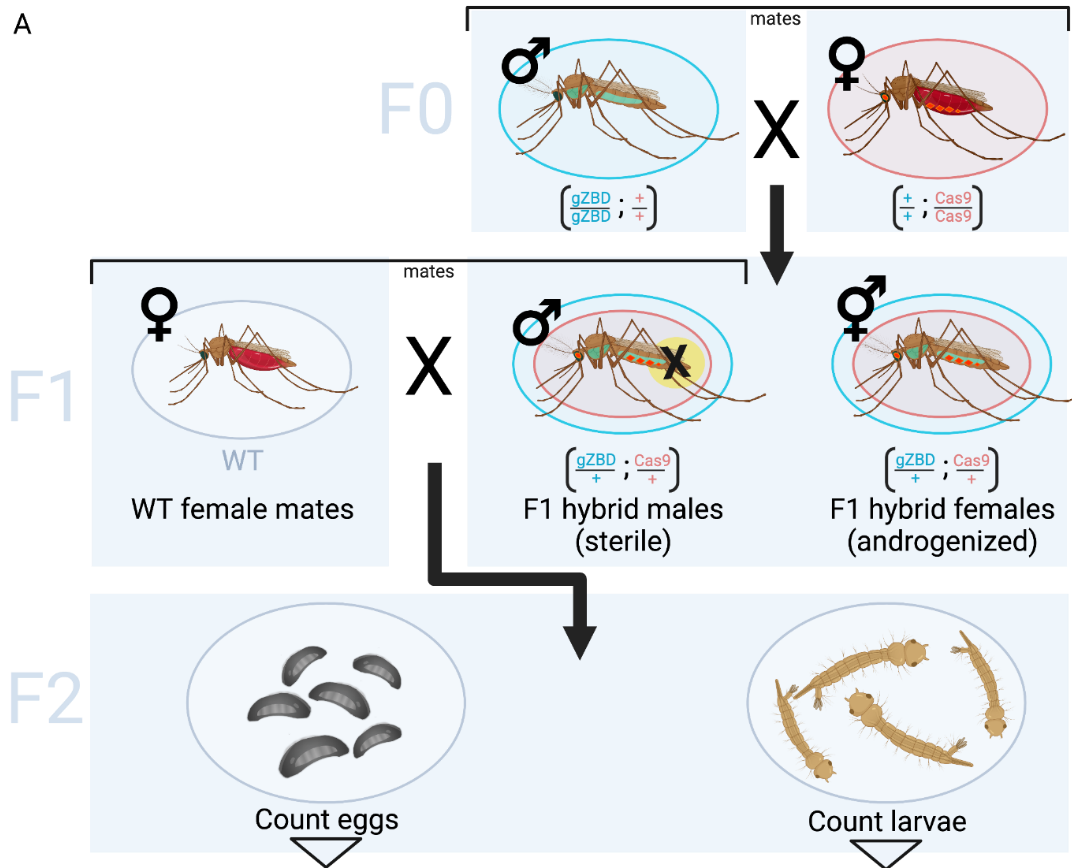
SUPPLEMENTARY FIGURES

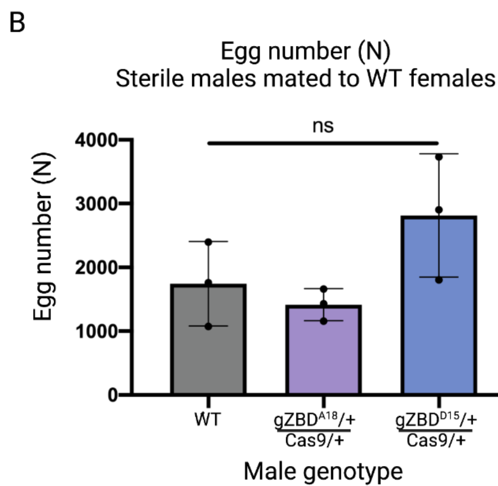
Figure S1: Images of DeltaDsx phenotype in (+/gZBD ; +/Cas9) hybrid F1 females
Figure S2. Sterility of (+/gZBD ; +/Cas9) F1 males
Figure S3. Summary of mutations under gRNA target sites
Figure S4. Testes dissections, mating plug transfer, and male survival curves of pgSIT^{D15} males.

SUPPLEMENTARY TEXT

Text S1. Calculation of sort:release ratio.

Figure S1. Intersex genitalia of F1 (+/gZBD ; +/Cas9) females. (+/gZBD ; +/Cas9) hybrid F1 female offspring from gZBD males crossed to Cas9 females. Evidence of *dsxF* mutagenesis as indicated by the presence of development of a male clasper (black arrow) on the female genital appendage. Scale bars indicate 1mm. 24 distinct intersex females shown.





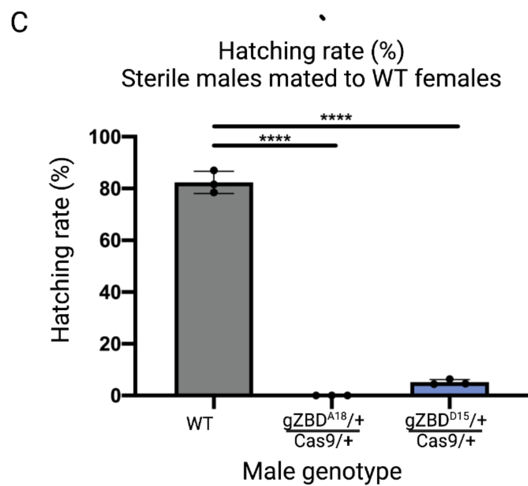


Figure S2. Sterility of (+/gZBD ; +/Cas9) F1 males. A) To generate F1 males, F0 gZBD males and Cas9 females are crossed together. In the F1 generation, females are expected to be

androgenized and males are expected to be sterilized. To test male sterility, F1 (+/gZBD ; +/Cas9) males are mated to wild type virgin females. Among the F2 offspring the number of larvae and eggs are counted to measure hatching rate (n% = 1 day old larvae / total number of eggs laid). **B)** Crosses of 50 (+/$gZBD^{A18}$; +/Cas9), (+/$gZBD^{D15}$; +/Cas9), or wild type control males mated to 50 wild type females were performed, and the number of eggs laid is reported. Egg number is nonsignificantly different between all groups (p=0.1057, One Way ANOVA). Mean and SD shown. **C)** The hatching rate of broods from the above described crosses is reported. Hatching rate is significantly lower than wild type controls for both sterile male groups (both p=<0.0001, One Way ANOVA, Dunnett's multiple comparisons test). Mean and SD shown. Observed larvae were present at the Mendelian ratios expected of transgenic fathers, and the mean hatch rate of these groups is reported in gray. Created with Biorender.com.

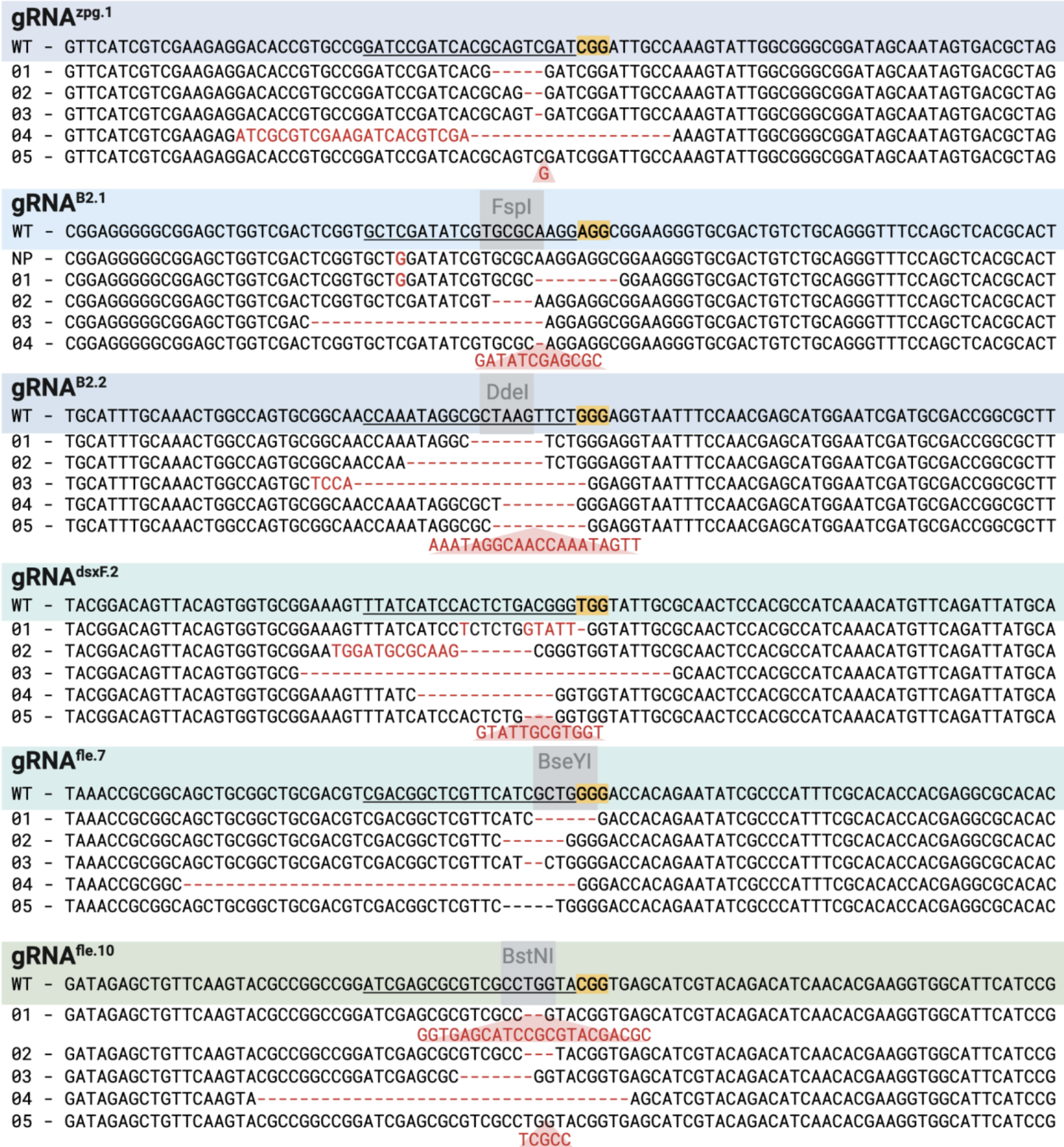
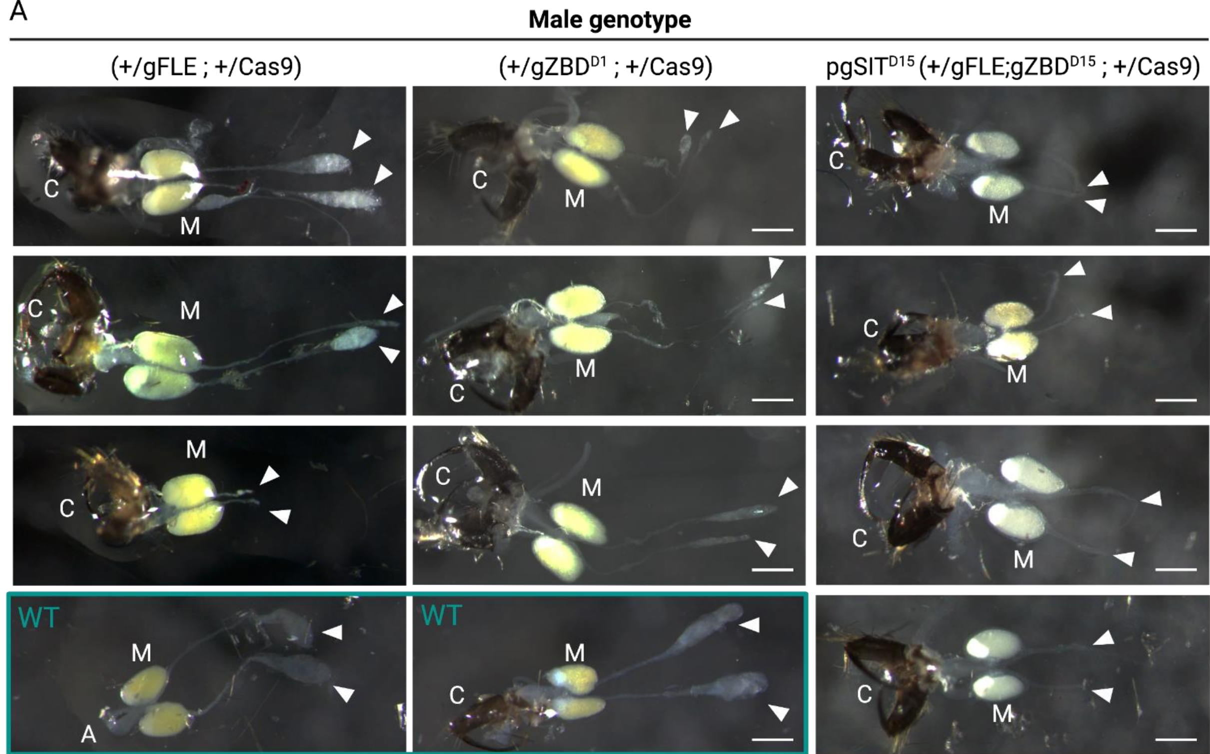
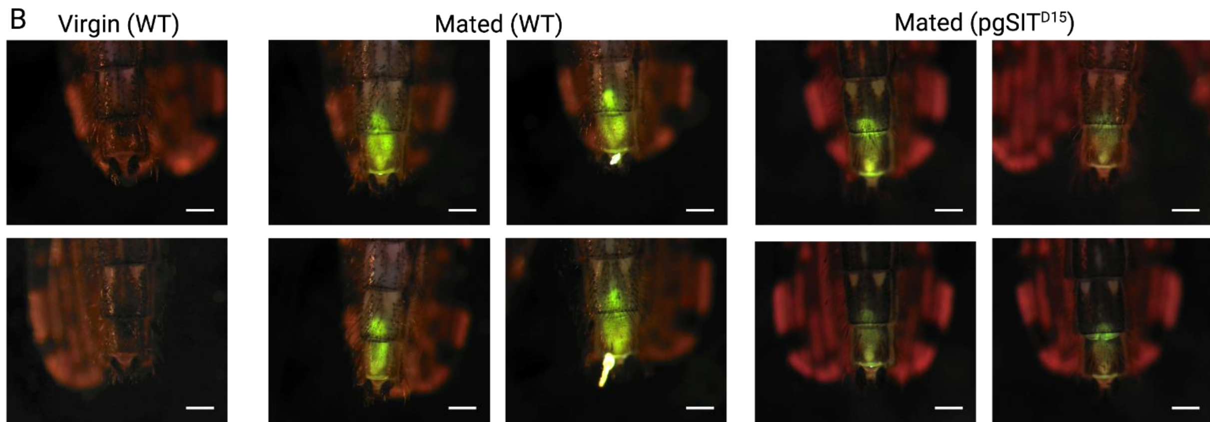


Figure S3. Target site mutations in pgSIT embryos. Each gRNA target site is underlined with the PAM highlighted in yellow. To isolate mutations under some gRNAs, the target site was PCR-amplified and digested with a restriction enzyme whose recognition sequence lies near the CRISPR cut site (grey box). Failure to cleave the PCR product was indicative of CRISPR mutations, and individual amplicon sequencing confirmed the sequence. Mutant reads are derived from (+/gFLE;gZBD^{D15} ; +/Cas9) individuals are marked with 0#. Naturally occurring polymorphisms are denoted by NP. Created with Biorender.com.


A
Male genotype
(+/gFLE ; +/Cas9)
(+/gZBD^D1 ; +/Cas9)
pgSIT^D15 (+/gFLE;gZBD^D15 ; +/Cas9)
C
M
WT
A


B
Virgin (WT)
Mated (WT)
Mated (pgSIT^D15)

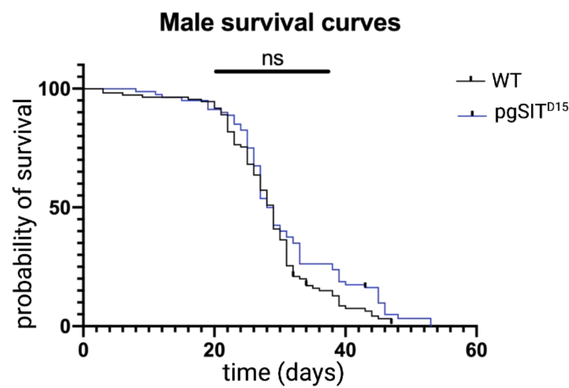

C
Male survival curves
ns
WT
pgSIT^D15
probability of survival
100
50
0
0
20
40
60
time (days)

Figure S4. pgSIT^{D15} male reproductive and fitness phenotypes. A) Dissections of lower reproductive tracts from three (+/gFLE ; +/Cas9), three (+/gZBD^{D15} ; +/Cas9) and four pgSIT^{D15} (+/gFLE;gZBD^{D15} ; +/Cas9) males shown, highlighting the presence or absence of testes. In the (+/gFLE; +/Cas9) group, tissues in which both, a single, and neither testes develop are shown. Wild type control reproductive tracts are shown boxed in teal. Both wild type reproductive tracts with attached and removed claspers are shown for reference; a clasper-less image is shown for reference to other published works, and an image with the claspers is included to show the presence of this important reproductive appendage, consistent with the other panels shown. Aedaegus is labeled with an A in the clasper-less sample. Claspers are denoted with C, Male Accessory Glands (MAGs) are denoted with M, the location at the end of the vas deferens where testicular tissue should be developed is marked with an arrow. Lighting brightness, position, white balance, and exposure time were not controlled for, therefore no conclusions regarding the brightness or color of tissues should be made. Scale bars denote 200 µm. **B)** The yellow-green autofluorescent mating plug is visible within the female lower reproductive tract and is sufficiently bright to be observed through the cuticle. In some females, a portion of the plug protrudes externally beyond the vagina. Virgin controls are unmated and therefore have no autofluorescent plug. Lighting brightness, position, and exposure time were not controlled for, therefore no conclusions regarding the brightness or color of tissues should be made. Scale bars denote 200 µm. **C)** Male survival curves. Male pgSIT^{D15} and wild type adults were monitored daily for death. pgSIT^{D15} survival differs non-significantly differently from wild type (Log-rank Mantel-Cox).

Text S1. Calculation of sort:release ratio and release sizes.

The rate limiting step for many vector control releases is the ability to sort releasable males from undesirable females. With current technology, sorting can be achieved two ways: fluorescently and optically. Fluorescence based sorting relies on a modified cell sorter machine, a COPAS, to sort up to 40,000 freshly hatched nascent larvae per hour (1). In contrast, optically-based sorting relies on computerized visual identification of adult males vs females, and has a slower sort speed (exact number per hour not disclosed). Not only do these technologies vary significantly in their sort speed, but optical sorters necessitate rearing of the undesirable sex to adulthood, wasting significant resources on husbandry.

If sex-specific fluorescent markers were incorporated into the stock strain (pgSIT 2.0), sorting via COPAS would facilitate generation of pgSIT 2.0 sterile males as follows: 40,000 larvae can conservatively be sorted per hour. Sorting 4 F0 mosquitoes (male from female for each line), would yield 1 fertilized female. Each fertilized female could conservatively produce 400 F1 eggs in her lifetime, half of which would be male. This yields a fairly conservative estimate of 2 million sterile males produced per one COPAS sort-hour.

Most vector control systems rely on direct isolation of males in the generation to be released (with the exception of RIDL systems). This yields a 2:1 sort:release ratio because one male and one female are sorted for every singular male released. This is the most common sorting:release ratio for most vector control systems, and is a ratio which requires a significant number of sorting machines to be able to achieve the scale required for mass releases. However this is in contrast with systems which automatically eliminate the females in the released generation, such as Ifegenia and pgSIT. In such systems, the F0 generation is sex sorted, and the F1 generation is released, improving throughput by orders of magnitude because the fecundity of F0 females is so high. Specifically, in pgSIT, 4 adult mosquitoes must be sorted - Cas9 males and gRNA females each from a pool of both sexes - to yield one fertilized F0 female, who can conservatively produce 400 eggs in her lifetime (though some say upwards of 1,000 eggs (2)). This yields a 4:400, or 1:100 sort:release ratio, and with half of the eggs being male, gives a final sort:release ratio of 1:50.

SUPPLEMENTARY DATASETS

Dataset S1: Quantification of *dsxF* knock-out phenotype in (+/gZBD ; +/Cas9) females from gZBD males X Cas9 females

Dataset S2: Raw larvae and egg counts from (+/gZBD ; +/Cas9) males X wild type females for male sterility assay (Presented in **Figure 2B**,**C**)

Dataset S3: Raw pupae counts from homozygous +/gFLE;gZBD female X Cas9 male for female elimination assay (Presented in **Figure 1B**)

Dataset S4: Raw larvae and egg counts from F1 pgSIT males (+/gFLE;gZBD ; +/Cas9) X wild type females for Male sterility assay (Presented in **Figure 1C**,**D**)

Dataset S5: Raw counts of dead F1 pgSIT adult males (+/gFLE;gZBD ; +/Cas9) for Male adult survival assay (Presented in **Figure S4C**)

Dataset S6: Raw larvae and egg counts from F1 pgSIT males X wild type males X wild type females (at different ratios) for Population Suppression Assays (Presented in **Figure 2B,C**)

Dataset S7: Model parameters describing pgSIT construct, mosquito bionomics and malaria epidemiology for simulated releases in Upper River region, The Gambia.

REFERENCES

1. C. Lutrat, *et al.*, Combining two Genetic Sexing Strains allows sorting of non-transgenic males for Aedes genetic control. *bioRxiv* 2022.03.11.483912 (2022).

2. A. N. Clements, “Metamorphosis” in *The Biology of Mosquitoes, Volume 1: Development, Nutrition and Reproduction*, (CABI, 1992), pp. 171–194.