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MECANISMOS MOLECULARES QUE LEVAM À FERTILIDADE REDUZIDA E AUMENTO DO SILENCIAMENTO DE TRANSGENES EM *CAENORHABDITIS ELEGANS* EM RESTRIÇÃO CALÓRICA

MOLECULAR MECHANISMS LEADING TO REDUCED FERTILITY AND INCREASED TRANSGENE SILENCING IN CALORIE RESTRICTED *CAENORHABDITIS ELEGANS*

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de Doutor em Genética e Biologia Molecular, na área de Genética Animal e Evolução.

Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Genetics and Molecular Biology, in the area of Animal Genetics and Evolution.

Orientador: MARCELO ALVES DA SILVA MORI

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Os membros da Comissão Examinadora acima assinaram a Ata de defesa, que se encontra no processo de vida acadêmica do aluno.

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Resumo

A restrição calórica (RC) promove longevidade em diversos organismos, incluindo *C. elegans*, porém também resulta em diminuição da fertilidade. Investigamos a causa dessa redução na fertilidade, com foco no papel da espermatogênese e oogênese. Nossos estudos demonstraram que a redução da fertilidade em um modelo genético de RC (mutantes *eat-2*) se deve, principalmente, a um defeito na linhagem germinativa feminina, possivelmente envolvendo oócitos. Surpreendentemente, o sequenciamento de RNA de células únicas revelou diferenças expressivas na espermatogênese em RC. Identificamos o fator de transcrição HLH-30 como essencial para regular a reprodução em vermes em RC, sendo sua atividade vital para manter a fertilidade adequada do espermatozoide e o desenvolvimento embrionário durante a RC. Adicionalmente, examinamos os padrões de herança da fertilidade reduzida em *C. elegans* submetidos à RC. Descobrimos que a diminuição da fertilidade em mutantes *eat-2* pode ser transmitida aos descendentes de uma forma dependente do sexo. Também observamos o silenciamento transgeracional de um transgene GFP em resposta ao cruzamento com machos mutantes *eat-2* ou selvagens, o qual depende dos sinais de ambos os pais para sua transmissão. Esse silenciamento não foi geral para todos os transgenes, sendo influenciado pelo contexto cromossômico. Apresentamos evidências que sugerem que o alvo principal do silenciamento é um transgene que expressa a argonauta RDE-1 sob um promotor muscular. O silenciamento se espalha para o GFP devido à proximidade genômica. Por último, descobrimos que o silenciamento do transgene está relacionado à redução da resistência ao estresse oxidativo e a uma expressão alterada de retrotransposons. Nossas descobertas fornecem informações valiosas sobre os mecanismos subjacentes às características reprodutivas e hereditárias de animais sob RC. Além disso, nossos resultados aprimoram nosso entendimento dos mecanismos moleculares por trás das mudanças na fertilidade sob RC, revelando um papel para HLH-30 nesse processo.

Abstract

Caloric restriction (CR) extends lifespan in various organisms, including *C. elegans*, but also leads to reduced fertility. We explored the cause of this fertility reduction, focusing on the role of spermatogenesis and oogenesis. Our experiments revealed that the fertility decline of a genetic model of CR (i.e., *eat-2* mutants) primarily stems from a defect in the female germline component, likely involving oocytes. Surprisingly, single-cell RNA sequencing exposed notable gene expression differences related to spermatogenesis between wild type and *eat-2* mutant sperm. We identified the transcription factor HLH-30 as crucial for regulating reproduction in *eat-2* mutant worms, as its activity is vital for maintaining proper sperm fertility and embryo development under CR. Furthermore, we investigated the inheritance patterns of reduced fertility in *C. elegans* subjected to CR. We discovered that reduced fertility in *eat-2* mutants can be transmitted to offspring with sex-specific differences in transmission. Additionally, we observed heritable silencing of a GFP transgene in response to crossing *eat-2* mutants or wild type males with GFP-expressing worms, dependent on signals from both parents. This silencing was not general to all transgenes and was influenced by the chromosomal location of the transgenes. We presented evidence suggesting the primary target of silencing is a transgene expressing RDE-1 under a muscle promoter, which spreads to GFP due to its genomic proximity. Lastly, we found that transgene silencing is linked to reduced oxidative stress resistance and altered transposon expression. Our findings offer valuable insights into the mechanisms underlying reproductive and inheritable traits in animals under CR. Additionally, our results advance our understanding of the molecular mechanisms behind fertility changes under CR, uncovering a role for HLH-30 in this process.

Summary

Introduction

Aging is a complex process characterized by the gradual accumulation of cellular and tissue damage over time, resulting in progressive dysfunction of cells and tissues and eventually leading to diseases and death [1]. Aging is influenced by a combination of genetic and environmental factors. The world's population is aging, with the number of individuals aged 65 or older increasing from 727 million in 2020 to an estimated 1.5 billion by 2050 [2]. This demographic shift is associated with challenges such as increased social security and healthcare expenditures and a shrinking workforce, considering that only 47% of the elderly man and 23.8% of the elderly women perform some work activity [2]. These issues stem from the prevalence of disabling diseases affecting 60% of individuals over 60 years of age [2]. Aging is the primary risk factor for diseases such as cancer, type 2 diabetes, cardiovascular disease, and neurodegenerative disorders [3].

Interventions aimed at promoting healthy aging and extending the disease-free period of life are of great interest. Calorie restriction (CR) has been shown to be effective in enhancing healthy aging and extending the lifespan of various organisms, from yeast to primates [4–6]. CR involves reducing calorie intake without compromising essential nutrient intake[7]. The beneficial effects of CR have been linked to multiple molecular pathways, including the activation of sirtuins [8], AMP-activated protein kinase (AMPK) [9], and the inhibition of insulin/insulin-like growth factor 1 (IGF-1) and the mechanistic target of rapamycin (mTOR) signaling [10,11].

Additionally implicated in the response to CR is the transcription factor HLH-30 in *C. elegans* and its human homologs, TFEB and TFE3. These transcription factors are activated by various stressors, including starvation [12,13], oxidative stress [14], DNA damage [15], and mitochondrial stress [16]. They regulate numerous genes and coordinate stress response pathways such as autophagy activation, lysosomal activity, and metabolic adaptation [12,17,18]. In *C. elegans*, HLH-30 nuclear translocation is triggered by CR through the *eat-2* mutation, insulin signaling inhibition via the *daf-2* mutation, or compromised mitochondrial function [19]. Interestingly, in mice, TFEB promotes cell migration in the testis [20], while in *C. elegans* HLH-30 expression in the *C. elegans* intestine protects the worms from mating-induced death [21], revealing an intricate relation of this transcription factor with reproduction.

Intriguingly, HLH-30 plays a critical role in normal recovery from various dietary stress scenarios in worms, including recovery from two development arrest situations induced by stress during development. These arrested developmental states resume once the environment becomes more conducive to a healthy life. Worms with *hlh-30* mutations are unable to recover from the developmental arrest situations of adult reproductive diapause (ARD) [22] and the *dauer* stage in *daf-2* mutants [23], both of which are associated with stress resistance and food scarcity.

Notably, autophagy activation has been demonstrated to be essential for the lifespan-extending effects elicited by CR, and it is dependent on the transcription factor PHA-4 in *C. elegans* [24]. Autophagy is activated by numerous pathways necessary for CR-induced longevity including HLH-30 [12], AMPK [25], and inhibition of IGF-1 [26], and mTOR signaling [27]. Thus, autophagy serves as one of the primary downstream effectors of CR's beneficial impact on longevity. Interestingly, autophagy activation alone is not sufficient to promote longevity, and it must be combined with other stimuli, such as CR or inhibition of insulin/IGF-1 signaling [24].

Nonetheless, implementing a caloric restriction (CR) diet presents difficulties, given the necessity for a persistent 20-40% reduction in caloric intake and potential adverse consequences, such as reproductive and behavioral abnormalities [28]. In light of these challenges, researchers have investigated alternative strategies, including intermittent fasting [29], time-restricted feeding [30], selective amino acid restriction [31,32], and the employment of CR mimetics like resveratrol [33] and rapamycin [34,35], with the aim of attaining comparable benefits without the undesirable repercussions typically associated with rigorous CR. Nevertheless, the efficacy of these interventions as CR mimetics remains a subject of debate, as it is uncertain whether their mechanisms of action closely parallel those of CR [36]

Reproductive defects are conserved across species, including mice [37], humans [38], and *C. elegans* [39], under CR. Understanding the mechanisms underlying these fertility decreases may help promote fertility in conditions where CR or similar pathways are activated.

Research has shown that human sperm is highly sensitive to dietary changes, exhibiting altered motility and tRNA expression after just two weeks of a sugar-rich diet [40]. Nevertheless, evidence suggests that human and monkey sperm maintain their function during fasting and CR [41,42], while sperm count decreases during fasting in mice and rats, accompanied by increased sperm head defects [37,43]. Interestingly, In men with obesity, weight loss results in increased sperm

concentration and sperm count [44]. This information indicates that sperm is highly sensitive to transcriptome changes due to diet, and these alterations, at least in humans and monkeys, seem to enable sperm to retain its function even during CR.

Conversely, in females, CR appears to extend the reproductive lifespan. However, this benefit comes with the trade-off of delayed fertility and reduced ovulation. In mice subjected to CR, fertility is almost entirely lost during the restrictive diet, but fertility resumes once they return to an *ad libitum* (AL) diet, and they remain fertile for a longer period than the AL control group [45]. Similarly, in *C. elegans*, CR animals exhibit an extended reproductive lifespan and decreased fertility [46], which might suggest that the female germline could be responsible for this reduction in fertility, although further investigation is needed to confirm this.

A trade-off between fertility and lifespan is often observed, where interventions that reduce fertility frequently lead to increased lifespan and vice-versa [47,48]. In line with this, CR extends lifespan while diminishing fertility. Remarkably, removing germline cells from *C. elegans* via laser ablation or through mutations results in an increased lifespan [48]. This germline removal interacts with CR; eliminating germ cell precursors, but not the somatic gonad, prevents the lifespan-extending effects of CR compared to AL conditions [49]. A potential explanation for this phenomenon is that under stress, organisms prioritize somatic maintenance over reproduction, thus preserving the soma's ability to reproduce when conditions become favorable [48].

The nematode *Caenorhabditis elegans* (*C. elegans*) is an exceptional model organism for investigating the impact of calorie restriction (CR) on fertility, owing to its large progeny (approximately 300 worms), brief generation time (around 3 days), and the availability of thousands of mutants for different genes [50]. *C. elegans* also enables the exploration of genetic factors influencing fertility, as gene interactions can be readily examined through gene silencing, epistasis studies, and visualization of fluorescently-labeled proteins.

C. elegans are hermaphroditic, containing both sperm and oocytes within a single worm capable of self-fertilization to produce self-progeny. However, a small percentage of worms are males (0.1%), and there are mutants lacking sperm that exhibit female-like characteristics. This array of tools renders *C. elegans* a compelling model for fertility research. Consequently, we have selected *C. elegans* as our model organism to examine the mechanisms by which CR influences longevity.

Dietary interventions can impact not only the fertility and aging of the organism, but also the traits displayed by its offspring. Starvation has been shown to change abundance of certain small RNAs and alter the pattern pf rRNA methylation in *C. elegans*, leading to longer lifespans, reduced fertility, and increased resilience to heat stress in subsequent generations[46,51]. Despite these intriguing findings, the mechanisms underlying these effects are not yet fully elucidated.

The phenomenon of epigenetic inheritance, defined as the transfer of information between generations without alterations in the DNA sequence, represents a fascinating dimension of biology, potentially enhancing adaptation to environmental conditions encountered by the parental generation, such as facilitating bacterial avoidance and chemotaxis in *C. elegans* [52,53]. A notable example of epigenetic inheritance is somatic transgenerational silencing, a process in which gene expression is downregulated across multiple generations due to epigenetic modifications, thereby influencing phenotypic outcomes [51] *C. elegans* serves as a widely used model for investigating epigenetic inheritance, including somatic transgenerational silencing, owing to its brief generational span and the relative simplicity of conducting genetic research with this organism.

Transgenerational silencing in *C. elegans* often involves small RNA molecules that silence genes with partially or entirely complementary sequences [54]. The role of small RNAs, such as piRNAs and endogenous siRNAs, in transgenerational epigenetic inheritance is an active area of research [55]. These small RNAs function by guiding argonaute proteins to target complementary sequences, leading to gene silencing or modulation of chromatin structure [56].

In particular, the role of small RNA molecules in transmitting starvation-induced epigenetic changes across generations has garnered significant attention. Recent studies have shown that under starvation conditions, the expression levels of certain small RNAs are altered in *C. elegans*, and these changes can persist for multiple generations, even after the return to normal feeding conditions [51]. These starvation-induced small RNAs appear to regulate the expression of target genes involved in metabolism, stress response, and development, potentially contributing to the observed phenotypic changes in the progeny [51].

Moreover, it has been posited that the intergenerational transmission of small RNAs, along with concomitant epigenetic alterations, might be enabled by germline-specific argonaute proteins and other constituents of the RNA interference (RNAi) machinery [57]. For instance, the nuclear argonaute protein HRDE-1 has been implicated in the transgenerational inheritance of small RNA-

mediated gene silencing in *C. elegans*. HRDE-1 binds to small RNAs and enlists histone-modifying enzymes, such as methyltransferases SET-25 and SET-32, to establish repressive chromatin marks at target sites [56]. Recent research suggests that the double stranded RNAs (dsRNAs) inducing silencing may originate in the soma and subsequently be transmitted to the germline [52]. These pathways are similarly employed for transposon silencing [58].

While numerous investigations have concentrated on germline-mediated transgenerational inheritance [59–62], limited attention has been given to somatic transgenerational silencing, which also possesses the capacity to influence offspring phenotypes. The precise molecular mechanisms governing the transfer of these epigenetic marks and their functional consequences to progeny health and disease susceptibility remain incompletely understood [55]. Additionally, the reasons behind the limited spread of germline silencing to the soma in most instances is a phenomenon that has not yet been fully elucidated [55].

Ultimately, exploring transgenerational epigenetic inheritance in both the germline and somatic lineages, particularly in relation to calorie restriction and starvation, has significant potential to enhance our comprehension of the intricate interplay between genetic and environmental factors in determining an organism's phenotype across generations. Further clarification of these mechanisms may lay the groundwork for the development of innovative approaches to promote healthy aging, preserve fertility, prevent inheritable diseases, support genomic stability through the inhibition of transposon activities, and avert transgene silencing in both experimental and clinical settings.

Hypothesis

In this study, we propose several hypotheses to better understand the effects of CR on fertility and inheritance patterns in *C. elegans*. The hypotheses are as follows:

1- The reduced fertility observed in *C. elegans* under CR conditions is primarily caused by alterations in the male and/or female germline, which may directly affect the overall fertility of the organism.
2- The transcriptome of spermatozoa and oocytes in animals under CR is significantly different from that of animals under *ad libitum* (AL) conditions, potentially revealing unique molecular responses to the CR diet that affects fertility.
3- Specific molecular regulators acting in the germline, such as transcription factors or signaling pathways, may explain the differences in the fertility of animals under CR, and their identification could provide insights into the underlying mechanisms.
4- The transmission of fertility information from a parent experiencing CR to its *ad libitum*-fed offspring may encompass sex-specific variations, potentially uncovering a multifaceted inheritance mechanism.
5- CR may have an impact on transgene silencing in *C. elegans*, influencing the expression of transgenic fluorescent proteins such as GFP and causing physiological changes in the worms.

By testing these hypotheses, we aim to provide a comprehensive understanding of the molecular mechanisms driving fertility changes under CR and advance our knowledge of reproductive biology and epigenetic inheritance in *C. elegans*.

Objectives

In this study, we aimed to address several key objectives to better comprehend the impact of caloric restriction (CR) on fertility and inheritance patterns in *C. elegans*. The objectives of this study include:

1- Examining the cellular mechanisms responsible for the observed decline in fertility in *C. elegans* under CR conditions, with a focus on the role of the male and female germline in the overall fertility of the organism.
2- Investigating the transcriptome of sperm cells and oocytes in animals under CR using single-cell RNA sequencing to determine their responses to the diet.
3- Identifying major molecular regulators that can explain the differences in the fertility of animals under CR.
4- Clarifying the inheritance patterns of fertility alterations under CR by investigating the transmission of fertility traits from parent to offspring and detecting any potential sex-specific differences.
5- Evaluating the impact of CR on transgene silencing in *C. elegans*. Using GFP as an indicator for transgene silencing, we aim to explore the possible effects of CR on the expression of transgenic fluorescent proteins and the subsequent consequences on worm physiology including stress resistance and gene expression.

By addressing these objectives, we hope to offer valuable insights into the molecular mechanisms underlying fertility changes under CR and enhance our understanding of reproductive biology and epigenetic inheritance in *C. elegans*.

Material and Methods

C. elegans maintenance and strains used

The nematodes were kept at different temperatures, including 15, 20, or 25°C, in Petri dishes with Nematode Growth Medium. The medium contained a layer of *E. coli* OP50-1 bacteria at an optical density of 10 and was supplemented with 100 µg/mL of streptomycin.

Strain	Genotype	Description	Source
WT/AL	N2	Ad libitum fed (control) strain	CGC – N2 Bristol
CR	*eat-2*(ad1116) II.	Caloric restricted strain Mutants with reduced food intake	CGC – DA1116
spe-9	spe-9(hc88) I	Mutants with non-viable sperm production at 25°C	CGC – BA671
fog-2	fog-2(q71) V.	Mutants without sperm production	CGC – JK574
hlh-30	*hlh-30*(tm1978) IV.	Mutants with loss of function for the transcription factor *hlh-30*	CGC – JIN1375
hlh-30 GFP	sqIs17 [*hlh-30*p::*hlh-30*::GFP + rol-6(su1006)].	*hlh-30* overexpression construct fused to GFP	CGC – MAH240

Strain	Genotype	Description	Source
RFP+ CR	otIs355 [rab-3p(prom1)::2xNLS::TagRFP] IV. *eat-2*(ad1116) II.	Pan-neuronal nuclear RFP expression. Caloric restricted strain.	CGC – AML5 crossed with DA1116
GPR-1	ccTi1594 [mex-5p::GFP::gpr-1::smu-1 3'UTR + Cbr-unc-119(+), III: 680195] III.	Overexpressing GPR-1 strain, generates chimeric progeny	CGC – PD1594
m*rde-1*	*rde-1*(ne219), kzIs20 [hlh-1p::*rde-1* + sur-5p::NLS::GFP]	Muscle-specific RNAi-sensitive strain	CGC – NR350
ges-1	zcIs18 [ges-1::GFP(cyt)]	Strain expressing GFP under the control of intestine-specific promoter	CGC - SJ4144
rde-1	*rde-1*(ne219)	RNAi-deficient (*rde-1*) mutant strain	CGC – WM27
myo-3	myo-3p::GFP::LacZ::NLS + (pSAK4) myo-3p::mitochondrial GFP	Strain expressing GFP under the control of muscle-specific promoter	CGC - ATU3301

Strain	Genotype	Description	Source
m*rde-1* (oc+)	*rde-1*(ne219), kzIs20 [hlh-1p::*rde-1* + sur-5p::NLS::GFP]	Unsilenced strain – prone to silencing by outcrossing two times with WT males	CGC – NR350
m*rde-1* (oc-)	*rde-1*(ne219), kzIs20 [hlh-1p::*rde-1* + sur-5p::NLS::GFP]	Silenced strain – prone to silencing by outcrossing two times with WT males	CGC – NR350
CR m*rde-1* (oc+)	*rde-1*(ne219), kzIs20 [hlh-1p::*rde-1* + sur-5p::NLS::GFP] *eat-2*(ad1116) II.	Unsilenced strain – prone to silencing by outcrossing two times with CR males. Caloric restricted strain.	CGC – NR350, DA1116
CR m*rde-1* (oc-)	*rde-1*(ne219), kzIs20 [hlh-1p::*rde-1* + sur-5p::NLS::GFP] *eat-2*(ad1116) II.	Silenced strain – prone to silencing by outcrossing two times with CR males. Caloric restricted strain.	CGC - NR350, DA1116
GFP *rde-1* m*rde-1*	ctIs40 [dbl-1(+) + sur-5::GFP] X. *rde-1*(ne300) V; neIs9 X. *eat-2*(ad1116) II.	*rde-1* mutant worms with ubiquitous GFP expression (sur-5 promoter) and muscle-specific *rde-1* rescue (mrde-1)	CGC – WM118, DA1116, BW1940
sur-5	ctIs40 [dbl-1(+) + sur-5::GFP] X.	Strain expressing GFP under the control of a ubiquitous promoter	CGC - BW1940

This table lists the *C. elegans* strains used in the study, detailing their strain name, genotype, a description of the strain, and the source from which the strain was obtained. The description provides insight into the specific characteristics or modifications present in each strain, such as caloric restriction, mutations affecting sperm production, or overexpression of certain genes. The source indicates the reference strain or lab from which the strain was acquired, with the Caenorhabditis Genetics Center (CGC) being a common provider for many strains.

RNA Interference through dsRNA Feeding

To silence specific genes in *C. elegans*, we used RNA interference (RNAi), in which the worms were fed with HT115 (DE3) *E. coli* bacteria that produce double-stranded RNA (dsRNA) complementary to the target gene. The worms were placed in Petri dishes with Nematode Growth Medium containing a layer of *E. coli* bacteria at an optical density of 10. The medium was supplemented with 50 μg/mL of ampicillin and 10 μg/mL tetracycline as antibiotics, as well as 1mM IPTG, which induces the production of the dsRNA under the IPTG inducible T7 polymerase. The HT115 (DE3) *E. coli* bacteria used in this protocol were obtained from an RNAi feeding library provided by Horizon Discovery.

Assessing C. elegans viable Progeny Production

To assess the number of viable progeny produced by individual *C. elegans* worms, a single mother at the L4 larval stage was placed in each well of a 24 well plate. After every 2 days, the mother was transferred to a new well, and the number of viable offspring in each well where eggs had been laid was counted 1 day later, at the L1-L2 stage. This transfer and counting process was repeated every other day until the mother stopped producing viable eggs. The viability of the eggs was determined by evaluating their morphology and observing the absence of larvae on the plate. To determine progeny production in crossed worms, the hermaphrodite parent was placed with six males in each well, which were maintained with the mother until the first transfer to a new well.

Quantifying Spermatozoa and Oocytes

To quantify the number of spermatozoa and oocytes in each worm, we subjected synchronized day 0 adult worms to three washes with M9 buffer followed by incubation in 4% PFA pH 7.2 for 45 minutes. The worms were then washed twice with PBS and incubated in 95% ethanol for 1 minute. Afterward, the worms were washed twice with PBS and incubated with Hoescht 33342 at a concentration of 1 μg/mL for 15 minutes in the dark to stain the nuclei of the cells. Finally, the

worms were washed with PBS, placed on a slide, and imaged using fluorescence microscopy. The number of spermatozoa in the spermatheca and oocytes was determined by counting the number of nuclei stained by Hoescht 33342.

Worm Lysis

In this protocol for worm lysis, fresh enzyme solutions were prepared, and all washes were performed in a microcentrifuge at approximately 600 g. Synchronized day 0 adult worms were first washed five times with M9 buffer followed by one wash with lysis buffer (200 mM DTT, 0.25% SDS, 20 mM HEPES pH 8.0, 3% sucrose). The lysis buffer was left in contact with the worms for 6 minutes and 30 seconds before they were washed five times with M9 buffer. Next, either pronase enzyme (Sigma, P6911, 20 mg/mL in ddH2O), trypsin-EDTA (ThermoFisher, 25200072), or collagenase type IV (Worthington LS004188, 3 mg/mL in PBS) was added and incubated for 15 minutes at room temperature (for pronase) or 30°C (for collagenase and trypsin). Before the trypsin and collagenase type IV incubation, the worms were washed once with PBS to adjust the pH of the solution. During incubation, the nematodes were pipetted against the bottom of the tube approximately 30 times every 2 minutes with a 200 µL pipette and a wide bore pipet tip. Afterwards, a PBS solution with 2% heat-inactivated fetal bovine serum was added to stop the reaction. The lysate was then filtered through a 20 µm cell strainer to remove large pieces of worms and eggs. In the adapted protocol using trypsin, the cells were further processed by centrifugation at 350 g for 10 minutes at 4°C. The trypsin was removed, and the cells were resuspended in PBS or, for dead cell removal using the Dead Cell Removal Kit (Miltenyi Biotec), resuspended in the beads from the kit. The dead cells were then removed according to the manufacturer's protocol.

Single cell sequencing

For the purpose of single cell RNA sequencing, day 0 adult *C. elegans* were obtained under two different conditions - normal feeding (AL) and caloric restriction (CR in *eat-2* mutants). The optimized protocol described in the methods section was used to obtain single cells, and the dead cell removal method was employed. The single cell cDNA library was generated using the Chromium Next GEM Single Cell 3′ Reagent Kit v3.1, which uses beads containing poly(dT) primer to capture poly-A tailed messenger RNA. The cDNA library was sequenced using Novaseq, and the reads were assigned to each cell and aligned using Cell Ranger.

Bioinformatic analysis

The Seurat package (version 4.3.0) in R (version 4.3.0) was utilized to process single-cell RNA sequencing (scRNA-seq) data (reference: https://www.nature.com/articles/s41576-019-0093-7). In brief, raw sequencing reads were filtered and aligned to the WBcel235 reference genome using Cell Ranger (version 6.0.1) from 10x Genomics. Subsequently, feature-barcode matrices were imported into R and filtered to eliminate low-quality cells and genes, maintaining a minimum number of features between 200 and 2500, and capping the expression of mitochondrial genes at 15%. The SCTransform method was employed for normalizing and scaling gene expression, followed by principal component analysis (PCA) to identify significant sources of variation. Known marker genes and cell type-specific gene expression signatures, such as those provided by WormBase (10.1093/genetics/iyac003.), served as the basis for cell type identification. Data visualization was carried out using Uniform Manifold Approximation and Projection (UMAP). The FindMarkers function facilitated differential gene expression analysis. Default parameters were used for all analyses unless otherwise stated.

Pathway analysis

To determine the potential implications of the differentially expressed genes between the spermatozoa populations of AL and CR *C. elegans*, pathway analysis was performed using the WormEnrichr platform. Specifically, the differentially expressed genes in the CR population were analyzed using the TF2DNA database to identify enriched transcription factors and the GO Biological Process AutoRIF to identify altered biological pathways with a p value < 0.01. The analysis was based on a comparison of the single cell transcriptome of spermatozoa from AL and CR hermaphrodites.

Oxidative stress resistance

To assess the oxidative stress resistance of *C. elegans*, worms were exposed to either 10 or 15 mM arsenite diluted in M9 buffer for six hours. Following the exposure, the number of live worms was determined by observing their movement in response to being touched by a platinum wire. Worms that did not respond were considered dead.

Cell sorting and flow cytometry analysis of C. elegans cells

For cell sorting and flow cytometry analysis of *C. elegans* cells, the BD FACSAria III apparatus and BD FACS Verse (BD Biosciences) were used. A minimum of 10,000 events per condition were

obtained for sample characterization. Propidium iodide was used to label cells by incubating lysates with 1 µg/ml of the dye for 10 minutes at room temperature. For labeling with Zombie Aqua™ (BioLegend) and MitoSOX™ (ThermoFisher), lysates were centrifuged at 300 x g for 10 minutes at 4°C to precipitate the cells, and diluted in 100 µL of phosphate buffer (157 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4) to remove the enzyme. Zombie Aqua™ was added at a concentration of 1/500. The samples were kept in the dark at room temperature for 15 minutes for labeling to occur, then 250 µl of PBS with 2% fetal bovine serum was added and the cells were kept on ice and immediately analyzed.

MitoSOX Staining Analysis of C. elegans Cells

The cells were lysed using a trypsin-based protocol developed in this project. Following incubation in L15 culture medium at 20°C for 1 hour and 30 minutes, certain groups of cells were treated with 0.6 µM of rotenone and 1 µM of antimycin A or vehicle, as shown in the accompanying figure. Afterwards, the cells were incubated with 2 or 5 µM of MitoSOX in HBSS-rich medium for 20 minutes at 20°C. The cells were then resuspended in PBS and analyzed via flow cytometry to assess MitoSOX staining.

RT-qPCR

RNA was extracted using Trizol (Invitrogen), which involved homogenizing 50 *C. elegans* worms with beads and a blender, followed by chloroform extraction and RNA precipitation using isopropyl alcohol and sodium acetate. 1 µg of the resulting RNA was used as starting material for reverse transcription, which was performed using the High Capacity cDNA Reverse Transcription kit. Real-time PCR was then performed using 250 nM of each primer, the cDNA, and 1x Maxima SYBR-Green Master Mix. Gene expression was evaluated in a CFX384 equipment with standardized primers for the genes of interest and a constitutive gene, his-10, used as an endogenous control. Gene expression data were calculated as $2^{-\Delta Ct}$, where ΔCt = Ct of the target-Ct endogenous.

The sequences of the primers used are as follows:

rde-1 Forward: AGGCCCACTGGTAAATGCGA Reverse: CCGATCGTAAATTTTGCTGGAGA

tc1 Forward: AACCGTTAAGCATGGAGGTG Reverse: CACACGACGACGTTGAAACC

tc3 Forward: GAGCGTTCACGGAGAAGAAG Reverse: AATAGTCGCGGGTTGAGTTG

alg-3 Forward: TGACTAGTTCAGGATCTGGTTCAC Reverse: GTCACGTGGCGAGTCAGAAT

alg-4 Forward: TGGATAACAACACGCTGACT Reverse: ATTGCGGTGGTGACAGTGAT

spe-46 Forward: GGTTTGGCTGGAGGTCAACT Reverse: AAACTGGCAATGCAGCAAGG

msp Forward: CATCGGCTCGTCGTATTGGA Reverse: AAAGGCAAAAGCATCGCAGG

his-10 Forward: GCAATTCGTCGTCCGC Reverse: GACTCCACGGGGTTTCCT

Observing GFP Transgene Silencing

To visualize the GFP transgene silencing in this study, we employed a ZEISS SteREO Discovery.V8 fluorescence microscope equipped with a ZEISS HXP 120 C illuminator and a GFP-specific filter.

Initially, a GFP-expressing animal was chosen as the founder of the first generation (P0). The animal was isolated on a plate for 24 hours to lay eggs and then removed. Once the offspring (F1) reached maturity, the GFP expression in each animal was examined. The eggs and larvae of more than 50 F2 offspring were subsequently transferred to a new dish, and this process was repeated for each successive generation.

To investigate the duration of GFP silencing, we performed a similar experiment with one modification: a GFP-negative animal was selected as the starting point in the F1 generation.

Pharyngeal pumping in C. elegans.

This phenotype was verified using a light stereoscope, observing the movement of the tissue. The number of contractions per minute was quantified.

Measurement of lifespan in C. elegans.

120 synchronized worms were monitored daily from day 0 of adulthood and considered dead when no motor response was obtained upon touching their extremities with a platinum wire. The plates contained 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B to prevent contamination, and 0.5 µg/mL of FuDR, a mitosis inhibitor that prevents progeny development and inhibits plate overcrowding.

Synchronization

Pregnant hermaphrodites were washed off NGM plates with sterile water. Then, 0.5mL of 5 M NaOH and 1 mL of 5% sodium hypochlorite were added, completing the volume with sterile water up to 5mL. After vortexing for approximately 10 minutes, the sample was centrifuged at 300 x g for 1 minute, and the eggs were washed with 15 mL of sterile ddH2O and pipetted onto NGM plates with food.

Microscopy of C. elegans cells

To evaluate the cellular viability of *C. elegans* isolated cells, we analyzed wild-type animals at day 0 of adulthood using both the original and optimized lysis protocols outlined in the methods section. We then utilized fluorescence microscopy (100x magnification) with Hoechst 33342 and propidium iodide to label live and dead cell nuclei, respectively, after a 10-minute incubation with Hoechst 33342 (1 µg/mL) and Propidium Iodide (1 µg/mL). For cell adhesion, *C. elegans* cells were attached to glass coverslips that were cleaned by incubating them with 1M HCl at 50°C for 12 hours, washed with distilled water twice and ethanol once, and then dried. To promote cell adhesion, coverslips were treated with 100 µg/mL poly-D-lysine (P6407, Merck) for 10 minutes, washed with PBS, and dried. *C. elegans* lysates were then incubated on the coverslips for 30 minutes, and images were obtained using a Cytation 5 microscope with the magnifications and filters described in the figure legends.

Results

1. Reduced Progeny in Caloric Restricted *C. elegans*

1.1 Spermatogenesis and Oogenesis Assessments

Initially, we investigated whether the *C. elegans* model of caloric restriction (CR), *eat-2* mutants, would exhibit a reduced progeny compared to wild type worms under normal ad libitum (AL) feeding, as previously reported [39]. Our observations confirmed the previous results (Figure 1A). Subsequently, we aimed to determine whether this reduction in progeny was due to defects in oogenesis or spermatogenesis. To examine this, we crossed AL and CR hermaphrodites with AL males. Since male sperm has a competitive advantage over hermaphrodite sperm, if spermatogenesis were impaired in CR, AL sperm would compensate for this defect. However, our results did not support this possibility (Figure 1A), indicating that a defect in spermatogenesis is not the issue in CR.

To further corroborate that spermatogenesis remains unaffected in CR, we crossed AL and CR males with two types of AL females: *spe-9* mutant hermaphrodites with non-functional sperm and *fog-2* mutants lacking sperm altogether. In both cases, the females exhibited a similar number of progeny when crossed with AL and CR males (Figure 1B), confirming that sperms are not compromised in CR animals.

Although spermatogenesis does not appear to be the cause of reduced fertility in CR, endogenous sperm from CR worms could still non-autonomously influence other cell types, such as oocytes or sperm from AL males crossed with CR hermaphrodites. To investigate this possibility, we crossed AL males with AL and CR *spe-9* and *fog-2* females. Our findings revealed that CR females exhibited reduced fertility in both cases (Figure 1C), ruling out a cell non-autonomous effect of sperm as the underlying cause of decreased fertility in CR.

1.2 Oocytes and Reduced Fertility in CR C. elegans

In line with a potential role for oocytes in the reduced fertility observed in *C. elegans* under CR, we noted that CR worms did not show a decrease in spermatozoa count (Figure 1D, E), but did exhibit a reduction in oocyte number (Figure 1F, G).

Based on these findings, we conclude that the primary factor contributing to reduced fertility in *C. elegans* under CR is a defect in the female germline component, most likely involving oocytes.

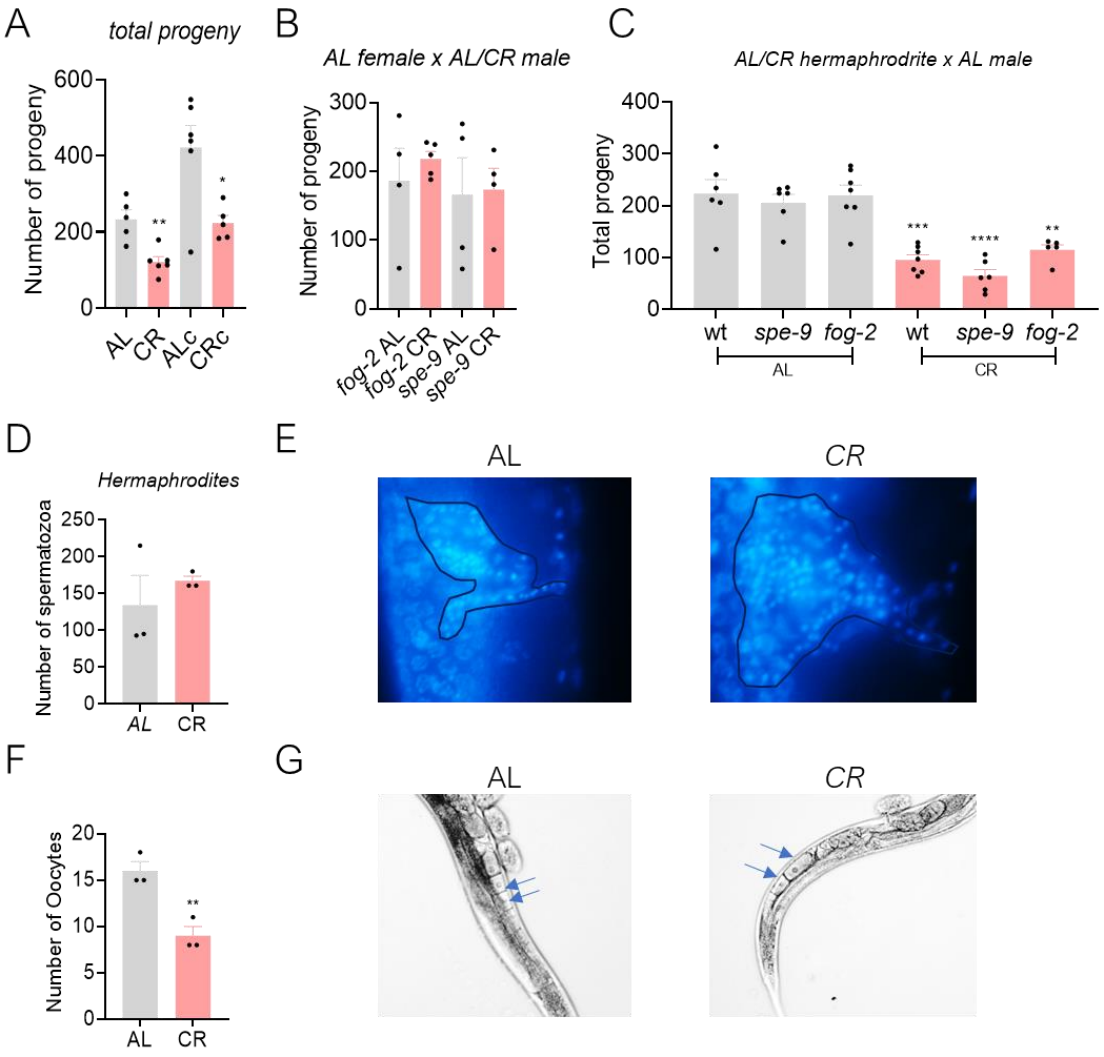

Figure 1 - *Reproductive Characteristics of C. elegans Under Ad Libitum Feeding and Dietary Restriction.* **(A)** Progeny quantification in AL and CR hermaphrodites, crossed (ALc, CRc) or not with AL males, individually monitored from L4 larval stage until cessation of egg laying. * = $p<0.05$ vs ALc, ** = $p < 0.01$ vs AL, unpaired T tests were performed. **(B)** Progeny quantification in AL hermaphrodites lacking spermatozoa (females, fog-2 or spe-9 mutants), paired with AL/CR males from L4 stage until day 2 of adulthood. **(C)** Progeny quantification in AL and CR hermaphrodites, with (wild type – wt) or without (females, fog-2 or spe-9 mutants) spermatozoa,

paired with AL males from L4 stage until day 2 of adulthood. *** = p< 0.001 **** = p< 0.0001 ** = p< 0.01 vs the corresponding AL group. Unpaired t tests were performed. **(D)** Spermatozoa quantification in AL and CR hermaphrodites on day 0 of adulthood, following fixation with PFA and nuclear staining with Hoechst 33342. **(E)** Fluorescent images of AL and CR worms after fixation with PFA and nuclear staining with Hoechst 33342; spermatheca outlined in blue, with spermatozoa nuclei inside counted for (D). **(F)** Oocyte quantification in hermaphrodites on day 0 of adulthood. ** = p<0.01. An unpaired t test was performed **(G)** Phase contrast microscopy of AL and CR hermaphrodites used to count oocytes in (F); oocytes indicated by blue arrows in images. Graphs display mean ± SEM.

2. Improved Cell Isolation Protocol for *C. elegans*

2.1 Optimizing Enzyme and pH Conditions

To gain a deeper understanding of how CR affects the germline of *C. elegans* worms, we isolated these cells for more detailed characterization. We initially attempted to use a published protocol for isolating *C. elegans* cells [63], employing 20 mg/mL pronase to lyse the worm cuticle and release its cells. However, upon microscopic examination using live and dead cell markers, we found that the majority or all of the obtained material consisted of dead cellular debris (Figure 2A). To create a gentler environment for cell isolation, we reduced the pronase concentration to 1 mg/mL, which increased cell viability as observed under the microscope but yielded a low number of cells (Figure 2A). To further optimize the cell isolation conditions, we employed milder enzymes commonly used in cell culture and isolation, such as type IV collagenase and trypsin. Additionally, we adjusted the solution's pH prior to enzyme incubation using PBS, thereby creating an even more favorable environment for cellular viability. Microscopy and flow cytometry analyses revealed that both enzymes significantly improved cell number and viability compared to the published protocol (Figure 2A, B).

2.2 Assessing Cellular Health and Metabolic Activity

To assess whether these cells also exhibited active metabolism, a key indicator of cellular health, we measured mitochondrial superoxide production using the MitoSox probe under basal conditions and in response to mitochondrial stress induced by respiration inhibitors rotenone and antimycin A. We found that the technique effectively detected both basal metabolism and the

increased superoxide production in response to the inhibitors (Figure 2C), supporting our hypothesis that the isolated cells were indeed healthy.

Thus, we successfully developed a cell isolation protocol that yielded much more viable cells than the published method, with cells that were both alive and metabolically active. We then proceeded to use these cells to investigate the differences between AL and CR.

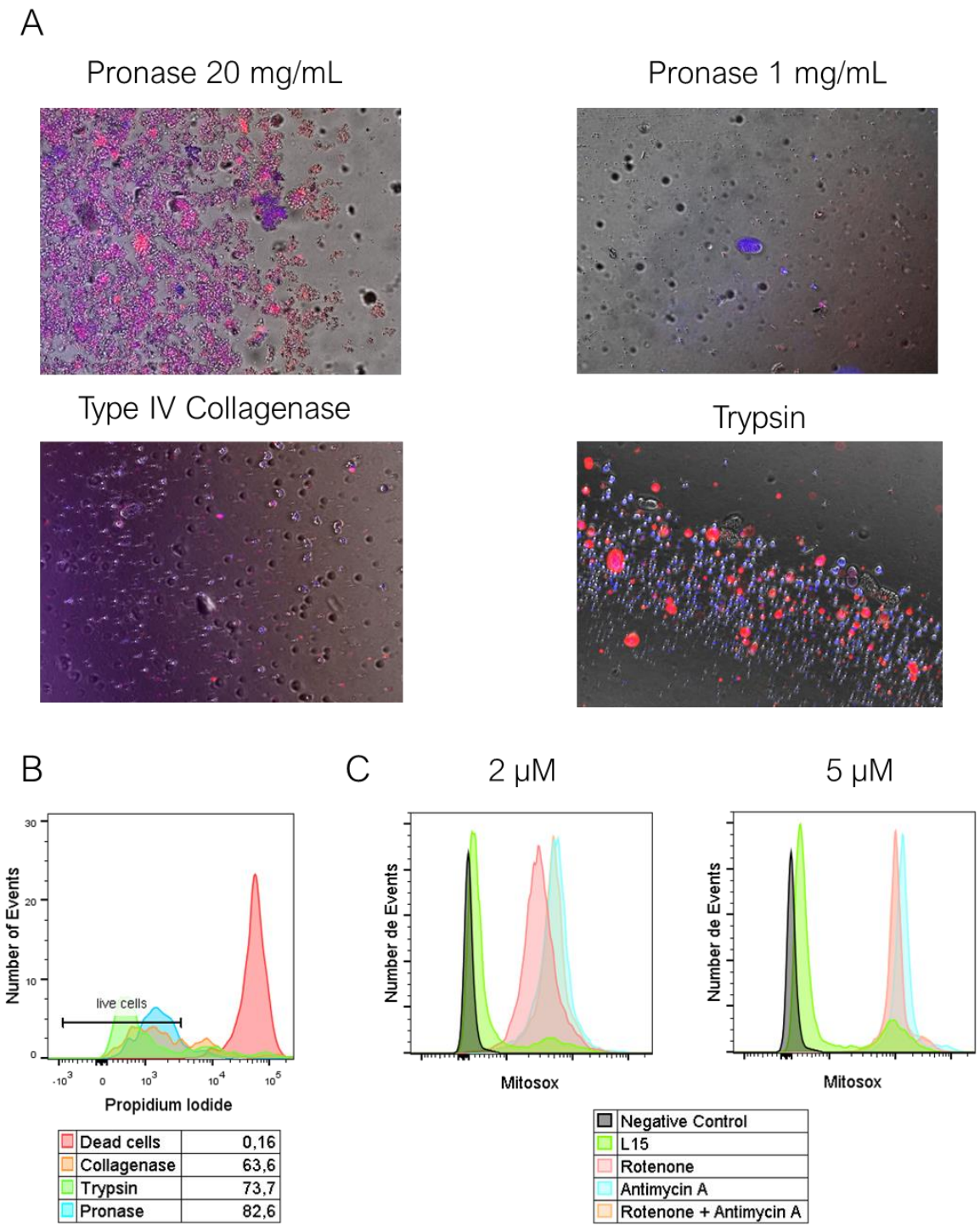

Figure 2 – *Cell Isolation and Viability Analysis Using Different Lysis Protocols in C. elegans*. **(A, B)** Wild-type *C. elegans* at day 0 of adulthood underwent cell isolation using either the original (using pronase) or optimized protocols (using collagenase or trypsin) detailed in the methods

section. **(A)** Cells were visualized with fluorescence microscopy (100x magnification) using Hoechst 33342 (blue) and propidium iodide (red) to label nuclei of live and dead cells, respectively. **(B)** Cells were stained with propidium iodide and analyzed using flow cytometry. The graph shows a histogram of cell count and propidium iodide staining intensity, with a gate outlining live cells. Below the graph is a quantification of live cells in each group; cells maintained at 65°C for 10 minutes were used as a positive control for dead cell population. **(C)** Wild-type *C. elegans* at day 0 of adulthood were lysed using the optimized protocol described in the methods section. Cells were then treated with either 0.6 µM rotenone, 1 µM antimycin A, or a vehicle for 1 hour and 30 minutes. Subsequently, cells were stained with 2 or 5 µM MitoSOX for 20 minutes. MitoSOX staining intensity was analyzed using flow cytometry, and the histogram displays the obtained results.

3. Single-cell RNA Sequencing of *C. elegans* Germline Cells

3.1 High-quality Sample Acquisition

Previous studies have indicated that cell isolation protocols in *C. elegans* primarily yield germline cells [64], likely due to their high abundance within the organism and the vulva's vulnerability as a point of contact between the worm's interior and exterior. In many instances, the vulva spontaneously ruptures during the worm's lifespan, further highlighting its vulnerability [65]. Our goal was to leverage the predominance of germline cells obtained during cell isolation to characterize cells from AL and CR worms using the protocol we developed for single-cell RNA sequencing. This approach may provide insights into how reproduction is affected by CR at the single-cell level and uncover the molecular underpinnings of fertility changes associated with this intervention.

To perform single-cell RNA sequencing, a protocol must produce samples with minimal cellular debris and eggs and maintain cellular viability above 90%. This is because debris and dead cells may be mistaken for single cells during analysis and can also release RNA, contaminating the reads from other cells. Our optimized protocol achieved a cellular viability of approximately 70% (Figure 2B), necessitating further improvement to meet single-cell RNA sequencing grade. To this end, we purified the cells obtained using our protocol by passing them through columns with the Dead Cell Removal Kit from Miltenyi Biotec. This treatment significantly enhanced sample

quality, as evidenced by a reduction in particles with low FSC and SSC (typical of cellular debris) when analyzed by flow cytometry (Figure 3A) and a cellular viability of 95% upon analysis using a dead cell stain (Zombie Aqua) and flow cytometry (Figure 3B). Phase contrast microscopy also revealed minimum debris (Figure 3C).

3.2 Filtering Process and Analysis

Having achieved a sample with cellular viability and purity consistent with single-cell sequencing requirements, we also needed to ensure the sample was free of aggregates, which could obstruct the flow during single-cell library preparation. We observed that eggs within the isolated material formed large aggregates (Figure 3D) that could hinder single-cell analysis. To eliminate these eggs, we used a filtering process, as eggs are significantly larger than *C. elegans* cells. We analyzed samples after filtering with cell strainers containing a 20 or 40 μm mesh and found that only the 20 μm mesh effectively removed the eggs (Figure 3E).

However, there was a concern that the smaller mesh might also remove cells along with the eggs. To address this concern, we analyzed the material obtained with both the 20 and 40 μm meshes and observed no significant differences in cellular profiles using phase contrast microscopy and flow cytometry (Figure 3F).

Having obtained a sample that met single cell RNA sequencing grade, we proceeded to perform this technique using cells from AL and CR worms.

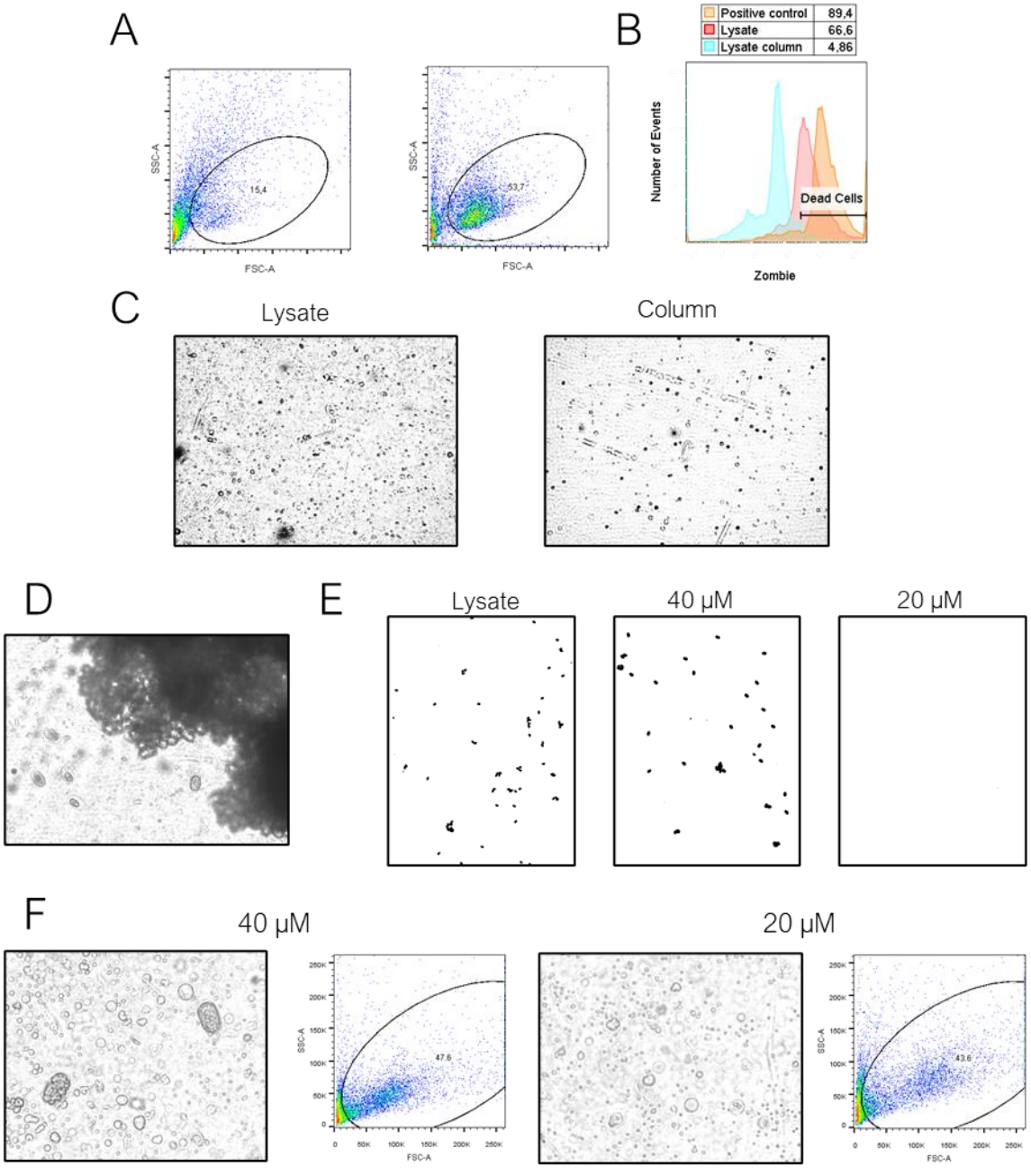

Figure 3 - *Optimized Lysis Protocol and Dead Cell Removal for C. elegans Cell Analysis.* Wild-type *C. elegans* at day 0 of adulthood were subjected to lysis using the optimized protocol detailed in the methods section. **(A-C)** Cells obtained using the optimized protocol were further purified by passing them through columns to remove dead cells with the Dead Cell Removal Kit from Miltenyi Biotec. **(A-B)** Resulting cells were analyzed using flow cytometry, with heat-killed cells as a positive control. **(A)** A graph displaying FSC and SSC parameters of cells before (Lysate) and after (Column) dead cell removal. **(B)** A histogram shows the intensity of Zombie Aqua staining, which

labels dead cells, with a legend indicating the percentage of dead cells in each group. **(C)** Lysate before and after dead cell removal was imaged using phase contrast microscopy. **(D)** A phase contrast image of cells and eggs from *C. elegans* subjected to lysis. **(E)** Light microscopy of eggs from *C. elegans* subjected to lysis before and after filtering with cell strainers with a 20 or 40 µM mesh. **(F)** Cells from *C. elegans* were analyzed using phase contrast microscopy after adhering to a glass surface treated with poly-D-lysine (left images of each group) or in a flow cytometer (right graphs in each group). Samples were analyzed after filtering with cell strainers with a 20 or 40 µm mesh, as indicated in the images. Flow cytometry graphs plot FSC x SSC axes for each sample, with a gate at the location of cells and the percentage of this population indicated.

Upon conducting single-cell analysis, we initially observed two major cell populations (Figure 4A). By examining the genes expressed by these cells, we determined that one cluster predominantly expressed genes with spermatozoon-restricted expression, while the other expressed genes with oocyte-restricted expression (Figure 4B). Consequently, we concluded that one cluster consisted of spermatozoa and the other of oocytes, confirming that we had successfully determined the single-cell profile of germline cells from AL and CR worms.

Interestingly, the oocytes from AL and CR worms showed minimal differences in the UMAP analysis, where similar cells cluster together. However, spermatozoa from AL and CR worms exhibited significant differences in gene expression according to the UMAP analysis (Figure 4A). This contrasted with the functional characterization of these cells, where the female germline appeared to be the causal factor for decreased fertility upon CR.

Intrigued by this result, we decided to investigate what was altered in these cells. We assessed the differentially expressed genes in the CR spermatozoa population compared to AL for enriched biological processes (using the GO Biological Process AutoRIF database). This analysis revealed that many genes involved in spermatid development and spermatogenesis were upregulated during CR (Figure 4C). We validated the differential expression of four of these strictly sperm-expressed genes using RT-qPCR on whole worms, confirming the single-cell findings (Figure 4D).

These results demonstrate that CR and AL sperm exhibit substantial differences in the expression of key genes involved in spermatogenesis. We then decided to explore what drives these gene expression changes and whether they have any functional consequences for sperm in a CR context.

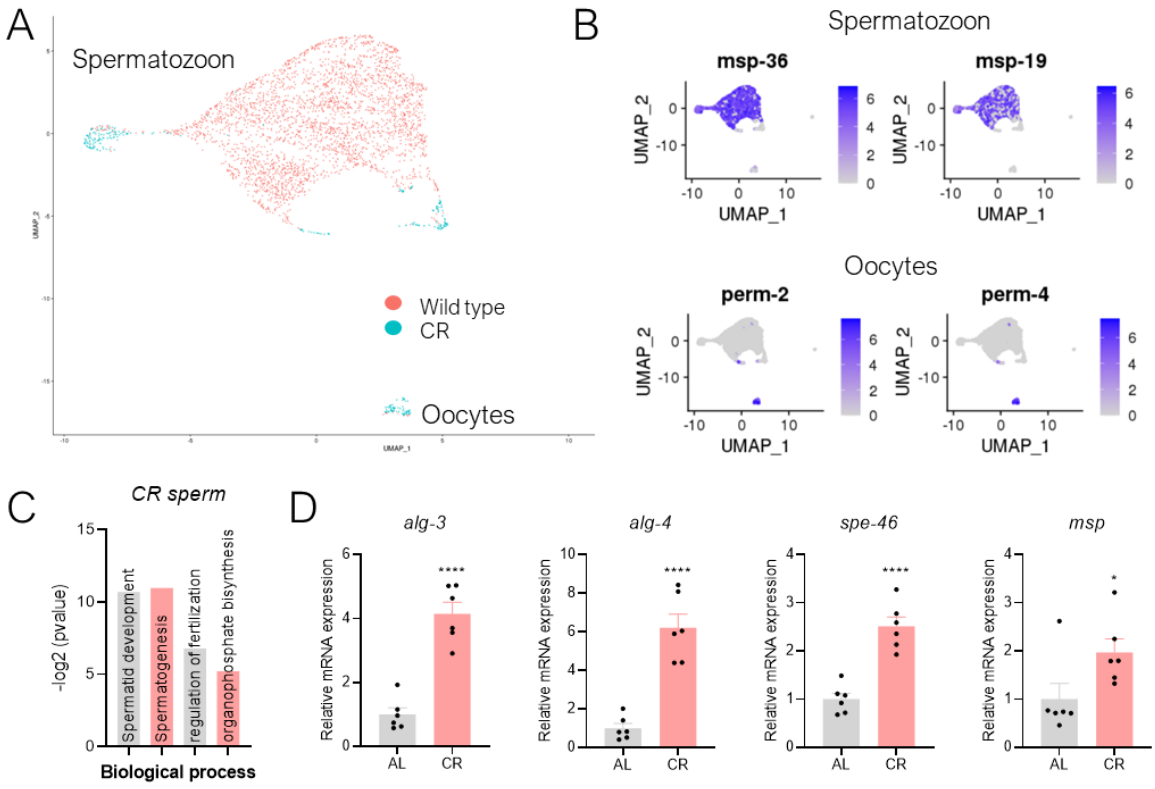

Figure 4 – *Single-cell RNA Sequencing and Gene Expression Analysis of germline cells of C. elegans Under Ad Libitum and Dietary Restriction.* **(A-C)** Single-cell RNA sequencing of *C. elegans* under ad libitum (AL) feeding and dietary restriction (*eat-2* mutants, CR) was performed on day 0 of adulthood. Worms were lysed using the optimized protocol detailed in the methods section. Dead cells and eggs were removed, and single-cell cDNA libraries were generated using the Chromium Next GEM Single Cell 3′ Reagent Kit v3.1 with poly(dT)-primed beads targeting poly-A containing mRNA. Libraries were sequenced on the NovaSeq platform. **(A)** A UMAP plot was generated to cluster cells from CR and wild-type animals, revealing two major clusters identified as spermatozoa and oocytes based on tissue-restricted gene expression patterns. Some of

the selected genes used for classification are depicted in **(B),** which shows the UMAP plot generated in A, but each dot representing each cell is stained according to the expression of each gene described in the figure, with blue indicating high expression and gray indicating lower expression. **(C)** Differentially expressed genes in the CR spermatozoa population, when compared to AL, were assessed for enriched biological processes (on GO Biological Process AutoRIF database) at a p value < 0.01. **(D)** Relative mRNA expression of the genes indicated in the figures measured on samples from AL or CR day 0 adults. **** = p< 0.0001, * = p<0.05 vs AL. Unpaired T tests were performed. The graphs show the mean +- SEM.

4. HLH-30's Role in CR Spermatozoa and Development

4.1 Differential Gene Expression in CR Spermatozoa

To identify key transcriptional regulators of the changes observed in sperm following CR, we evaluated differentially expressed genes in the CR spermatozoa population compared to AL and looked for enrichment of transcription factor targets using the TF2DNA database. Intriguingly, the top 2 hits were HLH-30 and MXL-3 targets (Figure 5A). These transcription factors exhibit antagonistic effects, acting in concert to regulate expression of genes related to lysosomal lipolysis during fasting [12]. During fasting, HLH-30 activity is induced while MXL-3 activity is repressed, resulting in increased lysosomal lipolysis [12]. Consequently, we hypothesized that alterations in the activity of these transcription factors may play a role in the transcriptional adaptations of the sperm during CR.

4.2 Investigating HLH-30's Function in CR Worm Fertility

To verify whether activation of HLH-30 occurs with CR, we crossed worms overexpressing HLH-30 tagged with the GFP fluorescent protein under the control of the *hlh-30* promoter with *eat-2* mutants. Using fluorescence microscopy, we observed a higher translocation of this transcription factor to the nuclei of intestinal cells in animals under CR (Figure 5B), consistent with previous results when worms were exposed to fasting [19]. Importantly, as previously described [19], we noticed that this transcription factor is expressed in the spermatheca of CR animals (Figure 5C), suggesting that it may be acting in sperm cells of animals under CR to regulate their transcriptome and activity.

One possibility is that HLH-30 activity induction serves as an adaptation to aid sperm function when oocyte is limited during CR. If this were the case, further increasing its activity

might promote enhanced sperm function in animals under CR and alter their fertility. However, we found that HLH-30 overexpression did not affect the fertility of AL or CR animals (Figure 5D).

We next investigated whether *hlh-30* activity is necessary for the fertility of AL and CR animals. Interestingly, we observed that while *hlh-30 (allele tm1978)* mutation had no effect on the fertility of AL animals, it rendered CR animals almost entirely sterile (Figure 5E). Remarkably, when crossed with wild-type males, the fertility of these *hlh-30* mutants under CR reverted to levels seen in CR worms which are wild type for *hlh-30* (Figure 5E), while the fertility of mutants and wild-type animals in AL conditions remained unchanged, regardless of whether the worms were crossed or not.

These findings reveal that HLH-30 is essential for fertility during CR. Interestingly, it is not the capacity of the worms to produce progeny that is affected, as crossing reverses this defect; rather, it is something that occurs after or during the fertilization of the oocyte. One possibility is that the sperm of CR *hlh-30* (tm1978) animals is incapable of fertilizing the oocytes, and that is why wild-type sperm restores fertility. Alternatively, CR *hlh-30* (tm1978) animals may not develop after the egg is laid, and when crossed with wild-type animals, the heterozygous progeny can develop normally.

To determine whether *hlh-30* (tm1978) CR worms are laying eggs that fail to develop, we counted the number of eggs laid by *hlh-30* (tm1978) AL and CR worms and the number of adults that developed from those eggs. Interestingly, while the number of eggs and adults obtained from AL worms were quite similar, indicating high egg viability, most of the eggs and larvae from *hlh-30* (tm1978) CR worms did not develop into adults, demonstrating their low viability (Figure 5F).

Next, our objective was to elucidate the cause of the low viability of these eggs and determine whether this phenotype is related to sperm function. Two possibilities were considered: either the eggs are not being fertilized by sperm due to a defect in the sperm of the *hlh-30* (tm1978) CR hermaphrodites, or the embryos of *hlh-30* (tm1978) CR worms fail to develop.

To investigate the developmental capacity of *hlh-30* (tm1978) CR worms, we utilized *eat-2* mutant males heterozygous for the *hlh-30* (tm1978) mutation, with one chromosome IV carrying the *hlh-30* mutation and the other expressing a red fluorescent protein (RFP). During meiosis, the

resulting sperm will either contain the RFP or the *hlh-30* mutation, except for rare instances of crossing over, which could lead to sperm carrying both or neither of the alterations.

We crossed these males with *hlh-30* (tm1978) CR and CR hermaphrodites and monitored the fluorescence of the progeny. Our observations showed that both RFP-containing and *hlh-30* (tm1978) -containing sperm were viable, as the progeny of CR worms included both RFP+ and RFP- offspring (Figure 5G). Intriguingly, the progeny from *hlh-30* (tm1978) CR worms were all RFP+, suggesting that embryos fertilized by *hlh-30* (tm1978) sperm, and hence *hlh-30* homozygous mutant, fail to develop (Figure 5G). This indicates that CR embryos lacking *hlh-30* predominantly do not develop into adulthood.

Consistent with this hypothesis, when we counted the total number of progeny from CR *hlh-30* (tm1978) worms crossed with heterozygous *hlh-30*(tm1978)/RFP CR males, we found that it was reduced by 70% in comparison to that of CR *hlh-30* (tm1978) worms crossed with AL males (Figure 5H). This result also indicates that worms derived from *hlh-30* mutant sperm do not develop, leading to a lower progeny production.

To assess whether the sperm carrying the *hlh-30* mutation differed from the RFP-containing sperm in terms of fertilization capacity, we counted the number of RFP+ and RFP- males resulting from the cross of the CR worms. We focused on males since they can only be produced as a result of the cross, while hermaphrodites may arise from self-fertilization. If both types of sperm had similar fertility, we would expect 50% of the males to be RFP+; however, our observations showed that nearly 80% of the males were RFP+ (Figure 5I). This finding suggests that the *hlh-30* mutation has a cell autonomous effect on the sperm of CR males, reducing their fertility compared to sperm without the mutation.

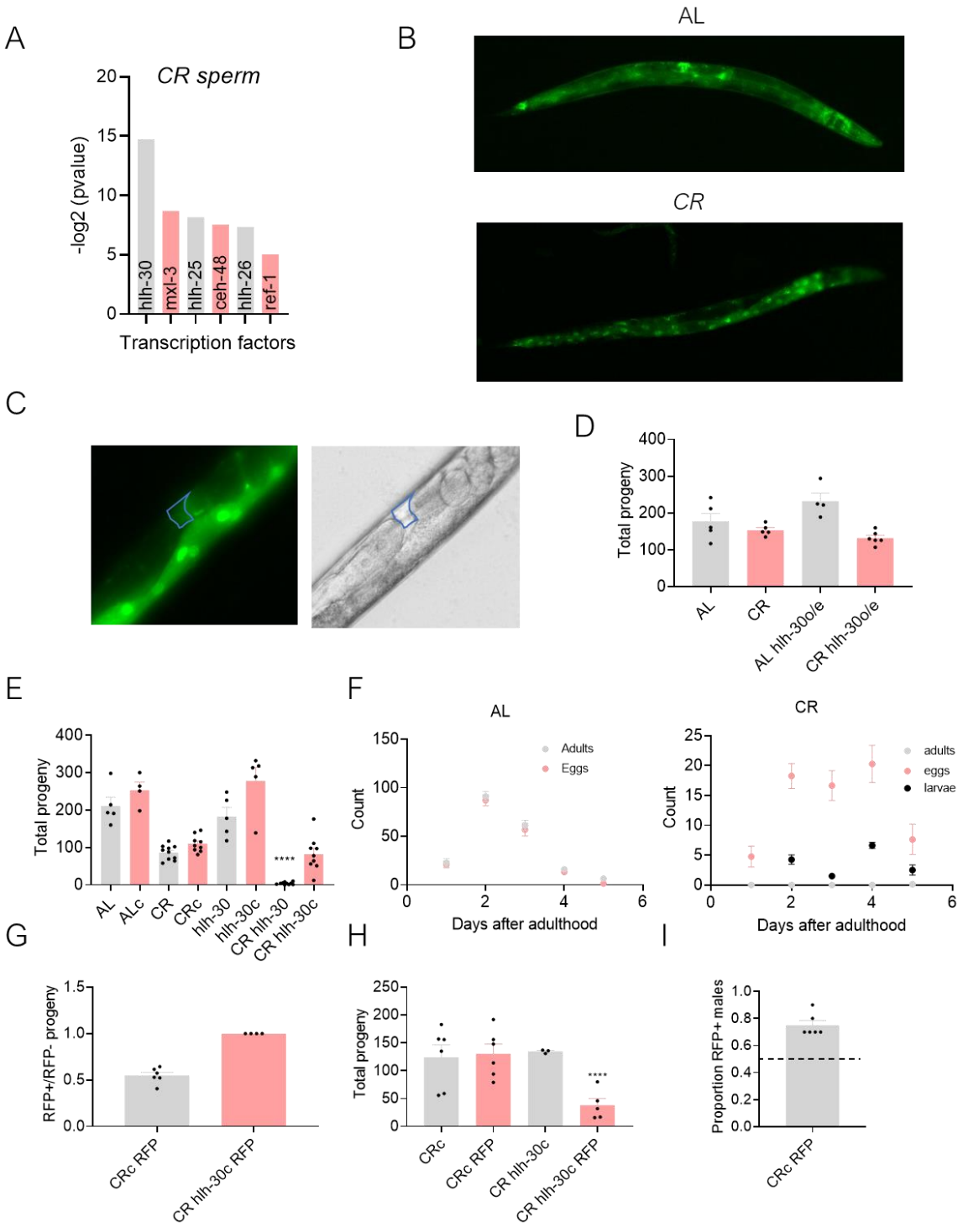

Figure 5 – *HLH-30 Effects on Progeny Production in C. elegans Under Ad Libitum and Dietary Restriction.* **(A)** Single-cell RNA sequencing of *C. elegans* under ad libitum (AL) feeding and dietary restriction (*eat-2* mutants, CR) was performed on day 0 of adulthood. Differentially

expressed genes in the CR spermatozoa population, when compared to AL, were assessed for enriched transcription factors (TF2DNA database) at a p value < 0.01. **(B,C)** AL and CR worms overexpressing a GFP tagged HLH-30 protein under the control of the *hlh-30* promoter were imaged using fluorescent microscopy. **(C)** A CR spermatheca is outlined in blue and the corresponding phase contrast image is shown below. **(D)** Progeny quantification of ad libitum (AL) feeding and dietary restriction (*eat-2* mutants, CR) with or without *hlh-30* overexpression monitored until cessation of egg laying. **(E)** Progeny quantification of ad libitum (AL) feeding and dietary restriction (*eat-2* mutants, CR) with or without *hlh-30* mutation, individually maintained from L4 stage until day 2 of adulthood with or without AL males (indicated by c) and monitored until cessation of egg laying. **** = p< 0.0001 vs CR, an unpaired T test was performed. **(F)** Individual *hlh-30* mutant worms, under AL or CR, were monitored and transferred to a new well daily. The number of eggs laid, larvae and adults produced by these eggs on each well was counted. **(G, H, I)** CR worms, with and without *hlh-30* mutations, were crossed with AL males (denoted by a lowercase 'c') or CR males heterozygous for the *hlh-30* mutation with an RFP marker on the other chromosome IV (denoted by 'c RFP'). **(G)** The quantity of RFP+ and RFP- offspring for each worm was determined. **(H)** The total number of offspring produced by each worm was measured. **** = p<0.0001 vs CR *hlh-30*c, an unpaired t test was performed. **(I)** The count of RFP+ and RFP- male offspring from each worm was assessed. Graphs show the mean ± SEM.

4.3 Analysis of HLH-30 Downstream Targets

Thus, HLH-30 appears to be a critical transcription factor for *C. elegans* under caloric restriction, playing an essential role in embryo development and influencing sperm fertility in CR conditions. We subsequently examined whether the known downstream effectors of HLH-30 involved in lysosomal lipolysis could be responsible for the observed effects.

Utilizing RNAi to silence genes previously identified as targets of HLH-30 in lysosomal lipolysis (Lipl-1, -2, -3, and -5, lgg-1 and -2, atg-16.2) [12], we found no impact on the progeny production of CR worms (Figure 6A). As a positive control, we employed RNAi for pha-4, which is known to decrease progeny production in *C. elegans* and inhibits autophagy under CR conditions [24]. Intriguingly, a more pronounced reduction of fertility was observed in CR worms following this RNAi treatment (Figure 6A), suggesting that autophagy might be involved in fertility reduction upon *hlh-30* mutation, as this transcription factor also targets autophagy [13]. These findings suggest

that either HLH-30 is not acting through its lysosomal lipolysis function to regulate fertility and development in CR worms, or that multiple factors need to be silenced to mimic the effects of *hlh-30* (tm1978) on worm fertility.

To further investigate the potential involvement of lipid metabolism in *hlh-30* (tm1978) impact on fertility and development in CR worms, we supplemented their diet with two types of lipid precursors (cholesterol and oleic acid) and assessed the worms fertility. However, we observed no effects on the fertility of AL, CR, AL *hlh-30* (tm1978) and CR *hlh-30* (tm1978) worms, indicating that reduced availability of these lipids is not the cause of decreased fertility in *hlh-30* (tm1978) CR worms.

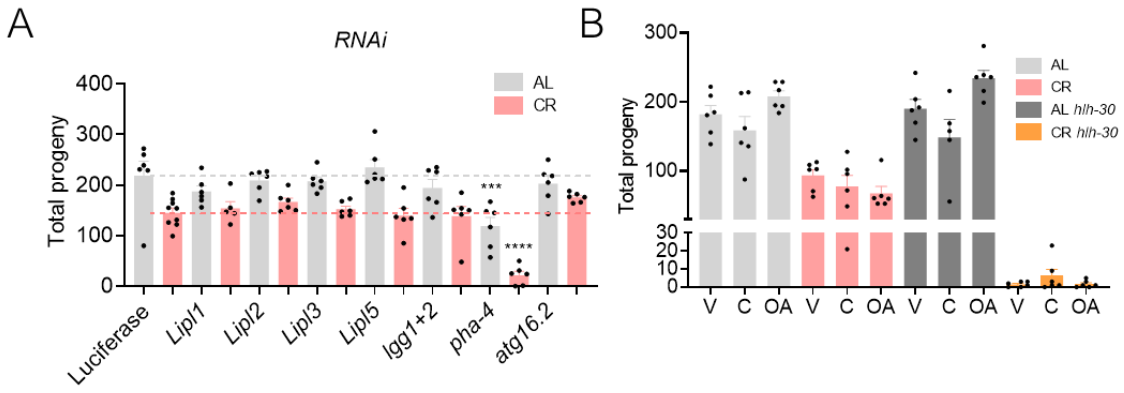

Figure 6 - *RNAi Effects on Progeny Production and Dietary Impact on C. elegans with Different Genetic Backgrounds.* **(A)** Worms were subjected to RNAi from the egg stage until cessation of egg laying, using bacterial HT1115 feeding producing dsRNA complementary to target genes regulated by transcription factor HLH-30, control dsRNA (luciferase), or dsRNA against pha-4, a transcription factor involved in autophagy regulation. Progeny production was quantified and plotted. *** = p<001 vs luciferase AL, **** = p<0.0001 vs Luciferase CR. An ordinary One Way ANOVA with Dunnett's as a multiple comparisons test was performed for AL and CR separately. The dashed line indicates the luciferase group expression as a reference. **(B)** Worms maintained on a normal diet (ad libitum - AL) or dietary restriction (*eat-2* mutants - CR), with or without mutations in the transcription factor HLH-30 were exposed to vehicle, double the normal amount of cholesterol, or oleic acid. Progeny production was quantified and plotted. Graphs display mean ± SEM.

5. Genetic and Environmental Factors Impacting Transgene Silencing and Fertility

5.1. Inheritance of Reduced Fertility in eat-2 Worms

Starvation not only affects the fertility of parent worms but also influences their progeny's fertility [46]; although the inheritance patterns involved remain incompletely understood. We investigated the inheritance of reduced fertility in *eat-2* worms by crossing AL or CR females with AL males. Intriguingly, we found that the heterozygous progeny displayed reduced fertility (Figure 7A), indicating that worms exposed to CR transmits their reduced fertility to the AL offspring.

We further explored this inheritance pattern by examining whether male *eat-2* worms could also transmit the reduced fertility of CR worms. Crossing AL females with AL or CR males revealed that the CR male worms' resulting AL progeny exhibited increased fertility (Figure 7B), demonstrating that the transmissible phenotypes of *eat-2* depend on whether they are transmitted by the male or female germline.

Lastly, we examined whether the germline of *eat-2* worms carries alterations that can transmit altered fertility to subsequent generations without reduced caloric intake. To achieve this, we utilized worms overexpressing the GPR-1 protein, which leads to a defect in cellular divisions during early embryogenesis, resulting in a chimera progeny. This chimera progeny comprises part of its cells containing only the male parent's genetic material and part containing the female's. We used AL GPR-1 overexpressing hermaphrodites crossed with CR male worms, which produced chimeric progeny with most of their somatic cells containing the genetic material from the AL parent and their germline containing the genetic material from the CR parent. These chimeric worms showed reduced progeny production, despite having a normal pumping rate (Figure 7C, D). This confirms that the reduced fertility inherited from the CR males can be passed onto the next generation regardless of the feeding behavior of the progeny, but the context in which this progeny is produced influences their phenotypes.

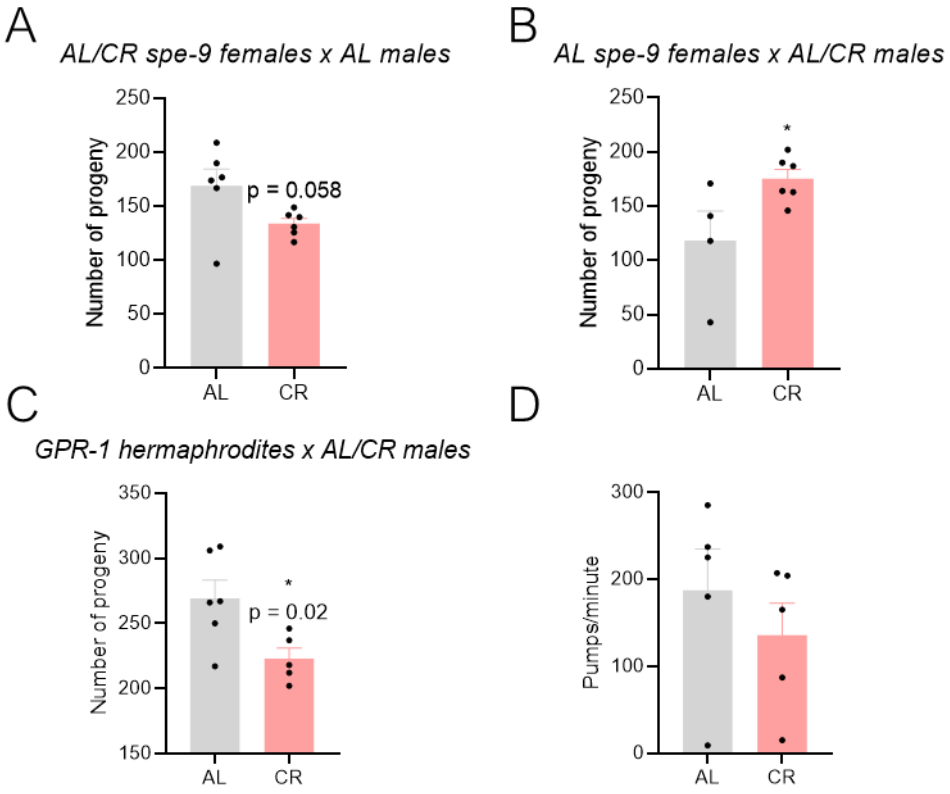

Figure 7 – *Transmission of reduced progeny phenotype due to caloric restriction (CR).* **(A, B)** AL or CR female *C. elegans*, *spe-9* mutants with non-viable sperm production at 25°C, were crossed with AL or CR wild-type males. **(A)** Progeny (F2) production from F1 progeny of AL or CR females crossed with AL males was quantified. **(B)** Progeny (F2) production from F1 progeny of AL females crossed with AL or CR males was quantified. **(C, D)** Non-Mendelian inheritance in *C. elegans* overexpressing GPR-1, generating chimeric progeny upon crossing, with germline and some somatic cells from one parent and the majority of soma from the other parent. AL GPR-1 worms were crossed with CR or AL males, resulting in progeny containing germline and some somatic cells from the male and most somatic cells from the hermaphrodite. **(C)** Progeny production of chimeric worms from the cross was quantified. **(D)** Food intake, assessed by pharyngeal pumping rate, was measured in the resulting chimeric worms. * = $p < 0.05$ vs AL. Unpaired T tests were performed. Graphs display mean ± SEM.

5.2. Heritable Effects on Somatic Transgene Expression

Besides the heritable impact of the *eat-2* mutation on fertility, we observed that crossing CR males with worms expressing a fluorescent protein transgene could produce heritable effects

on transgene expression. We crossed *eat-2* males twice with worms expressing GFP and having muscle-restricted RDE-1 expression (*rde-1* mutants with transgenic muscle rescue [66], referred to as m*rde-1* in this study). Interestingly, although we selected GFP homozygous worms, the proportion of fluorescent CR worms diminished across generations while maintaining 30 random progeny per generation (Figure 8A). Although the proportion of fluorescent worms decreases, GFP+ animals could be maintained through selection. This silencing was transmitted to future generations of silenced parents, with 100% GFP- progeny persisting for at least 14 generations (Figure 8B). Notably, the silencing effect did not depend on the *rde-1* mutation for maintenance, as removing the mutation did not prevent silencing in future generations (Figure 8C). The strains outcrossed with CR males were designated m*rde-1* CR (oc) with + or – indicating the presence (+) or absence (-) of GFP expression.

We next investigated whether the heritable phenotype resulted from crossing with a CR male or any male. After crossing the m*rde-1* strain with AL males twice we observed a similar silencing phenotype (Figure 8D), which was also independent of the *rde-1* mutation and transmitted throughout generations (Figure 8D, E). The strains outcrossed with AL males were designated m*rde-1* (oc) with + or – indicating the presence (+) or absence (-) of GFP expression.

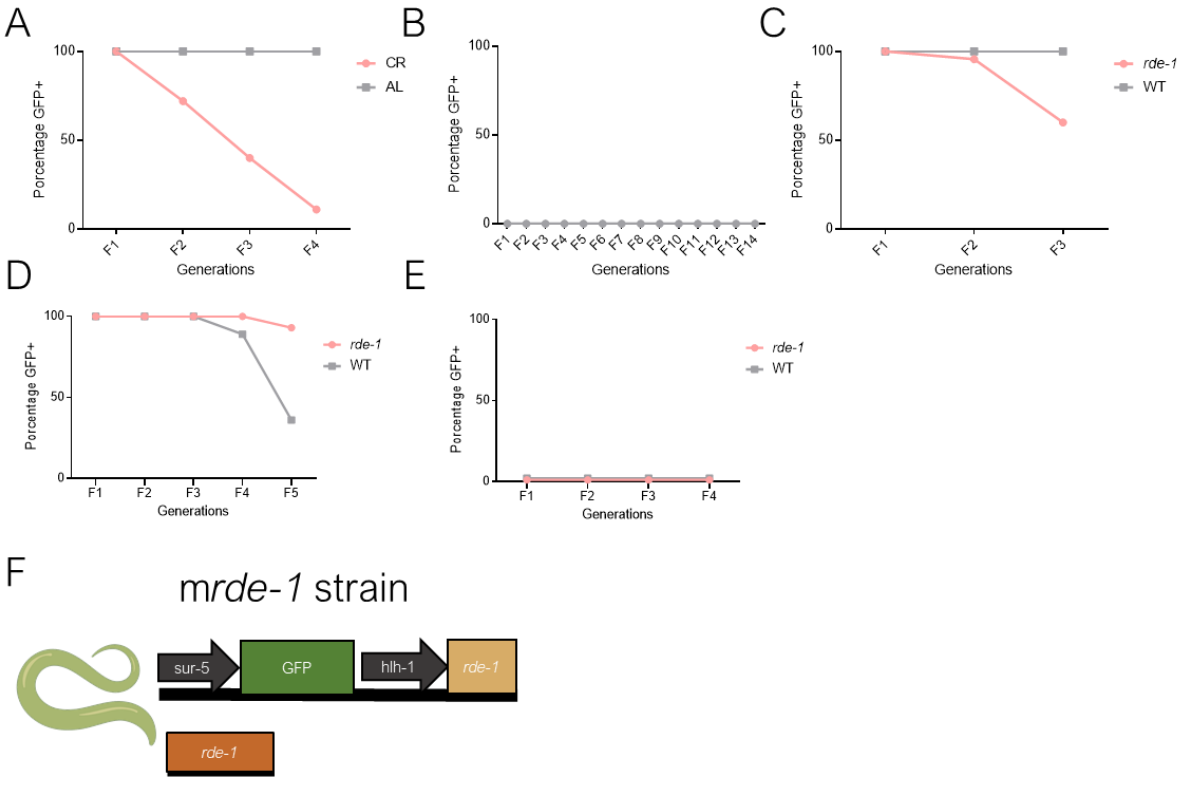

Figure 8 - *Inheritance of GFP Silencing in mrde-1 worms Crossed with CR and AL worms* **(A)** m*rde-1* worms were crossed (CR) or not (AL) twice with CR males, and the percentage of GFP+ offspring was assessed in subsequent generations after transferring 30 random worms between each generation. **(B)** A single silenced worm from the experiment in A was isolated, and the percentage of GFP+ offspring in subsequent generations was assessed after transferring 30 random worms between each generation. **(C)** CR worms from A were crossed to remove (WT) or not (*rde-1*) the *rde-1* mutation, a single WT or *rde-1* GFP+ animal was transferred, and the percentage of GFP+ offspring in subsequent generations was assessed after transferring 30 random worms between each generation. **(D)** m*rde-1* worms were crossed twice with AL males, a single GFP+ animal was transferred, and the percentage of GFP+ offspring in subsequent generations of *rde-1* mutant or wild-type (WT) parents was assessed after transferring 30 random worms between each generation. **(E)** A single silenced worm from the WT and *rde-1* mutant populations described in D was isolated, and the percentage of GFP+ offspring in subsequent generations was assessed after transferring 30 random worms between each generation. **(F)** A diagram illustrating the genetic modifications in the m*rde-1* strain, with black arrows denoting the promoters directing GFP and *rde-1* transgenic expression, and the box containing *rde-1* in red representing the *rde-1* loss-of-function mutation.

Prior studies investigating the inheritance of transgene silencing in *C. elegans* have mainly concentrated on germline-expressed transgenes due to their widespread silencing occurrence, while somatic silencing is infrequently observed [55,57]. Our research, however, focuses on a transgene expressed in the soma, offering a unique opportunity to delve further into the mechanisms underlying somatic transgene silencing.

In our initial investigation of the patterns leading to silencing transmission, we examined m*rde-1* worms after outcrossing with CR males. We observed these worms to be prone to GFP silencing, but their behavior was not uniform. Some displayed 100% GFP+ progeny, while others exhibited a mixed progeny (Figure 9A). The factors behind these differences remain uncertain, but our conclusion is that the factors leading to transgene silencing are stochastic. Nonetheless, we consistently found that when tracking the progeny of worms with 100% GFP+ F1 over multiple generations, silencing ultimately occurred (Figure 9B).

We proceeded to investigate the source of the silencing transmission signal within the worm. By employing chimeric GPR-1 overexpressing worms, which generated chimeric progeny

upon crossing [67], we discovered that germline cells derived from the genetic material of silenced animals transmitted silencing with complete penetrance to subsequent generations in the context of a soma primarily consisting of cells with genetic material from non-silenced worms. This finding indicates a germline origin for the silencing signal (Figure 9C).

Next, we wanted to find out if the male or female germline was responsible for passing on the silencing traits. We used worms with no GFP silencing (non-silenced), GFP+ worms with silencing triggered by outcrossing (partially silenced), and fully silenced GFP- animals (silenced). When non-silenced males were crossed with silenced hermaphrodites, we observed no silencing in the offspring, suggesting that the female germline alone could not transmit silencing (Figure 9D). The same result was seen when crossing a silenced male with a partially silenced hermaphrodite, showing that both parents' signals were needed for silencing transmission (Figure 9D). Silencing was passed on to offspring when crossing silenced hermaphrodites with silenced males, but not when crossing two partially silenced animals, as expected (Figure 9D). However, when a partially silenced male was crossed with a silenced hermaphrodite, silencing was only passed on to the hermaphrodite offspring, revealing sex-specific differences in silencing transmission (Figure 9D).

Following this, we investigated whether silencing could be transmitted to other chromosomes. We crossed silenced hermaphrodites with non-silenced males and found that silencing was not passed on to the offspring's non-silenced male chromosome, as it remained GFP+ (Figure 9E). Silencing did not reestablish for at least two generations (Figure 9E), suggesting that silencing is not transmitted to other chromosomes during the time period studied. This could mean that silencing only occurs when both chromosomes are present during silencing triggered by the cross, or the number of generations observed is insufficient to detect silencing transmission.

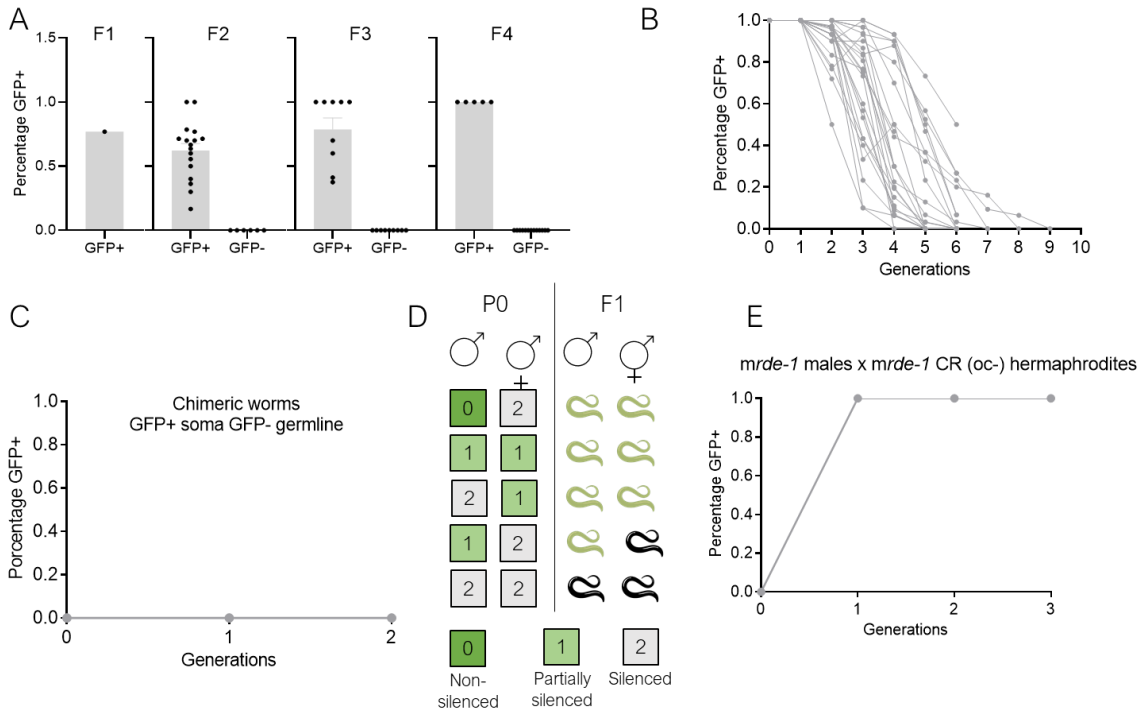

Figure 9 - *GFP Silencing Inheritance Dynamics in mrde-1 Worms.* **(A)** An unsilenced m*rde-1* CR (oc+) animal was isolated on a plate, and its progeny were assessed for silencing on day 0 of adulthood (F1). 24 F1 animals were individually placed on separate plates, and their offspring were analyzed on day 0 of adulthood (F2). Subsequently, 1 random F2 animal from each plate was transferred to generate F3, and the same process was repeated with F3 animals to produce F4. F2 and F3 offspring were analyzed on day 0 of adulthood. Below the graph, GFP+ and GFP- represent the silencing state of each parent that produced the assessed progeny. **(B)** An unsilenced m*rde-1* CR (oc+) animal was isolated on a plate, and about 30 worms from its progeny and subsequent generations were randomly transferred to new plates, with GFP silencing being assessed. The figure displays only the animals that did not silence GFP by F1, and each line represents a separate biological replicate. **(C)** Animals overexpressing GPR-1 generate mosaic offspring, with the germline lineage from one parent and the somatic cells from another. Utilizing this tool, we produced animals with only the germline lineage from silenced m*rde-1* CR (oc-) animals and the soma from wild type animals and examined the percentage of silenced animals in subsequent generations. **(D)** Animals at different stages of silencing were crossed (P0), and their progeny were analyzed (F1). Male and hermaphrodite symbols indicate the sex of animals below in P0 or F1. Beneath these symbols, it is described: P0 - the stages of P0 concerning the silencing as detailed in

the legend below. F1 - The silencing state of the animals exemplified by a figure. **(E)** m*rde-1* males were crossed with m*rde-1* CR (oc-) hermaphrodites in the P0 generation, and their progeny and subsequent generations were assessed for GFP silencing.

5.3. Investigating the Mechanism of Somatic Transgene Silencing

We then questioned the generality of somatic transgene silencing triggered by crossing and whether it would occur with other transgenes. We crossed CR males with worms expressing GFP under the control of muscle and intestinal promoters, but observed no silencing of the transgenes (Figure 10A), indicating that this process is not general to all transgenes.

We sought to understand what makes the GFP transgene in our study distinct in a way that allows silencing to be triggered by crossing. We first examined if the promoter driving GFP expression enabled silencing, so we crossed worms with the same GFP construct (with the sur-5 promoter) twice with CR males, but saw no silencing (Figure 10A).

Another possibility is that *rde-1* alterations in the m*rde-1*, leading to muscle-specific activity, might allow silencing. We had already demonstrated that the *rde-1* mutation was not essential for transmitting the GFP silencing status to subsequent generations, but we had not tested its necessity during initial crossing. To investigate this, we obtained worms with GFP expression driven by the sur-5 promoter and the *rde-1* mutation, and found no transgene silencing induced by crossing (Figure 10B).

We considered whether the rescue of *rde-1* expression in muscle was essential for triggering silencing. We obtained worms with GFP transgene expression, *rde-1* mutation, and muscle rescue of its expression, but still observed no silencing upon outcrossing with AL males (Figure 10C).

One difference between the generated strain and the m*rde-1* is that the GFP transgene and muscle promoter-driven *rde-1* were inserted at the same chromosomal locus in the latter, while they were at different loci in the generated strain. This could suggest that the original target for transgene silencing is the muscle *rde-1* transgene, and this silencing spreads to nearby genes, such as GFP.

The original paper describing the m*rde-1* creation states that it is sensitive to RNAi targeting muscle genes but not those in other tissues [66]. Consistent with this, we found the strain was not sensitive to RNAi targeting the germline gene pos-1 (Figure 10D). However, it exhibited low

sensitivity to RNAi targeting muscle genes (Figure 10D), supporting the notion that muscle *rde-1* might be silenced.

To further investigate whether muscle *rde-1* might be silenced, we measured its mRNA expression. While AL and CR worms showed no expression changes, the m*rde-1* strain exhibited increased *rde-1* expression, consistent with muscle transgenic expression (Figure 10E). However, when silencing was triggered by crossing with CR worms, *rde-1* expression decreased on GFP+ worms (Figure 10E). Once GFP was silenced, *rde-1* expression increased, although not to levels observed in the m*rde-1* strain. This occurred independently of the *rde-1* mutation (Figure 10E).

These results align with a model in which the m*rde-1* strain experiences muscle *rde-1* transgene silencing, making it muscle RNAi-insensitive. Crossing with AL or CR males intensifies muscle *rde-1* transgene silencing, which then spreads to GFP, silencing both transgenes. Subsequently, *rde-1* expression increases, which could be due to several reasons. One of them is due to the fact that the mRNA of a transgene is needed as a template for further small RNA production to amplify and maintain the RNA-mediated silencing process[68,69]. With the silencing of this transgene and nearby genes, the template amount decreases, releasing some silencing. Another possibility is that GFP silencing recruits components of small RNA silencing machinery, which frees these components from muscle *rde-1* silencing.

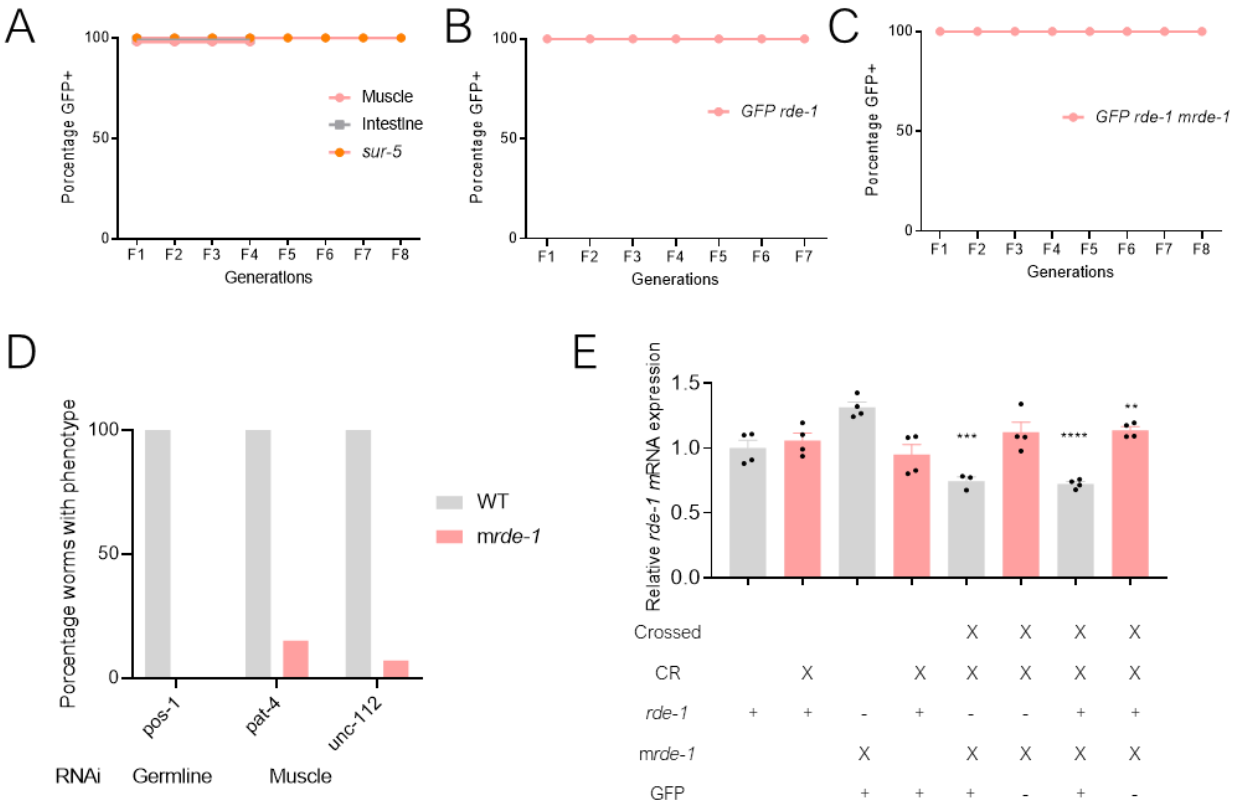

Figure 10 - *GFP and rde-1 Silencing and Inheritance in different genomic contexts* **(A)** Worms expressing GFP under the control of muscle (myo-3), intestine (ges-1), or ubiquitous (sur-5) promoters were crossed twice with CR males. A single GFP+ animal from each resulting strain was transferred, and the percentage of GFP+ offspring in subsequent generations was assessed after transferring 30 random worms between each generation. **(B)** *rde-1* mutant worms with ubiquitous GFP expression (sur-5 promoter) were crossed twice with CR males. A single GFP+ animal from the resulting strain was transferred, and the percentage of GFP+ offspring in subsequent generations was assessed after transferring 30 random worms between each generation. **(C)** *rde-1* mutant worms with ubiquitous GFP expression (sur-5 promoter) and muscle-specific *rde-1* rescue (m*rde-1*) were crossed twice with CR males. A single GFP+ animal from the resulting strain was transferred, and the percentage of GFP+ offspring in subsequent generations was assessed after transferring 30 random worms between each generation. **(D)** m*rde-1* or WT animals were exposed to RNAi targeting germline or muscle-expressed genes, as shown in the image, and the percentage of worms with RNAi phenotypes was assessed. **(E)** Animals with the genotypes described in the image were analyzed using RT-qPCR for *rde-1* mRNA expression. ** = $p < 0.01$ ** = $p<0.001$

**** = p<0.0001 vs m*rde-1*. An ordinary One Way ANOVA with Dunnett's as a multiple comparisons test was performed for m*rde-1* vs m*rde-1* (oc) wt and *rde-1* mutants separately. The graph shows the mean ± SEM.

5.4. Effects of Transgene Silencing on Worm Health

Interestingly, we observed that transgene silencing appears to impact worm health. Compared to the m*rde-1* strain, crossed GFP+ worms exhibit reduced oxidative stress resistance and transposon expression (Figure 11 A, B). Notably, these phenotypes revert upon GFP silencing. There are several possible interpretations for these results. One is that after crossing (which triggers silencing) and before GFP silencing, the components of the silencing pathway are recruited and activated, leading to the observed phenotypes. These phenotypes are then released once the silencing is completed.

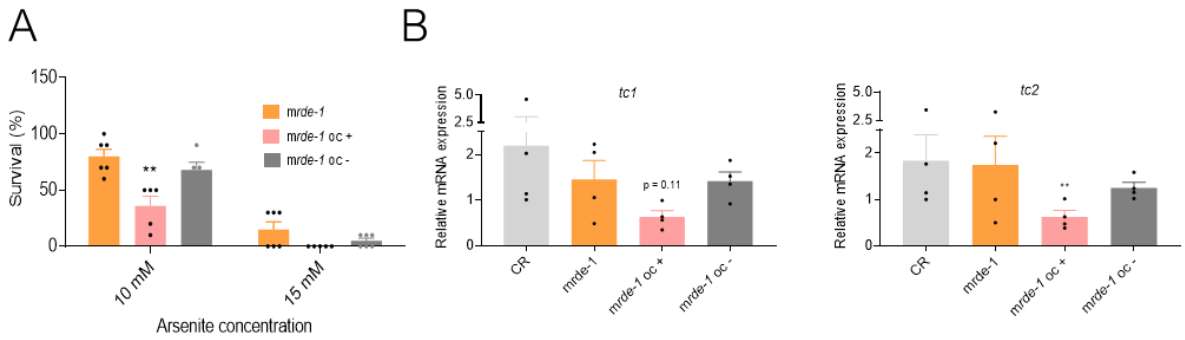

Figure 11 - *Sodium arsenite sensitivity and retrotransposon gene expression in CR, mrde-1, and CR mrde-1 worms* **(A)** Day 0 adult, m*rde-1* or CR m*rde-1* (oc+ and oc-) animals were exposed to 7.5 mM or 10 mM sodium arsenite dissolved in M9 for 8 hours, and survival was assessed. The number of surviving animals is displayed on the y-axis of the graph. ** = p < 0.01 vs m*rde-1*. An ordinary One Way ANOVA with Dunnett's as a multiple comparisons test was performed for m*rde-1* vs m*rde-1* (oc) + and -. **(B)** Relative mRNA expression of the indicated genes was measured through RT-qPCR for CR, m*rde-1*, or CR m*rde-1* (oc+ and oc-) animals. ** = p < 0.01 vs m*rde-1*. An ordinary One Way ANOVA with Dunnett's as a multiple comparisons test was performed for m*rde-1* vs m*rde-1* (oc) + and -. The graph shows the mean ± SEM.

Discussion

In this study, we aimed to investigate the impact of caloric restriction (CR) on the fertility of *C. elegans* and the molecular mechanisms underlying this effect. Our findings demonstrate that the primary factor contributing to reduced fertility in *C. elegans* under CR is a defect in the female germline component, most likely involving oocytes. Interestingly, while sperm function does not appear to be impaired in CR worms, we found significant differences in gene expression of key genes involved in spermatogenesis. Further analysis revealed that the transcription factor HLH-30 plays a critical role in ensuring sperm fertility and embryo development in CR worms.

In our quest to isolate and characterize germline cells, we employed single-cell RNA sequencing techniques. However, the pre-existing methods for cell isolation [63] failed to produce samples of sufficient quality for this analysis, and the available technique for single-cell examination of adult worms was unsuitable for germline cells, as it eliminated cells possessing a haploid genome [64]. Consequently, we were compelled to devise a new approach to obtain viable germline cells. In this research, we have established an improved cell isolation protocol for *C. elegans* germline cells, which yielded samples of superior viability and purity in comparison to previously published methods, thereby facilitating single-cell RNA sequencing with these specimens.

Notably, the isolated cells exhibited sustained viability in cell culture for over two hours, as evidenced by the absence of dead cell marker staining and the presence of active mitochondrial metabolism, as indicated by superoxide production following stimulation using mitochondrial respiration inhibitors. This methodological development enables in vitro examination of live germline cells derived from hermaphrodite *C. elegans*. Prior to our study, *in vitro* analysis was limited to sperm cells from males [70]; however, our findings have expanded the scope of investigation to include both oocytes and sperm cells from hermaphrodites, presenting numerous opportunities for research in the realm of reproduction.

Intriguingly, while our single-cell transcriptome analysis did not reveal significant differences in oocyte gene expression, we found that the female germline was responsible for the decline in fertility following caloric restriction (CR). This conclusion is supported by several

observations. Firstly, sperm function appears not to be limited under CR conditions, as evidenced by the preserved sperm count in CR hermaphrodites and the fact that mating them with ad libitum (AL) males does not restore their fertility to AL levels. Secondly, CR males exhibit fertility comparable to AL males. However, when hermaphrodites lacking sperm are subjected to CR, their fertility is decreased relative to AL when crossed with AL males, suggesting a defect in the female germline.

The absence of detectable differences in single-cell data from oocytes might imply that the factors contributing to this diminished fertility under CR could be located in the somatic gonad or the precursor germline cells responsible for producing oocytes. These cells were not discernible in our single-cell data, likely because they are characterized by multiploidy and connectivity, forming a large cell cluster that is challenging to isolate effectively [50].

Despite the seemingly efficient functionality of sperm from caloric-restricted (CR) animals, their transcriptome exhibits significant differences compared to sperm from ad libitum (AL) animals. Upon analysis of the enriched biological pathways in CR sperm, we discovered an overrepresentation of genes associated with spermatogenesis. We confirmed these changes using RT-qPCR for genes previously demonstrated as essential for spermatogenesis and found to be enriched in developing spermatocytes, such as the ones encoding argonaut proteins ALG-3 and ALG-4, which play a crucial role in 26G RNA production in developing spermatocytes [71], and SPE-46, which prevents premature sperm activation[72]. Hence, CR sperm express markers of immature dividing sperm, which may contribute to their stemness and explain the extended reproductive lifespan of CR animals [39].

Interestingly, when we looked for enrichment of transcription factor targets among the genes differentially expressed in CR sperm, we discovered an overrepresentation of genes regulated by HLH-30 and MXL-3, which exert opposing effects and cooperate to modulate gene expression related to lysosomal lipolysis during fasting. Under fasting conditions, HLH-30 activity increases while MXL-3 activity decreases, leading to enhanced lysosomal lipolysis[12]. HLH-30 is also important for *eat-2* induced longevity [19]. Notably, we observed that CR *hlh-30* (tm1978) mutant hermaphrodites exhibit significantly reduced fertility, yielding an average of only 4 offspring per adult, compared to the CR animal average of 86. We determined that this decline in progeny is primarily due to the decreased viability of mutant eggs. This reduced viability is

dependent on both the mother's CR status, as AL *hlh-30* (tm1978) mutants remain fertile, as well as on the complete loss of *hlh-30* (tm1978) in the offspring, since heterozygous progeny from CR *hlh-30* (tm1978) mutant worms develop normally. It is plausible that CR in the mother leads to a nutrient deficiency that the embryo can only overcome in an HLH-30-dependent manner.

Previous research has demonstrated that the offspring of CR *hlh-30* (tm1978) mutant worms exhibit reduced body size [23]. In line with these findings, we have observed that the majority of larvae developing from the eggs of CR *hlh-30* (tm1978) mutant worms fail to reach adulthood, ceasing development and ultimately dying. Interestingly, HLH-30 is also essential for normal recovery from other dietary stress situations in worms. Such conditions include recovery from adult reproductive diapause (ARD), during which food is withdrawn at the mid-L3 stage, prompting worms to enter a state of low metabolism and activity to ensure their survival [22], as well as recovery from the *dauer* stage in daf-2 mutants, which is a stress-resistant stage worms enter under various stressors, including food scarcity [23].

The finding that few larvae develop from CR *hlh-30* mutant worms under dietary stress implies they may enter a state similar to ARD or *dauer* during early larval stages and fail to recover due to the *hlh-30* mutation. However, the few that recover grow into typical CR adults in terms of size and fertility. Notably, CR and ARD worms share similarities like increased lifespan, reduced size, and fertility [22,73], supporting the idea that similar pathways are involved in both conditions.

HLH-30 and its human counterparts, TFEB and TFE3, are transcription factors triggered by various stresses such as starvation [12,13], oxidative stress [14], DNA damage [15], and mitochondrial stress [16]. They regulate numerous genes in the nucleus, orchestrating stress response pathways like autophagy activation, lysosomal activity, and metabolic remodeling [12,17,18]. In *C. elegans*, HLH-30 nuclear translocation is activated by CR caused by the *eat-2* mutation, insulin signaling inhibition caused by the daf-2 mutation, or impaired mitochondrial function[19].

Notably, we have found that RNAi targeting genes involved in the enhanced lysosomal lipolysis driven by HLH-30 [12] does not lead to a reduction in offspring for CR hermaphrodites. Additionally, supplementing the diet of CR *hlh-30* mutant worms with lipids fails to improve their fertility, suggesting that this may not be the pathway responsible for regulating embryonic lethality in this context. However, we observed that CR worms were more sensitive to sterility when subjected to RNAi targeting *pha-4*, a transcription factor that regulates autophagy in the context of

CR [24]. The observation that two transcription factors regulating autophagy can render CR worms almost sterile, points to the possibility that this may be the pathway regulated by HLH-30 during early embryogenesis, which is crucial for egg development under CR conditions. Another possibility is that there are parallel pathways controlled by PHA-4 and HLH-30 that acts in concert to regulate development in the context of CR.

We have also shown that HLH-30 plays a cell autonomous role in the sperm of CR males. When CR males heterozygous for the *hlh-30* (tm1978) mutation mate with a CR hermaphrodite, a higher number of offspring result from the wild-type sperm compared to the *hlh-30* (tm1978) mutant sperm. This outcome is not accounted for by the heterozygous mutation of *hlh-30* in the progeny, as we have demonstrated that these worms develop normally. The downstream effectors of HLH-30 in this context remain unclear, but they may involve the targets of HLH-30 identified as altered in CR sperm through single-cell analysis, which primarily includes uncharacterized genes specifically expressed in sperm (Supplementary information 1). Future research to characterize the functions of the proteins encoded by these genes could reveal whether they play a role in the cell autonomous effects of HLH-30 on sperm fertility.

In the next part of the study, we studied the complex inheritance patterns and transgenerational effects in *eat-2* mutant and transgenic *C. elegans*, providing novel insights into the transmission of somatic transgene silencing and its impact on worm health.

In summary, our research uncovers intricate inheritance patterns with transgenerational implications in *eat-2* mutant *C. elegans*, highlighting that the diminished fertility of these worms can be passed on to their descendants in a gender-specific manner. Additionally, we show that the heritable characteristics of *eat-2* rely on the parental germline, capable of transmitting fertility alterations to future generations without the need for reduced caloric intake.

These findings hold potential implications for comprehending and mitigating heritable conditions triggered by environmental factors in humans. Observational studies involving women who experienced famine during pregnancy have reported that their children exhibited increased incidences of obesity, diabetes, infectious diseases, and cardiovascular diseases [74–78]. Therefore, understanding the inheritance of dietary regimen effects could contribute to enhancing human health by inspiring the creation of interventions that promote healthspan traits in offspring.

We also uncovered a novel aspect of transgene silencing, showing that silencing of a somatic-expressed transgene can be inherited across generations. Our results suggest that both the male and female germline contribute to the transmission of transgene silencing phenotypes, and that the transmission is not a general feature for all transgenes but appears to be context-dependent. We propose that the muscle *rde-1* transgene may be a primary target for silencing, which then spreads to nearby genes, including GFP. This silencing may impact worm health and produce phenotypes such as reduced oxidative stress resistance and transposon expression.

Numerous studies have been conducted on the inheritance of transgene silencing in *C. elegans*, but the majority of these studies has focused on transgene silencing in the germline [59–62]. The *C. elegans* germline has specialized mechanisms to silence transgenes, such as the expression of piRNAs and specific argonautes [57]. However, the soma mostly lacks these mechanisms, and transgene silencing is seldomly observed in somatic cells, even when the same transgene is silenced in the germline [55]. The transgene we examine is expressed in the soma, presenting a unique opportunity to delve deeper into the processes governing somatic transgene silencing.

Interestingly, the gene we observed being silenced, *rde-1*, encodes an argonaute protein crucial for RNAi triggered by exogenous dsRNA [79]. RDE-1 binds to primary small RNAs and amplifies the signal into secondary siRNAs [79]. One of the few other examples of spontaneous somatic transgene silencing that spreads to an endogenous gene in the soma involves the protein SID-1 [80], which is also essential for systemic RNAi because it transports RNAs between cells. This suggests that somatic silencing may be facilitated in genes related to RNAi. High levels of endogenous small interfering RNAs targeting genes in this pathway have been demonstrated [59], potentially acting as a negative feedback mechanism to prevent uncontrolled activation that could be detrimental to the cell. It is possible that these high levels of small RNAs targeting genes involved in small RNA pathways predispose them to spontaneous silencing in the soma, triggered by the presence of a transgene.

The occurrence of somatic transgenerational silencing causing phenotypes in worms adds another layer of complexity. The activation of transgene silencing pathways may protect the worm in the context of silencing transgenes, viruses, or transposons that infiltrate the *C. elegans* genome. However, this may also have side effects on the worm's health, similar to the consequences of

hyperactivation of the immune system in humans, which can be detrimental in various contexts, such as during viral infections, autoimmune diseases, and obesity [81–84].

Conclusions

In conclusion, our study has uncovered novel insights into the impact of caloric restriction on the fertility of *C. elegans* and the molecular mechanisms underlying these effects. We found that the primary cause of reduced fertility under CR is a defect in the female germline, most likely involving oocytes, while sperm function is not impaired. Further investigation highlighted the critical role of transcription factor HLH-30 in sperm fertility and embryo development in CR worms. Additionally, we have developed an improved cell isolation protocol for adult *C. elegans* suitable for analyzing germline cells, which expands the scope of in vitro analysis to include both oocytes and sperm cells from hermaphrodites, offering new opportunities for research in reproduction. Our findings suggest that the HLH-30-dependent pathway is crucial for egg development under CR conditions. Furthermore, we have demonstrated a cell autonomous role for HLH-30 in the sperm of CR males, with future research required to elucidate the downstream effectors and functions of uncharacterized HLH-30 target genes specifically expressed in sperm. The knowledge gained from this study contributes significantly to our understanding of fertility regulation under caloric restriction and lays the foundation for future investigations in the field of reproductive biology.

Furthermore, our study reveals complex inheritance patterns with transgenerational consequences in *eat-2* mutant *C. elegans*, emphasizing that their reduced fertility can be transmitted to offspring in a sex-specific manner. We also demonstrate that the heritable traits of *eat-2* depend on the parental germline, which can convey fertility changes to future generations without the requirement for decreased caloric intake. Moreover, we discovered a new facet of transgene silencing, illustrating that silencing of a somatic-expressed transgene can be passed on through generations. Our findings indicate that both male and female germlines contribute to the transmission of transgene silencing phenotypes, and that this transmission is not a universal characteristic for all transgenes but seems to be context-dependent. We suggest that the muscle *rde-1* transgene might be a primary target for silencing, subsequently spreading to neighboring genes, including GFP. This silencing affects worm health and result in phenotypes such as diminished oxidative stress resistance and transposon expression.

Our results carry extensive implications for understanding the molecular mechanisms at play in epigenetic inheritance and transgene silencing in *C. elegans*, underscoring the significance

of examining both germline and somatic roles in these processes. Upcoming research should strive to pinpoint the specific factors and molecular pathways necessary for initiating and transmitting somatic transgene silencing between generations.

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Appendix

Y48B6A.5; nep-13; Y113G7C.1; Y69A2AR.19; C01G5.4; ZK1053.2; C50C3.2; F44G3.7; Y54E2A.9; ZK370.4; clp-6; M70.3; Y39B6A.30; tat-3; T08B6.4

Supplementary Information 1 – A compilation of HLH-30 target genes present in the differentially expressed genes between the AL and CR sperm populations, as identified through single-cell sequencing analysis using the TF2DNA2018 tool.

Annexes

Documento referente a Bioética e Biossegurança

UNICAMP

Of. CIBio/IB 05/2016

Cidade Universitária "Zeferino Vaz",
16 de maio de 2016.

Prof. Dr. MARCELO MORI
Departamento de Bioquímica e Biologia Tecidual
Instituto de Biologia
UNICAMP

Prezado Professor:

Informamos que o projeto CIBio **Interferindo com o Envelhecimento por Meio da Via de Interferência por RNA**, sob sua responsabilidade, envolvendo OGM do tipo I, foi aprovado pela CIBio-IB/Unicamp para ser desenvolvido nas dependências do Laboratório de Biologia do Envelhecimento, DBBT/IB/UNICAMP e Biotério do Laboratório de Neurobiologia Molecular, Área de Genética e Evolução, DGEB/IB/UNICAMP, ambos Nível de Biossegurança 1, protocolado sob o número **2016/03**:

> **Transcrição do parecer:**
> **O projeto visa estudar o papel da via de RNAi no envelhecimento e em doenças metabólicas. Para isso usará 2 modelos de animais geneticamente modificados (C. elegans e camundongos) que superexpressam ou apresentam perda de função de componentes-chave da via de RNAi (como Dicer) para esclarecer se essas modificações são suficientes para alterar o tempo e a qualidade de vida dos animais.**
> **Todos os OGMs e experimentos requerem apenas instalações laboratoriais de nível NB-1. O pessoal do laboratório já possui treinamento e serão supervisionados por cientistas com treinamento em biologia molecular e fisiologia animal. Há um Manual de Biossegurança disponível e todos os integrantes foram ou serão treinados para executar as técnicas e procedimentos dele constantes.**

Recomendamos que sejam observadas as instruções normativas referentes transporte e contenção da OGMs, disponíveis na webpage da CTNBio <www.ctnbio.gov.br>.

Informamos que toda documentação, assim como uma cópia desta aprovação deve estar impressa e assinada para futuras visitas/inspeções pela CIBio/IB-UNICAMP e pelos órgãos externos competentes.

Atenciosamente,

Atenciosamente,

Profa. Dra. Helena Coutinho Franco de Oliveira
Presidente da CIBio
Instituto de Biologia – UNICAMP

Cópia: chefia DBBT, Profa. Dra. Sarah Arana

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Campinas, 01 de junho de 2023.

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