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SHORT COMMUNICATION

Reproductive tradeoffs govern sexually dimorphic tubular lysosome induction in *Caenorhabditis elegans*

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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 found 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.

KEY WORDS: Autophagy, DAF-7, Dietary restriction, Sexual dimorphism, Spinster, Aging

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 *Caenorhabditis 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 gene *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 are 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 preprint). Upon starvation or dietary restriction, gut lysosomes transform from vesicles into expansive tubular networks that show high degradative activity (Dolese et al., 2021; Villalobos et al., 2021 preprint). 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 preprint). 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 that of the male, during aging. Using sperm-defective mutants, we found 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, endogenous *spin-1::mCherry* transgene expression was tracked by stereomicroscopy, and genetic mutations were verified by phenotypic characterization and/or sequencing.

Animal maintenance

Unless otherwise noted, worms were raised at 20°C on NGM agar (51.3 mmol l^{-1} NaCl, 0.25% peptone, 1.7% agar, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 25 mmol l^{-1} KPO_4, 12.9 µmol l^{-1} cholesterol, pH 6.0). For standard experiments, fed worms were maintained on NGM agar plates that had been seeded with *E. coli*

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OP50 bacteria. To obtain starved adult worms, worms were washed 5× 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 mol l^{-1} NaOH, 1.51% NaClO) for 5 min to isolate eggs, and eggs were then washed 3 times in M9 buffer (22 mmol l^{-1} KH_2PO_4, 42 mmol l^{-1} Na_2HPO_4, 85.5 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgSO_4$) before plating.

For RNAi experiments, synchronous populations of animals were grown on OP50-seeded NGM plates until late L4 or day 1 of adulthood, at which time they were transferred to RNAi plates (NGM plus 100 ng μl^{-1} carbenicillin and 1 mmol l^{-1} 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 15°C but, when raised at 25°C, produce sperm that signal appropriately but are defective in fertilizing oocytes. In experiments involving these mutants, strains were routinely maintained at 15°C 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 25°C 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 2 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 to adulthood) heat shock at 25°C, or, alternatively, L4 hermaphrodites were subjected to a briefer (4–6 h) heat shock at 30°C. 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 20°C 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. 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

Thirty 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 2 days, males were transferred off the plates, and *fog-2* feminized virgins were transferred onto the plates at day 1 of adulthood to expose them to the male-conditioned environment. *fog-2* feminized virgins were imaged 2 or 4 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 (Thermo Fisher Scientific); a small volume of 2 mmol l^{-1} 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 (Thermo Fisher Scientific) was placed on top to complete the mounting. Live-animal fluorescence microscopy was performed using a Leica DMi8 THUNDER imager, equipped with 10× (NA 0.32), 40× (NA 1.30) and 100× (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. The number of objects and junctions was 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 young *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 preprint). 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 food using endogenous *spin-1::mCherry*, which encodes a Spinster ortholog that robustly labels TLs (Villalobos et al., 2021 preprint). We found that young male worms, unlike young hermaphrodites (Villalobos et al., 2021 preprint), in fact exhibited TLs in the gut when food was abundant (Fig. 1A,B). As in starved hermaphrodites (Villalobos et al., 2021 preprint), TL induction in young males on food was accompanied by a relative increase in endogenous SPIN-1 protein fluorescence intensity (Fig. 1C,D), suggesting SPIN-1

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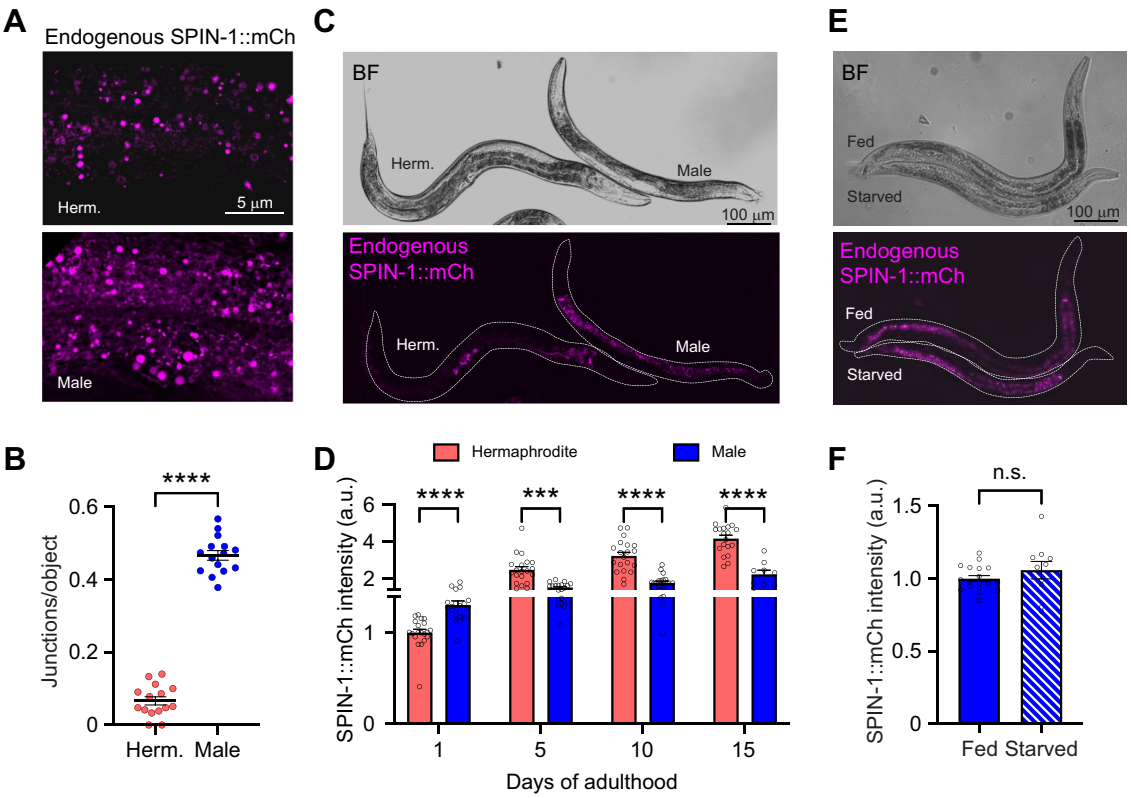

Fig. 1. Young male *Caenorhabditis elegans* show constitutive tubular lysosome (TL) induction in the gut, even under nutrient-rich conditions. (A) Representative images of endogenously tagged SPIN-1::mCherry in hermaphrodite and male worms on day 1 of adulthood. (B) Quantification of lysosome junctions/object in hermaphrodite and male worms (n=15 worms). Data are presented as means±s.e.m., and statistical significance was determined using Student's t-test (****P<0.0001). (C) Representative images of *spin-1* expression in hermaphrodite and male worms on day 1 of adulthood. BF, brightfield. (D) Quantification of SPIN-1::mCherry fluorescence intensity (a.u., arbitrary units) in hermaphrodite (n=20 worms for days 1, 5 and 10; n=19 worms for day 15) and male worms throughout adulthood (n=20 worms for days 1, 5 and 10; n=9 worms for day 15). Data are presented as means±s.e.m., 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 fluorescence intensity in fed (n=18) and starved (n=10) male worms. Data are presented as means±s.e.m., and statistical significance was determined using Student's t-test (n.s., not significant).

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 fluorescence intensity compared with males raised on food (Fig. 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 preprint). Given that young male worms exhibited TL induction and higher SPIN-1::mCherry fluorescence intensity compared with 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 5 of adulthood, SPIN-1::mCherry fluorescence intensity in hermaphrodites superseded that in males, and this trend continued into late life (Fig. 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 dietary restriction 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 (Fig. 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 intensity and TL induction in young males (Fig. 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 (Fig. 1D), we observed a significant increase in SPIN-1::mCherry fluorescence intensity from day 1 to 5 of adulthood when hermaphrodites were raised on control RNAi (Fig. 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 (Fig. 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

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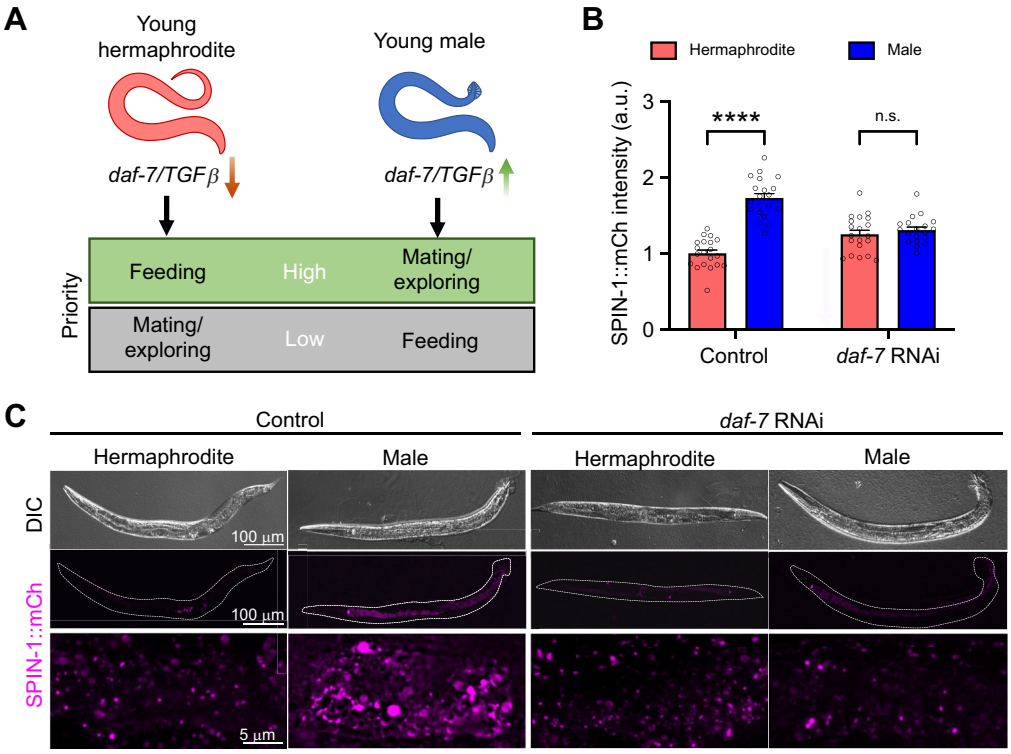

Fig. 2. Elevated TL induction in young males results from DAF-7-dependent prioritization of mating over feeding. (A) Schematic diagram illustrating the DAF-7-dependent sexually dimorphic feeding/exploring behavior in *C. elegans*. In young hermaphrodites, DAF-7 signaling is downregulated, which promotes feeding behaviors. In contrast, DAF-7 signaling is upregulated in young male worms to promote exploratory behaviors. (B) Quantification of SPIN-1::mCherry fluorescence intensity 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 means±s.e.m., and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (n.s., not significant, ****P<0.0001). (C) Representative images of *spin-1* expression and TL induction in control and *DAF-7* RNAi-treated worms. DIC, differential interference contrast.

a consequence of a self-imposed dietary restriction caused by DAF-7-dependent prioritized mate-searching behaviors.

Sperm signaling and embryo production contribute to increased SPIN-1 fluorescence intensity 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 (Fig. 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 fluorescence intensity levels as a proxy for TL induction, we compared SPIN-1::mCherry fluorescence intensity in hermaphrodites, virgin feminized animals and mated feminized animals throughout adulthood. At day 1, no significant differences were observed between the three groups (Fig. 3A,B). However, by days 5 and 10, SPIN-1::mCherry fluorescence intensity was significantly lower in virgin feminized animals compared with that in both hermaphrodite and mated feminized animals (Fig. 3A,B). Consistently, the increase in SPIN-1::mCherry fluorescence intensity in mated feminized animals at days 5 and 10 correlated with TL induction (Fig. 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 2–4 days failed to exhibit increased SPIN-1::mCherry fluorescence intensity compared with control virgin feminized worms (Fig. S2A,B). Thus, exposure to male-specific pheromones is insufficient to induce an increase in 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 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 fluorescence intensity 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 1 of adulthood, no significant increase in SPIN-1::mCherry fluorescence intensity was detected in either *spe-9* and *fer-1* mutants compared with virgin feminized worms (Fig. 3E,F). However, by days 3 and 5, SPIN-1::mCherry fluorescence intensity in *spe-9* and *fer-1* mutants increased significantly compared with that in virgin feminized worms, albeit not to the level of hermaphrodite worms (Fig. 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 dietary restriction

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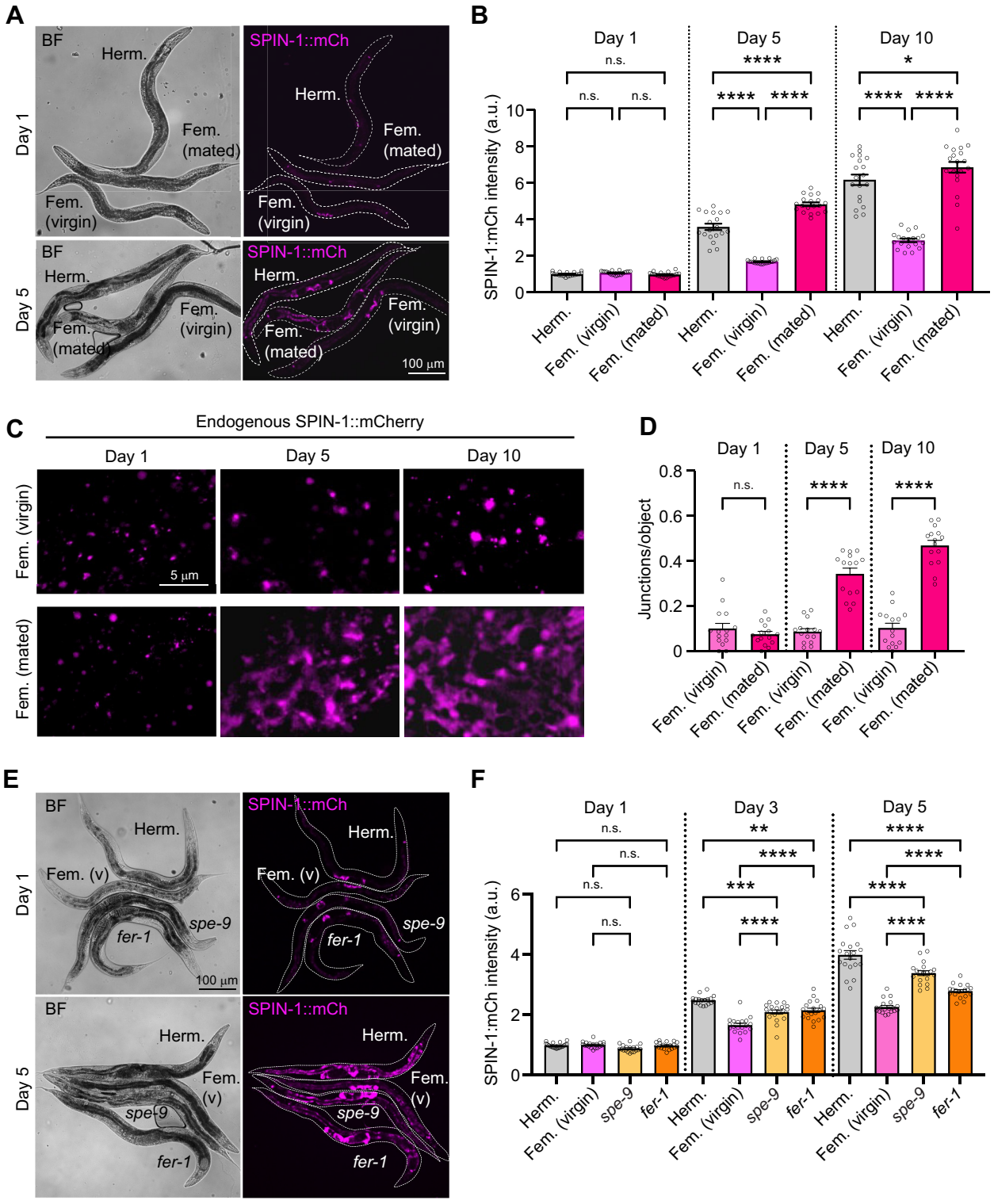

Fig. 3. Sperm signaling and embryo production contribute to increased SPIN-1 fluorescence intensity in mothers during early aging.
(A,B) Representative images of *spin-1* expression (A) and quantification of SPIN-1::mCherry fluorescence intensity (B) in hermaphrodite and feminized worms (virgin and mated) at days 1, 5 and 10 of adulthood (*n*=19 worms for all genotypes and conditions). Data are presented as means±s.e.m., and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (n.s., 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 days 1, 5 and 10 of adulthood (*n*=15 worms for all conditions). Data are presented as means±s.e.m., and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (n.s., not significant; *****P*<0.0001). (E,F) Representative images of *spin-1* expression (E) and quantification of SPIN-1::mCherry fluorescence intensity (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 means±s.e.m., and statistical significance was determined using a one-way ANOVA followed by Šídák's multiple comparisons test (n.s., not significant; ***P*<0.01, ****P*<0.001, *****P*<0.0001).

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 dietary restriction period to boost their reproductive fitness. Dietary restriction has long been known to confer health benefits and extend lifespan in many species; however, in *C. elegans*, lifespan is extended by dietary restriction 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 dietary restriction 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 preprint). 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 no competing or financial interests.

Author contributions
Conceptualization: K.A.B., A.E.J.; Methodology: C.D.R., K.A.B.; Formal analysis: C.D.R., K.A.B.; Investigation: C.D.R., K.A.B., A.E.J.; Resources: C.D.R.; Data curation: C.D.R.; Writing - original draft: K.A.B., A.E.J.; Writing - review & editing: C.D.R., K.A.B., A.E.J.; Visualization: C.D.R., K.A.B., A.E.J.; Supervision: K.A.B., A.E.J.; Project administration: K.A.B., A.E.J.; Funding acquisition: K.A.B., A.E.J.

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