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Males shorten the lifespan of *C. elegans* hermaphrodites via secreted compounds

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Abstract

In the nematode *C elegans*, the presence of males accelerated aging and shortened the lifespan of individuals of the opposite sex (hermaphrodites), including long-lived or sterile hermaphrodites. The male-induced demise could occur without mating and required only exposure of hermaphrodites to medium in which males were once present. Such communication through pheromones or other diffusible substances points to a non-individual autonomous mode of aging regulation. The male-induced demise also occurred in other species of nematodes, suggesting an evolutionary conserved process whereby males may induce the disposal of the opposite sex to save resources for the next generation or to prevent competition from other males.

Introduction

In species ranging from worms to non-human primates, the lifespan of individuals is nearly always assessed in conditions where males and females are kept separate. Yet in the wild, the opposite sexes coexist, at least during attraction and mating. In flies and worms, the presence of males decreases longevity in the opposite sex (1, 2). In *Drosophila*, males shorten the lifespan of females after mating through peptides present in seminal fluid (2). In *C. elegans*, male-induced lifespan shortening of the opposite sex (hermaphrodites) has been proposed to result from physical damage caused by copulation (1). Whether additional mechanisms of male-induced killing exist in *C. elegans*, and the extent to which such mechanisms may be evolutionarily conserved, is largely unknown.

Results

The continuous presence of young males significantly shortened the lifespan of hermaphrodites (>20% decrease) (Fig. 1A, Table S1). The male-induced shortening of lifespan was seen whether males were placed with hermaphrodites at the beginning of their life (day 1) or at sexual maturity (day 4) (Fig. 1A). This lifespan shortening was not a result

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of crowding because the total numbers of worms were the same in all conditions. Males also induced behavioral and morphological phenotypes characteristic of an advanced age in these hermaphrodites: movement was slowed, paralysis increased, and a general decrepitude was observed, as exemplified by increased incidence of vacuole-like structures and structural decline within the cuticle, muscle, pharynx, and intestine (Fig. 1B, fig. S1A to I; and movies S1 to S3) (3–6). We termed this phenomenon male-induced demise (MID).

Long-lived hermaphrodites, such as insulin receptor mutants *daf-2*, germline-deficient mutants *glp-1*, and wild-type worms subjected to dietary-deprivation (DD), also exhibited a shortened lifespan (Fig. 1C, D, Fig. S1J). *daf-2* mutant hermaphrodites displayed a large (>60 %) reduction in median lifespan in the presence of wild-type males (Fig. 1C). *glp-1* mutant hermaphrodites exhibited shortening of lifespan in response to males, even though they are sterile (Fig. 1D), in agreement with observations that sterile hermaphrodites are equally susceptible to male-induced lifespan shortening (1). Thus, the deterioration of hermaphrodites in the presence of males is not a simple result of increased progeny production from sexual reproduction (7), and extension of lifespan through several well-known longevity pathways is not sufficient to alleviate this form of demise.

To understand how males restrict the lifespan of the opposite sex, we assessed genome-wide changes in hermaphrodite gene expression triggered by males. To avoid expression changes due to fertilized embryos in the mother, we used sterile hermaphrodites (*glp-1)*. We placed *glp-1* young adult hermaphrodites with wild-type young males for 8 days, then removed the males and collected the hermaphrodites' RNA for microarray analysis (Fig. 2A). As a control, we collected RNA from *glp-1* hermaphrodites that were not placed in the presence of males but were grown at the same density with other hermaphrodites (Fig. 2A). Unbiased clustering of the microarray data revealed that the presence of males induced large changes in gene expression in hermaphrodites (Fig. 2B, Fig. S2A, Table S2). Genes whose expression was increased in response to males were enriched for insulin signaling ($P = 4.3\times10^{-3}$) (e.g. insulin peptides (*ins-4, ins-11, ins-23* and *ins-31*), which are expressed in neurons), transthyretin-related family members ($P = 4.3\times10^{-3}$) (which are involved in neurodegenerative diseases in mammals (8)), and G-protein coupled chemoreceptors ($P = 1.9\times10^{-3}$) (which are expressed in sensory neurons) (Fig. S2B). In contrast, genes whose expression was decreased in response to males were enriched for C-type lectins and the cuticle (Fig. S2B). That the presence of males triggered changes in the expression of neuronally-expressed genes suggests that mechanisms other than structural damage resulting from copulation also contribute to MID.

We next tested whether modulating the expression of genes whose expression was increased in hermaphrodites in response to males and expressed in neurons could rescue MID. We used RNAi to decrease expression of 10 hand-picked genes that are expressed in neurons and either belong to a significant functional annotation enrichment category or undergo large changes in message abundance in response to males. We used a strain of *C. elegans* that is sensitized for RNAi in neurons *Punc-119::sid-1*. Depletion of mRNA from three of these genes (*ins-11, F11A5.3*, and *utx-1*) partially rescued MID (Fig. 2C–E, Fig. S2C–L). *ins-11* encodes an insulin-like peptide that is expressed primarily in sensory neurons (9). *F11A5.3* encodes a conserved small guanosine triphosphatase (GTPase) of the Rab family of

little known function in worms, but whose human ortholog (*RAB2A*) functions in vesicular trafficking (10). *utx-1* encodes a histone H3 demethylase (H3K27me3 demethylase), depletion of which increases longevity in *C. elegans* (11, 12). Whereas decreased expression of *F11A5.3* and *utx-1* also extended the lifespan of hermaphrodites without males, decreased expression of *ins-11* specifically ameliorated MID without impacting the lifespan of hermaphrodites kept in the absence of males. The specific rescue of MID after depletion of *ins-11* likely results from the action of this gene in the hermaphrodite themselves and not in the males because *ins-11* mutant hermaphrodites were also partially resistant to demise induced by wild-type males (Fig. 2F). Thus, the shortening of lifespan induced by males can be ameliorated by depletion of the insulin peptide INS-11.

Because MID could be rescued by manipulating a single gene in the hermaphrodites, the phenomenon seems unlikely to solely result from structural damage caused by copulation. To more directly test whether males could shorten the lifespan of hermaphrodites without being in physical contact with them, we placed males on plates for 2 days, removed these males, and then added hermaphrodites to the male-conditioned plates (Fig. 3A). Conditioning the plates with males shortened the lifespan of wild-type hermaphrodites, in a manner that depended on the number of males used to make the conditioned medium (Fig. 3B). Hermaphrodites placed on male-conditioned plates underwent signs of MID (video S4, S5 and S6). While there may be a physical component to MID, one or more diffusible substances secreted or released by males on the plate is sufficient to decrease lifespan of hermaphrodites.

C. elegans secrete small molecules called ascarosides, which act as pheromones to regulate various processes, including development, behavior, and lifespan (13–18). Ascaroside production has been primarily studied in the context of hermaphrodites, but males also excrete a sex-specific blend of ascarosides (19). We therefore tested whether pheromone sensing by hermaphrodites and pheromone production by males were required for MID. Hermaphrodites deficient for processing a range of sensory signals, including those from pheromones (20, 21) (*che-13*) were not susceptible to MID (Fig. 3C, Table S1), even though they mated normally with wild-type males (Fig. S3A–B). Males deficient in ascaroside pheromone biogenesis (*daf-22*) triggered MID less effectively in both wild-type or long-lived hermaphrodites (Fig. 3D, Fig. 3E), although these *daf-22* males mated normally with wild-type hermaphrodites (Fig. S3A, B). Conditioned medium from males that are defective in pheromone production (*daf-22*) did not trigger MID in wild-type hermaphrodites (Fig. 3F). Thus, the ability to secrete and sense pheromones appears to be necessary for males to induce shortening of lifespan in hermaphrodites.

Is the male-induced demise a more general, conserved phenomenon? The genus *Caenorhabditis* includes several distantly related species, each of them with different strains (Fig. S4A). Similar to what we observed in the long-domesticated strain of *C. elegans* (N2), males from a wild *C. elegans* strain, AB1, also shortened the lifespan of hermaphrodites of that strain (Fig. S4B). Males from the species *C. briggsae*, which diverged from *C. elegans* about 20–30 million years ago (22), decreased the lifespan of hermaphrodites of two different *C. briggsae* isolates (Fig. 4A). Males from the species *C. remanei*, which has obligate males and females, led to lifespan shortening of females (Fig. 4B). Together, these

results indicate that MID is conserved at least over 20–30 million years of evolution and is not linked to hermaphroditism. The evolutionary conservation of MID raises the possibility that this phenomenon has adaptive value and may be caused by conserved mechanisms.

We find that continuous presence of mating competent males shortens the lifespan of the opposite sex, and triggers a phenotype that resembles progeria. In our study, *C. elegans* hermaphrodites were exposed to an unnaturally high percentage of males for the duration of their lifespan. The proportion of male *C. elegans* is usually low (0.01–0.1%), though this percentage can be increased in response to stress stimuli. However, MID does not appear to be solely due to an artificially high percentage of males, as *C. remanei* females, which naturally exist in a 1:1 ratio with males, also displayed MID in our experimental setting. Males shorten the lifespan of the opposite sex in part by releasing one or more diffusible substances, possibly a pheromone. Thus, while male secretions promote reproduction under normal circumstances, they might also accelerate demise, especially when these secretions are concentrated. In addition to male pheromones, the male-conditioned plates are also likely to contain sperm and seminal fluid from male-to-male copulation attempts (23). Copulation may help male secretions to be produced or act efficiently. Although male sperm has been previously ruled out as a cause of the male-induced shortening of lifespan in *C. elegans* (1), seminal fluid, which may itself contain pheromones, cannot be excluded as a causal agent.

In both worms and flies, sensory deficiencies in olfactory and gustatory neurons extend lifespan (24–26). Although the exact sensory neurons and specific chemoreceptors responsible for pheromone perception are just beginning to be identified (16, 18, 27, 28), pheromones activate conserved downstream signaling pathways, including those activated by transforming growth factor (TGF)-β and insulin (29). Male-induced demise could be influenced by similar neuronal circuits and signaling pathways. Indeed, interfering with the gene encoding the insulin peptide INS-11, which is expressed in sensory neurons, specifically rescued the male-induced demise of hermaphrodites. INS-11 and other genes identified in our microarray analysis could provide a handle on the dissection of sensory or intersexual interactions. If males in wild worm populations shortened lifespan of the opposite sex after reproduction occurred, this might have the evolutionary advantage of preserving limited resources for the offspring (30) or preventing competition from other males.

Material and Methods

Maintenance and strains

Unless otherwise noted, worms were maintained on NGM using *Escherichia coli* OP50 as a food source at 20°C. Wild-type (N2) worms were provided by Dr Man-Wah Tan. The *daf-2(e1370)* strain was provided by Dr. Cynthia Kenyon. The *C. briggsae* strain, HK104, was provided by Dr Abby Dernburg. The mutant *C. elegans* strains *glp-1(e2141), che-13(e1805), daf-22(m130), ins-11(tm1053),* TU3311 [*Punc-119::sid-1*] were provided by the Caenorhabditis Genome Center (CGC). All mutant strains were backcrossed 3 times to our lab's N2 strain, with the exception of TU3311, which was backcrossed 3x previously (32). The *C. elegans* strain, AB1, the *C. briggsae* strain, AF16, and the *C. remanei* strain,

PB4641, were also provided by the CGC. To generate numbers of males sufficient for these experiments, mating plates were set up every other day with 12 males (N2 or *daf-22(m130)*) and 4 adult hermaphrodites (N2 or *daf-22(m130)*). Mating plates were not necessary to generate *C. remanei* males as they naturally exist in a 1:1 ratio with females.

Hermaphrodite lifespan assays

Worm lifespan assays were performed at 20°C as described previously (33) unless noted otherwise. For each lifespan assay, worms were transferred to new plates at least every other day and were scored dead or alive. Worms were scored dead if they did not respond to repeated prodding with a worm pick. Worms were scored as censored if they died because of bagging, vulval rupture, or if they crawled off the plate. Data from the censored worms were included up to the day of censorship. For each hermaphrodite lifespan assay, at least 90 hermaphrodite worms per condition were divided evenly among three plates (≥30 hermaphrodites/plate) unless otherwise noted.

In assays in which the lifespan of hermaphrodites was assessed in the presence of males, at least 45 hermaphrodite worms and an equal number of male worms were divided evenly among three plates (≥15 hermaphrodites/plate) as to control for total number of worms/plate. Each mixed sex condition begins with an equal number of hermaphrodite to male ratio (ex. 15 hermaphrodites: 15 males). The number of males on the plate stayed fixed throughout the assay. Wild-type male worms between adult day 1 and adult day 4 were used and they were freshly replaced every other day at the time the hermaphrodites were transferred to new plates. Male stocks were set up every other day for the entirety of the lifespan assay. Males were added to the hermaphrodites upon hatching for all experiments besides the RNAi screen, in which males were added to young adult hermaphrodites.

For the RNAi screen, adult worms were placed on NGM plates containing ampicillin (100 $mg.ml^{-1}$) and IPTG (0.4 mM) seeded with the respective bacteria and removed after 4–6 h to obtain synchronized populations of worms. HT115 bacteria transformed with vectors expressing RNAi to the genes of interest were all obtained from the Ahringer library (a gift from A. Fire). To avoid potential developmental defects, RNAi was initiated after larval development in young adult worms obtained from these synchronized populations. To each “hermaphrodite only” plate, 35 *Punc-119::sid-1* young adult hermaphrodites were added. To each “hermaphrodite + male” plate, 15 wild-type males were added to 20 *Punc-119::sid-1* young adult hermaphrodites. 15 wild-type male worms between adult day 1 and adult day 4 were replaced every other day at the time the living hermaphrodites were transferred to new plates. The lifespan assay for the RNAi screen was performed and scored as described above.

In lifespan assays in which the plates were conditioned with males prior to adding hermaphrodites (Fig 3A for schematic), each time a plate is conditioned, 30 males (day 1–4 of adulthood) were placed on an OP50 seeded plate and maintained at 20°C for 2 days. Males were removed from the plates (red tildes in Fig 3A) and at least 30 L1 hermaphrodites were added to the male-conditioned plates (black tildes in Fig 3A). These hermaphrodites were transferred to a male-conditioned plate every other day until the completion of the lifespan assay.

Statistical analysis of lifespan assays

Statistical analyses of lifespan were performed on Kaplan-Meier survival curves in Prism 6 by Logrank (Mantel-Cox) tests (Table S1).

Mating assays

Mating assays were performed to assess several metrics of sexual behavior in *C. elegans*. 50 μl of OP50 was spotted on a standard 5 cm diameter plate containing NGM agar. A single adult day 1 wild-type or mutant hermaphrodite was placed in the center of the plate and left to acclimate to new environment for 30 min. A single adult day 1 wild-type or mutant male was placed on the same plate as the hermaphrodite and observed for 2 hours. The number of mating attempts as well as the duration of attempt(s) were assessed. The duration of mating was started upon initiation of male mating behavior and stopped upon physical separation of the male and hermaphrodite, upon successful completion of mating or otherwise

Microarray analysis

To identify male-induced expression using microarray analysis, 250 *glp-1(e2141)* L1 hermaphrodites were placed in the presence of 250 wild-type males (L4-adult day 1) for 8 days on a 10 cm NGM plate seeded with OP50. As a control for worm number, 500 L1 *glp-1(e2141)* hermaphrodites were placed on a 10 cm NGM plate seeded with OP50 for 8 days. Both conditions were incubated at 25°C, the restrictive temperature for the *glp-1(e2141)* worms throughout the experiment. On day 8, male worms were removed from the plate and RNA was extracted from 200 hermaphrodites from each condition. RNA from 3 independent experiments was used for the microarray hybridization.

Total RNA was isolated using an RNeasy (Qiagen). Microarray hybridization was performed at the Stanford Protein and Nucleic Acid facility with oligonucleotide arrays (Affymetrix, GeneChip*C. elegans* Genome Arrays). The raw microarray data are deposited at the Gene Expression Omnibus (GEO) under the Subseries entry (GEO number pending).

Data processing and analysis from the worm transcriptomes from the three independent experiments was performed in the R software (http://www.r-project.org/), using packages ‘affy’ (34) and recently published batch-correction algorithm ComBat (Johnson et al 2007). Briefly, raw probe fluorescence values were subjected to RMA normalization, and then the batch covariates were used to adjust for batch effects as previously described (35). To estimate the reliability of biological replicates after the processing, sample clustering was performed using the Pvclust algorithm using its R package ‘pvclust’ implementation (36). Parameters chosen for the clustering step were the Euclidean distance as a similarity measure, and the use of complete linkage hierarchical clustering. To estimate robustness and P-values, experiments were conducted with 10, 000 bootstrap replications of the clustering step.

To identify significantly regulated genes between the −male/+male conditions, we used the Rank Products methods (R package ‘RankProd’) (36), using the batch information to adujst for experiment specific biases, and set a False Discovery Rate (FDR) threshold for discovery of genes of 5%. From this analysis, 341 probes on the array were significantly up-regulated

in the male treated samples, and 289 were significantly down-regulated. Functional annotation enrichment was performed using DAVID software portal as described previously (37).

Whole-worm immunofluorescence

To assess structural integrity of body wall muscles, worms from 2 independent experiments were washed several times to remove bacteria and resuspended in fixing solution (160 mM KCl, 100 mM Tris HCl pH 7.4, 40 mM NaCl, 20 mM Na2EGTA, 1 mM EDTA, 10 mM spermidine HCl, 30 mM Pipes pH 7.4, 1% Triton X-100, 50% methanol, 2% formaldehyde) and subjected to two rounds of snap freezing in liquid N2. The worms were fixed at 4 °C for 30 min and washed briefly in T buffer (100 mM Tris HCl pH 7.4, 1 mM EDTA, 1% Triton X-100) before a 1-h incubation in T buffer supplemented with 1% β-mercaptoethanol at 37 °C. The worms were washed with borate buffer (25 mM H3BO3, 12.5 mM NaOH, pH 9.5) and then incubated in borate buffer containing 10 mM DTT for 15 min. Worms were blocked in PBST (PBS, pH 7.4, 0.5% Triton X-100, 1 mM EDTA) containing 1% BSA for 30 min and incubated in 1/20 dilution of fluorescently tagged (Alexa 594) phalloidin (Invitrogen, Carlsbad, CA, USA) to label actin and DAPI (2 $g.ml^{-1}$) to label nuclei. The worms were mounted on a microscope slide and visualized using a Leica DMRXA2 microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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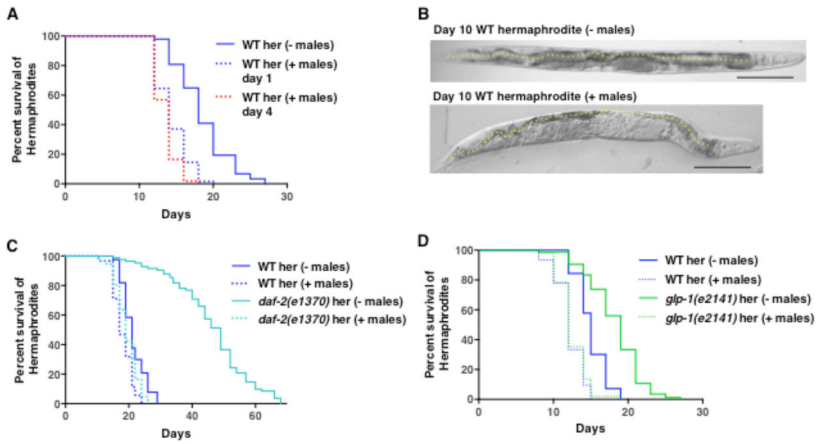
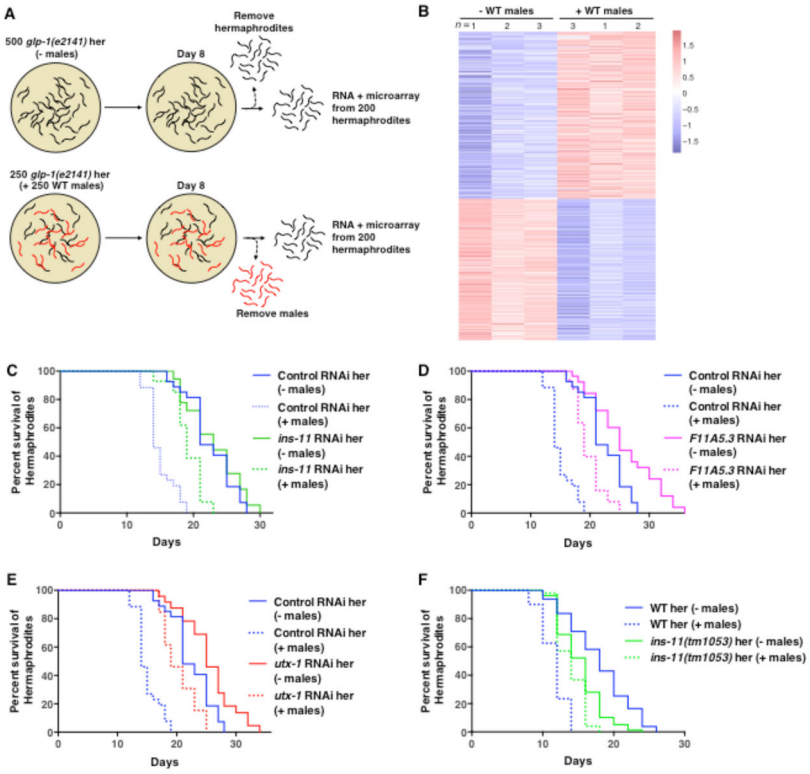


Figure 1.



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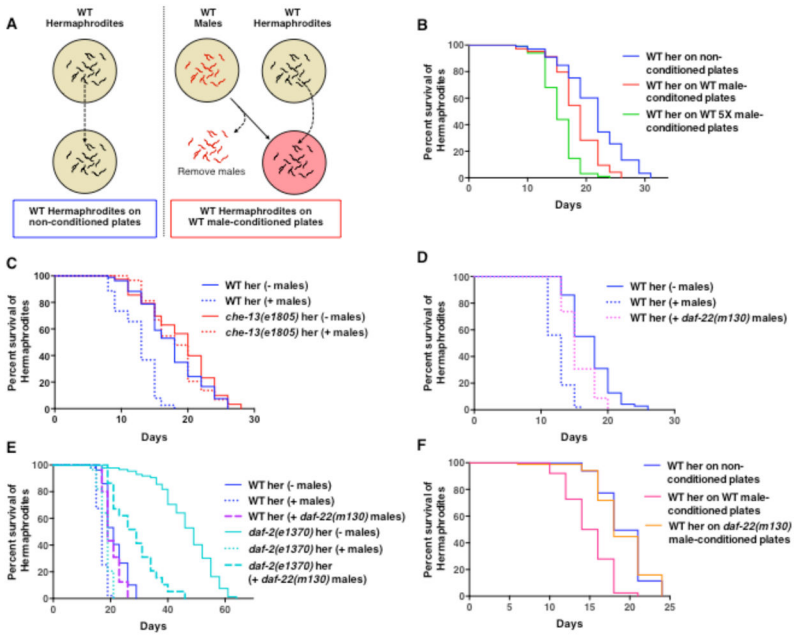

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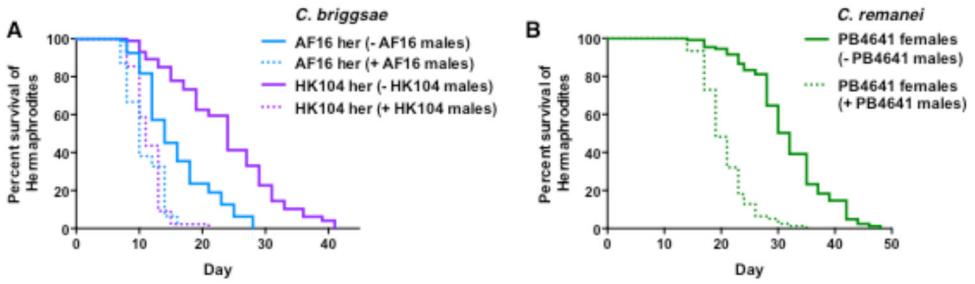

Figure 4.