

A newly evolved gene is essential for efficient sperm entry into eggs in *Drosophila melanogaster*

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Abstract

New genes arise through a variety of evolutionary processes and provide raw material for adaptation in the face of both natural and sexual selection. *De novo* evolved genes emerge from previously non-protein-coding DNA sequences, and many such genes are expressed in male reproductive structures. In *Drosophila melanogaster*, several putative *de novo* genes have evolved essential roles in spermatogenesis, but whether such genes can also impact sperm function beyond the male has not been investigated. We identified a putative *de novo* gene, *katherine johnson* (*kj*), that is required for high levels of male fertility. Males that do not express *kj* produce and transfer sperm that are stored normally in females, but sperm from these males enter eggs with severely reduced efficiency. Using a tagged transgenic rescue construct, we observed that KJ protein localizes to the nuclear periphery in various stages of spermatogenesis, but is not detectable in mature sperm. These data suggest that *kj* exerts an effect on sperm development, the loss of which results in reduced fertilization ability. While previous bioinformatic analyses suggested the *kj* gene was restricted to the *melanogaster* group of *Drosophila*, we identified putative orthologs with conserved synteny, male-biased expression, and predicted protein features across the genus, as well as instances of gene loss in some lineages. Thus, *kj* potentially arose in the *Drosophila* common ancestor and subsequently evolved an essential role in *D. melanogaster*. Our results demonstrate a new aspect of male reproduction that has been shaped by new gene evolution and provide a molecular foothold for further investigating the mechanism of sperm entry into eggs in *Drosophila*.

Article Summary

How fruit fly sperm enter eggs is poorly understood. Here, we identify a gene that potentially arose from non-protein-coding DNA and is required for efficient fertilization. Sperm from males lacking this gene's function cannot enter eggs. The gene appears to act during sperm production, rather than in mature sperm. This study illustrates how newly evolved genes can affect important aspects of reproduction and provides insights into sperm-egg interactions.

Introduction

From gamete shape to the mechanisms of chromatin packaging, reproductive traits and the genes that contribute to them often evolve rapidly between closely related species (Lüpold and Pitnick 2018; Chang et al. 2023). At the genetic level, this rapid evolution can take many forms: the adaptive evolution of protein-coding (Wilburn and Swanson 2016) or regulatory (VanKuren and Long 2018) sequences; relaxed selection on such genes due to their sex-limited expression patterns (Dapper and Wade 2020); gene duplication events followed by divergence that give rise to genes with more specialized reproductive roles (Schroeder et al. 2020); and the origination of new genes through non-duplication processes, such as horizontal gene transfer and *de novo* gene evolution (Blondel et al. 2020).

De novo evolved genes arise from mutations to previously non-protein-coding regions of the genome. This type of new gene origination was initially considered implausible but is now known to play important roles in a variety of biological processes (Van Oss and Carvunis 2019; Zhao et al. 2024). Recent examples include a gene (*GSE9*) that affects rice grain shape in *Oryza sativa* (Chen et al. 2023), a novel microprotein that became expressed specifically in heart cells of humans and chimpanzees (Vakirlis et al. 2022), anti-freeze glycoproteins in several fish lineages (Baalsrud et al. 2018; Rives et al. 2024), and an adaptively evolving transmembrane protein whose overexpression increases yeast colony size (Vakirlis et al. 2020). Notably, what constitutes evidence of a *de novo* gene's function varies across studies (Keeling et al. 2019), and the experiments that are feasible to interrogate function depend on the organism in question: some studies consider transcription and translation in a specific cell type to be evidence of function, while others use genetic approaches to reveal a specific phenotypic change when gene expression is altered.

Since many *de novo* genes are expressed specifically in reproductive cells and organs, there has been considerable interest in investigating how these genes affect reproductive traits (Levine et al. 2006; Begun et al. 2007; Knowles and McLysaght 2009; Ruiz-Orera et al. 2015; Xie et al. 2019; Zhang et al. 2019; Chen et al. 2023). Some studies focus on identifying recent new mutations that give rise to expressed, protein-coding sequences. In *Drosophila*, for example, these studies have shown that *de novo* genes have rapid rates of birth and death (Palmieri et al. 2014), are more likely to be expressed at specific stages of gametogenesis (Witt et al. 2019), are often polymorphic within a population (Zhao et al. 2014; Cridland et al. 2022), and can first become expressed in female reproductive organs (Lombardo et al. 2023). Such research has suggested a model in which genetic changes at the early stages of *de novo* gene emergence are selected not primarily for their effects on the novel gene itself, but rather for their effects on the expression of established genes in the same genomic region (Lee et al. 2024). It has been challenging, however, to identify specific functional roles for these youngest genes, perhaps because they are first expressed in reproductive cells that are already well functioning. Thus, the novel genes are unlikely to initially have non-redundant roles. Other research has examined the functions of putative *de novo* genes that are somewhat older. Because of limitations in sequence-based methods for detecting remote homology, it can be harder to definitively determine the *de novo* status of such genes (Weisman et al. 2020), though newer

methods based on whole-genome alignments show promise (Peng and Zhao 2024). However, putative *de novo* genes may be fruitful targets for functional investigation because their longer retention in host genomes makes them more likely to have evolved essential functions.

We recently performed genetic screens of putative *de novo* genes with testis-enriched expression in *Drosophila melanogaster* to investigate whether any had become essential for male fertility (Gubala et al. 2017; Rivard et al. 2021). We found three genes, *saturn*, *goddard* and *atlas*, that have each evolved roles in different steps in spermatogenesis. For example, Goddard protein localizes to developing sperm axonemes and is required for proper spermatid individualization (Lange et al. 2021), while Atlas localizes to spermatid nuclei and appears to transiently bind DNA during the process of nuclear condensation (Rivard et al. 2021). While these genes vary in their rates of protein-coding sequence evolution, each is well-conserved within the *melanogaster* group of *Drosophila* species. Orthologs of each gene are also found in more divergent *Drosophila* species, but each gene has been lost in at least one lineage, including in species for which sequence- and synteny-based homology detection should be robust. This pattern suggests that the essential functions performed by these putatively newly evolved genes in *D. melanogaster* might have evolved slowly over several millions of years.

These three putative *de novo* genes have evolved important functions in making sperm, a process that is well characterized in various animal systems (White-Cooper and Bausek 2010). What happens after sperm leave the male, but before development begins, is an active area of study, about which less is collectively known. Upon transfer to females, sperm must navigate through the reproductive tract to reach specialized site(s) at which they can be stored (Wolfner et al. 2023). In mammals, sperm storage typically involves binding to specialized regions of the oviduct epithelium (Suarez 2008), while in insects, specialized sperm storage organs are used (Pitnick et al. 1999). Stored sperm must then be released at a rate appropriate to fertilize oocytes when the latter are ovulated (Bloch Qazi et al. 2003; Manier et al. 2010). Upon release, sperm must find the egg and then fertilize it. In many taxa, including mammals and marine invertebrates, initial interactions between sperm and egg include the sperm's acrosome reaction (Okabe 2016), which facilitates the fusion of the sperm and egg plasma membranes and allows the contents of the sperm nucleus to enter the egg (Deneke and Pauli 2021; Elofsson et al. 2024). In *Drosophila* and some fish species, however, the whole sperm cell enters the egg through a small hole called the micropyle (Horne-Badovinac 2020). How *Drosophila* sperm locate the micropyle is unknown, as is the mechanism through which the sperm cell passes through the egg plasma membrane. The identification of a fly mutant in which sperm were unable to enter eggs (Perotti et al. 2001) suggested the possibility that specific gene products could be responsible for either of these steps, but that fly line is no longer available, and its affected gene was never identified molecularly. After a fly sperm enters an egg, the sperm plasma membrane breaks down, releasing a lysosome (the former acrosome), the nucleus, and centrioles. This membrane breakdown is mediated by a sperm transmembrane protein, sneaky, and is required for the subsequent unpackaging of the paternal genome (Fitch and Wakimoto 1998; Wilson et al. 2006). After the paternal genome is released, additional male- and female-derived proteins are required for proper chromatin decondensation and use (Loppin et al. 2001; Loppin, Bonnefoy, et al. 2005; Sakai et al. 2009; Tirmarche et al. 2016; Yamaki et al.

2016; Dubruille et al. 2023); mutations in the genes encoding these proteins lead to paternal- or maternal-effect lethality, respectively. Although *Drosophila* genetics has enabled the identification of many of these components (Loppin et al. 2015), our understanding of the processes between spermatogenesis and the onset of development remains incomplete.

Here, we report our initial functional and evolutionary characterization of a putative *de novo* evolved gene that we have named *katherine johnson* (*kj*). Knockdown or knockout of *kj* results in a severe reduction in male fertility in *D. melanogaster*. Knockout males produce sperm that are stored at normal levels in females' seminal receptacles, but the sperm enter eggs at much reduced rates. Because the encoded protein is detectable in various stages of spermatogenesis, but not in mature sperm, we suggest that *kj* exerts its effect during sperm development, and that in its absence, the ability of sperm to fertilize eggs is significantly impaired. Bioinformatic analyses initially detected *kj* only in the *melanogaster* group of flies (Heames et al. 2020; Peng and Zhao 2024). Across these species, it has maintained a male-biased pattern of expression but shown an elevated rate of sequence evolution. However, by analyzing gene synteny, expression patterns, and predicted protein features, we identified putative orthologs in outgroup *Drosophila* species, as well as evidence for gene loss in at least two lineages. Thus, *kj* provides both a striking example of a newly evolved gene becoming essential for male fitness and a potential foothold from which to further our understanding of the events surrounding *Drosophila* fertilization.

Methods

Drosophila stocks and experiments

Please see the Reagents Table for a full list of fly strains used in this study. Unless otherwise noted, *in vivo* experiments in *Drosophila* were performed at 25°C using standard molasses media consisting of agar (6.5 g/L), brewers yeast (23.5 g/L), cornmeal (60 g/L), molasses (60 mL/L), acid mix (4 mL/L; propionic and phosphoric acids), and tegosept (0.13%; antifungal agent).

Genetic ablation of CG43167

We first constructed a TRiP-style RNAi line (Ni et al. 2011) targeting *CG43167* expression and used RT-PCR to assess the degree of knockdown. The oligos used for creating the pValium20 plasmid and for RT-PCR are provided in Fig. S1. Fertility of small groups of knockdown and control male flies was assessed as previously described (Rivard et al. 2021).

We used the co-CRISPR method as previously described (Ge et al. 2016; Lange et al. 2021; Rivard et al. 2021) to engineer a complete deletion of *CG43167*. Guide RNA sequences used to target the gene and PCR primers used to verify the deletion are provided in Fig. S2. Flies carrying a deletion allele (Δkj) were crossed into the w^{1118} background and balanced over CyO. We generated trans-heterozygotes with no functional copies of *kj* using Bloomington Stock Center deficiency line #9717, with genotype $w^{1118}; Df(2L)BSC243/CyO$.

Unless otherwise stated, heterozygous control flies used in experiments were generated by crossing the Δkj line to w^{1118} ; we refer to these controls as $\Delta kj/+$.

*Cloning and transformation of tagged *kj* rescue constructs*

C-terminally tagged *kj:HA* rescue construct and N-terminally tagged *HA:kj* rescue constructs were generated using Gibson Assembly (Gibson et al. 2009). The *kj* coding sequence and putative upstream and downstream regulatory sequences were amplified from *Canton S* genomic DNA (prepared using Genra Puregene Cell Kit, Qiagen) using Q5 High Fidelity Polymerase (NEB). Primers used for making all constructs are listed in the Reagents Table. The 3x-HA tag was similarly amplified using pTWH plasmids (T. Murphy, *Drosophila* Genomics Resource Center plasmids 1100 and 1076). Amplified DNA fragments were then assembled into a XbaI/AscI-linearized w^+ attB plasmid (a gift of Jeff Sekelsky, Addgene plasmid 30326). Assembled constructs were integrated into the attP docking site of PBac{y⁺-attP-3B}VK00037 (Bloomington *Drosophila* Stock Center stock #24872) using PhiC31 integrase (Rainbow Transgenics).

Fertility assays

Male fertility of *kj* nulls, flies carrying rescue constructs, and controls was assessed using matings between single unmated males of each genotype and single unmated Canton S

females. Males and females were collected and isolated for a period of 72-96 hours prior to mating. During this period, females were reared in yeasted vials to encourage egg production. Each pair mating was then allowed to proceed for 72 hours before the parents were removed from the vial. Fertility was determined by counting pupal cases on the side of vials 10 days after the initial crossing. Twenty matings were set up for each male genotype; vials with any dead parents or atypical bacterial growth at the end of the mating period were excluded from analysis.

Sperm counts

We crossed the *Mst35Bb*-GFP (“protamine-GFP”) marker of mature sperm nuclei (Manier et al. 2010) into the *kj* null background and used it to quantify levels of sperm in the seminal vesicles of sexually mature, unmated males (3-5 days old), in the bursae of females 30 minutes after the start of mating (ASM), in the female seminal receptacle 2 hours ASM, and in the female seminal receptacle 4 days ASM. Matings, dissections, imaging and counting were performed as previously described (Gubala et al. 2017). Experimenters were blinded to the male genotype while counting sperm. Two-sample *t*-tests with unequal variances were used to compare sperm levels.

Egg-production and egg-to-pupae viability assay

We measured the amount of egg-laying, the rate at which eggs developed into pupae, and the total progeny production of Canton S females mated singly to either a *kj* null male or a heterozygous control ($\Delta kj/+$) using standard assays largely as previously described (Ravi Ram and Wolfner 2007; LaFlamme et al. 2012; Findlay et al. 2014). However, because the effects of *kj* knockout were large and consistent across days, we modified these procedures by: measuring egg-laying over four days (with one vial per female per day) instead of 10; analyzing pooled data across all four days of the assay (after observing that each individual day showed the same pattern); and using two-sample *t*-tests with unequal variances to compare knockout and control genotypes for each set of pooled data.

Sperm entry into eggs and early embryonic development

We recombined the *Dj*-GFP sperm tail marker (Santel et al. 1997) into the Δkj null background. For experiments examining sperm entry and early development, fly strains were maintained on yeast-glucose-agar media (Hu et al. 2020).

Embryo collections

All embryo collections were performed at room temperature. For each embryo collection cage, approximately 30 2-7 day-old males were mated overnight to approximately 40 3-6 day-old Canton S females. Embryos were collected on grape juice agar plates (2.15% agar, 49% grape juice, 0.5% propionic acid solution (86.3% acid/water mix)) with yeast paste smeared on top. To assess embryo development, plates with embryos were collected after approximately 18 hours. For *Dj*-GFP detection, embryos were pre-collected for 1 hour to allow flies to lay any retained eggs. Then, fresh grape juice plates with yeast paste were replaced in 1 hour intervals.

Sperm tail detection using Dj-GFP

Embryos from 1 hour collection plates were immediately dechorionated by treating with 50% bleach for 2 minutes. Embryos were then washed thoroughly with egg wash buffer (0.4% NaCl, 0.03% Triton-X100) and transferred to a 22x60mm coverslip prepared with a thin strip of heptane glue (stabilizes embryos lined up in a row to prevent double counting). Excess egg wash buffer was added to the slide to prevent embryo dehydration. Embryos were then imaged live on an Echo Revolve at 10X magnification to determine the proportion with detectable Dj-GFP sperm tails. For display purposes, some embryos were also fixed and imaged with confocal microscopy as described below. To ensure mating occurred, females from embryo collection cages were dissected and reproductive tracts were imaged to confirm presence of Dj-GFP sperm in the storage organs.

Embryo development assay

Embryos collected overnight were dechorionated with 50% bleach for 2 minutes and washed thoroughly with egg wash buffer. Embryos were then fixed for 20 minutes at room temperature in 1:1 mixture of 4% paraformaldehyde in 1X PBS and heptane. Embryos were devitellinized in a 1:1 mixture of heptane and methanol by shaking vigorously for 30 seconds. Embryos were then washed three times in both pure methanol followed by 1X PBS-T (0.1% Triton-X100). To detect nuclei, embryos were stained for 20 minutes at room temperature with 10mM Hoechst 33342 diluted 1:1000 and then washed thrice with 1X PBS-T. Embryos were then mounted on 22x22mm coverslips in Aqua Polymount. Embryos were imaged on a Zeiss LSM710 confocal microscope. Images were captured using either EC-Plan Neofluar 10x/0.45 Air or Plan-Apochromat 63x/1.4 oil objectives.

Cytology of KJ subcellular localization

We performed whole testis staining as described in Lange et al. (2021). See the Reagents Table for details on primary and secondary antibodies. Analysis of KJ expression in isolated cysts was performed as described in Rivard et al. (2021). Labeled samples were imaged using a TCS SP8 X confocal microscope (Leica Microsystems). Images were captured using HC PL APO CS2 20x/0.75 ILL and HC PL APO CS2 63x/1.40 oil objectives. Post-acquisition processing was performed using ImageJ Fiji (version 1.0).

Sperm nuclei decondensation assay

Nuclear decondensation was performed using a modified protocol described by Tirmarche et al. (2016). Isolated male flies were aged for 10 to 14 days prior to dissection to allow for an accumulation of sperm in seminal vesicles. Seminal vesicles were dissected on 0.01% poly-L-lysine treated slides and then pierced to release their sperm content. Sperm nuclei were subsequently decondensed by pretreating sperm with 1X PBS (phosphate buffered saline) supplemented with 1% Triton X-100 for 30 minutes prior to subjecting sperm to decondensation buffer (10 mM DTT and 500 ug/mL heparin sodium salt in 1X PBS). Following treatment, slides

were labeled with anti-HA antibodies using the immunohistochemistry protocol described (Rivard et al. 2021).

Molecular evolutionary analyses

We extracted the *kj* protein-coding DNA sequence and predicted amino acid sequence for *D. melanogaster* from FlyBase (Öztürk-Çolak et al. 2024). We used the protein as a query in iterative PSI-BLAST searches, which identified annotated orthologs across the *melanogaster* group of *Drosophila*. Because these orthologs varied in the quality of their annotations, we manually checked all orthologs for which genome browsers and RNA-seq data were available through the Genomics Education Partnership (thegep.org). Briefly, we BLASTed the predicted protein sequence of each PSI-BLAST hit against the corresponding species' genome assembly, then manually examined that species' genome in the GEP's implementation of the UCSC Genome Browser (Rele et al. 2022). This allowed us to visualize adult male and adult female RNA-seq reads (Brown et al. 2014; Chen et al. 2014) that mapped to the region so that we could assess expression patterns. To search for orthologs outside of the *melanogaster* group, we examined the syntenic region in outgroup species (Rivard et al. 2021; Rele et al. 2022) as demarcated by three conserved genes with conserved positions relative to each other and to *kj*: *CG6614*, *CG4983* and *Vha100-5*. Any unannotated location in the syntenic region that showed adult male expression by RNA-seq was examined for potential open reading frames, and potential proteins so identified were compared to *D. melanogaster* (and other) KJ orthologs and to the full *D. melanogaster* proteome by BLASTP. We examined the predicted membrane topology of potential orthologs with DeepTMHMM (Hallgren et al. 2022). Finally, potential orthologs found in non-*melanogaster* group species were compared by BLASTP to other *Drosophila* orthologs and by BLASTP and PSI-BLAST to all known proteins in GenBank.

We examined the molecular evolution of *kj* protein-coding sequences from the *melanogaster* group as described previously (Rivard et al. 2021). In addition to those PAML-based tests of positive selection, we implemented HyPhy-based tests for recurrent (Kosakovsky Pond and Frost 2005) and episodic (Murrell et al. 2015; Wisotsky et al. 2020) positive selection as implemented in the Datamonkey 2.0 web server (Weaver et al. 2018). The sequence alignment used in these analyses was checked for recombination using GARD (Kosakovsky Pond et al. 2006), but none was detected.

Results

CG43167 is required for full male fertility

CG43167 was identified as a potential *de novo* evolved gene in two previous bioinformatic analyses (Heames et al. 2020; Peng and Zhao 2024) and shows a highly testis-biased pattern of expression (Vedelek et al. 2018). We found that expression of a short hairpin targeting CG43167 using the *Bam-GAL4*, *UAS-Dicer2* driver had a marked effect on male fertility. Crude fertility assays in which seven knockdown or control males were mated with five unmated wild-type (Canton S) females for 2 days showed knockdown male fertility to be only 7-19% the level of controls. RT-PCR analysis of cDNA synthesized from controls and knockdown males showed virtually no detectable expression in knockdown males, suggesting that the transgenic line efficiently targets CG43167 transcripts (Fig. S1). Consistent with our previous rocket-themed nomenclature for testis-expressed, putative *de novo* genes (Gubala et al. 2017; Rivard et al. 2021), we named the CG43167 gene *katherine johnson* (*kj*), after the NASA mathematician who calculated rocket orbital mechanics for the Mercury and subsequent crewed missions (Shetterly 2016).

To confirm these data and to generate a null allele for genetic analysis, we engineered a deletion of the *kj/CG43167* gene region using CRISPR/Cas9. The resulting deletion allele (Δkj) eliminated the entirety of the protein-coding and untranslated regions and thus most likely constitutes a functional null (Fig. S2). Single pair fertility assays, in which either single control males (w^{1118}) or single Δkj homozygous null males were individually mated to single, wildtype, unmated females, revealed that Δkj null males have a fertility defect of a similar magnitude to that observed in the RNAi assay (Fig. 1). To rule out the effects of off-target mutations generated during CRISPR/Cas9 genome editing, we assessed the fertility of heterozygous males carrying a single copy of the Δkj allele in trans with *Df(2L)BSC243* (henceforth abbreviated as "*Df*"), a large genomic deficiency that uncovers several genes including the *kj* locus. In single pair fertility assays, $\Delta kj/Df$ trans-heterozygous males showed a fertility defect equivalent to Δkj null males, indicating that the severe loss-of-function phenotype in Δkj homozygotes reflects a full loss of *kj* function (Fig. 1). To further characterize the Δkj allele, we determined the fertility of male flies carrying only one copy of the Δkj allele. Removing a single copy of the *kj* gene had no effect on male fertility, ruling out dominance by haploinsufficiency (Fig. 1). Altogether, these experiments show that the Δkj allele acts as a recessive null allele.

We confirmed that the fertility defects associated with Δkj are due to loss of the *kj/CG43167* gene by complementing the loss of function phenotype with genomic rescue constructs. We integrated the 5.4-kb *kj* locus, which contained the 583 bp CG43167 transcript-encoding sequence along with putative upstream and downstream regulatory regions. No other annotated genes are present in this stretch of DNA. Two different constructs were produced for this analysis, differing in either the N-terminal or C-terminal location of an introduced hemagglutinin (3xHA) tag. Reintroducing either construct into $\Delta kj/Df$ males restored fertility (Fig. 1). However, the degree of rescue with the C-terminally (*kj*:HA) tagged construct was weaker than that of the N-terminally tagged construct (HA:*kj*), which showed full fertility restoration (Fig. 1). Thus, for

the remainder of the study, we focused on the N-terminally-tagged rescue construct. Collectively, these data indicate that the *kj* gene has an essential function in *Drosophila melanogaster* male fertility.

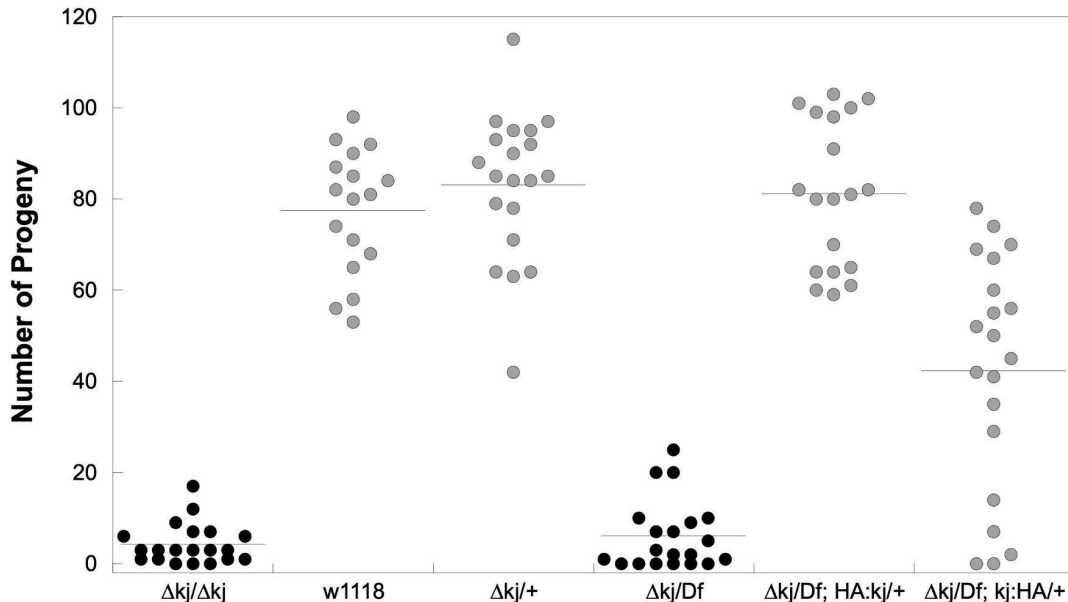


Figure 1. The *katherine johnson* gene (*CG43167*) is required for maximal male fertility in *D. melanogaster*. Males homozygous for a complete deletion allele (Δkj) had significantly lower fertility than w^{1118} controls and $\Delta kj/+$ heterozygotes (both $p < 10^{-13}$). $\Delta kj/+$ heterozygotes had no significant fertility difference from w^{1118} ($p = 0.26$), indicating the Δkj allele is fully recessive. Trans-heterozygote males ($\Delta kj/Df$) with no functional copies of *kj* showed no significant difference in fertility relative to Δkj homozygotes ($p = 0.37$). Fertility of $\Delta kj/Df$ heterozygotes was significantly increased upon addition of either of two tagged rescue constructs, HA:*kj* ($p < 10^{-15}$) or *kj*:HA ($p < 10^{-5}$). The N-terminally tagged construct had significantly higher fertility than the C-terminally tagged construct ($p < 10^{-5}$) and showed no significant fertility difference from w^{1118} controls ($p = 0.46$). Progeny number was counted as the number of pupal cases produced by females mated to males of a specific genotype. All p -values are from two-tailed t -tests with unequal variances. Horizontal lines show means. Samples sizes were $n = 17-20$ per genotype.

kj null males produce, transfer and store sperm normally, but the sperm enter eggs inefficiently

We next examined gross testis morphology in *kj* null males. To our surprise, dissected male reproductive tracts appeared equivalent to tracts from controls (Fig. S3). Furthermore, sperm with apparently normal morphology were present in the seminal vesicles (SV) of both control and mutant tracts, suggesting that spermatogenesis can proceed to completion in the absence of *kj*. We used the *Mst35Bb*-GFP sperm head marker (Manier et al. 2010) to quantify sperm present in SVs of sexually mature, unmated males. We found a slight decrease in the number of sperm per SV (null males mean \pm SEM: 1667.5 ± 81.0 , heterozygous control: 1927.2 ± 96.2 , two-sample t -test with unequal variances $p = 0.049$). While statistically significant, this difference

was not of the same magnitude as the observed fertility difference (Fig. 1), and therefore cannot account for the observed fertility defects in Δkj males.

In addition to producing mature sperm, *D. melanogaster* males must also transfer sperm into females and generate functional sperm that can swim to female storage organs (Manier et al. 2010). We assessed sperm transfer by counting sperm in the female bursa 30 minutes after the start of mating (ASM), and observed the opposite pattern, a slight but significant increase in sperm transferred by *kj* null males (null: 1436.0 ± 120.3 , control 1082.3 ± 76.0 , *t*-test $p = 0.021$). Again, this difference was not of a comparable magnitude to the null fertility defect, nor was it in the expected direction. Thus, while *kj* null males may exhibit minor differences from controls in sperm production and sperm transfer to females, neither is likely to be the primary cause of the *kj* null fertility defect.

Since *D. melanogaster* sperm must enter specialized sperm storage organs before they can be used for fertilization, we next quantified sperm levels in the female's primary storage organ, the seminal receptacle (SR), at two timepoints. The level of sperm in the SR at 2 hrs ASM indicates the ability of sperm to enter storage, while sperm levels at 4 days ASM provide a readout of sperm persistence in storage and the rate of sperm release during the initial days after mating. Females mated to *kj* null males showed no significant differences in the levels of stored sperm at either time point (Fig. 2). Thus, sperm from *kj* null males migrate to and enter the SR normally and appear to be released from the SR at a comparable rate to sperm from heterozygous controls.

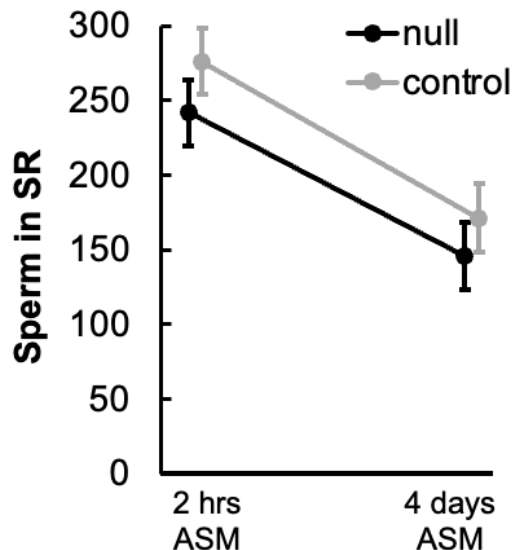


Figure 2. Loss of *kj* has no effect on sperm entry into or maintenance in the female seminal receptacle. Females mated to *kj* null males or heterozygous controls ($\Delta kj/+$) showed no significant differences in the number of sperm stored in the seminal receptacle (SR) at either 2 hours or 4 days after the start of mating (ASM). Error bars indicate one standard error of the mean. At each timepoint, the two groups were compared with a two-sample *t*-test with unequal variances: 2 hrs ASM, $p = 0.29$; 4 days ASM, $p = 0.43$. Sample sizes for each group ranged from $n = 11-14$.

We next assessed the rates of egg laying and egg-to-pupal viability in females mated singly to either *kj* null or control males. In the four days following mating, females mated to *kj* null males laid a slightly, but not statistically significantly, lower number of eggs compared to females mated to controls (Fig. 3A). However, a much lower percentage of these eggs hatched (i.e., developed to pupae) (Fig. 3B), and accordingly, mates of *kj* nulls produced lower levels of progeny (Fig. 3C). Taken together with the sperm storage data (Fig. 2), these results suggest that the *kj* null fertility defect arises within a narrow, but critical, window of time between the release of sperm from storage and the onset of development.

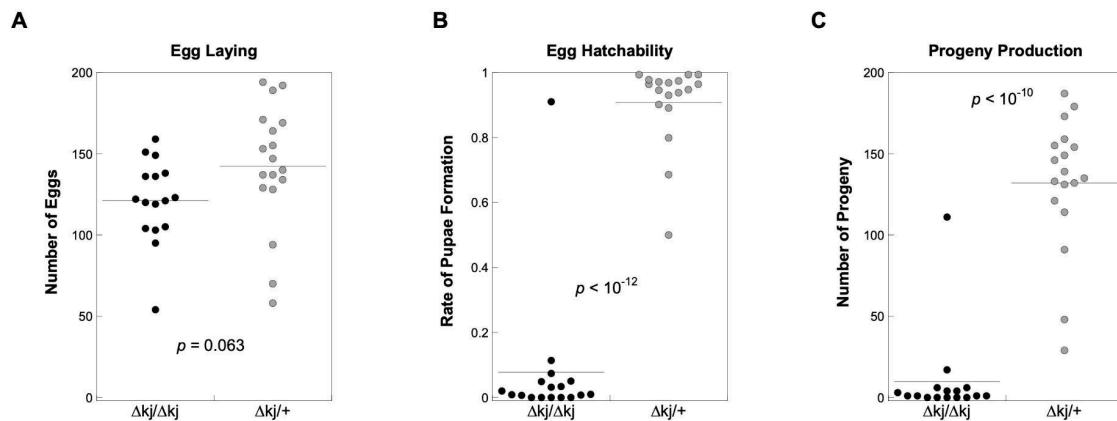


Figure 3. The fertility defect of *kj* null males results from an egg hatching defect. A) Egg-laying over a four-day assay by females mated to *kj* null males or heterozygous ($\Delta kj/+$) controls. The groups showed no significant difference. B) The proportion of eggs from panel (A) that developed to pupae. Eggs laid by mates of *kj* null males had a significantly lower hatching rate. C) Progeny production for females mated to *kj* null males is correspondingly lower. The single high outlier for the *kj* null genotype in panels B and C might have resulted from the use of a mis-identified $\Delta kj/+$ heterozygous male in the *kj* null group. In each panel, the two genotypes were compared by two-sample *t*-tests with unequal variances, with *p*-values given in the graphs.

As Δkj males produced sperm that can be maintained in storage and do not hamper egg laying in females, we reasoned that the *kj* fertility defect may be due to either an inability of mutant sperm to enter eggs (Perotti et al. 2001) or a defect in a step immediately following sperm entry. Sperm with defects in the latter process fall into the category of paternal effect lethals and reflect aberrations in post-fertilizations events, such as failures in sperm plasma membrane breakdown (Wilson et al. 2006) or in the proper decondensation or initial use of the paternal chromatin inside the embryo (Loppin, Lepetit, et al. 2005; Dubruille et al. 2023).

To distinguish these possibilities, we crossed the *don juan*-GFP (*Dj*-GFP) marker (Santel et al. 1997) into the *kj* null background. This marker labels mature sperm tails and allows for the visualization of sperm entry into eggs. Canton S (wild-type) females were mated to either $\Delta kj/CyO$ or $\Delta kj/\Delta kj$ males expressing *Dj*-GFP and allowed to lay eggs on grape juice plates in one-hour intervals. Eggs were then immediately dechorionated and imaged live by epifluorescence to assess sperm presence in the anterior end of the embryo (for examples of

embryos with and without sperm, see fixed confocal images in Fig. 4A-B; example epifluorescence images used for quantification are in Fig. S4). While nearly 80% of embryos laid by females mated to heterozygous males had detectable sperm tails, *Dj*-GFP was detected in only 0.74% of embryos laid by females mated to *kj* null males (Fig. 4A-C). This significant decrease in sperm entry rate was consistent with the magnitude of the fertility differences observed above (Fig. 1, Fig. 3C), so we concluded that the inability of sperm to enter eggs efficiently is the major factor driving the *kj* null subfertility phenotype.

To evaluate the possibility of an additional defect in embryos successfully fertilized by $\Delta kj/\Delta kj$ sperm, mated females were allowed to lay eggs onto grape juice plates for an 18-hour overnight period. Embryos were then collected and stained for DNA to allow us to assess embryonic development. Over 97% of embryos laid by females mated to control males developed normally, with a mix of developing stages up to Stage 16 present as expected (Fig. 4D, F; exact stages not quantified) (Foe et al. 1993). However, embryos laid by females mated to $\Delta kj/\Delta kj$ males showed normally developing embryos only 11.6% of the time (Fig. 4E, magenta arrowhead, Fig. 4F), with similar stages present as controls. The remaining 88.4% of embryos were devoid of DNA staining and appeared to have deteriorated (Fig. 4E, cyan arrowhead), consistent with the embryos being successfully laid and activated, but not fertilized (Horner and Wolfner 2008). These experiments indicate that the few eggs that are successfully fertilized by sperm from $\Delta kj/\Delta kj$ males can progress normally through embryogenesis, consistent with the outcomes of our fertility assays. Thus, *kj* expression in the male germline appears not to affect development (i.e., *kj* is not a paternal effect gene), and the *kj* null fertility defect occurs between the time of sperm exit from storage and entry into eggs.

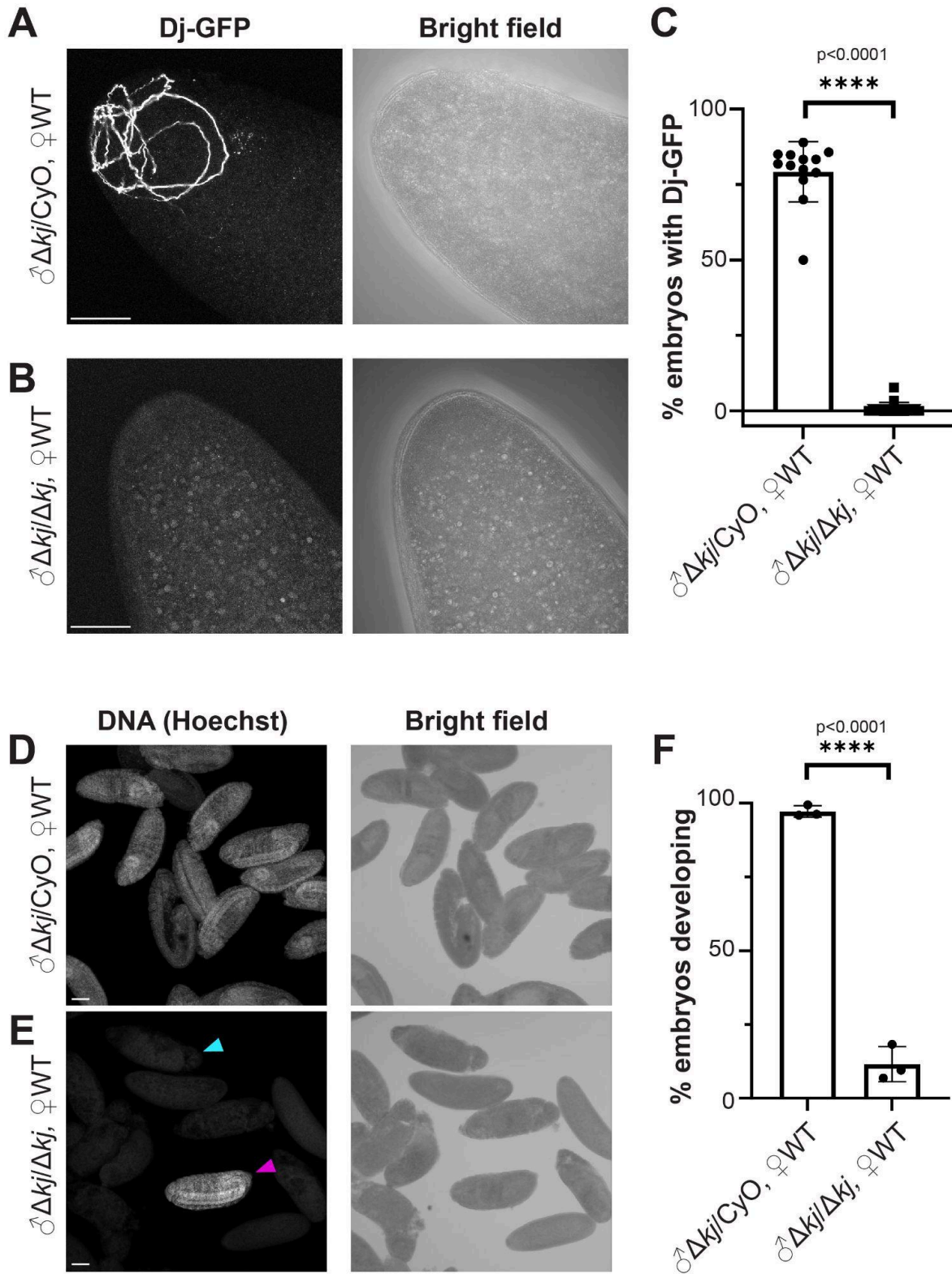


Figure 4: Sperm from *kj* null males fertilize eggs inefficiently. A-C) Max projection confocal images of fixed <1 hour old embryos laid by Canton S (WT) females mated to either $\Delta kj/\text{CyO}$ controls or $\Delta kj/\Delta kj$

males expressing *Dj*-GFP (scale bars = 50 μ m). A) *Dj*-GFP sperm from $\Delta kj/CyO$ flies were frequently detected in the anterior of <1 hour old WT embryos. B) *Dj*-GFP sperm from $\Delta kj/\Delta kj$ flies were rarely detected in the anterior of <1 hour old WT embryos. C) Quantification of A,B. Embryos fathered by $\Delta kj/CyO$ flies are positive for *Dj*-GFP 79.2% of the time (n=212 embryos), compared to 0.7% when fathered by $\Delta kj/\Delta kj$ flies (n=275 embryos). D-F) Max projection confocal images of fixed, Hoechst-stained embryos collected overnight from WT females mated to either $\Delta kj/CyO$ or $\Delta kj/\Delta kj$ males (scale bars = 100 μ m). D) Embryos fertilized by $\Delta kj/CyO$ males develop normally and reach up to Stage 16 of embryonic development during the collection period. E) When fertilized by $\Delta kj/\Delta kj$ males, embryos appear to develop normally (magenta arrowhead). Unfertilized embryos deteriorate during the collection period (cyan arrowhead). F) Quantification of D,E. Embryos from females mated to $\Delta kj/CyO$ males appear to develop normally 97.3% of the time (n=504 embryos), compared to 11.6% of the time when mated to $\Delta kj/\Delta kj$ males (n=544 embryos). ****p<0.0001, unpaired t-test, two-tailed. At least three biological replicates were performed for each experiment.

KJ protein localizes to the nuclear periphery during spermatogenesis

To investigate potential KJ protein functions, we used our rescue constructs in a *kj* null background to examine the expression pattern and subcellular localization of HA-tagged KJ proteins within male reproductive tracts. Since N-terminally tagged HA:KJ produced complete rescue (Fig. 1), we focused on its localization. Although *kj* mutants show no major defect in sperm production, we detected HA:KJ in the testes at specific stages of spermatogenesis (Fig. 5A). In spermatocytes (pre-meiotic cells), HA:KJ was enriched around the nuclear periphery and was found diffusely in the cytoplasm (Fig. 5B). HA:KJ was also present in post-meiotic spermatids. In these cells, bundled nuclei synchronously proceed through a stepwise condensation process that ultimately produces the thin sperm heads found in mature sperm (Rathke et al. 2014). Round and canoe shaped nuclear bundles reflect elongating stages of spermiogenesis, while needle shaped nuclei, with their fully condensed chromosomes, characterize spermatids undergoing individualization. Analysis of spermatid cysts revealed that HA:KJ localizes transiently to the nuclear periphery during the canoe stages before disappearing at the onset of individualization (Fig. 5C). HA:KJ showed an asymmetric localization in these cells, with enrichment along one long edge of each nucleus. This pattern is reminiscent of proteins that localize the dense body, a structure that develops during elongation and disappears from the nucleus at the onset of individualization (Fabian and Brill 2012; Li et al. 2023). Consistent with the disappearance of HA:KJ from nuclei at individualization, anti-HA staining of mature sperm isolated from SVs did not detect HA:KJ around the nucleus (Fig. 5D). To confirm that the lack of HA:KJ around mature sperm nuclei was not due to the protein becoming inaccessible to our antibody due to the extreme condensation of these nuclei (Eren-Ghiani et al. 2015; Kaur et al. 2022), we performed the same staining after decondensing mature sperm nuclei *in vitro*, but again did not detect HA:KJ (Fig. 5D). Finally, we examined the localization of C-terminally tagged KJ:HA (Fig. S5). This protein showed a similar localization to the nuclear periphery in spermatocytes but was not detectable in post-meiotic cells. This could reflect the potential cleavage or inaccessibility of the HA tag in later stages of spermatogenesis or lower overall levels of expression from this transgene, consistent with its reduced ability to rescue the null mutant. Overall, these data suggest that KJ plays a role in sperm development that affects later sperm function in females.

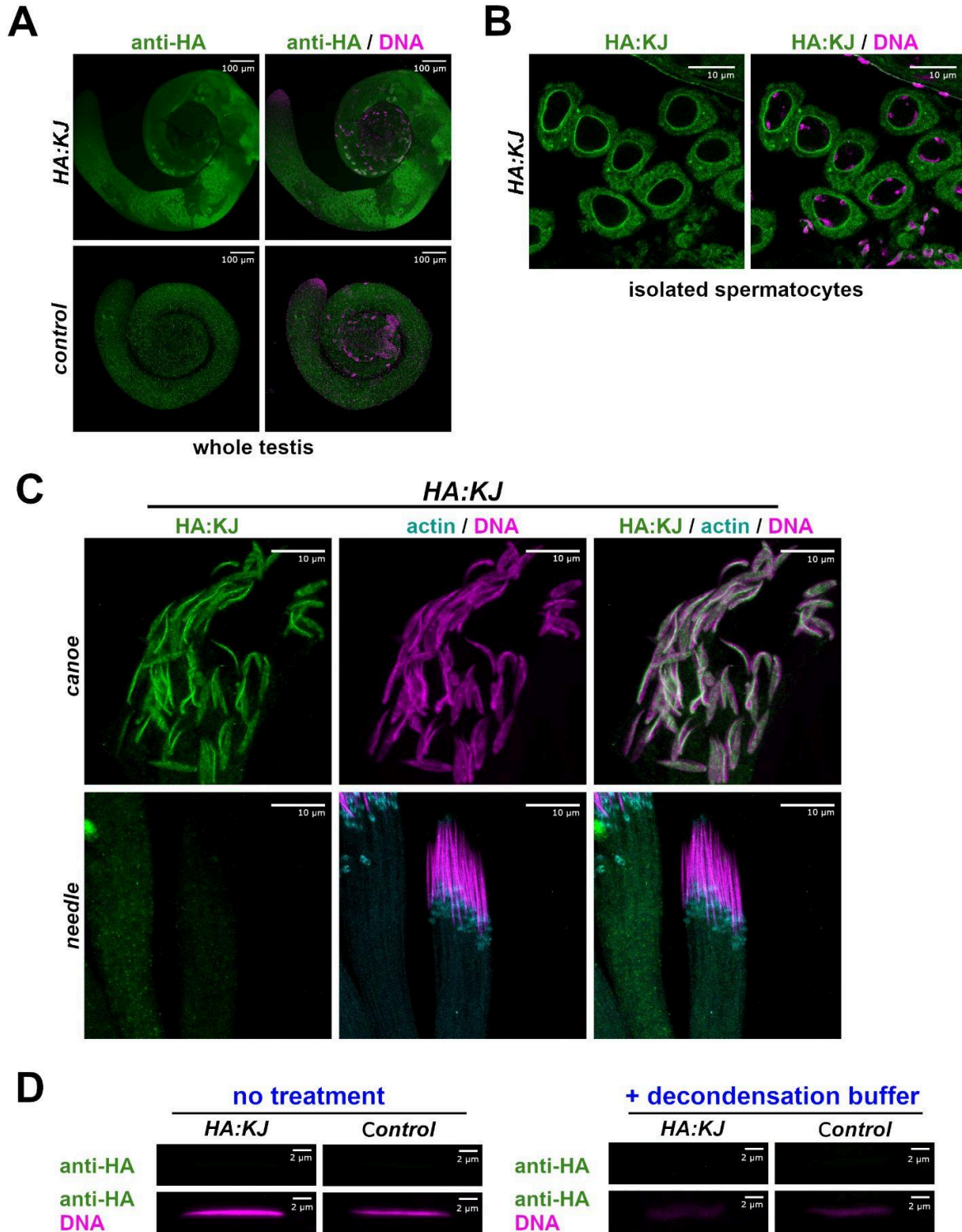


Figure 5. KJ associates with nuclei in both spermatocytes and spermatids but is undetectable in mature sperm A) In whole mount testes, HA:KJ (full genotype: $\Delta kj/\Delta k$; $HA:KJ/+$) is enriched in spermatocytes and condensing spermatid nuclei. A low level of background is present w¹¹¹⁸ control testes

labeled with anti-HA. B) In labeled isolated spermatocytes cysts, HA:KJ has a diffuse localization throughout the cytoplasm but is enriched at the nuclear periphery and at large punctate structures of unknown identity. C) In canoe-stage spermatid nuclei, HA:KJ is localized to condensing nuclei, with an enrichment on one side of each nucleus reminiscent of dense bodies. By the needle stage of condensation, marked by the presence of actin-rich investment cones at the base of nuclei, HA:KJ is no longer detectable at nuclei but instead shows a faint distribution throughout the sperm tail cytoplasm. D) Staining of mature sperm isolated from seminal vesicles shows no detectable HA:KJ around sperm nuclei. HA:KJ was also undetectable on sperm treated with a buffer to decondense the nuclei. Control sperm are from *w¹¹¹⁸* males.

Predicted biochemical properties of KJ protein

The *D. melanogaster kj* gene is located on chromosome 2L (Muller element B), and its single exon is predicted to encode a 126-amino acid protein of predicted molecular weight 15 kDa and a predicted isoelectric point of 8.7. DeepTMHMM (Hallgren et al. 2022) predicts the protein to have one transmembrane domain spanning residues 21-36, with the N-terminus predicted to be outside the membrane and the C-terminus predicted to be inside. AlphaFold (Jumper et al. 2021) predicts the protein to have two alpha helices: one spanning residues 1-60, and another spanning residues 82-106; the other regions are predicted disordered (Fig. 6A). Of these predicted helices, the portion of the longer helix corresponding with the DeepTMHMM-predicted transmembrane domain is predicted at the “very confident” level (pLDDT > 90), while the other helical portions are predicted at the “confident” (70 > pLDDT > 90) level.

Molecular evolution of *kj* in *melanogaster* group species

Because of its lack of identifiable homologs outside of *Drosophila* and lack of identifiable protein domains, the *kj* gene and its encoded protein were characterized as putatively *de novo* evolved in a previous bioinformatic analysis (Heames et al. 2020). Further support for the gene's *de novo* status came from a comprehensive investigation of *de novo* genes in *D. melanogaster*, which used a whole-genome alignment approach to assess the age of each gene (Peng and Zhao 2024). That analysis determined that *kj* was restricted to the *melanogaster* group of the *Drosophila* genus (Fig. 6B). Consistent with the gene's putative *de novo* status and the previous results, our BLASTP and iterative PSI-BLAST searches showed no detectable homology to any other protein. PSI-BLAST (and subsequent manual annotation of hits) identified 22 additional full-length orthologs throughout the *melanogaster* group, but not outside of it (Table S1, File S1). We identified partially annotated ortholog fragments in four additional species. Another species, *D. eugracilis*, initially appeared to have a pseudogenized copy of *kj* due to a 1-nucleotide insertion in the ORF, but upon manual inspection we found that this nucleotide was not present in RNA-seq reads that mapped to this location and thus likely represented an error in the reference genome. Based on TimeTree estimates (Kumar et al. 2022), these results would suggest the gene arose ~25-30 million years ago in the common ancestor of this group. RNA-seq data (Brown et al. 2014; Chen et al. 2014) were available through the Genomics Education Partnership for 16 of the 24 species with putatively functional, full-length orthologs. All 16 of these orthologs are expressed in adult males, and nearly all in a

male-specific or heavily male-biased pattern (Table S1). Thus, *kj* may function in male reproduction across the *melanogaster* group.

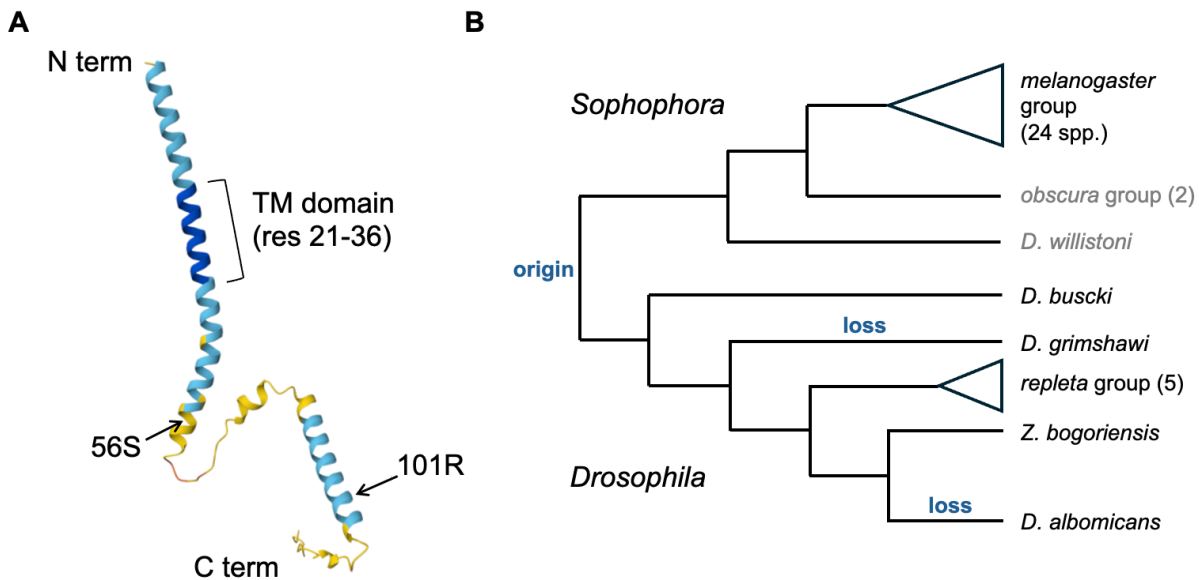


Figure 6. Predicted protein structure and molecular evolution of KJ in *Drosophila*. A)

AlphaFold-predicted structure of the 126-residue *D. melanogaster* KJ protein. The position of the predicted transmembrane (TM) domain and residues identified by FEL to have experienced recurrent positive selection (arrows) are indicated. Color indicates the degree of model confidence (dark blue: very high confidence, pLDDT > 90; light blue: high confidence, 90 > pLDDT > 70; yellow: low confidence, 70 > pLDDT > 50). B) The hypothesized origin, phylogenetic distribution and gene loss events for *kj* in genus *Drosophila*. Orthologs of *kj* were detected in both subgenera, *Sophophora* and *Drosophila*, but not outside of genus *Drosophila*, implying that *kj* arose at the base of the genus. Two independent gene loss events likely occurred in the lineages leading to *D. grimshawi* and *D. albomicans*. Gray text indicates some uncertainty about the orthologs identified in the *obscura* group and *D. willistoni*. For clarity, some species are collapsed into groups; the number of species from the group for which full-length orthologs were detected is shown in parentheses. Branch lengths are not to scale; tree topology shows species relationships inferred by Suvorov et al. (2022).

Genes that mediate reproduction often evolve at elevated rates (Wilburn and Swanson 2016), as do *de novo* evolved genes (Van Oss and Carvunis 2019; Zhao et al. 2024). We therefore used an alignment of 22 *melanogaster* group orthologs (Table S1; File S3) to examine the molecular evolution of the *kj* protein-coding sequence and to ask whether any KJ residues had experienced recurrent adaptive evolution. PAML model M0 (Yang et al. 2000) estimated the overall d_N/d_S ratio across the whole gene as 0.42. When similar whole-gene d_N/d_S estimates were calculated genome-wide for six representative species of the *melanogaster* group (*Drosophila* 12 Genomes Consortium 2007; Chang et al. 2023), a value of 0.42 fell into the top 1-2%, suggesting that *kj* evolves more rapidly than most *D. melanogaster* genes.

When we asked whether specific residues of the KJ protein had experienced adaptive evolution, the results were ambiguous. The PAML sites test (Yang et al. 2000) compares the likelihood of

a model of molecular evolution (M7) that allows only purifying and neutral evolution to a model (M8) that additionally allows a subset of sites to evolve adaptively with $d_N/d_S > 1$. This test found no difference in likelihood between the models ($\chi^2 = 0$, 2 df, $p = 1.00$) and thus found no evidence of recurrent, adaptive evolution on any KJ residue. An analogous method to detect this type of recurrent selection, the Fixed Effects Likelihood (FEL) analysis in the DataMonkey suite of programs (Kosakovsky Pond and Frost 2005), identified three positions (each with $p < 0.1$) in the alignment as having significant evidence for recurrent, adaptive evolution: positions that aligned to residues 56S and 101R in the *D. melanogaster* protein (Fig. 6A), as well as residues from other species that aligned (less reliably, in our judgment) to a gap between *D. melanogaster* residues 15A and 16F. The BUSTED-HM algorithm (Murrell et al. 2015; Wisotsky et al. 2020) found no significant evidence for episodic (as opposed to recurrent) positive selection on specific residues. Thus, we conclude that *kj* evolves rapidly, but with only limited evidence for recurrent adaptive evolution on a few of its sites. Its high overall rate of evolution may instead be due to relaxed constraint (Dapper and Wade 2020) on at least some portions of the protein, as has been observed for a high fraction of fly seminal proteins (Patlar et al. 2021), despite its essential function. Consistent with these results, Peng and Zhao (2024) determined for a different set of *melanogaster* group species that most non-synonymous substitutions in KJ were non-adaptive.

Identification of potential *kj* orthologs outside of the *melanogaster* group

Strong evidence for *kj* orthologs in the *Drosophila* subgenus. Our previous studies of putative *de novo* genes (Gubala et al. 2017; Rivard et al. 2021) have sometimes identified more distantly related orthologs that were not detectable by BLAST and/or not previously annotated as genes. To investigate the possibility of such orthologs for *kj*, we queried *Drosophila* genomes outside of the *melanogaster* group using TBLASTN with relaxed parameters (e-value threshold < 10, word size = 3). Any hits from these searches were evaluated for their genomic location, their expression pattern based on available RNA-seq data, and whether the inferred potential protein showed homology to *D. melanogaster* KJ. This process identified a potential *kj* ortholog in a *repleta* group species of subgenus *Drosophila*, *D. virilis* (Fig. S6). The initial TBLASTN search identified a 75-nt stretch in this species predicted to encode 25 amino acids with 52% identity (72% similarity) to a region of *D. melanogaster* KJ, producing an e-value of 8.3. This hit's position in the *D. virilis* genome is syntenic to the position of *kj* in *D. melanogaster* because it is flanked by three of the same genes that surround *kj* in *D. melanogaster* (orthologs of CG6614 and CG4983 upstream, and the ortholog of *Vha100-5* downstream). The region identified by TBLASTN exists within a potential open reading frame (ORF) that could encode 171 amino acids. The genomic region encoding this ORF showed signals of expression in RNA-seq data from both sexes of adult *D. virilis*. The maximum read depth was 43-fold higher in males, consistent with a gene that functions in male reproduction. A pairwise BLASTP comparison of the full *D. virilis* ORF to *D. melanogaster* KJ produced a significant e-value of 10^{-7} , and DeepTMHMM predicted a single transmembrane domain with the same orientation with respect to the membrane (N terminus outside, C terminus inside) as *D. melanogaster* KJ. A small, duplicated amino acid motif in the C terminus of the putative *D. virilis* ortholog contributes to this ortholog's longer length (Fig S6).

The presence of a likely *kj* ortholog in the *repleta* group of the *Drosophila* subgenus implied that the origin of the *kj* gene could be earlier than the previously estimated 25-30 million years ago. To determine the phylogenetic distribution of *kj* across the genus, we used a combination of BLASTP, TBLASTN and synteny to search for additional orthologs in a variety of species and groups (Fig. 6B). These methods identified proteins of similar length and the same DeepTMHMM-predicted topology in other *repleta* group species (*D. novamexicana*, *D. hydei*, *D. mojavensis*, *D. arizonae*), *D. busckii* and *Zaprionus bogoriensis* (File S2). (*Zaprionus* is a genus within the paraphyletic *Drosophila* genus; see Fig. 6B.) The syntenic region of *D. navojoa* contained a much shorter ORF (60 a.a.) with male-specific expression and sequence identity to these orthologs, but the predicted protein did not contain a transmembrane domain, so this region may represent a pseudogene or a gene with altered function. Table S2 lists the genomic locations and biochemical properties of the likely orthologs outside of the *melanogaster* group.

Somewhat strong evidence for kj orthologs in the obscura group. Since we detected *kj* orthologs in both the *Sophophora* and *Drosophila* subgenera, we wondered whether *kj* was present in the *obscura* group, a part of the *Sophophora* subgenus distinct from the *melanogaster* group (Fig. 6B). Using *D. pseudoobscura* and *D. subobscura* as representative species, we identified in their syntenic regions ORFs supported as male-expressed by RNA-seq data that could encode proteins of similar length to *D. melanogaster* KJ (Table S2). These ORFs were predicted by DeepTMHMM to have a single transmembrane domain in the same approximate position as the KJ orthologs described above, though the predicted topology (N terminus inside the membrane, C terminus outside) was inverted. The predicted proteins showed significant identity to each other across their full lengths. Pairwise BLASTP homology to the above-detected KJ orthologs was marginal. The *D. pseudoobscura* ORF, for example, matched three orthologs from the *melanogaster* (*D. erecta*, *D. setifemur*) and *repleta* groups (*D. arizonae*) with $0.01 < e < 0.05$, and sixteen other orthologs with $e < 5$. Most of these matches corresponded to the predicted transmembrane domain. The data were similar for *D. subobscura*: its predicted ORF hit nine other KJ orthologs with e values ranging from 0.003 to 0.53, with most regions of homology falling in the predicted transmembrane domain. Thus, the evidence for orthologs in the *obscura* group is somewhat strong but not definitive.

Marginal evidence for a kj ortholog in D. willistoni. We identified a potential *kj* ortholog in *D. willistoni*, a *Sophophora* subgenus species that is an outgroup to both the *melanogaster* and *obscura* groups, by examining regions with male gonad RNA-seq expression data within the syntenic region (Fig. S7). One such region showed the potential to encode a protein of 138 amino acids, with one predicted transmembrane domain (though only a single residue, the first methionine of the polypeptide, is predicted to be outside the membrane). The full-length ORF had marginal BLASTP similarity to potential KJ orthologs from the *obscura* group (e -values between 0.5 and 1). Thus, *D. willistoni* may have a *kj* ortholog, but the evidence is ambiguous.

Somewhat strong evidence for loss or absence of kj in D. grimshawi and D. albomicans. Two remaining *Drosophila* subgenus species for which good RNA-seq and genome browser data were available were *D. grimshawi* and *D. albomicans*. TBLASTN searches of the orthologs

above against the whole genomes of either species did not produce any meaningful hits, so we focused on the syntenic region. For *D. grimshawi* (Fig. S8), the *D. virilis* ortholog produced a reasonably strong TBLASTN hit within the syntenic region ($e < 10^{-6}$ across a 65-residue region of homology toward the C-terminus of the protein). However, this region had stop codons immediately upstream of it in all three reading frames, and RNA-seq coverage was spotty and at a much lower level than we observed for better-supported orthologs. Thus, we find no evidence of a functional *kj* in the *D. grimshawi* syntenic region; instead, the evidence may be consistent with a somewhat recent pseudogenization event.

For *D. albomicans*, we identified three regions with male-specific/biased expression in the syntenic region (Fig. S8). None were predicted to encode an ORF of >65 amino acids, and none of the potential ORFs had predicted transmembrane domains. When we used TBLASTN to query the entire syntenic region (150,000 bp) for regions of potential homology to any *Drosophila* subgenus KJ ortholog (Table S2), only the *D. virilis* ortholog had faint homology ($e = 0.69$) to a single, 20-codon region of *D. albomicans*. However, this region had no evidence of being transcribed and contained a stop codon in the relevant reading frame just upstream of the potential homologous region. We suspect this BLAST hit is spurious, but if it did represent true homology, the other evidence would suggest pseudogenization. Thus, we conclude there is no detectable *kj* ortholog in the *D. albomicans* syntenic region.

Conclusions about kj age and phylogenetic distribution. Collectively, these phylogenetic data suggest that *kj* likely originated at the base of the *Drosophila* genus, estimated by TimeTree to be ~43 million years ago (Kumar et al. 2022). Our evolutionary analyses suggest the gene has been fairly well conserved but was likely lost at least twice (Fig. 6B). The clearest evidence for loss is along the lineages leading to *D. grimshawi* and *D. albomicans*. The gene could also have been lost along the lineages leading to *D. willistoni* and the *obscura* subgroup, but given the difficulty of detecting short, rapidly evolving, potential *de novo* genes (Weisman et al. 2020), we favor the hypothesis that these taxa have highly diverged, putative orthologs based on the evidence outlined above. Within the *melanogaster* group, however, the gene and its male-specific expression pattern are well conserved. This pattern of a potential origin at the base of the *Drosophila* genus, followed by independent loss events outside of the *melanogaster* group, is similar to the evolutionary patterns of other putative *de novo* genes with essential functions in *D. melanogaster* (Gubala et al. 2017; Rivard et al. 2021). However, because of the even higher degrees of sequence divergence and the breakdown of synteny outside of the genus, it is also possible that *kj* is present in related Dipteran species but not detectable by available methods. Regardless of the exact mechanism and timing of *kj*'s origin, however, the KJ protein is novel in that it has no detectable resemblance to other annotated *Drosophila* proteins and has evolved a function required for *D. melanogaster* fertilization.

Discussion

While initial studies of *de novo* evolved genes focused on their identification across taxa and timescales, recently more progress has been made in determining their functions (Zhao et al. 2024). Three other putative *de novo* genes are essential for making sperm in *D. melanogaster* (Gubala et al. 2017; Lange et al. 2021; Rivard et al. 2021), but *kj* is the first such gene discovered to affect sperm function around the time of fertilization. *kj* thus showcases a different way in which newly evolved genes have integrated into, and evolved critical roles within, the complex process of producing functional sperm. Indeed, this pattern is mirrored by new genes that evolved through other mechanisms (Loppin, Lepetit, et al. 2005; Ding et al. 2010; VanKuren and Long 2018; Stromberg et al. 2023; Najera et al. 2024).

Relatively little is known about the molecules required for sperm-egg interactions in *Drosophila* (Loppin et al. 2015). Mutations in several other genes result in normal sperm production and transfer, but low hatchability, as we observe for *kj*. However, the cellular causes of their fertility defects are distinct. Mutants in genes like *wasted* and *Nep4* cause abnormal sperm storage or release, resulting in lower rates of fertilization (Ohsako and Yamamoto 2011; Ohsako et al. 2021), but sperm from *kj* nulls appear to be stored and released normally (Fig. 2). Other paternal effect mutants cause abnormalities in processes such as sperm membrane dissolution (Fitch and Wakimoto 1998; Wilson et al. 2006) or paternal chromatin unpacking or reorganization (Loppin, Lepetit, et al. 2005; Dubruille et al. 2023), but sperm from these mutant males are proficient at egg entry, unlike sperm from *kj* nulls (Fig. 4). Thus, *kj* is the only extant and molecularly characterized gene that distinctly affects sperm entry into eggs.

One other gene, *casanova* (*csn*), had been reported to have a mutant phenotype similar to what we find for *kj*: *csn* mutant males produce and transfer motile, morphologically normal sperm that are stored properly, but are unable to fertilize eggs (Perotti et al. 2001). Unfortunately, *csn* mutants are no longer available, and the molecular nature of the gene is unknown. It is clear that *csn* is distinct from *kj*, since they map to different chromosomal positions (*kj* is at cytological region 34F4 on chromosome arm 2L; *csn* was mapped to cytological region 95E8-F7 on chromosome arm 3R). It has been proposed that sperm interact with and/or cleave β -N-acetylglucosamine and α -mannose sugars that are present on the egg at the site of sperm entry but are no longer detected after fertilization (reviewed by Loppin et al. 2015). Sperm plasma membranes have been reported to contain glycosidic enzymes that cleave these sugars (Cattaneo et al. 2002; Intra et al. 2006), and sperm β -N-acetylglucosaminidase activity is reduced in *csn* mutants (Perotti et al. 2001). Our data suggest *KJ* is not involved in such carbohydrate interactions between egg and sperm, as it is not detected by immunofluorescence on mature sperm in seminal vesicles (Fig. 5D). *KJ* was also not detected in the mature sperm proteome determined by mass spectrometry (Garlovsky et al. 2022). While we recognize the limitations to negative results with both of these detection methods, the lack of any sequence similarity of *KJ* to any glycolytic enzyme supports our view that *KJ* is unlikely to participate directly in sperm-egg carbohydrate interactions.

The localization pattern of HA:KJ in the testes (Fig. 5) suggests other possible roles for KJ in spermatogenesis. In spermatocytes, KJ is enriched around the entire periphery of the nucleus. In spermatids, however, KJ enrichment becomes skewed toward one side of the elongating nuclei. Interestingly, KJ's localization pattern in spermatids resembles the distribution of proteins that localize to the dense body, a structure that forms through close physical interactions between nuclear membrane proteins, microtubules, and actin-based structures (Fabian and Brill 2012). Mutations in genes that alter dense body formation cause defects in nuclear shaping at late stages of spermatogenesis (Kracklauer et al. 2010; Augière et al. 2019; Li et al. 2023), although some of these genes also have functions before and after nuclear shaping. The localization of KJ in spermatids might thus suggest a role in nuclear and sperm head shaping. In this case, although we do not see gross changes in sperm nucleus/head shape in the absence of *kj*, its loss might cause subtle abnormalities in these regions that make it more difficult for sperm to enter the micropyle, the size of which coevolves with the diameter of insect sperm (Soulsbury and Iossa 2024). Alternatively, it is possible that *kj* mediates some other aspect of sperm head organization (such as ensuring correct protein placement on sperm heads) or acts in another process required to prepare sperm for efficient egg entry or to release sperm from storage in a way that facilitates their interaction with the egg.

Similar to Peng and Zhao (2024), we found that the *kj* gene is well conserved in the *melanogaster* group. We also observed that these orthologs show strongly male-biased expression. This pattern is consistent with the hypothesis that *kj* may play an important role in male reproduction across species in this clade and, thus, that it might have already evolved its essential function in the common ancestor of this group. However, the *kj* protein-coding sequence has evolved considerably faster than most genes do in this group of species, with limited evidence of recurrent adaptation. This pattern could indicate that only some regions of the KJ protein are important for its essential function (while others evolve under relaxed constraint) and/or that the protein's essential function arose in a more recent ancestor of *D. melanogaster*.

kj was initially identified as a putative *de novo* evolved gene because of the lack of detectable orthologs outside of *Drosophila* and the lack of identifiable protein domains (Heames et al. 2020). A sophisticated analysis using whole-genome alignments similarly concluded that *kj* was unique to *melanogaster* group species (Peng and Zhao 2024). Since our approach to ortholog detection was tailored to the *kj* gene, we were able to use features specific to *kj* (such as synteny, expression pattern and predicted protein features) and a relaxed threshold for initial BLAST searches to identify potential *kj* orthologs beyond the *melanogaster* group. This gene-specific approach would not have been feasible for the previous genome-scale studies. Our results highlight the utility of considering gene-specific parameters when searching for orthologs of putative *de novo* genes. We also found at least two likely instances of independent gene loss events (Fig. 6B). This pattern implies that *kj* was probably present at the origin of the *Drosophila* genus but was dispensable in some lineages. This phylogenetic distribution is similar to those observed for two other putative *de novo* genes with essential functions in *D. melanogaster*, *saturn* and *atlas* (Gubala et al. 2017; Rivard et al. 2021), which are also well conserved in the *melanogaster* group and detectable in only some outgroup species. Our

general hypothesis for this pattern is that these genes could have had slight, positive effects on fertility in the most ancient ancestors of the *Drosophila* genus before evolving more essential, non-redundant roles in the lineage leading to the *melanogaster* group. It is also possible that larger-scale changes to the process of spermatogenesis in specific lineages could have rendered once-beneficial genes superfluous. Indeed, several instances of major, lineage-specific changes in spermatogenesis are known, such as the evolution of three types of sperm, only one of which is fertilization competent, in *D. pseudoobscura* and related species (Alpern et al. 2019), and the evolution of new sex chromosomes, which can affect processes such as the regulation of sex-linked genes in germline cells (Wei et al. 2024) and sex chromosome meiotic drive (Chang et al. 2023).

While we identified likely *kj* orthologs across *Drosophila* species, neither BLAST, PSI-BLAST nor HMMER (hmmer.org) could detect homologs outside the genus. We thus conclude, tentatively, that *kj* could have arisen *de novo* in the *Drosophila* common ancestor. However, because sequence-based detection of rapidly evolving proteins outside of the genus is difficult (Weisman et al. 2020), and synteny breaks down as well, we are unable to identify a specific non-coding sequence in an outgroup species from which *kj* arose. Thus, we consider *kj* a putative *de novo* gene (McLysaght and Hurst 2016), or an orphan gene in the parlance of Zhao et al. (2024). It is possible that more sensitive sequence- or structure-based methods will at some point identify a *kj* ortholog outside of *Drosophila*. Even if such an ortholog is detected, however, a gene that is required for efficient fertilization and that has evolved within *Drosophila* to the point that it is currently unrecognizable in outgroup species would remain of considerable functional and evolutionary interest. This study demonstrates how focusing on the functions of putative *de novo* genes can provide a foothold to understanding the largely mysterious process of sperm entry into eggs in *D. melanogaster*. As genome editing becomes easier to perform in non-model species (Bubnell et al. 2022), it should also be possible to test whether and how *kj* is required for male fertility in other *Drosophila* species.

Data Availability Statement

Fly strains are available on request. Files S1 and S2 contain the inferred protein sequences of predicted KJ orthologs. File S3 contains the DNA sequence alignment used in the molecular evolutionary analyses. File S4 contains the phylogenetic tree used for PAML analysis. File S5 contains the raw data underlying the graphs and statistical analyses in Figures 1-4. Other supporting information is provided in either the supplemental figures and tables or in the Reagents Table.

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List of Supplemental Material

Figure S1: **RNAi reagents and assessment of knockdown.** A) Oligos to create a hairpin siRNA to be cloned into pValium20, which created a siRNA targeting the *CG43167* gene. B) RT-PCR of cDNA from whole males that were either knocked down for *kj* (*Bam*-GAL4, *UAS-Dicer2* > *UAS:CG43167* RNAi) or control (*Bam*-GAL4, *UAS-Dicer2*) shows that *kj* transcript levels are essentially undetectable in knockdown males. C) RT-PCR of *RpL32* is shown as a positive control for successful cDNA synthesis. D) RT-PCR primers for detecting *kj* expression.

Figure S2: **CRISPR reagents and confirmation of *kj* deletion allele.** A) Chromosomal coordinates of guide RNA (gRNA) target sites flanking *CG43167*. Coordinates of the region that was ultimately deleted in the Δkj allele are shown. The deletion was initially detected by PCR screening using the primers indicated. B) UCSC genome browser graphic showing the BLAT result when the primers were used to amplify and sequence genomic DNA from Δkj homozygous males. The entire *kj/CG43167* gene is deleted.

Figure S3: **Gross testis morphology of *kj* null males appears normal under phase contrast microscopy.** A) Testis and seminal vesicle from a *kj+/kj+* male. B) Testis and seminal vesicle from a $\Delta kj/\Delta kj$ male.

Figure S4: **Epifluorescence images used to quantify presence of Dj-GFP in embryos.** Representative epifluorescence images used to quantify presence of Dj-GFP in Fig. 4C. Cyan arrows indicate sperm marked by dj-GFP. Scale bar = 100 μ m.

Figure S5: **Localization of KJ:HA in whole mount testes and spermatocytes.** A) Whole mount testis from males expressing KJ:HA in the *kj* null background. B) KJ:HA is enriched around spermatocyte nuclei. Unlike HA:KJ, however, KJ:HA is not detected around condensing spermatid nuclei.

Figure S6: **Evidence for a *kj* ortholog in *Drosophila virilis*.** A) In *D. melanogaster*, the *kj* gene (*CG43167*) is flanked by three genes that are well conserved in other *Drosophila* species: *CG6614*, *CG4983* and *Vha100-5*. B) The syntenic region of the *D. virilis* genome contains orthologs of these same three genes. The orange box indicates the stretch within the syntenic region that had faint TBLASTN homology to *D. melanogaster* KJ. C) TBLASTN hit details and RNA-seq evidence of male-biased expression at the putative orthologous region of *D. virilis*. D) The full-length ORF surrounding the *D. virilis* TBLASTN hit (original peptide matching *D. melanogaster* KJ is in orange; two potential start codons are in green). The full-length *D. virilis* ORF has significant identity to *D. melanogaster* KJ in a pairwise BLASTP comparison. This comparison shows a small region of potential duplication toward the end of the *D. virilis* ORF. The pairwise BLASTP gave a significant e-value while the genome-wide TBLASTN did not because the latter considered a much larger set of possible subject sequences. E) DeepTMHMM predictions of transmembrane domains and protein topology for KJ in *D. melanogaster* (left) and the putative *D. virilis* ortholog (right).

Figure S7: **Evidence for a possible *kj* ortholog in *Drosophila willistoni*.** A) Unannotated regions of the *kj* syntenic region that showed RNA-seq expression in adult males were evaluated to determine whether they could encode a *kj* ortholog. Most expressed regions were ruled out for reasons listed at bottom left. Region B, however, encoded an ORF with a predicted transmembrane domain that showed marginal BLASTP similarity to *kj* orthologs from the *obscura* group. B-C) Details of RNA-seq expression, transmembrane prediction and BLASTP results of the possible ortholog.

Figure S8: **Evidence for *kj* gene absence in *Drosophila albomicans* and *Drosophila grimshawi*.** A) The *kj* syntenic region of *D. albomicans* contains three unannotated regions (A-C) with RNA-seq evidence of male expression. None of these regions is likely to encode a *kj*-like ORF for reasons listed at the bottom left. B) One stretch of the *D. albomicans* syntenic region had very faint TBLASTN homology to *D. virilis* KJ, but the region has no extended ORF and no evidence for adult expression. C) In *D. grimshawi*, TBLASTN identified one stretch of the *kj* syntenic region that has homology to *D. virilis* KJ. There is very low, spotty coverage of this region from adult male RNA-seq data, but no extended ORFs that would include the TBLASTN hit. These data may be consistent with *kj* pseudogenization in *D. grimshawi*.

Table S1: Coordinates, protein lengths, predicted protein topologies, and expression data of putative *kj* orthologs in *melanogaster* group species.

Table S2: Coordinates, protein lengths, predicted protein topologies, and expression data of putative *kj* orthologs outside of the *melanogaster* group.

File S1: Protein sequences of *kj* orthologs from *melanogaster* group *Drosophila* species.

File S2: Protein sequences from putative *kj* orthologs from non-*melanogaster* group *Drosophila* species.

File S3: Alignment of protein-coding DNA sequences of *kj* orthologs from *melanogaster* group *Drosophila* species.

File S4: Phylogenetic tree of protein-coding DNA sequences of *kj* orthologs from *melanogaster* group *Drosophila* species.

File S5: Data underlying the statistics and graphs in Figures 1-4.

Reagents Table

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