# A Male Expressed Copulatory Plug Increases Female Fertility in *Caenorhabditis* Nematodes

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**Short Title:** Mate plugging benefits females

## ABSTRACT

The genomes of self-fertilizing hermaphrodites offer a unique opportunity to probe why some genes with sex-specific functions are lost while others are maintained. In the androdiecious species, *Caenorhabditis elegans*, the presence of mate plugging is polymorphic across genetic backgrounds. The production of a mating plug is controlled by a single gene, *plg-1*, which has male-specific expression. Given that males are present less than 1% of the time, this gene is likely lost through relaxed selection or purifying selection, especially if mate plugging is maintained through intrasexual selection in males as is often hypothesized. We capitalized on a diverse panel of almost 2,000 *C. elegans* natural strains to show that *plg-1* is maintained in at least 61% of genetic backgrounds, despite the androdiecious mating system. Genetic diversity estimates suggest that the loss of *plg-1* is correlated with species globalization. We show that hermaphrodite total fecundity is not correlated with *plg-1* genotype, indicating that this gene is not being maintained through linkage to a hermaphrodite beneficial gene. We then demonstrate in both *C. elegans* and the obligate outcrossing species, *C. remanei*, that the presence of a mating plug does not assure paternity by preventing subsequent males from mating. Instead, females with a mating plug have an extended peak reproductive window and higher fecundity than females without a mating plug. Together, these results indicate that mate plugging is female beneficial. We suggest that this benefit is derived from mating plugs acting as a vulval cover that can inhibit sperm loss during egg laying, thus reducing sperm limitation. Our work indicates that mate plugging may not always invoke male competition and can instead represent reproductive cooperation. More broadly, it demonstrates that male-specific gene expression does not equate to male-specific function, especially in the context of reproductive interactions.

**Keywords:** Mate plugging, Mating signal, Reproductive benefit, *Caenorhabditis* nematodes

## INTRODUCTION

Evolutionary transitions in mating system offer an opportunity to link changes in genome content and function with reproductive traits that benefit one sexual function over another. The transition from outcrossing to self-fertilizing is recurrent across plant and animal taxa (Shimizu & Tsuchimatsu, 2014). A hallmark of self-fertilization is a streamlining and shrinking of the hermaphrodite genome because of inbreeding depression, the purging of deleterious alleles, and severe population bottlenecks (Oyama *et al.*, 2008; Wright *et al.*, 2008; Thomas *et al.*, 2012). Additionally, populations with increased rates of self-fertilization have reduced intragenomic conflict due to the increased efficacy of purifying selection (Wright *et al.*, 2008). Although typically observed as a reduction in selfish genetic elements (Wright *et al.*, 2008; Thomas *et al.*, 2012), it can also lead to a reduction in sexual conflict. In self-fertilizing hermaphroditic species, sexual conflict arises as interlocus conflict between the genes underlying female and male functions (Abbott, 2010). If there is greater opportunity for selection to act on one sexual function over the other, then sex-specific selection should maintain the genes of that sexual function. Genes of the other sexual function are expected to be lost due to purifying selection or genetic drift. Thus, self-fertilizing hermaphrodites represent an evolutionary model testing of why, how, and what classes of genes are lost under reproductive shifts.

*Caenorhabditis* nematodes are an excellent model for studying the genomics underlying mating system transitions. The genus contains three independent lineage transitions – *C. elegans*, *C. briggsae*, and *C. tropicalis* – to androdiecious mating systems comprised predominantly of self-fertilizing hermaphrodites and rare (on average less than 1%) males (Cutter *et al.*, 2019). The hermaphroditic species genomes are smaller than their gonochoristic sister species (Yin *et al.*, 2018; Noble *et al.*, 2021; Teterina *et al.*, 2023; Wang *et al.*, 2026), which is driven by a loss of genes with male-specific function. For example, the *C. briggsae* genome has 23.5% fewer protein coding genes than its gonochoristic sister species *C. nigoni*, and the *C. nigoni* genes lacking orthologs in *C. briggsae* are disproportionately male-biased in expression (Yin *et al.*, 2018). This loss of male-specific genes underlies the degradation of successful male mating behaviors (Chasnov *et al.*, 2007; Garcia *et al.*, 2007; Thomas *et al.*, 2012). Together, these patterns suggest a repeated evolutionary history of male gene loss due to weaker stabilizing selection on male function coupled with reduced sexual selection and sexual conflict.

An interesting case study for testing these processes is the ancestral and largely conserved gene *plg-1*. This gene is expressed exclusively in the male vas deferens and produces a mucin protein that is deposited over the female/hermaphrodite vulva after sperm transfer (Barker, 1994; Palopoli *et al.*, 2008). Such mating plugs are common across insects, reptiles, and

some mammals (Parker, 1970; Schneider *et al.*, 2016). Mating plugs are often hypothesized to assure a male's paternity by deterring another male from attempting to mate or physically preventing another mating. This paternity assurance leads to strong intrasexual selection and can have a neutral effect on female fecundity or can cause sexual conflict if male competition limits female choice and access to sperm (Eberhard, 2015; Schneider *et al.*, 2016). Alternatively, mating plugs can directly benefit female fecundity by acting as a nuptial gift (Perry & Rowe, 2008; Schneider *et al.*, 2016), increasing ejaculate retention (Avila *et al.*, 2015; McDonough-Goldstein *et al.*, 2022), or increasing female remating rate (Perry & Rowe, 2008; Yun *et al.*, 2024). Correspondingly, male fecundity would also increase. Under the paternity assurance hypothesis alone, *plg-1* would be expected to be lost in hermaphroditic *Caenorhabditis* species either because of relaxed selection on any male functions or selection on hermaphrodites against mating with mate-plugging males. Conversely, the female benefit hypothesis would indicate that *plg-1* would be maintained in hermaphrodites if the fitness benefit provided by a mating plug was sufficient to overcome the rarity of males and potential loss through stochastic events. Here, *plg-1* would be favored in males because the plug increases male fecundity and in hermaphrodites because their sons will express *plg-1* and therefore have higher fecundity. Previous work in *C. remanei*, found no evidence for paternity assurance and nominal evidence for female benefit (Timmermeyer *et al.*, 2010), suggesting that *plg-1* could be selectively maintained in hermaphroditic species, though relaxed constraint is more likely.

In *C. elegans*, *plg-1* is polymorphic across genetic backgrounds (*i.e.*, strains) with some strains maintaining a functional copy and others having a nonfunctional gene copy. The loss of function is driven by retrotransposon insertion of a *Cer1* element and its long terminal repeats (LTRs) into the third exon of *plg-1* (Palopoli *et al.*, 2008; Fig. 1A). Here, we connect the polymorphic nature of *plg-1* in *C. elegans* with its reproductive fitness effect to better understand if this gene is being purged from the genome due to male-specific function or if instead it is being selectively maintained. First, we expand the genotyping of *plg-1* across *C. elegans* strains to better understand the phylogenetic context and evolutionary history of this locus. Then, we explicitly test the paternity assurance hypothesis in both *C. elegans* and *C. remanei* in a robust, repeatable manner. We support these data by quantifying hermaphrodite and female reproductive fitness is test the female benefit hypothesis. Together, our work indicates that even a small fitness benefit to hermaphrodites can maintain a male-specific gene when males are rare.

## MATERIALS AND METHODS

### Worm Culture and Strains

Distinct *C. elegans* genetic backgrounds (*i.e.*, strains) were selected to capture a spectrum of genetic diversity present in the species. We initially screened the 12 strains included in the *Caenorhabditis* Natural Diversity Resource (CaeNDR) divergent set (Crombie *et al.*, 2023). Additional strains were then selected to expand our sample of strains isolated from the Hawaiian Islands (N = 50 strains total) because the highest genetic diversity is found in these strains (Crombie *et al.*, 2019). To support our findings in *C. elegans*, we also included three *C. remanei* strains collected in the eastern United States: QX1539, QX1557, and KRK1. *Caenorhabditis remanei* is an obligate outcrossing species with fixed mate plugging. A complete list of *C. elegans* and *C. remanei* strain information is listed in File S1.

For both species, strains were cultured on 10 cm NGM-agar plates seeded with OP50-1 *Escherichia coli* and maintained at 20ºC (Brenner, 1974; Kenyon, 1988). Prior to all experiments strains were cleaned and age synchronized using hypochlorite treatment (Kenyon, 1988). Male-rich *C. elegans* strains were maintained by transferring an additional 30-40 young adult males during the transfer of worms to plates with fresh bacteria.

### ***plg-1*** **Genotyping**

We used polymerase chain reaction (PCR) to test for the presence or absence of *Cer1* insertion in *plg-1* using the primers developed in Palopoli *et al.* (2008). Genotype confirmation was performed for the 72 strains listed in File S1. Genomic DNA was purified by isolating two adult worms in 4 μL of 20 mg/μL Proteinase K in elution buffer. Samples were freeze-cracked in liquid nitrogen three times. They were then incubated at 58ºC for 60 minutes followed by 10 minutes at 95ºC to inactivate the Proteinase K. PCR products were generated using the One-Taq Quick Load 2X Master Mix with Standard Buffer (NEB #M0486) and 2 μL of purified DNA in accordance with manufacturer instructions. Each sample was run for two reactions. One reaction amplified a functional copy of *plg-1* and produced a 599 bp product (forward primer: CGCATAAAACGTCAGCAGAA; reverse primer: ATTCGGAGTAGTCGGGTCCT). The other reaction amplified a non-functional copy of *plg-1* as indicated by a retrotransposon insertion and produced a 658 bp product (forward primer: TCCACAAAACCTGCTGACTG; reverse primer: ATCCACTCGATTTTCGCAAC). Strain genotype was classified based on having the presence of the correct product size in one reaction and no product in the other. Genotype calls were verified with plate-based observations of a mating plug.

### **Computational Inference of** ***plg-1*** **Genotype**

To expand the genotype information to all 1,953 sequenced strains, we developed a computational pipeline based on the read depth for *plg-1* and *Cer1*. For each of the 1,953 strains

with sequencing reads available from the CaeNDR 20260625 data release, we identified paired-end reads that mapped around the *plg-1* insertion site. Reads were considered if one end was mapped between 100 and 2,000 bp away from the *Cer1* insertion boundary and oriented towards the insertion site. Reads were classified as *plg-1* orphans if the other read end was unmapped, hybrids if the other end mapped within the *Cer1* sequence, or *plg-1* only if the other end mapped to sequence on the opposite side of the insertion site. Similarly, reads were identified in which one end mapped between 100 and 2,000 bp inside of the *Cer1* sequence oriented towards the closest *Cer1* boundary. Reads were classified as *Cer1* orphans if the other read end was unmapped, hybrids if the other end mapped outside of *Cer1* with an insert size less than or equal to 2.5 kb, and other if the insert size was unknown (not mapped in a forward-reverse pairing or to a different chromosome) or greater than 2.5 kb. In addition, we found the mean read coverage of a 10 kb non-repetitive stretch of *plg-1* 2.6 kb upstream of the *Cer1* insertion site and the mean read coverage of a 10 kb region centered in the inserted *Cer1* sequence.

Strains were classified as non-plugging if the *Cer1* coverage was greater than five reads/bp, there were fewer than five *plg-1* orphan reads, at least five hybrid reads, and less than five *plg-1* only reads. These criteria were used to ensure that *Cer1* was present in the genome and inserted within *plg-1*. Strains were classified as plugging if the mean insert size of *plg-1* only reads was between 9-9.5 kb and there were at least five *plg-1* only reads, suggesting that reads were mapping across the entire *Cer1* insertion site. Any strain not meeting one of these sets of criteria was considered to have an ambiguous plugging status. *Cer1* genomic copy number was inferred by rounding the ratio of mean *Cer1* coverage to mean *plg-1 coverage.*

**Inferring Evolutionary History**

We calculated nucleotide diversity ($\pi$) for strains classified as having a functional copy of *plg-1*, a non-functional copy of *plg-1*, or being computationally ambiguous. Genomic data (hard-filter VCF) were accessed from the CaeNDR 20250625 data release and $\pi$ was calculated in 10 kb sliding windows using vcftools (Danecek *et al.*, 2011). Genome-wide and chromosome III diversity results were analyzed in the R Statistical Language (Team, 2020). We overlaid *plg-1* genotype on the phylogenetic tree of all 1,953 *C. elegans* (CaeNDR 20250625 data release) using the package phytools (Revell, 2024) in R (R Core Team, 2020).

**Fecundity Assays**

We assayed the overall reproductive success of 17 *C. elegans* hermaphrodites with a functional (N = 11 strains) or non-functional (N = 6 strains) copy of *plg-1*. These strains included the 12 in the CaeNDR divergent set along with one additional strain with a functional copy of *plg-1* and four

additional strains with a non-functional copy of *plg-1*. Individual hermaphrodites were isolated during late larval stage 4 (L4) onto small NGM-agar plates (35 mm diameter) seeded with 20 μL *E. coli* OP50-1. After 24 hours, each hermaphrodite was transferred to a new small plate to continue laying eggs. Transfers continued every 24 hours until day 7 of adulthood when progeny production had ceased. The total number of progeny were counted as a measure of each hermaphrodite's reproductive success (File S5). The late L4 progeny were screened for the presence of males as an estimate of the non-disjunction rate for each strain. Three independent assays were conducted for each strain for a total of at least 30 hermaphrodites examined, except for JU346 (N = 29). Fecundity data were analyzed using a phylogenetic least squares regression (PGLS) to account for phylogenetic relationships. The full strain phylogeny was used to represent the strongest inference of node relationships and tips were dropped to represent the 17 strains of interest using phytools (Revell, 2024) in R (R Core Team, 2020). Differences in the frequency of males between *plg-1* genotypes was analyzed using a proportions test.

From this group, we selected three pairs of closely related strains with alternative *plg-1* genotypes to represent the different plugging states in an otherwise similar genetic background. Specifically, we selected sister strains ED3017 and MY920, and EG4725 and NIC266, which have functional and non-functional *plg-1* genes, respectively. Additionally, we selected the closely related, but paraphyletic, pair JU346 and JT11398 as there were no other alternative genotype sister strains in this group of 17 strains.

We assayed the reproductive success of males from each of the strain pairs when mated to a common pseudo-female background (JK574). We isolated individual JK574 pseudo-females during late L4 onto small NGM-agar plates (35 mm diameter) seeded with 10 μL *E. coli* OP50-1 and maintained them as virgins until the start of the assay. A single male was added to each plate and allowed to mate with the female for 24 hours. After the mating period, males were removed, and females were transferred to a new small plate to continue laying eggs. Transfers continued every 24 hours until day 7 of adulthood when progeny production had ceased. The total number of progeny were counted as a measure of each male's early adulthood reproductive success (File S6). Strain pairs were assayed at the same time with three independent assays conducted for each pair. At least 25 mated females were examined per strain. Fecundity data were analyzed using R (R Core Team, 2020), using a generalized linear model that fit the effect of genotype and replicate on fecundity using a Poisson distribution.

**Male Signaling Assay**

To test the paternity assurance hypothesis, we performed two sequential matings using a full factorial design of *plg-1* genotypes for each of the strain pairs. Thus, for each strain pair there

were four mating combinations of first and second male genotypes. Individual JK574 pseudo-females were isolated during late L4 onto small NGM-agar plates (35 mm diameter) seeded with 10 μL *E. coli* OP50-1 and maintained them as virgins until the start of the assay. A single male was added to each plate and allowed to mate with the female for 24 hours. After 24 hours, the plate was screened for the presence of eggs to ensure that mating had occurred with the first male. Those females were then moved to a new plate, and a second male was added. To identify sperm transferred from the second male, we stained males with 10 μM MitoTracker Red CMXRos (Invitrogen M7512) on medium NGM-agar plates (60 mm diameter) for approximately 20 hours. After a 24-hour mating period with the second, stained male, females were mounted on 1% agarose pads with 400 μM sodium azide. Females were scored in real time using a Nikon Ti2 inverted microscope (20× DIC microscope objective, 150 ms fluorescence exposure time) for the presence of second male sperm in the spermathecae, uterus, and mating plug. Three independent assays were conducted for each strain pair (File S7).

We repeated these assays in *C. remanei*. Since mate plugging is a fixed trait, sequential matings were performed within a strain (KRK1, QX1539, and QX1557) and thus there was only a single mating combination. Again, three independent assays were conducted for each strain (File S8).

Sperm presence data were analyzed using R (R Core Team, 2020). A proportions test was performed to determine if the observed number of second male matings deviated from 50% (*i.e.*, random choice). Additionally, within each strain pair, we performed generalized linear models that fit the effect of first male genotype and replicate on second male sperm presence. Because we found qualitatively the same results across all strain pairs, we pooled all strains together and present the combined findings.

**Plug Area Measurements Over Time**

Individual females were isolated during late L4 onto small NGM-agar plates (35 mm diameter) seeded with 10 μL *E. coli* OP50-1 and maintained as virgins until the start of the assay. A single plugging male was added to each plate and allowed to mate with the female for 24 hours. After 24 hours, the plate was screened for the presence of eggs to ensure that mating had occurred. The male was then removed and females were imaged 24-, 48-, and 72-hours post-mating period. This experimental procedure was done in *C. elegans* using JK574 pseudo-females mated with either ED3017, EG4725, or JU346 males. In *C. remanei*, females of KRK1, QX1539, and QX1557 were mated with males from the same strain.

Mating plug size was quantified in *C. elegans* and *C. remanei* using Nikon NIS-Elements software (Nikon Instruments Inc., Tokyo, Japan). Images were captured using a Nikon Ti2 inverted microscope equipped with a 20× DIC microscope objective and 43 ms exposure time. For each image, the mating plug was outlined and measured using the Polygonal ROI tool in NIS-Elements. Each traced ROI was converted to a binary layer, and only the reference binary layer was displayed during subsequent tracing to prevent duplicate or overlapping measurements. The boundary of each mating plug was delineated manually, and area ($\mu m^2$) was obtained using the ROI Statistics tool.

Each plug was measured six times in total – three independent measurements by each of two trained analysts – to minimize observer bias and assess measurement repeatability. A total of 276 plugs were analyzed across the three *C. elegans* strains, and 606 plugs were analyzed across the three *C. remanei* strains (File S9). The number of individuals analyzed per strain and per day varied depending on sample availability. The change in plug area over time was analyzed for each species using a linear model that fit time and the interactions between strain and observer. Potential species effects were also modeled in a general linear framework. All analyses were done using R (R Core Team, 2020).

## RESULTS

### *plg-1* Loss is Correlated with Globalization in *C. elegans*

We expanded the genotyping of Palopoli *et al.* (Palopoli *et al.*, 2008) to capture the increase in *C. elegans* sampling (Figure S1). Specifically, we focused on genotyping strains from the Hawaiian Islands, which are likely the ancestral location for *C. elegans* due to the high genetic diversity of strains isolated from this region (Crombie *et al.*, 2019; Rockman *et al.*, 2025). If *plg-1* was lost early in the lineage transition to hermaphroditism, then we would expect few Hawaiian strains to maintain a functional copy of *plg-1*. Our new genotyping included 23 strains from Hawaii, 14 strains from Kauai, 6 strains from Maui, 2 strains from Molokai, and 5 strains from O'ahu. Additionally, we expanded the sampling across the continental United States, Australia, and Europe. In total, we genotyped 72 new strains at the *plg-1* locus (File S1), which brings the total number of PCR confirmed *plg-1* genotypes to 112 strains across datasets. Of these, 96 strains have a functional copy of *plg-1* (*plg-1*+) and 16 have a non-functional copy (*plg-1*–). Importantly, all the strains from the Hawaiian Islands are *plg-1*+, suggesting that *plg-1* was not lost early in the transition to hermaphroditism.

Next, we developed a computational pipeline to classify the *plg-1* genotype in the 1,953 sequenced *C. elegans* strains. Genotypes were inferred based on the counts of reads aligning to

*plg-1*, to the retrotransposon *Cer1*, and to the exon 3 insertion overlap (Fig. 1A). The results were validated against the PCR genotyped strains. We found that 61% of strains are *plg-1*+ (N = 1,194) and 10% of strains are *plg-1*– (N = 187). Model results for 572 strains were ambiguous. Mapping genotype information to the strain phylogeny again supports that *plg-1* was not lost in the mating system transition because no strains isolated from the Hawaiian Islands were classified as *plg-1*– (Fig. S1). Instead, the phylogeny supports the conclusions in Palopoli *et al.* (2008) that *plg-1* has been lost during globalization. Specifically, the majority of *plg-1*– strains form monophyletic clades that are sampled from specific regions, such as France, Germany, the Netherlands, and Australia. These phylogenetic relationships suggest a history of retrotransposon insertion into *plg-1* occurs during population bottleneck events that accompany globalization followed by local population expansion and diversification.

To test this globalization hypothesis, we measured nucleotide diversity ($\pi$) in strains grouped by each genotype. If *plg-1* loss is correlated with bottleneck events, then we would expect to see a lower genomic diversity both genome-wide and on chromosome III, which contains *plg-1*, in *plg-1*– strains than in *plg-1*+ strains. Conversely, if *plg-1* were selected against in hermaphrodites due to antagonistic function, then we would not expect a correlation between genotype at this locus and genome-wide patterns of diversity, while local diversity at the *plg-1* locus would decrease. Genome-wide the mean nucleotide diversity was an order of magnitude larger in *plg-1*+ strains ($\pi$ = 0.002) compared to *plg-1*– strains ($\pi$ = 0.0006). Both the median (MW-test: W = 87302342, $p < 0.001$) and distribution of $\pi$ values (KS-test: D = 0.64, $p < 0.001$) were significantly different between genotype groups, even though the maximum diversity was the same ($\pi$ = 0.02). Nucleotide diversity on chromosome III recapitulated these patterns (Fig. 1). The median nucleotide diversity on chromosome III in *plg-1*+ strains ($\pi$ = 0.002) was significantly larger (MW-test: W = 1677014, $p < 0.001$) than that in *plg-1*– strains ($\pi$ = 0.0005). This difference was driven by the complete lack of nucleotide diversity in the chromosome center of *plg-1*– strains, overlapping with the *plg-1* locus (Fig. 1A). Both genotype groups show the characteristic hyperdivergent chromosome arms (Lee *et al.*, 2021; Moya *et al.*, 2025). The counts of variants were also significantly higher in *plg-1*+ strains (KS-test: D = 0.89, $p < 0.001$; Fig. 1B). These results indicate a genome-wide loss of nucleotide diversity in *plg-1*– strains as expected after a bottleneck.

To confirm that *plg-1* is not maintained simply because the *Cer1* element was lost, we also quantified the number of *Cer1* element copies between genotype groups. We found that *Cer1* copy number ranged from 0-16 with only 36 *plg-1*+ strains inferred to lack any *Cer1* elements (Fig. 1C). Although the mean number of *Cer1* elements is significantly higher in *plg-1*+ strains (t-

test: t = 8.6, df = 379, p < 0.001), it is questionable if this is biologically significant. Taken together, these results support that the loss of *plg-1* function is correlated with population bottlenecks during globalization rather than selection against gene function. Instead, they suggest that *plg-1* is maintained despite it being a male-specific gene in an almost entirely hermaphroditic lineage.

## ***plg-1* is Maintained in *C. elegans* by Selection on Males**

A possible mechanism maintaining *plg-1* is linkage to a hermaphrodite beneficial gene. Here, purifying selection would not effectively remove *plg-1* from the genome if the benefit of the haplotype exceeded any potential costs of mate plugging when males occurred. Thus, hermaphrodites with a functional *plg-1* would have higher fitness than those without, representing the beneficial haplotype rather than a benefit of *plg-1* itself. To test this hypothesis, we measured the total reproductive success of hermaphrodites from 11 *plg-1*+ strains and six *plg-1*– strains (Fig. 2A). The mean fecundity of *plg-1*– hermaphrodites ($x \pm SE = 270 \pm 3.5$, N = 258) trended higher than that of *plg-1*+ hermaphrodites ($x \pm SE = 245 \pm 3.8$, N = 391) (Fig. 2B). However, this trend was not significant when accounting for phylogenetic relationships (PGLS: $F_{1,15} = 1.5$, p = 0.24). These results indicate that *plg-1* does not exist on a reproductive beneficial hermaphroditic haplotype and thus linked selection is not maintaining *plg-1* in the genome.

Alternatively, *plg-1* could be maintained simply due to differences in male frequency between strains genotypes. Because *C. elegans* has a XX hermaphrodite and XO male sex determination system, males arise in the population through X chromosome non-disjunction events. This explanation would suggest a positive correlation between the maintenance of *plg-1* and non-disjunction rate leading to male production. We quantified the presence of males resulting from non-disjunction during self-fertilization in the same 11 *plg-1*+ strains and six *plg-1*– strains. Male frequency ranged from 0% in JU258 to 2% in both LKC34 and MY16 (Fig. 2A). The proportion of male progeny did not differ significantly between genotypes ($\chi^2 = 0.17$, df = 1, p = 0.68). Thus, *plg-1* is not maintained through increased male frequency in *plg-1*+ populations. Rather, the maintenance of *plg-1* must be due to a selective advantage of its function during reproduction.

## **Mating Plugs Do Not Function To Prevent Future Reproductive Events**

Next, we tested that paternity assurance hypothesis to determine if the presence of a mating plug either chemically or physically prevents a second male from mating. We sequentially mated feminized *C. elegans* hermaphrodites (*i.e.*, pseudo-females that cannot produce self-sperm) with *plg-1*+ and *plg-1*– males from three strain pairs using a full factorial design. We then quantified the presence of fluorescently dyed sperm from the second male across three anatomical regions:

spermathecae, uterus, and mating plug (Fig. S2A). Under the paternity assurance hypothesis, we would expect to see less sperm from the second male in the female reproductive tract if a mating plug was present.

The presence of second male sperm ranged from 81% to 94% (Fig. 3A; Fig. S2B-C). The proportion of females with second male sperm is significantly higher than expected under random mating ($\chi^2$ = 361.6, df = 1, p < 0.001), demonstrating the males are not deterred from mating with non-virgin females. Moreover, we found no effect of first male genotype on second male sperm presence within any region of the female reproductive tract for each strain pair (LM: $F_{3,620}$ = 0.37, p = 0.77). The lack of effect did not change when analyzing sperm presence separately for each anatomical region ($LM_{spermathecae}$: $F_{1,622}$ = 0.58, p = 0.45; $LM_{uterus}$: $F_{1,622}$ = 0.81, p = 0.37; $LM_{plug}$: $F_{1,622}$ = 0.58, p = 0.44). These results show that mating frequency does not depend on whether a female has a mating plug. Thus, mating plugs do not enhance a male's paternity and do not limit a female's access to sperm. Interestingly, we detected an effect of second male genotype on sperm presence such that when the second male produced a mating plug a higher frequency of females were observed with second male sperm (LM: $F_{1,620}$ = 4.3, p < 0.01). Although this effect could be an experimental artifact, it suggests a potential role of the mating plug in keeping sperm within the reproductive tract.

We repeated these assays in *C. remanei*, a gonochoristic species where mate plugging is a fixed trait. In all three strains examined the second male again mated more frequently than expected under random mating ($\chi^2$ = 98.23, df = 1, p < 0.001). The frequency of second male matings was higher than in *C. elegans* and ranged from 97% to 100% (Fig. 3B). Again, we found no effect of first male genotype on second male sperm transfer to any region of the female reproductive tract (LM: $F_{1,105}$ = 0.50, p = 0.61), providing more statistically conclusive evidence than previous work (Timmermeyer *et al.*, 2010). Together, these data do not support that mating plugs in *Caenorhabditis* nematodes act as a paternity assurance and provide no evidence that they limit female access to future matings.

**Females with a Mating Plug Have Higher Fecundity**

Given the lack of evidence for the paternity hypothesis, we then tested if instead mate plugging provides a female benefit. *Caenorhabditis elegans* pseudo-females were mated with a single male from each of the three strain pairs and the progeny produced was quantified across days of adulthood. The total progeny produced ranged from 184 offspring (N = 39) in JT11398 to 343 offspring (N = 42) in EG4725 (Fig. 4). In females mated with *plg-1*+ males, reproduction peaked on day 3 of adulthood and was consistently higher than females mated with *plg-1*– males (Fig. 4A). We found a significant effect of genotype (GLM: z-value = 5.0, p < 0.001), day (GLM: z-value

= -90.8, $p < 0.001$), and the interaction between them (GLM: z-value = 20.9, $p < 0.001$) on female fecundity, which translated to females producing on average 100 more offspring when mated with *plg-1*+ males ($x \pm SE = 354 \pm 1.3$, N = 97) than when mated with *plg-1*– males ($x \pm SE = 246 \pm 1.4$, N = 107) (Fig. 4B). Thus, the presence of a mating plug benefits female reproductive success by lengthening peak reproduction and total reproductive success. These fitness data suggest that the reproductive benefit of *plg-1* is sufficient to maintain this male-specific gene despite the rarity of males in the population.

**Plug Size Does Not Decrease with Time Post-Mating**

Mating plugs have been shown to benefit female fitness by acting as a nuptial gift (Perry & Rowe, 2008) or preventing sperm loss (Avila *et al.*, 2015). If the mating plug in *Caenorhabditis* nematodes were acting a nutrient resource, then we might expect the size of the plug to decrease post-mating as it was broken down to macromolecular components. Alternatively, if the plug were instead acting as a vulval cover to retain sperm, then we would expect to see no change in plug size over time. To examine these alternative forms of benefits, we mated *C. elegans* pseudo-females and *C. remanei* females with *plg-1*+ males of the respective species and then measured plug area 24-, 48-, and 72-hours post-mating.

In *C. elegans*, plug area was greatest 72-hours post-mating ($x \pm SE = 2485 \pm 13.6um^2$, N = 102) and smallest 48-hours post-mating ($x \pm SE = 2085 \pm 40.2um^2$, N = 60), though sample size was also smallest on this day (Fig. 5A). Overall, we found no significant change in plug area over time, nor did plugs differ between male genotypes (LM: $F_{6,269} = 1.10$, $p = 0.36$). In *C. remanei*, plug area increased from 24-hours post-mating ($x \pm SE = 2625 \pm 7.8um^2$, N = 180) to 72-hours post-mating ($x \pm SE = 3884 \pm 11.3um^2$, N = 216) in a significant fashion (Fig. 4B; LM: $F_{6,591} = 41.9$, $p < 0.001$). Although, we detected differences in plug area between genotypes, suggesting that expression level could vary across genetic backgrounds (Fig. 5B), each strain independently had a significant increase in area over time ($LM_{KRK1}$: $F_{1,226} = 18.2$, $p < 0.001$; $LM_{QX1539}$: $F_{1,184} = 35.1$, $p < 0.001$; $LM_{QX1557}$: $F_{1,184} = 12.0$, $p < 0.001$). Finally, we examined the species effect and found a significant interaction between species and time (LM: t-value = 3.14, $p < 0.01$), despite each individual component not contributing significantly to plug area. Together, these results show that plug area is maintained or increased with time post-mating in a species-specific manner, which is contrary to a nuptial gift expectation. Rather, they support that mating plugs might act as a vulval cover and help to retain sperm in the uterus, which would positively impact reproductive fitness.

## DISCUSSION

Studying the evolutionary transition to self-fertilizing hermaphroditism provides a comparative framework to probe why and what types of genes with sex-specific functions are lost versus maintained. Here, we examined *plg-1,* a male-expressed gene that underlies mate plugging, which is often hypothesized to ensure male paternity. These characteristics would suggest genome streamlining to lose *plg-1* during the lineage transition to self-fertilizing hermaphroditism. Instead, we found that *plg-1* has been maintained in at least 61% of *C. elegans* strains, including all the ancestral Hawaiian Island strains. The loss of mate plugging likely occurred several independent times during globalization. We find no evidence that mate plugging assures male paternity or limits female mating choice in nematodes. Rather, the mating plug appears to act as a beneficial cover that prolongs female reproductive success post-mating and increases overall fecundity. Together, these results indicate that male-specific expression does not equate to male-specific function and demonstrate the role male-specific genes play during reproduction even when genetic males are not required for reproduction to occur.

Both genetic-diversity estimates and phylogenetic relationships indicate that the loss of *plg-1* is correlated with range expansion out of the Hawaiian Islands. During such globalization genetic diversity decreases due to the coupling of population bottlenecks and serial founder effects (Henn *et al.*, 2012). In *C. elegans*, a single hermaphrodite can found a new population. Thus, if the *Cer1* retrotransposon were to insert into *plg-1* in a single founder, then all subsequent generations would contain the insertion and lack a function copy of *plg-1*. This scenario likely explains the 10% loss of *plg-1* across strains. A loss of the *Cer1* element is not responsible for the maintenance of *plg-1* (Fig. 1C). Interestingly, although we annotated a median of four *Cer1* copies in *plg-1*+ strains, the maximum copy number was four times higher. Previous work in on these elements in a lab-adapted *C. elegans* strain annotated only four *Cer1* elements (Ganko *et al.*, 2001), suggesting that this retrotransposon family could have a more complex evolutionary history in the genetically diverse Hawaiian strains.

Early observations of mate plugging suggested that it was a paternity assurance trait under intrasexual selection and sexual conflict. Although this characterization holds true particularly in some mammalian species (see Mangels *et al.*, 2016), most studies find that the function is more complex. Recent studies have shifted this paradigm such that reproductive benefits of mate plugging to their mating partners could be the predominant function (Perry & Rowe, 2008; Avila *et al.*, 2015; Schneider *et al.*, 2016; McDonough-Goldstein *et al.*, 2022; Yun *et al.*, 2024). Supporting this perspective, we find no evidence for the paternity assurance hypothesis (Fig. 3) and instead show that females with a mating plug have increased fertility. This result

supports and expands on previous work in *C. remanei* that showed that plugged females produced more eggs and offspring than non-plugged females (Timmermeyer *et al.*, 2010). Although we cannot rule out the potential that the mating plug acts as a nutrient gift, we find no evidence that plug area decreases post-mating as would be expected if it were actively broken down into its component macromolecular parts. Additionally, the nutrient content of a mucin protein is not immediately clear. Instead, we hypothesize that the mating plug acts as vulval cover that allows males to still find the vulva opening for copulation but does not allow sperm cells to pass out of the reproductive tract. Previous studies showed that females pressurize the reproductive tract to push out the male spicule after mating and that sperm can also be lost during this process (Barker, 1994; Kleemann & Basolo, 2007). This sperm loss can lead to females being sperm limited (Kleemann & Basolo, 2007). We show two lines of circumstantial evidence that the mating plug retains sperm. First, the higher late-life fecundity of plugged females (Fig. 4) coupled with visualized sperm within the mating plug (Fig. S2B) suggest that the plug prevents sperm from being pushed out of the uterus during vulval muscle contractions. Second, plug area was maintained post-mating or even increased in size, likely from taking in moisture from the environment, suggesting the cover itself is important. Together, these results support that at least one function of mating plugs is to prevent sperm loss and represent a promising avenue for future research.

As the field moves away from a male-biased perspective on reproductive interactions – particularly in regard to the role of seminal fluid proteins – we have opportunities to reassess the roles that male-expressed genes play in female function and fitness. Transitions in mating systems, as seen across plant and animal taxa, offer an excellent comparative genomic framework to address these questions from this new perspective.

**Data Accessibility:** The complete list of strains analyzed in this study along with their *plg-1* genotype are given in File S1. Computationally inferred *plg-1* genotype and *Cer1* copy number are given in File S2. Genome-wide nucleotide diversity statistics are given in File S3 and chromosome III nucleotide diversity statistics are given in File S4. The hermaphrodite fertility data are available in File S5, and the pseudo-female fertility data are available in File S6. The sperm signaling data are available in File S7 (*C. elegans*) and File S8 (*C. remanei*). Plug area data are available in File S9. All scripts are available via the Kasimatis Lab GitHub repository plg-1 (https://github.com/Kasimatis-Lab/plg-1). All *C. elegans* strains are available from CaeNDR, the *Caenorhabditis* Natural Diversity Resource. Strains QX1539 and QX1557 were generously provided by the Andersen lab. Strain KRK1 is available from the Kasimatis lab upon request.

**Author Contributions:** KRK devised the project. LJB performed the PCR genotyping, and MS performed the computational genotype inference. LJB and KRK performed the fecundity assays. LJB performed the plug signaling assays with assistance from KRK. LJB and AG quantified plug area over time. KRK analyzed the data. KRK wrote the manuscript with the support of the other authors.

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The authors declare no conflicts of interest.

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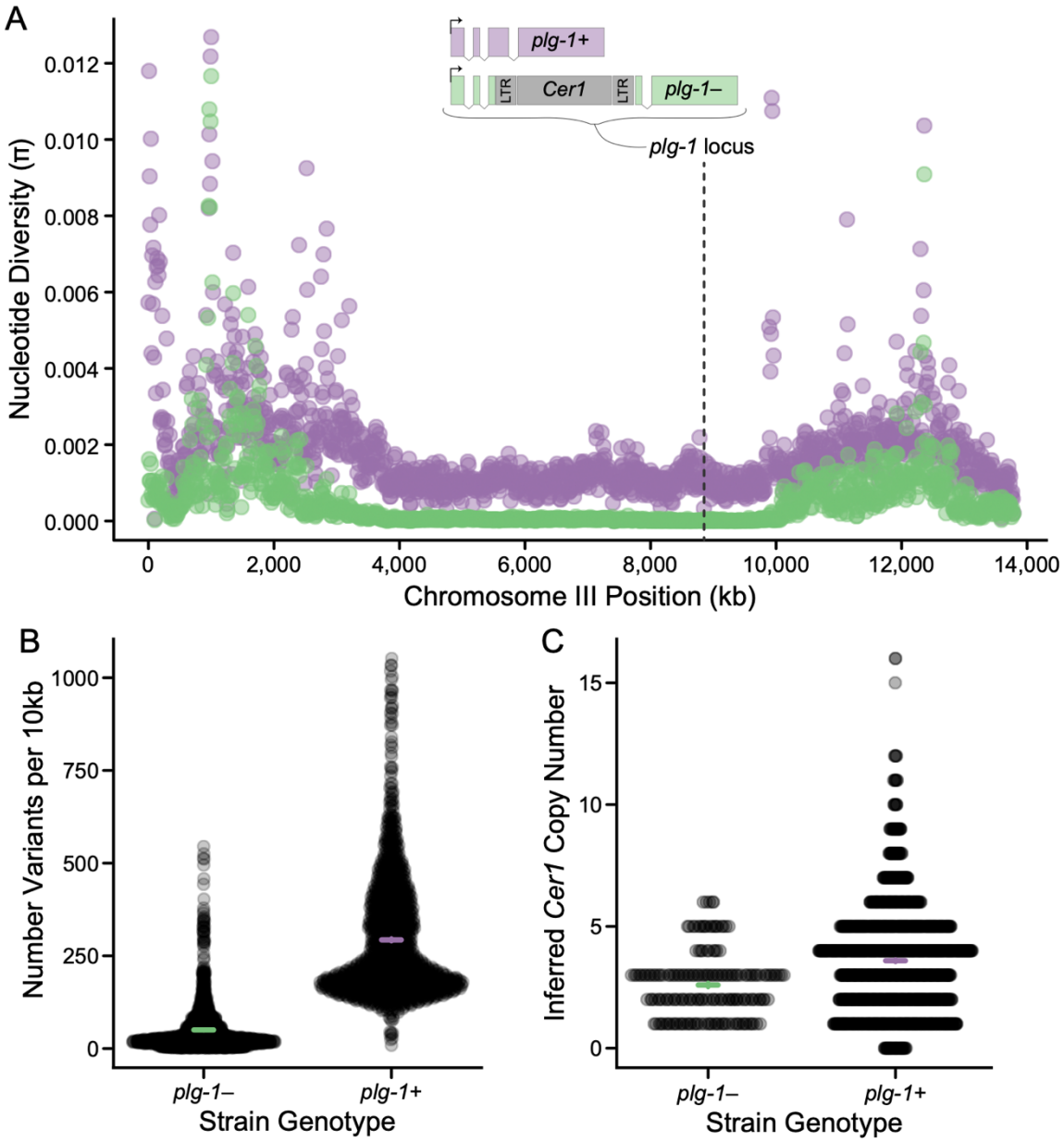


**Figure 1.** Chromosome III nucleotide diversity in 1,194 *plg-1*+ and 187 *plg-1*– strains. **A)** Nucleotide diversity calculated in 10 kb sliding windows along chromosome III for *plg-1*+ (purple) and *plg-1*– (green) strains. The dashed line shows the location of the *plg-1* locus with the inset representing the gene models of for functional and non-functional *plg-1* copies. Nucleotide diversity is highest on the chromosome arms in both genotype groups, though overall diversity is higher in *plg-1*+ strains. **B)** The number of variants falling into each 10 kb sliding window on chromosome 3 for *plg-1*+ and *plg-1*– strains. The mean and standard error for each genotype is given by the purple (*plg-1*+) and green (*plg-1*–) crosses. There are on average more variants per window in *plg-1*+ strains. **C)** The number of *Cer1* element copies (at any genomic location) in *plg-1*+ and *plg-1*– strains. The mean and standard error for each genotype is given by the purple (*plg-1*+) and green (*plg-1*–) crosses.

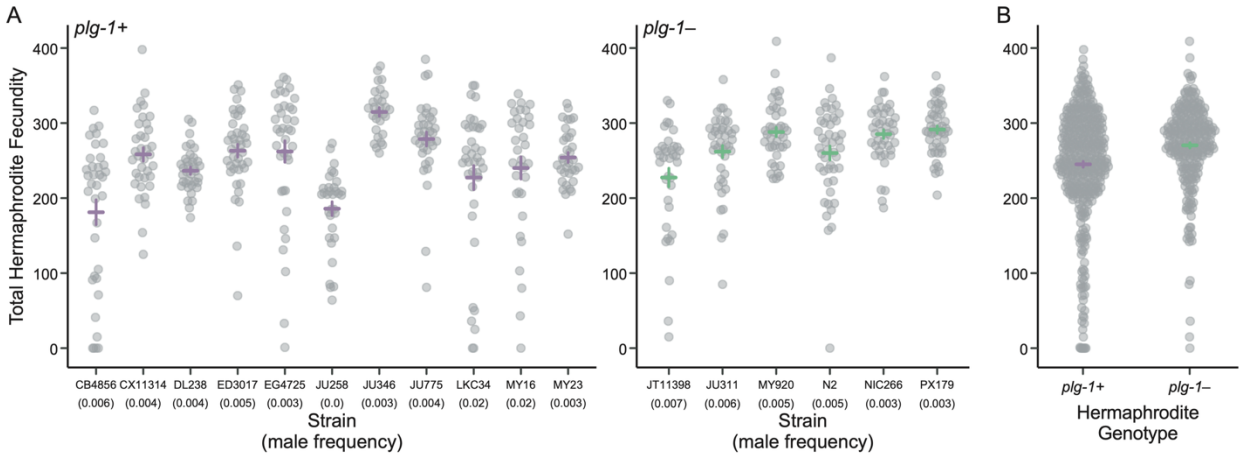


**Figure 2.** The lifetime fecundity for self-fertilizing *C. elegans* hermaphrodites. **A)** Total fecundity for each of the 11 *plg-1*+ and six *plg-1*– strains analyzed. Each point represents the total number of viable offspring for an individual hermaphrodite. At least 33 individual hermaphrodites were examined for each strain. The mean and standard error for the strain is given by the purple (*plg-1*+) and green (*plg-1*–) crosses. The frequency of males observed in each strain due to non-disjunction events are given in parentheses. **B)** Total fecundity grouped by hermaphrodite *plg-1* genotype. Again, each point represents the fecundity of an individual hermaphrodite. The mean and standard error for the strain is given by the purple (*plg-1*+) and green (*plg-1*–) crosses.

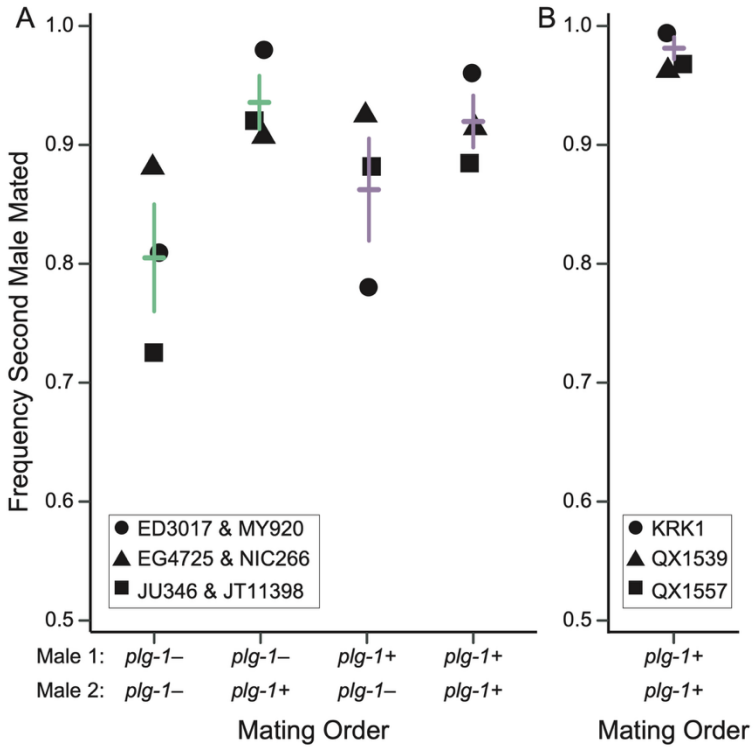


**Figure 3.** The frequency that sperm from the second mating male in a sequential mating is observed within the female reproductive tract. **A)** Factorial crosses in *C. elegans* for each of the three strain pairs examined are given by the different shaped points. Each point represents the frequency observed in at least 45 mated females. The mean and standard error across strains is shown and colored based on the first male genotype (green is *plg-1*– and purple is *plg-1*+). **B)** Sequential cross within three *C. remanei* strains. Each point represents the frequency observed in at least 35 scored females from each strain with the shapes representing the different strains. The mean and standard error across strains is shown.

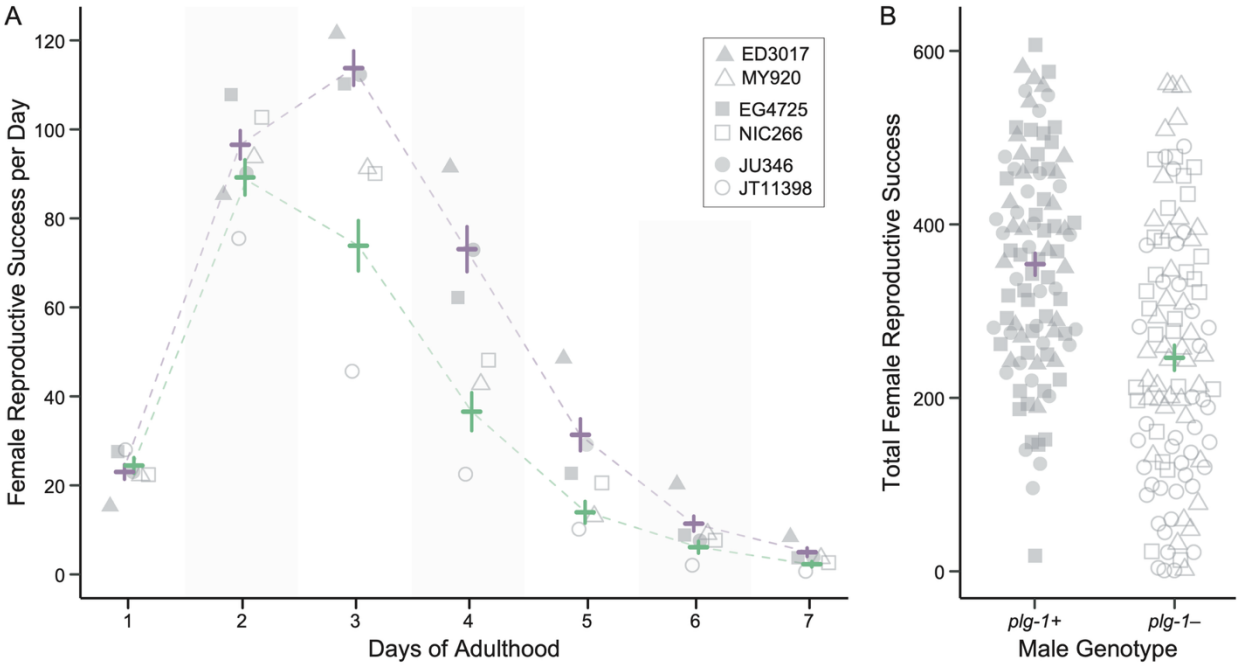


**Figure 4.** The fecundity of *C. elegans* pseudo-females mated with either *plg-1*+ or *plg-1*– males. **A)** Female reproductive success across days of adulthood. Each point represents the mean fecundity of at least 25 mated females. The point shapes represent the male genotype for the sister strain pairs with filled shapes corresponding to the *plg-1*+ genotype and the open shapes corresponding to the *plg-1*– genotype. The mean and standard error across strains of the same genotype is shown (green is *plg-1*– and purple is *plg-1*+). **B)** Total female fecundity summed across all of adulthood and grouped by male *plg-1* genotype. The point shapes are the same as in panel A. The mean and standard error is shown.

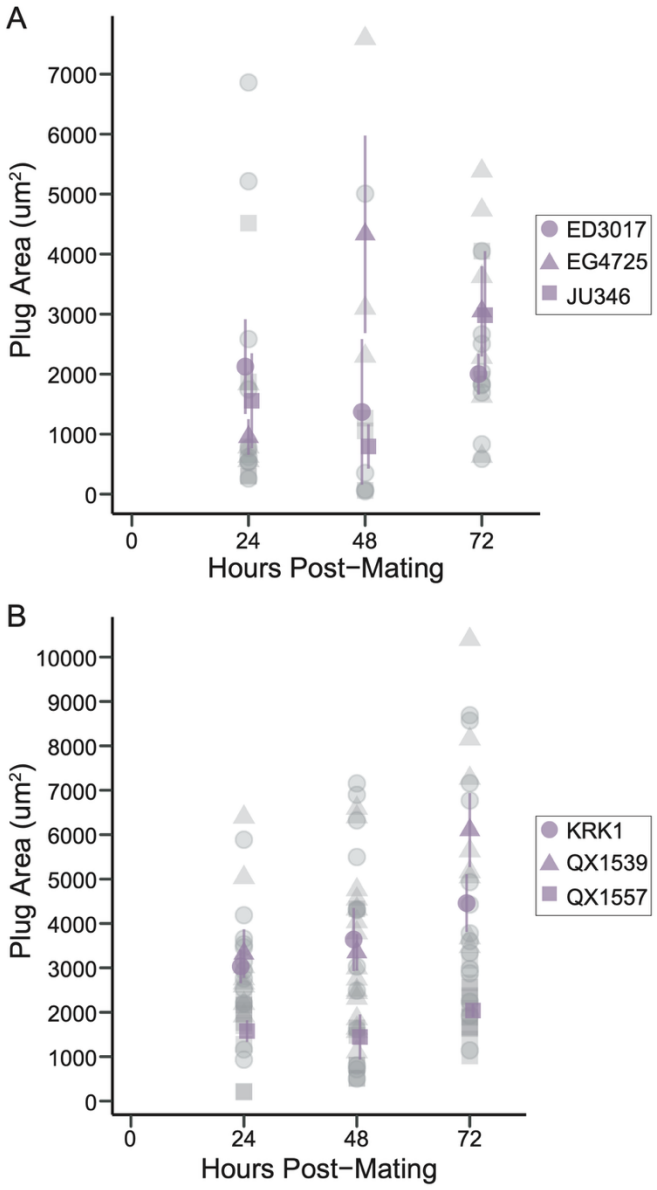


**Figure 5.** Mating plug area 24-, 48-, and 72-hours post-mating. **A)** Plug area measured on *C. elegans* pseudo-females mated with three different strains of *plg-1*+ males (represented by the different shapes). Each point represents the mean area of plug from six measurements (three technical replicate measures from each of two independent observers). The mean and standard error for females mated with each male genotype are shown in purple. **B)** Plug area measured on mated *C. remanei* females from three different strains (represented by the different shapes). Again, each point represents the mean area of plug from six measurements (three technical replicate measures from each of two independent observers). The mean and standard error for females mated with each male genotype are shown in purple.

**Figure S1.** Phylogenetic relationships between *C. elegans* strains. Tip color corresponds to the computationally inferred *plg-1* genotype, where *plg-1*+ strains are purple, *plg-1*– strains are green, and ambiguous genotypes are gray. The phylogeny was generated in the CaeNDR 20250625 data release.

**Figure S2.** Representative images of scoring second male sperm presence using MitoTracker Red CMXRos. **A)** A schematic diagram of a female nematode with the spermathecae, uterus, and mating plug labeled. The colored dots represent dyed sperm cells. **B)** A representative female with second male sperm in the spermathecae (S), uterus (U), and mating plug (P). From left to right the images were taking using DIC brightfield light and red fluorescence using a Nikon Ti2 inverted microscope (20× DIC microscope objective, 150 ms fluorescence exposure time). The merged image is shown on the right. This female was mated first with a JU346 (*plg-1*+) male and second with another JU346 male. **C)** Representative images of the factorial crossing design between ED3017 (*plg-1*+) and MY920 (*plg-1*–). The merged brightfield and fluorescence image is shown.

## Supplemental Files

**File S1. Strain data.** The complete list of strains analyzed in this study along with their *plg-1* genotype.

**File S2: Inferred genotype for 1,953 *C. elegans* strains.** The strain, inferred *plg-1* genotype, inferred number of *Cer1* elements along with their location, *plg-1* coverage, *Cer1* coverage, and orphan reads are given.

**File S3: Genome-wide nucleotide diversity statistics.** The chromosome, window start (in bp), window end (in bp), number of variants, nucleotide diversity, and *plg-1* genotype are given.

**File S4: Chromosome III nucleotide diversity statistics.** The chromosome, window start (in bp), window end (in bp), number of variants, nucleotide diversity, and *plg-1* genotype are given.

**File S5: Hermaphrodite total fertility.** The total reproductive success for hermaphrodites from 11 *plg-1*+ strains and 6 *plg-1*– strains.

**File S6: Pseudo-female fecundity.** The reproductive success of *C. elegans* pseudo-females mated with either a *plg-1*+ male or a *plg-1*– male for 24 hours.

**File S7: Sperm signaling in *C. elegans*.** The frequency of observed sperm transferred from a second male during sequential factorial crosses in three *C. elegans* sister strain pairs.

**File S8: Sperm signaling in *C. remanei*.** The frequency of observed sperm transferred from a second male during sequential crosses in three *C. remanei* strains.

**File S9: Plug area post-mating.** The change in plug area 24-, 48-, and 72-hours post-mating in three *C. elegans* strains and three *C. remanei* strains.