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Dramatic fertility decline in aging *C. elegans* males is associated with mating execution deficits rather than diminished sperm quality

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Abstract

Although much is known about female reproductive aging, fairly little is known about the causes of male reproductive senescence. We developed a method that facilitates culture maintenance of *Caenorhabditis elegans* adult males, which enabled us to measure male fertility as populations age, without profound loss of males from the growth plate. We find that the ability of males to sire progeny declines rapidly in the first half of adult lifespan and we examined potential factors that contribute towards reproductive success, including physical vigor, sperm quality, mating apparatus morphology, and mating ability. Of these, we find little evidence of general physical decline in males or changes in sperm number, morphology, or capacity for activation, at time points when reproductive senescence is markedly evident. Rather, it is the loss of efficient mating ability that correlates most strongly with reproductive senescence. Low insulin signaling can extend male ability to sire progeny later in life, although insulin impact on individual facets of mating behavior is complex. Overall, we suggest that combined modest deficits, predominantly affecting the complex mating behavior rather than sperm quality, sum up to block effective *C. elegans* male reproduction in middle adult life.

Keywords

Aging Sperm; *C. elegans*; Males Fertility

1. Introduction

In today's society it has become increasingly common for couples to wait until later in life to have children. In this context, much attention has been devoted to the study of female reproductive decline with age (Balasch and Gratacós, 2011; Djahanbakhch et al., 2007). In

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Conflict of Interest

No conflicts of interests.

women, fertility declines in the fourth decade followed by reproductive cessation after menopause. Advanced maternal age increases the risk of birth defects, especially those related to chromosomal abnormalities such as the well-known Down's syndrome (Cleary-Goldman et al., 2005; Djahanbakhch et al., 2007). The risks of advanced paternal age, which include miscarriages, birth deformities and neurodevelopmental disorders (Saha et al., 2009), as well as a possible enhanced incidence in schizophrenia and autism (Kong et al., 2012), are also of medical concern. However, male reproductive aging has generally received relatively little experimental attention.

Reproductive fitness is an important determinant of the survival of species, and hence age-related decline in fertility is a factor in evolution itself. The factors that contribute to fertility decline across species are likely to involve those proposed for aging and its evolution in general –including mutation accumulation (Medawar, 1952); positive natural selection for genes critical for development and reproduction but that confer a long-term, later-life deleterious effect (antagonistic pleiotropy, Williams, 1957); and the resource tradeoffs in maintenance of the soma that are made to sustain a high quality germline (disposable soma theory Kirkwood, 1977). Indeed, for reproductive aging, the issue of maintained integrity of germline vs. soma is of central interest, with sustained function of both needed for successful reproduction.

Caenorhabditis elegans is a facile model organism for the studies of aging and reproduction (Kenyon, 2010a; Partridge, 2011; Singson, 2001; Singson et al., 2008). This animal exists mainly as a hermaphrodite form, although infrequent meiotic non-disjunction gives rise to males, which can cross-fertilize hermaphrodites (Brenner, 1974). In *C. elegans* studies on reproductive lifespan, emphasis has been placed on the hermaphrodite and potential tradeoffs between reproduction and lifespan. Under standard lab culture conditions, wild type and a variety of genetic mutants do not exhibit significant correlation between longevity and self-reproductive span (Gems et al., 1998; Huang et al., 2004; Johnson et al., 1993; Kenyon et al., 1993; Tissenbaum and Ruvkun, 1998), and the production of progeny by itself does not shorten life span (Gems and Riddle, 1996; Hsin and Kenyon, 1999). Interestingly, germ-line stem cells and the somatic gonad components of the reproductive system produce signals that influence *C. elegans* lifespan in opposite directions (Arantes-Oliveira et al., 2002; Ghazi, 2013; Hsin and Kenyon, 1999; Kenyon, 2010b), and have no net effect on longevity when the entire reproductive system is removed (Hsin and Kenyon, 1999; Kenyon et al., 1993). Although the length of the self-reproductive span is not correlated with lifespan (Anderson et al., 2011; Gems and Riddle, 1996; Huang et al., 2004; Johnson, 1987), the length of the reproductive span of mated hermaphrodites is (Hughes et al., 2007; Mendenhall et al., 2011; Wu et al., 2012). (It should be noted, however, that the lifespan of mated hermaphrodites is shortened by copulation as the male to hermaphrodite ratio increases Gems and Riddle, 1996). Environmental conditions such as dietary restriction do reveal a strong tradeoff between reproduction and lifespan in *C. elegans* (Huang et al., 2004; Hughes et al., 2007; Klass, 1977), an observation supported in fly and rodent models (Ball et al., 1947; Berg, 1960; Berg and Simms, 1960; Merry and Holehan, 1979; Partridge et al., 2005). Moreover, higher resistance to stress confers longevity with a fitness cost

(Casanueva et al., 2012; Rea et al., 2005). Thus, integration of environmental conditions is a clear factor on the reproductive output/lifespan equation of hermaphrodites.

Male lifespan is also influenced by culture conditions and mating experience (Gems and Riddle, 2000; McCulloch and Gems, 2003). It should be noted that male lifespan analysis is complicated by practical limitations of long-term culture of males. *C. elegans* males have a mate-searching drive that causes them to leave growth plates/food in large numbers—searches often bring them to encounter plate edges, where they desiccate. Thus, extraordinary culture conditions must be used to study male lifespan (McCulloch and Gems, 2003). In this work, we present novel use of garlic chemo-avoidance strategies to maintain aging male populations for later life study.

We have had a long-term interest in differential age-associated tissue decline, a phenomenon not extensively addressed in *C. elegans* males to date. In contrast, some elegant studies have characterized the decline of the aging *C. elegans* oocyte population (Luo et al., 2010). Although unmated hermaphrodites live approximately 3 weeks, they are fertile for about five days of their adult life using their own sperm supply. The period of fertility can be extended as long as 15 days by cross fertilization (Hughes et al., 2007; Luo et al., 2009; Mendenhall et al., 2011), revealing some late-age maintenance of germline, previously suggested by electron microscopy data in Herndon et al. (2002). Still, a marked decline in hermaphrodite fertility, attributed to the decline in oocyte quality with age (Luo et al., 2010), occurs irrespective of the presence of sperm. Oocyte decline is independent of the rate of oocyte use and the number of progeny produced (Andux and Ellis, 2008).

With an interest in characterizing aspects of male healthspan, we studied the fertility of the *C. elegans* male over adult life. We found that male reproductive lifespan is strikingly short, with the ability to sire cross progeny falling off dramatically by day six of adult life, similar to a recent report (Guo et al., 2012). Male fertility decline could be the outcome of elimination/decline of functional germ cells; alternatively, infertility could follow from the failure of somatic tissues and functions required for successful reproduction. We asked whether germline integrity or somatic integrity was a significant factor in the end of male reproduction. We find that male mating behavior, rather than sperm quality, is associated with the mid-life failure to sire progeny. In *C. elegans*, the spirit is willing, but the flesh is weak.

2. Materials and methods

2.1. Nematode strains and growth

The *C. elegans* strains used in this work were: N2 (wild-type variation from Bristol), DR466 [*him-5(e1490)* V], CB61 [*dpy-5(e61)* I], CB169 [*unc-31(e169)* IV], ZB2604 [*daf-2(e1370)* III; *him-5(e1490)* V], and ZB2605 [*daf-16(mgDf50)* I; *him-5(e1490)* V]. All strains were grown on NGM (nematode growth media) agar plates (35-mm Petri dishes) with *Escherichia coli* (strain OP50) as food, and were maintained at 20 °C with the exception of the *daf-2(e1370);him-5(e1490)* mutant strain, which was grown at 15 °C, prior to shifting to restrictive temperature, along with like-treated *him-5* control in some experiments.

2.2. Garlic-ring technique

2.2.1. Preparation of garlic extract—Garlic cloves (from the grocery shop) were peeled and crushed with the aid of a mortar and pestle (alternatively, the cloves can be placed in folded Saran wrap to gently crush them with a hammer). 50 g of crushed garlic was placed in a flask containing 50 ml of 95% ethanol and left to soak in the flask (covered with parafilm) for about an hour. Two such flasks were arranged. The mixture was then poured through a 150 ml disposable, vacuum-driven filter and stored in a sterile container, sealed and refrigerated.

2.2.2. Application of garlic extract to plates—Garlic extract was allowed to reach room temperature and then applied to plates under a hood to minimize the spread of garlic odor. A ring of garlic extract was created by adding a drop with a Pasteur pipette at the boundary of the NGM media touching the lateral side of the plate. The plate had to be held slightly tilted in one hand to spread the drop around the NGM boundary by rotation. Sometimes an additional drop was needed to complete the ring. Treated plates were left to dry under a hood.

2.3. Lifespan of *him-5(e1490)* nematodes exposed to garlic extract

Adult lifespans of *him-5(e1490)* males and hermaphrodites were separately measured in garlic-treated and non-treated plates (control). *him-5(e1490)* nematodes were age synchronized by collecting L1 larvae from bleached hermaphrodites. Cohorts were separated into genders at the L4 larval stage. Adult males were aged in isolation and kept on the same plate for the duration of their lifespan. Adult hermaphrodites were transferred onto new plates every other day during their reproductive period to prevent contamination by younger growing progeny. The ring of garlic-extract was re-applied once a week when male adults were most active to improve retention. Adults that got lost and hermaphrodites that died due to vulva disruption or internal hatching of progeny were censored. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. Two trials were performed for each gender.

2.4. Mating efficiency assay

Males were synchronized and aged on NGM plates containing OP50 and garlic extract to prevent male loss. Groups of four age-matched males were crossed to one L4 *dpy-5(e61)* hermaphrodite. The parents were removed 48 h after the initial mate and the F1 progeny were scored two days after the removal of the F0 generation. The % cross progeny was calculated based on the number of non-Dpy males and total number of progeny. The mating efficiency patterns observed in *him-5* males (used as controls) crossed with young *dpy-5* hermaphrodites (the carriers of the genetic marker for outcross progeny) were corroborated by performing crosses between WT males and WT hermaphrodites (in ratios 4 males:1 hermaphrodite, 1 male:1 hermaphrodite and on 10 mm and 60 mm culture plates) to validate the approach we used to assay mating efficiency and to address any concerns on the potential confounding effects of the *him-5(e1490)* and *dpy-5(e61)* mutations, the concentration of males, and the size of the mate searching field.

2.5. Vigor assay

To score the animals' physical capacity, individual male worms previously washed in $1 \times$ M9 buffer were transferred to either a drop of viscous halocarbon oil (series hc-700 by Halocarbon Productions Corp., River Edge, NJ) or a drop of a liquid salt solution ($1 \times$ M9). Full body thrashes were counted over a period of 30 s.

2.6. Tail morphology assay

The male to be imaged was washed in $1 \times$ M9 buffer and placed on an agarose slide containing 7 μ l of levamisole solution. A cover slip was placed over the slide and gently rolled until the tail fan laid flat. Images were captured using DIC microscopy for days 1, 3, and 5 post-L4, and each one was scored for morphological irregularities like collapsed rays or rough cuticular edges (Emmons, 2005).

2.7. Sperm activation assay

3 age-matched males, washed in $1 \times$ M9 buffer, were placed on a microscope slide containing 12 μ l of Pronase/Sperm Media solution. The solution consisted of 0.066 μ g of Pronase per 1 μ l of Sperm Media. The males were dissected to release sperm, and the slide was then left uncovered in a humid chamber for 7 min, after which a cover slip was placed on the slide and the image was taken. The number of sperm that activated (showed pseudopods) out of the total number of sperm present was reported as % activation. Sperm were observed using polarized light and a $40 \times$ lens.

2.8. Mating interest: Response and vulva location assay

Response assay plates were prepared by spotting 12 μ l of OP50 culture on a fresh NGM plate the previous night. 20 *unc-31* hermaphrodites were placed onto the mating dot and allowed to acclimate for 10 min. Response and vulva location were measured as described (Barr and Garcia, 2006) on male mating behavior for a time period of 4 min for each animal. Three separate trials with 15 males each were conducted for all genotypes.

2.9. Statistical analysis

All statistical analyses (Fisher's exact test, Student's *T* test, ANOVA and the post-hoc Bonferroni's multiple comparison test) were performed using the GraphPad Prism 5 software (La Jolla, CA).

3. Results

3.1. Garlic extract prevents loss of males due to mate searching behavior

C. elegans males indulge in mate searching behavior, such that in the absence of hermaphrodites, males leave the food source and crawl towards the edge of the culture plate (Emmons and Sternberg, 1997; Lipton et al., 2004). As a result, when males are segregated and aged in isolation for the quantification of mating behaviors, many encounter the sides of the culture plates and desiccate on the plastic plate walls. Male mate searching exodus in *C. elegans* leads to a significant reduction in the number of older males (past 6 days) available for experiments (on the order of ~50%, Fig. 1A). Traditionally, the way to compensate for

losses due to male searching behavior has been to start with large numbers of males per plate, but persisting males can be physically damaged by wall encounters.

To establish a more efficient method for maintaining aging male populations, to minimize physical stress on aging males, and to avoid selection for lack of mate searching behavior in aging male populations, we developed a chemical avoidance protocol. We noted that *C. elegans* males avoided patches that contained garlic extracted in ethanol (Fig. 1B). To contain males, we therefore created a ring of garlic extracted in ethanol around the entire edge of the petri dish on which animals were cultured. *him-5(e1490)* males isolated on such garlic-surrounded plates showed drastically lower rates of leaving the food source (Fig. 1A). The *him-5(e1490)* mutation generates a high frequency of males and is commonly used for male studies. *him-5(e1490)* does not affect male mating behaviors (Geldziler et al., 2011; L'Hernault and Roberts, 1995; Nelson and Ward, 1980; Zannoni et al., 2003). The presence of a garlic ring on plates greatly increases male retention and mildly extends *him-5(e1490)* male median and maximum lifespan (Supplementary Fig. 1). Indeed, the garlic-retained males have better maintenance of body integrity, possibly due to diminished time away from food or fewer desiccating interactions with the edge of culture plates that typically occur for searching males. Interestingly, our garlic ring method slightly shortens the median and maximum lifespan of *him-5(e1490)* hermaphrodites (two trials, data not shown). This is in contrast to the lifespan extension observed in hermaphrodites treated with low concentrations of diallyl trisulfide (an organosulfur compound present in garlic) in DMSO (Powolny et al., 2011). The difference between our method and that of the Fisher lab is that we used the whole extract of garlic, prepared the garlic solution in ethanol, and did not expose adult animals to continuous physical contact with the garlic extract. Given that we observe opposing lifespan effects in males and hermaphrodites and that male body integrity is better maintained on our plates, we conclude that the garlic ring technique described here not only significantly improves the retention of males but also confers some protection against the health-compromising effects of male searching behavior. It is important to note that diminishing these deleterious culture effects minimizes the confounding variables in male aging experiments.

3.2. Fertility in male worms decreases with age

Upon *C. elegans* mating, male sperm compete with hermaphrodite sperm to give rise to outcrossed progeny, and sperm competition occurs even in the absence of fertilization (Birkhead, 1996; Singson et al., 1999). This sperm competition phenomenon and the possibility to easily identify cross progeny, render the study of *C. elegans* male fertility feasible. In this report, we assayed male fertility by determining the percentage of outcrossed progeny by mating wild-type (WT) males to a recessive, genetically marked hermaphrodite strain (m/m). Male sperm can outcompete hermaphrodite sperm to give rise to m/+ cross progeny that are physically distinct from the hermaphrodite parent and m/m self-progeny (Brenner, 1974). The effect of male age on progeny production can thus be assayed by counting cross progeny in a simple mating assay in which male age varies but hermaphrodite partners are always young adults (Material and methods) (Supplementary Tables 1–4).

We observed that the percentage of cross progeny decreased as WT males aged, starting from the first day of adulthood until the reproductive period effectively ended at adult day 7 (Fig. 2). Young WT males show high fertility rates 1–3 days after the L4 larval stage. Beyond this point, the drop in cross progeny is dramatic, such that no cross progeny are produced after 7 days, although males can live for many more days. Importantly, we find that *him-5* males exhibit a similar decline in fertility as WT males (differences are not significant using one-way ANOVA and Bonferroni's multiple comparison test) indicating that the *him-5(e1490)* mutation does not alter male reproductive span. Crosses of WT males with WT hermaphrodites showed outcomes similar to crosses using *dpy-5* mutant hermaphrodites (with male cross progeny scored, Supplementary Fig. 2). Furthermore, in one-to-one mating assays, mating success sharply decreased on day 2 and dropped to less than 50% by day 3 (Supplementary Fig. 3A) (for total progeny counts see Supplementary Tables). Plate size did not affect mating efficiency scores (Supplementary Fig. 3B). Thus, outcomes on male fertility decline are not changed by mating couple genotypes, expanse of mating domain, or number of animals available for mating. Our data establish that male fertility in *C. elegans* declines with age and that the reproductive span is significantly shorter than lifespan.

3.3. Male physical capacity decreases minimally relative to reproductive decline

In *C. elegans* hermaphrodites, aging is associated with a decline of motor activity. With age, adults become progressively less coordinated and show less vigor, ultimately ceasing movement except shuddering in response to poking (Collins et al., 2008; Croll et al., 1977; Herndon et al., 2002; Hosono et al., 1980; Huang et al., 2004). Since male reproductive success depends on the physical ability to approach and interact with a hermaphrodite, we measured male vigor over the period of fertility decline. We submerged individual males of different ages in either drops of viscous halocarbon oil or M9 buffer to count the number of body thrashes in a 30 s interval. We find that both N2 and *him-5* males continue to move vigorously in both media after reaching infertility (Fig. 3A and Supplementary Fig. 4). When we directly compare the physical capacity of *him-5* males with their mating efficiency scores (Fig. 3B), it is evident that the ability to sire progeny declines much more rapidly than general locomotory ability. We thus suggest that age-related locomotory capability has a minor impact, if any, on *C. elegans* male reproductive aging.

3.4. Sperm activation in aging *C. elegans* males

In several species, sperm counts and quality contribute significantly to fertility (Jensen et al., 2002; Moller et al., 2009). To address whether poor sperm quality or low sperm numbers underlie the decline in male fertility, we evaluated spermatid morphology, capacity for activation, and number of sperm produced, well established measures in *C. elegans* (Geldziler et al., 2011). Note that in *C. elegans* males, spermatids are produced throughout adult life and are stored in the seminal vesicle (L'Hernault and Roberts, 1995; Singson, 2001). During mating, spermatids are ejaculated into the uterus of the hermaphrodite, where they undergo activation upon contact with seminal fluid. Activation results in the formation of amoeboid spermatozoa that can migrate to the spermatheca, the site of fertilization.

To check spermatid morphology, we dissected *him-5* male gonads at different ages and characterized the spermatids as either normal (round and smooth) or abnormal (irregular in shape and spiky), (see L'Hernault et al., 1988 for published examination criteria). We do not find any striking change in sperm morphology with age (Fig. 4A), suggesting that overall integrity of sperm is well maintained during reproductive aging.

To test for maintained capacity for spermatid activation, we used an in vitro activation assay using pronase E (Shakes and Ward, 1989; Singaravelu et al., 2011). Spermatid activation involves the conversion of round, sessile spermatids into amoeboid spermatozoa that have a pseudopod that confers sperm motility. We calculated the percent spermatid activation for spermatid populations from D1 to D5 males (Fig. 4B). Our data show that sperm from aged, barely fertile males (D5) are comparable to sperm derived from young animals in their capacity to be activated.

To address the possibility that sperm number might decline with age, we fixed reproductively aging males in methanol and stained sperm nuclei with DAPI. We find that sperm abundance does not decline significantly during reproductive aging (Supplementary Fig. 6).

In sum, sperm morphology, activation capacity, and numbers do not change markedly during the period of dramatic male reproductive decline. We conclude that a decline in sperm quality is unlikely to be a major factor driving *C. elegans* male reproductive aging.

3.5. Physical changes in male mating structures may contribute to reproductive decline

We hypothesized that the accumulation of physical damage/ deterioration of the key mating structure, the male tail, might be a contributing factor to the reproductive decline in males. We examined the male tail structure, including the extending nine rays of the cuticular fan, the hook and the two sensory spicules, which serve to locate and hold open the hermaphrodite vulva during mating and sperm transfer (Garcia et al., 2001; Liu and Sternberg, 1995). We examined male tails at D1, D3 and D5 to search for any general patterns of morphological decline. Note that damage and desiccation of males from interaction with plate plastic should be minimal using our garlic-ring protocol, so that age-associated decline, rather than plate-interaction decline, should be evident.

Based on overall appearance, we visually characterized each tail as being normal, semi-normal, or abnormal. Normal tails are those with all nine rays intact, a cuticular fan with a smooth, regular edge, and a generally healthy appearance. Semi-normal and abnormal tails are distinguished based on the severity of their irregularities, which include poorly defined or collapsed-looking rays and non-uniform cuticular edge. We find that numbers of normal tail structures in *him-5* are not significantly different from day 1 to day 5 (Fig. 5; $p = 0.2767$, Fisher's exact test). Thus, although a trend toward physical decline might cause some functional changes in the structures that are essential for successful mating, reproductive senescence transpires in the absence of a major physical deterioration.

3.6. Male mating ability decreases with age in a pattern similar to the decline in reproductive success

Male mating involves a complex set of behaviors that includes responding to chemosensory cues from the hermaphrodite (Simon and Sternberg, 2002), running the tail along the hermaphrodite body to locate the vulva (which often requires a sharp turn at the head or tail of the hermaphrodite), and finally insertion of spicules into the vulva and ejaculation (Barr and Garcia, 2006) (Fig. 6A). To test if the “desire” to mate and mating ability might contribute to the reduction in fertility observed in *C. elegans* males, we conducted well-established simple mating interest and execution assays (Barr and Garcia, 2006). We placed well-fed males that were either adult D1 or D5 on plates that contained a central spot of bacteria that was surrounded by immobile *unc-31* hermaphrodites. We recorded mating responses for each male, and quantitated specific mating behaviors that transpired during a 4-minute interval.

The response to hermaphrodites is assayed as purposeful contact between the male tail and hermaphrodite, such that the male begins to run its tail along the hermaphrodite body. When the male is committed to scanning the hermaphrodite’s body, this was scored as a positive response and given a score of 1, whereas a “touch and go” (really touch and leave) response was scored as 0. We find that older *him-5* males exhibit a lowered response to hermaphrodites (Fig. 6B; $p = 0.0496$, Fisher’s Exact test), and the time taken to respond to hermaphrodites, scored as time from male placement on the plate to time of purposeful contact, trends toward increase but it is not significant in our sample size (Fig. 6C; $p = 0.0911$, Student’s *T* test). Thus it appears that male “interest” (involving chemoattraction and mechano-attraction) diminishes somewhat during the reproductive period. Since the extent of diminished response to hermaphrodites is less than the reproductive decline overall, we infer that the failure to detect or respond to a hermaphrodite is not the sole or major factor in lowering fertility over the male reproductive lifespan.

We next focused on potential differences in the mechanics of mating behavior. As males age, they exhibit significantly increased difficulty in effective turning while scanning the hermaphrodite body, which is measured by failing to turn and remain on the hermaphrodite body upon reaching its head or tail (Fig. 6D; $p = 0.0003$, Fisher’s Exact test). In addition, older responding males exhibit significant difficulty in locating the vulva (Fig. 6E; $p < 0.0001$, Student’s *T* test). We also noted a deficit in sustaining contact that would be needed for spicule insertion and sperm transfer (Fig. 6F; $p = 0.0137$, Fisher’s Exact test). We conclude that challenges in mechanics of the act of mating increase significantly with age, and suggest that diminished efficacy of mating, compounded by declines in multiple steps of mating, is a significant factor in decreased fertility outcomes for middle-aged males.

3.7. The insulin pathway affects reproductive aging, with impact on the behavior segments that change most in wild-type aging

One of the best-studied genetic regulatory networks that modulate *C. elegans* life span is the conserved insulin/insulin growth factor 1 (IGF-1)-like signaling (IIS) pathway (Kaletsky and Murphy, 2010). The IIS pathway involves signaling through insulin/IGF-like receptor DAF-2, which activates a kinase cascade, including the AGE-1 PI3 kinase that ultimately

phosphorylates the DAF-16/FOXO transcription factor to prevent its nuclear access. Low IIS pathway signaling diminishes DAF-16/FOXO phosphorylation, allowing nuclear translocation and consequent up-regulation of genes involved in metabolism, developmental control and longevity (Hsu et al., 2003; Lee et al., 2003). Reduction of function mutations in IGFR *daf-2* cause a doubling of *C. elegans* lifespan (Kenyon et al., 1993; Kimura et al., 1997), whereas loss of function of FOXO *daf-16* suppresses the lifespan extension caused by *daf-2* mutations (Lin et al., 1997; Ogg et al., 1997). Although insulin signaling influences many aspects of *C. elegans* aging biology, impact on male reproduction has not been extensively addressed.

To study the effect of IIS on *C. elegans* male fertility, we examined progeny production in long-lived *daf-2(e1370)* reduction-of-function (rf) mutants and in short-lived progeric *daf-16(mgDf50)* null mutants. We find that although fertility declines during the reproductive period for *daf-2;him-5* mutants, *daf-2;him-5* males maintained at 16 °C sire an increased number of progeny in early adulthood and also exhibit an extended period of fertility (Fig. 7A). *daf-2;him-5* mutants shifted to 20 °C at young adult life had less dramatic effects than the same mutants kept at 16 °C their whole life (Supplementary Fig. 7), which suggests that either a milder downregulation of the insulin/IGF-1 pathway or a compounded effect of both lower signaling and temperature may be beneficial for male reproductive span. On the other hand, we find that *daf-16;him-5* males grown at the standard temperature of 20 °C have a shortened reproductive period, with *daf-16;him-5* males unable to sire progeny after day 4 (Fig. 7B). Although different temperature manipulations preclude direct comparison of absolute scores for *daf-2* and *daf-16*, our data reveal that IIS strength can influence male fertility: in long-lived *daf-2(e1370)* mutants, male fertility is maintained longer into adulthood; whereas in short-lived *daf-16(mgDf50)* (which mimics strong pathway signaling with full DAF-16 inhibition) the period of reproductive fitness is shortened.

3.8. Effects of insulin signaling changes on male reproductive span are likely complex

We wondered if any specific components of sperm integrity or mating behavior might be changed in longevity mutants of the IIS pathway. We did not find a clear correlation between sperm morphology or activation and fertility decline when insulin signaling strength was manipulated (Supplementary Fig. 5). For example, at day 5 of adulthood, both *daf-16*() and *daf-2(rf)* exhibit decreased sperm activation (68% and 76% respectively, $p < 0.0001$, compared to virtually no change in *him-5*, $p = 0.6013$, Fisher's Exact test; Supplementary Fig. 5B), although *daf-16*() mutants are infertile by this day and *daf-2(rf)* mutants have enhanced fertility at the corresponding timepoint. No obvious morphological defects develop in either *daf-2(rf)* or *daf-16*() sperm on day 5 (Supplementary Fig. 5A). Our data are consistent with our conclusion that sperm morphology and activation do not change much in our control *him-5* during reproductive decline.

In terms of behavior, we compared 5 day old scores to 1 day old scores among *him-5*, *daf-2*, and *daf-16* mutants in an attempt to reveal disproportionate changes during early aging. For the *daf-16* null mutant, which was measured with the same constant 20 °C growth regimen as *him-5* in Fig. 8, impairment of turning behavior and location of vulva tend to early onset

(Fig. 8C, D) and decrease in the ability to maintain contact with the vulva is significantly diminished by day5 (Fig. 8E). *daf-2(rf)* mutants (grown here under different temperature regimens and therefore not directly comparable to *him-5* and *daf-16* scores in Fig. 8), maintain better relative responsiveness to hermaphrodites, have late onset turning defects (Fig. 8C) and a smaller proportionate decline in location of vulva defects (Fig. 8D), which might contribute to better maintained reproductive success. Our data thus suggest that insulin signaling may impact different components of mating behavior to different extents. We note that mating behaviors that change most with age in *him-5* males (turning defects and vulva location/spicule insertion) can be altered by IIS status.

Overall, our data suggest that “end game” behavioral stages of male mating-turning, location of vulva, and spicule insertion, may be most susceptible to age-associated decline; poor mating execution could reduce progeny production. A loss in the ability to mate appears to be a significant factor in male reproductive decline in *C. elegans*.

4. Discussion

Here we report that the ability of *C. elegans* males to sire progeny diminishes markedly early in adult life—the ability to cross-fertilize disappears by 7 days of adulthood, which is only ~ 1/3 of the average adult lifespan. Functional reproductive senescence thus occurs long before death of the animal. Our studies on sperm morphology, in vitro sperm activation capacity, and overall maintenance of sperm number did not support that a decline in male gamete quantity or quality confers a major impact on reproductive success in the paradigm we studied. Rather, diminished efficacy of executing mating behavior seems to convey the most significant impact on loss of reproductive prowess: although worms maintain a fair amount of interest and the spirit appears willing (tracking to the hermaphrodite to initiate interaction), cumulative behavioral performance diminishes.

4.1. A method for maintaining aging male populations on plates

Males kept in groups have been reported to have shorter lifespan than mating males and solitary males (Gems and Riddle, 2000). Previous studies of male lifespan have been challenging in part because in the absence of hermaphrodites, males leave the central food area to search for mates. This “leaving behavior” involves sensory transduction by the male tail rays (Barrios et al., 2008). In the lab, mate-searching males crawl onto plastic plate edges, where they desiccate and die. Large proportions of male populations are therefore rapidly lost in adult cultures. Furthermore, the “leaving” males look physically stressed, such that their tails appear somewhat tattered. Tattering may be the result of encounters with the plate edges, encounters with self or with other males, or might be the consequence of food limitation conditions during searching. We improved male retention and male tail structural integrity by using garlic as a chemo-repellant. Our retention approach now enables the study of very old males, which had been previously nearly impossible to keep on plates.

4.2. Sperm quality is well maintained despite dramatic functional reproductive senescence

Some evidence supports that sperm quality diminishes in older human males (Silva et al., 2012; Templado et al., 2011). Still, catastrophic events associated with aged male parents

are relatively rare, and human males can be considered to remain fertile throughout lifespan. In contrast, *C. elegans* males exhibit a “male menopause”—their functional reproductive lives are markedly diminished only partway through their adult lives (about 1/3–1/2 through the adult lifespan). A diminution of sperm quality does not appear to be a major factor in the decline of reproductive success since sperm activation (the most critical function), sperm number, and sperm morphology seem to be maintained over the period in which reproductive success drops. Perhaps there still may be more insidious sperm defects in older males that could contribute, in a way reminiscent of humans, to the mild shortening of the mean life span of their progeny described by Klass (1977), but such abnormalities were not detected to the degree we tested in this study. Nevertheless, the strong sperm maintenance into mid-life we observed in males contrasts with the aging of oocytes, in which declining oocyte quality has been documented with age (Andux and Ellis, 2008; Hughes et al., 2007; Luo et al., 2010).

For males, soma, rather than germline, appears to be the first to suffer age-induced functional decline that impairs reproductive success. The relationship between somatic and reproductive aging is intriguing. A recent genetic screen identified mutants with both delayed reproductive and somatic aging (Hughes et al., 2011). Identification of these genes may provide insights on core aging pathways or define totally new ones and help uncover the molecular underpinnings of reproductive aging in both male and hermaphrodite nematodes.

4.3. The complex *C. elegans* mating behavior pattern becomes inefficient in early adulthood

We find that the biggest measurable changes during early adult aging are in the execution of mating behavior. Older males have modestly diminished interest, as scored by male approach toward young hermaphrodites and initiation of interaction. Execution of the complex physical behaviors that constitute successful mating, however, is more heavily impacted during the reproductive lifespan, with successful turns, location of vulva and spicule insertion particularly susceptible to failure. These behavioral changes might have a neuronal focus, a muscle focus, or both. Indeed, in a recent paper, Guo et al. (2012) show that muscle excitability is enhanced as males age, which disrupts the complex mating behavior (Barr and Garcia, 2006). Recent work by Liu et al. (2011) has shown that a cholinergic circuit involving the cloacal sensory/motor neurons and the posterior sex muscles is responsible for maintaining genital contact. Morphological aging of specific neuronal types has recently been documented (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). It remains to be seen what specific deficits occur with age in the neuronal mating circuits and how these influence mating behavior and reproduction.

The relatively early manifestation of defects in siring progeny is striking. Although several *C. elegans* components decline with age, few are so dramatic so early in adulthood. The end of reproductive proficiency declines at a rate roughly similar to that of pumping of the muscular pharynx (Huang et al., 2004). Mating muscle excitability changes as males age, which disrupts the complex mating behavior of the male (Guo et al., 2012). The observations on male reproductive senescence thus appear to underscore a general theme

that muscle dysfunction/deterioration (sarcopenia) contributes in a major way to the earlier age-associated decline in functional behavior.

4.4. Insulin signaling and maintenance of mating behavior

Insulin signaling can influence *C. elegans* healthspan, as well as overall lifespan. Low insulin signaling levels can maintain muscle (Glenn et al., 2004; Herndon et al., 2002) and neuronal (Tank et al., 2011; Toth et al., 2012) integrity, but less is understood about the detailed consequences of insulin signaling levels on behavior. Insulin signaling can influence associative learning (Lin et al., 2010; Murakami and Murakami, 2005). We have found that changing insulin signaling levels modulates distinct steps of mating behavior, and that outcomes of low signaling are not necessarily all beneficial, when individual behavioral components are examined. Interestingly, complex outcomes in insulin signaling mutants have been previously reported for male lifespans. Gems and Riddle (2000) found that the greater lifespan of solitary males as compared to grouped males was influenced by *daf-16*, but not by *daf-2*. In sum, low insulin signaling in the *daf-2(rf)* mutant extends the reproductive period of males in our assay system, and mimicking high signaling, as with the *daf-16* null mutant, limits male reproductive lifespan. Overall, insulin signaling does not appear to have major effects on sperm number, morphology or capacity for activation. Instead, the ability to execute complex mating behavior may be changed directly or indirectly via insulin signaling, with *daf-16* possibly more critical for young adult behavioral maintenance. It is also worth noting that many influences on healthspan have been documented, and it remains possible that pathways that have the strongest impact on male mating decline remain to be identified. Our development of a male-retention strategy may facilitate future identification of such factors.

4.5. Summed modest deficits in a complex behavior may impair reproductive success in aging males

Overall, we suggest that it is the combination of incremental declines that we have documented—physically damaged or diminished mating structures, somewhat lowered interest in hermaphrodites, poor execution of turns that maintain body contact during mating, and ineffective location of vulva behavior—that can sum up to failed copulation and a dramatic overall decline in the ability to produce progeny late into life.

C. elegans are predominantly self-fertilizing hermaphrodites in their natural environments, and thus males are not thought to play major roles in propagation of the species. It would be of interest to compare male reproductive aging in closely related species such as *Caenorhabditis remanei*, which must reproduce by male and female matings and therefore depend more strongly on maintained male mating proficiency. Mating ability and fertility may be differentially critical in gonochoristic nematode species, in which males are indispensable for reproductive success.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Garlic rings on aging culture plates markedly limit loss of males. A. At 6 days post L4 more males are retained on plates encircled with garlic ($p = 0.0067$, Fisher's exact test) (total $n = 300$ in each scenario; 20 culture plates ringed with garlic and 20 control plates each with $n = 15$). B. Worm tracks are left on the bacterial lawn as animals move across plates; note the lack of tracks near the center patch of garlic extract (squared region). Error bars are standard error of the mean percentage.

Fig. 2.

Percent of cross progeny decreases with age for both WT and *him-5* male worms. Males aged on garlic plates ($n = 12-15$ per data point, 1 day post L4 to 7 days post-L4) at 20° C were mated to L4 *dpy-5* hermaphrodites. Non-Dpy progeny were scored as cross progeny. The WT and the *him-5* strains are similar in % cross progeny (differences are non significant ($p > 0.05$) using one-way ANOVA and Bonferroni's multiple comparison test, selected pairs), and both strains reach sterility at the end of day 7. Error bars are standard error of the mean.

Fig. 3.

Male physical capacity decreases only slightly compared to reproductive decline. A. Movement in aging WT and *him-5* worms as recorded by the number of thrashes per 30 s in halocarbon oil decreases only slightly during the first week of adult life ($n = 10$ per data point). Note that assays in M9 buffer lead to the same conclusion ($n = 53$ – 56 per data point; Supplementary Fig. 4). B. Comparison of movement decline in *him-5* males with percent cross progeny produced shows that the degree of locomotory decline does not parallel the

dramatic drop in progeny production during male aging. Error bars are standard error of the mean.

Fig. 4.

Sperm morphology and activation do not change dramatically during the period of reproductive senescence. A. Spermatid morphology remains unchanged at day 5. B. Graph showing that sperm activation does not change significantly ($p > 0.05$, Fisher's Exact Test) between day 1 ($n = 372$) and day 5 ($n = 629$) for *him-5*. Sperm from day 5 males were activated in vitro using Pronase and the number of sperm with or without pseudopods (arrow) was scored. Error bars represent standard error of the proportion.

Fig. 5.

Male tail morphology deteriorates slowly during reproductive aging. Representative images of day 1, day 3, and day 5 tails taken from a complete sample set ($n \approx 20$) for *him-5* males. A. Tail image for *him-5* day 1 is scored as normal while *him-5* day 5 would be scored as abnormal because of irregularities such as missing or deformed rays and non-uniform cuticular edges B. Graph showing that the percent of males with normal tails decreases by 26% in *him-5*, which is not statistically significant for our sample size ($p = 0.2767$, Fisher's exact test). Error bars represent standard error of the proportion. Male mating structures tend

to show mild to moderate damage with time, although changes are not proportional to the rapid decline in the capacity to sire progeny.

Fig. 6.

Components of male mating behavior decline significantly by adult Day 5. A. Steps involved in male mating behavior. The male responds to the hermaphrodite chemosensory cues, moves to the hermaphrodite, and makes contact with the tail. The male scans the hermaphrodite's body with his tail and makes a turn as he reaches the end of the hermaphrodite. Once the vulva is located, contact at this position is maintained, followed by spicule insertion and insemination. B. Day 5 males have a significantly lowered response to hermaphrodites compared to Day 1 males ($p = 0.0496$, Fisher's Exact test). A positive

response to hermaphrodites is scored if the male begins to run its tail along the hermaphrodite body upon an encounter (score 1); no response is “touch and go”, 0. C. The time taken to respond to a hermaphrodite, scored as time from male placement on the plate to time of purposeful contact, slightly increased in 5 day old males but it is not significant in our sample size ($p = 0.0911$, Student’s T test). D. Older males showed turning difficulties during mating behavior ($p = 0.0003$, Fisher’s Exact test), as assayed by failing to turn and remain on the hermaphrodite body upon reaching its head or tail. E. While executing mating behavior, typically a male will scan for the hermaphrodite’s vulva and pass over it once or twice before attempting to insert its spicules. This behavior called Lov efficiency is calculated as Vulva located (1 or 0)/number of passes. Lov efficiency in Day 5 males is significantly decreased as animals have to pass over the vulva several times before locating it, in contrast to Day 1 males ($p < 0.0001$, Student’s T test). F. In aging males, the ability to maintain position over the vulva once it is located, and subsequent spicule insertion, is reduced. This maintained positioning directly determines the male ability to inseminate the hermaphrodite ($p = 0.0137$ Fisher’s Exact test for day 1 vs. day 5 males). $n = 15$ per data point, three trials. Error bars are standard error of the mean percentage. Panel A is redrawn from Worm Atlas and Altun et al., 2002–2012.

Fig. 7.

Reproductive aging in IIS mutants indicates that low insulin signaling can extend male reproductive lifespan. A. In long lived *daf-2(e1370)* mutants, fertility is higher than in *him-5* males and continues to remain high on day 6 at 16 °C ($p < 0.05$, Student's *T* test). The *daf-2(e1370)* lesion is temperature sensitive and causes developmental arrest at 20 °C. The beneficial effects of the *daf-2(e1370)* mutation are less prominent when the mutant males developed at the permissive temperature of 15 °C and switched to 20 °C to test adult reproductive span at the standard growth temperature (see supplementary Fig. 7). B. At 20

°C, fertility in *daf-16(mgDf50)* mutants is elevated relative to young *him-5* males but declines rapidly from day 1, with the biggest drop from days 3 to 4, when *him-5* males are still fertile. n = 15 to 20 crosses were performed for each day tested. Error bars are standard error of the mean.

Fig. 8.

Effect of insulin signaling strength on components of mating behavior. A. % responding males. Similar to *him-5* males, older *daf-16*(^{old}) males were less responsive to hermaphrodites ($p = 0.0496$ and $p = 0.0184$ for *him-5* and *daf-16*(^{old}) males, Fisher's Exact Test), whereas the responsiveness of younger vs. older *daf-2*(*rf*) males was not statistically significant ($p = 0.0859$, Fisher's Exact test). B. Time to respond. *him-5* D1 vs. D5 difference is not significant ($p = 0.0911$, Student's *T* test), *daf-16*(^{old}) D1 vs. D5 difference is not significant ($p = 0.3501$, Student's *T* test), and *daf-2*(*rf*) D1 vs. D5 difference is also not

significant ($p = 0.1$, Student's T test). C. Turning difficulty. *daf-16*(Δ) show some progeric turning difficulties although they are not statistically significant for our sample size ($p = 0.1225$, Fisher's Exact Test), whereas *daf-2(rf)* mutants have no defects early on. *him-5*, *daf-2(rf)* and *daf-16*(Δ) older males have increased difficulty in turning when mating but differences between ages for a given strain are only statistically significant for *him-5* and *daf-2(rf)* males in our sample size ($p = 0.0003$, Fisher's Exact test for D1 vs. D5 *him-5* and D1 vs. D5 *daf-2(rf)* males; $p = 0.1225$ for D1 vs. D5 *daf-16*(Δ) males), possibly since *daf-16* is already diminished in this behavior early in adult life. D. LOV behavior. All the male strains tested deteriorate in this aspect of mating behavior by day 5 (~40% of day 1 for *him-5* and *daf-2(rf)* males) ($p < 0.0001$, Student's T test). E. *daf-2(rf)* males show a strong ability to maintain contact with the vulva and insert spicules on day 1 ($p = 0.0001$, Fisher's Exact test). This behavior is severely impaired in *daf-16*(Δ) males on day 5 ($p = 0.0018$, Fisher's Exact test); *him-5* D1 vs. D5 ($p = 0.0137$, Fisher's Exact test). $n = 15$ for each data point, three trials. *him-5* and *daf-16*(Δ) males were tested at 20 °C; *daf-2(rf)* at 16 °C. Error bars are standard error of the mean percentage.