Reproductive tradeoffs govern sexually dimorphic tubular lysosome induction in *C. elegans*

Cara D. Ramos, K. Adam Bohnert* and Alyssa E. Johnson*

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803

*Corresponding authors: johnsona@lsu.edu; bohnerta@lsu.edu

Abstract

Sex-specific differences in animal behavior commonly reflect unique reproductive interests. In the nematode *Caenorhabditis elegans*, hermaphrodites can reproduce without a mate and thus prioritize feeding to satisfy the high energetic costs of reproduction. However, males, which must mate to reproduce, sacrifice feeding to prioritize mate-searching behavior. Here, we demonstrate that these behavioral differences influence sexual dimorphism at the organelle level; young males raised on a rich food source show constitutive induction of gut tubular lysosomes, a non-canonical lysosome morphology that forms in the gut of hermaphrodites when food is limited or as animals age. We find that constitutive induction of gut tubular lysosomes in males results from self-imposed dietary restriction through *daf-7*/TGFβ, which promotes exploratory behavior. In contrast, age-dependent induction of gut tubular lysosomes in hermaphrodites is stimulated by self-fertilization activity. Thus, separate reproductive tradeoffs influence tubular lysosome induction in each sex, potentially supporting different requirements for reproductive success.

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Introduction

Many animal traits and behaviors, especially those linked to reproduction, display sexual dimorphism (Portman, 2007; Yamamoto, 2007; Zilkha et al., 2021). Such phenotypes vary from one sex to another within a single species, and maintenance of these differences is often vital for efficient reproduction and, ultimately, species survival. In the nematode *C. elegans*, the choice between feeding and mate searching presents an interesting example of a sexually dimorphic behavior; young male worms prioritize mate searching over feeding, whereas hermaphrodites, which reproduce on their own using self-sperm and oocytes, constantly prioritize feeding (Lipton et al., 2004; Ryan et al., 2014). The male-specific preference for mating over feeding is controlled by the *daf-7*/TGFβ neuroendocrine signaling axis. In well-fed young males, elevated DAF-7 inhibits expression of the odorant receptor *odr-10*, thereby reducing the preference to feed (Hilbert and Kim, 2017; Wexler et al., 2020). In contrast, inhibiting *daf-7* in young males is sufficient to prevent mate-searching behavior and to promote feeding behavior instead (Wexler et al., 2020). While sacrificing feeding for exploratory behavior is a critical element of male reproductive behavior and success, its effects on other aspects of animal physiology is less clear. In principle, metabolic parameters linked to nutritional status could be impacted in males. To what degree this occurs, and how it compares to changes in hermaphrodites, which assume high metabolic costs in producing embryos, is unknown.

In previous work, we demonstrated that nutritional cues govern the induction of autophagic tubular lysosomes (TLs) in the digestive tissues of worms and flies (Dolese et al., 2021; Villalobos et al., 2021). Upon starvation or dietary restriction (DR), gut lysosomes transform from vesicles into expansive tubular networks that show high degradative activity (Dolese et al., 2021; Villalobos et al., 2021). Importantly, this morphological transformation in

lysosome structure supports lifespan extension in nutrient deprived conditions, and can even be artificially mimicked in well-fed animals for health benefits (Villalobos et al., 2021). Given that nutritional cues are intimately linked to sexually dimorphic feeding/mating behaviors in *C. elegans*, it is conceivable that TL induction might naturally vary between the biological sexes in this species. If so, this could contribute to sex-specific differences in animal health and physiology.

Here, we demonstrate that young male *C. elegans* induce TLs in their gut even in the presence of abundant nutrient sources. We further demonstrate that this is linked to the self-imposed dietary avoidance that permits male worms to spend more time searching for a mate. In contrast, hermaphrodites show lower TL-related signaling in young adulthood, but this increases dramatically, surpassing even the male, during aging. Using sperm-defective mutants, we find that elevated TL induction with age in hermaphrodites relates to both the presence of sperm and embryo production, potentially as a mechanism to supply nutrients to the developing progeny and/or the mother. Collectively, our results suggest that reproductive tradeoffs dictate TL induction in *C. elegans* and may provide physiological support to animals as they prioritize distinct modes of reproductive success.

Materials and Methods

Strains

Table S1 provides a complete list of strains used in this study. Endogenously-tagged *spin-1::mCherry* was generated by *In Vivo* Biosystems using CRISPR technology. For genetic crosses, the endogenous *spin-1::mCherry* transgene was tracked by stereomicroscopy, and genetic mutations were verified by phenotypic characterization and/or sequencing.

Animal maintenance

Unless otherwise noted, worms were raised at 20ºC on NGM agar (51.3 mM NaCl, 0.25% peptone, 1.7% agar, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KPO_4, 12.9 µM cholesterol, pH 6.0). For standard experiments, fed worms were maintained on NGM agar plates that had been seeded with *E. coli* OP50 bacteria. To obtain starved adult worms, worms were washed 5x in 5 mL M9 buffer and transferred onto NGM agar that lacked OP50 bacteria. Synchronous populations of worms were obtained by bleaching gravid hermaphrodites. Briefly, adult hermaphrodites were vortexed in 1 mL bleaching solution (0.5 M NaOH, 1.51% NaClO) for 5 minutes to isolate eggs, and eggs were then washed three times in M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85.5 mM NaCl, 1 mM $MgSO_4$) before plating.

For RNAi experiments, synchronous populations of animals were grown on OP50-seeded NGM plates until late L4 or day one of adulthood, at which time they were transferred to RNAi plates (NGM plus 100 ng/µl carbenicillin and 1 mM IPTG) that had been seeded with bacteria expressing *daf-7* RNAi, which was obtained from the Julie Ahringer collection (Kamath et al., 2003) provided by Source Bioscience. An empty L4440 vector was used as a negative control.

In experiments involving the *fog-2* strain, virgin females were isolated by transferring male-sterile hermaphrodites onto NGM plates seeded with OP50 bacteria without males. Populations of mated feminized worms were maintained on plates with a source of young males throughout the experiments to ensure continuous mating during their adult lifespan.

Sperm-defective *fer-1* and *spe-9* strains are fertile at 15ºC but, when raised at 25ºC, produce sperm that signal appropriately but are defective in fertilizing oocytes. In experiments involving these mutants, strains were routinely maintained at 15ºC until the experiment was conducted. To obtain experimental, synchronous populations of *fer-1* and *spe-9* mutants, strains

were bleached, and NGM agar plates with eggs and OP50 bacteria were shifted to 25ºC to render animals self-sterile. Control strains for these experiments were treated identically at the same time.

For aging experiments, synchronous populations of worms were obtained by bleaching gravid hermaphrodites and plating their eggs onto NGM plates seeded with OP50 bacteria. Animals were allowed to develop to L4 stage, and subsequently L4 worms were picked and transferred to fresh NGM plates to begin aging experiments (4 plates of 10-15 worms each were used for each experiment). Mated feminized or hermaphrodite strains were picked and transferred to NGM plates seeded with fresh OP50 every two days to isolate adults from their progeny.

Male generation and propagation

Males were generated by heat shocking hermaphrodites to induce non-disjunction of the X chromosome. Specifically, hermaphrodites were subjected to a persistent (L1 through adulthood) heat shock at 25ºC, or, alternatively, L4 hermaphrodites were subjected to a briefer 4-6 hour heat shock at 30ºC. Male progeny isolated in the next generation were propagated by mating. For mating, 8-10 hermaphrodites were placed on a 35 mm NGM plate with roughly 20 males and maintained at 20ºC overnight. This plate was seeded with a small scoop of OP50 bacteria at the center of the plate to increase the likelihood of mating encounters. On the following day, hermaphrodites were transferred to 60 mm NGM plates and allowed to lay eggs. The mating process was repeated in subsequent generations to maintain a consistent population of males.

Male-conditioned plates

30 male worms were transferred onto NGM plates seeded with OP50 bacteria to allow males to secrete pheromones onto the plates (Maures et al., 2014). After two days, males were transferred off the plates, and *fog-2* feminized virgins were transferred onto the plates at day one of adulthood to expose them to the male-conditioned environment. *fog-2* feminized virgins were imaged two or four days after exposure.

Microscopy

For imaging experiments, worms were mounted onto agarose pads as follows. 4% agarose (Fisher Bioreagents) pads were dried on a Kimwipe (Kimtech) and then placed on top of a Gold Seal™ glass microscope slide (ThermoFisher Scientific). A small volume of 2 mM levamisole (Acros Organics) was spotted on the agarose pad as a paralyzing agent. Worms were transferred to the levamisole spot, and a glass cover slip (Fisher Scientific) was placed on top to complete the mounting. Live-animal fluorescence microscopy was performed using a Leica DMi8 THUNDER imager, equipped with 10X (NA 0.32), 40X (NA 1.30), and 100X (NA 1.40) objectives and GFP and Texas Red filter sets.

Image analysis

Images were processed using LAS X software (Leica) and FIJI/ImageJ (NIH). Lysosome networks were analyzed using "Skeleton" analysis plugins in FIJI. Briefly, images were converted to binary 8-bit images and then to skeleton images using the "Skeletonize" plugin. Skeleton images were then quantified using the "Analyze Skeleton" plugin. Numbers of objects and junctions were scored. An "object" is defined by the Analyze Skeleton plugin as a branch

connecting two endpoints, an endpoint and a junction, or two junctions. Junctions/object was used as a parameter to quantify network integrity. For SPIN-1:mCherry fluorescence quantification, the gut tissue was outlined using the free-draw tool in FIJI/ImageJ, and average fluorescence intensity of the outlined area was measured. For all fluorescence intensity experiments, the same laser intensity (50%), exposure time (300 ms), and FIM (100%) were used.

Statistical analyses

Data were statistically analyzed using GraphPad Prism (version 9.3.1). For two sample comparisons, an unpaired t-test was used to determine significance (α=0.05). For three or more samples, a one-way ANOVA followed by Šídák's multiple comparisons test was used to determine significance (α=0.05).

Results and Discussion

Young male worms show constitutive TL induction in the gut, even under nutrient rich conditions

Previously, we demonstrated that well-fed *C. elegans* hermaphrodites show gut lysosomes that are morphologically static and predominantly vesicular in structure; however, upon starvation, these lysosomes transform into dynamic, autophagic, tubular networks (Dolese et al., 2021; Villalobos et al., 2021). Thus, starvation acts as a natural trigger for TL induction in the gut of *C. elegans* hermaphrodites. To extend these studies, we explored whether male animals, which normally sacrifice feeding for mating, show differences in TL induction, perhaps

even in the presence of food. We tracked lysosomes in males on and off of food using endogenous *spin-1::mCherry*, which encodes a Spinster ortholog that robustly labels TLs (Villalobos et al., 2021). We found that young male worms, unlike young hermaphrodites (Villalobos et al., 2021), in fact exhibited TLs in the gut when food was abundant (Figure 1A-B). As in starved hermaphrodites (Villalobos et al., 2021), TL induction in young males on food was accompanied by a relative increase in endogenous SPIN-1 protein intensity (Figure 1C-D), suggesting SPIN-1 protein expression serves as a proxy for TL induction. Additionally, young male worms that were raised without food exhibited no further increase in SPIN-1::mCherry protein intensity compared to males raised on food (Figure 1E-F). Thus, in young males, TLs appear to be constitutively induced in the gut, regardless of food status.

Like starvation, aging also induces gut TLs in hermaphrodite worms (Dolese et al., 2021; Villalobos et al., 2021). Given that young male worms exhibited TL induction and higher SPIN-1::mCherry fluorescence intensity compared to young hermaphrodites, we surmised that male worms might have a higher basal level of SPIN-1, which would continue to increase relative to hermaphrodite levels during aging. However, this was not the case; by day five of adulthood, SPIN-1::mCherry intensity in hermaphrodites superseded that in males, and this trend continued into late life (Figure 1D). Thus, the stronger TL induction in nutrient rich conditions was specific to young male worms. Moreover, reproductive activities specific to self-fertilizing hermaphrodites in young adulthood may contribute to their relatively fast increase in SPIN-1 protein levels with age.

Elevated TL induction in young males results from* daf-7*-dependent prioritization of mating over feeding

Our observation that TLs were induced in male worms even on a rich food source could suggest that the same starvation-based mechanisms of TL induction seen in hermaphrodites do not apply to the male sex. Yet, given the consideration that young male worms trade off feeding in order to spend more time searching for a mate (Lipton et al., 2004; Ryan et al., 2014), we reasoned that a self-imposed DR due to prioritization of exploratory behavior may explain the constitutive TL induction in males raised on food. To test this hypothesis, we manipulated the *daf-7*/TGFβ signaling axis that differentially regulates feeding/mating decision-making in *C. elegans* hermaphrodites and males (Figure 2A) (Hilbert and Kim, 2017; Milward et al., 2011; Wexler et al., 2020; You et al., 2008). Strikingly, inhibition of *daf-7* by RNAi prevented the male-specific increase in SPIN-1::mCherry fluorescence intensities and TL induction during young age (Figure 2B-C). We further examined whether the age-dependent SPIN-1 increase in hermaphrodites was also dependent on *daf-7* signaling. Consistent with our previous findings (Figure 1D), we observed a significant increase in SPIN-1::mCherry intensities from day one to five of adulthood when hermaphrodites were raised on control RNAi (Figure S1A-B). Notably, inhibition of *daf-7* by RNAi had no significant effect on this trend; SPIN-1::mCherry fluorescence likewise increased with age to a similar extent when hermaphrodites were treated with *daf-7* RNAi (Figure S1A-B), indicating that the age-dependent increase in SPIN-1 protein levels in hermaphrodites does not require *daf-7* signaling. Collectively, these data support the model that TL induction in young male worms, but not in aging hermaphrodites, is a consequence of a self-imposed DR caused by *daf*-7-dependent prioritized mate-searching behaviors.

Sperm signaling and embryo production contribute to increased SPIN-1 intensities in mothers during early aging

Given that the age-dependent increase in SPIN-1 protein levels in hermaphrodites is not influenced by *daf-7* signaling (Figure S1A-B), we next considered alternative mechanisms that could contribute to elevated SPIN-1 protein expression and TL induction in hermaphrodites with age. Although the two natural sexes of *C. elegans* are hermaphrodite (XX) and male (XO), "feminized" hermaphrodites are obtained from XX animals incapable of producing sperm (Barton and Kimble, 1990). For example, in *fog-2* mutant animals, germ cells that would normally differentiate into sperm instead differentiate into oocytes (Schedl and Kimble, 1988). Using SPIN-1::mCherry intensity levels as a proxy for TL induction, we compared SPIN-1::mCherry intensities in hermaphrodites, virgin feminized animals, and mated feminized animals throughout adulthood. At day one, no significant differences were observed between the three groups (Figure 3A-B). However, by days five and ten, SPIN-1::mCherry intensities were significantly lower in virgin feminized animals compared to both hermaphrodite and mated feminized animals (Figure 3A-B). Consistently, the increase in SPIN-1::mCherry intensity in mated feminized animals at days five and ten correlated with TL induction (Figure 3C-D). These data suggest that the presence of sperm might drive the steep increase in hermaphrodite SPIN-1 expression during adulthood.

Intriguingly, the mere presence of mating-competent male worms has been demonstrated to depreciate physiological health and lifespan of hermaphrodite worms cultured in the same environment (Maures et al., 2014). Moreover, pre-conditioning plates with male pheromones alone is sufficient to cause reduced lifespan in hermaphrodites, indicating that exposure to male pheromones rather than mating triggers accelerated aging phenotypes in hermaphrodite worms

(Maures et al., 2014). These studies prompted us to test whether exposure to male pheromones was also sufficient to induce age-related changes to *spin-1* expression levels in feminized animals. We found that virgin feminized worms exposed to the male-conditioned plates for two or four days failed to exhibit increased SPIN-1::mCherry intensities compared to control virgin feminized worms (Figure S2A-B). Thus, exposure to male-specific pheromones is insufficient to induce SPIN-1 levels, consistent with sperm instead playing a causal role.

The above results suggested three possibilities: (i) signals emanating from sperm trigger an age-related increase in SPIN-1 and TLs in the mother, independent of fertilization; (ii) production of embryos upon fertilization of oocytes by sperm instead triggers TL induction; or (iii) signals from both sperm and embryo production contribute to increased SPIN-1 and TL induction. To distinguish between these possibilities, we examined SPIN-1::mCherry intensities in *spe-9* and *fer-1* mutants, which can produce both gametes (sperm and oocytes) but have mutations that render the sperm incapable of fertilization (L'Hernault et al., 1988; Singson et al., 1998; Ward and Miwa, 1978; Ward et al., 1981). These sperm-defective mutants allowed us to examine a biological scenario in which sperm signals are present, but embryo production is disabled. At day one of adulthood, no significant increase in SPIN-1::mCherry intensity was detected in either *spe-9* and *fer-1* mutants compared to virgin feminized worms (Figure 3E-F). However, by days three and five, SPIN-1::mCherry intensity in *spe-9* and *fer-1* mutants increased significantly compared to virgin feminized worms, albeit not to the level of hermaphrodite worms (Figure 3E-F). These results suggest that signals from both sperm and embryo production contribute to increasing SPIN-1::mCherry levels and TL induction in the mother.

In conclusion, we have uncovered two sexually dimorphic properties of TL induction in *C. elegans*: (1) young males show constitutive TL induction due to a self-imposed DR that permits enhanced mate-searching behavior; and (2) TL induction in hermaphrodites commences later during aging, dependent on previous reproductive signaling and activity. We propose that TLs are induced by different mechanisms in each sex to meet the nutritional demands imposed by their distinct reproductive activities. In young males, the induction of TLs could provide health benefits during this self-imposed DR period to boost their reproductive fitness. DR has been long known to confer health benefits and extend lifespan in many species; however, in *C. elegans*, lifespan is extended by DR in hermaphrodites, but not in males (Honjoh et al., 2017). This supports the notion that male worms, which are calorically restricted by choice, already exhibit the health benefits of DR as a natural consequence of this behavior and, thus, do not exhibit any further lifespan extension when put under experimental dietary constraints. Moreover, we have shown previously that artificial induction of TLs allows worms to sustain their mobility longer in life (Villalobos et al., 2021). Thus, it is interesting to speculate whether induction of TLs in young males improves their physical fitness and ability to find a mate. In the case of hermaphrodites, developing embryos inside the uterus require significant nutritional support, which must come from the mother. Thus, TL induction might allow mothers to recycle nutrients, such that they can provide additional nutritional sustenance to the developing embryos and/or themselves during reproduction. Collectively, these findings add to growing evidence indicating that different sexes have distinct nutritional requirements during their reproductive lifespan, and they also suggest that TL induction may contribute to sustaining reproductive fitness by different mechanisms in each sex.

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Competing interests

The authors declare that they have no competing interests.

Author contributions

Conceptualization: KAB, AEJ; Methodology: CDR; Investigation: CDR, KAB, AEJ; Visualization: CDR, KAB, AEJ; Funding acquisition: KAB, AEJ; Project administration: KAB, AEJ; Supervision: KAB, AEJ; Writing – original draft: KAB, AEJ; Writing – review & editing: CDR, KAB, AEJ

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Figures

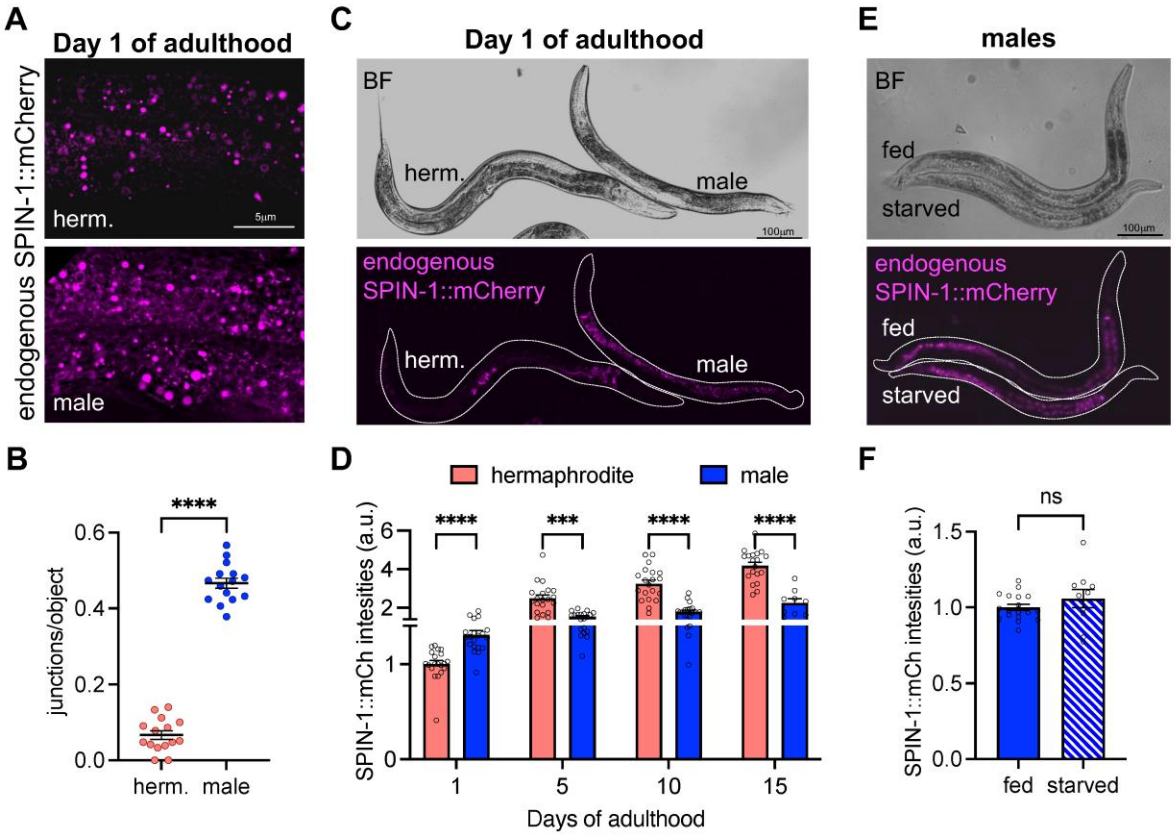

Figure 1: Young male worms show constitutive TL induction in the gut, even under nutrient rich conditions.

A. Representative images of endogenously tagged SPIN-1::mCherry in hermaphrodite and male worms. **B.** Quantification of lysosome junctions/object in hermaphrodite and male worms (*n*=15 worms). Data are presented as mean ± SEM, and statistical significance was determined using a student's t-test (****p<0.0001). **C.** Representative images of *spin-1* expression in hermaphrodite and male worms. **D.** Quantification of SPIN-1::mCherry intensities in hermaphrodite (*n*=20 worms for days one, five and ten; *n*=19 worms for day fifteen) and male

worms throughout adulthood (*n*=20 worms for days one, five and ten; *n*=9 worms for day fifteen). Data are presented as mean ± SEM, and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (***p<0.001, ****p<0.0001). **E.** Representative images of *spin-1* expression in fed and starved male worms. **F.** Quantification of SPIN-1::mCherry intensities in fed (*n*=18 worms) and starved (*n*=10 worms) male worms. Data are presented as mean ± SEM, and statistical significance was determined using a student's t-test (ns- not significant).

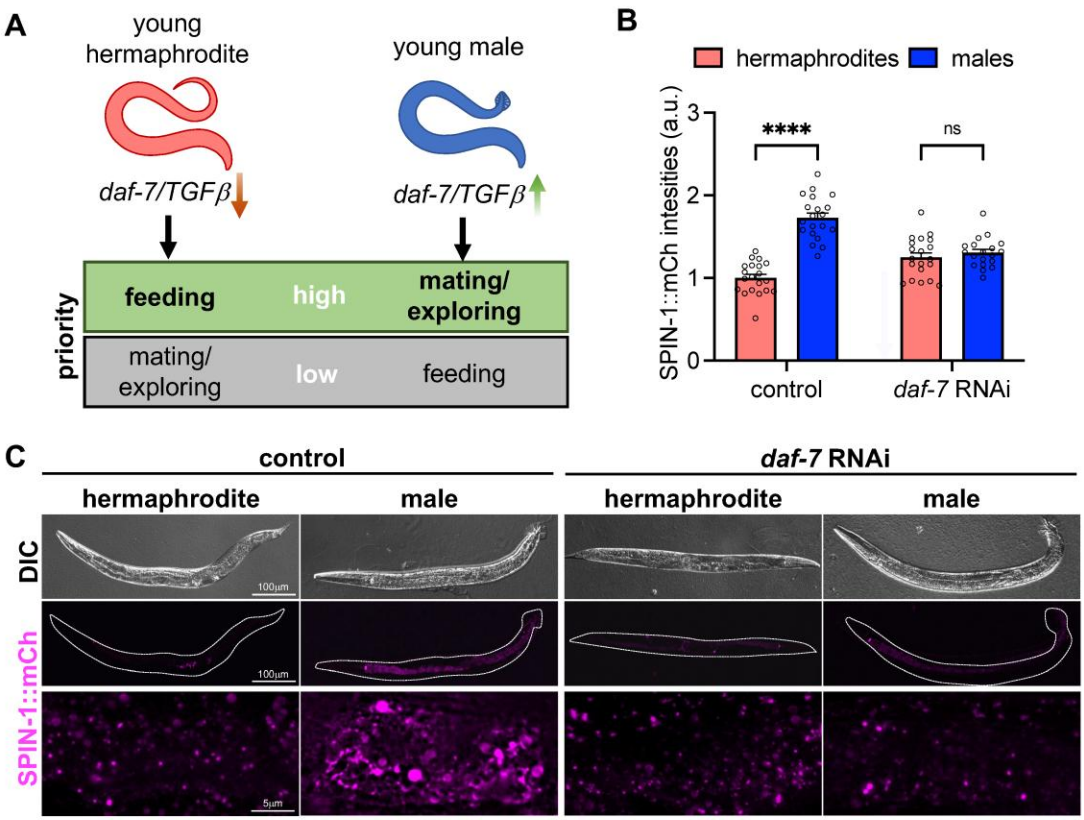

Figure 2: Elevated TL induction in young males results from *daf-7*-dependent prioritization of mating over feeding.

A. Schematic diagram illustrating the *daf-7*-dependent sexually dimorphic feeding/exploring behavior in *C. elegans*. In young hermaphrodites, *daf-7* signaling is downregulated, which promotes feeding behaviors. In contrast, *daf-7* signaling is upregulated in young male worms to promote exploratory behaviors. **B.** Quantification of SPIN-1::mCherry intensities in control (*n*=20 worms for both sexes) and *daf-7* RNAi treated worms (*n*=20 worms for hermaphrodites, *n*=19 worms for males). Data are presented as mean ± SEM, and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (ns- not significant, ****p<0.0001). **C.** Representative images of *spin-1* expression and TL induction in control and *daf-7* RNAi treated worms.

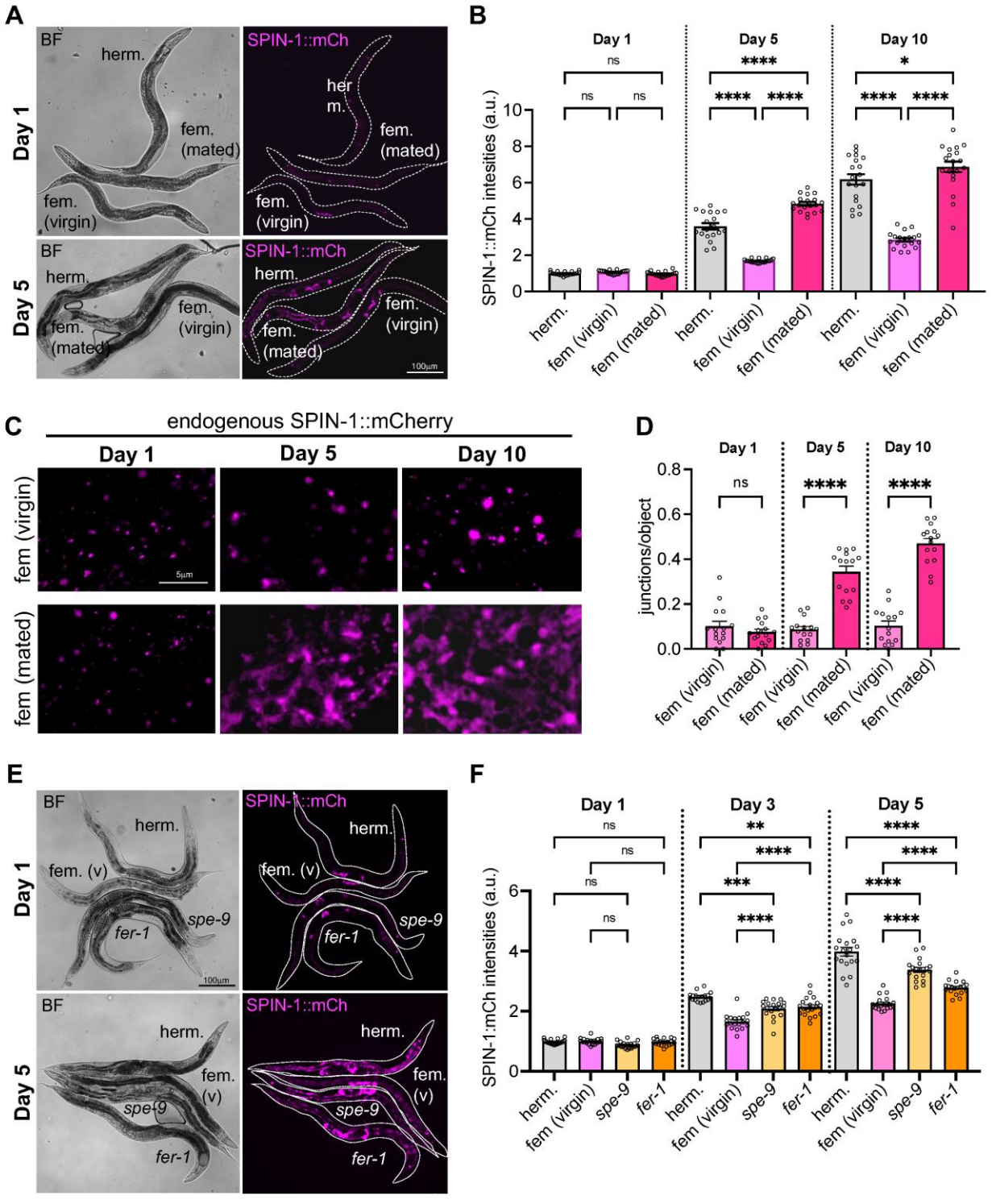

Figure 3: Sperm signaling, and embryo production contribute to increased SPIN-1 intensities in mothers during early aging.

A-B. Representative images of *spin-1* expression (A) and quantification of SPIN-1::mCherry intensities (B) in hermaphrodite and feminized worms (virgin and mated) at days one, five and ten of adulthood (*n*=19 worms for all genotypes and conditions). Data are presented as mean ± SEM, and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (ns-not significant, *p<0.05, ****p<0.0001). **C-D.** Representative images of endogenously tagged SPIN-1::mCherry (C) and quantification of lysosome junctions/object (D) in feminized worms (virgin and mated) at the ages indicated (*n*=15 worms for all conditions). Data are presented as mean ± SEM, and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (ns-not significant, ****p<0.0001). **E-F.** Representative images of *spin-1* expression (E) and quantification of SPIN-1::mCherry intensities (F) in hermaphrodites, virgin feminized worms, and mutants with fertilization-incompetent sperm (*spe-9* and *fer-1*) during early adulthood (*n*=19 worms for all genotypes and conditions). Data are presented as mean ± SEM, and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (ns-not significant, **p<0.01, ***p<0.001, ****p<0.0001).

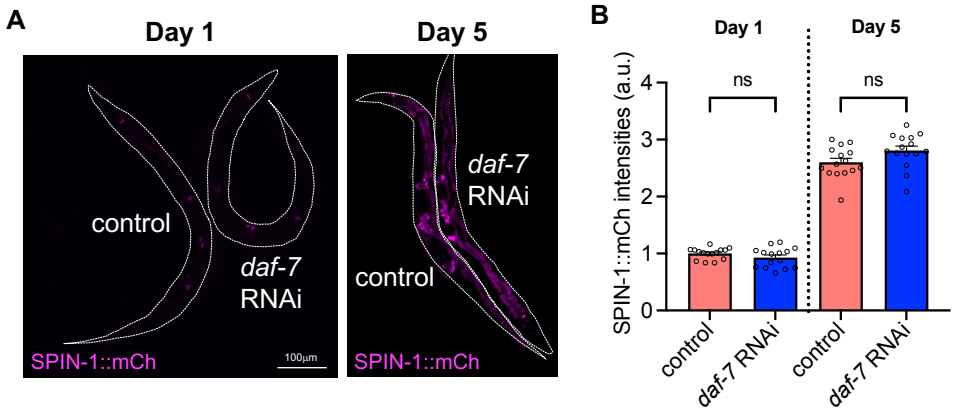

Fig. S1. Age-dependent increase in *spin-1* expression in hermaphrodites is not dependent on *daf-7*/TGFβ signaling.

A. Representative images of *spin-1* expression in hermaphrodites at days one and five of adulthood after treatment with control or *daf-7* RNAi. **B.** Quantification of SPIN-1::mCherry intensities in hermaphrodites at days one and five of adulthood after treatment with control or *daf-7* RNAi (*n*=15 worms for all conditions). Data are presented as mean ± SEM, and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (ns- not significant).

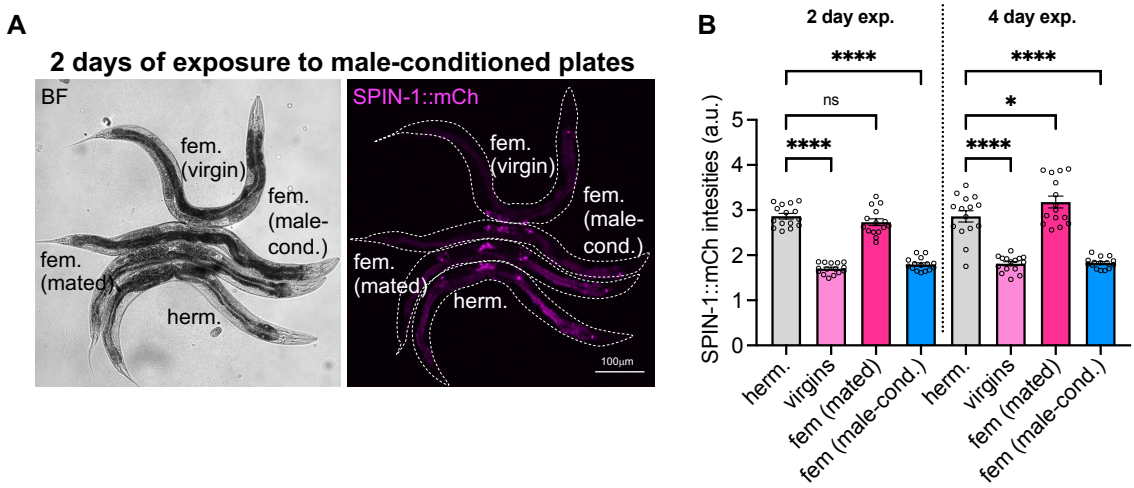

Fig. S2. Exposure to male pheromones is not sufficient to induce TLs in feminized worms.

A. Representative images of *spin-1* expression in feminized worms that were virgin, mated, or exposed to male-conditioned plates for two days. **B.** Quantification of SPIN-1::mCherry intensities in age-matched strains in the conditions indicated (*n*=15 worms for all conditions). Data are presented as mean ± SEM, and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (ns- not significant, *p<0.05, ****p<0.0001).

Table S1. Strains used in this study.

Strain name	Genotype	Source
COP2331	*spin-1(knu1010[spin-1::mCherry::loxP::HygR::loxP])* V	Villalobos et al, 2021; (by *In Vivo* Biosystems)
KAB74	*fog-2(q71)* *spin-1(knu1010[spin-1::mCherry::loxP::HygR::loxP])* V	this study
KAB98	*spe-9(hc52) I;* *spin-1(knu1010[spin-1::mCherry::loxP::HygR::loxP])* V	this study
KAB99	*fer-1(b232) I;* *spin-1(knu1010[spin-1::mCherry::loxP::HygR::loxP])* V	this study