteria in liquid cultures (bDR) and the *eat-2* mutation. Interestingly, *pha-4* mRNA levels are increased in *eat-2(ad1116)* mutants (Panowski, 2007). Later work showed that *pha-4*/FOXA is not necessary for the response induced by sDR (Greer and Brunnet, 2009).

*skn-1*/NRF-2 is another gene shown to mediate DR-induced longevity (Bishop and Guarente, 2007) in *C. elegans*. *skn-1* encodes the SKN-1/NRF2 zinc-finger transcription factor, and induction of the b isoform of the protein in amphid sensilla I ASI neurons is necessary for DR-mediated longevity (Bishop and Guarente, 2007). Two strains carrying *skn-1* mutations, *zu135* and *zu169*, had normal basal lifespans but failed to respond to DR (Bishop and Guarente, 2007). A simple model proposed by Bishop and Guarente suggested that DR induces *skn-1* expression in ASI neurons, which then promotes cell non-autonomous signaling in the peripheral tissues. In turn, this increases respiration and extends lifespan. However, it is also possible that other neurons or genetic pathways act in parallel with *skn-1* to mediate the DR-induced longevity response.

The TOR intracellular signal-transduction signaling pathway is conserved in eukaryotes from yeast to mammals (Wullschleger, 2006). In mammals, mTOR (known as mTOR) integrates signals derived from (nutrients, hormones, growth factors, and stressors) through a complex signaling network. Certain nutrients also increase levels of insulin, which additionally activates mTOR. TOR signaling increases protein synthesis and inhibits autophagy, driving cellular mass growth (Hands, 2009; Blagosklonny, 2009). TOR also activates a wide variety of cellular processes, including functions, ranging from the bone absorption by osteoclasts, and the contractility in smooth muscle cells, to insulin secretion by  $\beta$ -cells of the pancreas, and lipoprotein synthesis in the liver. mTOR by activation of S6 kinase causes insulin resistance. All of these cellular alterations can be linked to organismal aging and age-related diseases such as benign and malignant tumors, cardiac hypertrophy, osteoporosis, metabolic syndrome, atherosclerosis, hypertension, neurodegeneration, and age-related macular degeneration (Blagosklonny, 2009). Like in yeast (Kaeberlein, 2005), serum starvation and the TOR inhibitor rapamycin suppress

mammalian cell senescence in mammals (Demidenko, 2009). and yeast (Kaeberlein, 2005). In addition, It is well known that, rapamycin extends the lifespan of mice (Harrison, 2009) by inhibiting the mTOR. Figure III-A shows a simple scheme proposed by Greer and Brunnet, (2009), which shows how distinct signaling pathways are activated by the different DR regimens, is shown below (Fig III-A).



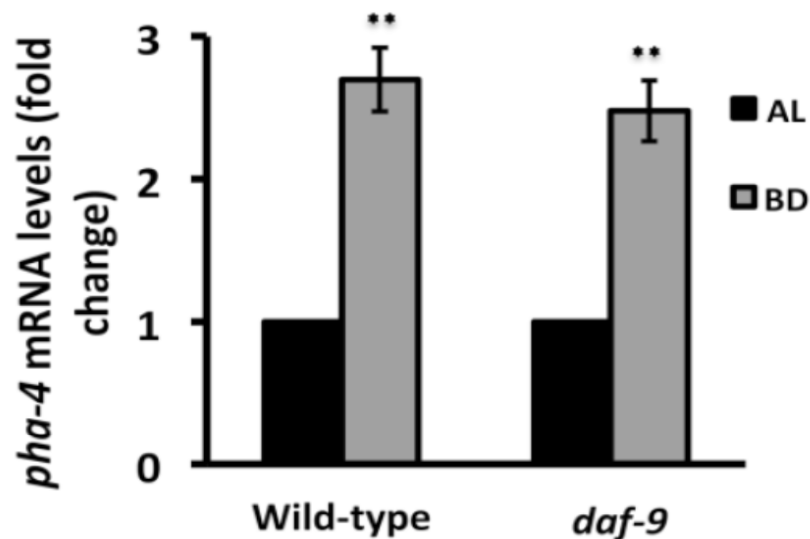
**Figure III: Different regimens of DR:** Based on DR method, distinct signalling pathways are activate. Molecules presented in scheme have been shown to play a role in mediating the longevity extension effects of DR methods. Question marks indicate conflicting reports (Henderson et al., 2006; Wang & Tissenbaum, 2006; Hansen et al., 2007). Reproduced from Greer and Brunnet, 2009.

Following on from our work showing that *daf-9* and *nhr-8* are required for lifespan extension induced by DR, we next sought to understand the mechanisms responsible. Therefore, we examined the involvement of several of the key genes described above, which have previously been shown or suggested to be DR mediators: *pha-4*/FOXO, *skn-1*/NRF2, *aak-2*/AMPK, and *let-263*/mTOR.

## III-2 Results

### III-2.1 *pha-1* transcription in response to bacterial deprivation is unaffected by *daf-9* mutation

As previously described, *pha-4*/FOXA is required for DR-mediated lifespan extension, and *pha-4* mRNA levels are increased in *eat-2(ad1116)* mutants. Because *daf-9(rh50)* mutants fail to show long lifespans in response to DR conditions, we asked whether induction of *pha-4* is compromised in these animals. For this, *pha-4* transcript levels in animals under AL and BD conditions were quantified by real-time PCR. We found that *pha-4* transcript levels were induced similarly in *daf-9(rh50)* mutants and wildtype animals under both BD and AL feeding conditions (Fig. III-1). These data suggest that *pha-4* induction by DR is not affected by steroid signaling.



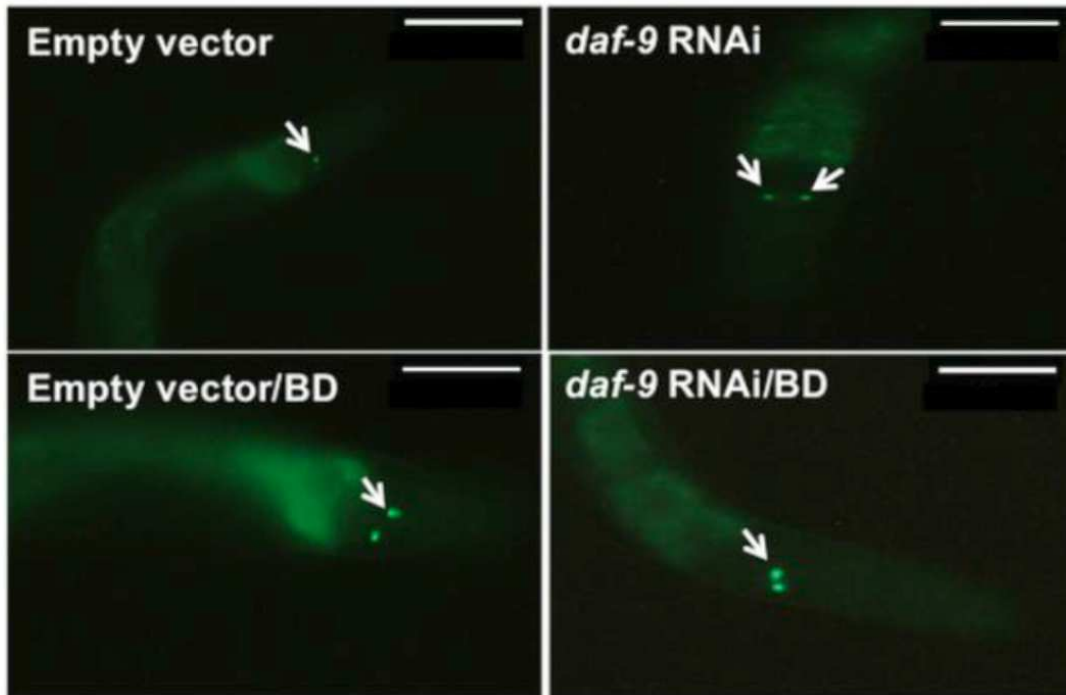
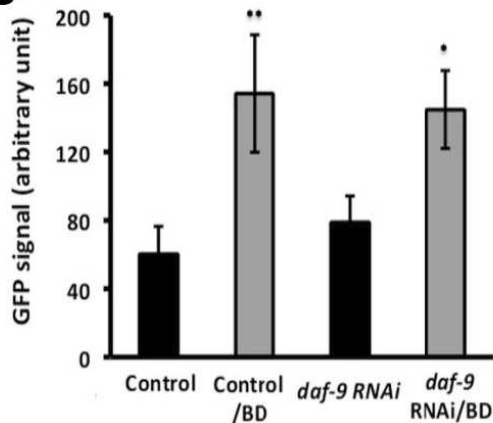
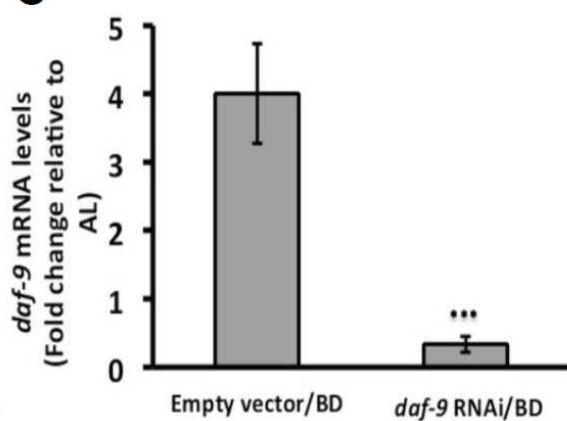
**Figure III-1: DR induces *pha-4* mRNA levels to similar extents in wild-type and *daf-9(rh50)* mutant animals:** Bacterial deprivation increased the transcript levels of the forkhead transcription factor PHA-4/FOXA in both wild-type animals (2.7-fold  $\pm$  0.64,  $P < 0.001$ ,  $n = 5$  independent biological samples for AL and BD conditions each) and *daf-9(rh50)* mutants (2.5-fold  $\pm$  0.58,  $P < 0.008$ ,  $n = 6$  independent biological samples for AL and BD conditions each).  $P$  values were calculated using the two-tailed Student's t-test. Error bars, SD.

### **III-2.2 *skn-1b*::GFP levels are not altered in *daf-9* mutants subjected to bacterial deprivation**

We next examined induction of the b isoform of SKN-1/NRF2 in ASI neurons, which is also necessary for DR-mediated longevity in *C. elegans*. For this, we expressed GFP under the control of the *skn-1b* promoter and monitored induction by fluorescence microscopy and quantification of GFP signal intensity. *daf-9* expression in these animals was suppressed by RNAi. As we had observed with *pha-4* induction, we found that lowering *daf-9* mRNA levels had no effect on GFP induction in nutrient-deprived *skn-1b*::GFP animals (Fig. III-2). As a control, we confirmed RNAi efficiency by measuring *daf-9* mRNA levels in animals fed with bacteria expressing empty vector or *daf-9*-targeting dsRNA (Fig. III-2C). Taken together, these data suggest that DR induction of *skn-1b* is not affected by steroid signaling.

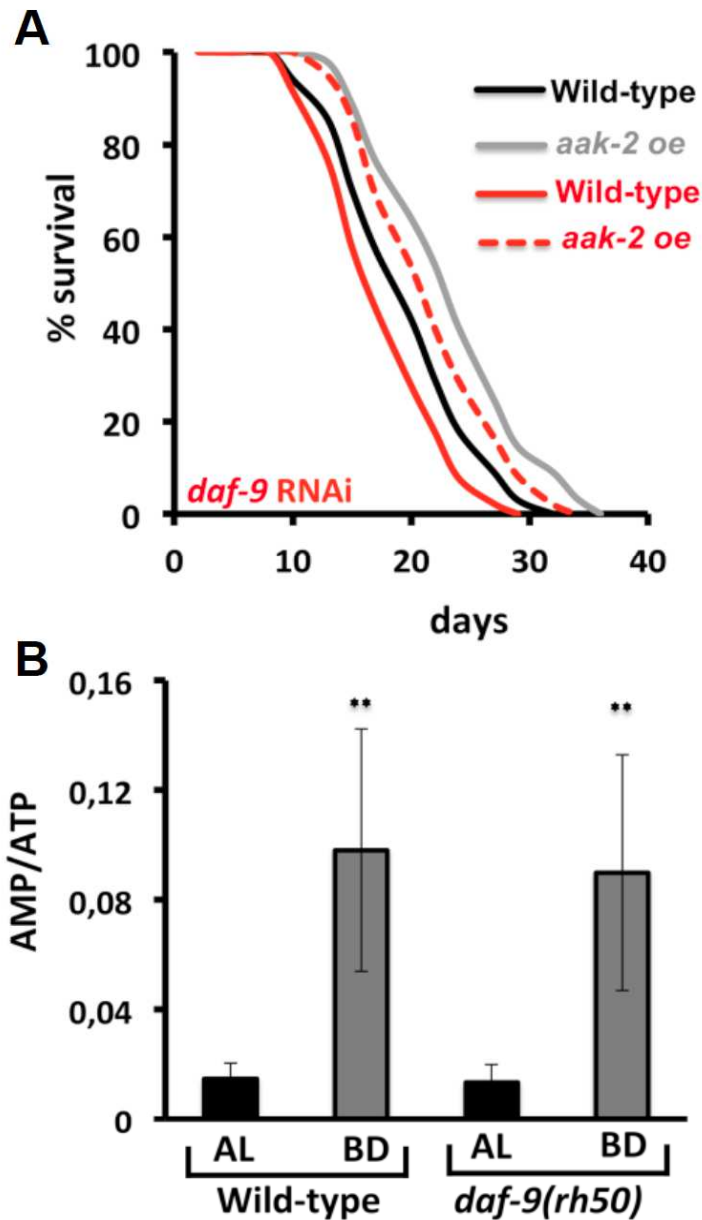
### **III-2.3 *aak-2*/AMPK activation in response to bacterial deprivation is not affected in *daf-9(rh50)* mutants**

The energy sensor *aak-2*/AMPK is required for lifespan extension in response to DR (Greer, 2007) and overexpression results in significant lifespan extension (Mair, 2011). We asked whether *aak-2*/AMPK activation is altered in *daf-9(rh50)* mutants under AL or BD conditions. First, we measured the ratio of AMP to ATP in wildtype and *daf-9(rh50)* animals, and found no significant differences in fed or starved animals of either genotype (Fig. III-3B). Next, we asked whether induction of lifespan extension by *aak-2*/AMPK overexpression is affected by RNAi-mediated downregulation of *daf-9*. We found that *daf-9* downregulation reduced the lifespan of wildtype animals and *aak-2*/AMPK overexpression mutants to similar extents (Fig. III-3A, Table 2).

**A****B****C**

**Figure III-2: *daf-9* RNAi does not affect the induction of *skn-1b::GFP* by starvation:**

(A) *skn-1b::GFP* is expressed in the ASI neurons of *C. elegans* and is induced normally by DR in worms subjected to *daf-9* RNAi. (B) Quantification of GFP signal. GFP expression was induced 2.2-fold by bacterial deprivation in animals expressing control vector (n = 10 and 9 for AL and BD conditions respectively) and 2.0-fold in animals expressing *daf-9* dsRNA (n = 14 and 11 for AL and BD conditions respectively). (C) RNAi reduced *daf-9* mRNA levels by 77%, and levels remained low after 24 h of starvation, the time at which *skn-1b::GFP* expression was measured. Arrows indicate foci of GFP. Scale bar 100  $\mu$ m. Error bars represent SD.



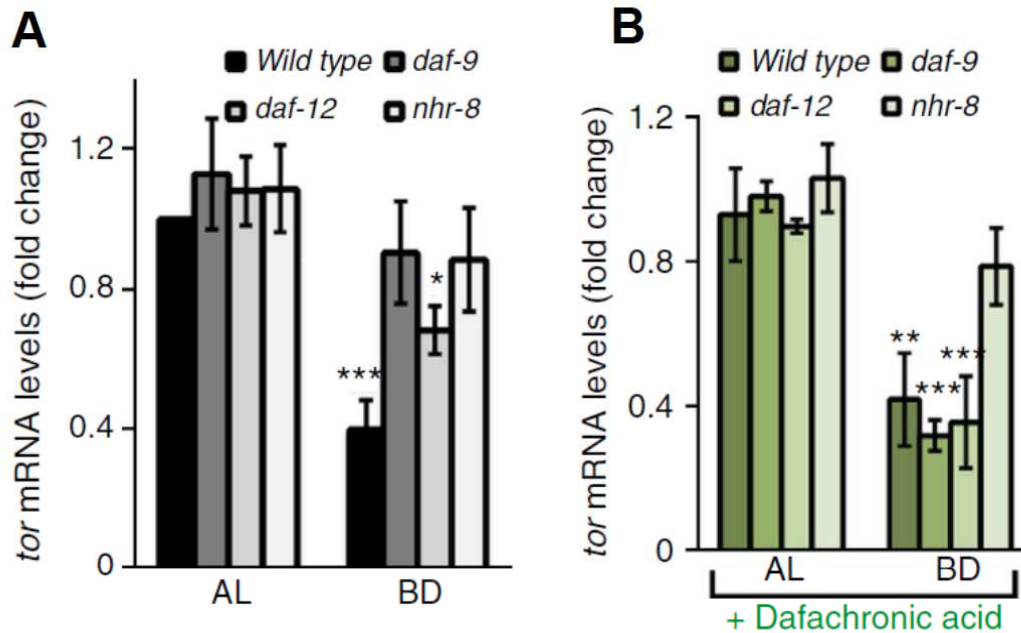
**Figure III-3: *daf-9* RNAi does not affect *aak-2*/AMPK mediated lifespan extension:** (A) The overexpression of *aak-2*/AMPK significantly extends lifespan in *C. elegans* (in black; mean lifespan increase of 19%,  $P < 0.0001$ ) and this is not affected by the downregulation of *daf-9* by RNAi (in red; mean lifespan increase of 22%,  $P < 0.0001$ ). (B) In wild type and *daf-9(rh50)* mutants, the AMP/ATP ratio is induced 7-fold and 6.8-fold by nutrient deprivation, respectively.  $P < 0.0001$ .  $n = 3$  independent biological samples.  $P$  values were calculated using the log-rank (Mantel-Cox) test for survival analysis and, the two-tailed Student's t-test was performed for AMP/ATP fold change. Error bars, SD.

### **III-2.4 *let-363*/mTOR transcript levels are not reduced in *daf-9(rh50)* and *nhr-8(ok186)* mutants subjected to bacterial deprivation**

Downregulation of the TOR gene (*let-363* in *C. elegans*) through genetic ablation (Powers, 2006; Vellai, 2003, Kapahi, 2004), RNAi (Vellai, 2003) or chemical inactivation (Harrison, 2009) has been shown to decrease TOR signaling and extend the lifespans of yeast (Powers, 2006), flies (Kapahi, 2004), worms (Vellai, 2003), and mice (Harrison, 2009; Selman, 2009). Because TOR is a nutrient-sensing kinase, we considered that it may play a role in promoting the longevity response to DR. We first quantified the relative levels of *let-363*/mTOR transcripts in wildtype, *daf-9(rh50)*, and *nhr-8(ok186)* animals under food-deprived and fed conditions. We found that nutrient deprivation did indeed markedly decrease *let-363*/mTOR mRNA levels in wildtype animals (Fig. III-4A) but not in either *daf-9(rh50)* mutants or *nhr-8(ok186)* mutants (Fig. III-4A). We also quantified *let-363*/mTOR mRNA levels in *daf-12(rh61rh411)* mutant animals. We found that *let-363*/mTOR transcript levels were modestly reduced in food-deprived compared with fed *daf-12(rh61rh411)* mutants (Fig. III-4A).

Having shown that BD did not reduce *let-363*/mTOR transcript levels in *daf-9(rh50)* and *nhr-8(ok186)* mutant animals, we next investigated the effects of supplementing with  $\Delta^7$ -DA (100 nM) on *let-363*/mTOR transcription. Consistent with the results of the lifespan analyses,  $\Delta^7$ -DA had no additional effects on *let-363*/mTOR mRNA levels in wildtype animals subjected to BD (Fig. III-4B). However,  $\Delta^7$ -DA did reduce *let-363*/mTOR transcripts in nutrient-deprived *daf-9(rh50)* and *daf-12(rh61rh411)* mutants to the levels observed in wildtype animals (Fig. III-4B). In contrast,  $\Delta^7$ -DA had no effect on *let-363*/mTOR levels in *nhr-8(ok186)* mutants (Fig. III-4B). These results suggest that  $\Delta^7$ -DA may regulate *let-363*/mTOR mRNA levels through NHR-8/NHR.





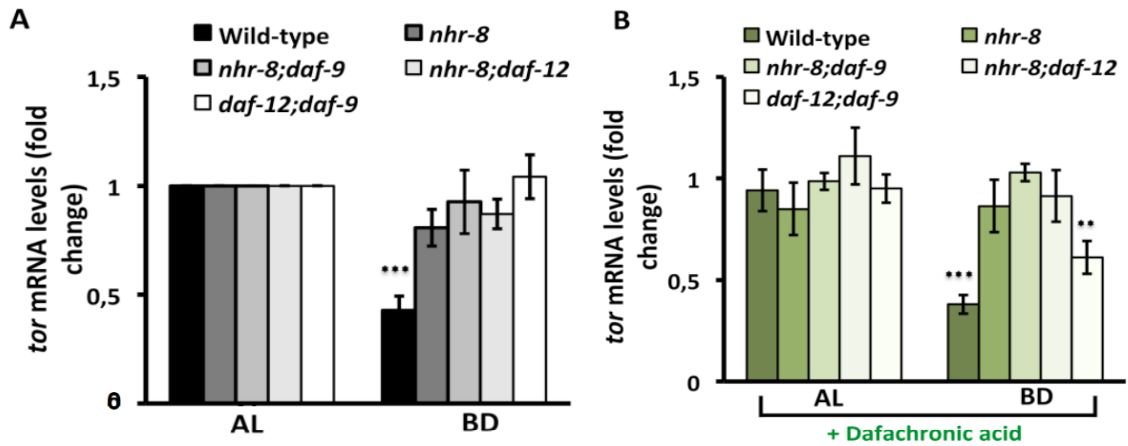
**Figure III-4: *let-363*/mTOR responds to nutrient availability in a steroid hormone-dependent manner: (A) *let-363* mRNA levels are reduced upon nutrient deprivation in wild-type worms (60%,  $P < 0.001$ ,  $n = 6$  independent biological samples) but not in *daf-9(rh50)* or *nhr-8(ok186)* mutant animals ( $P = 0.362$  and  $P = 0.068$ , respectively,  $n = 6$  independent biological samples for each). Nutrient deprivation has an intermediate effect on *let-363*/mTOR mRNA levels in *daf-12(rh61rh411)* mutants (37.25%,  $P < 0.01$ ,  $n = 6$  independent biological samples). (B) Supplementation with 100nM  $\Delta^7$ -DA decreases *let-363* mRNA levels by 50%, 66%, and 54% in wild-type, *daf-9(rh50)* mutant, and *daf-12(rh61rh411)* mutant animals, respectively, but had no effect on *nhr-8(ok186)* mutants ( $P < 0.054$ ,  $n = 6$  independent biological samples).**

### **III-2.5 Dafachronic acid reduces *let-363*/mTOR transcript levels and extends the lifespan of *daf-9(rh50);daf-12(rh61rh411)* mutants but not *daf-9(rh50);nhr-8(ok186)* double mutants**

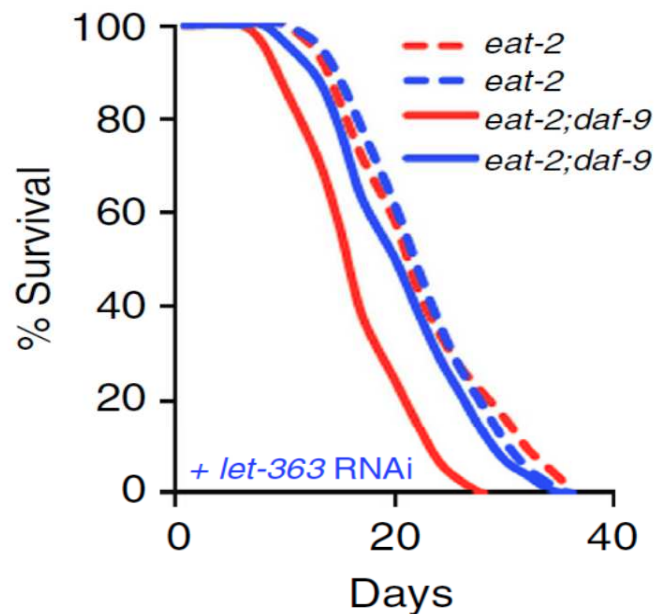
To determine whether steroid signaling-mediated DR-induced lifespan extension requires NHR-8, we quantified *let-363*/mTOR transcript levels in *daf-9(rh50);daf-12(rh61rh411)* and *daf-9(rh50);nhr-8(ok186)* mutant animals.. We found that,  $\Delta^7$ -DA supplementation in *daf-9(rh50);daf-12(rh61rh411)* double mutants subjected to DR exhibited low *let-363*/mTOR levels (Fig III-5A, B), whereas *daf-9(rh50);nhr-8(ok186)* double mutants harboured high *let-363*/mTOR mRNA levels (Fig III-5A, B). These data are consistent with the lifespan results (see section II-11), wherein,  $\Delta^7$ -DA supplementation increased the lifespan in *daf-9(rh50);daf-12(rh61rh411)* double mutant animals that were food deprived in comparison to fed animals (Fig II-11A, Table 2), but failed to do so in *daf-9(rh50);nhr-8(ok186)* and *daf-9(rh50);nhr-8(ok186)* double mutant animals (Fig 11B, Table 2).

### **III-2.6 Downregulation of *let-363*/ mTOR is sufficient to restore the dietary restriction longevity response**

The results of our *let-363*/mTOR transcriptional analysis raised the possibility that the high *let-363*/TOR mRNA levels in *daf-9* and *nhr-8* mutants prevent their response to DR. To test this hypothesis, we determined whether reducing *let-363*/mTOR expression would extend the lifespan of *eat-2(ad1116);daf-9(rh50)* double mutants, which are normally unresponsive to DR. Indeed, *let-363* RNAi increased the mean lifespan of the double mutants to that of the *eat-2* single mutants (Fig. III-6, Table 2). These data suggest that high *let-363*/mTOR levels prevented DR-mediated lifespan extension in these animals. Collectively, these results suggest that TOR acts downstream of steroid signaling pathway to mediate the DR-induced lifespan extension in *C. elegans*.



**Figure III-5: *let-363/mTOR* mRNA levels in *daf-9(rh50);nhr-8(ok186)* and *daf-12(rh61rh411);daf-9(rh50)* double mutants: (A), Bacterial deprivation reduced *let-363* transcript levels in wild-type animals (60%,  $P < 0.0001$ ,  $n = 6$  independent biological samples for each condition) but not in *nhr-8(ok186)* single mutants ( $P = 0.065$ ,  $n = 5$  independent biological samples), *daf-9(rh50);nhr-8(ok186)* double mutants ( $P = 0.201$ ,  $n = 6$  independent biological samples), or *daf-12(rh61rh411);daf-9(rh50)* double mutants ( $P = 0.72$ ,  $n = 6$  independent biological samples). (B), Addition of DA did not alter *let-363/mTOR* mRNA levels in nutrient-deprived *nhr-8(ok186)* or *daf-9(rh50);nhr-8(ok186)* double mutants ( $P = 0.492$ , and  $P = 0.685$ , respectively;  $n = 5$  independent biological samples for each condition and genotype). However, DA significantly reduced *let-363* mRNA levels in nutrient-deprived *daf-12(rh61rh411);daf-9(rh50)* double mutants ( $P < 0.01$ ,  $n = 6$  independent biological samples).  $P$  values were calculated using the two-tailed Student's t-test. Error bars, SD.**



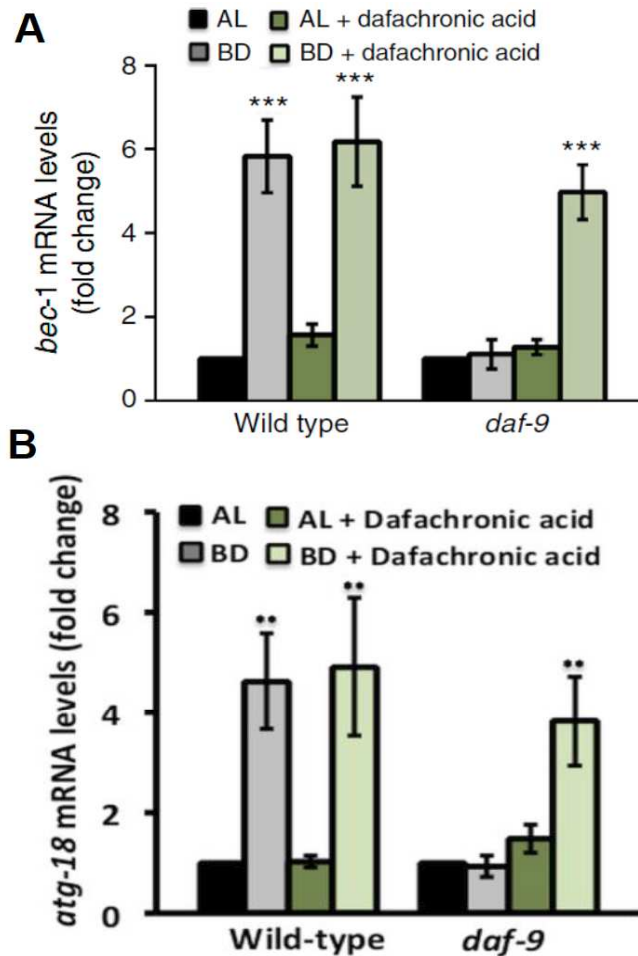
**Figure III-6: Downregulation of *let-363/mTOR* is sufficient to restore the DR effect: RNAi mediated downregulation of *let-363* extends the lifespan of *eat-2(ad1116);daf-9(rh50)* double mutants (29%,  $P < 0.001$ ) to the levels of *eat-2(ad1116)* single mutants but fails to extend the lifespan of *eat-2(ad1116)* single mutants.  $P$  values for mRNA quantification and lifespan analyses were calculated using the two-tailed Student's t-test and log-rank (Mantel–Cox) test, respectively.**

### III-2.7 TOR signaling is perturbed in *daf-9(rh50)* and *nhr-8(ok186)* mutants

As previously mentioned, TOR activity has been shown to regulate many metabolic and physiological processes such as protein synthesis, autophagy, and cell growth (Hands, 2009; Blagosklonny, 2009). Having shown that *let-363/mTOR* transcription is altered in *daf-9(rh50)* and *nhr-8(ok186)* mutant animals subjected to DR, we next asked whether altered *let-363/TOR* expression is sufficient to perturb TOR signaling in *daf-9(rh50)* mutant animals. For this, we measured transcription of the autophagy genes *bec-1* and *atg-18*, which are induced by DR in a TOR signaling-dependent manner. Low levels of TOR generally induce the autophagy in animals. We found that *bec-1* and *atg-18* mRNA levels were enhanced by nutrient deprivation in wildtype animals but not in *daf-9(rh50)* mutants, consistent with aberrant TOR signaling in these animals (Fig. III-7A, B). Similar to the *let-363/mTOR* expression data, addition of exogenous  $\Delta^7$ -DA was sufficient to restore wildtype-like levels of *bec-1* and *atg-18* in *daf-9(rh50)* mutants (Fig. III-7A, B). We also analyzed animals expressing a GFP-tagged version of the LC3 ortholog, LGG-1, and confirmed that *daf-9* RNAi impairs autophagosome formation in these animals under DR conditions (Fig. III-8A).

Another important metabolic process controlled by TOR signaling is protein synthesis. TOR positively regulates translation and hence, low TOR activity reduces protein synthesis. TOR is known to regulate the expression of the DR-response gene (*drr-2*), which encodes an ortholog of human eukaryotic translation initiation factor 4H (eIF4H) that mediates the initiation step of mRNA translation. Genetic analyses suggest that eIF4H/DRR-2 functions downstream of TOR but in parallel to the S6K/PHA-4 pathway to mediate the lifespan effects of DR (Hensen, 2005 and Ching, 2010). We found that *drr-2* mRNA levels were

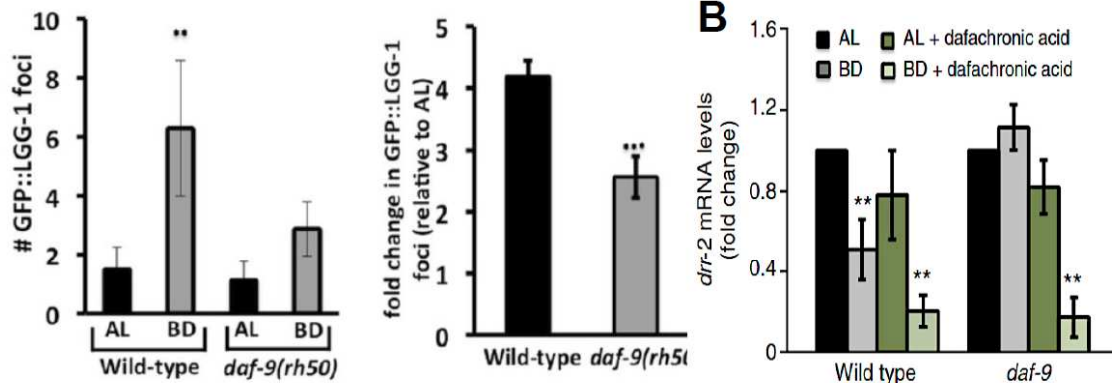
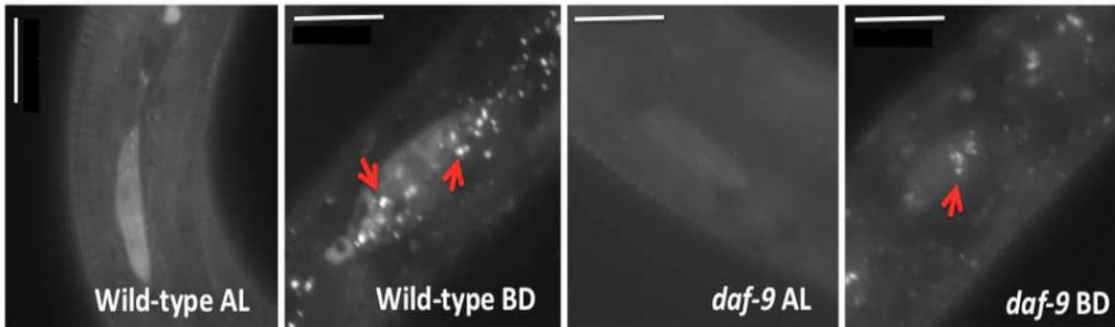
decreased by nutrient deprivation in wildtype animals but not in *daf-9(rh50)* and *nhr-8(ok186)* mutants (Fig. III-8B). Consistent with the *bec-1* and *atg-18* data, addition of  $\Delta^7$ -DA reduced *drr-2* mRNA levels in *daf-9(rh50)* but not in *nhr-8(ok186)* mutant animals (Fig. XX). Collectively, these data suggest that autophagy and protein synthesis are both affected.



**Figure III-7: DR induces transcript levels of autophagy genes in DA dependent manner: (A)** Nutrient deprivation induces TOR signalling, as shown by increased transcription of the autophagy gene *bec-1*, in wild-type animals (600%,  $P < 0.001$ ,  $n = 5$  independent biological samples) but not in *daf-9(rh50)* mutants (6%,  $P < 0.474$ ,  $n = 6$  independent biological samples). Supplementation with 100nM  $\Delta^7$ -DA restores wild type-like induction of *bec-1* transcription in *daf-9(rh50)* mutants (500%,  $P < 0.001$ ,  $n = 6$  independent biological samples). **(B)** Bacterial deprivation induced the transcript levels of autophagy gene *atg-18* in wild-type animals (400%,  $P < 0.0001$ ,  $n = 6$  independent biological samples for each condition) but not in *daf-9(rh50)* mutants (-7%,  $P < 0.802$ ,  $n = 6$  independent biological samples for each condition). Supplementing 100 nM  $\Delta^7$ -DA restores wild-type-like induction of *atg-18* transcription in *daf-9(rh50)* mutants (380%,  $P < 0.0001$ ,  $n = 5$  independent biological samples for each condition). Scale bars, 60  $\mu\text{m}$ . Error bars, SD.



**A**



**Figure III-8: Steroid signalling controls TOR signalling: (A)** *lgg-1::GFP* forms punctate in the intestine of wild type L3 larvae upon nutrient deprivation (left upper panels). This increase is hampered in *daf-9(rh50)* mutants (left lower panels). A quantification of the *GFP::LGG-1* signal quantified on the right upper panel (n = 8, 7, 8, and 8 for wild-type AL, wild-type BD, *daf-9(rh50)* AL and *daf-9(rh50)* BD conditions respectively) . The induction by nutrient deprivation is of 4.2-fold in wild type larvae while it is of 2.5-fold in *daf-9(rh50)* mutants ( $P < 0.005$ , two-tailed Student's t-test). This is depicted in a graph on the right lower panel. **(B)** Nutrient deprivation reduces expression of the elongation factor *drr-2* in wild-type animals (50%,  $P < 0.01$ , n=5 independent biological samples) but not in *daf-9(rh50)* mutants ( $P = 0.386$ , n.6 independent biological samples). Addition of 100nM  $\Delta^7$ -DA restores wild type-like *drr-2* transcript levels in *daf-9(rh50)* mutants (80%,  $P < 0.001$ , n=6 independent biological samples). P values were calculated using the two-tailed Student's t-test. Error bars represent s.d.

### III-3 Conclusions and future perspectives

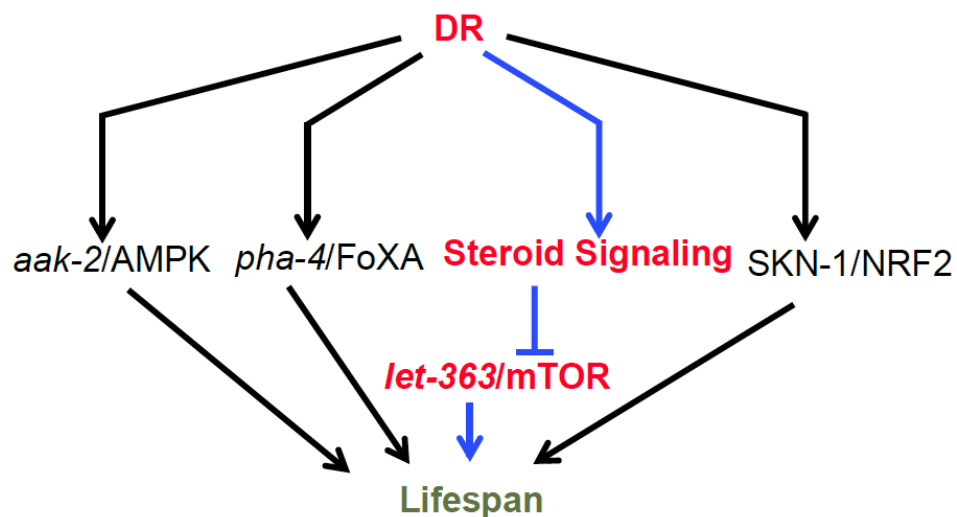
Collectively, the results presented in this chapter strongly suggest that the DR responses of *pha-4*, *skn-1b*, and *aak-2* mutants are not influenced by steroid signaling. Since these genes have each been shown to mediate DR-induced lifespan extension, our data raise the possibility that this response can also occur independently of steroid signaling. We therefore searched for other genes that might link lifespan regulation with steroid signaling. The primary candidate was *let-363/mTOR*, a major nutrient sensor. Our findings suggest that high *let-363/mTOR* levels prevent DR-mediated lifespan extension in *C. elegans*.

We showed that *let-363/mTOR* expression is regulated by both nutrients and  $\Delta 7$ -DA (or a derived metabolite), suggesting that integration of the nutritional and steroidal signals occurs via NHR-8/NHR. Whether NHR-8/NHR controls *let-363/mTOR* expression directly or indirectly remains to be determined. Our data also demonstrate that  $\Delta 7$ -DA (or a derived metabolite) acts upstream of the nuclear receptor NHR-8/NHR, but not DAF-12, to regulate *let-363/mTOR* expression and downstream processes. TOR is therefore capable of regulating longevity by integrating both nutrient-derived and steroidal signals in *C. elegans* at the transcriptional level. Taken together, the results presented here demonstrate that steroid signaling is activated in low nutrient conditions and is required for DR-induced lifespan extension through TOR signaling.

Our results demonstrate that two crucial biological processes regulated by TOR signaling; namely, autophagy and protein synthesis, are altered in *daf-9(rh50)* and *nhr-8(ok186)* mutant animals. We do not rule out the possibility that other TOR signaling-dependent processes are also affected in these animals. It would be interesting to see which of these processes could rescue the lifespan phenotype in *daf-9(rh50)* and *nhr-8(ok186)*

mutants. Also, it would be interesting to see how steroid signalling regulates TOR activity in response to low nutrient conditions. It is shown that TOR activity is regulated at posttranslational levels such as phosphorylation. TOR phosphorylates S6K (S6 kinase), which controls global translation process. It would be interesting to measure S6K phosphorylation levels in *daf-9(rh50)* and *nhr-8(ok186)* animals.

It would also be interesting to investigate how *nhr-8/NHR* regulates TOR activity in response to DR. Though, our results demonstrate that *let-363/mTOR* mRNA levels are reduced in animals under food deprivation in a *daf-9* and *nhr-8* dependent manner, the actual mechanism by which this happens is yet to be investigated. Chromatin Immunoprecipitation experiments may yield useful data to find whether NHR-8 directly regulate *let-363* expression. It would also be possible that NHR-8 may heterodimerize with other nuclear receptor which may then regulate TOR signalling to mediate lifespan under DR conditions,



**Figure III-B:** Known mediators of DR response (*pha-4/FoXA*, *aak-2/AMPK*, and *SKN-1/NRF2*) are not altered in animals lacking steroid signalling. However, steroid signalling regulated TOR signalling to mediated the DR effect to induce the lifespan in *C. elegans*.



## Chapter IV

# Germline Plasticity is Linked to Lifespan Extension

## **Chapter IV Germline Plasticity is Linked to Lifespan Extension**

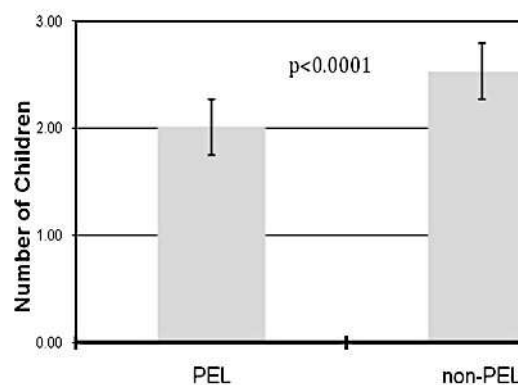
### **IV-1 Introduction**

As discussed in Chapter I, a number of leading theories of aging suggest the existence of a trade-off between longevity and reproduction; these include the antagonistic pleiotropy theory (Williams, 1957), the disposable soma theory (Kirkwood, 1977), and more recently, the reproductive–cell cycle theory (Bowen and Atwood, 2004, 2010). Although there is an abundance of data linking longevity with reduced fertility in lower life forms, the data in humans is conflicting. The disposable soma theory of aging argues that investment in reproduction deprives organisms of resources required for self-maintenance, thus reducing longevity. This effect can easily be explained in females that carry the direct physical burdens of pregnancy, childbirth, and breast-feeding. In most animals, reproduction trades off with maintenance and survival so that individuals with reduced reproduction live longer than those with higher reproductive effort (Bell and Koufopanou, 1986; Partridge, 2005).

The reproductive–cell cycle theory posits that hormones that regulate reproduction act in an antagonistic pleiotropic manner to control aging via cell cycle signaling. Thus, growth and development are promoted early in life to ensure reproduction, but later in life, in a futile attempt to maintain reproduction, become dysregulated and drive senescence (Bowen and Atwood, 2004, 2010). Since reproduction is the most important function of an organism from the perspective of the survival of the species, if reproductive–cell cycle signaling factors determine the rates of growth, development, reproduction, and senescence, then by definition they determine the rate of aging and thus lifespan. (Bowen and Atwood, 2004, 2010). Support for this theory is attributed to the fact that a genetic predisposition to hinder and/or delay some hormonal mechanism that might reduce reproductive success and at the

same time delay aging and mortality in women and men alike (Westendorp, 1998; Perls, 1997).

Several studies have suggested that genes involved in cholesterol metabolism may also be involved in bridging reproduction and longevity (Barzilai, 2001; Atzmon 2002). In 2011, a study on genetically and socially homogenous Ashkenazi Jewish centenarians (average age ~100 years) showed that individuals who achieve exceptional longevity have fewer children and tend to reproduce later in life than a contemporaneous population with normal lifespans (Fig. IV-A; Tabatabaie, 2011).



**Fig IV-A.** The number of children born to people with exceptional longevity (PEL) and a non-PEL control group. (Reproduced from Tabatabaie, 2011)

DR during adulthood is known to reduce or eliminate female reproduction while also extending the lifespan (Masoro, 2005; Merry, 1995). However, whether a reduction in reproduction is necessary to achieve lifespan extension remains an open question (Carey, 2008; Grandison, 2009). The combined effects of increased lifespan and reduced fecundity that typically result from DR have long been thought to reflect a re-allocation of resources from reproductive effort to somatic maintenance, perhaps as an adaptive strategy that maximizes the individual's chances of surviving periods of resource shortage (Shanley and

Kirkwood, 2000). Recent studies on the neriid fly *Telostylinus angusticollis* have found that the lifespan–reproduction trade-off is both context- and sex-dependent; DR extended the lifespans of both sexes by 65% and rendered females completely infertile, but the costs of DR on male fecundity were subtle and evident only in particular environmental conditions (Adler, 2013). Their study therefore suggests that reproductive capacity need not trade off with lifespan, but rather, suggests that optimization of reproduction vs. longevity may be environment- and sex-dependent (Adler, 2013).

The impact of germline ablation on lifespan was first demonstrated in *C. elegans* and was shown to be mediated by *daf-16* and *daf-12* (Hsin and Kenyon, 1999). Another component of the IIS pathway, *daf-18/PTEN*, is also required for lifespan extension due to germline ablation (Hsin and Kenyon, 1999; Larsen, 1995). Later, many factors unrelated to the IIS pathway were linked to germline-mediated lifespan extension, including *kri-1*, an ankyrin repeat protein orthologous to the human gene KRIT1, and *tcer-1*, a transcription elongation factor related to TCERG1. These factors promote lifespan extension in germline-less animals by interacting with *daf-16/FOXO* independently of canonical insulin signaling (Ghazi, 2009).

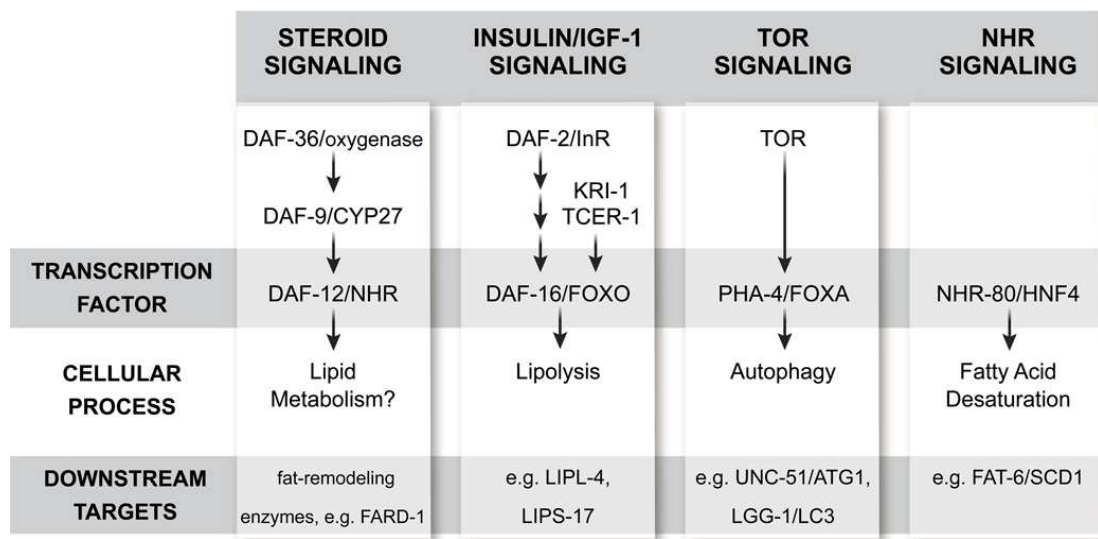
Steroid signaling is known to mediate lifespan extension in response to germline loss, at least in *C. elegans*. A study by Yamawaki (2010) suggested that DA might be produced in the somatic gonad, which could explain the requirement for these tissues in lifespan extension in response to germline ablation (Hsin and Kenyon, 1999). The requirement for the somatic gonad is diminished in animals carrying hypomorphic *daf-2/InR* alleles that display markedly reduced IIS (Hsin and Kenyon, 1999; Yamawaki, 2008). One possible explanation for this observation is that low levels of IIS might induce ectopic production of

longevity-promoting steroid hormones in tissues other than the somatic gonad.

In *C. elegans*, removal of the germline promotes longevity in part by modulating lipid metabolism via fatty acid desaturation, lipolysis, and autophagy. Interestingly, in a variety of organisms, increased lifespan is associated with reduced reproduction but markedly increased lipid storage and thus improved survival under starvation conditions (Gems, 1998; Judd, 2011; Rion and Kawecki, 2007; Tatar, 2001). Despite these observations, the mechanisms connecting reproduction, fat metabolism, and lifespan remain poorly understood. Recent evidence suggests that the three processes might be causally linked through a reproductive–endocrine signaling axis. In *C. elegans* and *Drosophila*, for example, ablation of the germline increases lifespan (Flatt, 2008; Hsin and Kenyon, 1999) and significantly alters lipid metabolism (O'Rourke, 2009; Parisi, 2010). Recent work has begun to elucidate the molecular mechanisms by which signals from the reproductive system regulate lipid metabolism and lifespan (Goudeau, 2011; Lapierre, 2011; McCormick, 2011; Wang, 2008).

Many investigators have focused on the possible involvement of the TOR signaling pathway as a link between longevity and reproductive capacity (Blagosklonny, 2010; Blagosklonny, 2010). In addition to its roles in nutrient sensing, cell growth, and proliferation (Weichhart, 2012), mTOR is also believed to play a role in central regulation of puberty. Acute activation of mTOR in pubertal female rats stimulates luteinizing hormone (LH) secretion, whereas blockade by rapamycin inhibits gonadotropic axis and delays puberty (Roa, 2009). Others have shown that inhibition of mTOR extends the lifespans of invertebrates (Hands, 2009) and mammals (Harrison, 2009). Therefore, reduced mTOR signaling might result in enhanced longevity at the expense of delayed puberty or limited fertility. Germline-less *C. elegans* have lower intrinsic TOR levels, and TOR inactivation

upregulates *lipl-4* expression in a *daf-16*/FOXO-dependent fashion (Lapierre, 2011). Future work examining TOR pathway components will shed light on the dual regulatory role of TOR signaling in germline-less animals. A simple model for various signaling pathways and their impact on fat metabolism is shown in the scheme below (Fig. IV-B: reproduced from Aguilaniu, 2013).



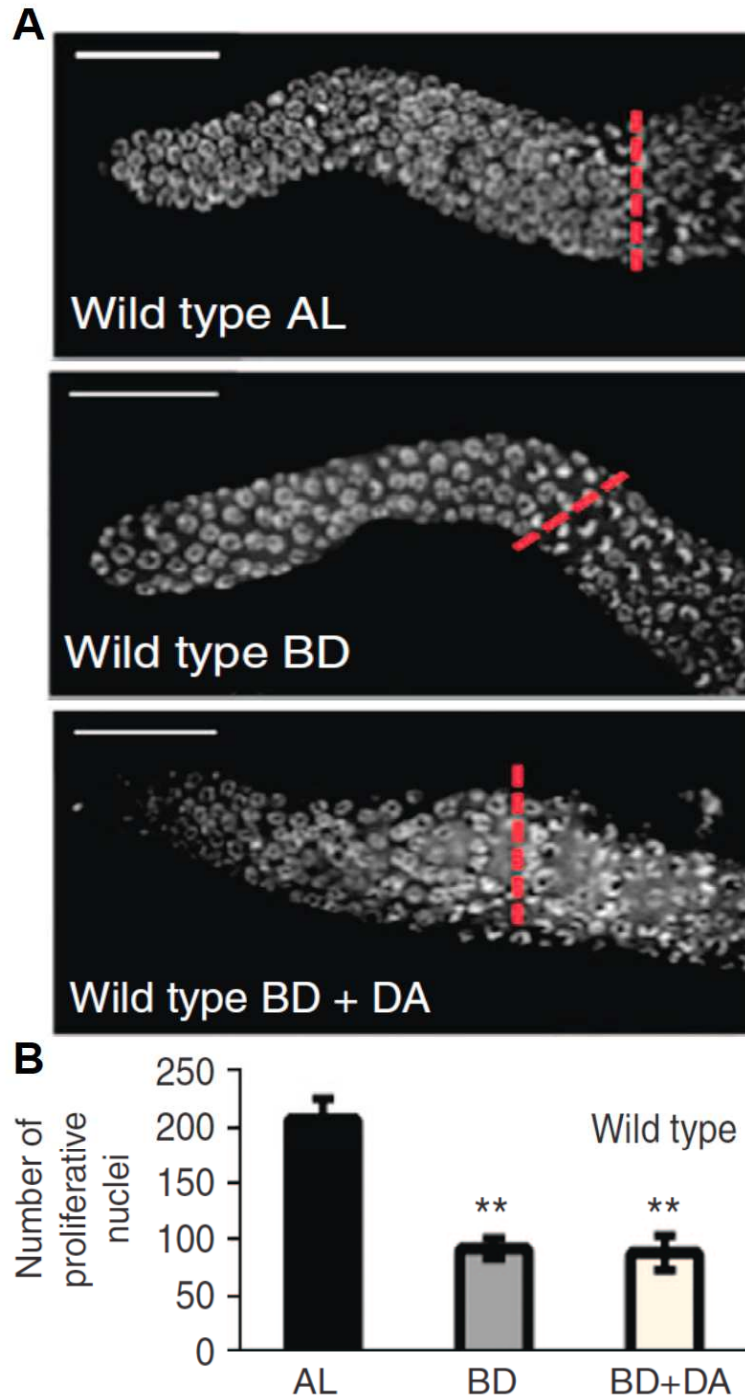
**Figure IV-B:** In *C. elegans* and *D. melanogaster*, removal of germline increases lifespan and alters fat metabolism. Lifespan extension through removal of the germline therefore provides insights into the mechanisms that link reproduction, fat metabolism, and lifespan. (Reproduced from Aguilaniu, 2013).

Recent work in *C. elegans* showed that TOR signaling controls germ cell replication in the proliferative zone of the gonad and entry of the cells into meiosis in response to nutrient availability (Korta, 2012). We therefore asked whether steroid signaling via the DAF-9/ $\Delta^7$ -DA/NHR-8 pathway might play a role in the response of the germline to DR. The main goal of the studies in this chapter was to understand how germline plasticity is affected in dietary-restricted *C. elegans* in the presence and absence of steroid signaling.

## IV-2 Results

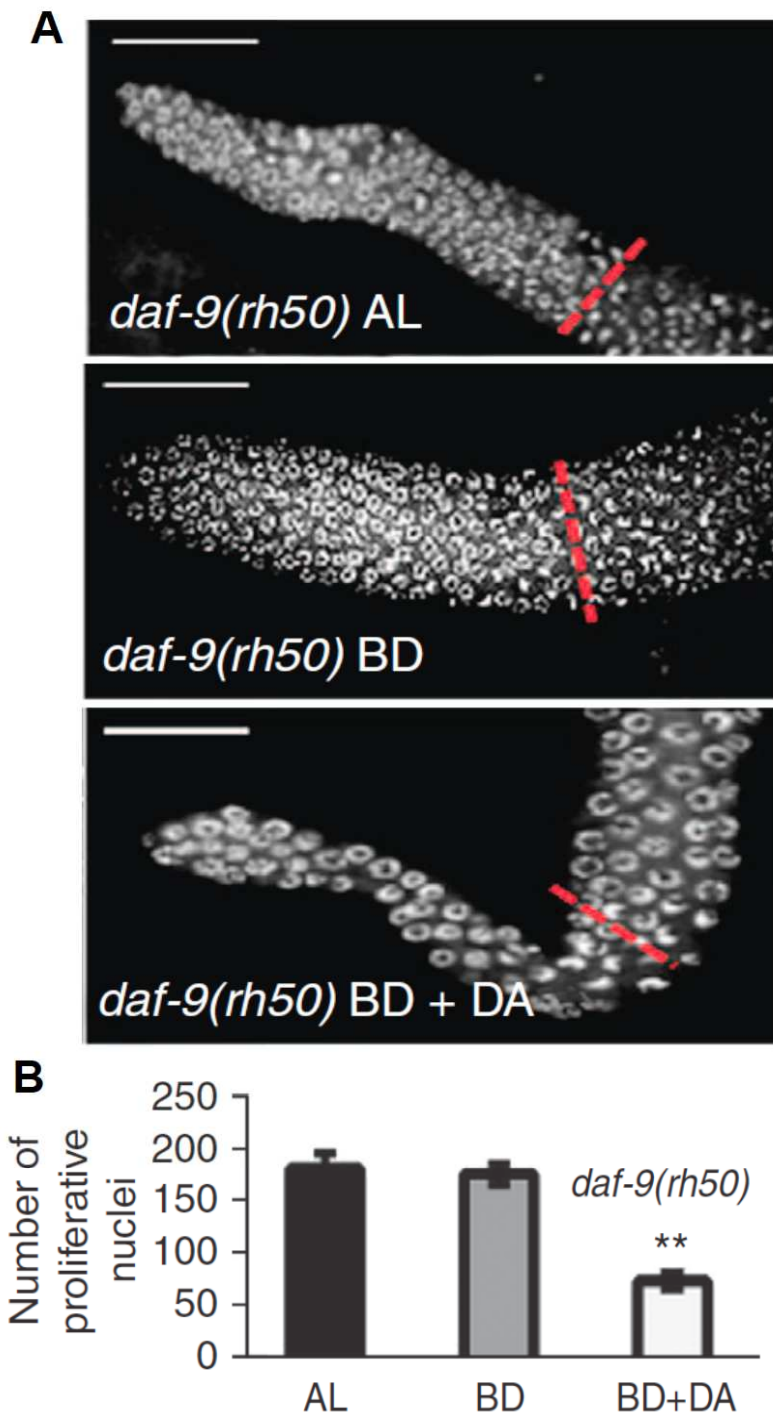
### IV-2.1 Bacterial deprivation reduces the number of proliferative nuclei in the germline of wildtype *C. elegans*

In chapters II and III, we demonstrated a requirement for active steroid signaling and steroid signaling-dependent control of TOR for DR-induced lifespan extension in *C. elegans*. TOR signaling has also been shown to control the proliferation and maturation of germ cells in the *C. elegans* gonad in response to environmental stimuli such as temperature and nutrition (Korta, 2012). We therefore asked whether altered steroid signaling affects germline plasticity in *C. elegans* subjected to BD. When we quantified the number of germ cells in the proliferative zone of the gonad, we observed that nutrient deprivation significantly decreased the number of nuclei in the germline of wildtype animals but had no effect in either *daf-9(rh50)* or *nhr-8(ok186)* mutants (Fig. IV-1–3). Next, we asked whether  $\Delta^7$ -DA supplementation could reduce the number of proliferative nuclei in the germline of starved *daf-9(rh50)* mutants. Indeed,  $\Delta^7$ -DA reduced the number of nuclei in *daf-9(rh50)* mutants, but not in *nhr-8(ok186)* mutants, to the levels seen in wildtype animals (Fig. IV-1–3). These results suggest that DR-induced germline plasticity requires steroid signaling.

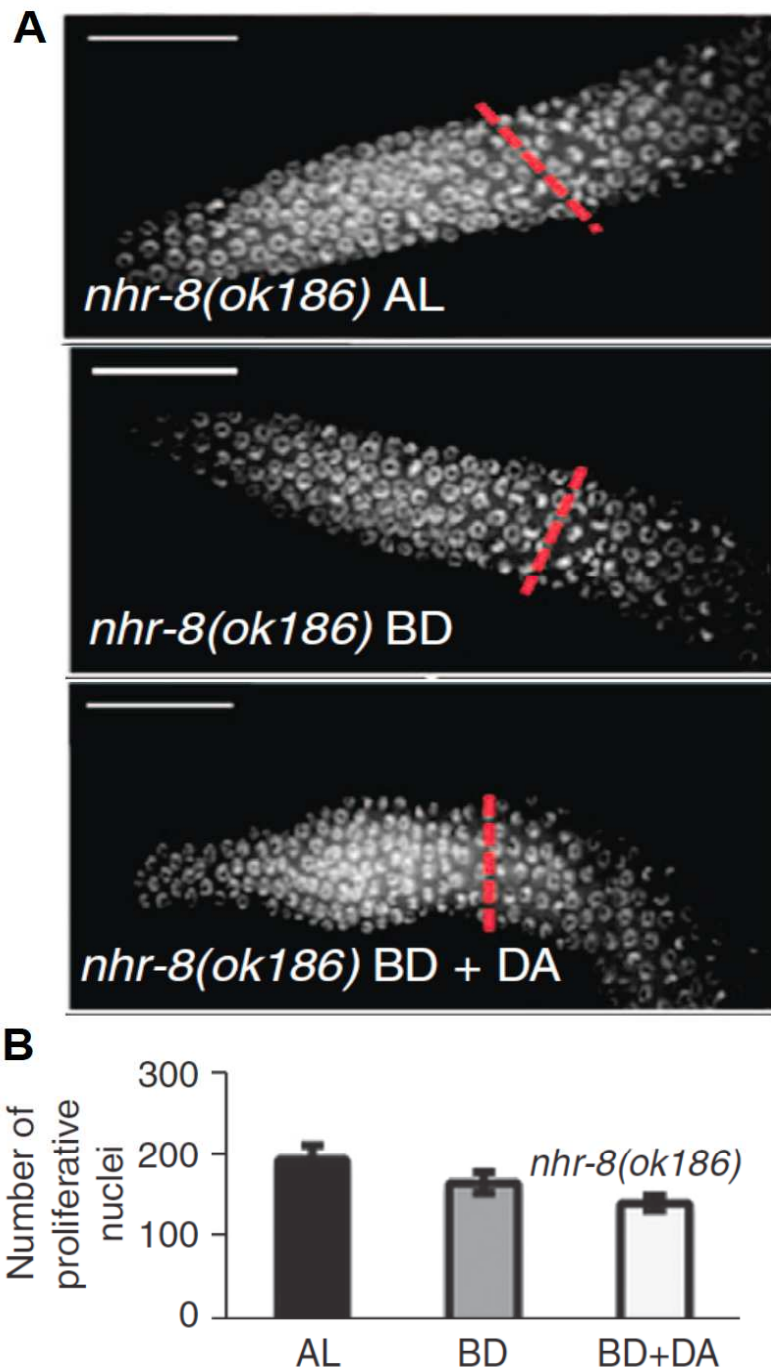


**Figure IV-1: Nutrient deprivation reduces germ cells number in wild-type: (A)** Imaging of the proliferative zone of the germline of wild-type animals fed ad libitum or subjected to bacterial deprivation in the presence or absence of DA. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). **(B)** Quantification of germ cells in the animals shown in A, indicating the DR reduced the germ cells numbers by 56% in wild-type animals (AL, n=11, BD, n=10,  $p < 0.0001$ ). Adding  $\Delta^7$ -DA did not further reduce the germ cells number in DR (BD+DA, n=9,  $P = 0.2010$ ). P values were calculated using the two-tailed Student's t-test. Scale bar, 40  $\mu$ m. Error bars represent s.e.m.





**Figure IV-2: Nutrient deprivation does not reduce germ cell number in *daf-9(rh50)* mutants:** (A) Imaging of the proliferative zone of the germline of *nhr-8(ok186)* animals fed ad libitum or subjected to bacterial deprivation in the presence or absence of DA. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). (B) Quantification of germ cells in the animals shown in A, indicating that in *nhr-8(ok186)* DR failed to reduce the germ cell numbers (AL, n=13, BD, n=12, p=0.192). Adding  $\Delta^7$ -DA did restore the germ cell number in DR (BD+DA, n=11, P=0.001) to wild-type levels. P values were calculated using the two-tailed Student's t-test. Scale bar, 40  $\mu$ m. Error bars represent s.e.m.

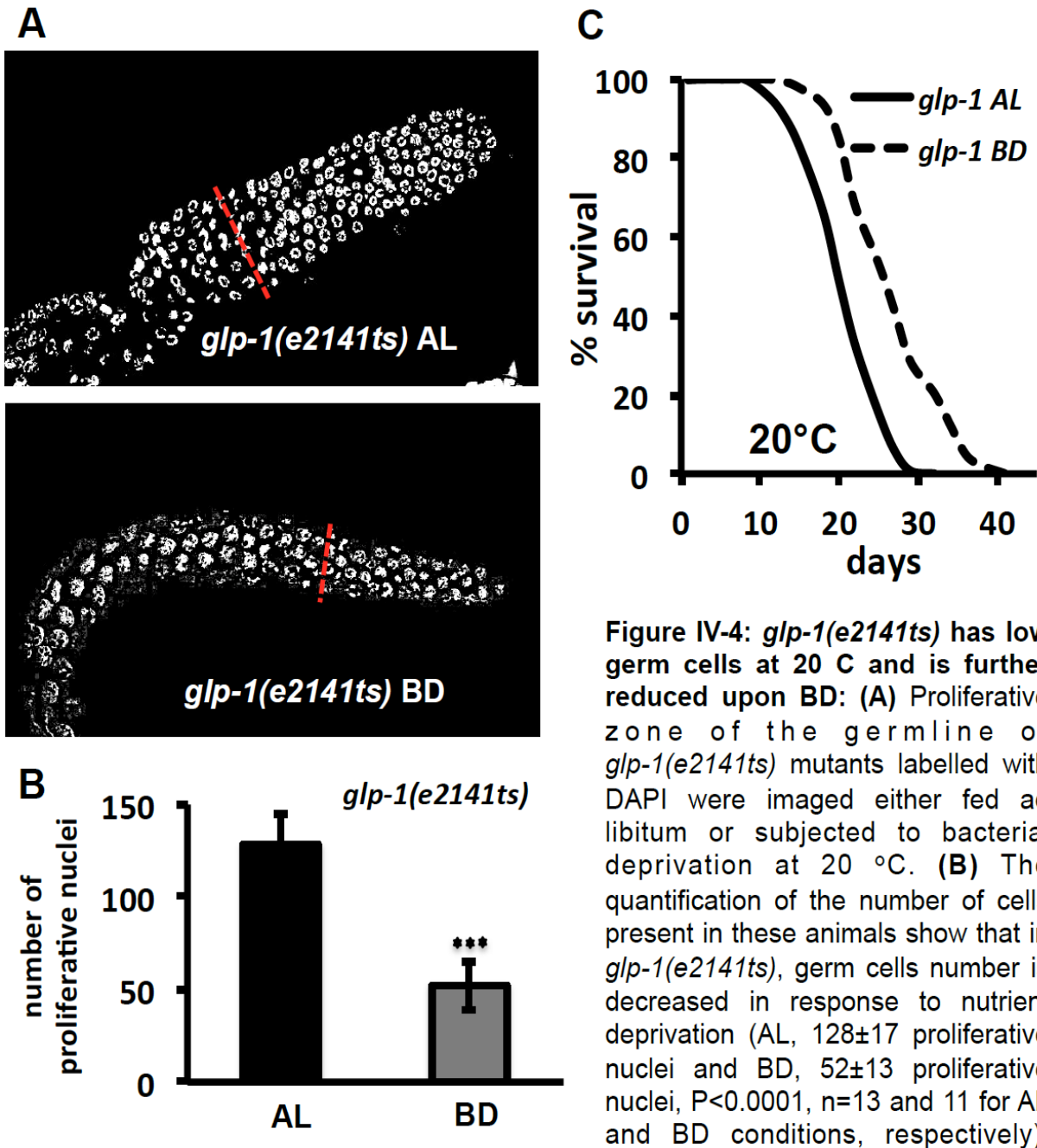


**Figure IV-3: Nutrient deprivation does not reduce germ cell number in *nhr-8(ok186)* mutants:** (A) Imaging of the proliferative zone of the germline of *nhr-8(ok186)* animals fed ad libitum or subjected to bacterial deprivation in the presence or absence of DA. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). (B) Quantification of germ cells in the animals shown in A, indicating that in *nhr-8(ok186)* DR failed to reduce the germ cell numbers (AL, n=9, BD, n=10, p=0.07). Adding  $\Delta^7$ -DA did not reduce the germ cell number in DR (BD+DA, n=9, P=0.058). P values were calculated using the two-tailed Student's t-test. Scale bar, 40  $\mu$ m. Error bars represent s.e.m.

## **IV-2.2 Reduction of germ cell numbers restores bacterial deprivation-induced lifespan extension in *daf-9(rh50)* and *nhr-8(ok186)* mutants**

To further probe the relationship between steroid signaling, germline plasticity, and lifespan, we examined worms carrying the *glp-1(e2141ts)* or *daf-7(e1372)* alleles, which alter germline stem cell proliferation through distinct pathways. The *glp-1(e2141ts)* allele completely arrests germline stem cell proliferation at 25°C but has only a partial effect at 20°C. At 25°C, germline signaling is not active and *glp-1(e2141ts)* mutant animals display a wildtype-like lifespan under both BD and AL feeding conditions (Fig. IV-4 and Table 2). Interestingly, when we combined either the *daf-9(rh50)* or *nhr-8(ok186)* alleles with the *glp-1(e2141ts)* allele we found that DR enhanced the lifespans of the double mutants normally (Fig. IV-5A, B; Fig. IV-6A, B; and Table 2).

The second method we used to reduce germ cell numbers was mutation of *daf-7*, which encodes TGF- $\beta$ . Downregulation of TGF- $\beta$  reduces the number of germ cells in the proliferative zone by promoting their differentiation, but this occurs in a *glp-1*-independent manner (Dalfo, 2012). Similar to the observations with the combined *glp-1(e2141ts)* mutants, we found that *daf-7(e1372);nhr-8(ok186)* double mutants did indeed have fewer nuclei in the distal part of the germline, and starvation further reduced this number (Fig. IV-7A, B). Interestingly, the *daf-7(e1372);nhr-8(ok186)* animals also showed an extended lifespan when subjected to DR (Fig. IV-7C and Table 2).



**Figure IV-4: *glp-1(e2141ts)* has low germ cells at 20 C and is further reduced upon BD: (A)** Proliferative zone of the germline of *glp-1(e2141ts)* mutants labelled with DAPI were imaged either fed ad libitum or subjected to bacterial deprivation at 20 °C. **(B)** The quantification of the number of cells present in these animals show that in *glp-1(e2141ts)*, germ cells number is decreased in response to nutrient deprivation (AL, 128±17 proliferative nuclei and BD, 52±13 proliferative nuclei,  $P < 0.0001$ ,  $n = 13$  and 11 for AL and BD conditions, respectively).

P values were calculated using the two-tailed Student's t-test. Scale bar, 40  $\mu$ m. Error bars represent s.e.m. **(C)** *glp-1(e2141ts)*-mutant animals at 20 C exhibit wild type-like mean lifespans under ad libitum feeding or BD conditions (mean lifespan: AL, 21±0.7 days and BD, 27±1 days,  $P < 0.0001$ ). P values were calculated using the log-rank (Mantel–Cox) test for lifespan. Lifespan values indicate mean±95% CI.

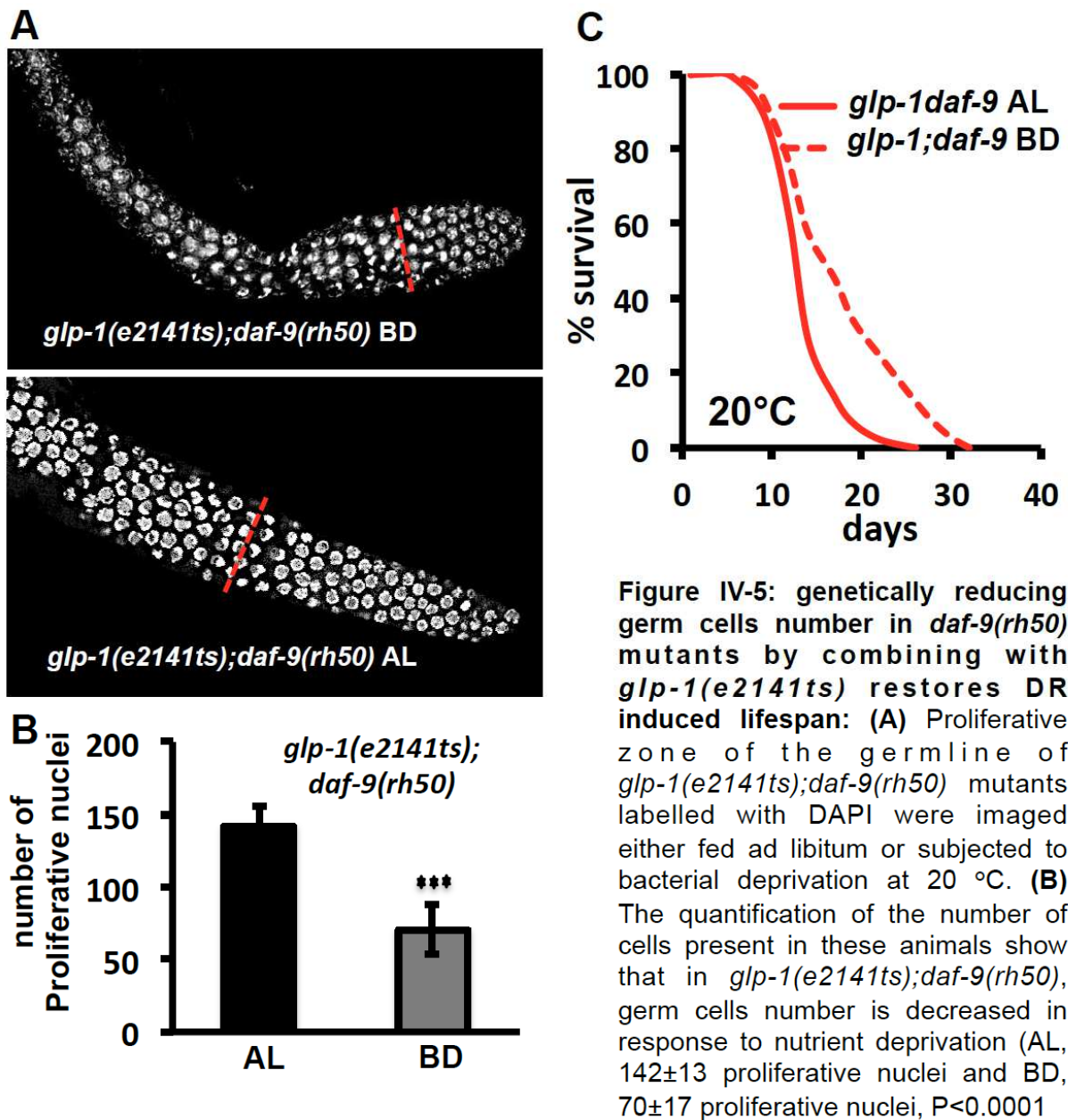


Figure IV-5: genetically reducing germ cells number in *daf-9(rh50)* mutants by combining with *glp-1(e2141ts)* restores DR induced lifespan: (A) Proliferative zone of the germline of *glp-1(e2141ts);daf-9(rh50)* mutants labelled with DAPI were imaged either fed ad libitum or subjected to bacterial deprivation at 20 °C. (B) The quantification of the number of cells present in these animals show that in *glp-1(e2141ts);daf-9(rh50)*, germ cells number is decreased in response to nutrient deprivation (AL, 142±13 proliferative nuclei and BD, 70±17 proliferative nuclei, P<0.0001

for *glp-1(e2141ts);daf-9(rh50)*, n=9 and 12 for AL and BD conditions, respectively). P values were calculated using the two-tailed Student's t-test. Scale bar, 40 mm. Error bars represent s.e.m. (C) DR-induced lifespan was restored by genetically mimicking the effects of DR on germline plasticity with the *glp-1(e2141ts)* allele at 20 °C in combination with *daf-9(rh50)* mutants. DR induced the lifespan in *glp-1(e2141ts);daf-9(rh50)* double mutants (mean lifespan: AL, 14±1 days and BD, 18±1.5 days, P<0001). P values were calculated using the log-rank (Mantel–Cox) test for lifespan. Lifespan values indicate mean±95% CI.



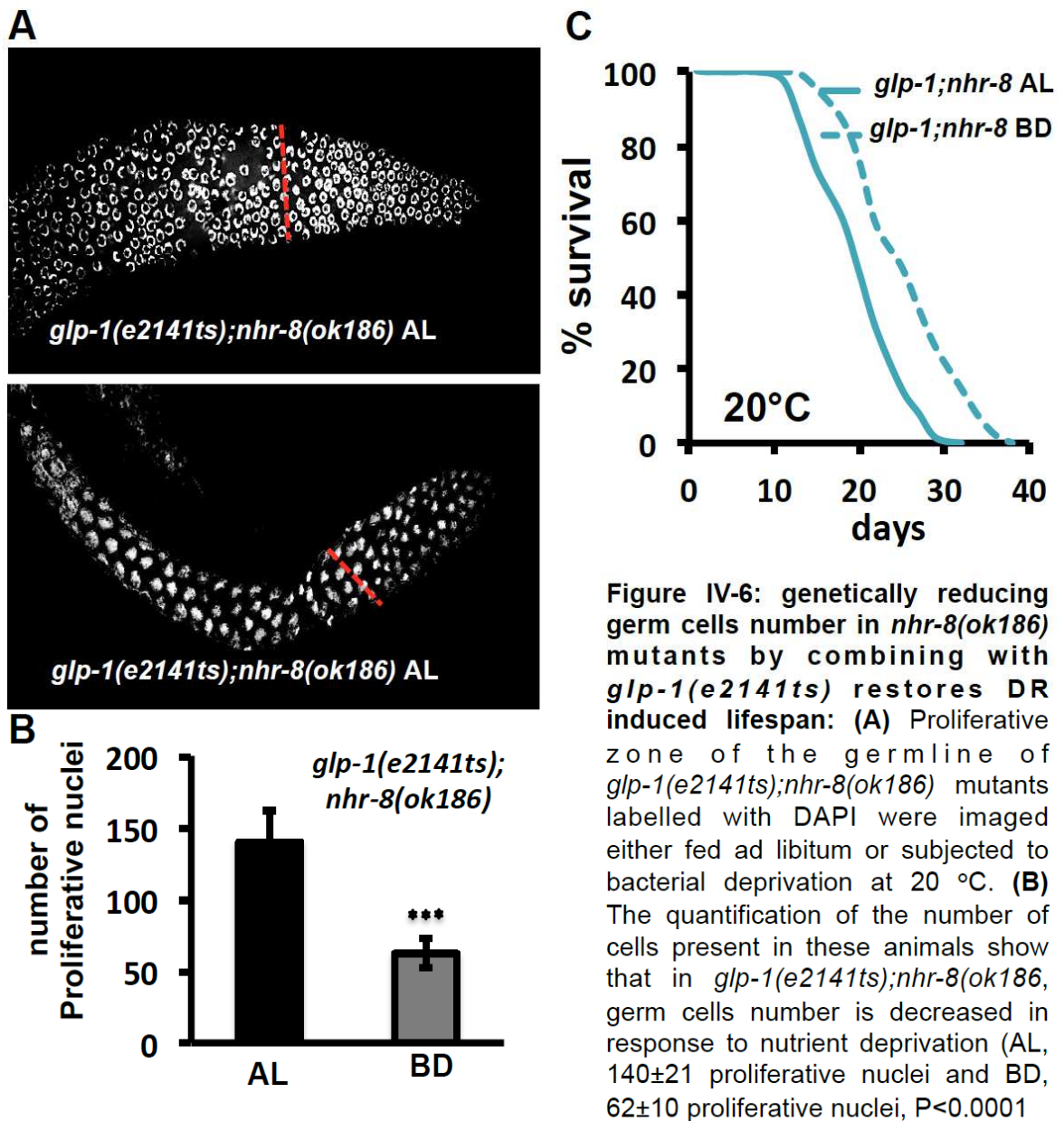
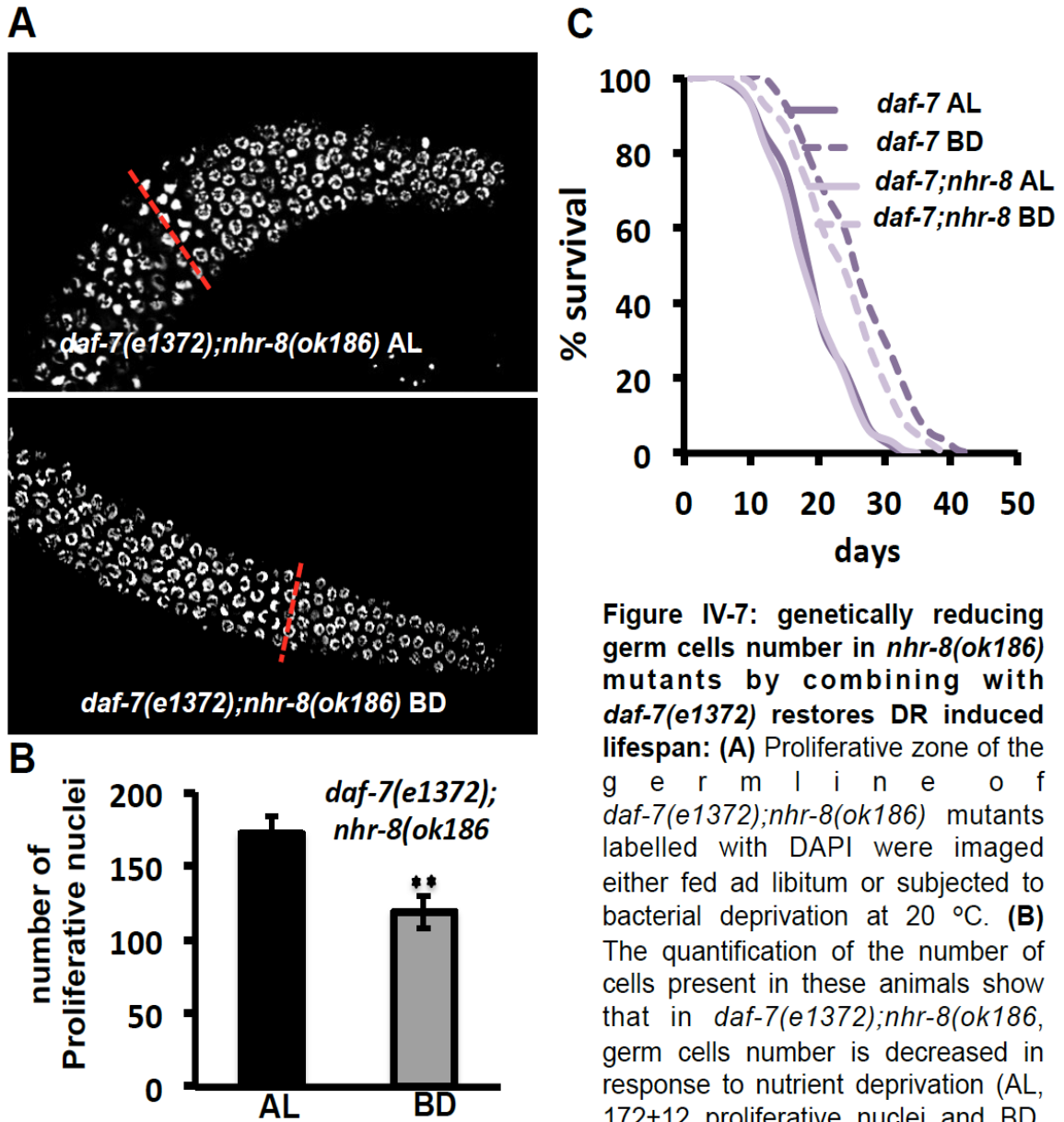


Figure IV-6: genetically reducing germ cells number in *nhr-8(ok186)* mutants by combining with *glp-1(e2141ts)* restores DR induced lifespan: (A) Proliferative zone of the germline of *glp-1(e2141ts);nhr-8(ok186)* mutants labelled with DAPI were imaged either fed ad libitum or subjected to bacterial deprivation at 20 °C. (B) The quantification of the number of cells present in these animals show that in *glp-1(e2141ts);nhr-8(ok186)*, germ cells number is decreased in response to nutrient deprivation (AL, 140±21 proliferative nuclei and BD, 62±10 proliferative nuclei, P<0.0001

for *glp-1(e2141ts);nhr-8(ok186)*, n=11 and 9 for AL and BD conditions, respectively). P values were calculated using the two-tailed Student's t-test. Scale bar, 40 mm. Error bars represent s.e.m. (C) DR-induced lifespan was restored by genetically mimicking the effects of DR on germline plasticity with the *glp-1(e2141ts)* allele at 20 °C in combination with *nhr-8(ok186)* mutants. DR induced the lifespan in *glp-1(e2141ts);nhr-8(ok186)* double mutants (mean lifespan: AL, 20±1 days and BD, 26±1 days, P<0.0001). P values were calculated using the log-rank (Mantel–Cox) test for lifespan. Lifespan values indicate mean±95% CI.

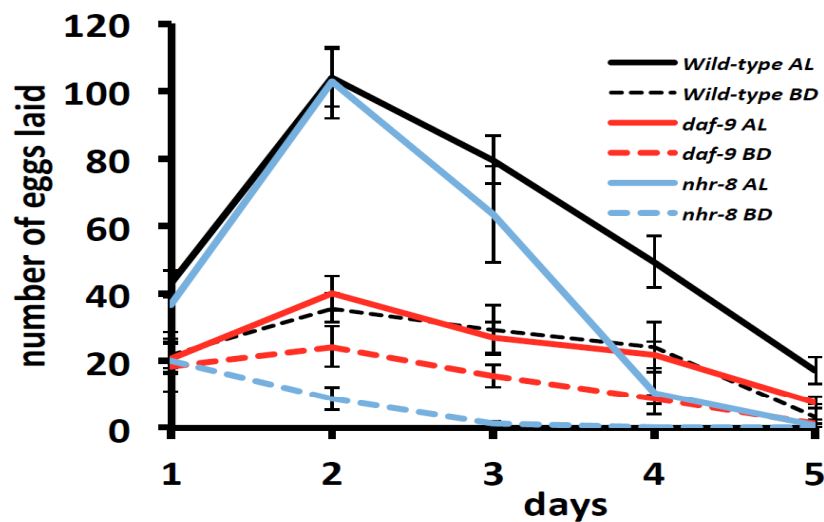


**Figure IV-7: genetically reducing germ cells number in *nhr-8(ok186)* mutants by combining with *daf-7(e1372)* restores DR induced lifespan: (A) Proliferative zone of the germline of *daf-7(e1372);nhr-8(ok186)* mutants labelled with DAPI were imaged either fed ad libitum or subjected to bacterial deprivation at 20 °C. (B) The quantification of the number of cells present in these animals show that in *daf-7(e1372);nhr-8(ok186)*, germ cells number is decreased in response to nutrient deprivation (AL, 172±12 proliferative nuclei and BD, 119±11 proliferative nuclei, P<0.01**

for *daf-7(e1372);nhr-8(ok186)*, n=10 and 8 for AL and BD conditions, respectively). P values were calculated using the two-tailed Student's t-test. Scale bar, 40 mm. Error bars represent s.e.m. (C) DR-induced lifespan was restored by genetically mimicking the effects of DR on germline plasticity with the *daf-7(e1372)* allele at 20 °C in combination with *nhr-8(ok186)* mutants. DR induced the lifespan in *daf-7(e1372);nhr-8(ok186)* double mutants (mean lifespan: AL, 19±1 days and BD, 24±1 days, P<0.0001). P values were calculated using the log-rank (Mantel-Cox) test for lifespan. Lifespan values indicate mean±95% CI.

### IV-2.3 The reproductive response to dietary restriction is not affected by *daf-9* or *nhr-8* mutation

The finding that germline plasticity is altered in *daf-9(rh50)* and *nhr-8(ok186)* mutants prompted us to examine the reproductive response of these mutants to DR. Because both mutants failed to reduce germ cell numbers, we assumed that they would have larger brood sizes than wildtype animals under DR conditions. Surprisingly, we found that the brood size of both mutants was actually reduced by BD, similar to the observations with wildtype animals (Fig. IV-8). Even though *daf-9(rh50)* mutants had smaller brood sizes than either wildtype animals or *nhr-8(ok186)* mutants under AL feeding conditions, BD further reduced the brood size in these mutants (Fig. IV-8). These results suggest that reproduction *per se* is not altered by starvation of *daf-9(rh50)* and *nhr-8(ok186)* mutant animals, despite the changes in germline plasticity.



**Figure IV-8: Reproductive profiles of wild-type, *daf-9(rh50)*, and *nhr-8(ok186)* mutants in AL and BD conditions:** Bacterial deprivation severely reduced the brood size in wild-type, *daf-9(rh50)*, and *nhr-8(ok186)* mutant animals (Brood size,  $231 \pm 7.1$  in AL vs.  $111 \pm 5.2$  in BD for wild-type animals,  $P < 0.001$ ;  $110 \pm 8.6$  in AL vs.  $67 \pm 8$  in BD for *daf-9(rh50)* mutants,  $P < 0.01$ ;  $271 \pm 14.5$  in AL vs.  $52 \pm 7.3$  in BD for *nhr-8(ok186)* mutants,  $P < 0.001$ ;  $n = 16, 17, 14$  in AL and 15, 15, and 12 in BD, for wild-type, *daf-9(rh50)*, and *nhr-8(ok186)* mutants respectively). P values were calculated using two-tailed t-test.



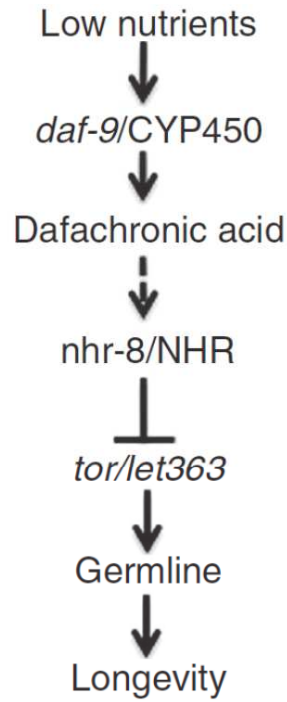
### IV-3 Conclusions and future perspectives

In this chapter, we demonstrated that mutation of *daf-9* and *nhr-8* reduces the germline plasticity response to DR. In the absence of steroid signaling, the number of germ cells within the proliferative zone of the germline is no longer affected by nutrient availability (Fig. 4). Interestingly, genetic reduction of germ cells alleviates the requirement for steroid signaling for DR-mediated lifespan extension, even though the reproductive responses of *daf-9(rh50)* and *nhr-8(ok186)* mutants to food deprivation were not affected. These data are consistent with previous reports that reproduction *per se* does not regulate the lifespan of adult *C. elegans*, but rather that the somatic tissue is required to integrate signals from the germline (Yamawaki, 2010).

We speculate that genetically lowering the germ cell count mimics the response of the germline to DR and induces a signal that is usually emitted when nutrients are scarce and germline activity is reduced. In wildtype animals, this would normally be achieved through the DAF-9/ $\Delta^7$ -DA/NHR-8 pathway. However, further work will be necessary to confirm the identity of this signal. It is important to note that BD also extends the lifespan of sterile or post-reproductive animals (Lee, 2006; Smith, 2008). In these animals, the putative signal for low germline activity might be constitutively produced, resulting in activated steroid signaling. Alternative mechanisms may also contribute to DR-induced longevity when reproduction is fully extinguished.

Interestingly, recent studies have shown that the mammalian lifespan-promoting hormone FGF21 (Zhang, 2012) also controls female reproduction. Furthermore, FGF21 expression is regulated by the NHR-8 homologs, FXR and LXR, and is induced by bile

acids similar to  $\Delta^7$ -DA (Cyphert, 2012; Archer, 2012). Our findings therefore establish the molecular basis for the interplay between nutrition and reproduction in regulating lifespan.



**Figure IV-C:** We propose a model in which nutrient scarcity induces DAF-9-mediated production of DA. In turn, DA activates NHR-8/NHR and TOR signalling, culminating in lifespan extension through the germline. The precise nature of the NHR-8/NHR ligand(s) and interactor(s) remains to be determined.

## **Materials and Methods**

## Chapter V Materials and Methods

### V-1 Strains

Strains used in this study include N2, *daf-9(rh50)*, *daf-9(m540)*, *daf-12(rh61rh411)*, *nhr-8(ok186)*, *daf-9(e1406);mgEx662[daf-9p::daf-9 genomic::GFP]*, *eat-2(ad1116)*, HGA8015 [*eat-2(ad1116);daf-9(rh50)*], HGA8016 [*nhr-8(ok186);daf-9(rh50)*], HGA8017 [*daf-12(rh61rh411);nhr-8(ok186)*], and *daf-12(rh61rh411);daf-9(rh50)*.

### V-2 Lifespan analyses, microscopy, and dafachronic acid treatment

Lifespan analyses were performed as previously described (Goudeau, 2011). In brief, synchronized L1 larvae were raised at 20°C until they reached the late L4 larval stage or early day 1 of adulthood. Worms were then transferred to NGM plates containing 5-fluorouracil (to prevent development of progeny) with or without bacteria. Statistical analysis of lifespan data was performed with XLSTAT software (Addinsoft). RNAi experiments were performed as previously described (Goudeau, 2011). Images of SKN-1::GFP worms were acquired with a Nikon fluorescent microscope at 400× magnification, and signals were quantified using ImageJ software (NIH). For DA treatment,  $\Delta^7$ -DA (Adipogen) was dissolved in ethanol and added to NGM plates at a final concentration of 100 nM.

### V-3 Dafachronic acid measurement

DA was measured by reverse-phase UPLC followed by mass spectrometry in negative ionization mode. Approximately 5000 worms were washed from the cultivation plates with cold PBS. After addition of 1 ml methanol, worms were lysed by sonication in an ice cold bath for 15 min. Samples were centrifuged at 14,000 rpm and 4°C for 10 min, the supernatant was transferred to a fresh Eppendorf tube, and the sample was concentrated 10-fold using a SpeedVac concentrator. The samples were separated on a Kinetex C18 column

(50 mm × 2.1 mm, 1.3 μm particle size) with an acetonitrile gradient. Separation was carried out on a Waters Acquity UPLC (Waters, Eschborn, Germany) coupled to a Bruker maXis UHR-ToF-MS (Bruker Daltonics, Bremen Germany). The system was calibrated using Low Concentration Tuning Mix (Agilent, Waldbronn, Germany).

#### **V-4 AMP/ATP measurement**

Worms were washed twice with S-buffer to remove bacteria and waste products, transferred to a 15 ml tube, and resuspended in 4 ml of S-buffer. Remaining bacteria were removed by addition of 8 ml of 60% sucrose followed by thorough shaking and centrifugation for 1 min at 3000 rpm. The nematodes were then transferred to a fresh 15 ml tube and washed twice more with S-buffer. The supernatant was removed and the worm pellet (approximately 100 μl) was mixed with glass beads (0.2 g) and homogenized using a mechanical bead beater. The homogenate was centrifuged (8–10 min, 4°C, 14,000 rpm) and the supernatant was transferred to a fresh 1.5 ml tube. The ADP/ATP ratio in 10 μl aliquots of worm extract was measured with an EnzyLight™ ADP/ATP Ratio Assay kit (ELDT-100, BioAssay Systems), according to the manufacturer's procedure. EnzyLight™ assay data were normalized to the total protein content of worm samples, as determined with the Bradford protein assay. The AMP/ATP ratio was approximated as the square of the ADP/ATP ratio (Hardie, 2001).

#### **V-5 Germ cell count**

The number of cells in the proliferative zone was assessed as described (2, 3). Briefly, adult worms (day 2) were immobilized on a microscope slide using levamisole solution (0.2 mM in M9). Gonads were extruded by cutting the worms at the head near the pharyngeal bulb or at the anal region. Worms with extruded gonads were fixed in cold methanol for 5 min and washed by the addition of PBTw (1× PBS with 0.1% Tween 20) followed by centrifugation

at 3000 rpm for 1 min. The supernatant was discarded and the worms were washed twice more. The worm pellet was then incubated with DAPI solution (100 ng/ml) for 1 min, transferred onto a large 2% agarose pad, and covered with a coverslip. Images were acquired using a confocal Leica TCS SP5 microscope with 40× or 63× zoom lenses (final zoom 400× or 630×). To enumerate germ cells, the boundary between the mitotic and transition zones was marked as described by Crittenden et al., in 2006. Cells in the proliferative zone were counted at different focal planes through the width of the germline.

### **V-6 Pumping rate**

Animals were raised at 20°C until they reached late L4 larval stage or early day 1 of adulthood. Animals were then transferred to NGM plates with or without bacteria, and pharyngeal pumping was evaluated on the second day of adulthood. Animals were left undisturbed for 1 h and pharyngeal pumping was counted for 20 s in at least 20 worms for each condition. Results are presented as the mean  $\pm$  s.d. and statistical significance was analyzed using Student's t-test.

### **V-7 Quantitative PCR**

Synchronized L1 larvae were grown at 20°C on HT115 *E. coli* until they reached late L4 stage or early day 1 of adulthood. Animals were then transferred to NGM plates with or without bacteria. For RNAi experiments, animals were raised at 20°C on HT115 *E. coli* bacteria and were transferred onto plates seeded with control bacteria or bacteria expressing dsRNA against the gene of interest. On day 2 of adulthood, animals were collected and washed three times with M9 solution.

Washed animals (~1,000 per point) were frozen at -80°C for at least 24 h. RNA was extracted using TRIzol (Invitrogen) and RNeasy Mini Kits (Qiagen). The concentration and

purity of RNA was determined using a NanoDrop spectrophotometer. cDNA was synthesized from samples of 1 µg of RNA using an iScript Advanced cDNA Synthesis Kit (Bio-Rad). Q-PCR was performed using the StepOne Plus system (Applied Biosystems) according to the manufacturer's recommended protocol. Relative mRNA levels were normalized against the housekeeping genes *cdc-42*, *pmp-3*, and *ama-1*. Primers sequences used in this study are listed in below (see section I-14.8). Results are expressed as the mean ± s.d. of triplicates. All Q-PCR experiments were repeated at least twice. Statistical significance was determined using the two-tailed Student's t-test. \* $P < 0.1$ , \*\* $P < 0.05$ , \*\*\* $P < 0.01$ .

### V-8 Primer Sequences

LET-363 F	TATAAGAAAGCACGCGGCAC
LET-363 R	CAGATACGGTATTAGTTCGCC
AMA-1 F	TGGA ACTCTGGAGTCACACC
AMA-1 R	CATCCTCCTTCATTGAACGG
BEC-1 F	TGCAGTTGCTCGAGTTTTTG
BEC-1 R	TGACACCATTGTCAACCAGTG
DAF-9 F	GGGCAAATATCGTGTTATGG
DAF-9 R	GATTGGATCTCCTCTGTTCA
DAF-12 F	ATGACTCCAACACATGGTTTT
DAF-12 R	TTCTCCTGGCAGCTCTTCG
DRR-2 F	TGAAGCCCCGTACCACAGA
DRR-2 R	CTTGGTCTCCTCTTCTTCTTGCT
CDC-42 F	CCAGGAATTGCTGATCGTATGCAGAA
CDC-42 R	TGGAGAGGGAAGCGAGGATAGA
PMP-3 F	GTTCCCGTGTTCACTCAT
PMP-3 R	ACACCGTCGAGAAGCTGTAGA
PHA-4 F	GCCAATTTTCATGCAAGGAGG
PHA-4 R	GCCAGTGGTAAAACCAAGAGGT
ATG-18 F	ACAAATCCCTGTCGTTCCAG
ATG-18 R	AATGAGCCGTTGGATAATGC

**Table 2: Life-span analysis**

Genotype	Condition	Mean $\pm$ 95% CI	# worms	% Change	p Value
Wild-type	AL	19.15 $\pm$ 0.95	130/149		
	BD	27.43 $\pm$ 0.87	179/212	43.21	< 0.0001
<i>daf-9 (rh50)</i>	AL	15.81 $\pm$ 0.830	147/158		
	BD	17.14 $\pm$ 0.69	147/166	8.37	0.13
<i>daf-9 (m540)</i>	AL	15.33 $\pm$ 0.90	151/160		
	BD	17.05 $\pm$ 0.82	132/145	10.07	0.02
Wild-type	AL	18.96 $\pm$ 0.83	149/154		
	BD	26.76 $\pm$ 0.82	177/204	41.20	< 0.0001
<i>daf-9 (rh50)</i>	AL	16.57 $\pm$ 0.64	138/149		
	BD	17.62 $\pm$ 0.94	178/193	6.34	0.29
<i>daf-9 (m540)</i>	AL	17.88 $\pm$ 1.31	153/161		
	BD	19.06 $\pm$ 0.74	144/165	7.00	0.20
Wild-type	AL	18.81 $\pm$ 0.90	121/129		
<i>eat-2 (ad1116)</i>		21.98 $\pm$ 0.99	110/120	16.85	< 0.001
<i>daf-9 (rh50)</i>		15.87 $\pm$ 0.64	121/126		
<i>eat-2 (ad1116); daf-9 (rh50)</i>		17.48 $\pm$ 0.98	122/130	9.72	0.04
Wild-type	AL	19.26 $\pm$ 0.60	102/110		
<i>eat-2 (ad1116)</i>		23.84 $\pm$ 0.84	108/119	23.78	< 0.0001
<i>daf-9 (rh50)</i>		16.95 $\pm$ 0.53	112/121		
<i>eat-2 (ad1116); daf-9 (rh50)</i>		18.18 $\pm$ 0.69	104/116	7.26	0.10
Wild-type	AL+Ethanol	19.89 $\pm$ 0.94	110/112		
	BD+Ethanol	25.16 $\pm$ 1.0	112/129	26.50 (wrt AL+Ethanol)	< 0.0001
	AL+DA	21.31 $\pm$ 0.94	121/122	7.14 (wrt AL+Ethanol)	0.11
	BD+DA	26.27 $\pm$ 1.02	118/127	23.28 (wrt AL+Daf.acid)	< 0.0001
<i>daf-9 (rh50)</i>	AL+Ethanol	17.5 $\pm$ 0.90	117/126		
	BD+Ethanol	18.7 $\pm$ 0.98	98/121	6.86 (wrt AL+Ethanol)	0.13
	AL+DA	18.51 $\pm$ 0.94	119/123	5.77 (wrt AL+Ethanol)	0.28
	BD+DA	25 $\pm$ 0.92	115/123	35.06 (wrt AL+Daf.acid)	< 0.0001
				4.41 (wrt BD+Ethanol)	0.30
				33.69 (wrt BD+Ethanol)	< 0.0001
Wild-type	AL	19.31 $\pm$ 1.08	103/108		
	BD	32.89 $\pm$ 1.17	142/173	70.00	< 0.0001
<i>daf-36(k114)</i>	AL	14.95 $\pm$ 0.93	140/146		
	BD	22.69 $\pm$ 1.34	136/148	52.00	< 0.0001
<i>hsd-1(mg433)</i>	AL	22.15 $\pm$ 1.1	170/190		
	BD	25.3 $\pm$ 1.15	117/143	20.00	< 0.0001
<i>eat-2(ad1116)</i>	empty vector	25.38 $\pm$ 1.16	119/132		



<i>dhs-16(gk236319)</i>	<i>emb-8 RNAi</i>	27.04 ± 1.22	125/138	7.00	0.24
	AL	17.63 ± 0.82	113/116		
	BD	22.83 ± 1.03	121/140	29.00	< 0.0001
Wild-type	AL	17.31 ± 0.88	97/108		
<i>daf-36(k114)</i>	BD	30.09 ± 0.86	121/142	74.00	< 0.0001
	AL	16.95 ± 0.74	104/113		
<i>eat-2(ad1116)</i>	BD	24.54 ± 0.94	118/144	44.00	< 0.0001
	empty vector	27.18 ± 1.02	98/110		
<i>dhs-16(gk236319)</i>	<i>emb-8 RNAi</i>	25.84 ± 0.96	107/119	5.00	0.453
	AL	16.39 ± 0.80	111/118		
	BD	21.36 ± 0.96	128/147	30.00	< 0.0001
<i>daf-12(rh61rh411)</i>	AL	13.69 ± 0.76	132/140		
	BD	17.39 ± 1.2	121/155	27.03	< 0.0001
<i>daf-12(m20)</i>	AL	14.89 ± 0.33	115/152		
	BD	17.53 ± 0.66	157/181	17.73	< 0.0001
<i>daf-12(rh61rh411)</i>	AL	14.05 ± 0.82	129/133		
	BD	16.23 ± 0.82	144/174	15.52	< 0.0001
<i>daf-12(m20)</i>	AL	15.35 ± 0.78	132/151		
	BD	18.58 ± 0.86	151/178	21.04	< 0.0001
<i>nhr-8(ok186)</i>	AL	17.88 ± 0.96	107/116		
	BD	19.11 ± 0.99	99/112	6.88	0.22
<i>nhr-8(ok186)</i>	AL+Ethanol	13.88 ± 1.1	88/96		
	BD+Ethanol	14.8 ± 1.1	93/103	6.63(wrt AL+Ethanol)	0.69
	AL+DA	15.29 ± 1.16	96/99	10.16(wrt AL+Ethanol)	0.09
	BD+DA	15.92 ± 1.2	95/108	4.12(wrt AL+Daf.acid) 7.57(wrt BD+Ethanol)	1.00 0.40
<i>daf-12(rh61rh411); daf-9(rh50)</i>	AL+Ethanol	17.81 ± 1.02	102/113		
	BD+Ethanol	19.18 ± 1.04	98/121	7.69(wrt AL+Ethanol)	0.29
	AL+DA	18.86 ± 1.08	108/118	5.89(wrt AL+Ethanol)	0.47
	BD+DA	22.07 ± 1.36	101/122	17.02(wrt AL+Daf.acid) 15.07(wrt BD+Ethanol)	< 0.0001 < 0.0001
<i>nhr-8(ok186)</i>	AL+Ethanol	16.21 ± 0.96	103/112		
	BD+Ethanol	17.41 ± 0.90	108/131	7.40(wrt AL+Ethanol)	0.36
	AL+DA	16.04 ± 1.0	99/108	1.05(wrt AL+Ethanol)	1.00
	BD+DA	17.79 ± 0.90	109/136	10.91(wrt AL+Daf.acid) 2.18(wrt BD+Ethanol)	0.08 1.00
<i>daf-9(rh50); nhr-8(ok186)</i>	AL+Ethanol	15.94 ± 1.02	101/109		
	BD+Ethanol	17.13 ± 0.92	111/138	7.47(wrt AL+Ethanol)	0.629
	AL+DA	16.21 ± 1.06	98/106	1.69(wrt AL+Ethanol)	1.00
	BD+DA	17.88 ± 0.98	105/121	10.30(wrt AL+Daf.acid) 4.38(wrt BD+Ethanol)	0.19 0.85
Wild-type	empty vector	19.89 ± 0.94	117/126		

<i>daf-9(rh50)</i>	<i>daf-2 RNAi</i>	30.86 ± 1.04	103/121	55.15	< 0.0001
	empty vector	18.63 ± 0.9	113/119		
	<i>daf-2 RNAi</i>	28.07 ± 1.16	101/109	50.67	< 0.0001
Wild-type	empty vector	19.89 ± 1.1	100/113		
	<i>daf-9 RNAi</i>	17.95 ± 1	91/108	-9.75	< 0.007
aak-2 OE	Empty vector	23.78 ± 1.25	89/98	19.55(wrt wild-type)	< 0.0001
	<i>daf-9 RNAi</i>	22.04 ± 1.1	97/106	-7.31	< 0.03
				22.78(wrt wild-type)	< 0.0001
<i>daf-9(e1406); mgEx662[daf-9p:: genomic::GFP]</i>	AL	18.02 ± 0.9	115/118		
	BD	22.05 ± 0.9	113/118	22.36	< 0.0001
<i>daf-9(e1406); mgEx662[daf-9p:: genomic::GFP]</i>	AL	19.68 ± 1.06	104/112		
	BD	24.56 ± 0.98	119/138	24.80	< 0.0001
<i>glp-1(e2141ts); daf-9(rh50)</i>	at 20 °C AL	14.46 ± 0.76	92/110		
	at 20 °C BD	18.27 ± 1.43	87/106	26.34	< 0.0001
<i>glp-1(e2141ts); nhr-8(ok186)</i>	at 20 °C AL	20.45 ± 0.86	136/139		
	at 20 °C BD	25.80 ± 0.90	165/189	26.16	< 0.0001
<i>glp-1(e2141ts)</i>	at 20 °C AL	20.92 ± 0.782	131/137		
	at 20 °C BD	27.08 ± 0.90	163/188	29.44	< 0.0001
Wild-type	empty vector	19.89 ± 0.94	117/126		
	<i>daf-2 RNAi</i>	30.86 ± 1.04	103/121	55.15	< 0.0001
<i>nhr-8(ok186)</i>	empty vector	13.72 ± 0.70	88/96		
	<i>daf-2 RNAi</i>	25.39 ± 1.06	108/124	85.08	< 0.0001
<i>daf-7(e1372)</i>	AL	19.97 ± 1.16	99/103		
	BD	26.79 ± 1.30	114/137	34.15	< 0.0001
<i>daf-7(e1372);nhr-8(ok186)</i>	AL	19.41 ± 1.07	99/108		
	BD	24.32 ± 1.27	127/128	25.29	< 0.0001

# worms = number of dead worms observed/total number of worms at start

DA = Dafachronic acid

# Article

ARTICLE

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# Steroid hormone signalling links reproduction to lifespan in dietary-restricted *Caenorhabditis elegans*

Manjunatha Thondamal<sup>1</sup>, Michael Witting<sup>2</sup>, Philippe Schmitt-Kopplin<sup>2</sup> & Hugo Aguilaniu<sup>1</sup>

Dietary restriction (DR) increases healthspan and longevity in many species, including primates, but it is often accompanied by impaired reproductive function. Whether signals associated with the reproductive system contribute to or are required for DR effects on lifespan has not been established. Here we show that expression of the cytochrome P450 DAF-9/CYP450 and production of the steroid hormone  $\Delta^7$ -dafachronic acid (DA) are increased in *C. elegans* subjected to DR. DA signalling through the non-canonical nuclear hormone receptor NHR-8/NHR and the nutrient-responsive kinase *let-363*/mTOR is essential for DR-mediated longevity. Steroid signalling also affects germline plasticity in response to nutrient deprivation and this is required to achieve lifespan extension. These data demonstrate that steroid signalling links germline physiology to lifespan when nutrients are limited, and establish a central role for *let-363*/mTOR in integrating signals derived from nutrients and steroid hormones.

<sup>1</sup> Molecular Biology of the Cell Laboratory, Ecole Normale Supérieure de Lyon—CNRS—Université de Lyon Claude Bernard, UMR5239, 46, Allée d'Italie, Lyon, Cedex 07 69364, France. <sup>2</sup> Helmholtz Zentrum Muenchen-Department of Environmental Sciences (DES)-Analytical BioGeoChemistry (BGC), Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany. Correspondence and requests for materials should be addressed to H.A. (email: hugo.aguilaniu@ens-lyon.fr).

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- [Manjunatha Thondamal](#),
- [Michael Witting](#),
- [Philippe Schmitt-Kopplin](#)
- [Hugo Aguilaniu](#)

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