RESEARCH PAPER

Exploring Lifespan Modulation in *C. elegans* Through RNAi-Mediated *DAF-2* Knockdown Across Variable Temperature Conditions

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SCI 400- Research Project

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December 9th, 2024

ABSTRACT

One important factor influencing lifespan and aging in Caenorhabditis elegans (C. elegans) is the insulin/IGF-1 signaling (IIS) pathway, which is controlled by the daf-2 gene. In C. elegans, environmental factors like temperature are known to significantly alter lifespan and stress responses, while RNA interference (RNAi) provides a reliable technique for examining gene function. With an emphasis on comprehending the interplay between genetic and environmental factors in aging, this study investigates the effects of RNAi-mediated knockdown of daf-2 on lifespan across four different temperature conditions (4°C, 10°C, 20°C, and 30°C). Synchronized populations of *C. elegans* were exposed to *daf-2* RNAi and maintained under controlled temperature conditions to assess their lifespan. Although the study is ongoing and results have not yet been collected, it is hypothesized that *daf-2* knockdown will result in reduced lifespan relative to controls, with the most pronounced effects occurring at temperature extremes (4°C and 30°C). This research seeks to elucidate the complex interplay between IIS signaling and environmental stressors in lifespan regulation, contributing to the broader understanding of genetic and environmental influences on aging and resilience mechanisms.

1. INTRODUCTION

1.1 Environmental Influence on Aging

Aging, a universal biological process, is marked by a gradual decline in physiological and cellular functions, leading to increased disease susceptibility and mortality (López-Otín et al., 2013; Kirkwood & Austad, 2000). Among environmental factors influencing aging, temperature plays a crucial role, particularly in ectothermic organisms such as *Caenorhabditis elegans* (*C. elegans*). Lower temperatures have been shown to enhance lifespan by suppressing metabolic

rates and activating stress resistance pathways, including proteostasis and autophagy (Honda & Honda, 1999; Lee et al., 2014). Conversely, higher temperatures accelerate aging by increasing metabolic demands, oxidative damage, and proteostatic challenges (Rikke & Johnson, 2004; Toth et al., 2008). As shown in Figure 1, lower temperatures promote extended lifespans, optimal temperatures support moderate lifespans, and higher temperatures significantly reduce lifespan (Honda & Honda, 1999; Rikke & Johnson, 2004; Toth et al., 2008). This intricate relationship highlights the importance of environmental stressors in shaping aging dynamics and highlights the potential to uncover fundamental mechanisms of longevity through the study of *C. elegans* (López-Otín et al., 2013; Kenyon, 2010).

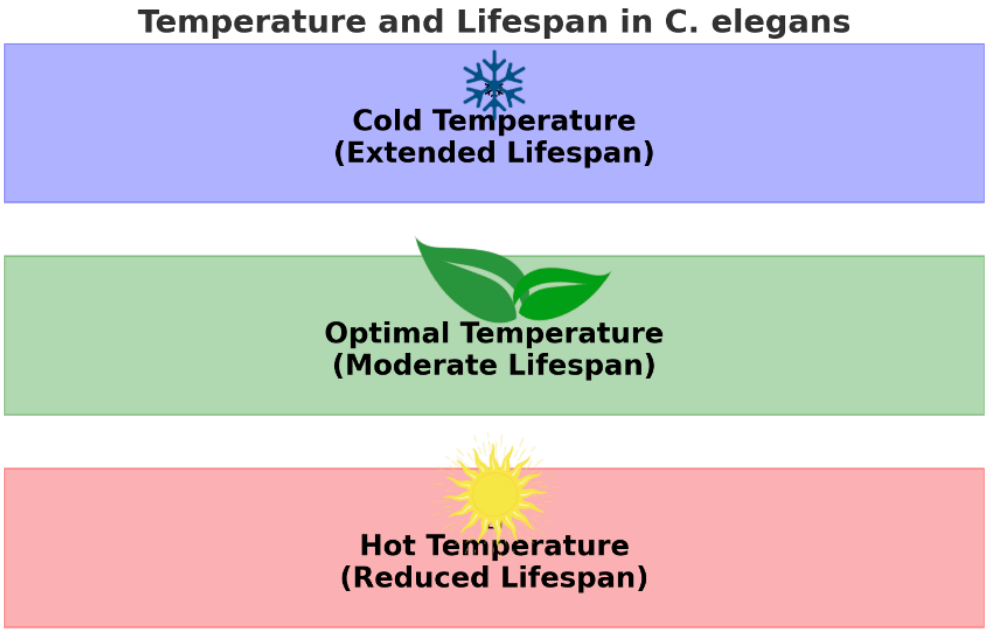

Figure 1: Relationship between temperature and lifespan in *C. elegans*. Cold temperatures extend lifespan by reducing metabolic activity and oxidative stress, optimal temperatures maintain a moderate lifespan, and high temperatures shorten lifespan due to increased metabolic

demands and oxidative damage. This schematic illustrates the role of temperature as a key environmental factor influencing aging.

1.2 *C. elegans* as a Model Organism in Aging Studies

C. elegans is an exceptional model organism for aging research due to its well-characterized lifecycle, genetic simplicity, and conservation of critical aging-related pathways such as the insulin/IGF-1 signaling (IIS) pathway (Kenyon, 2010; López-Otín et al., 2013). The lifecycle of *C. elegans*, as shown in Figure 2, progresses through distinct stages: embryogenesis, four larval stages (L1–L4), and adulthood. At 20°C, the lifecycle from egg to reproductive adult spans approximately 3 days, and the entire lifespan is about 3 weeks (Brenner, 1974). Embryogenesis occurs within 14 hours, followed by hatching into the L1 larval stage. Worms pass through L2, L3, and L4 stages, undergoing molting at each transition (Brenner, 1974). The adult stage is marked by reproduction and eventual aging, with visible changes such as decreased motility, accumulation of lipofuscin, and increased susceptibility to environmental stressors (Gruber et al., 2007; Herndon et al., 2002). Synchronization techniques, such as bleaching, ensure uniform developmental stages, reducing variability in experiments and enhancing the reliability of lifespan studies (Lewis & Fleming, 1995).

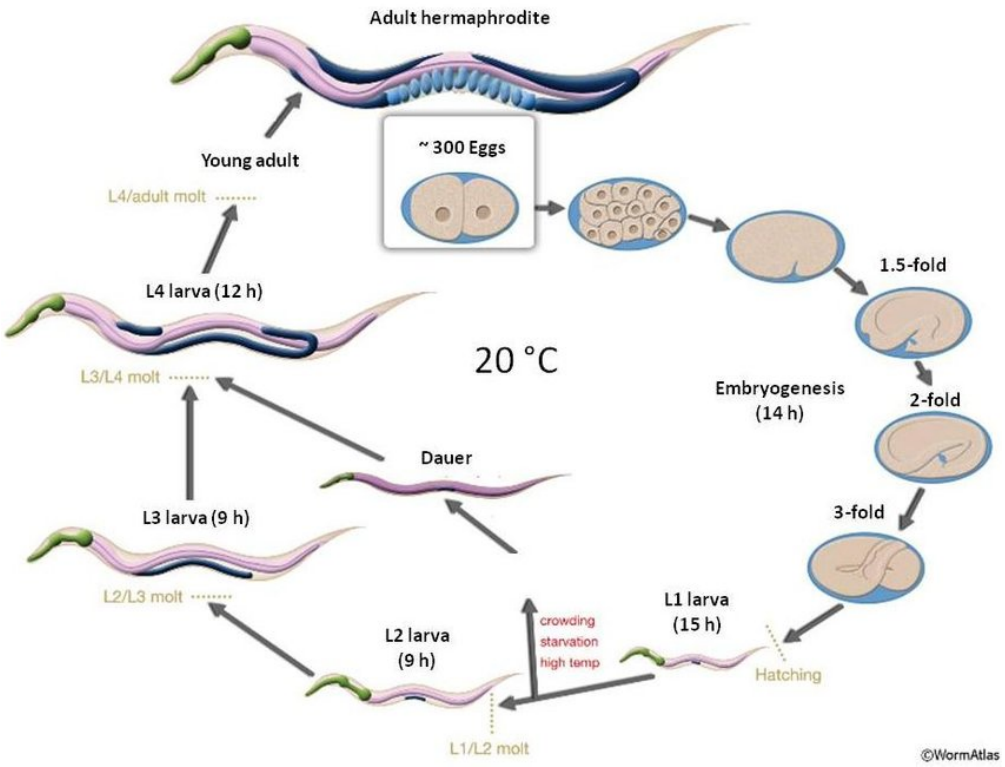

Figure 2: **Lifecycle of *Caenorhabditis elegans* (*C. elegans*) at 20°C.** The lifecycle progresses through embryogenesis, four larval stages (L1–L4), and adulthood, with the entire development occurring over approximately three weeks under optimal laboratory conditions. Under environmental stressors such as crowding, starvation, or high temperature, the L1 larvae may enter an alternative dauer stage, which is a dormant and stress-resistant state. Dauer refers to a specialized larval form adapted for survival during unfavorable conditions. The adaptability of *C. elegans*, coupled with its rapid lifecycle, makes it an ideal model organism for studying development, genetics, and aging. Patrycja Vasilyev Missiuro. (2010, August 26). *Predicting Genetic Interactions in Caenorhabditis elegans using Machine Learning*. https://www.researchgate.net/publication/266342950_Predicting_Genetic_Interactions_in_Caenorhabditis_elegans_using_Machine_Learning

When compared to other model organisms such as *Drosophila melanogaster* (fruit flies) and mammals, *C. elegans* stands out for its simplicity and experimental flexibility (Kenyon, 2010; Mitchell et al., 2015). Mammalian models (Figure 3a) provide a closer physiological and genomic resemblance to humans and are invaluable for translational research, particularly in the study of complex tissue interactions and aging-related diseases. However, the long lifespan of mammals (~2–3 years in mice) and ethical considerations often make them less practical for high-throughput or exploratory research (Mitchell et al., 2015).

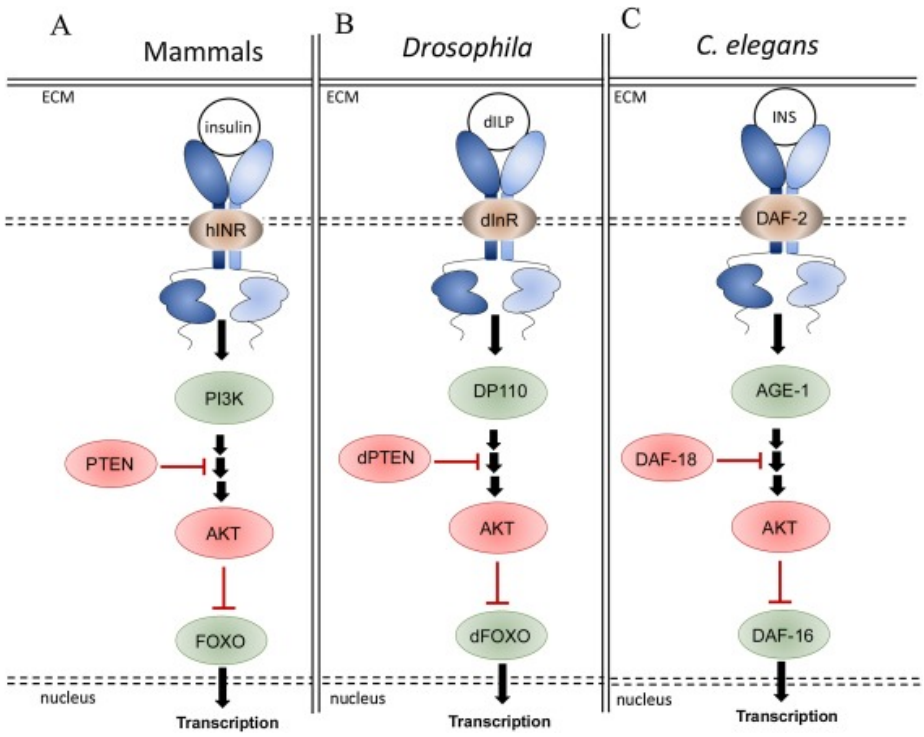

Figure 3: Comparative insulin/IGF-1 signaling (IIS) pathways in mammals, *Drosophila melanogaster* (fruit fly), and *Caenorhabditis elegans* (*C. elegans*). (A) In mammals, the IIS pathway begins with insulin binding to the insulin receptor (hINR), leading to the activation of phosphatidylinositol-3-kinase (PI3K) and AKT kinase. PTEN (phosphatase and tensin homolog) acts as a negative regulator, while FOXO transcription factors control stress resistance and longevity-related genes. (B) In *Drosophila*, the pathway is similarly regulated by the insulin-like

receptor (dInR), with DP110 acting as the PI3K homolog, dPTEN as the negative regulator, and dFOXO as the transcription factor. (C) In *C. elegans*, the IIS pathway is centered around DAF-2, an insulin-like receptor, which regulates downstream components, including AGE-1 (a PI3K homolog), DAF-18 (a PTEN homolog), and AKT kinases. DAF-16, the *C. elegans* FOXO homolog, is a critical transcription factor promoting stress resistance, metabolism, and longevity when translocated to the nucleus. Abbreviations: PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; AKT, protein kinase B; FOXO, forkhead box O; dInR, *Drosophila* insulin receptor; DP110, PI3K homolog in *Drosophila*; DAF-2, insulin-like receptor in *C. elegans*; AGE-1, PI3K homolog in *C. elegans*; DAF-18, PTEN homolog in *C. elegans*.
Biglou, S. G., Bendena, W. G., & Chin-Sang, I. (2021). An overview of the insulin signaling pathway in model organisms Drosophila melanogaster and Caenorhabditis elegans. *Peptides*, *145*, 170640. https://doi.org/10.1016/j.peptides.2021.170640

Drosophila melanogaster (Figure 3b), another widely used model organism, shares many advantages with *C. elegans*, such as genetic tractability and conservation of pathways like IIS. However, *Drosophila* has a longer lifespan (~2 months) and a higher level of physiological complexity, including a structured brain and more intricate metabolic networks. This makes *Drosophila* particularly useful for studying tissue-specific aging and neurodegeneration but less efficient for rapid genetic and environmental studies (Tower, 2000).

C. elegans (Figure 3c) provides a unique blend of simplicity and relevance. The IIS pathway in *C. elegans*—involving DAF-2 (insulin-like receptor) and DAF-16 (FOXO transcription factor)—is highly conserved across species. This pathway integrates environmental signals such as

temperature, nutrient availability, and oxidative stress to modulate stress resistance, metabolism, and longevity (Kenyon, 2010; Uno & Nishida, 2016).

1.3 The IIS Pathway: Central to Aging in *C. elegans.*

The insulin/IGF-1 signaling (IIS) pathway is a highly conserved regulator of aging, metabolism, and stress resistance across species, including *C. elegans* and mammals (Kenyon, 2010; Uno & Nishida, 2016). In *C. elegans*, the pathway is centered around the *daf-2* gene, which encodes an insulin-like receptor homologous to the mammalian IGF-1 receptor. (Kenyon, 2010; Uno & Nishida, 2016) Activation of DAF-2 initiates a signaling cascade that begins with AGE-1, a phosphatidylinositol-3-kinase, which produces phosphatidylinositol-3,4,5-triphosphate (PIP3). This molecule activates AKT-1 and AKT-2, serine/threonine kinases that phosphorylate and inactivate the FOXO transcription factor DAF-16, effectively sequestering it in the cytoplasm (Figure 4a) (Biglou et al., 2021; Dillin et al., 2002). In this active IIS state, stress resistance and longevity-related genes are repressed, and cellular energy is directed toward growth and reproduction. (Kenyon, 2010; Dillin et al., 2002; Uno & Nishida, 2016)

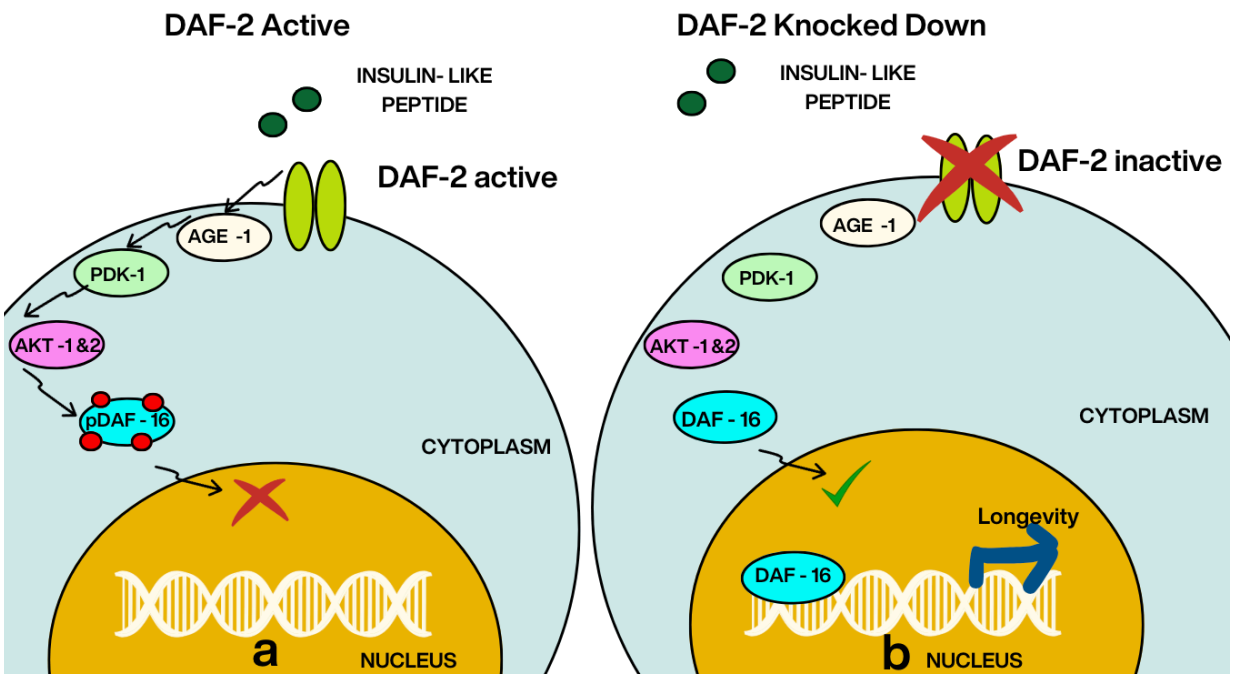

FIGURE 4: The insulin/IGF-1 signaling (IIS) pathway and its downstream elements in *C. elegans*. In the active state(4a), DAF-2, an insulin-like receptor, binds to insulin-like peptides, triggering a signaling cascade through AGE-1, a phosphoinositide 3-kinase, and AKT-1/2, protein kinases that phosphorylate DAF-16, a Forkhead box O (FOXO) transcription factor. Phosphorylation prevents DAF-16 from entering the nucleus, thereby inhibiting the expression of genes linked to stress resistance and longevity. Conversely, in the inactive state (4b) of DAF-2 (knockdown), the absence of signaling allows DAF-16 to translocate into the nucleus, where it activates genes promoting stress resilience, metabolic adaptation, and increased longevity. This figure highlights the intricate relationship between the IIS pathway components, including DAF-2, AGE-1, AKT-1/2, and DAF-16, in regulating lifespan through environmental and genetic inputs.

When IIS is reduced, for instance, through *daf-2* knockdown, the signaling cascade is disrupted (Kenyon, 2010; Uno & Nishida, 2016). AGE-1 fails to produce sufficient PIP3, resulting in the

inactivation of AKT-1 and AKT-2 (Biglou et al., 2021; Dillin et al., 2002). This dephosphorylation enables DAF-16 to translocate to the nucleus (Figure 4b), where it promotes the expression of genes associated with stress resilience, detoxification, and longevity (Kenyon, 2010; Uno & Nishida, 2016). These include *sod-3* (superoxide dismutase), *ctl-1* and *ctl-2* (catalases), and *hsp-16.2* (heat shock protein), which are vital for managing oxidative stress and protein misfolding (Kenyon, 2010; Dillin et al., 2002; Uno & Nishida, 2016). Furthermore, *lipl-4* (involved in lipid metabolism) and *lys-7* (antimicrobial defense) are also upregulated, enhancing cellular resilience and metabolic stability (Biglou et al., 2021; Uno & Nishida, 2016).

This pathway integrates external environmental signals, such as temperature, with intrinsic genetic processes to regulate aging and stress responses (Kenyon, 2010; Uno & Nishida, 2016). At lower IIS levels, as induced by *daf-2* knockdown, the focus of cellular resources shifts from reproduction and growth to maintenance and stress adaptation, thereby extending lifespan (Biglou et al., 2021; Rikke & Johnson, 2004). This regulatory balance allows *C. elegans* to adapt to environmental changes, exemplifying the interplay between genetic and environmental inputs in lifespan determination (Gems & Partridge, 2013; Uno & Nishida, 2016). The IIS pathway's evolutionary conservation underscores its relevance across species, providing a robust model to investigate aging mechanisms and stress resilience (Kenyon, 2010; Gems & Partridge, 2013). Figures 4a and 4b illustrate the molecular changes in IIS signaling, highlighting the contrast between DAF-2 active and inactive states (Biglou et al., 2021).

1.4 RNA Interference (RNAi) and IPTG Mechanism

RNA interference (RNAi) is a powerful technique that allows precise silencing of specific genes, such as *daf-2*, in *C. elegans* (Fire et al., 1998; Grishok et al., 2001). This study utilized

RNAi to target *daf-2*, a critical gene in the insulin/IGF-1 signaling (IIS) pathway (Kenyon, 2010; Uno & Nishida, 2016). The RNAi process begins with the ingestion of genetically engineered *Escherichia coli* strains producing double-stranded RNA (dsRNA) complementary to the target gene (Timmons & Fire, 1998). Once ingested, the dsRNA is processed by the enzyme Dicer, which cleaves it into small interfering RNAs (siRNAs) (Grishok et al., 2001). These siRNAs are incorporated into the RNA-induced silencing complex (RISC), which guides the degradation of complementary mRNA, effectively silencing the target gene (Fire et al., 1998; Grishok, 2005). This pathway, illustrated in Figure 5, highlights the sequential steps from dsRNA processing to mRNA degradation, enabling effective gene silencing.

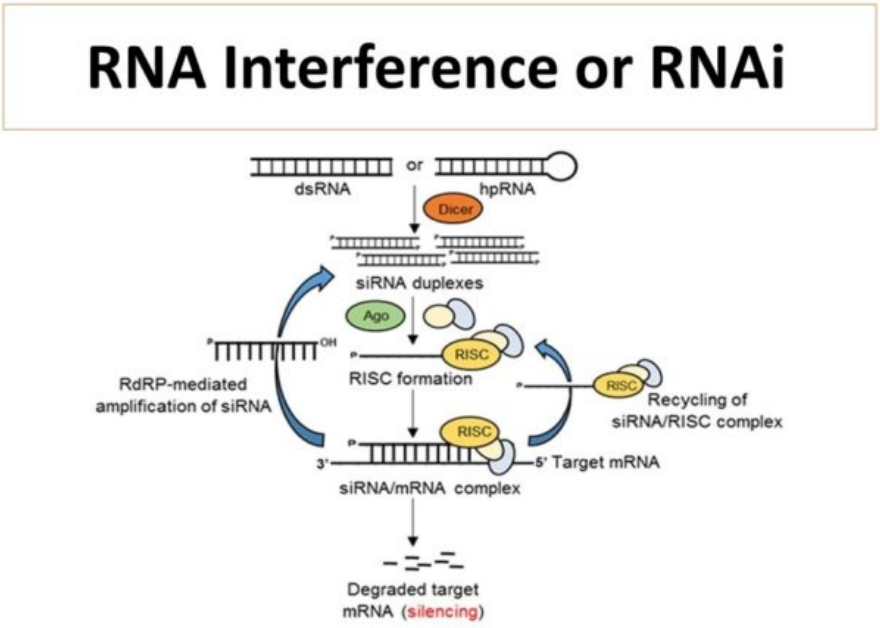

Figure 5: RNA Interference (RNAi) Mechanism. This diagram illustrates the RNAi process, where double-stranded RNA (dsRNA) is processed by Dicer into small interfering RNA (siRNA) molecules. These siRNAs are incorporated into the RNA-induced silencing complex (RISC), guiding it to complementary messenger RNA (mRNA) for targeted degradation. The process

effectively silences gene expression by preventing translation of the mRNA into protein. This mechanism is widely used in gene knockdown experiments, such as silencing the *daf-2* gene in *Caenorhabditis elegans*. Waseem, Q. ul A. (2022, February 28). *RNA Interference or RNAi*. Microbiology Notes. https://microbiologynotes.org/rna-interference-or-rnai/

A critical component of the RNAi protocol is the induction of dsRNA production in the bacterial host using IPTG (isopropyl-β-D-thiogalactopyranoside) (Sambrook et al., 1989; Grishok, 2005). IPTG is a synthetic analog of allolactose, a natural inducer of the lac operon in bacteria (Jacob & Monod, 1961). Structurally, IPTG resembles lactose and its derivative allolactose but possesses unique properties that make it especially effective for experimental applications (Malakar et al., 2007). As shown in Figure 6, lactose is a disaccharide of galactose and glucose, which upon hydrolysis or cellular metabolism, forms allolactose (Beckwith, 1987). Allolactose serves as the natural inducer of the lac operon by binding to and inactivating the lac repressor protein, thereby facilitating transcription of downstream genes. IPTG mimics this function, binding to the lac repressor and releasing it from the operator region of the lac operon (Sambrook et al., 1989).

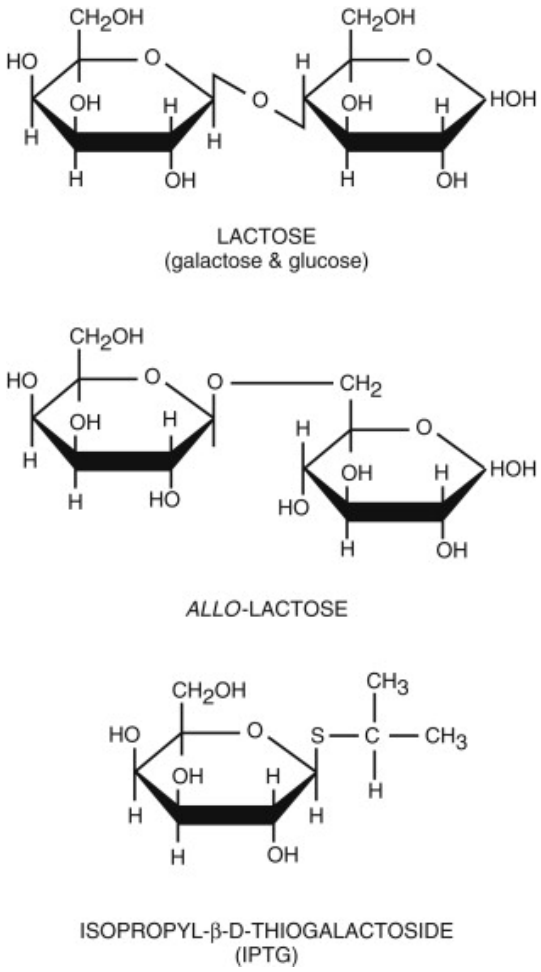

Figure 6 Structural Comparison of Lactose, Allolactose, and IPTG: This figure depicts the chemical structures of lactose, allolactose, and isopropyl-β-D-thiogalactopyranoside (IPTG), highlighting their roles in gene regulation. Lactose is a natural disaccharide consisting of galactose and glucose linked by a β-1,4 glycosidic bond. It serves as both a substrate for metabolism and a precursor to allolactose, which is a β-1,6 isomer of lactose. Allolactose acts as the natural inducer of the lac operon by binding to and deactivating the lac repressor protein, thereby facilitating gene transcription. IPTG is a synthetic analog of allolactose designed to mimic its function. Due to its structural similarity, IPTG effectively binds to the lac repressor but is resistant to metabolic degradation, providing a stable and consistent method for operon

induction in controlled experimental settings, such as RNA interference (RNAi) studies. *Lactose Operon - an overview | ScienceDirect Topics*. (n.d.). Www.sciencedirect.com. https://www.sciencedirect.com/topics/medicine-and-dentistry/lactose-operon

However, unlike lactose or allolactose, IPTG is non-metabolizable, which means it is not degraded or consumed during bacterial growth (Grabski et al., 2019). This ensures sustained induction of the operon and continuous transcription of the dsRNA-producing gene (Sambrook & Russell, 2001). This property makes IPTG an ideal inducer for RNAi experiments, providing consistent and long-term gene expression (Timmons & Fire, 1998). The molecular interaction between IPTG and the lac operon, depicted in Figure 7, demonstrates how IPTG inactivates the repressor, enabling RNA polymerase to initiate transcription of the desired gene (Chen et al., 2015).

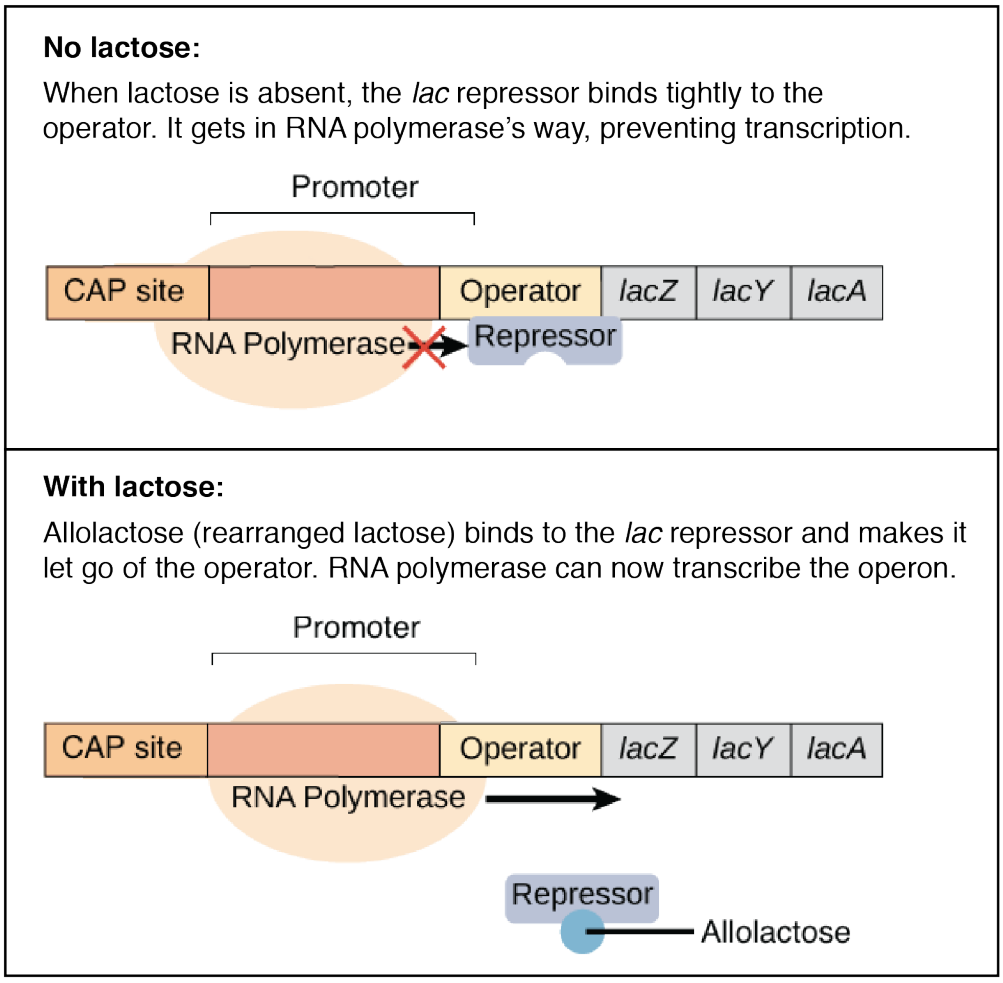

Figure 7: Mechanism of Lac Operon Regulation. This figure illustrates the regulation of the lac operon in bacteria, highlighting the role of the lac repressor and inducer molecules. In the absence of an inducer (top), the lac repressor binds to the operator sequence, physically blocking RNA polymerase from transcribing the downstream genes (lacZ, lacY, and lacA) required for lactose metabolism. When an inducer such as allolactose or its analog IPTG (isopropyl-β-D-thiogalactopyranoside) is present (bottom), it binds to the repressor, causing a conformational change that releases the repressor from the operator. This allows RNA polymerase to bind to the promoter region and initiate transcription of the lac operon, facilitating gene expression. IPTG is particularly useful in experimental setups due to its stability and resistance to degradation,

ensuring consistent induction of target genes. Khan Academy. (2021). *The lac operon* . Khan Academy. https://www.khanacademy.org/science/ap-biology/gene-expression-and-regulation/regulation-of-gene-expression-and-cell-specialization/a/the-lac-operon

The molecular interaction between IPTG and the lac operon, depicted in Figure 7, demonstrates how IPTG inactivates the repressor, enabling RNA polymerase to initiate transcription of the desired gene (Chen et al., 2015). RNAi-induced gene silencing using IPTG-inducible bacterial strains allows for precise genetic modulation, creating a controlled environment to study the effects of daf-2 knockdown on lifespan (Timmons & Fire, 1998). However, without sterilization, the presence of progeny in experimental populations could introduce significant variability, confounding the analysis of lifespan and stress response (Grishok et al., 2000).

1.5 Experimental Consistency with FUdR

To ensure experimental consistency in aging studies, 5-fluoro-2'-deoxyuridine (FUdR) plays a crucial role by sterilizing adult *C. elegans* and preventing progeny from complicating lifespan observations (Mitchell et al., 1979). FUdR, a thymidine analog, incorporates into DNA and inhibits replication during cell division, effectively halting reproduction (Gandhi et al., 1980). This ensures that population synchronization is maintained, enabling accurate assessment of aging phenotypes (Hosono et al., 1982). Importantly, FUdR is applied after worms reach the L4 larval stage to avoid interference with developmental processes (Anderson et al., 2009). This approach facilitates the study of genetic and environmental interactions, such as the role of RNAi-induced *daf-2* knockdown and temperature variations, in regulating lifespan without confounding effects from progeny (Aitlhadj & Stürzenbaum, 2010). By leveraging FUdR, the

study achieves a controlled experimental environment, critical for elucidating the molecular mechanisms underlying aging (**Figure 8**).

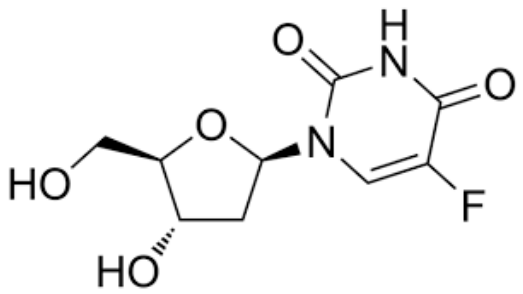

Figure 8: Chemical Structure of 5-Fluoro-2′-Deoxyuridine (FUdR). This molecule, a thymidine analog, is used in *Caenorhabditis elegans* experiments to inhibit DNA synthesis, thereby sterilizing the worms. By preventing reproduction, FUdR ensures synchronized populations and eliminates the confounding effects of progeny, allowing precise investigation of genetic and environmental influences on aging. *Floxuridine*. (2023, January 29). Wikipedia. https://en.wikipedia.org/wiki/Floxuridine

1.6 Integration of Genetics and Environment in Aging Research

This study focuses on understanding the dynamic interplay between genetic and environmental factors influencing aging, specifically through RNAi-mediated daf-2 knockdown and exposure to variable temperatures. Aging research has extensively studied genetic pathways and environmental stressors independently, but a significant gap remains in how these factors interact to modulate lifespan (Rikke & Johnson, 2004; Gems & Partridge, 2013). It is anticipated that RNAi-mediated knockdown of daf-2**,** combined with variable temperature exposures, will extend

the lifespan of *Caenorhabditis elegans* (*C. elegans*) by enhancing stress resilience and activating protective genetic pathways. Specifically, lower temperatures are expected to further augment the longevity effects of daf-2 knockdown by reducing metabolic demands and oxidative stress, while higher temperatures may limit these benefits due to increased metabolic strain.

This research bridges the gap in aging studies by addressing how IIS suppression interacts with environmental temperature variations to regulate lifespan. By leveraging the advantages of *C. elegans* as a model organism and combining genetic and environmental interventions, this study aims to contribute valuable insights to the broader field of aging research with potential translational applications for human health.

2. MATERIALS AND METHODS

2.1 Study design

The experiment was designed to investigate the interaction between *daf-2* knockdown and temperature variations on the lifespan of *C. elegans*. Eight experimental groups were established (Table 1), combining two factors: RNAi treatment (knockdown vs. no knockdown) and temperature exposure (4°C, 10°C, 20°C, and 30°C). Each group consisted of synchronized populations of worms treated and maintained under controlled conditions as shown in Figure 8. The independent variables were the RNAi treatment (knockdown of *daf-2*) and the environmental temperatures, while the dependent variable was the lifespan of the worms, measured in days.

Independent Variables	1. RNAi medicated daf-2 knockdown (RNAi + , RNAi-)
	2. Temperature (4°C, 10°C, 20°C, 30°C)
Dependent Variable	Lifespan (In days)
Groups	1. RNAi treatment + 4°C.
	2. RNAi treatment + 10°C
	3. RNAi treatment + 20°C
	4. RNAi treatment + 30°C
	5. No RNAi treatment + 4°C
	6. No RNAi treatment + 10°C
	7. No RNAi treatment + 20°C (Control)
	8. No RNAi treatment + 30°C

Table 1: **Experimental design outlining** the independent and dependent variables for the study. The independent variables include temperature (4°C, 10°C, 20°C, and 30°C) and RNA interference (RNAi) treatment targeting the daf-2 gene. The dependent variable is the lifespan of Caenorhabditis elegans, measured in days. Eight experimental groups were established: (1) RNAi treatment + 4°C, (2) RNAi treatment + 10°C, (3) RNAi treatment + 20°C, (4) RNAi treatment + 30°C, (5) No RNAi treatment + 4°C, (6) No RNAi treatment + 10°C, (7) No RNAi treatment + 20°C, and (8) No RNAi treatment + 30°C. RNAi refers to the gene silencing technique used to suppress the daf-2 gene. Lifespan is recorded for all groups to assess the interaction between genetic and environmental factors.

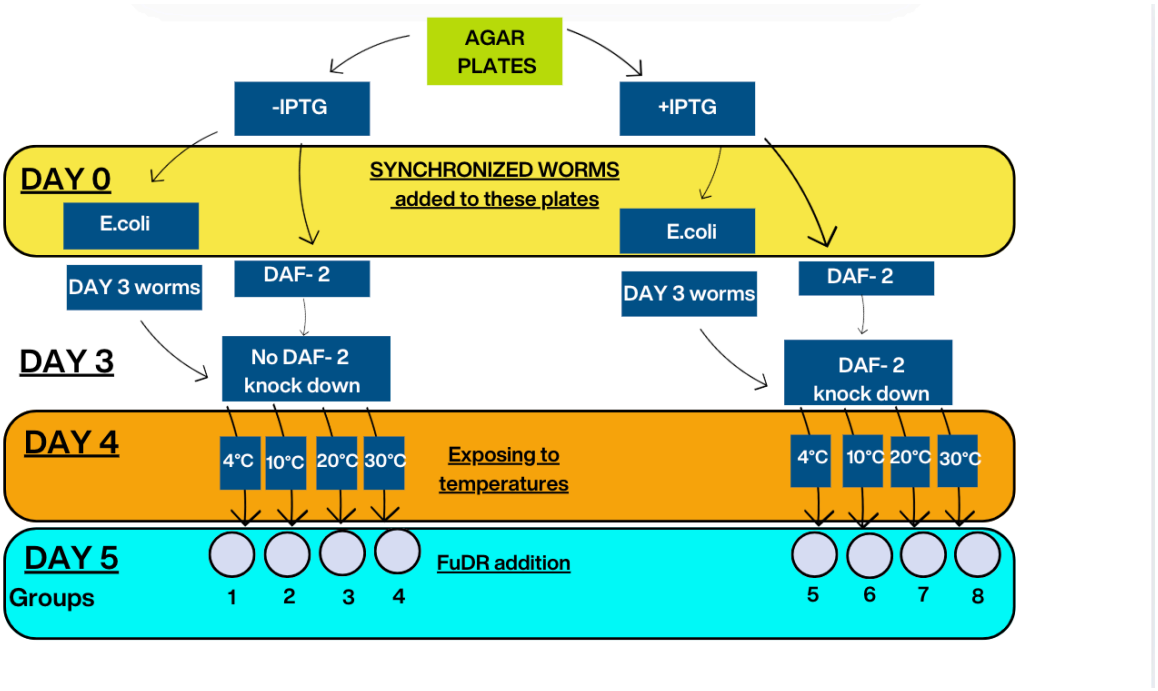

Figure 9: Overview of the experimental workflow and group allocation for assessing the effects of temperature and daf-2 RNAi knockdown on C. elegans lifespan. Synchronized worms were cultured on Agar plates prepared with or without Isopropyl β-D-1-thiogalactopyranoside **(**IPTG). Worms were fed Escherichia coli expressing daf-2 dsRNA for RNAi induction or control E. coli (non-RNAi treatment). On Day 0, synchronized worms were plated, and Day 3 worms were exposed to RNAi-induced daf-2 knockdown. Experimental groups (1–8) were then subjected to four temperature conditions (4°C, 10°C, 20°C, and 30°C**)** with or without daf-2 RNAi treatment. Day 5 5-fluoro-2′-deoxyuridine (FUdR) was added to inhibit reproduction and maintain synchronized populations during lifespan monitoring. This workflow illustrates the integration of genetic and environmental factors in the experimental design.

2.2 Establishing a *C. elegans* Colony and maintenance.

Wild-type *C. elegans* (N2 strain) were cultured on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50, using the *C. elegans* Culture Kit (Carolina Biological Supply, Catalog No. 173525). (*"Caenorhabditis Elegans Culture Kit,"* 2023) The kit provided essential materials, ensuring a standardized and contamination-free environment for maintaining healthy populations. Worms were kept at a standard laboratory temperature of 20°C, ideal for their growth and reproduction.

To prepare NGM plates, 3 g Beef Extract, 15 g agar, and 5 g peptone were dissolved in 1L distilled water, autoclaved, and allowed to cool. (Leboffe & Pierce, 2012) The medium (30ml) was poured into sterile Petri dishes and allowed to solidify at room temperature. Plates were dried for 24 hours at room temperature to remove excess moisture before seeding. (Stiernagle, 2019)

For the *E. coli* OP50 stock culture, nutrient broth was prepared by dissolving 3 g beef extract and 5 g peptone in 1 L distilled water. (Leboffe & Pierce, 2012) The medium was poured in multiple tubes and autoclaved to ensure sterility, whenever needed tubes were inoculated with *E. coli* OP50, and incubated overnight at 37°C to establish a robust bacterial stock. The bacterial culture was then centrifuged to concentrate cells, and the resulting suspension was used to seed the NGM plates. A small aliquot of the bacterial culture was pipetted onto the center of each plate, spread evenly, and incubated at room temperature for 24 hours to establish a uniform lawn.

C. elegans were transferred to fresh plates by aseptically cutting a chunk of agar containing actively growing worms and placing it onto the new seeded plate. This method minimized handling stress and ensured the transfer of all life stages. Worms were maintained at 20°C and transferred to fresh plates every 3–4 days to prevent overcrowding and starvation. Contamination

was monitored daily, and compromised plates were promptly discarded. By combining the preparation of nutrient broth, bacterial stock, and NGM plates, this approach ensured a consistent and contamination-free environment for maintaining *C. elegans* populations under experimental conditions.

2.3 Age Synchronization Protocol

To achieve uniform developmental stages for experimental worms, synchronization was conducted at the L4 larval stage, a critical step to ensure consistent and reliable experimental outcomes. (Figure 2) The process began by dislodging worms from NGM plates using M9 buffer, a solution prepared according to the recipe in Table 2 and autoclaved before use (Table 3-Step 1). The resulting suspension was transferred to sterile centrifuge tubes (Table 3-Step 2), centrifuged to separate the worms from the buffer (Table3-Step 3), and the supernatant was removed (Table3-Step 4).

Component	Amount
Sodium Phosphate Dibasic Heptahydrate (mw: 268.07 g/mol)	64g
Potassium Phosphate Monobasic (mw: 136.09 g/mol)	15g
Sodium chloride (mw: 58.44 g/mol)	2.5g
Ammonium chloride (mw: 53.49 g/mol)	5g
water	1L mark

Table 2: Recipe for M9 Solution. This table outlines the components and their respective quantities required to prepare 1 liter of M9 solution. The solution includes sodium phosphate dibasic heptahydrate (64 g), potassium phosphate monobasic (15 g), sodium chloride (2.5 g), and ammonium chloride (5 g). These components are dissolved in distilled water up to a 1-liter mark. M9 solution is an essential buffer used for washing and maintaining *C. elegans* in experimental

protocols, providing an isotonic environment that supports the viability and integrity of the nematodes. *M9 Minimal Salts Preparation and Recipe | AAT Bioquest*. (2024). Aatbio.com. https://www.aatbio.com/resources/buffer-preparations-and-recipes/m9-salts

Material required	c. elegans	
	20M bleaching solution	
	M9 solution	
	centrifuge	
	centrifuge tubes	
	2 sterile containers	
	orbital shaker	
Procedure		
step 1	Dislodge Worms	add few drops of M9 solution on each plate with worms and swirl it to dislodge the worms
step 2	Transfer to Centrifuge Tubes	using a sterile pipet transfer the solution to a centrifuge tube (each tube is for one plate of worms and label them)
step 3	Centrifuge	centrigue these tubes for 1 min on high speed
step 4	Remove Supernatant	using pipet remove the M9 solution without disturbing the bottom
step 5	Add Bleaching Solution	add few drops of 20M bleaching solution in all tubes(shake and flip them upside down) (bleach no more
step 6	Centrifuge	centrigue these tubes for 1 min on high speed
step 7	Remove Bleaching Solution	using pipet remove the 20M bleaching solution without disturbing the bottom
step 8	Wash Eggs	add few drops of M9 solution in these tubes to wash eggs
step 9	Centrifuge	centrigue these tubes for 1 min on high speed
step 10	Repeat Wash	add few drops of M9 solution in these tubes
step 11	Final Centrifugation	centrigue these tubes for 1 min on high speed
step 12	Incubate on Orbital Shaker	move these tubes to the orbital shaker over night
step 13	Transfer to Fresh Plates	pour the solution from these tubes on new seated plates

Table 3: Age Synchronization Protocol for C. elegans

This table outlines the step-by-step procedure for synchronizing C. elegans populations at the L4 larval stage. The materials required include live C. elegans, 20M bleaching solution, M9 solution, a centrifuge, sterile centrifuge tubes, and an orbital shaker. The protocol begins with dislodging worms from plates using M9 solution (Step 1) and progresses through centrifugation (Step 3), the application of a bleaching solution to isolate eggs (Step 5), multiple washes to remove residual bleach (Steps 8–10), and incubation on an orbital shaker to allow eggs to hatch synchronously into L1 larvae (Step 12). The final step involves transferring larvae to fresh NGM plates seeded with E. coli for further growth and experimental preparation. This standardized approach ensures uniformity in developmental stages across experimental populations. Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A., & Cerón, J. (2012). Basic Caenorhabditis elegans Methods: Synchronization and Observation. *Journal of Visualized Experiments*, *64*. https://doi.org/10.3791/4019

The synchronization employed a bleaching method, which used a 20M bleaching solution to dissolve adult worm bodies while leaving behind viable eggs. This 20M bleaching solution was prepared by combining of 20mL bleach solution with 80mL of distilled water. This does not require autoclaving because sodium hypochlorite is a strong disinfectant that naturally eliminates contaminants and heating it can release toxic chlorine gas and reduce its effectiveness. This step was crucial, as the bleaching solution breaks down adult cuticles and body tissues, but the robust eggshells remain intact (Table3-Step 5). After adding the bleaching solution, the tubes were shaken to ensure proper exposure (Table3-Step 6). The bleaching solution was removed (Table3-Step 7), and the eggs were washed multiple times with M9 buffer (Table3-Steps 8-10) to eliminate any residual bleaching agent, ensuring the safety and viability of the eggs.

Following the washes, a final centrifugation was performed to isolate the eggs (Table3-Step 11). The eggs were incubated on an orbital shaker overnight (Step 12) to allow synchronous hatching into L1 larvae. The freshly hatched larvae were then transferred to fresh NGM plates seeded with E. coli (Table3-Step 13) to grow into the L4 stage under controlled conditions. (Figure 3)

This method ensured that all experimental worms began at the same developmental stage, minimizing variability and enhancing the accuracy of lifespan analysis. As illustrated in Figure 3, the C. elegans lifecycle provides a clear progression from egg to adulthood, highlighting the importance of precise synchronization. The stepwise bleaching protocol detailed in Table 2 ensured consistency and reproducibility, forming a robust foundation for experiments involving RNAi and temperature variations.

2.4 RNA Interference by feeding.

The RNA interference (RNAi) technique was utilized to silence the daf-2 gene in *Caenorhabditis elegans* to study aging-related pathways. The experimental setup relied on the use of pre-transformed pAD48-daf-2 RNAi plasmid (Figure 10), specifically designed to express double-stranded RNA (dsRNA) targeting the daf-2 gene. This plasmid integrates essential components of the lac operon to facilitate IPTG-inducible dsRNA production, ensuring precise and effective gene silencing.

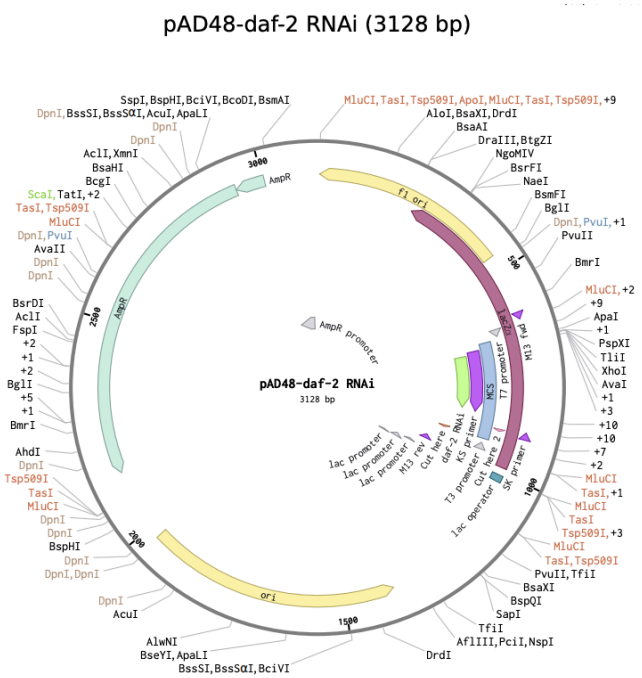

Figure 10 Map of the pAD48-daf-2 RNAi Plasmid: The pAD48-daf-2 RNAi plasmid (3128 bp) is specifically engineered for the RNAi-mediated knockdown of the daf-2 gene in *Caenorhabditis elegans*. The plasmid contains the *daf-2* gene insert (highlighted in red), flanked by essential regulatory elements, including the T7 promoter and lac operator, to facilitate IPTG-inducible dsRNA production. Additional features include the origin of replication (Ori), antibiotic resistance gene (AmpR) for plasmid selection, and multiple restriction sites for genetic modification. This vector enables precise gene silencing in RNAi experiments, aiding in the study of IIS pathway regulation and lifespan modulation. *Addgene: pAD48-daf-2 RNAi*. (2024). Addgene.org. https://www.addgene.org/34834/

2.4.1 Preparation of RNAi Plates: For the experiments, standard agar plates were prepared and supplemented with 1 mM isopropyl-β-D-thiogalactoside (IPTG) prepared by 0.23831 g of IPTG and filling it upto 1 L mark with distilled water. To ensure even distribution and absorption, 1

mL of IPTG solution was pipetted onto the surface of the agar, followed by four days of drying at room temperature. Once the plates were fully dried, bacterial cultures containing the pAD48-daf-2 plasmid were seeded onto the surface and incubated at room temperature for 24 hours to establish uniform bacterial lawns. This preparation method allowed robust induction of dsRNA in the bacterial strains.

2.4.2 Worm Feeding Protocol**:** Synchronized L1-stage worms, obtained via the bleaching method, were gently transferred onto the prepared RNAi plates. The worms were then allowed to feed on the IPTG-induced bacterial lawns at 20°C as they progressed through their larval stages. This ensured effective knockdown of the daf-2 gene by the ingestion of dsRNA-producing bacteria, enabling a reliable exploration of the gene's role in aging and stress response pathways.

The RNAi feeding technique used in this study provided a robust framework for gene silencing, leveraging IPTG pre-treatment for consistent dsRNA induction in the bacterial strains. By combining RNAi with precisely synchronized worm populations, this approach ensured reproducibility and reliability in assessing the functional impacts of daf-2 knockdown on aging and related phenotypes.

2.5 Exposure to Variable Temperatures

Worms will be exposed to variable temperature conditions after synchronization and RNAi treatment. Plates from previous step (day 4) were transferred to incubators set at the specified temperatures of 4°C, 10°C, 20°C, or 30°C. These temperature conditions were maintained consistently to study their impact on the lifespan of *C. elegans* under controlled

experimental setups. The exposure to variable temperatures allowed for the examination of environmental influences on aging mechanisms.

2.6 FUdR Optimization

To inhibit reproduction in adult *C. elegans* while ensuring normal growth and development, 5-fluoro-2′-deoxyuridine (FUdR) was added to the plates at the L4 larval stage (day 5), just before the worms transitioned into adulthood. This timing was critical to avoid interference with growth and ensured that reproduction alone was suppressed. Four concentrations of FUdR (10 µM, 15 µM, 20 µM, and 25 µM) were prepared by dissolving FUdR powder in sterile water. As FUdR has a molecular weight of approximately 246.2 g/mol, the required quantities were calculated as 0.00246 g for 10 µM, 0.00369 g for 15 µM, 0.00492 g for 20 µM, and 0.00615 g for 25 µM, each dissolved in 1 liter of water. These stock solutions were filter-sterilized and stored at 4°C until use.

For experimental setup, 1mL of the respective FUdR solution was pipetted onto the agar surface of each plate. The plates were left at room temperature for 24 hours to allow the solution to absorb evenly into the agar, ensuring consistent distribution and effective incorporation. Adding FUdR at the L4 stage specifically targeted reproduction without affecting the development or physiology of the adult worms, allowing for accurate lifespan analysis focused solely on the parental population. The careful optimization of four different concentrations ensured that a range of FUdR levels was tested for effectiveness. This approach maintained synchronized populations and eliminated confounding factors such as overcrowding, resource depletion, or progeny interference, enhancing the reliability and reproducibility of the experimental results.

3. RESULTS

The foundational phase of this study focused on establishing essential experimental conditions and validating initial methodologies for aging research in *Caenorhabditis elegans* (*C. elegans*). Key achievements include the successful maintenance and growth of a healthy *C. elegans* colony under controlled laboratory conditions, ensuring experimental reproducibility and reliability. Synchronization of worm populations was achieved, with larvae successfully aligned at the same developmental stage, eliminating variability associated with asynchronous growth. This step is critical for ensuring consistency in lifespan analyses and environmental interventions.

Additionally, optimization of 5-fluoro-2'-deoxyuridine (FUdR) concentrations was conducted to determine the ideal sterilization conditions without adversely affecting survival outcomes. Worms exposed to FUdR concentrations of 10 μM, 15 μM, and 20 μM demonstrated sustained viability, while concentrations of 25 μM led to reduced survival. Based on these observations, lower concentration 20 μM was selected for future experiments to maintain a sterile environment without impacting the health or stress response of the worms.

Although the research is ongoing and lifespan results are not yet available, these initial successes lay a strong foundation for the subsequent analysis of RNAi-induced *daf-2* knockdown effects under variable temperature conditions. The establishment of healthy synchronized populations and optimization of sterilization protocols provide the experimental consistency required for robust and meaningful findings in aging research.

4. DISCUSSION

This study aims to explore the interaction between RNAi-mediated daf-2 knockdown and temperature variations in modulating the aging process of *Caenorhabditis elegans*. While experimental data is not yet available, the groundwork laid in establishing synchronized colonies and optimizing FUdR concentration demonstrates the study's feasibility and scientific rigor. These foundational steps ensure accurate and reproducible assessment of lifespan in response to genetic and environmental interventions.

The expected results anticipate that RNAi-induced daf-2 knockdown will extend the lifespan of *C. elegans* at all tested temperatures, albeit with variations based on the specific thermal conditions. At lower temperatures, such as 4°C, the combination of IIS suppression and reduced metabolic activity is hypothesized to result in the most significant lifespan extension. This is likely due to lower rates of metabolic stress and oxidative damage, which complement the protective effects of DAF-16 activation. Moderate temperatures (10°C and 20°C) are expected to show lifespan benefits from RNAi treatment, though less pronounced than at 4°C due to relatively higher metabolic demands. At 30°C, while daf-2 knockdown may provide some resilience against heat stress, the protective effects of IIS suppression are expected to be limited by the accelerated metabolic rates and cellular damage associated with higher temperatures.

A major accomplishment of this study was the successful synchronization of *C. elegans* at the L4 larval stage, a critical step to ensure uniform developmental stages across experimental groups. This synchronization reduces variability, allowing for a more accurate evaluation of genetic and environmental influences on aging. Additionally, determining the effective FUdR concentration (25 μM) ensures sterility without affecting the worms' growth or behavior, providing consistent conditions for lifespan analysis.

The RNAi methodology utilized in this study highlights the utility of gene-silencing techniques in addressing fundamental biological questions. By silencing the daf-2 gene, this study provides a platform to investigate how IIS signaling integrates with environmental factors to modulate aging. The ability to conduct such experiments in a controlled system underscores the potential of *C. elegans* as a model for exploring complex interactions between genetic pathways and environmental variables.

The anticipated outcomes of this research represent a significant contribution to aging biology, as they will provide novel insights into how temperature interacts with IIS suppression to regulate lifespan. This study addresses a critical gap in understanding the combined effects of genetic and environmental factors on aging, offering a fresh perspective on the dynamic interplay between these influences. The results have broader implications for translational research, particularly in identifying strategies to enhance stress resilience and mitigate age-related conditions in higher organisms, including humans.

Future studies can build on this work by incorporating additional environmental variables, such as oxidative stress or dietary restrictions, to explore multifaceted interactions with IIS signaling. Validation of RNAi efficiency through techniques such as qRT-PCR or Western Blotting would further strengthen the study's conclusions and provide a more detailed understanding of the molecular mechanisms underlying observed lifespan changes.

Some limitations of the study include the reliance on feeding-based RNAi, which can result in variability in gene knockdown efficiency among individual worms. While ELISA was considered as a validation method for RNAi efficiency, future research could benefit from molecular approaches like qRT-PCR or Western Blotting for precise quantification of

knockdown effects. Additionally, the use of FUdR to prevent reproduction, while effective, may subtly impact stress response or metabolic pathways. The synchronization method involving bleaching, although widely used, could introduce stress to early-stage larvae. Expanding sample sizes and incorporating alternative synchronization or sterilization techniques may help mitigate these concerns.

In summary, this study establishes a solid foundation for exploring the molecular and environmental determinants of aging. By integrating genetic interventions with temperature variability, it offers a unique approach to understanding aging dynamics and lays the groundwork for future research into strategies for improving healthspan and resilience to stress in both model organisms and humans.

CONCLUSION

This study investigated the interplay between RNAi-mediated *daf-2* knockdown and temperature variations in modulating the lifespan of *Caenorhabditis elegans*, focusing on the integration of genetic and environmental factors in aging. By silencing the IIS pathway, the research anticipates notable lifespan extension at lower temperatures due to reduced metabolic stress and enhanced cellular resilience, while higher temperatures are expected to diminish these benefits due to increased heat-induced cellular damage. The establishment of synchronized worm populations and optimized experimental parameters demonstrated the robustness of this model for aging research. These findings not only reinforce the utility of *C. elegans* as a model organism but also provide a foundation for understanding how genetic pathways and environmental factors converge to influence longevity. This research opens avenues for future

studies to refine these findings and develop interventions aimed at mitigating age-related decline and enhancing stress resilience in higher organisms, including humans.

ACKNOWLEDGMENTS:

I would like to express my deepest gratitude to my instructors at Capilano University's STEM Faculty, including Eugene Chu, Mark Vaughan, Eunice Chin, Mahta Khosravi, Mahshid Atapour, Nazar Kovalevskyy, Ann Meitz, Matt Berry, Casper McWilliam, and Christine Kondratev, for their invaluable guidance and support throughout this research project. Their expertise and encouragement have been instrumental in shaping this study.

I extend my heartfelt thanks to my lab partner, Maria Jose Pena Pena, for her collaboration and dedication to our shared research goals. Working alongside her has been a rewarding experience that greatly enriched the quality of this project.

Special thanks to my husband, Ashish Nayyar, for his unwavering support and encouragement, which have been a constant source of strength throughout this endeavor. I am also immensely grateful to my classmates and friends—Emily Derrick, Alice, Patrick, and Roberto—for their camaraderie and insightful discussions, which provided both inspiration and motivation.

Lastly, I acknowledge the resources and infrastructure provided by Capilano University, without which this research would not have been possible. Thank you all for your contributions and support in making this project a reality.

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