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Regulation of male fertility and accessory gland gene expression by the *Drosophila* HR39 nuclear receptor

Sophia A. Praggastis [a,1], Hyuck-Jin Nam [a], Geanette Lam [a], Myron B. Child VI [b], Dean M. Castillo [b], Carl S. Thummel [a,*]

[a] Department of Human Genetics, University of Utah School of Medicine, 15 North 2030 East, Rm 5100, Salt Lake City, UT, 84112, USA
[b] School of Biological Sciences, University of Utah, 257 South 1400 East, Rm. 201, Salt Lake City, UT, 84112, USA

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ABSTRACT

Successful reproduction is dependent on the transfer of male seminal proteins to females upon mating. These proteins arise from secretory tissues in the male reproductive tract, including the prostate and seminal vesicles in mammals and the accessory gland in insects. Although detailed functional studies have provided important insights into the mechanisms by which accessory gland proteins support reproduction, much less is known about the molecular mechanisms that regulate their expression within this tissue. Here we show that the *Drosophila* HR39 nuclear receptor is required for the proper expression of most genes that encode male accessory gland proteins. Consistent with this role, *HR39* mutant males are infertile. In addition, tissue-specific RNAi and genetic rescue experiments indicate that *HR39* acts within the accessory glands to regulate gene expression and male fertility. These results provide new directions for characterizing the mammalian orthologs of HR39, the SF-1 and LRH-1 nuclear receptors, both of which are required for glandular secretions and reproduction. In addition, our studies provide a molecular mechanism to explain how the accessory glands can maintain the abundant levels of seminal fluid production required to support fertility.

1. Introduction

A critical facet of insect reproduction is the dramatic reprogramming of female physiology and behavior following mating (Chapman, 2001; Gillott, 2003; Wolfner, 1997). This transition is initiated by the transfer of seminal proteins from males to females, providing a key link between mating and reproductive success. Seminal proteins originate from the accessory glands, which are located along the male ejaculatory duct between the testes and the ejaculatory bulb (Wolfner, 1997). This tissue produces at least 292 seminal proteins in *Drosophila*, including a large number of proteases and protease inhibitors as well as proteins involved in innate immunity, redox, and lipid metabolism (Wigby et al., 2020). These seminal proteins have dramatic effects on female reproductive physiology, including inducing ovulation, stimulating egg production, facilitating sperm storage and utilization, and suppressing remating – all critical steps in ensuring efficient reproduction of the species (Wolfner, 1997, 2009). Several accessory gland proteins have been characterized in detail, including those encoded by *ovulin* (*Acp26Aa*) and *sex peptide* (*SP*). Ovulin is synthesized as a prohormone that is processed after mating and required for efficient egg production (Heifetz et al., 2005; Herndon and Wolfner, 1995; Park and Wolfner, 1995). Sex peptide also regulates egg production in females (Chapman et al., 2003; Liu and Kubli, 2003). Unlike ovulin, however, sex peptide binds to sperm and its continued presence in females after mating induces more long-term changes including mating receptivity, feeding, and sperm storage (Misra and Wolfner, 2020; Peng et al., 2005; Singh et al., 2018). Several accessory gland proteins are required for sex peptide to bind sperm and exert its effects, however, the mechanisms involved in this regulation remain to be defined (Singh et al., 2018). Functional studies of this tight interplay between males and females have provided critical insights into the mechanisms of insect reproduction, with implications for our understanding of transmissible diseases and crop protection. These studies also provide far-ranging insights into the basic biology of reproduction and a framework for understanding the mechanisms of sperm competition and the evolutionary forces that shape the survival of a species.

Several transcription factors play critical roles in accessory gland

* Corresponding author.
E-mail address: cthummel@genetics.utah.edu (C.S. Thummel).
[1] Current address: Regeneron, 777 Old Saw Mill River Road, Tarrytown, NY 10591.

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development and function. These include Paired (Prd) and the homeodomain protein Defective proventriculus (DveA), each of which is required for male fertility and the proper formation of accessory glands (Minami et al., 2012; Xue and Noll, 2002). Consistent with this, *sex peptide* and *ovulin* mRNA are expressed at reduced levels in *prd* mutant males (Xue and Noll, 2002). The Abd-B homeodomain protein is also required for proper accessory gland development, as revealed by genetic studies of the *iab-6* cis-regulatory element (Gligorov et al., 2013; Sitnik et al., 2016). This work showed that *iab-6* mutants display abnormal secondary cell morphology in the accessory glands and are unable to maintain the long term mating response in females. RNA-seq analysis, however, demonstrated that only 3% of genes misregulated in *iab-6* mutant accessory glands are expressed primarily in this tissue, consistent with *Abd-B* having more wide-ranging effects on development (Sitnik et al., 2016). Transcriptional control also mediates the hormone regulation of accessory gland function by the major insect hormones ecdysone and juvenile hormone (JH) (Chen, 1984). Mutants for the JH receptor display reduced male fertility and reduced accumulation of accessory gland proteins (Wilson et al., 2003). Similarly, accessory gland-specific depletion of the ecdysone receptor (EcR) by RNAi resulted in reduced male fertility and small accessory glands with defects in cellular organization (Sharma et al., 2017). Consistent with these defects in accessory gland development, all eight genes encoding seminal proteins examined in *EcR* RNAi accessory glands were expressed at reduced levels.

Here we describe a critical role for the HR39 nuclear receptor in male fertility and the regulation of accessory gland gene expression in *Drosophila*. Nuclear receptors comprise a large family of ligand-regulated transcription factors that contain a conserved zinc finger DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD). HR39 is a member of the NR5A subfamily of nuclear receptors with one *Drosophila* homolog, FTZ-F1, and two mammalian orthologs, Steroidogenic Factor-1 (SF-1) and Liver Receptor Homolog-1 (LRH-1). Both SF-1 and LRH-1 play important roles in regulating cholesterol homeostasis, steroidogenesis, and tissue-specific cell proliferation (Fayard et al., 2004; Meinsohn et al., 2019; Parker, 1998). Both are expressed in endocrine and gonadal tissues and required for proper steroidogenesis and reproduction. This includes the male reproductive system where LRH-1 regulates spermatogenesis, while SF-1 is required for testicular development (Fayard et al., 2004; Meinsohn et al., 2019; Parker, 1998). Both receptors are also essential for proper ovarian development and function. Consistent with this, mutations in human *SF-1* are associated with reproductive disorders (El-Khairi and Achermann, 2012). Studies of the *Drosophila* orthologs of these factors could provide new insights into the regulation and function of NR5A nuclear receptors, with implications for our understanding of mammalian endocrinology and reproduction.

Functional studies of *HR39* in *Drosophila* have demonstrated that it regulates female reproductive development much like its mammalian counterparts. *HR39* acts early during sexual maturation for female reproductive gland protrusion and the formation of spermathecae and parovaria in the reproductive tract (Allen and Spradling, 2008; Sun and Spradling, 2012). These are secretory glands that support fertilization and maintain sperm and seminal fluid. The spermathecae and parovaria are also required for ovulation and sperm storage in females (Sun and Spradling, 2013). Interestingly, mammalian LRH-1 can rescue spermathecal development in *HR39* mutant females, supporting the proposal that NR5A nuclear receptors have conserved roles in female reproduction (Sun and Spradling, 2012).

The published studies of *HR39* in *Drosophila* reproductive development were all done in females using partially penetrant loss-of-function alleles (Allen and Spradling, 2008; Sun and Spradling, 2012, 2013). Here we use CRISPR/Cas9 technology to engineer predicted null alleles in *HR39* and use these to conduct initial studies of its reproductive functions in males. We show that *HR39* mutants develop normally and have a normal lifespan, similar to the results of earlier studies (Allen and Spradling, 2008; Horner and Thummel, 1997). Despite their apparently normal reproductive development, including the formation of motile sperm, mutant males are functionally sterile. Expression profiling using RNA-seq revealed that many transcripts expressed in the accessory glands are present at reduced levels in *HR39* mutants. Of the 292 genes identified as encoding proteins that comprise the accessory gland proteome (Wigby et al., 2020), 52% are expressed at reduced levels in mutant males, including *ovulin* and *sex peptide*. In addition, noncoding RNAs that are specifically expressed in the accessory glands are present at reduced levels in *HR39* mutants, suggesting that they may contribute to accessory gland function. Using tissue-specific genetic rescue studies and RNAi we show that *HR39* is required in the accessory glands for fecundity and accessory gland gene expression. Our studies establish *HR39* as required for male fertility and as a broad regulator of accessory gland transcription. This role in a reproductive secretory tissue parallels its functions in *Drosophila* females and also provides evidence of evolutionarily conserved roles for NR5A nuclear receptors in reproduction.

2. Materials and methods

2.1. Drosophila strains and media

Drosophila were reared on a diet containing 8% yeast, 9% sugar, 1% agar, 0.05% $MgSO_4$, and 0.05% $CaCl_2$. Tegosept (10 ml/L) and propionic acid (6 ml/L) were added prior to pouring. Mutations in *HR39* were generated using CRISPR-Cas9 as described (Gratz et al., 2014). The guide RNA sequence GAGCTTGCAAGCTTACACCCTGG was used to make the $HR39^{\Delta 48}$ mutation and guide RNAs GAGCTTGCAAGCTTACACCCTGG and GTGGAAATGCTGACCTGACATGG were used to make the $HR39^{\Delta 3}$ mutation. Stocks were established using single males and homozygous mutants were sequenced to confirm the presence of the deletions (Fig. S1A). These mutant stocks were outcrossed to w^{1118} using balancers to provide a consistent genetic background on the X and third chromosomes. We used w^{1118} as a control for all studies. Genetic studies of *HR39* mutants were performed using a transheterozygous combination of the $HR39^{\Delta 3}$ (Bloomington 91373) and $HR39^{\Delta 48}$ (Bloomington 91374) alleles. Tissue-specific genetic studies were performed using *prd-GAL4* (Bloomington, 1947), *UAS-HR39* (FlyORF F000605), *UAS-HR39-RNAi* (VDRC 37694), and *UAS-HR39-RNAi* (TRiP collection, Bloomington 27086). Fourteen day males were used for all studies, and transferred every 2–4 days, except where noted.

2.2. Developmental timing and lifespan assays

Vials were established containing either w^{1118} controls or $HR39^{\Delta 48}$/*CyO, twi-GAL4, UAS-GFP* females and $HR39^{\Delta 3}$/*CyO, twi-GAL4, UAS-GFP* males. After two days, flies were transferred to fresh vials for 7–8 h intervals to collect fertilized eggs. Larvae that did not express GFP were selected two days after egg collection to isolate $HR39^{\Delta 3}/HR39^{\Delta 48}$ mutants. Control and mutant animals were moved to new vials and allowed to develop. Seven vials of each genotype were established containing approximately 20 animals/vial. Developmental timing was determined by recording the fraction of control and *HR39* mutant prepupae formed per day after animals began pupariating. Adult eclosion rates were determined by recording the fraction of control and *HR39* mutant flies that emerged from pupal cases per day, counted after animals began eclosing. For lifespan assays, adult males were maintained on the 8% yeast, 9% sugar diet and allowed to mature and mate for 3–16 days. Ten animals were placed in individual vials at 25 °C and transferred to fresh media every 2–4 days. Lethality was assayed at the time of transfer.

2.3. Fecundity assays

Fecundity was assayed using single male-female mating pairs. Newly-eclosed flies were separated by sex and reared on the control diet until 4–10 days of adulthood. Mating pairs were established in fresh vials by transferring flies using either a mouth pipette or 24 h after CO_2 treatment. Single control w^{1118} virgins were then crossed to single control,

HR39 mutant, *prd > HR39-RNAi,* or *prd > HR39*, *HR39*$^{-}$ males. Following a 24 h mating period, pairs were transferred to fresh vials and the egg lays were quantified at 24 h. Following a 24 h or 7 day mating period, the number of adults that eclosed was quantified and recorded.

2.4. Accessory gland imaging

Accessory glands were dissected in PBS, transferred to a microscope slide, and mounted in glycerol. Half coverslips were used as spacers on either side of the samples. A coverslip was then deposited on top of the dissected tissues before observation by bright-field microscopy using a Zeiss Axioskop Plus microscope equipped with an Infinity 3 Lumenera camera and Infinity Analyze software. Images were processed for contrast and assembled using Fiji (ImageJ) and Photoshop CS (Adobe, USA). The sizes of accessory glands and ejaculatory bulbs were measured using Fiji, after which accessory gland size was divided by the size of the ejaculatory bulb to generate an accessory gland ratio. Independent dissections were performed two times (n = 11–13), with representative images shown in Fig. 3.

2.5. Metabolite assays

Assays for protein, glycogen, glucose, and triglycerides were performed as described (Tennessen et al., 2014). Five adult male flies were placed into microcentrifuge tubes and each sample was homogenized in 100 μL of PBS. Samples of 10 μL were set aside prior to heat-treatment to assay protein levels, while the remaining lysate was treated for 10 min at 70 °C. Triglycerides, glycogen, and glucose levels were normalized to protein and the data are presented relative to the metabolite level in controls.

2.6. Feeding rate assays

CAFE assays were performed as described (Butts et al., 2019). Four-well plates were utilized that contained two holes for 20 μL capillary tubes (VWR). Ten adult males were placed into the plates by mouth pipette and the capillary tubes were filled with an 8% yeast 9% sugar liquid solution with a small, black mineral overlay to reduce evaporation and facilitate measurements. A time-lapse camera was used to collect images at 5 min intervals (Brinno) and assays were run for 24 h periods. Total consumption of each pair of capillary tubes per plate was quantified, summed, and recorded by the change in position of the mineral dye overlay.

2.7. RNA-seq transcriptional profiling

RNA was isolated from samples of fifteen control or *HR39* mutant male flies using a Direct-zol RNA Miniprep Kit (Zymo Research). Four to five independent control and mutant samples from 7 to 8 day old mated males were submitted for sequencing. Library generation of poly(A)-selected RNAs (Illumina RNA TruSeq Stranded mRNA Library Prep kit with oligo dT selection), quality control assays (ScreenTape Assay, Kapa qPCR), and sequencing (Illumina NovaSeq 6000 flow cell using the NovaSeq XP chemistry workflow, 2 × 51 cycle paired end sequence run) were performed by the High-Throughput Genomics core facility at the University of Utah. The Bioinformatics Core Facility at the University of Utah aligned this dataset to the *Drosophila melanogaster* dm3 genome assembly. We restricted our analysis to identify differentially expressed genes by using a cut-off for significance of Log2 ratio ±0.5 and p-value≤0.05. RNA-Seq data from this study can be accessed at NCBI GEO (GSE152031). Gene lists for accessory gland-specific genes and testes-specific genes were generated using the RNA-seq profile tool from FlyBase FB 2020_04 (https://flybase.org/rnaseq/profile_search). Genes were identified with peak expression levels not less than “high” in the accessory gland or testes, while not having a peak expression of more than “low” in all other tissues (Supp. Tables 3 and 4).

2.8. RT-qPCR

RNA was extracted from samples of 10–15 mated males at 7–8 days of age using a Direct-zol RNA Miniprep Kit (Zymo Research) or NucleoSpin RNA Kit (Macherey-Nagel 740955-50). First-strand cDNA was synthesized using 0.5 μg RNA, Superscript Reverse Transcriptase II (ThermoFisher Scientific, 18064-014), and oligo (dT) primers (Invitrogen, 18418012). qPCR experiments were performed on cDNA as described using an Applied Biosystem Quantstudio 3 device and the SYBR GreenER qPCR SuperMix Universal kit (ThermoFisher Scientific 11762100) (Storelli et al., 2019). ROX Reference Dye was diluted ten times before use and 0.1 μL was added for a final reaction volume of 20 μL. Fold inductions in transcript level were determined using the ΔΔCt method. Transcript levels were normalized to *rp49*. The forward and reverse primers used for qPCR experiments are as follows:

HR39: AAATCGTCAAATGGTGCAAGAG; AGCAAGCAGATTTGGTCAT;

sex peptide: GGAATGGCCGTGGAATAGGA; TAACATCTTCCACCCC AGGC;

ovulin: TGCTCTCCAATTTTACTGCTGC; GGTGCATAGCTGAATCGAG TTG;

CG43350: AGCTTCTGTCACTGTCAGTTGT; CACTTGTCCACGGGA GGTTT;

rp49: GACGCTTCAAGGGACAGTATCTG; AAACGCGGTTCTGCATGA.

Sequences for the *HR39, sex peptide, ovulin,* and *CG43350* primer sets were obtained from FlyPrimerBank. qPCR experiments were conducted using five independent *Drosophila* cDNA samples, and primers were used after confirming an efficiency between 90 and 110%.

2.9. Statistical analysis

GraphPad PRISM 6 software was used for graphical representation and statistical analysis throughout. For bar graphs, the top of the bar represents the mean and the error bars represent the SEM. Statistical comparisons were performed using a Student T-test with Welch's correction for unequal variances and a Mann-Whitney test for datasets with unequal variances that do not have a Gaussian distribution. For multiple comparisons, one-way ANOVA was performed followed by Tukey's multiple comparisons test. Kruskal Wallis tests were performed for multiple comparisons of data with significantly different standard deviations and nonnormal distributions. Lifespan data are presented as Kaplan-Meier survival plots. Statistical analysis of RNA-seq data is described above.

3. Results

3.1. Characterization of HR39 mutants

Most genetic studies of *HR39* have been conducted using P element insertions that act as partial loss-of-function alleles (Allen and Spradling, 2008; Horner and Thummel, 1997; Sun and Spradling, 2012, 2013). In an effort to generate stronger loss-of-function mutants, we used CRISPR/-Cas9 technology to create specific deletions in the *HR39* locus. *HR39*$^{\Delta 48}$ is a two base pair deletion in the first protein-coding exon of the locus, residing in the middle of the region that encodes the DBD (Fig. 1A, Supp. Fig. 1A). This deletion results in a frameshift, an early stop codon, and a predicted truncated protein that affects all *HR39* isoforms. *HR39*$^{\Delta 3}$ is a ~2.25 kb deletion spanning approximately half of the wild-type protein-coding region (Fig. 1A, Supp. Fig. 1A). The presence of these deletions was validated using DNA sequencing of PCR fragments amplified from homozygous mutants for each allele (Supp. Fig. 1A). These mutant alleles were outcrossed to *w*^{1118} animals to provide a consistent genetic background and used in transheterozygous combination throughout these studies (*HR39*$^{\Delta 3}$/*HR39*$^{\Delta 48}$). These animals will be referred to as *HR39* mutants, with control animals corresponding to *w*^{1118} animals. The level of *HR39* mRNA is significantly reduced in mutant adults, likely due to nonsense-mediated decay of prematurely truncated transcripts

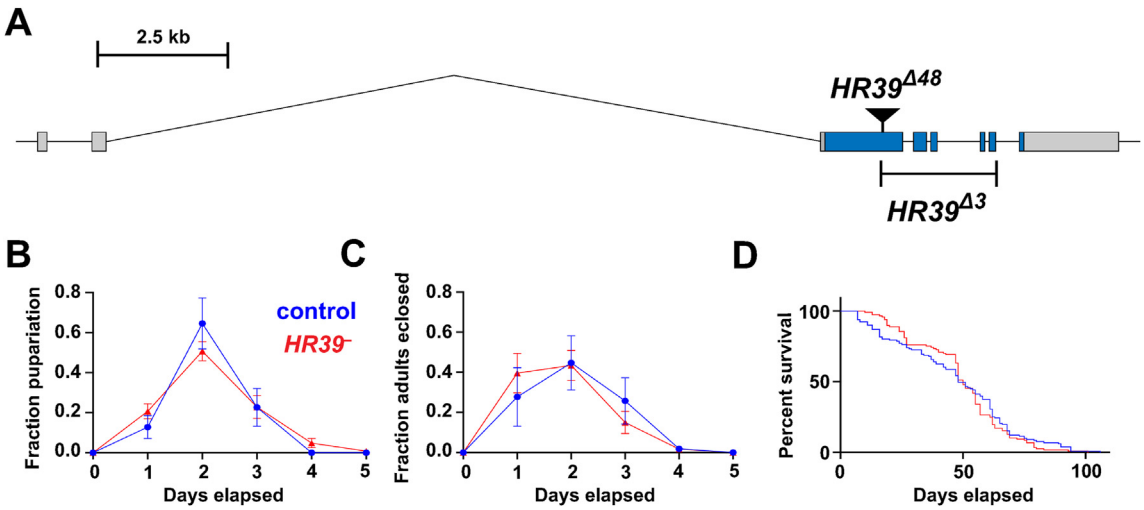


Fig. 1. *HR39* mutants develop at normal rates. (A) A schematic representation of the *HR39* genomic locus is depicted, including the protein-coding regions (blue) and untranslated regions (grey), along with the *HR39* mutations $HR39^{\Delta 3}$ and $HR39^{\Delta 48}$. (B) *HR39* mutants progress normally through larval stages. Developmental timing at pupariation was measured by recording the fraction of control (blue) and *HR39* mutant (red) prepupae formed per day after animals began pupariating. n = 99–125. Data represents the mean ± SEM. (C) *HR39* mutant adults eclose normally. Adult eclosion rates were assayed by recording the fraction of control (blue) and *HR39* mutant (red) flies that emerged from pupal cases per day, counted after animals began eclosing. n = 94–126. Data represents the mean ± SEM. (D) Lifespan was scored for controls (blue) and *HR39* mutants (red) on a normal diet. Median lifespans: controls = 48 days (n = 131); *HR39* mutants = 50 days (n = 117).

(Fig. 3A).

HR39 mutants appear to develop normally. They have a normal length of larval development, as assayed by the timing of puparium formation (Fig. 1B). In addition, *HR39* mutant adults eclose at normal rates compared to controls (Fig. 1C). *HR39* mutants also have a normal lifespan, with similar survival rates as controls (Fig. 1D). As part of this initial characterization, we also analyzed the levels of the major energy stores in mutants and controls at eight days of adulthood. Free glucose, stored lipids in the form of triglycerides, and stored carbohydrates in the form of glycogen, are all normal in *HR39* mutant males (Supp. Fig. 1 B–D). Consistent with this, these animals feed at similar rates as controls (Supp. Fig. 1E). Metabolite levels remain unaffected in mutant males at more advanced ages and under various dietary conditions.

3.2. *HR39 regulates the expression of most accessory gland genes*

As an initial step toward understanding the molecular basis of *HR39* function, we performed RNA-seq analysis on *HR39* mutant males at eight days of adulthood. This study identified 759 protein-coding genes that are significantly misregulated in mutants, with 513 genes expressed at reduced levels and 246 genes expressed at elevated levels (Supp. Table 1). The larger number of genes expressed at reduced levels is consistent with the predominant role of nuclear receptors as transcriptional activators. Interestingly, many genes involved in male fertility were revealed by gene ontology (GO) analysis of the down-regulated gene set (Fig. 2A). These include genes involved in sperm storage, sperm competition, insemination, and female receptivity to mating. GO analysis of significantly up-regulated genes revealed an enrichment of immune response genes, suggesting a possible role for *HR39* in immune suppression (Supp. Fig. 2A).

Many of the genes affected in the *HR39* mutants are expressed in the male accessory glands and encode proteins that are transferred to the female during copulation. To examine this in more detail, we compared our list of protein-coding genes expressed at reduced levels in *HR39* mutants with a recent list of 292 ″high confidence” genes expressed in the accessory glands (Wigby et al., 2020). Remarkably, this showed that 52% of the predicted accessory gland proteome is encoded by genes that are regulated by *HR39* (Fig. 2B). Our RNA-seq analysis also detected 263 non-coding RNAs that are expressed at reduced levels in *HR39* mutants (Supp. Table 2). To determine if these RNAs might also be preferentially expressed in the accessory glands, we assembled a list of all transcripts that are expressed at moderate to high levels in the accessory glands and at low or not detectable levels in other tissues using the FlyBase RNA-Seq Expression Profile tool (Supp. Table S3). Comparison of these 190 accessory gland transcripts with the non-coding RNAs that are expressed at reduced levels in *HR39* mutants revealed that 14% of these transcripts are expressed in the accessory gland (Supp. Fig. S2B). More significantly, comparison of this accessory gland gene list with all transcripts expressed at reduced levels in *HR39* mutants (coding and non-coding) revealed that most accessory gland RNAs are affected by the *HR39* mutation (84%, Fig. 2C). Taken together, these studies demonstrate that *HR39* plays an important role in maintaining the normal levels of accessory gland gene expression in males.

3.3. *HR39 mutants are infertile and display reduced expression of key fertility genes*

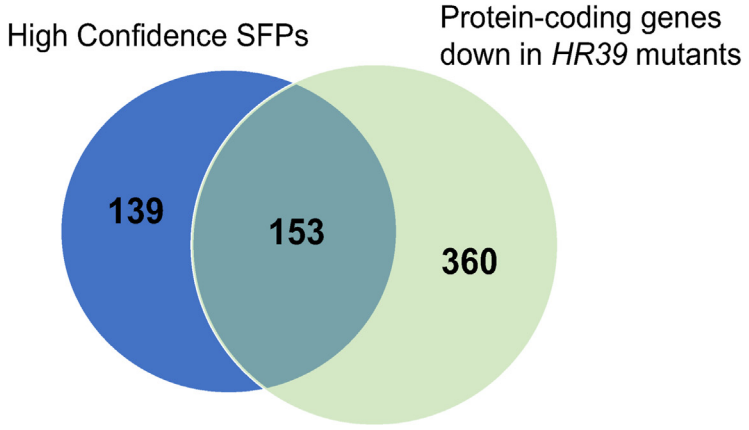
We used RT-qPCR to validate the reduced mRNA levels for three genes in our RNA-seq dataset: *sex peptide* and *ovulin*, each of which is important for male fertility, as well as *CG43350*, which is the fourth most affected gene in our dataset (Supp. Table 1). In agreement with the RNA-seq results, all three of these genes are expressed at significantly reduced levels in *HR39* mutants (Fig. 3B–D). This effect on accessory gland gene expression indicates that *HR39* mutant males should be infertile because they should not stimulate oviposition and sperm will not be stored in female sperm storage organs, which are necessary prerequisites for fertilization. Consistent with this, we found that control females mated with *HR39* mutant males resulted in reduced egg lays relative to females mated to control males (Fig. 3E). In addition, only a few of the eggs laid during a 24 h mating period with *HR39* mutant males developed into adults (Fig. 3F). Similar results were seen when the mating period was extended to seven days (Fig. 3G). Taken together, these observations are consistent with the broad reduced expression of accessory gland gene expression and indicate that this results in male infertility.

A simple explanation for this reduction in both accessory gland gene expression and male fertility in *HR39* mutants would be that the mutants display a defect in accessory gland development. However, no overt morphological defects were observed in *HR39* mutant accessory glands compared to controls (Fig. 3H–J). The accessory glands appear to be of normal size and contain visible material in their lumen. In addition,

A

Gene Ontology Process	Number of genes (total)	Fold Enrichment	P value
sperm storage	7 (11)	22.9	8.59E-04
sperm competition	14 (24)	20.99	1.65E-09
reproduction	132 (1374)	3.46	1.91E-33
multicellular organism reproduction	132 (1241)	3.83	7.27E-38
multicellular organismal process	153 (3439)	1.6	9.28E-07
multi-multicellular organism process	14 (25)	20.15	2.51E-09
insemination	14 (25)	20.15	2.51E-09
copulation	14 (31)	16.25	2.40E-08
mating	19 (135)	5.06	1.21E-04
negative regulation of female receptivity, post-mating	10 (16)	22.49	2.34E-06
negative regulation of female receptivity	10 (18)	19.99	5.52E-06
regulation of female receptivity	15 (34)	15.87	5.48E-09
female mating behavior	15 (41)	13.16	4.76E-08
mating behavior	15 (120)	4.5	1.29E-02
reproductive behavior	18 (130)	4.98	3.48E-04
biological regulation	61 (4374)	0.5	1.35E-08
regulation of female receptivity, post-mating	15 (22)	24.53	4.38E-11

B



C

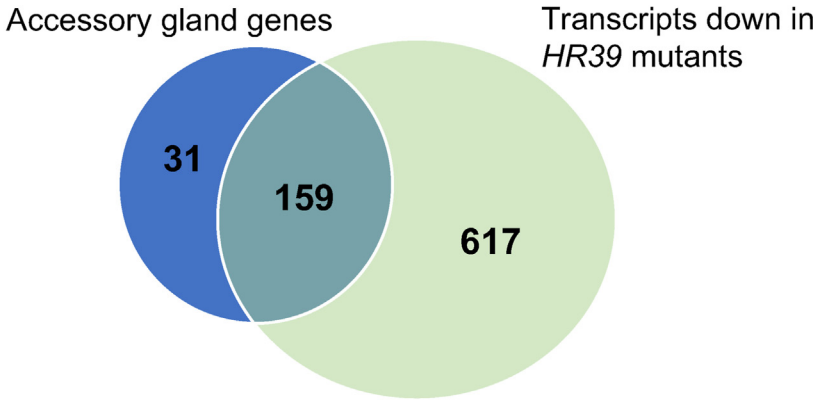


Fig. 2. *HR39* regulates accessory gland gene expression. **(A)** Gene ontology (GO) enrichment analysis of the genes that display significantly reduced expression in *HR39* mutants as determined by RNA-seq. The GO process categories are ordered by their fold enrichment. Analysis was performed using PANTHER GO-slim Biological Process. (B) Venn diagram depicting the overlap between protein-coding genes expressed at reduced levels in *HR39* mutants (total = 513; Supp. Table 1) and 292 genes encoding high confidence seminal fluid proteins (SFPs) (Wigby et al., 2020). (C) Venn diagram depicting the overlap between transcripts expressed at a significantly reduced level in *HR39* mutants (total = 776; Supp. Tables 1 and 2) and transcripts expressed in the accessory glands (total = 190; Supp. Table 3). The list of genes expressed in the accessory glands was generated using the FlyBase RNAseq Profile tool using tissue filters to select for "high" expression in the accessory glands and "low" in other tissues.

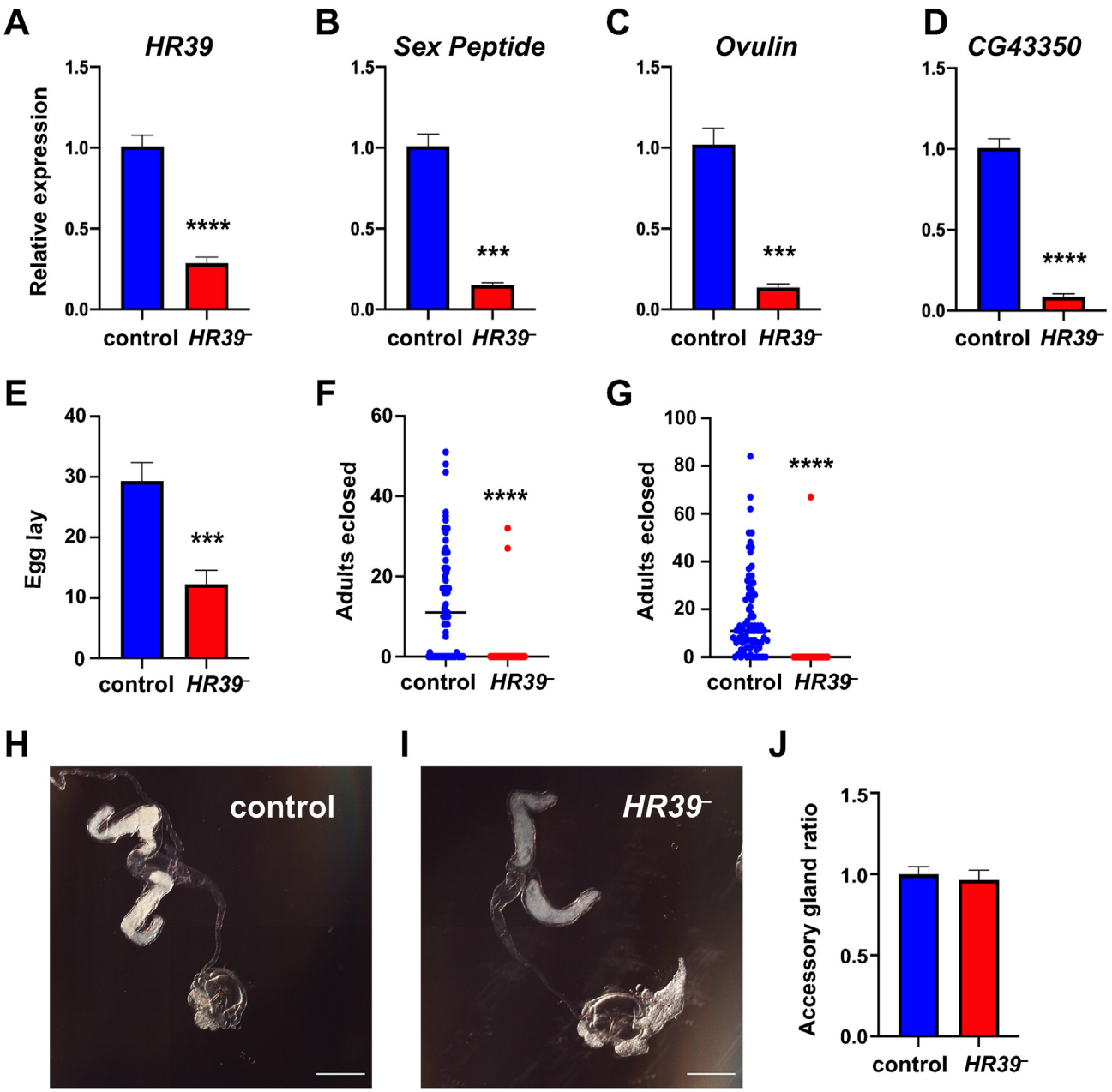


Fig. 3. *HR39* mutant males are infertile and display reduced expression of key fertility genes. (A–D) RT-qPCR measurements of *HR39, sex peptide* (*Acp70A*)*, ovulin,* and *CG43350* mRNA levels in controls (blue) and *HR39* mutants (red). Transcript levels are normalized to *rp49* and presented relative to controls. n = 5 independent samples with 7–10 flies per sample. (E) Egg lays of single control females mated to a single control male (blue) or a single *HR39* mutant male (red) over a 24 h mating period. n = 27–59 single mating pairs. Data are presented as a bar graph of the mean with error bars representing the SEM. (F) Fecundity was assayed by mating a single control female to a single control male (blue) or an *HR39* mutant male (red). Following a 24 h mating period, pairs were cleared and adult progeny were quantified. n = 42–65 single mating pairs. Different collections of animals were used to gather the data for panels E and F. (G) Fecundity over an extended period of time was assayed by mating a single control female to a single control male (blue) or an *HR39* mutant male (red). Following a 7 day mating period, pairs were cleared and adult progeny were quantified. n = 42–65 single mating pairs. (H–I) Representative brightfield images of control and *HR39* mutant accessory glands dissected from five day old unmated males. Scale bar, 200 μm. (J) The accessory gland ratio was calculated using images of control (blue) and *HR39* mutant (red) accessory glands dissected from 5 to 14 day unmated males. The average size of accessory gland pairs was divided by the ejaculatory bulb size to determine the ratio. Data are normalized to the ratio in controls. n = 11–12 (***) $P \leq 0.001$; (****) $P \leq 0.0001$.

dissection and microscopic analysis of the testes and seminal vesicles revealed that all control and *HR39* mutant males have motile sperm, with no obvious qualitative differences observed in the amount of motile sperm between the two (n = 5 males per genotype; Supp. Movie 1, 2). Taken together, these observations support the model that *HR39* is not required for accessory gland development or sperm motility but rather acts to maintain the appropriate levels of gene expression in mature accessory glands that are required for male fertility.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ydbio.2021.07.011

3.4. *HR39 functions in the accessory gland to regulate male fertility and transcription*

Our studies suggest that *HR39* acts within the accessory glands to maintain normal levels of seminal gland transcription. To test this model,

we used the *prd-GAL4* driver to perform RNAi and genetic rescue studies of *HR39* specifically in the accessory glands and assayed the effects on target gene expression using RT-qPCR (Fig. 4). Two *UAS-HR39-RNAi* constructs that target distinct regions in the *HR39* locus, from the VDRC and TRiP collections, were used for the tissue-specific RNAi studies. This approach resulted in a reduction in *sex peptide*, *ovulin*, and *CG43350* transcript levels (Fig. 4A,B,D,E,G,H). The overall effect, however, was less than that seen in *HR39* mutant males (compare with Fig. 3B–D). Several factors could be contributing to the relatively weak response to *HR39* RNAi. One of these could be that these RNAi constructs do not efficiently target *HR39* mRNA for degradation. In addition, it has been shown that the *prd-GAL4* driver is strongly expressed in the accessory glands of newly eclosed males but has only low scattered expression by 10 days of adulthood (Xue and Noll, 2002), an observation that we have confirmed using a *prd > GFP* reporter. Our experiments were conducted using 7-8 day-old animals, which have relatively low levels of *prd-GAL4* driver expression. Consistent with these possibilities, we see only an ~2-fold reduction in *HR39* mRNA levels in *prd > HR39-RNAi* animals (Fig. 4J and K). In spite of this relatively inefficient RNAi, however, we still detect a significant effect on target gene mRNA levels, supporting the proposal that *HR39* acts specifically in the accessory glands to regulate gene expression. It is interesting to note that, although the VDRC RNAi line has a slightly greater impact on *HR39* mRNA levels than the TRiP line, no effect is seen on *sex peptide* mRNA levels in *prd > HR39* (VDRC) RNAi animals (Fig. 4A, J, K). In contrast, *ovulin* mRNA levels are more reduced in *prd > HR39* (VDRC) RNAi animals relative to the TRiP RNAi animals (Fig. 4D). It would be useful to use an additional *HR39* RNAi line to address these apparent discrepancies. Nonetheless, the overall reduction in *sex peptide*, *ovulin*, and *CG43350* mRNAs in both *prd > HR39* (VDRC) and *prd > HR39* (TRiP) RNAi animals are consistent with our RNA-seq analysis and support the proposal that *HR39* plays an important role in maintaining appropriate levels of accessory gland gene expression.

To further explore the role of *HR39* in supporting accessory gland gene transcription, we performed the converse experiment in which we restored wild-type *HR39* expression specifically in the accessory glands of *HR39* mutant males and assayed for effects on transcription. Under these conditions, *sex peptide*, *ovulin*, and *CG43350* mRNA levels are partially restored in *HR39* mutants (Fig. 4C,F,I). The effect on target gene expression, however, is only about 2-fold above the baseline seen in *HR39* mutants and not statistically significant for *ovulin* or *CG43350* transcription. As discussed above, this relatively modest effect could be due to the reduced expression of the *prd-GAL4* driver in older males. Nonetheless, in combination with the results of our RNAi studies, we conclude that *HR39* is acting in the accessory glands to maintain normal levels of gene expression. Temporal requirements for *HR39* function during earlier stages of development, however, will require further investigation.

Finally, if the role of *HR39* in regulating accessory gland gene expression is of functional consequence then we would expect that accessory gland-specific expression of wild-type *HR39* should be sufficient to at least partially restore fertility in an *HR39* mutant male. Consistent with this prediction, while *HR39* mutant males are normally infertile, male fertility is partially rescued in *HR39* mutants that express *HR39* in the accessory glands using the *prd-GAL4* driver (Fig. 5). Taken together with our tissue-specific RNAi study, these results support the proposal that *HR39* acts specifically within the accessory glands to maintain accessory gland gene expression and male fertility.

4. Discussion

Reproductive success in both mammals and insects is dependent on the transfer of seminal fluid proteins to female recipients. In mammals, the prostrate and seminal vesicles are responsible for this protein production, while in *Drosophila* an analogous structure, the accessory gland, produces the seminal proteins required for successful reproduction. Although a number of transcription factors have been identified that are required for the development and function of the *Drosophila* accessory glands, a broad regulator of accessory gland gene expression has not yet been identified. Here we show that the HR39 nuclear receptor is required for male fertility and the proper expression of many accessory gland-specific genes. Tissue-specific RNAi and genetic rescue studies indicate that *HR39* acts within the accessory gland to exert its functions, consistent with its predicted role as a transcription factor. Taken together, our work identifies *HR39* as a key regulator of male accessory gland function in *Drosophila*. This activity raises the interesting possibility that *HR39* might be a good candidate for targeted sterile male approaches, which are being used to control insect pest populations, including those that threaten agricultural production as well as human disease vectors (Alphey, 2002; Krafsur, 1998). Furthering our insight into insect reproduction may lead to the development of more advanced tools to control these populations.

4.1. Conserved roles for HR39 in reproduction

Interestingly, prior studies of *HR39* in females have defined a critical role for this factor in the spermathecae and parovaria of the reproductive system (Allen and Spradling, 2008; Sun and Spradling, 2012, 2013). Like the male accessory glands, these structures provide secretory functions that support fertilization, suppress infections, and maintain male sperm. *HR39* mutant females have reduced fertility and lack one or more spermathecae and parovaria. They also display reduced expression of genes that appear to function in spermathecal secretions (Allen and Spradling, 2008). Thus, *HR39* plays a similar role in both female and male reproduction - supporting the key secretory functions that facilitate fertility. These studies in females, however, were done using partial loss-of-function alleles for *HR39*. It would be interesting to revisit this work using the likely null alleles described here to see if they result in a more severe female phenotype.

It is also interesting to note that the functions described here for *HR39* in *Drosophila* male fertility are conserved through evolution. A functional survey of nuclear receptors in the red flour beetle, *Tribolium castaneum,* revealed that *HR39* is required for the proper expression of eight genes expressed in the accessory glands as well as normal sperm production (Xu et al., 2012). Similarly, the mammalian orthologs of *HR39*, SF-1 and LRH-1, are central regulators of secretory glands and reproduction (Fayard et al., 2004; Meinsohn et al., 2019; Parker, 1998). LRH-1 regulates testicular steroidogenesis and SF-1 is required for male sexual development and fertility in mice and humans (Ahn et al., 2013; El-Khairi and Achermann, 2012; Meinsohn et al., 2019; Parker, 1998). In addition, expression of mouse *LRH-1* can partially rescue *HR39* function in female *Drosophila* reproductive glands (Sun and Spradling, 2012). These observations suggest that the functions of NR5A nuclear receptors are conserved through evolution and that further studies in *Drosophila* might provide new insights into their roles in reproductive biology.

4.2. Temporal and spatial regulation of HR39 expression

HR39 is expressed throughout most of development with some peaks corresponding to pulses of the steroid hormone 20-hydroxyecdysone (Sullivan and Thummel, 2003). In spite of this broad temporal pattern of expression, however, mutant phenotypes have only been observed in adult reproduction. It is possible that *HR39* exerts other roles during earlier stages of development. This could include earlier roles for *HR39* in accessory gland development that are not apparent when we conduct our studies in adult males. In addition, *HR39* may act in a redundant manner with its sister NR5A nuclear receptor in *Drosophila*, FTZ-F1 (King-Jones and Thummel, 2005). Double mutant studies might reveal shared regulatory functions for these two nuclear receptors. Further studies could also address how *HR39* might interact with other transcriptional regulators of accessory gland development and function, such as *Prd* and *EcR* (Xue and Noll, 2002; Sharma et al., 2017). In addition, characterization

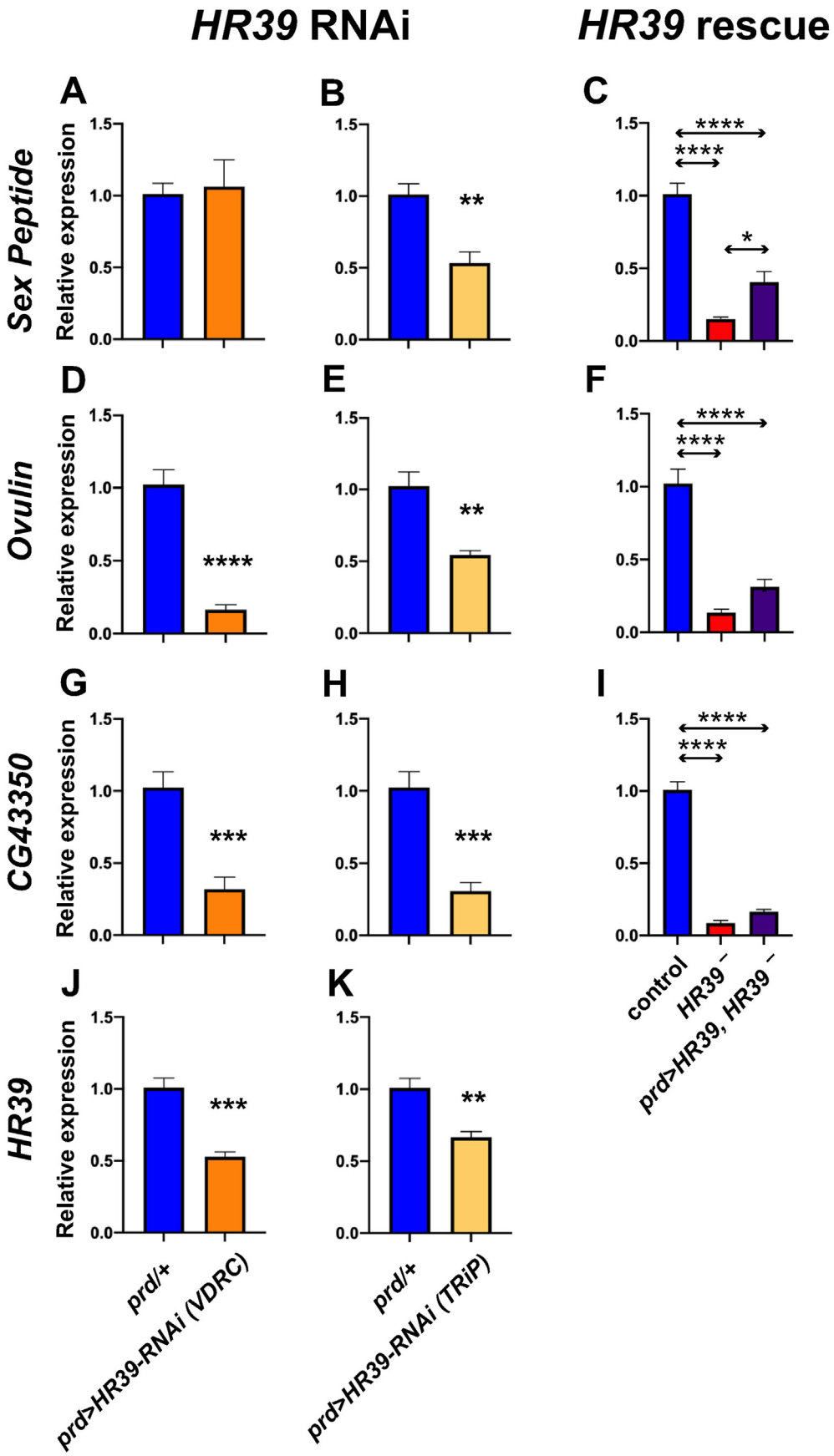


Fig. 4. *HR39* is required in the accessory glands for gene expression. Accessory gland specific *HR39* RNAi (A,B,D,E,G,H,J,K) and genetic rescue (C,F,I) were performed using the *prd-GAL4* driver, assaying for effects on target gene transcription. For RNAi studies, RT-qPCR was used to measure levels of *sex peptide* (A,B), *ovulin* (D,E), *CG43350* (G,H), or *HR39* (J,K) mRNA in *prd-GAL4/ +* controls (blue), *prd > HR39-RNAi* males (VDRC, orange), or *prd > HR39-RNAi* males (TRiP, yellow), all staged at 7–8 days of adulthood. For genetic rescue studies, RT-qPCR was used to measure levels of *sex peptide* (C), *ovulin* (F), or *CG43350* (I) mRNA in controls (blue), *HR39* mutant males (red), or males with accessory gland-specific expression of *HR39* in a *HR39* mutant background (*prd > HR39, HR39⁻*, purple), all staged at 7–8 days of adulthood. Transcript levels are normalized to *rp49* mRNA and presented relative to control levels. All experiments are n = 5 independent samples with 7–10 flies per sample. (*) $P \leq 0.05$; (**) $P \leq 0.01$; (***) $P \leq 0.001$; (****) $P \leq 0.0001$.

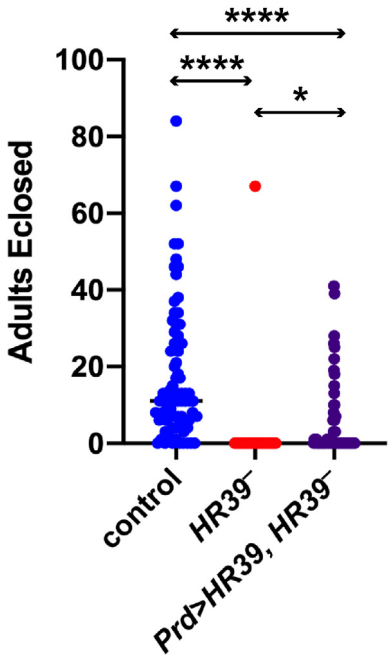


Fig. 5. Genetic rescue of infertility in *HR39* mutants. Fecundity was assayed by mating a single control female to a single control male (blue), a single *HR39* mutant male (red), or a single male with accessory gland-specific expression of *HR39* in a *HR39* mutant background (*prd* > *HR39, HR39*⁻, purple). Adults at 6–10 days of age were used for mating pairs. Following a seven day mating period, pairs were cleared and adult progeny were quantified. n = 42–76 single mating pairs.

of the spatial patterns of *HR39* expression in *Drosophila* could provide directions for future functional studies. Efforts by our lab and another group, however, were unable to raise antibodies against HR39 protein, which appears to be present at relatively low levels. Further work along these lines, such as generating a tagged version of *HR39*, could facilitate our understanding of *HR39* functions during development. It is also interesting to note that 19% of the genes annotated as expressed in the testis are reduced in their expression in *HR39* mutants, raising the interesting possibility that *HR39* may also function in this tissue (Supp. Table 4; Supp. Fig. 2C). Further studies are required to address roles for *HR39* in the testis and its possible contribution to male fecundity. In addition, *Est-6*, which is expressed in the ejaculatory duct and transferred to females upon mating, is expressed at reduced levels in *HR39* mutants (Supp. Table 1). Taken together, these observations suggest that *HR39* plays a broader role in regulating male fertility, outside of the accessory glands. This possibility could also explain why *prd* > *HR39* does not fully rescue male fertility. Further studies of *HR39* function in different tissues could provide more insights into its roles in regulating reproduction.

It is also important to further study the effects of *HR39* mutations on accessory gland development and function. Despite the importance of *HR39* within the accessory gland, this tissue appears morphologically normal (Fig. 3H and I). Further characterization of the development and morphology of the main and secondary cells that comprise the secretory cells of the accessory glands could provide new insights into the roles of *HR39* in this tissue. In addition, it is interesting to note that 37 noncoding RNAs appear to be expressed at reduced levels in *HR39* mutant males (Supp. Figure 2B). The transfer of small RNAs in sperm has been proposed to be a mechanism for regulating fertility and inheritance (Holman and Price, 2014; Hosken and Hodgson, 2014). It would be interesting to see if *HR39* mutants display any defects in these relatively poorly understood pathways.

4.3. A possible role for HR39 in suppressing inflammation

In addition to the widely reduced expression of accessory gland gene expression in *HR39* mutant males, we discovered that key components of the innate immune and inflammatory pathways are expressed at elevated levels in these animals (Supp. Fig. 2A). These include *Unpaired-3*, which is related to the mammalian IL-6 proinflammatory cytokine, and *Socs36E*, which is an indicator of active JAK/STAT signaling in *Drosophila* (Zeidler et al., 2000). In addition, key markers of antimicrobial innate immunity are expressed at higher levels in *HR39* mutant males, including *Metchnikowin, Drosocin, Defensin, Diptericin B,* and *Drosomycin*, which are regulated by the Toll and Imd pathways (Leclerc and Reichhart, 2004; Lemaitre and Hoffmann, 2007). Interestingly, *Drosomycin* and *Defensin* are among the top 15 up-regulated genes in *HR39* mutants, suggesting that this represents a significant response (Supp. Table 1). These inflammatory and innate immune pathways have been detected in male reproductive tissues, with JAK/STAT signaling playing an important role in testis homeostasis and regeneration (Ferrandon et al., 1998; Herrera and Bach, 2019). This raises the interesting possibility that *HR39* might regulate fertility in part through its role in these pathways. In addition, inflammation and innate immune responses can arise from the *Drosophila* fat body or intestine and signal remotely to other tissues. In this regard, *LRH-1* plays an important role in regulating inflammatory responses in the mammalian intestine (Fernandez-Marcos et al., 2011). It would be interesting to determine where JAK/STAT signaling and the Toll/Imd pathways are being activated in *HR39* mutants as a first step toward determining whether they contribute to new regulatory functions for this nuclear receptor in *Drosophila*.

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Appendix A. Supplementary data

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