Targeting mosquito X-chromosomes reveals complex transmission dynamics of sex ratio distorting gene drives

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Abstract

Engineered sex ratio distorters (SRDs) have been proposed as a powerful component of genetic control strategies designed to suppress harmful insect pests. Two types of CRISPR-based SRD mechanisms have been proposed: X-shredding eliminates X-bearing sperm, while X-poisoning eliminates daughters inheriting disrupted X-chromosomes. These differences can have a profound impact on the population dynamics of SRDs when linked to the Y-chromosome: an X-shredder is invasive constituting a classical meiotic Y-drive, whereas X-poisoning is self-limiting, unable to invade but also insulated from selection. Here, we established the first X-poisoning strains in the malaria vector *Anopheles gambiae* targeting three

X-linked genes during spermatogenesis resulting in male bias. Surprisingly, we found that sex distortion was primarily driven by a loss of X-bearing sperm with limited evidence for postzygotic lethality of daughters. By leveraging a *Drosophila melanogaster* model, we show unambiguously that engineered SRD traits can operate differently in these two insects. Unlike X-shredding, X-poisoning could theoretically operate at early stages of spermatogenesis. We therefore explored premeiotic Cas9 expression to target the mosquito X-chromosome. We find that, by pre-empting the onset of meiotic sex chromosome inactivation, this approach may enable the development of Y-linked SRDs if mutagenesis of spermatogenesis-essential genes is functionally balanced.

Keywords

Sex ratio distortion, CRISPR gene drive, X-shredding, X-poisoning, genetic control, meiotic drive

Introduction

In 1967, Hamilton proposed that sex ratio distorting (SRD) alleles could represent a component of a highly efficient genetic control method for the suppression of pest species, highlighting malaria mosquitoes as a possible target[1]. Approximately forty years later, insect molecular biology and genetics had matured sufficiently to begin engineering Hamilton's meiotic drives in the malaria vector *Anopheles gambiae*. Using the I-*Ppo*I meganuclease from a slime mold, we targeted the repetitive and X-chromosome specific 28S rDNA gene cluster during male meiosis, a strategy we called X-shredding. As a result, X-bearing gametes were almost entirely excluded from successfully fertilized eggs, resulting in a highly male-biased sex ratio within offspring of transgenic males[2,3]. Subsequently, CRISPR/Cas9 was used instead of I-*Ppo*I, targeting the same 28S rDNA genes, and also resulted in high sex ratio distortion without affecting male fertility[4]. This opened new possibilities to transfer this system to other pests, which do not share the unique X-chromosome specificity of the rDNA cluster of *An. gambiae*, by targeting other repetitive X-chromosome specific sequences. To test this, we previously identified[5] and then targeted suitable repetitive X-linked sequences first in the *Drosophila melanogaster* model[6], and soon after in the agricultural pest *Ceratitis*

capitata[7], thereby confirming that CRISPR-based SRDs can be transferred to additional important pests. Insertion of an active X-shredding SRD into an insect Y-chromosome in a following step would result in its shielding from negative selection in females and ensure transmission to all sons, realizing the population suppression meiotic drive originally described by Hamilton in his models.

However, because a highly invasive Y-linked X-shredder may not always be desirable, for example by spreading to non-target areas, or simply because gene drives are controversial due to their novelty and implications, Burt and Deredec[8] proposed an alternative Y-linked SRD design based on the targeting of X-chromosome genes with a haploinsufficient (HI) phenotype. Since X-chromosomes are exclusively transmitted to daughters from males with XY karyotypes, developmental lethality would be confined to females, resulting in a postzygotic mortality of daughters. Alternatively autosomal HI genes could be targeted, provided they are female-specific. Similarly to X-shredding, Y-linkage would also shield such a postzygotic SRD from negative selection in females and ensure transmission to all sons, but unlike X-shredding, lethality of all daughters would theoretically limit the invasiveness of this system. More specifically, when the dynamics of suppression and transmission of such a Y-linked SRD are modeled, its behavior reflects a self-limiting system that does not spread but which can persist at its initial release frequency. This persistence is unlike other self-limiting constructs such as female-specific RIDL, which decline in frequency and ultimately disappear without additional releases[8]. Given these interesting dynamics, an autosomal proof-of-concept for such a system was demonstrated in *D. melanogaster*[6]. To distinguish it from X-shredding we called this approach X-poisoning, reflecting the postzygotic lethality of daughters inheriting mutated X-chromosomes from their fathers (**Figure 1**). X-poisoning transgenic males were designed to express Cas9 in sperm and sgRNAs targeting X-linked ribosomal protein (RP) genes with a known HI phenotype. Postzygotic lethality of a significant fraction of the offspring from X-poisoning males was observed and corresponded with a highly male-biased sex ratio among survivors reaching adulthood (92% sons), indicative of developmental lethality of daughters receiving mutant X-linked alleles. In a second *Drosophila* study, targeting a different X-linked HI gene involved in wing development also

resulted in significant postzygotic lethality of daughters[9]. Here, we develop and test the X-poisoning system in the malaria vector *An. gambiae* using proof-of-concept autosomal strains. We discover significant shifts of normal sex ratios, but through unexpected mechanisms that modify transmission dynamics of sex chromosomes. Using our *Drosophila* system we perform direct comparisons between X-poisoning in mosquitoes and flies and confirm that Cas9-targeted X-chromosomes are transmitted differently to the next generation between these two related insect species. Based on these results we demonstrate how this unexpected mechanism can help to close a current technological barrier for the engineering of invasive Y-chromosome drives in mosquitoes.

Results

Generation of transgenic mosquito X-poisoning strains

Cytosolic ribosomal protein (RP) genes have known haploinsufficient (HI) mutant phenotypes in many species, including in *Saccharomyces* yeast and *D. melanogaster* and were the basis of our first X-poisoning designs in flies[6,10]. *An. gambiae* and *D. melanogaster* do not share an ancestral dipteran X-chromosome, with the ancestral dipteran X being the dot chromosome in *D. melanogaster*[11,12]. Hence, we did not assume that we could directly target mosquito RP orthologs of validated *Drosophila* X-poisoning RPs, as these would likely not be X-linked in mosquitoes. We therefore cataloged all mosquito RP genes identifying six candidate loci on the X-chromosome (**Figure 2A**). Among the six candidates, we designed X-poisoning sgRNAs to target AGAP000739 (*AgRpS10*), AGAP000952 (*AgRpL37*) and AGAP000953 (*AgRpL10-1*) (**Figure S1A**). We prioritized AGAP000952 and AGAP000739 because both have sperm-specific, autosomal paralogs (AGAP011501 and AGAP005469, respectively) (**Figure S1B**) that may have evolved to substitute the function of the X-linked RP genes in meiotic cells, as has been observed in other species[13,14]. We reasoned that autosomal paralogs may be able to functionally rescue the targeting of the X-linked gene by Cas9, if the targeted X-linked gene is essential for spermatogenesis. This could mitigate trade-offs between Cas9 activity and male fertility and enabling the X-poisoning effect to manifest only in

the fertilized embryo. We also compared the relative expression of the non-targeted autosomal paralogs versus the targeted X-linked RP genes throughout stages of sperm development (**Figure S1C**). AGAP000953 was selected for its proximity to AGAP000952 and because its autosomal paralog (AGAP002395) is identical in protein sequence to the X-linked gene (**Figure S2**). AGAP002395 is also expressed in sperm, but also in other tissues (**Figure S1B**). *piggyBac* transformation constructs were designed to express either one or two sgRNAs per X-linked target RP gene using the U6 promoter[15]. Cas9 expression was driven using the regulatory regions of the spermatogenesis-specific *β2-tubulin* gene, to restrict Cas9 activity to spermatogenesis similarly to previous X-shredding constructs[2–4,6,16]. In addition, each construct contained a 3xP3-DsRed transformation marker[17]. For the single sgRNA constructs, we were able to isolate three independent transgenic strains targeting AGAP000739 (739_1_a, 739_1_b, and 739_1_c), two targeting AGAP000952 (952_2_a and 952_2_b), and one targeting AGAP000953 (953_1_a). For the double sgRNA construct, we generated a construct combining the two top-ranking sgRNAs for AGAP000952, resulting in independent strains 952_1;952_2_a, and 952_1;952_2_b. Finally, to test the combined effects of X-poisoning and X-shredding, we generated a strain expressing an sgRNA targeting AGAP000952 along with the previously validated X-shredding sgRNA targeting the 28S rDNA[4] (strain Xshrd;952_1). In total, we generated nine transgenic strains harboring multiple designs (**Figure 2B**).

Evaluating the sex ratio in offspring of X-targeting strains

To test the performance of our X-targeting strains, transgenic heterozygous males were crossed to wild type females and offspring were reared to adulthood to measure the sex ratio. Reciprocal crosses of transgenic heterozygous females and wild type intercrosses served as controls (**Figure 2C**). Of the nine strains, three exhibited a significant male-bias among offspring reaching adulthood compared to both the wild type cross and the reciprocal female cross (Tukey-Kremer, $p<0.0001$) (**Figure 2D**). The first strain, 739_1_c, corresponds to a single sgRNA construct targeting AGAP000739. Among the three independent strains expressing the same sgRNA (739_1), only 739_1_c demonstrated a significant male bias in the F1 populations (67.2%). The second strain, 952_1;952_2, corresponds to the double sgRNA construct targeting

AGAP000952. Among the two independent strains expressing the same sgRNAs, only 952_1;952_2_a exhibited substantial sex ratio distortion (73%). The distortion levels observed in the two X-poisoning strains, 739_1_c and 952_1;952_2_a, were not statistically different (Tukey-Kremer, p=0.2652). The third strain, 952_1;Xshred, corresponds to a double sgRNA construct combining both X-poisoning and X-shredding. With an average of 80.8% male F1 adults, the sex ratio distortion in 952_1;Xshred was statistically different from both 739_1_c (Tukey-Kremer, p<0.0001) and 952_1;952_2_a (Tukey-Kremer, p=0.0154). Next, we dissected testes from transgenic male adults and ran total mRNA sequencing to quantify Cas9 expression levels and to correlate these to distortion phenotypes (**Figure S3A**). Cas9 expression levels in testes were generally predictive of sex ratio distortion, where strains of the same construct resulting in significant distortion levels consistently had higher Cas9 expression compared to those that did not display sex ratio distortion. For strains displaying male bias, we also performed amplicon sequencing from pools of dissected testes and surviving daughters (**Figure S3B and Figure S4**). Amplicon sequencing for males of all living transgenic strains, regardless of the SRD phenotype, was also performed using genomic DNA extracted from posterior adult male abdomens that contain testes, but also other tissues, instead of dissected testes, given the larger number of strains (8 of 9). The frequencies of unique alleles not perfectly matching the native sgRNA target sites and occurring at least 10 times in the data were quantified. Mutant alleles were found at all sgRNA target sites, suggesting that all selected sgRNAs can guide Cas9 activity against their respective target sites (**Figure S3B**). Mutant alleles were consistently more abundant in transgenic testes or terminal segment samples than those occurring in wild type samples and were composed of substitutions and both in- and out-of-frame edits. When mutant alleles were identified in surviving daughters, they were exclusively composed of substitutions and in-frame deletions, suggesting that null or deleterious alleles of the X-linked target genes produce HI phenotypes, as expected, and that mutated X-chromosomes are transmitted to daughters from transgenic males (**Figure S4**).

Evaluating the timing of female elimination by X-poisoning

According to our model and data in *Drosophila* (**Figure 1**), X-poisoning should result in female-specific lethality during development, as an outcome of Cas9-induced mutagenesis of X-linked RP genes that are transmitted exclusively to daughters from transgenic males. To evaluate whether, and at what developmental stage, female-specific lethality occurs in our mosquito strains, we designed an experiment to track offspring survival throughout development in male crosses of the two active X-poisoning strains compared with their reciprocal female crosses. Surprisingly, we did not observe a decrease in relative offspring survival between male and female crosses that could account for final adult sex ratios (**Figure 3A**). More specifically, when comparing the male cross of the X-poisoning strain 739_1_c with the reciprocal female cross, we observed only a slight reduction in hatching and adult emergence (t-test, 6% with p=0.0416 and 9.6% with p=0.0089, respectively). Even more strikingly, in offspring from male crosses of the 952_1;952_2_a strain there was no difference in survival between male and female crosses at any stage of development (**Figure 3A)**. The sex ratios at the adult stage in stains 739_1_c and 952_1;952_2_a were consistent with our previous findings, exhibiting a strong male bias in all male crosses and no sex bias in female crosses (t-test, p<0.0001 and p=0.0382 for 739_1_c and 952_1;952_2_a, respectively) (**Figure 3A**). We observed relatively low survival of larvae that became pupae in offspring of 952_1;952_2_a parents, but concluded that it was independent of SRD activity, as it manifested in both the male and female reciprocal control cross and not in subsequent experiments (see below).

The absence of obvious postzygotic lethality during development coupled with the effective sex ratio distortion led us to consider the possibility that targeting loci on the X-chromosomes, even at single site, could result in meiotic drive through the removal of X-bearing gametes similarly to X-shredding, favoring the transmission of Y-bearing sperm (**Figure 1**). To investigate this, we genotyped individual embryos from crosses between SRD males and wild type females using diagnostic PCR primers that bind to abundant sex chromosome linked satellite DNA, producing amplicons of distinctive size from male and female genomic DNA[18] (**Figure 3C**). A total of 352 embryos were individually PCR-genotyped, over two sets of

experiments that were performed for all crosses on consecutive days using developmentally staged cages. For both X-poisoning strains, the proportion of male embryos out of the total number of embryos tested was significantly greater than in the wild type cross (Fisher's Exact test, p=0.0003 and p=0.0006 for 739_1_c and 952_1;952_2_a, respectively), suggesting that biased transmission of Y-bearing sperm may be the mechanism of sex ratio distortion, resulting in the absence of clear postzygotic lethality (**Figure 3B**). We also performed this analysis with the combined X-poisoning/X-shredding strain Xshrd;952_1 and observed similar results (Fisher's Exact Test, p<0.0001).

Tracking Y-chromosome transmission and sex-specific survival in mosquito and fly SRD strains

Given these unexpected results, we sought to examine further how Cas9 targeting of mosquito X-linked genes affects sex chromosome transmission dynamics, and to track in more detail sex-specific survival of offspring. More specifically, we were interested in precisely quantifying sex-specific survival of sons and daughters from each of the male crosses, rather than measuring overall survival rates agnostic of offspring sex. To discriminate between male and female individuals from first-instar larval stage (L1), we crossed males from a transgenic strain carrying a Y-linked eGFP marker (Ygfp) to females of the 739_1_c, 952_1;952_2_a, and Xshrd;952_1 strains, to obtain males carrying both a Y-chromosome linked marker and the CRISPR SRDs. We then established male and control (Ygfp-only males) crosses and tracked lethality of both sexes throughout development. A significantly higher frequency of males (eGFP+) was detected among newly hatched L1 larvae with all strains compared to control (Dunnett's Method, p=0.0029, p<0.0001 and p<0.0001 for 739_1_c, 952_1;952_2_a, and Xshrd;952_1, respectively) (**Figure 4A**). Hatching rates, as well as the number of eggs laid per female, were normal and did not differ from the control (**Figures 4B and Figure S5A**). Sex ratio distortion levels observed in L1 larvae in both 739_1_c (66.9%) and 952_1;952_2_a (75.8%) were not statistically different than frequencies of adult males completing development in our previous experiment (**Figure 2D)**, indicating there is no substantial additional female-specific mortality occurring during post-embryonic stages of development (**Figure 4C**). This was confirmed by directly comparing pupation and emergence rates of male and female F1s revealing

no significant differences in relative survival (**Figure S5B**). Interestingly, in the combined X-shredding-poisoning strain, the percentage of male L1s (95.4%) was higher than at the adult stage (t-test, $p<0.0001$) (**Figure 4C**), corresponding to a small but significant reduction in the emergence rate of males from this strain compared to females (t-test, $p=0.0368$) (**Figure S5B**) and is possibly due to a decrease in the emergence rate of transgenic males in this strain (t-test, $p=0.0362$) (**Figure S5C**).

To compare these results to previous observations in the *Drosophila* model, we re-created the best performing split Cas9 and X-poisoning sgRNA fly strains (βtub85D-cas9 and RpS6_2)[6]. We crossed the sgRNA strain to a Y-chromosome linked tdTomato marker (AByG[19]) to create a trans-homozygous sgRNA stock with a marked Y and males were then crossed to virgin Cas9 females. Triple-heterozygous males containing all X-poisoning components and the marked Y-chromosome were then crossed to wild type virgin females. Unlike mosquito strains and as previously described, we observed significant decrease in embryo hatching rates (63%) compared to βtub85D-cas9-only (82.8%) and wild type (89.9%) controls (Tukey-Kremer, $p=0.0019$ and $p=0.0001$, respectively) coupled with a male bias within freshly hatched fly larvae (58.6% compared to 47.2% and 48.9%, Tukey-Kremer, $p=0.0076$ and $p=0.0183$) (**Figures 4A and 4B**). The frequency of males among neonate larvae was significantly lower than male frequencies of adults (74.8%) (t-test, $p=0.0048$) (**Figure 4C**), indicating compounding female-specific mortality occurring during larval and/or pupal stages of development, in agreement with our original results[6]. Our results confirm that, contrary to our previous model (**Figure 1**), targeting of X-linked genes in mosquito sperm induces meiotic drive resulting in enhanced transmission of Y-bearing gametes at the cost of X-chromosome inheritance, unlike in *Drosophila* where similar activity against the X-chromosome does not affect sex chromosome inheritance and postzygotic induction of female-lethality indeed forms the basis for the observed sex ratio imbalance.

Exploring the compatibility of premeiotic Cas9 expression for meiotic drives targeting RP genes

When considering ultimate deployment of SRDs in the field, linkage to the Y-chromosome is an essential step, affording SRDs an evolutionary advantage by shielding them from negative selection and enabling Y-chromosome drive in the case of X-shredding, or stable post-release dynamics in the case of X-poisoning. Since early expression of transgenes is similar between Y and autosomal constructs[19,20], we sought to explore how earlier sperm Cas9 expression would impact the mechanism of sex ratio distortion and male fitness in our strains targeting RP genes. To leverage the mosquito strains already at hand, we generated an additional transgenic strain expressing Cas9 from the germline-specific *zpg* regulatory elements[21] that are active from spermatogonia stages[22]. We did not include additional sgRNAs in this construct, which was marked with the eCFP transformation marker to distinguish it from our X-poisoning constructs, marked with DsRed. We inserted the zpgCas9 transgene in the X1 attP site[23] on chromosome 2L and then crossed zpgCas9 males to females of the two active X-targeting strains (739_1_c and 952_1;952_2_a) and also one inactive strain, which did not induce sex ratio distortion in our earlier experiments (952_1;952_2_b).

We were not able to isolate any adult trans-heterozygotes for the cross of zpgCas9 and our best SRD strain 952_1;952_2_a, as all died at the larval stage. Trans-heterozygotes expressing both markers were significantly underrepresented already at L1 larvae, compared to single marker individuals and when found were substantially smaller than larvae carrying single transgenes, reminiscent of the *Drosophila* minute phenotype that is typical of mutants of ribosomal protein genes[24] (data not shown). By contrast, trans-heterozygotes containing the zpgCas9 and either the 739_1_c or 952_1;952_2_b transgenes were viable to adulthood and occurring at the expected Mendelian frequencies. Trans-heterozygous zpgCas9;739_1_c males crossed to wild type females sired no offspring (**Figure 5A**). When these males were dissected, we found abnormal, undeveloped testes that lacked mature sperm (**Figure S6**). On the other hand, trans-heterozygous zpgCas9;952_1;952_2_b were viable and fertile. Egg hatching rates fluctuated between replicate crosses and were lower on average (34.6%) compared to the control (93%) (Tukey-Kremer, p=0.0014) (**Figure 5A**). When reared to adulthood, offspring from these males displayed a significant male

bias (74.4%, t-test, $p<0.0001$) (**Figure 5B**). We found no obvious correlation between replicates with low hatching rates and those with higher male frequencies ($\rho=-3.714$, $p=0.4685$), suggesting that the lower average hatching rates are not linked to the sex ratio distortion. Since on its own the 952_1;952_2_b strain had not shown sex bias in the offspring of transgenic males (**Figure 2D**) and testes of this strain had low Cas9 expression according to our RNA-seq data (**Figure S3A**), we conclude that the activation of sex ratio distortion in trans-heterozygous males must have been driven by *zpg* driven Cas9 expression early in spermatogenesis.

Discussion

In this study we sought to transfer the X-poisoning system to the malaria vector *An. gambiae*, aiming to test a novel approach to control mosquito populations. Following the original design from *D. melanogaster*[6], our system targeted three X-chromosome linked ribosomal protein (RP) genes. We also targeted the X-chromosome with a combination of X-poisoning and X-shredding sgRNAs, predicting that sex ratio distortion levels could be boosted by eliminating daughters at both prezygotic and postzygotic stages. To do this, we generated and then characterized multiple transgenic mosquito strains harboring both the Cas9 and single or dual sgRNAs and uncovered unexpected patterns of female elimination and sex ratio distortion. Our findings demonstrate that the X-poisoning system can be used to induce a male-biased sex ratio distortion in *An. gambiae*; however, the mechanism underlying this distortion appears to differ fundamentally from what has been described in *D. melanogaster*.

We found that independent transgenic strains demonstrated varied levels of sex ratio distortion, with three strains showing a significant male bias. Male bias among strains carrying the same construct was consistently linked with the level of Cas9 expression in male testes. Since all constructs shared the *β2-tubulin* regulatory elements to drive expression of Cas9, differences between strains in total Cas9 expression were most likely an outcome of the chromosomal location that the transgenic construct landed in. Using amplicon sequencing, we measured the relative activity of every sgRNA used and found that all were able

to generate mutant alleles in the testes of transgenic sperm. The frequency of mutant alleles represented a minor fraction of all sequenced amplicons, but this was not surprising given that the *β2-tubulin* promoter is only activated late in spermatogenesis[25], around meiosis, and that therefore most X-chromosome alleles assayed in genomic DNA from whole testes would be derived from premeiotic sperm and further diluted somatic testicular epithelial cells. Similarly, mutant alleles were recovered only rarely from surviving adult daughters and not for all strains and sgRNA sites. Since our X-linked target genes should be haplolethal in nature, this was not surprising because daughters harboring mutation at these genes would not survive to adulthood. Indeed, when mutant alleles were present in adult daughters, they exclusively contained in-frame deletions.

Our initial experiments indicated a surprising absence of female-specific mortality during development in the strains displaying sex ratio distortion and targeting single RP genes. This is in contradiction with the expected outcome of the X-poisoning mechanism, whereby mutant X-chromosomes harboring non-functional alleles of haploinsufficient genes are transmitted from transgenic males to daughters, preventing them from completing development (**Figure 1)**. This unexpected observation compelled us to use a number of independent methods to explore the mechanism underlying the observed sex bias in mosquito male crosses, including single embryo genotyping and tracking of sex-specific lethality during development using Y-chromosome linked transgenic markers. We also re-generated our best performing X-poisoning components in transgenic flies[6] to precisely measure sex-specific lethality during development and re-testing our previous findings. Doing so validated also the use of a marked Y-chromosome enabling direct comparisons to our mosquito data. In flies, our experiments confirmed our initial conclusion that X-poisoning sex ratio distortion results from strong postzygotic lethality of daughters during embryogenesis and later during pre-adult stages[6]. Conversely, the mosquito experiments supported a mechanism of sex bias predominantly acting as a meiotic drive, i.e., resulting in the preferential loss of X-bearing gametes and favoring the transmission of Y-bearing sperm. As such, our results are analogous to the observations for X-shredding in *An. gambiae*[2-4], *D. melanogaster*[6] and *C. capitata*[7]. Overall, our findings suggest

fundamental differences exist in the pathways associated with X-chromosome breaks and repair during spermatogenesis between *An. gambiae* and *D. melanogaster*. When trying to account for these differences, it could be interesting to consider whether the lack of recombination in the male fly germline somehow protects from the loss of targeted X-gametes and enables their transmission to daughters. While many questions regarding the underlying mechanisms of preferential inheritance remain unanswered and should be further explored, our work underscores the importance of carefully accounting for the basis of sex bias when designing SRD-based genetic control systems, as these can have significant practical implications[8].

In the context of an engineered SRD, our results suggest that meiotic drives in *An. gambiae* could theoretically be built entirely agnostic of the target sequence's function or copy number on the X-chromosome. This could be explored in future studies by targeting sequences with no expected function or that are non-essential to development, for example intronic or intergenic X-linked sequences, or an X-linked phenotypic marker. It may also be interesting to explore whether the effect we observed depends on the specific location of the target site along the X-chromosome.

Regardless of the timing of female elimination, the ultimate goal for any engineered SRD alleles would be to be physically linked to the Y-chromosome, shielding the allele from negative selection in females and ensuring transmission to all sons. Previous studies have shown that expression from the *An. gambiae* Y-chromosome using the *β2-tubulin* promoter is suppressed, likely due to meiotic sex chromosome inactivation (MSCI)[26], whereas expression from promoters that are active earlier during spermatogenesis is possible from the Y, both in *An. gambiae* and *Dr. melanogaster*[19,20]. However, despite being conceptually the simplest way to circumvent MSCI, early expression of Cas9 may not be compatible with meiotic drive and/or male fertility. In *Drosophila*, transgenic males expressing X-poisoning sgRNAs and Cas9 driven by the *nanos* (*nos)* promoter, which is active in early germline stem cells, resulted in male sterility[6]. When combined with X-shredding, nosCas9 did not result in significant sex ratio distortion although high levels of activity could be detected[6]. In mosquitoes, despite significant efforts focused on generating X-shredding SRDs using I-*Ppo*I or Cas9 driven by premeiotic regulatory elements like *vasa*, which are also active in

early germline stem cells, a stable transgenic strain has never been isolated, likely as an outcome of induced male sterility or leaky expression in somatic tissues.

We therefore sought to test whether the meiotic drive dynamics we observed with X-poisoning sgRNAs might be compatible with early Cas9 expression. When we crossed several X-chromosome targeting strains to an autosomal Cas9 driven by the regulatory regions of *zpg*, which drive expression in the male germline prior to *β2-tubulin*, we observed distinct outcomes: when combined with the X-poisoning strain that consistently yielded the highest levels of sex ratio distortion (952_1;952_2_a), trans-heterozygous offspring inheriting both elements were unviable with few surviving larvae displaying minute-like phenotypes. Since zpgCas9 was introgressed from males, it is more likely that this phenotype was caused by leaky somatic Cas9 expression from one or both transgenes leading to somatic mutagenesis of *RpL37*, and not because of maternal deposition of Cas9. With the second active X-poisoning strain 739_1_c, trans-heterozygous males were sterile and contained morphologically atrophic testes without developing sperm, resembling the morphology of testes from *zpg* null or RNAi mutants[27,28]. zpgCas9/739_1_c trans-heterozygous males were sterile, similarly to nosCas9/RPS6 trans-heterozygous males in *Drosophila*, suggesting that disruption of the target gene *RpS10* and ribosome function is intolerable at this stage of spermatogenesis. However, the addition of zpgCas9 to the previously inactive strain 952_1;952_2_b, resulted in significant male-bias in the offspring. Since there was no correlation between hatching rates and sex ratio across replicates, we conclude that male-bias is likely an outcome of prezygotic meiotic drive and not by transmission of mutated X-chromosomes resulting in postzygotic lethality of daughters.

We hypothesized that fertility of SRD males expressing premeiotic Cas9 could depend on whether their autosomal paralogs can functionally rescue the depletion of ribosomal components encoded by the X-chromosome. Our results suggest that the autosomal AGAP0011501 can rescue *AgRpL37e* functions, when the X-linked copy (AGAP000952) is targeted by a *zpg* driven Cas9-SRD, but that the autosomal AGAP005469 gene cannot rescue the targeting of the X-linked *AgRpS10* (AGAP000739). This conclusion is supported by RNA-seq data measuring genome-wide expression at four stages of sperm development,

namely premeiotic, early & late meiotic and postmeiotic sperm cells[29] (**Figure S1C)**. For *AgRpL37e*, the autosomal gene (AGAP0011501) is more abundantly expressed throughout spermatogenesis compared to its X-linked copy (AGAP000952). The inverse was the case for *AgRpS10*, at least during early spermatogenesis, when the expression of the X-linked RP gene (AGAP000739) is around 6-fold higher than its autosomal paralog. Furthermore, at the sequence level, *AgRpL37e* paralogs are more similar to each other (67% similarity, 63% identity), than the paralogs of *AgRpS10* (61% similarity, 54% identity) (**Figure S2**). To the best of our knowledge, this is the first demonstration of an autosomal endonuclease-based SRD expressed in early spermatogenesis, before the onset of MSCI, resulting in significant male-biased sex distortion. This finding may enable the generation of active Y-linked SRDs leveraging zpgCas9 meiotic drives.

Materials and Methods

Mosquito Rearing

An. gambiae used in this work was derived from the G3 laboratory colony of Imperial College London in November 2019. All transgenic mosquito strains were reared under standard conditions at 28°C and 80% relative humidity at ACL-2+ containment. Larvae were provided with commercial fish food, while adult mosquitoes were given a 10% (wt/vol) glucose solution. Adult mosquitoes were allowed to mate for 4-7 days post-emergence and blood-fed on bovine blood using the Hemotek membrane feeding system (Hemotek, Ltd). 72 hours post blood-feeding, an egg-bowl was provided overnight to collect eggs. Laid eggs were placed in plastic trays containing 500 mL of demineralized water for hatching. 48 hours later, L1 larvae were transferred to clean trays containing 500ml of demineralized water at a density of 250 larvae per tray and provided with fish food solution daily until pupation, according to standard protocols[30].

Transgenic constructs

All cloning was performed using the Gibson assembly (NEBuilder HiFi DNA Assembly, New England Biolabs). PCR reactions were performed utilizing the Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs). Plasmids were sanger-sequenced to confirm the presence of all inserts. Primer and oligo sequences utilized for plasmid construction can be found in Table S1. To create the mosquito X-poisoning constructs, we utilized the previously described *piggyBac* vector p167[4], which includes a 3xP3-DsRed transformation marker, hCas9 driven by the β2tubulin promoter and 3' UTR regulatory sequence, along with a U6:sgRNA cassette housing a spacer cloning site bordered by BsaI restriction sites on both ends. Selection of sgRNA target sites was done with CHOPCHOP[31], using the AgamP4 assembly and parameters for Cas9 knock-out. All single guide RNAs constructs were generated by annealing complementary oligos carrying unidirectional BsaI compatible overhangs. The resulting plasmids, which targeted AGAP000952, AGAP000953, and AGAP000739, were named 952_2, 953_1 and 739_1 respectively. To construct the plasmids containing two distinct sgRNAs, one targeting AGAP000952 and the other of the X-poisoning and X-shredding sgRNAs, fragments containing both sgRNAs and the internal U6 promoter was generated separately by gene synthesis (Genewiz), amplified and and cloned into a BsaI-digested p167 backbone, yielding the final vectors: 952_1, 952_2, and Xshrd,952_1, respectively. The zpgCas9 construct was cloned by Gibson assembly of amplified fragments of hCas9, 3xP3 promoter, eCFP (from Addgene #117209) into an XhoI-digested *piggyBac* vector containing also an attB site. The intermediate plasmid was digested by AscI and DNA fragments containing the *zpg* 5' and 3' regulatory regions (~1 kbp each) that were amplified from the genomic DNA were cloned. To re-build the *Drosophila* X-poisoning constructs, DNA fragments of βtub85D 5' and the βtub56D 3' regulatory region were amplified from genomic DNA of the w^{1118} strain. The fragments were inserted into an XhoI and PsyI linearized plasmid (Addgene #112685) harboring the Cas9-T2A-eGFP expression cassette and an Opie2-DsRed marker. The RpS6_2 sgRNA plasmid was generated following the method described in Fasulo et al. (2020)[6]. Annealed oligos were assembled into

BbsI-linearized plasmid (Addgene #49410) containing the U6 promoter and the 3xP3-eGFP transformation marker.

Generation of transgenic strains

To generate mosquito transgenic strains, we performed microinjections of *An. gambiae* G3 embryos as described in Volohonsky et al. (2015)[23]. Embryos were injected with a mixture consisting of 0.4 μg/μl transformation plasmid and 0.2 μg/μl helper plasmid containing a *vasa*-driven *piggyBac* transposase. Injected larvae were screened for transient expression of the 3xP3-DsRed marker, and positives were individually back-crossed to reciprocal wild types to obtain transgenic F1 individuals. Since *β2-tubulin* is only active in the male germline, all transgenic strains were maintained by crossing heterozygous transgenic females to wild type males. The zpgCas9 strain was generated by microinjections into embryos of the X1 docking strain[23] with injection mixtures consisting of 0.33 μg/μl of the transformation plasmid and 0.08 μg/μl *vasa*-driven phiC31 integrase helper plasmid[32] (Addgene #62299). In this case, all surviving larvae were reared, and pupae were separated into male and female cages and crossed with X1 males or females respectively. To obtain the F1 transgenic population, larvae were screened for eCFP and separated accordingly after which transgenics were intercrossed to establish a homozygous population.

Measuring sex ratio of mosquito offspring

Crosses of 50 heterozygous transgenic males from each strain and 80 wild type females were established to measure sex ratio of adult offspring. The reciprocal female crosses (80 heterozygous transgenic females with 50 wild type males) and a wild type cross were also performed. For each cross three replicates of 250 random neonate larvae were reared to adulthood, and the total number of male and female mosquitoes was subsequently counted. To assess the stability of observed sex ratios, the experiment was repeated for at least three generations per cross. Statistical analysis was done using a One-Way ANOVA of the percentage of F1 adult males by cross, followed by Tukey-Kramer HSD for comparisons between all pairs ($p \leq 0.05$).

Quantification of Cas9 expression by RNA-seq

Virgin 2-4 day-old transgenic males were dissected to obtain a sample of 60-80 testes, which were separated from accessory glands and other somatic tissues. As a control, a sample of 80 testes dissected from wild type males was used. Samples were frozen in liquid nitrogen and mechanically homogenized before total RNA extraction was performed using the Quick-RNA™ Microprep Kit (Zymo Research). 200ng from each sample were transferred to GenTegra-RNA tubes (Syntezza Bioscience, Ltd) and lyophilized with an Alfha1-4 lyophilizer (Martin Christ). mRNA libraries were prepared using poly-A capture (Syntezza Bioscience, Ltd), and samples were sequenced using the NovaSeq NGS Sequencer with paired-end reads of 150 nucleotides, generating a total of 20 million reads (Novogene Co., Ltd). Reads were mapped using HiSat2 to Cas9 coding sequence to quantify abundance in each sample and reads were normalized to Transcripts Per Million (TPM).

Amplicon sequencing of targeted X-chromosomes in sperm and daughters

Genomic DNA was extracted from a pool of 25 posterior abdomen sections containing testes derived from 3-5 day-old adult transgenic heterozygous males. As controls, genomic DNA was also extracted in the same manner from wild type males. For testes samples, genomic DNA was extracted from a pool of 40 individual testes dissected from transgenic and wild type males. For surviving female samples, genomic DNA was extracted from a pool of four non-transgenic virgin daughters, from a cross between transgenic males with wild type females and wild type females were used as control. Amplification of target regions, spanning 369-428bp, was performed across all samples using primers incorporating Illumina adapters (see Table S2 for primer sequences). Amplicons were purified using the DNA Clean & Concentrator kit (Zymo Research Corp.) and sent for additional 15 cycles of amplification and sequencing using Illumina NGS with paired-end reads and a reading depth of 100 million reads. To detect mutations at the target site, CRISPResso[33] was used on raw sequencing data. The frequency of unique alleles not matching the native sgRNA target sites in terminal segments and testes was obtained by removing alleles with a perfect match to reference

sgRNA sequence, as well as alleles that appear less than 10 times in the dataset. We also removed any alleles that did not match the reference but present in wild type samples, to obtain sequences and frequencies of CRISPR-induced mutant alleles.

Measuring egg to adult survival

Eggs obtained from a cross between 30 heterozygous transgenic males and 40 wild type females were divided into ten replicates, with each replicate containing 50 eggs. As a control, eggs from the reciprocal female cross were included. Survival was measured daily by counting live individuals in each batch until the emergence of adults. Survival rates were calculated for three stages: hatching (% larvae from eggs), pupation (% pupae from larvae), and adult emergence (% adults from pupae). Adults were separated by sex and counted to determine the sex ratios in each replicate. For statistical analysis we performed one-sided t-tests, assuming equal or unequal variances, comparing the survival rates of the F1 population from the male cross to those from the female cross at each stage ($p \leq 0.05$), and comparing the percentage of F1 adults between the male and female crosses ($p \leq 0.05$).

PCR-based single embryo genotyping

To determine the sex of individual embryos, we used PCR primers 124678F2 and 124678R2 from Krzywinski et al. (2005)[18] binding to the AgY477 and AgX367 satellites located on the Y and the X-chromosomes, respectively. Genomic DNA samples were collected from 24-hour-old eggs from a cross of 10 heterozygous transgenic males and 20 wild type females. For each cross, eggs were obtained from a single cage over two sets of experiments conducted on consecutive days. Each egg was individually probed with a sterile tip to obtain the DNA sample, which was then immediately placed into a numbered PCR reaction (Platinum™ Green Hot Start PCR 2X Master Mix - Invitrogen). The sex-specific banding pattern following gel electrophoresis was evaluated for each egg to determine its sex. Samples that did not exhibit the 367 bp non-sex-specific product were labeled as unsuccessful reactions and were excluded from

downstream analysis. For the statistical analysis of this experiment, we employed a left-sided Fisher's Exact Test to compare the proportion of male eggs in each transgenic cross to the wild type control ($p \leq 0.05$).

Tracking sex-specific survival using fluorescently marked Y-chromosome

To obtain males carrying both the CRISPR SRD alleles and a fluorescently marked Y-chromosome, we crossed 20 transgenic females from each active SRD strain with 10 males from a strain harboring a Y-linked 3xP3-eGFP marker (Ygfp), generously provided by Dr. Jaroslaw Krzywinski. Groups of 5 trans-heterozygous males were then mated with 5 G3 virgin females in 4-7 replicates per cross. Eggs from each replicate were manually counted and then normalized to the number of living females within the cage, to calculate the average eggs per female number. Hatching rates were calculated by manually counting all hatching larvae from each replicate. To determine the proportion of male (eGFP+) and SRD+ (DsRed+) larvae were screened for both markers. For statistical analysis, we used a One-Way ANOVA to evaluate hatching rates and the percentage of males for each cross, following post-hoc comparisons of each strain against the Ygfp/+ control using Dunnet's method ($p<0.05$). Two-sided t-tests, assuming equal or unequal variances, were used to compare the percentage of males at the L1 larval stage with the percentage of males at the adult stage observed in the previous experiments ($p<0.05$). To track the survival rates of male versus female offspring, 30 random larvae of each phenotype (males, females, SRD+, SRD-) for each cross were reared together in trays until adulthood. Survival rates were assessed at two stages: pupation (% pupae from larvae) and adult emergence (% adults from pupae). For statistical analysis, paired t-tests, assuming equal or unequal variances, were used to compare the survival rates of male individuals with that of female individuals at both pupation and adult emergence stages. Additionally, same paired t-tests were conducted to compare the survival rates of SRD+ male individuals with those of SRD- male individuals at both pupation and adult emergence stages ($p<0.05$).

Re-examining distortion and female lethality from X-poisoning in D. melanogaster

Fly husbandry and crosses were conducted under standard conditions at a temperature of 25°C with a 12/12-hour day and night cycle. Fly injections were performed by Rainbow Transgenics (Camarillo, CA, USA). The βtub85D_cas9 construct was introduced into the third chromosome using attP site VK00033 (BDSC #9750) and included an opie2-DsRed marker. The RpS6_2 sgRNA construct was integrated into the genome at the P{CaryP}attP40 site on the second chromosome (BDSC #25709), containing a 3xP3-eGFP marker. Transgenic strains were balanced using w^{1118};Cyo;Sb/TM6B and maintained as homozygotes. Female virgins carrying the βtub85D-cas9 construct were crossed with male from the RpS6_2 sgRNA strain. At least ten trans-heterozygous RpS6_2/βtub85D_cas9 males crossed with an equal number of w^{1118} females. Sex ratio distortion was evaluated by determining the percentage of males and females among adult progeny. This experiment was replicated eight times each. To ascertain the developmental stage at which sex-specific mortality occurred, female virgins from the RpS6_2 strain were mated with males carrying a tdTomato marker linked to the Y-chromosome under the control of the 3xP3 promoter (AByG, BDSC #78567) to generate RpS6_2 males with the Y-chromosome marker. Subsequently, these males were crossed with female virgins carrying the βtub85D-cas9 construct to obtain triple-heterozygous males carrying both the X-poisoning components and the marked Y-chromosome. To assess egg hatching rates and the male ratio among L1 larvae, 20–30 triple-heterozygous males AByG/βtub85D/RpS6_2 and 20–30 w^{1118} virgin females were placed in embryo collection cups with grape juice agar plates coated with yeast paste for 1-2 hours. The number of eggs and hatching larvae were subsequently counted to calculate the hatching rate. Progeny were scored for 3xP3 fluorescence to identify males at the L1 stage. Two additional crosses involving AByG/+/+ and AByG/βtub85D_cas9/+ males crossed with w^{1118} females were conducted as controls, with each cross replicated four times. Statistical analysis of hatching rates and percentage of L1 males was done using a One-Way ANOVA, following post-hoc comparisons of all pairs using Tukey-Kramer HSD ($p<0.05$). A two-sided t-test, assuming equal variance, was used to compare the percentage of males at the L1 larval stage with the percentage of males at the adult stage ($p<0.05$).

Assessing sex distortion from zpgCas9 and X-poisoning trans-heterozygotes

To obtain trans-heterozygous males expressing both zpgCas9 and the X-poisoning constructs, 40 zpgCas9 males were crossed to 50 X-poisoning females from each strain. When trans-heterozygous males could be generated expressing both DsRed and eCFP markers, they were crossed in groups of 40 to 50 G3 females. As a control, a cross between male zpgCas9 and G3 females was established. 48 hours after blood feeding females were separated into subgroups of 5 individuals and provided egg-bowls. Hatching rates were calculated by manually counting all eggs and hatching larvae from each replicate. All hatched larvae were reared to adulthood, measuring sex at the pupal and adult stage. Statistical analysis of hatching rates was done using a One-Way ANOVA, following post-hoc comparisons of all pairs using Tukey-Kramer HSD ($p<0.05$). Statistical analysis of percent adult F1 males was done using a one-sided t-test assuming equal variance.

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Authors contributions - by Contributor Role Taxonomy (CRediT)

Conceptualization – DAH, YA1, NW, PAP; **Methodology –** DAH, YA1, YA2, PAP; **Resources** – EK, JK, EM, PAP; **Validation –** DAH, YA1, PAP; **Formal Analysis –** DAH, YA1, PAP; **Investigation –** DAH, YA1, LBL, YA2, CZ, FK, ESY, RDA, PAP; **Writing - Original Draft Preparation –** DAH, YA1, LBL, PAP; **Writing - review and editing –** DAH, YA1, NW, PAP; **Visualization –** DAH, YA1, PAP; **Supervision –** PAP; **Funding Acquisition –** PAP.

Ethics

All animals were handled in accordance and under the supervision of the ARO Institutional Animal Care and Use Committee approval number 2307-118-2-VOL-IL. All insect work was performed in facilities maintaining Arthropod Containment Level 2. This work received Institutional Approval and relevant authorizations from the Israel Ministry of Environmental Protection and Ministry of Agriculture (#31/2019).

Figure captions

Figure 1. Mechanisms of synthetic CRISPR-based sex ratio distorters. A sex ratio distorting CRISPR endonuclease targeting the X-chromosome during spermatogenesis could act in one of two modes: (1) by mutating X-linked essential haploinsufficient genes resulting in postzygotic lethality of daughters inheriting targeted chromosomes (X-poisoning, left) or (2) by repeatedly cutting the X-chromosome, leading to a prezygotic meiotic drive through the loss of X-bearing sperm (X-shredding - right).

Figure 2. Development of X-chromosome targeting SRD strains in *An. gambiae.* (A) Relative position of candidate haploinsufficient target genes and the 28S rDNA cluster on the *An. gambiae* X-chromosome. **(B)** Schematic of transformation constructs. (pBac) *piggyBac* inverted repeats; (3xP3-DsRed) the 3xP3 promoter driving DsRed fluorescent marker; (*β2tub*-Cas9) codon-optimized CDS of Cas9 endonuclease under the control of the male germline specific *β2tubulin* promoter; (U6-sgRNA) sgRNA under the control of the ubiquitous U6 Pol III promoter. **(C)** Overview of experimental crosses. **(D)** Mean percentage of F1 adult males observed over at least three generations. n indicates the total number of adults counted in the F1 progeny. Different letters indicate statistical differences using Tukey-Kramer HSD. Error bars represent the standard error (SEM).

Figure 3. Sex ratio distortion and the timing of female elimination. (A) Egg-adult survival analysis of F1 populations from active X-poisoning strains, showing the mean survival rate at each developmental stage and the percentage of male adults in male and female crosses. n indicates the total number of eggs,

hatching larvae, pupae, and adults, respectively. Error bars represent the standard error (SEM). E-L: Egg to Larva (hatching), L-P: Larva to Pupa (pupation) and P-A: Pupa to Adult (adult emergence). **(B)** PCR-based genotyping of individual *An. gambiae* eggs using sex-specific markers. Proportions of male (XY) and female (XX) eggs in F1 populations of the wild type cross (WT) and male crosses from active SRD strains. **(C)** Examples of male and female PCR patterns used for genotyping analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 4. Comparison of sex distortion mechanisms between *An. gambiae* and *D. melanogaster*. (A) Mean percentage of male L1 larvae among offspring based on expression of a Y-chromosome linked fluorescent marker. n indicates the total number of larvae screened in each cross. Error bars represent the standard error (SEM). **(B)** Mean hatching rates of embryos from transgenic males and controls. n indicates the total number of eggs counted. Error bars represent the standard error (SEM). **(C)** Mean percentage of males at the L1 stage and at the adult stage. Error bars represent the standard error (SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 5. Premeiotic expression targeting of X-chromosome targets in *An. gambiae*. (A) Mean hatching rate and (**B**) sex ratios of offspring from X-poisoning strains crossed with zpgCas9 targeting X-linked ribosomal proteins. n represents the total number of eggs counted. n indicates the total number of adults counted in the F1 progeny. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure S1. Transcriptional dynamics of X-linked ribosomal proteins genes and autosomal paralogs. (A) Structure of targeted X-linked ribosomal protein genes indicating exons, introns, CDS and location and sequence of sgRNA target sites. **(B)** For each ribosomal gene, normalized expression in the male carcass and in male reproductive tissues (RT), obtained from Papa et al. (2017)[34], is plotted as a percentage of the sum of expression of both tissues combined. The functional name of each ribosomal protein gene is indicated in the corresponding facet subtitle. Gene IDs are shown within the plot (omitting “AGAP00”) and colored according to chromosomal arm. The diagonal intercept represents equal expression in somatic and

germline male tissues. Genes labeled "*sperm*" are expressed exclusively in sperm according to data from additional tissues. **(C)** For each ribosomal gene, normalized mean expression is shown (in Transcripts Per Million, TPM) at four stages of sperm development collected by FACS sorting in cell populations (CP); premeiotic (CP1), early (CP2) & late meiotic (CP3) and postmeiotic (CP4) sperm cells. RNA-seq data for this analysis was obtained from Taxiarchi et al. (2019)[29].

Figure S2. Similarity of *An. gambiae* X-chromosome ribosomal proteins and their autosomal paralogs. Protein sequence similarity (shown as % identity) matrix of mosquito ribosomal protein genes based on Clustal-Omega alignment.

Figure S3. Analysis of CRISPR activity (A) Relationship between Cas9 expression in testes (in Transcripts Per Million, TPM) and induced male bias at the adult stage in offspring of transgenic males of every X-targeting strain. **(B)** Frequency of unique alleles not matching the reference sgRNA target sites from amplicon sequencing of genomic DNA extracted from posterior, terminal abdomens (top) and dissected testes (bottom). n indicates the total number of unique alleles occurring at least 10 times in the data.

Figure S4. Analysis of CRISPR-induced mutant alleles in testes and daughters. This figure presents the top 10 most prevalent CRISPR-induced alleles (i.e., alleles which do not perfectly match the reference sgRNA target sites and were absent in wild type samples) in both testes and surviving daughters. The sample type, strain ID, and the reference sequence at the corresponding site, with the amino acid sequence highlighted in green, are shown. The Cas9 cut site is denoted by a red arrow, and the PAM site is highlighted in blue. Deletions are represented by dashes, while substitutions are shown in red. The number of deleted amino acids is indicated by Δ and the impact on the reading frame in red or green, for out- and in-frame mutations, respectively. Substitutions that result in amino acid replacement are indicated in red. n represents the total reads for all alleles occurring at least 10 times in the dataset. Additionally, the percentage of reads for each CRISPR-induced allele, relative to the total reads, is provided in brackets. **(A)** 739_1 target site

(B) 952_1 target site **(C)** 952_1 target site **(D)** 953_1 target site.

Figure S5. Supporting data of sex-specific mortality assays in *An. gambiae*. (A) Average number of eggs per female. **(B)** Survival rates at pupation (pupa-larva) and adult emergence (pupa-adult) of male (blue) vs. female (red) F1 progeny. **(C)** Survival rates at pupation (pupa-larva) and adult emergence (pupa-adult) of DsRed+ transgenic (red) vs. DsRed- non-transgenic (gray) male F1 progeny. *$p<0.05$.

Figure S6. Testes morphology of zpgCas9/739_1_c sterile males and controls. Male reproductive tissues of **(A)** wild type male **(B)** zpgCas9 homozygous male and (**C**) zpgCas9/739_1_c sterile male. Testes are indicated with a black arrowhead. Male accessory glands are indicated in white (MAGs).

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Figure 1

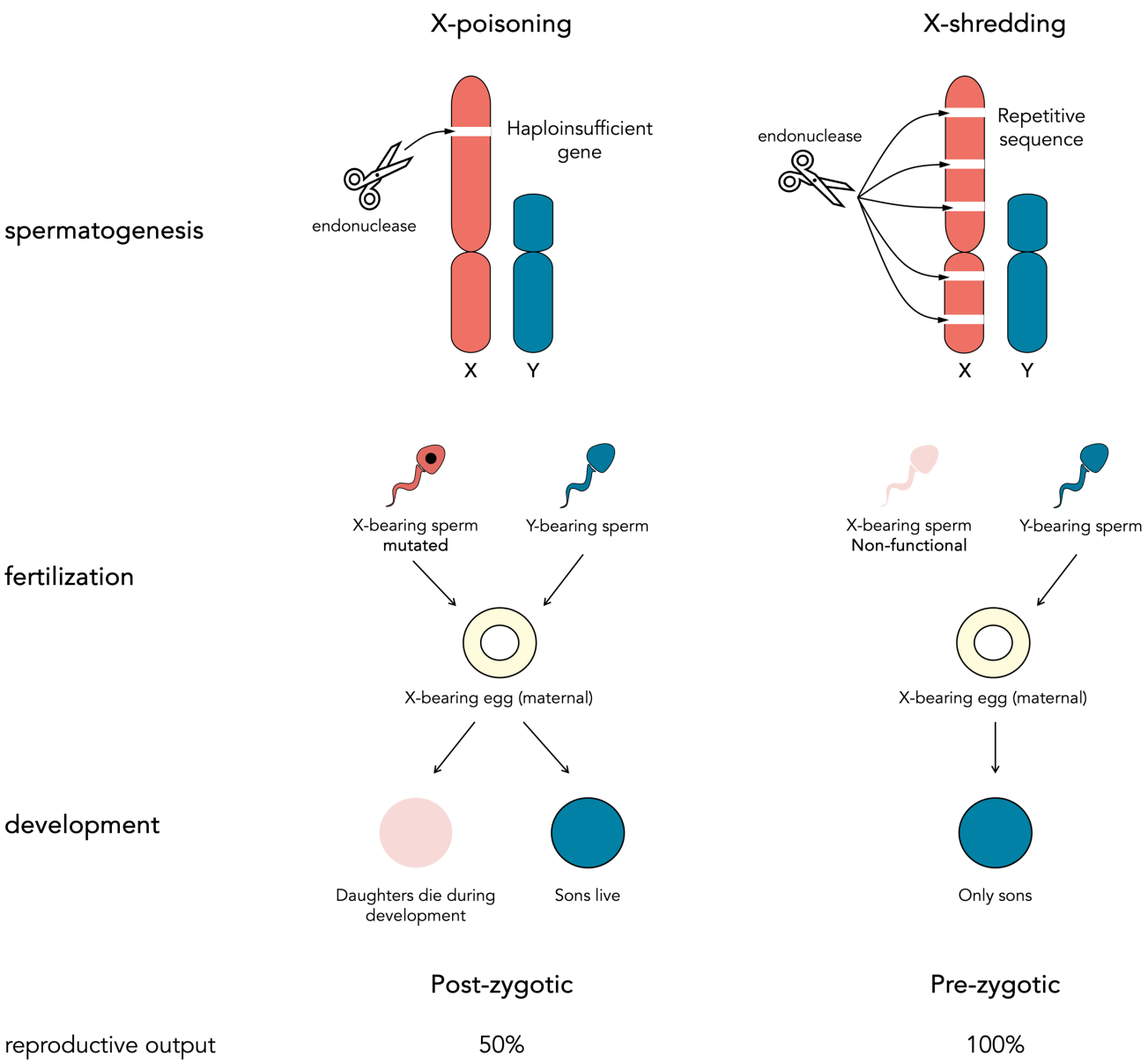

X-poisoning
X-shredding
spermatogenesis
endonuclease
Haploinsufficient gene
X
Y
endonuclease
Repetitive sequence
X
Y
fertilization
X-bearing sperm mutated
Y-bearing sperm
X-bearing egg (maternal)
X-bearing sperm Non-functional
Y-bearing sperm
X-bearing egg (maternal)
development
Daughters die during development
Sons live
Only sons
Post-zygotic
Pre-zygotic
reproductive output
50%
100%

Figure 2

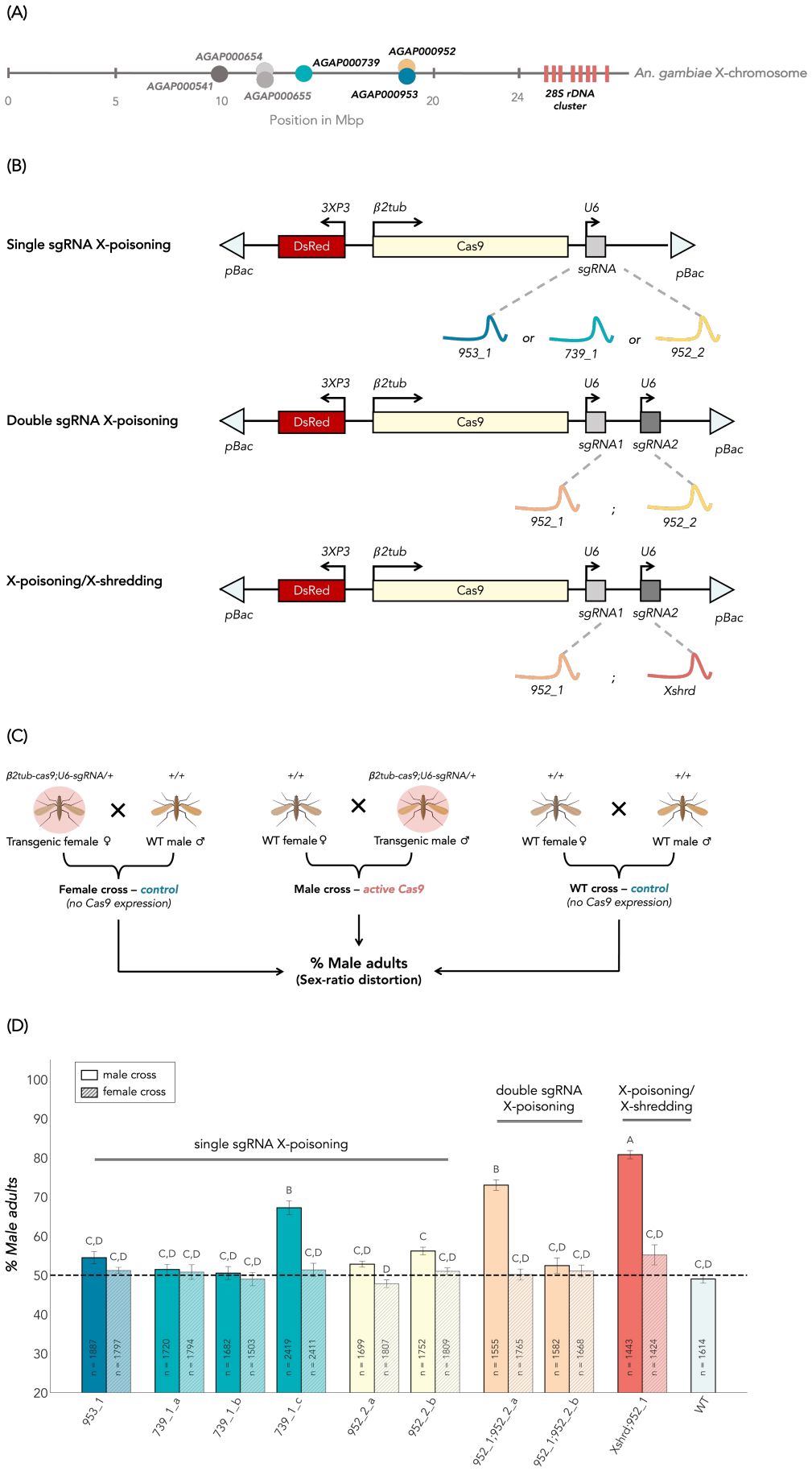

(A)
AGAP000654
AGAP000541
AGAP000655
AGAP000739
AGAP000953
AGAP000952
0
5
10
20
24
28S rDNA cluster
An. gambiae X-chromosome
Position in Mbp
(B)
Single sgRNA X-poisoning
3XP3
β2tub
U6
DsRed
Cas9
pBac
sgRNA
953_1
or
739_1
952_2
Double sgRNA X-poisoning
sgRNA1
sgRNA2
952_1
;
X-poisoning/X-shredding
Xshrd
(C)
β2tub-cas9;U6-sgRNA/+
+/+
Transgenic female ♀
WT male ♂
WT female ♀
Transgenic male ♂
Female cross – control
(no Cas9 expression)
Male cross – active Cas9
WT cross – control
(no Cas9 expression)
% Male adults
(Sex-ratio distortion)
(D)
male cross
female cross
% Male adults
single sgRNA X-poisoning
double sgRNA X-poisoning
X-poisoning/ X-shredding
953_1
739_1_a
739_1_b
739_1_c
952_2_a
952_2_b
952_1;952_2_a
952_1;952_2_b
Xshrd;952_1
WT

Figure 3

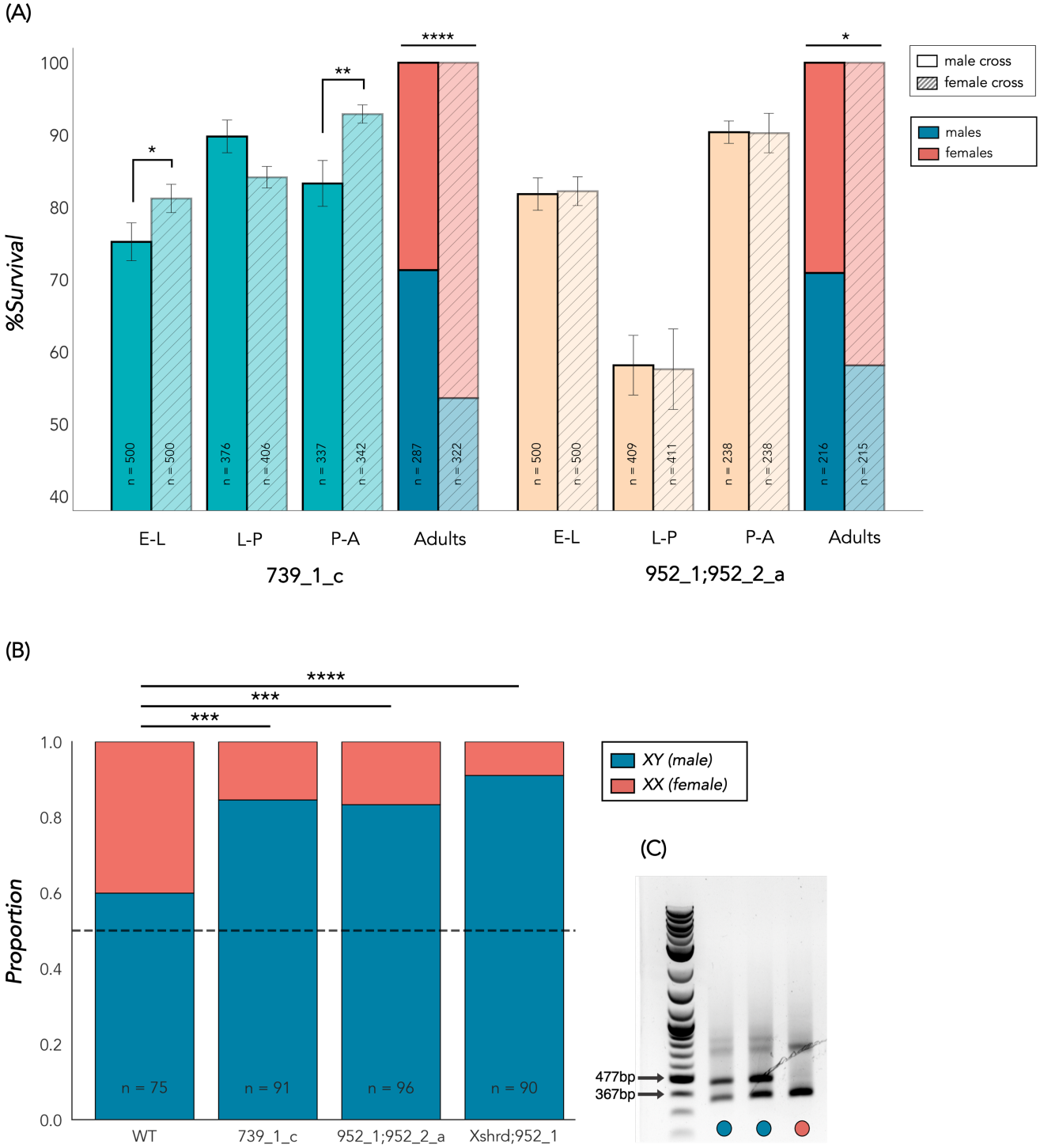

(A)
%Survival
100
90
80
70
60
50
40
*
**
****
*
male cross
female cross
males
females
n = 500
n = 500
n = 376
n = 406
n = 337
n = 342
n = 287
n = 322
n = 500
n = 500
n = 409
n = 411
n = 238
n = 238
n = 216
n = 215
E-L
L-P
P-A
Adults
E-L
L-P
P-A
Adults
739_1_c
952_1;952_2_a
(B)
Proportion
1.0
0.8
0.6
0.4
0.2
0.0
****
***
***
XY (male)
XX (female)
n = 75
n = 91
n = 96
n = 90
WT
739_1_c
952_1;952_2_a
Xshrd;952_1
(C)
477bp
367bp

Figure 4

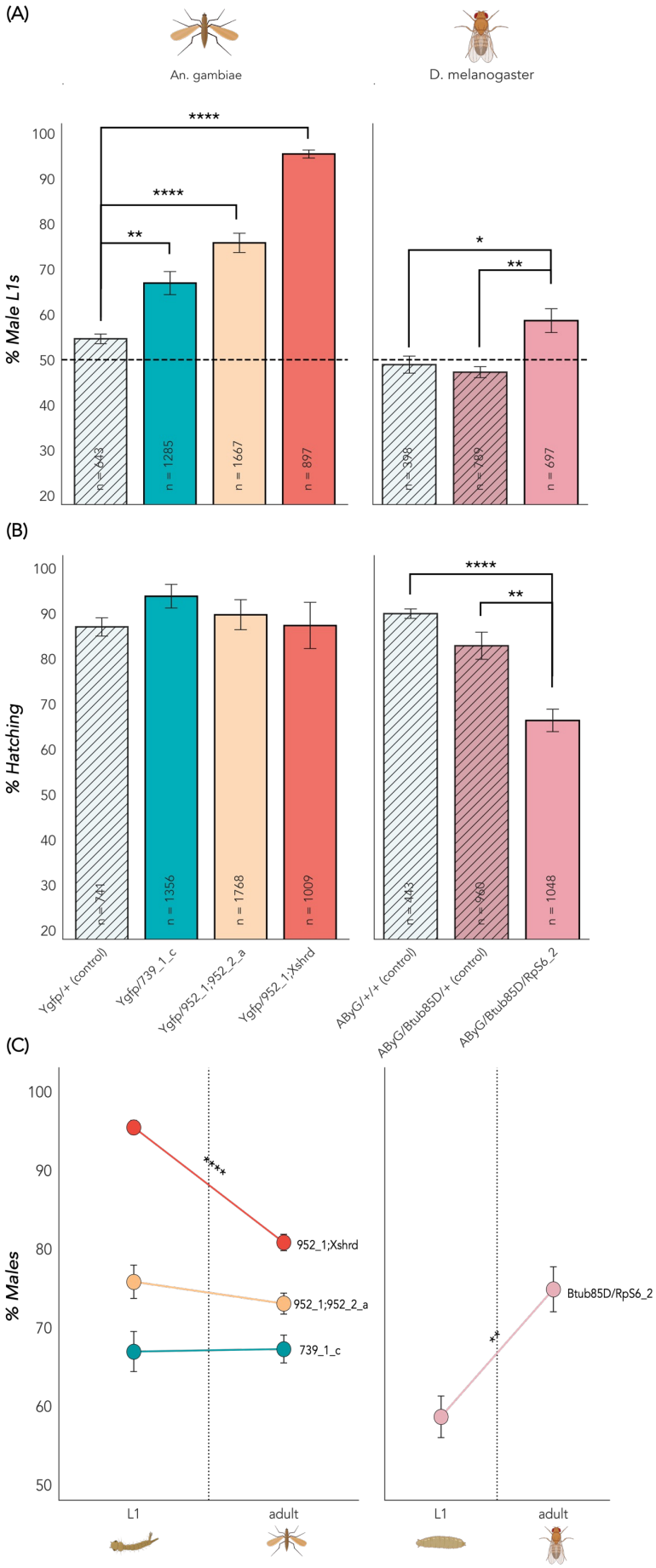

(A)
An. gambiae
D. melanogaster
% Male L1s
****
****
**
*
**
n = 643
n = 1285
n = 1667
n = 897
n = 398
n = 789
n = 697
(B)
% Hatching
****
**
n = 741
n = 1356
n = 1768
n = 1009
n = 443
n = 960
n = 1048
Ygfp/+ (control)
Ygfp/739_1_c
Ygfp/952_1;952_2_a
Ygfp/952_1;Xshrd
AByG/+/+ (control)
AByG/Btub85D/+ (control)
AByG/Btub85D/RpS6_2
(C)
% Males
****
**
952_1;Xshrd
952_1;952_2_a
739_1_c
Btub85D/RpS6_2
L1
adult
L1
adult

Figure 5

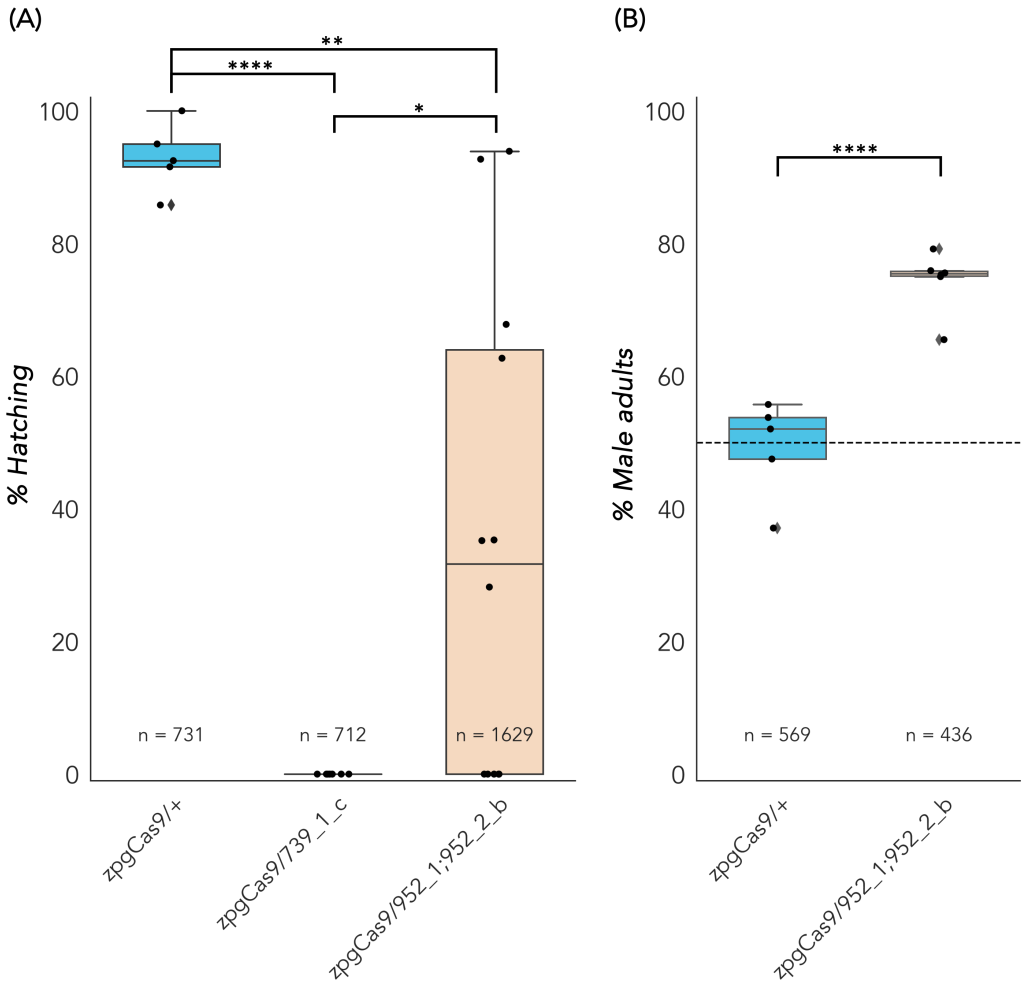

(A)
**
****
*
100
80
60
40
20
0
% Hatching
n = 731
n = 712
n = 1629
zpgCas9/+
zpgCas9/739_1_c
zpgCas9/952_1;952_2_b
(B)
****
100
80
60
40
20
0
% Male adults
n = 569
n = 436
zpgCas9/+
zpgCas9/952_1;952_2_b

Figure S1

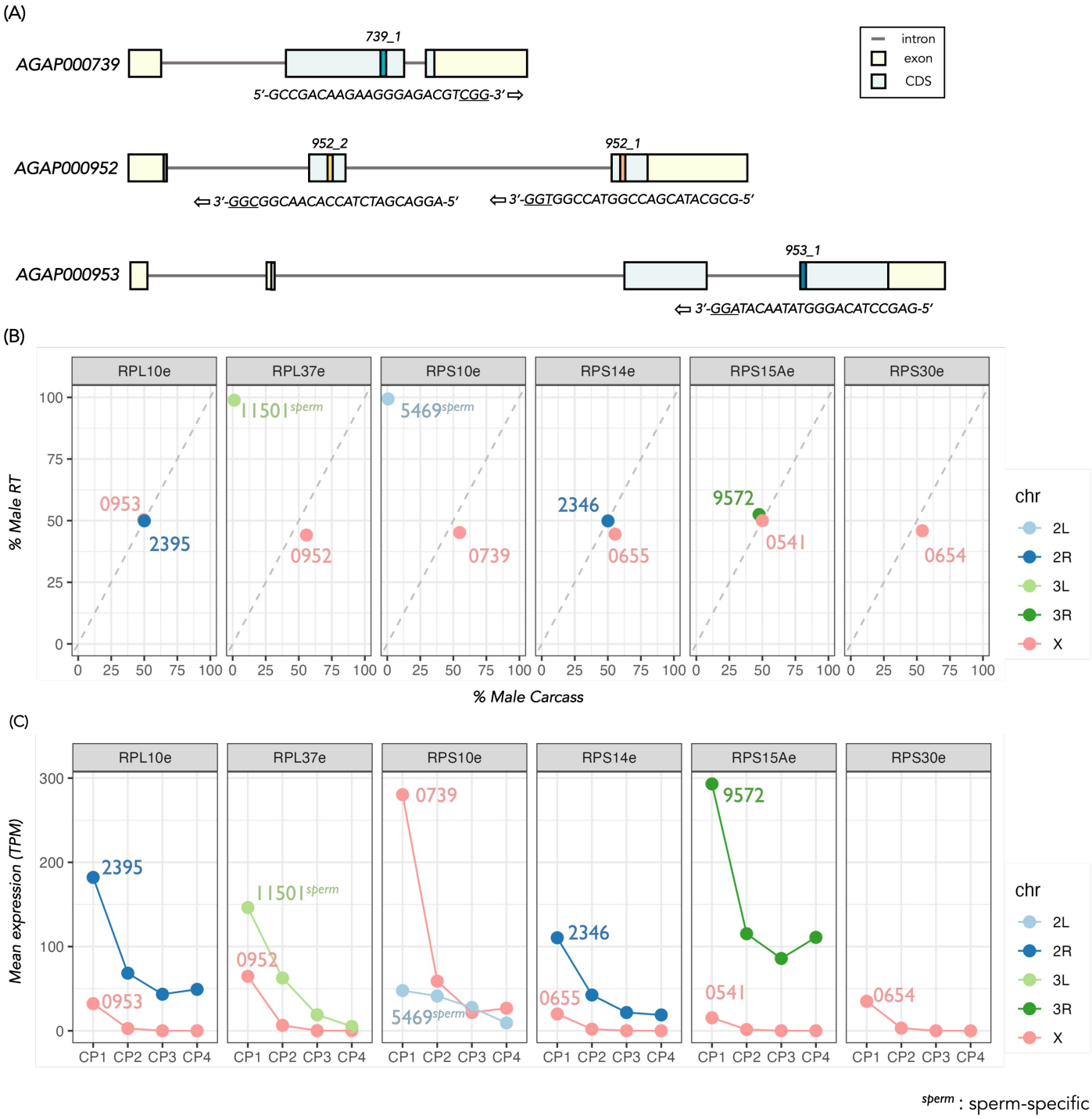

(A)
AGAP000739
739_1
5′-GCCGACAAGAAGGGAGACGTCGG-3′
AGAP000952
952_2
952_1
3′-GGCGGCAACACCATCTAGCAGGA-5′
3′-GGTGGCCATGGCCAGCATACGCG-5′
AGAP000953
953_1
3′-GGATACAATATGGGACATCCGAG-5′
intron
exon
CDS
(B)
RPL10e
RPL37e
RPS10e
RPS14e
RPS15Ae
RPS30e
% Male RT
% Male Carcass
0953
2395
11501 sperm
0952
5469 sperm
0739
2346
0655
9572
0541
0654
chr
2L
2R
3L
3R
X
(C)
Mean expression (TPM)
CP1
CP2
CP3
CP4
sperm : sperm-specific

Figure S2

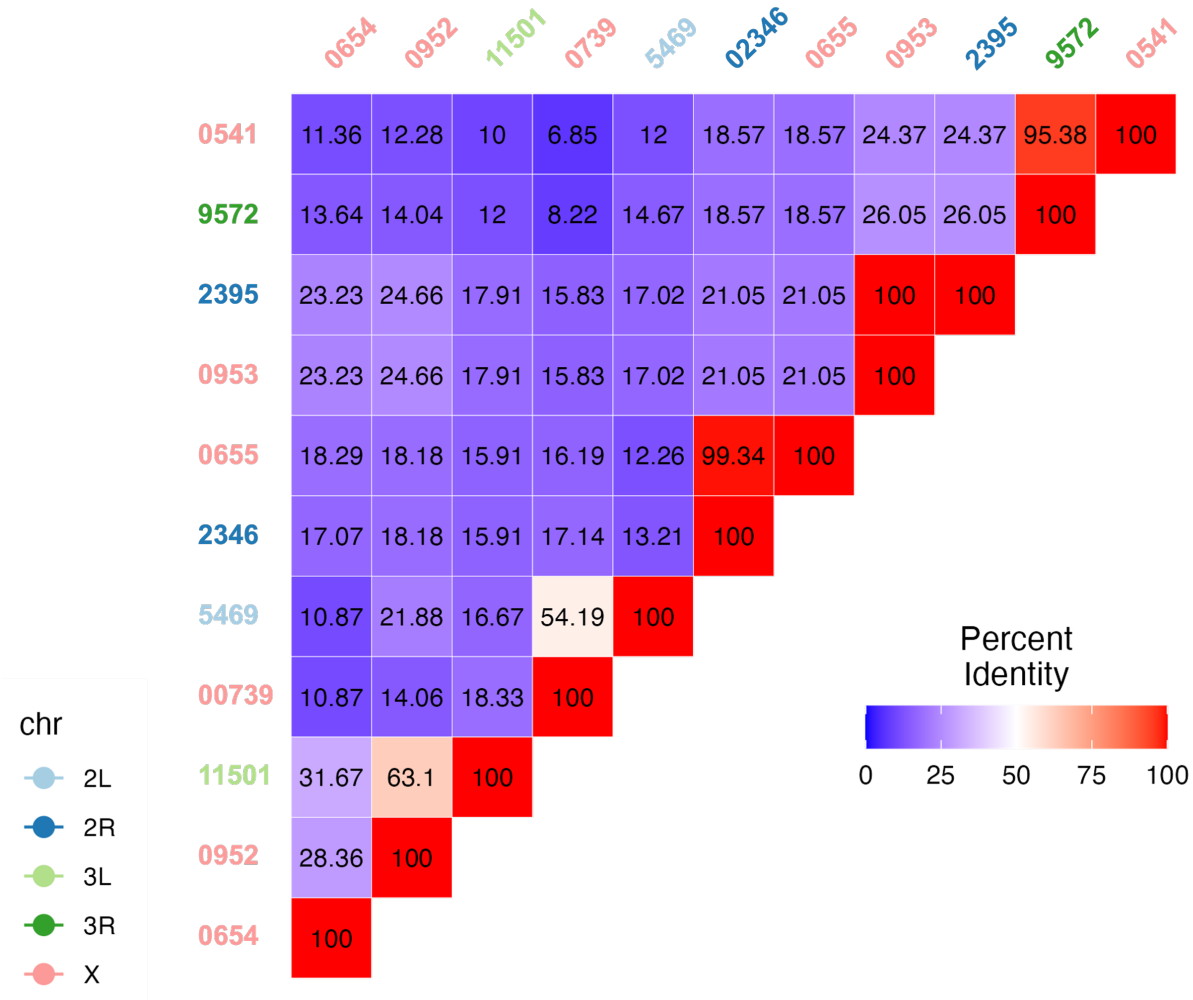

0654
0952
11501
0739
5469
02346
0655
0953
2395
9572
0541
0541 11.36 12.28 10 6.85 12 18.57 18.57 24.37 24.37 95.38 100
9572 13.64 14.04 12 8.22 14.67 18.57 18.57 26.05 26.05 100
2395 23.23 24.66 17.91 15.83 17.02 21.05 21.05 100 100
0953 23.23 24.66 17.91 15.83 17.02 21.05 21.05 100
0655 18.29 18.18 15.91 16.19 12.26 99.34 100
2346 17.07 18.18 15.91 17.14 13.21 100
5469 10.87 21.88 16.67 54.19 100
00739 10.87 14.06 18.33 100
11501 31.67 63.1 100
0952 28.36 100
0654 100
Percent Identity
0 25 50 75 100
chr
2L
2R
3L
3R
X

Figure S3

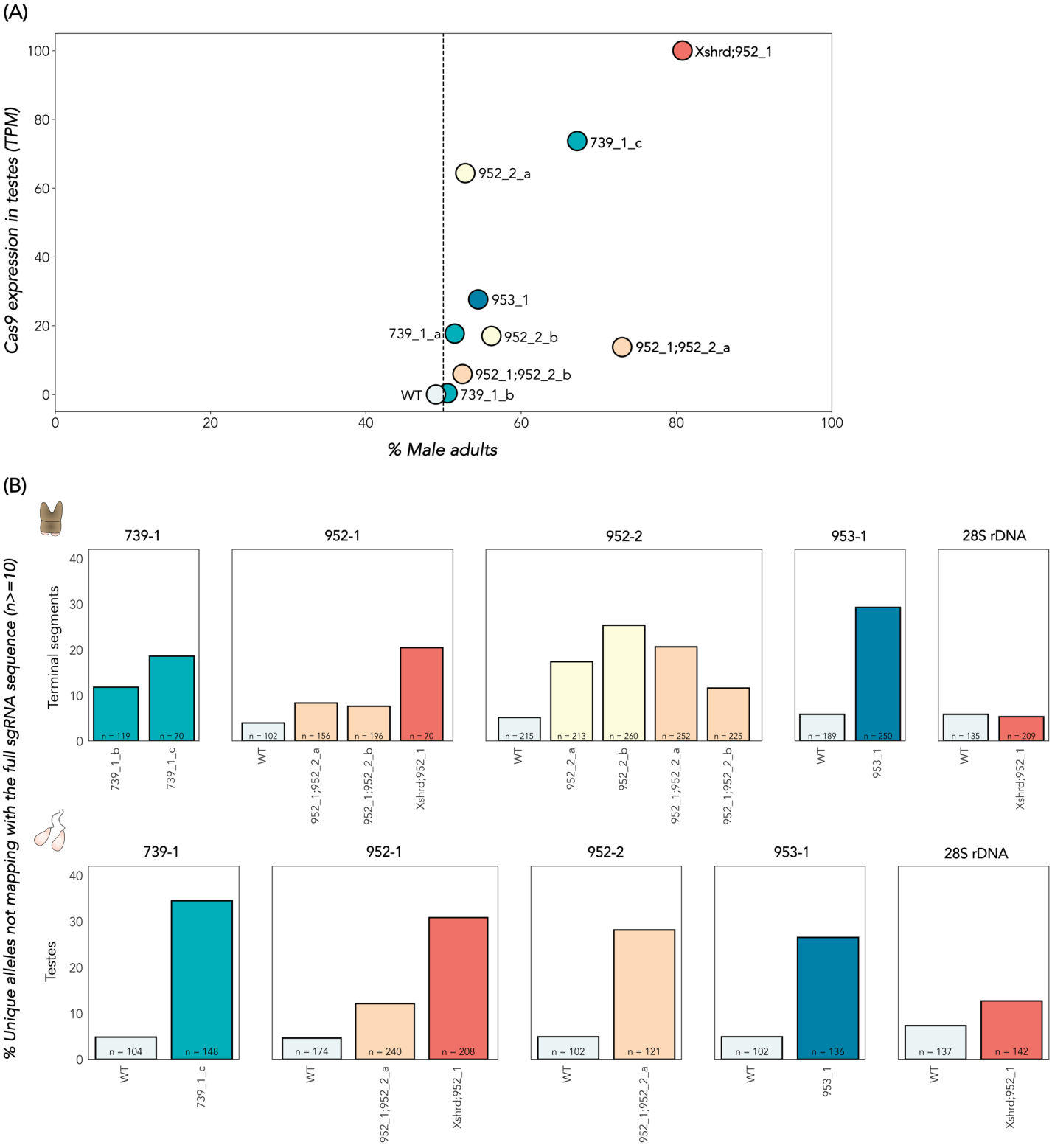

(A)
Cas9 expression in testes (TPM)
% Male adults
Xshrd;952_1
739_1_c
952_2_a
953_1
739_1_a
952_2_b
952_1;952_2_a
952_1;952_2_b
WT
739_1_b
(B)
% Unique alleles not mapping with the full sgRNA sequence (n>=10)
Terminal segments
Testes
739-1
952-1
952-2
953-1
28S rDNA
n = 119
n = 70
n = 102
n = 156
n = 196
n = 70
n = 215
n = 213
n = 260
n = 252
n = 225
n = 189
n = 250
n = 135
n = 209
n = 104
n = 148
n = 174
n = 240
n = 208
n = 102
n = 121
n = 102
n = 136
n = 137
n = 142

Figure S4

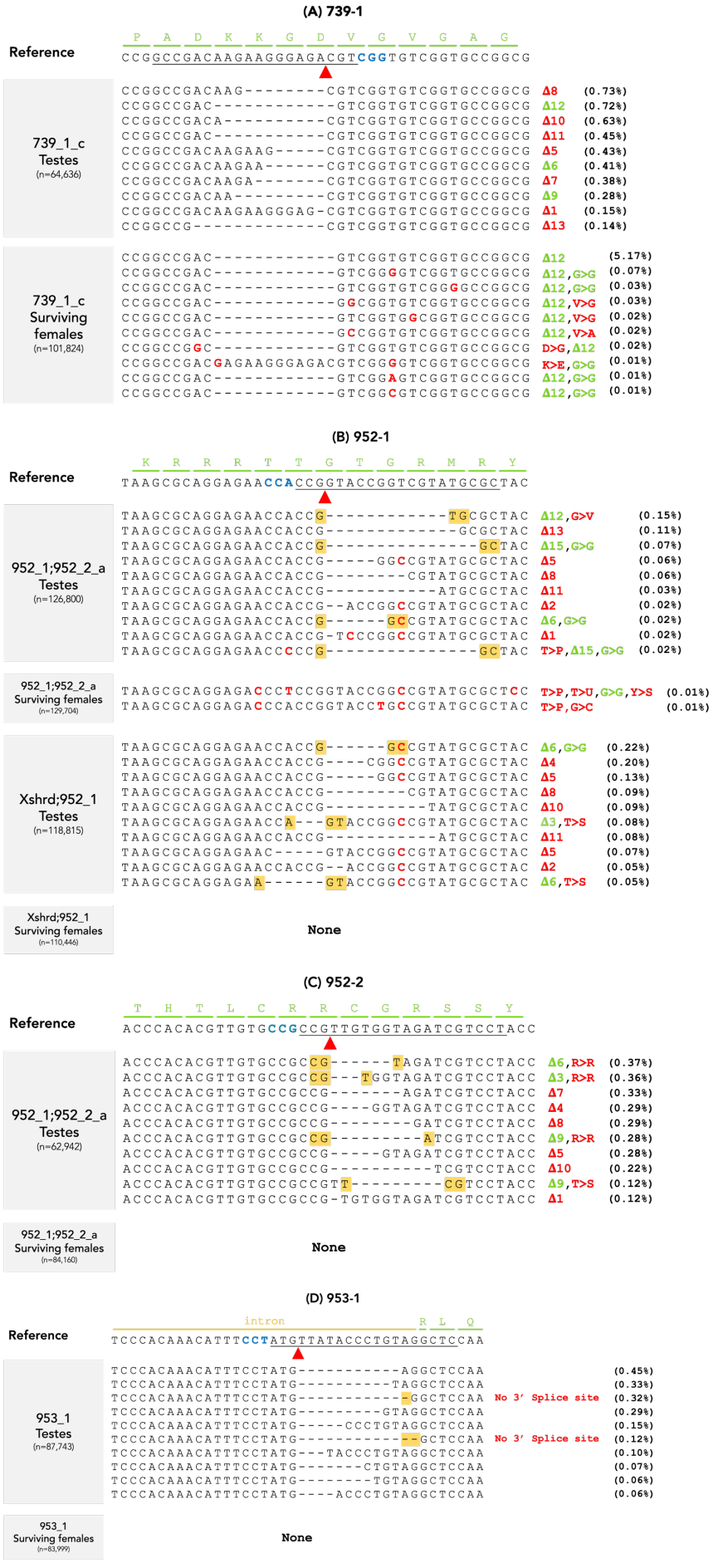

(A) 739-1
Reference
P A D K K G D V G V G A G
CCGGCCGACAAGAAGGGAGACGTCGGTGTCGGTGCCGGCG
739_1_c
Testes
(n=64,636)
CCGGCCGACAAG--------CGTCGGTGTCGGTGCCGGCG Δ8 (0.73%)
CCGGCCGAC----------GTCGGTGTCGGTGCCGGCG Δ12 (0.72%)
CCGGCCGACA----------CGTCGGTGTCGGTGCCGGCG Δ10 (0.63%)
CCGGCCGAC----------CGTCGGTGTCGGTGCCGGCG Δ11 (0.45%)
CCGGCCGACAAGAAG-----CGTCGGTGTCGGTGCCGGCG Δ5 (0.43%)
CCGGCCGACAAGAA------CGTCGGTGTCGGTGCCGGCG Δ6 (0.41%)
CCGGCCGACAAGA-------CGTCGGTGTCGGTGCCGGCG Δ7 (0.38%)
CCGGCCGACAA---------CGTCGGTGTCGGTGCCGGCG Δ9 (0.28%)
CCGGCCGACAAGAAGGGAG-CGTCGGTGTCGGTGCCGGCG Δ1 (0.15%)
CCGGCCG----------CGTCGGTGTCGGTGCCGGCG Δ13 (0.14%)
739_1_c
Surviving
females
(n=101,824)
CCGGCCGAC----------GTCGGTGTCGGTGCCGGCG Δ12 (5.17%)
CCGGCCGAC----------GTCGGGGTCGGTGCCGGCG Δ12,G>G (0.07%)
CCGGCCGAC----------GTCGGTGTCGGGGCCGGCG Δ12,G>G (0.03%)
CCGGCCGAC----------GGCGGTGTCGGTGCCGGCG Δ12,V>G (0.03%)
CCGGCCGAC----------GTCGGTGGCGGTGCCGGCG Δ12,V>G (0.02%)
CCGGCCGAC----------GCCGGTGTCGGTGCCGGCG Δ12,V>A (0.02%)
CCGGCCGGC----------GTCGGTGTCGGTGCCGGCG D>G,Δ12 (0.02%)
CCGGCCGACGAGAAGGGAGACGTCGGGGTCGGTGCCGGCG K>E,G>G (0.01%)
CCGGCCGAC----------GTCGGAGTCGGTGCCGGCG Δ12,G>G (0.01%)
CCGGCCGAC----------GTCGGCGTCGGTGCCGGCG Δ12,G>G (0.01%)
(B) 952-1
Reference
K R R R T T G T G R M R Y
TAAGCGCAGGAGAACCACCGGTACCGGTCGTATGCGCTAC
952_1;952_2_a
Testes
(n=126,800)
TAAGCGCAGGAGAACCACCG----------TGCGCTAC Δ12,G>V (0.15%)
TAAGCGCAGGAGAACCACCG----------GCGCTAC Δ13 (0.11%)
TAAGCGCAGGAGAACCACCG----------GCTAC Δ15,G>G (0.07%)
TAAGCGCAGGAGAACCACCG-----GGCCGTATGCGCTAC Δ5 (0.06%)
TAAGCGCAGGAGAACCACCG--------CGTATGCGCTAC Δ8 (0.06%)
TAAGCGCAGGAGAACCACCG----------ATGCGCTAC Δ11 (0.03%)
TAAGCGCAGGAGAACCACCG--ACCGGCCGTATGCGCTAC Δ2 (0.02%)
TAAGCGCAGGAGAACCACCG------GCCGTATGCGCTAC Δ6,G>G (0.02%)
TAAGCGCAGGAGAACCACCG-TCCCGGCCGTATGCGCTAC Δ1 (0.02%)
TAAGCGCAGGAGAACCCCCG----------GCTAC T>P,Δ15,G>G (0.02%)
952_1;952_2_a
Surviving females
(n=129,704)
TAAGCGCAGGAGACCCTCCGGTACCGGCCGTATGCGCTCC T>P,T>U,G>G,Y>S (0.01%)
TAAGCGCAGGAGACCCACCGGTACCTGCCGTATGCGCTAC T>P,G>C (0.01%)
Xshrd;952_1
Testes
(n=118,815)
TAAGCGCAGGAGAACCACCG------GCCGTATGCGCTAC Δ6,G>G (0.22%)
TAAGCGCAGGAGAACCACCG----CGGCCGTATGCGCTAC Δ4 (0.20%)
TAAGCGCAGGAGAACCACCG-----GGCCGTATGCGCTAC Δ5 (0.13%)
TAAGCGCAGGAGAACCACCG--------CGTATGCGCTAC Δ8 (0.09%)
TAAGCGCAGGAGAACCACCG----------TATGCGCTAC Δ10 (0.09%)
TAAGCGCAGGAGAACCA---GTACCGGCCGTATGCGCTAC Δ3,T>S (0.08%)
TAAGCGCAGGAGAACCACCG----------ATGCGCTAC Δ11 (0.08%)
TAAGCGCAGGAGAAC-----GTACCGGCCGTATGCGCTAC Δ5 (0.07%)
TAAGCGCAGGAGAACCACCG--ACCGGCCGTATGCGCTAC Δ2 (0.05%)
TAAGCGCAGGAGAA------GTACCGGCCGTATGCGCTAC Δ6,T>S (0.05%)
Xshrd;952_1
Surviving females
(n=110,446)
None
(C) 952-2
Reference
T H T L C R R C G R S S Y
ACCCACACGTTGTGCCGCCGTTGTGGTAGATCGTCCTACC
952_1;952_2_a
Testes
(n=62,942)
ACCCACACGTTGTGCCGCCG------TAGATCGTCCTACC Δ6,R>R (0.37%)
ACCCACACGTTGTGCCGCCG---TGGTAGATCGTCCTACC Δ3,R>R (0.36%)
ACCCACACGTTGTGCCGCCG-------AGATCGTCCTACC Δ7 (0.33%)
ACCCACACGTTGTGCCGCCG----GGTAGATCGTCCTACC Δ4 (0.29%)
ACCCACACGTTGTGCCGCCG--------GATCGTCCTACC Δ8 (0.29%)
ACCCACACGTTGTGCCGCCG---------ATCGTCCTACC Δ9,R>R (0.28%)
ACCCACACGTTGTGCCGCCG-----GTAGATCGTCCTACC Δ5 (0.28%)
ACCCACACGTTGTGCCGCCG----------TCGTCCTACC Δ10 (0.22%)
ACCCACACGTTGTGCCGCCGTT---------CGTCCTACC Δ9,T>S (0.12%)
ACCCACACGTTGTGCCGCCG-TGTGGTAGATCGTCCTACC Δ1 (0.12%)
952_1;952_2_a
Surviving females
(n=84,160)
None
(D) 953-1
Reference
intron R L Q
TCCCACAAACATTTCCTATGTTATACCCTGTAGGCTCCAA
953_1
Testes
(n=87,743)
TCCCACAAACATTTCCTATG----------AGGCTCCAA (0.45%)
TCCCACAAACATTTCCTATG----------TAGGCTCCAA (0.33%)
TCCCACAAACATTTCCTATG----------GGCTCCAA No 3' Splice site (0.32%)
TCCCACAAACATTTCCTATG---------GTAGGCTCCAA (0.29%)
TCCCACAAACATTTCCTATG-----CCCTGTAGGCTCCAA (0.15%)
TCCCACAAACATTTCCTATG----------GCTCCAA No 3' Splice site (0.12%)
TCCCACAAACATTTCCTATG---TACCCTGTAGGCTCCAA (0.10%)
TCCCACAAACATTTCCTATG-------CTGTAGGCTCCAA (0.07%)
TCCCACAAACATTTCCTATG--------TGTAGGCTCCAA (0.06%)
TCCCACAAACATTTCCTATG----ACCCTGTAGGCTCCAA (0.06%)
953_1
Surviving females
(n=83,999)
None

Figure S5

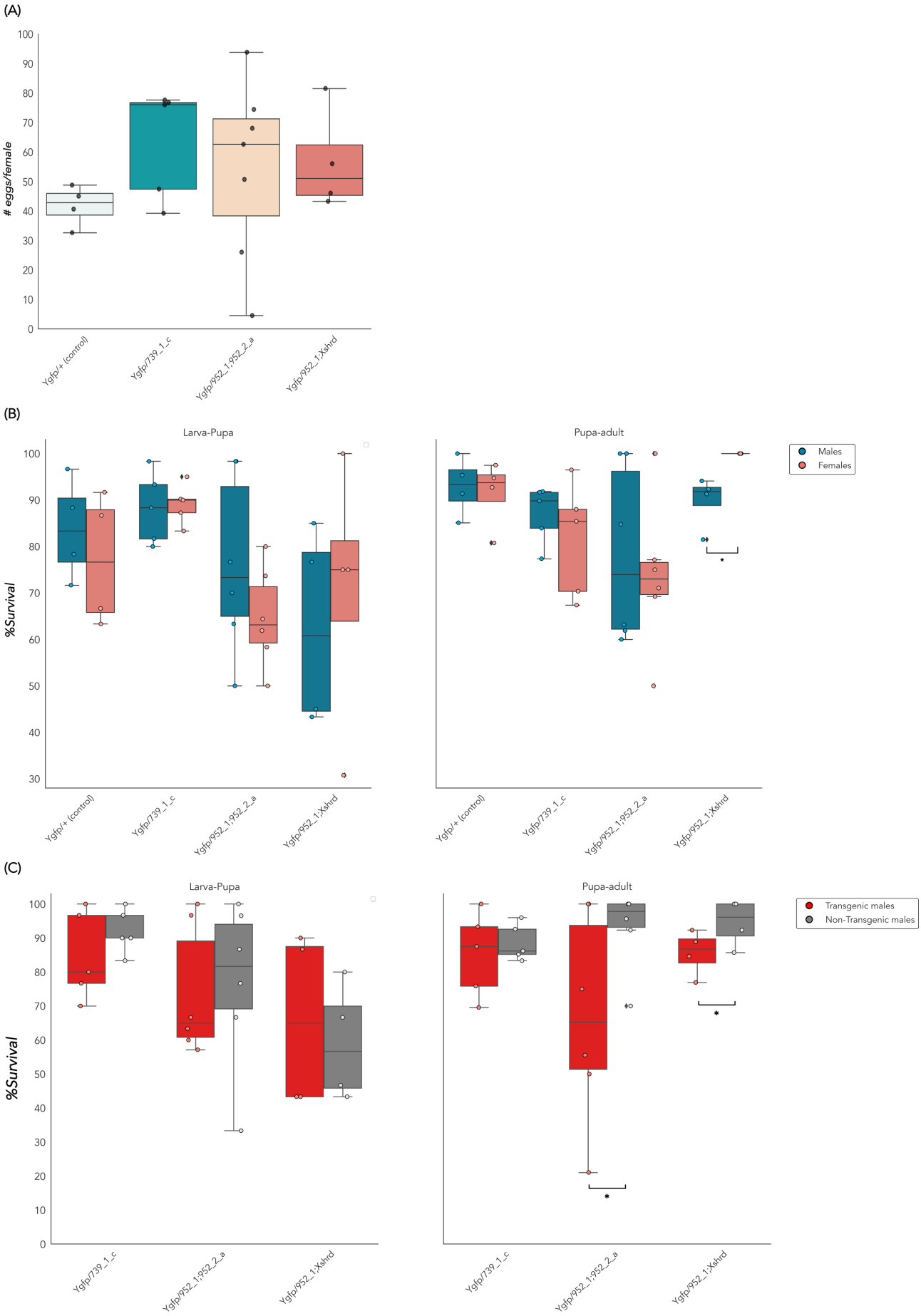
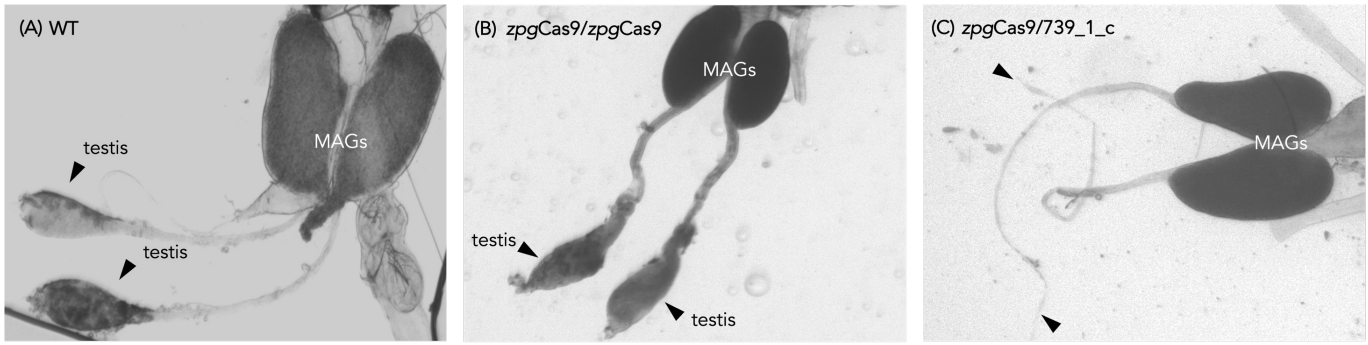

(A)
eggs/female
Ygfp/+ (control)
Ygfp/739_1_c
Ygfp/952_1;952_2_a
Ygfp/952_1;Xshrd
(B)
Larva-Pupa
Pupa-adult
%Survival
Males
Females
(C)
Transgenic males
Non-Transgenic males

Figure S6


(A) WT
testis
testis
MAGs
(B) zpgCas9/zpgCas9
MAGs
testis
testis
(C) zpgCas9/739_1_c
MAGs