UNIVERSITY OF CALIFORNIA SAN DIEGO

Development of pgSIT And Gynecider, Confinable Population Suppression Systems in *Anopheles gambiae*

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

James Pai

Committee in charge:
Professor Omar Akbari, Chair
Professor Kimberly Cooper,
Professor Alexis Komor

2022

The Thesis of James Pai is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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TABLE OF CONTENTS

LIST OF FIGURES

Survial curval, targets x2, transgenesis

LIST OF TABLES

LIST OF SCHEMAS

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The introduction section, Schema 1 is currently being prepared for submission for publication of the material. Reema Apte, Andrea L. Smidler, Neha Thakre, Omar S. Akbari. The thesis author was the primary investigator and author of this material.

The result section, Figure 2 is currently being prepared for submission for publication of the material. Reema Apte, Andrea L. Smidler, Neha Thakre, Omar S. Akbari. The thesis author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

Development of pgSIT and Gynecider, Confinable Population Suppression Systems in *Anopheles gambiae*

By

James Pai

Master of Science in Biology

University of California San Diego, 2022

Professor Omar Akbari, Chair

Malaria is among the world's deadliest diseases, killing over half a million people annually. Due to mosquito genetic plasticity, traditional vector control methods are growing less effective. Also, current genetic control systems, e.g Homing-based Gene Drives (HGD) or Female-Specific Release of Insects Carrying a Dominant Lethal gene (fsRIDL), face resistance and efficiency challenges. In order to overcome these challenges, we are adapting the low resistance genetic vector control system, precision guided Sterile Insect Technique (pgSIT) to *Anopheles gambiae*. Furthermore, we develop a unique Genetic Sexing System (GSS) we term **Gynecider (G**enetical**Y** e**N**cod**E**d **C**RISPR **I**nduced **D**aughter **ER**adicator) and demonstrate that

mosaic CRISPR-knockout of *femaleless (fle)* in F1 results in 100% female lethality in all individuals scored thus far (n = 2573 mutants). We believe that our systems will help overcome the current genetic vector control challenges and push genetic control to real-life implementation.

Introduction:

In 2020 alone, malaria caused 5.7 million cases and over 12,000 deaths worldwide (*1*). Malaria is caused by infection of the parasite *Plasmodium*, which is transmitted by many *Anopheline* mosquitoes but primarily *Anopheles gambiae* in Africa. The current strategies to counter malaria - mainly insecticides, bed nets, and drugs- are expensive, impose logistic burdens, and are rapidly growing ineffective due to the plasticity of *Plasmodium* and the mosquito vectors (*2*, *3*). Therefore, there is an urgent need to develop a resistance-proof, cost-effective, and sustainable technology that attenuates malaria transmission.

In response, novel genetic modification (GM) technologies have been developed over the past decade to suppress the wild *A. gambiae* vector population. For example, Homing-based Gene Drives (HGD) aim to modify or suppress the population by releasing mosquitoes that carry a selfish transgene, which is driven by expression of Cas9 and gRNA that are sandwiched between 2 homology arms complementing the target gene (*4–6*). The transgene biases its heritance by copying and pasting itself into the second allele through Cas9 cleavage followed by Homology Directed Repair (HDR). Despite their demonstrated ability for population modification/suppression, HGDs are currently undesirable for field release because they spread unconfineably and are highly prone to resistance (*7*, *8*). Furthermore, lines with a severe sex bias, including some gene drive lines, are difficult to maintain because they must be constantly spiked with wildtype mates (*9*, *10*).

Other sex-biasing GM technologies involve releasing mosquitoes that carry a transgene which induces female-specific lethality but allows male to survive and pass down the transgene. The elimination of females results in population decline. One of the sex-biasing GM technologies, X-Shredder, expresses the X-chromosome-specific endonuclease I-*Ppo*I during

spermatogenesis so that X-carrying sperm die and only Y-carrying sperm survive (*11–13*). However, this technology is not only cumbersome to rear due to its sex bias, but also has incomplete female-eliminating penetrance and thus requires females to be manually sorted out before release. Furthermore, a trial in Africa showed that transgenic X-Shredder mosquitoes had lower fitness than wild types due to low transgene expression in undesired tissues (*13–15*), and similar to HGDs, surviving X-Shredder females add the risk of developing resistance.

In effort to develop a GM sex-biasing population control technology that is easier to rear, Labbé et al. developed a GM technology called Female-Specific Release of Insects Carrying a Dominant Lethal gene (fsRIDL) (*16*, *17*). fsRIDL involves releasing male mosquitoes who carry a transgene that induces female-specific lethality when expressed. Females that inherit the transgene fail to survive and reproduce, resulting in a population crash. Because the fsRIDL system uses tetracycline as a molecular switch to turn off expression of the female lethal gene, the lines can be reared without spiking in wild types when fed tetracycline. While fsRIDL mosquitoes are easier to rear and less prone to resistance than HGD, it suffers from incomplete female-killing penetrance, making the system unsuitable for release (*16*, *18*). Furthermore, fsRIDL has not yet been adapted to *A. gambiae.*

Other GM technologies have employed releasing sterile male mosquitoes into the wild to mate. The cross between a sterile male and a wild female fails to produce viable offspring, resulting in population suppression. Because female *A. gambiae* can only mate once in their lifetime, releasing large amounts of sterile males can suppress local mosquito populations. In Insect Incompatibility Technique (IIT), *Wolbachia*-infected male mosquitoes, which cannot produce fertile offspring with wild type females due to *Wolbachia*-induced Cytoplasmic Incompatibility (CI), are released (*19*). However, *Wolbachia*-infected males can produce fertile

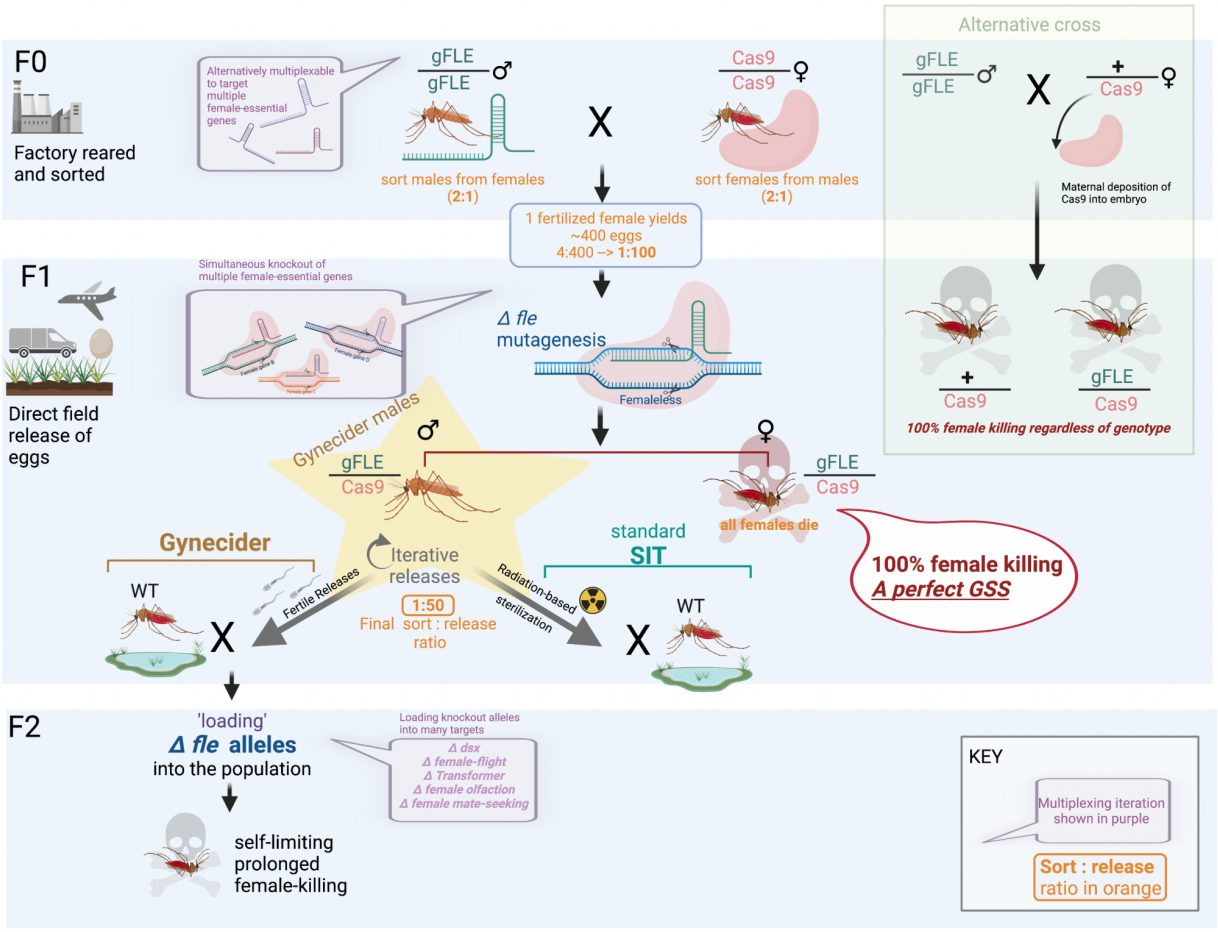
offspring with *Wolbachia*-infected females, so accidental release of *Wolbachia*-infected females can compromise population suppression. In fact, females were indeed accidentally released in the *Ae. aegypti* IIT trial in Singapore and reduced suppression efficiency (*20*). Moreover, IIT may pose unknown risks because the evolutionary stability of Wolbachia is yet to be fully characterized. Finally, stable IIT have not yet been developed in *A. gambiae*, and further research is needed to ensure the release efficacy before implementation.

Another sterile male technology, pgSIT, produces sterile males in mass by crossing a Cas9 line with a gRNA line targeting female-essential genes and male fertility genes. Unlike HGD, pgSIT has a very low chance of resistance because it is a dead end system in which the released generation cannot reproduce. Therefore, it does not self-propagate in the population or select for resistant alleles. Rearing Cas9 and gRNA lines separately obviates the need to spike in wild type. Therefore, pgSIT is a confiable technology that is easier to rear and faces less resistance compared to other genetic control methods. Previously, our lab developed pgSIT in *Aedes aegypti* (*21*, *22*), but because the gene targets used in the *A. aegypti* pgSIT design are not conserved in *A. gambiae*, new gene targets must be identified and tested in *A. gambiae*.

Currently, as part of a team, I am adapting pgSIT to *A. gambiae*. The gRNA targets in our *A. gambiae* pgSIT construct are *Doublesex (Dsx), Zero population growth (Zpg), and Beta-2 (B2)*. *Dsx* is a highly-conserved insect sex determination gene necessary for somatic sex determination in *Drosophila melanogaster*, *Aedes aegypti*, and *A.gambiae* (*23–25*). *Zero Population Growth* (*Zpg*) is responsible for forming gap junctions during *Drosophila* germline stem cell differentiation, and knockout in *A. gambiae* results in failure to develop germline tissues (*26–29*). *B2-tubulin* is a globular structural protein that polymerizes to form microtubules. Specifically, *B2-tubulin* is expressed exclusively in the tails of sperm to provide propulsion.

Because *A. gambiae* spermatogenesis is similar to that of many other insects, we believe that knocking out *B2-tubulin* will produce males with deformed and nonfunctional sperm, rendering them infertile (*30*).

In addition to developing pgSIT in *A. gambiae*, I am also testing the female-essential gene *femaleless* (*fle*) as a knockout gene target. Krzywinska et al. used RNAi to characterize the function of the novel gene *fle* in the *A. gambiae* sex differentiation pathway. The group observed that *fle* knockdown results in a range of female androgenization and potentially some female lethality (*31*). To characterize *fle* knockout and its potential to be implemented in vector control technologies, we crossed separate Cas9 and gRNA lines- as done in pgSIT- and observed strong female-specific lethality at the larval stage in F1 transheterozygotes, mosquito with both Cas9 and gRNA, while males remain reproductively viable. Our results show that *fle* is a powerful gene target to incorporate in *A. gambiae* control technologies such as pgSIT. We further developed a CRISPR-based transgenic system termed Gynecider (GeneticallY eNcodEd CRISPR Induced Daughter ERadicator), in which viable transheterozygous males with mutated *fle* are released to load the population with mutant *fle* alleles. Our data demonstrates that Gynecider can be a safe, scaleable, confinable, and sustainable *A.gambiae* population suppression technology.



Schema 1: Establishing Transgenic CRISPR Lines to Target *fle*
In F0 generation, homozygous gFLE males are crossed with homozygous Cas9 females to generate only Gynecider males, the transheterozygous male offspring. The Gynecider males will further mate with wild type females to load *Δfle* alleles into the population. Because *Δfle* alleles kill females, the *fle* mutant alleles will eventually be eliminated in the population.

Acknowledgement:

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Results:

Establishing Transgenic pgSIT gRNA lines

We hypothesized that crossing the Cas9 line with a gRNA line targeting *Dsx, Zpg,* and *B2* would generate sterile males(*22*). We used the Cas9 line that was previously established in the Catteruccia lab (*26*). To generate the gRNA lines, we designed and cloned a plasmid encoding 5 gRNA's (2 targeting *Dsx*, 1 targeting *Zpg*, and 2 targeting B2), and a *Act5-CFP* fluorescent reporter. All gRNA's were designed to target the N-terminal region of the gene as to bias towards loss-of-function knockout. We established 4 gRNA-expressing lines (families A,B,D,E) using piggyBac transgenesis and confirmed insertion by fluorescence (Figure 1A). Families B and E were discarded because family B had the transgene inserted in the X chromosome and Family E had rearing issues. Additionally, inverse PCR results suggested that families A and D had multiple insertion sites. In effort to separate the insertion sites, each family was crossed to WT for a few generations and then split into subfamilies, which we termed Families A1, A10, A16, A18, D3, D9, D13, D15, and D21.

The Current pgSIT System Efficiently Produces Sterile Males, but Still Produces Some Phenotypic Females

To test the efficiency of pgSIT in producing sterile males, we crossed our mixed homozygous/heterozygous gRNA lines to the Cas9 lines and scored the F1 transhterozygous offspring (individuals inheriting both Cas9 and gRNA, as determined by fluorescence) for fertility and sex. To test transheterozygote fertility, we mated F1 transheterozygotes of both sexes to WT of the opposite sex, and found that none of these crosses produced viable offspring. We also observed partial female-killing and female androgenization in the F1 transheterozygotes

(Table 1, Figure 1B), indicating partial loss-of-female penetrance. Our data suggests that the current pgSIT design efficiently generates sterile males but does not completely eliminate functional females. We selected A18, D3, and D15 for further analysis because they showed the highest intersex rate and transheterozygote survival rate.

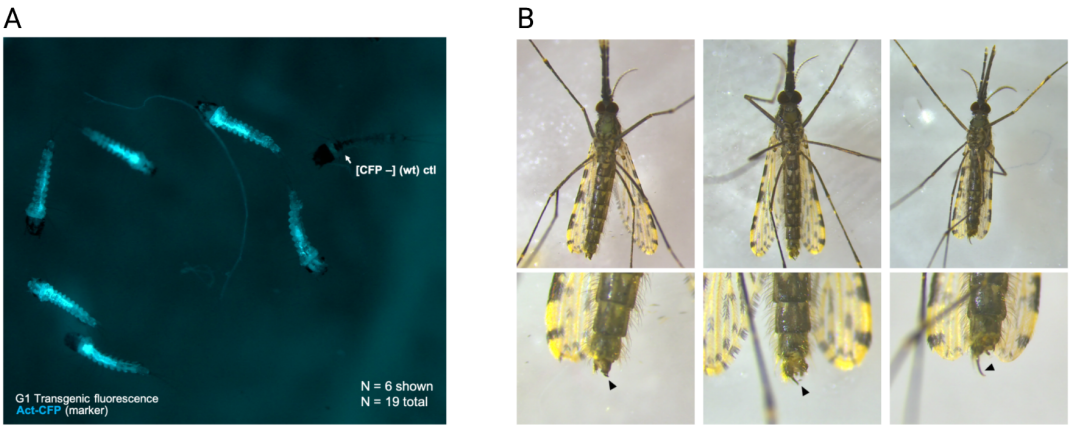


Figure 1: G1 Fully Transgenic pgSIT gRNA Larvae and Intersex Adult Phenotype.
(A) We established gRNA lines by injecting G0 embryos with the gRNA plasmid to produce mosaic adults, and outcrossing these mosaic individuals to WT. The offspring of this cross are transgenic throughout their body (see image). (B) Intersex F1 transheterozygous individuals have deformed claspers.

Table 1: Loss-of-female Penetrance in F1 Transheterozygotes
The 3 pgSIT gRNA lines (A18, D3, and D15) that we have selected due to higher phenotypic penetrance. Each line yields some female and intersex transheterozygous offspring, indicating incomplete loss-of-female penetrance.

Family	# intersex and females scored	% intersex among all females
A18	n=139	**77%** (n=108/139)
D3	n=145	**75%** (n=109/145)
D15	n=140	**68%** (n=96/140)

Establishing transgenic *fle*-targeting gRNA lines

While the current pgSIT design efficiently generates sterile males, additional gene targets are needed to increase the loss-of-female penetrance. Recently, Krzywinska et. al identified *fle* as a sex determination gene that is important for female development in *A. gambiae* using RNAi. Based on this, we believed that *fle* could be a potential pgSIT gene target. We hypothesized that crossing the Cas9 line with the *fle*-targeting gRNA line (gFLE) will generate mostly male offspring (*31*) (Fig 2A,2B). We designed and cloned plasmid with 2 N-terminal *fle*-targeting gRNA. 3 gFLE families (G,I,and J) were established by piggyBac transgenesis and confirmed by *ACT-GFP* fluorescence marker. The same Cas9 line as the pgSIT project is used.

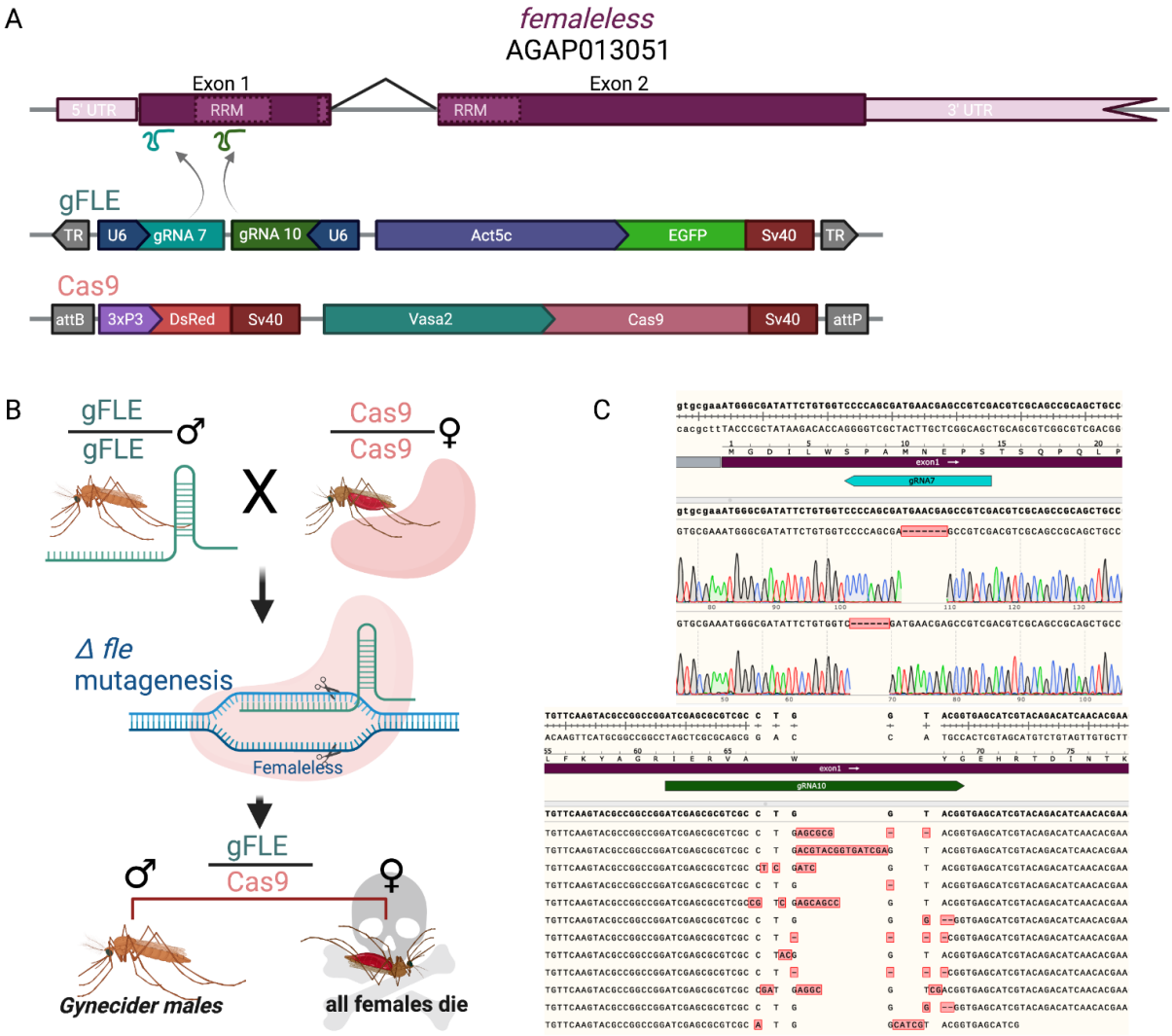


Figure 2: *fle* Knockout Completely Eliminates Female Transheterozygotes.
(A) gFLE and Cas9 plasmid constructs. The gFLE construct includes 2 gRNA's target the N terminal region of *fle*. (B) Crossing homozygous *fle* gRNA males to homozygous Cas9 females exclusively produces *Δfle* Gynecider male offspring. (C) Sanger sequencing confirms that mutagenesis occurs at both gRNA cut sites.

To determine the *Δfle* phenotype, we cross the gFLE-positive males with homozygous Cas9 females. Notably, we observed no F1 transheterozygous female pupae across all 3 gFle families (n=2573) (Figure 3A).

To determine if the loss-of-female phenotype was caused by female lethality or androgenization, we set up experimental crosses between heterozygous gFLE males and +/Cas9 females, and scored the offspring by sex and fluorescence/genotype. As expected, no transheterozygous F1 females were observed across all 3 gFLE families, and similar numbers of males were scored across all genotypes, supporting complete female killing and no androgenization. Significantly, there was not a single F1 +/gFle female pupae in Family G (n=0/2582) and very few F1 +/gFle female pupae in Families I and J (n=27/5645). This suggests that maternal deposition of Cas9 and inheritance of the gFLE-transgene is sufficient to eliminate F1 females (Figure 3B, 3C). PCR on transhterozygotes and +/gFle adults confirmed that all individuals amplify Y-chromosomal DNA, further supporting that females were killed and not androgenized (Figure 3D).

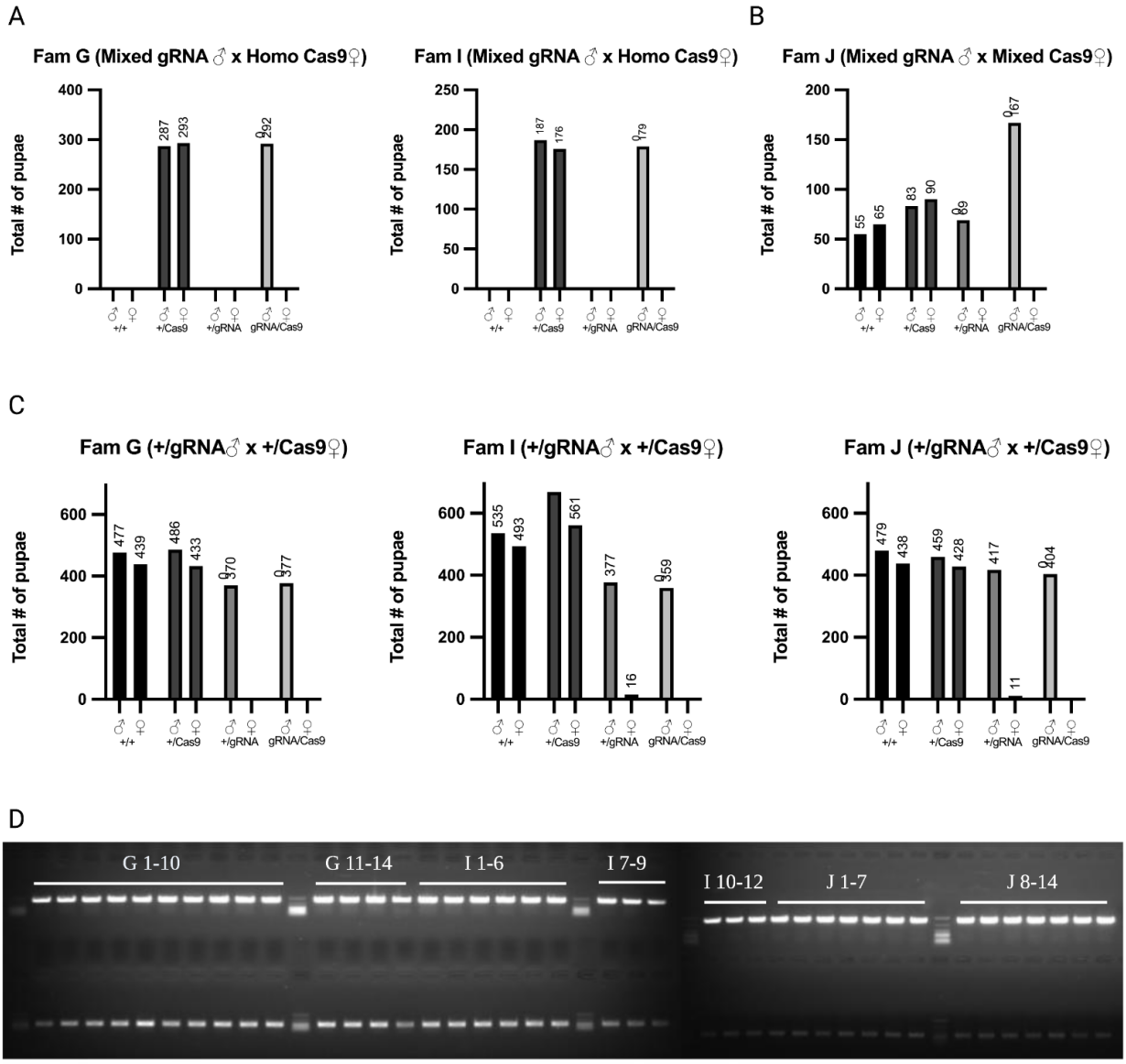


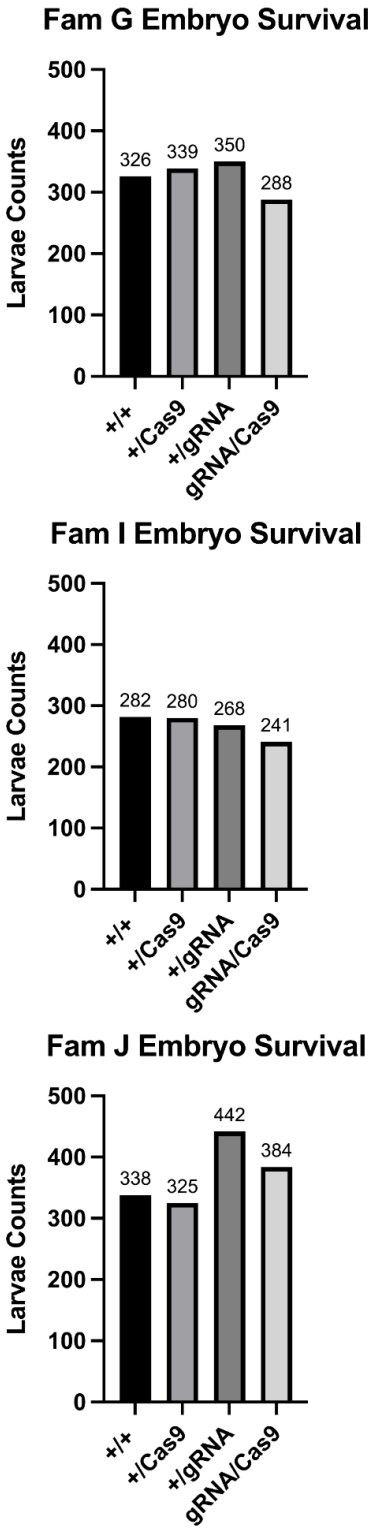
Figure 3: *fle* Knockout Results in Complete Female Lethality.
(A) Preliminary crosses between mixed gFle/gFle and +/gFle males (Families G and I) and **homozygous** Cas9 females shows complete loss-of-female in F1 transheterozygotes. (B) Preliminary crosses between mixed gFLE-positive males (Famy J) and heterozygous Cas9 females shows complete loss-of-female in F1 transheterozygotes. (C) An experimental cross between heterozygous gRNA male and Cas9 female suggests complete female killing in transheterozygotes and highly-penetrant female-killing in +/gFle group in F1 generation. (D) Y chromosome and control 28S gene PCR of F1 transheterozygous males. The top row shows Y chromosome PCR, and the bottom row shows control 28S gene PCR. All transheterozygous male samples have Y chromosome bands, which indicate all samples are genetically male.

***Δfle* Shows Early Stage Female Killing.**

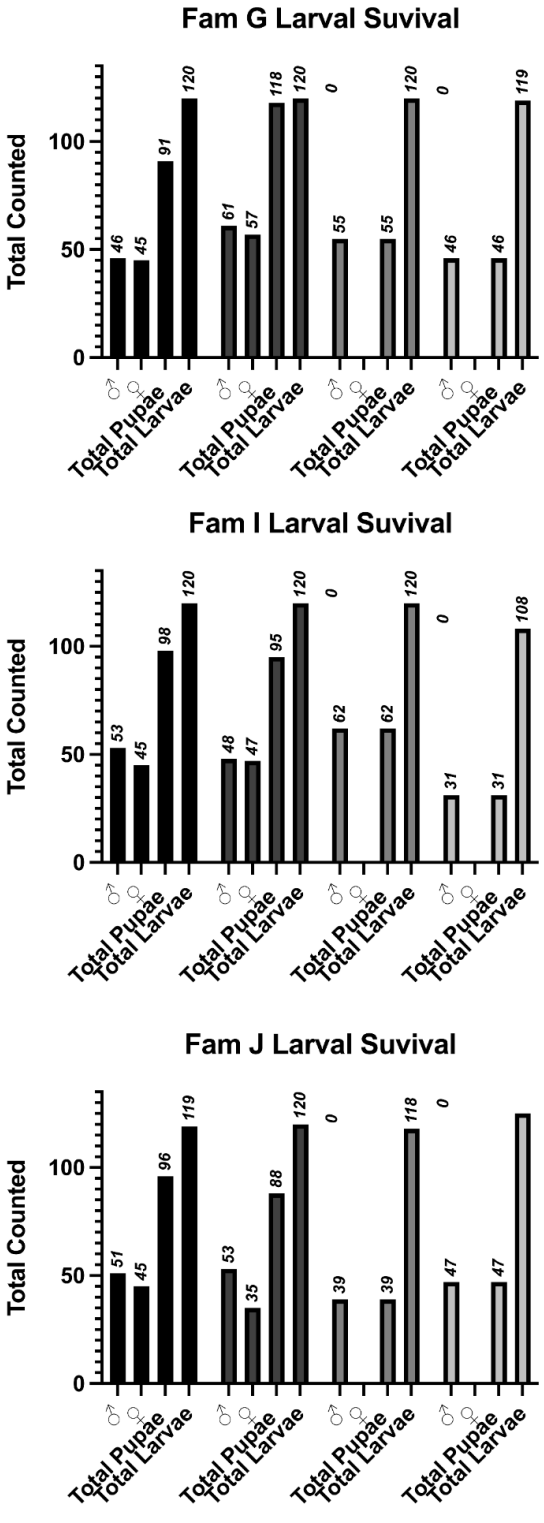
Based on these initial experiments, we concluded that female death occurs before pupaehood- either during embryogenesis or larvaehood. In the freshly-hatched L1 offspring of the +/gFLE X +/Cas9 cross, we observed an approximately 1:1:1:1 genotypic ratio, suggesting that most female lethality happens after embryogenesis (Figure 4A). To identify death at the larval stage, we sorted freshly-hatched L1 larvae by fluorescence and reared them separately in broods of 40 individuals. Then, we scored the pupae in each tray by sex and genotype. Significantly, only about half of the F1 +/gFle and transheterozygous larvae survived to pupaehood, all of which were male, whereas a majority of the +/+ and +/Cas9 sibling controls survived to pupaehood, suggesting that most female death was occuring during the larval stage (Figure 4B).

Figure 4: Embryo Survival and Larvae Survival Assays Show that Female Death Occurs in the Larval Stage.
(A) F1 freshly-hatched larvae were sorted by their fluorescence and counted into respective genotypes. (B) Around 120 L1 larvae from each genotype were raised separately in trays of about 40 individuals. The rightmost bar in each genotype shows the numbers of larvae placed in trays. The second rightmost bar shows the number pupae from each genotype. The leftmost and second leftmost bar shows the number of male and female successfully pupated in each genotype.

A


Fam G Embryo Survival
Larvae Counts
500
400
300
200
100
0
326
339
350
288
+/+
+/Cas9
+/gRNA
gRNA/Cas9
Fam I Embryo Survival
Larvae Counts
282
280
268
241
Fam J Embryo Survival
Larvae Counts
338
325
442
384

B


Fam G Larval Suvival
Total Counted
100
50
0
46
45
91
120
61
57
118
120
0
55
55
120
0
46
46
0
119
Total Pupae
Total Larvae
Fam I Larval Suvival
53
45
98
120
48
47
95
120
0
62
62
120
0
31
31
0
108
Fam J Larval Suvival
51
45
96
119
53
35
88
120
0
39
39
118
0
47
47

Transheterozygous, *Δfle* Gynecider Males Have Minimal Fitness Cost and Pass Down *Δfle* Alleles.

Due to the complete loss-of-female penetrance, knocking out *fle* in the gFle male x Cas9 female crossing scheme can be a Genetic Sex Sorting technology (GSS) candidate for *A.gambiae* vector control. It can also be used as an independent vector control technology with iterative releases of F1 transheterozygotes, the population can be 'loaded' with *Δfle* alleles, leading to sustained population suppression. We term this novel system Gynecider (**G**eneticall**Y** e**N**cod**E**d **C**RISPR **I**nduced **D**aughter **ER**adicator).

In Gynecider, the released F1 transheterozygous males must be able to pass down *Δfle* alleles. To characterize the transmission frequency of *Δfle* alleles to the F2 generation, as well as the sex-biasing properties of *Δfle* alleles, we crossed F1 transheterozygous males to WT females and scored the F2 offspring by sex and genotype. In the F2 generation, we observed complete lethality of transheterozygous females in Family G and very few transheterozygous females in Families I and J. The other genotypes show roughly normal Mendelian distribution (Figure 5A). Sequencing at the gRNA cut sites in F2 adults from all 4 genotypes demonstrated that mutant alleles were inherited (Figure 2C). We then crossed the F2 transheterozygous males to WT females, yielding an F3 generation that had consistent results with the F2 generation: no F3 transheterozygous females were found in Family G, and few F3 transheterozygous females were found in Family J. As of writing this thesis, the F3 family I cross is currently undergoing. Interestingly, we observed that the genotypes of the pupae were skewed towards WT and transheterozygote individuals (Figure 5B), potentially due to a WT contamination when the cross was set up. More replicates of data are ongoing to validate the result.

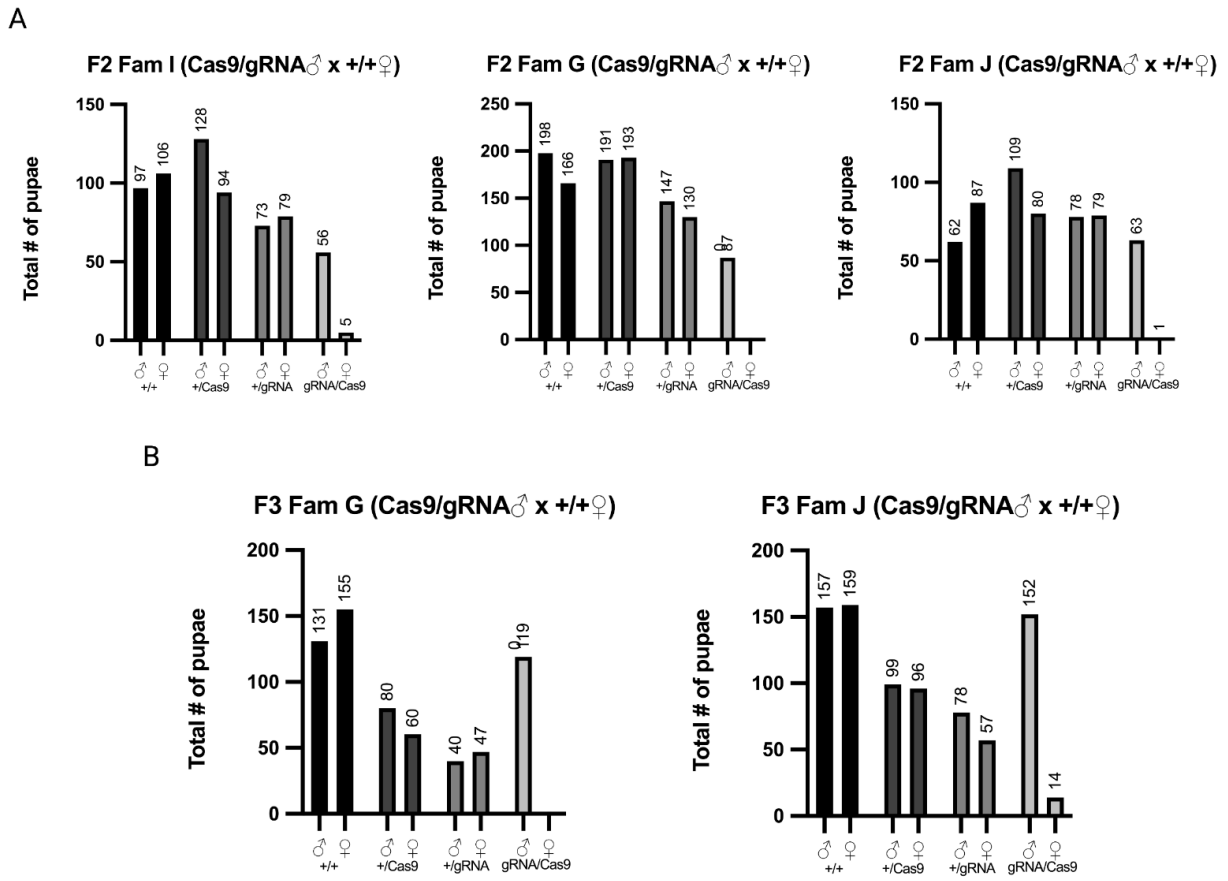


Figure 5: Transheterozygous Females Arise in F2 Generations, but Family G Still Shows Complete Transheterozygous Female Killing.
(A) The F2 offspring from experimental cross between Gynecider males with wild type females shows high transheterozygous female killing efficiency, but show females transheterozygous females arised in family I and J. (B) The F3 offspring from experimental cross between F2 transheterozygous males cross with wild type females still show high female killing efficiency. As expected, transheterozygous females arised in family J.

Furthermore, Gynecider requires that transheterozygous males are able to survive and be healthy enough to mate. To quantitatively analyze the lifespan of Gynecider males, we set up cages with transheterozygous F1 adult males and WT control siblings, and counted the number of adults that died each day. We observed that male mosquitoes from both the transhteterzogyous and WT control group start dying around the 25th day, suggesting that Gynecider males have

minimal fitness cost. As of writing this thesis, most male survival assays are ongoing. More male survival assays in other gFle families will be performed.

A.

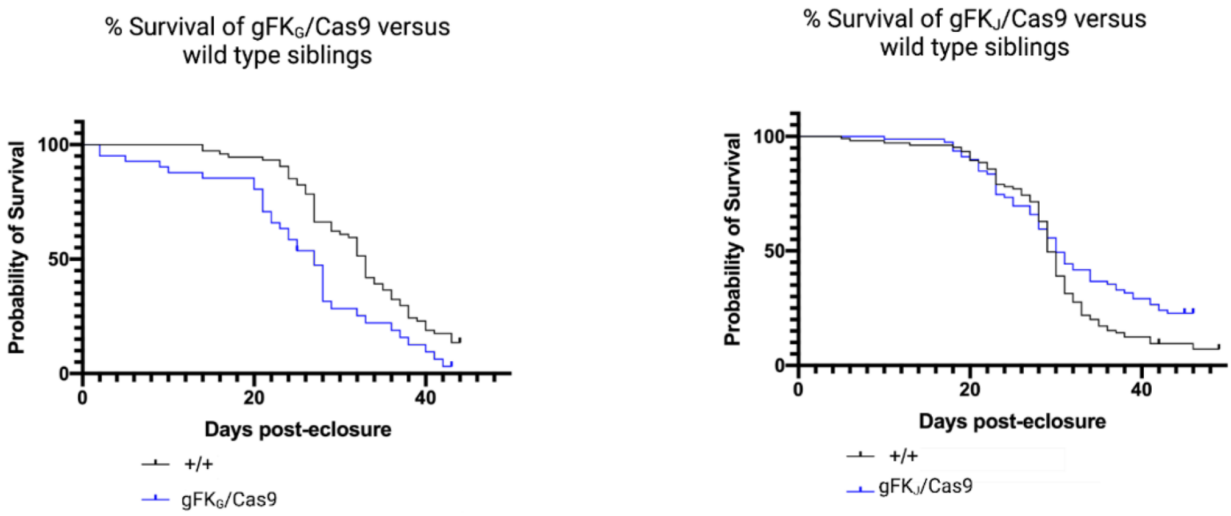


Figure 6: Ongoing family G WT and Transheterozygous Male Survival Assay
The 2 figures show the % of surviving adult males in the cage over time. Adults are placed in a cage and counted on day 0. Each day, a number of dead individuals are counted and removed from the cage.

Acknowledgement:

The result section, figure 1 is currently being prepared for submission for publication of the material. Reema Apte, Andrea L. Smidler, Neha Thakre, Omar S. Akbari. The thesis author was the primary investigator and author of this material.

Discussion:

Here, we have demonstrated that the current pgSIT gRNA design, consisting of the gRNA targets *zpg, B2* , and *dsx*, efficiently sterilizes males but has insufficient loss-of-female penetrance (Table 1), which has the risk of releasing of female mosquitoes and increase disease transmission.

In effort to find a highly female-specific gene target to increase the female-killing penetrance of pgSIT, we found that knocking out *fle*, an essential sex determination gene in *A. gambiae* identified by Krzywinska et al., in a pgSIT crossing scheme shows complete transheterozygous female killing and almost complete female killing in +/gFle female in F1 generation. Thus, incorporating *fle* as a gRNA target offers potential to greatly increase the female-killing efficiency of pgSIT. Because of this unexpectedly highly-penetrant phenotype, we built a novel GSS technology that we term Gynecider. Ultimately in the Gynecider system, homozygous gFLE males will be crossed with homozygous Cas9 females to exclusively generate transheterozygous F1 males that have 1 allele of Cas9 and gRNA. Releasing the F1 transheterozygous males will load the population with *Δfle* alleles to cause female lethality, as well as yield more transheterozygotes in the following generation because gFLE and Cas9 are on different chromosomes. With the combined effect of passing down *Δfle* alleles and generating more transheterozygous males, the release of a single generation will sustain multiple generations of female-killing in the wild. The F2 transheterozygous females from the F2 generation in Families I and J (**Figure 5A**) have not been processed to date. We speculate that these surviving F2 transheterozygous females have silent mutations in their alleles. Furthermore, we only observed transheterozygous females in certain crosses, suggesting that resistance is

individual dependent. Therefore, the formation of the resistance allele might be random, and while no transheterozygous females to date, more might appear in family G when more generations are tested. Compared to pgSIT, which is a dead-end single-generation system, Gynecider might have a higher efficiency for population control, but the few F2 transheterzogyous females show a possibility of developing resistance.

Compared to other genetic vector control systems, Gynecider is efficient because it has a lower sort:release ratio (ratio of mosquitoes that must be sorted to those that can be released). Currently, incomplete loss-of-female in fsRIDL and pgSIT in *A. gambiae* means that an additional sex sorting step is required prior to release. Because males and females can be separated at the pupae stage at earliest, this significantly reduces system efficiency. Because Gynecider kills females completely, Gynecider individuals can be released at the egg stage. Furthermore, compared to traditional fluorescent sexing strains that have a 2:1 ratio, Gynecider is 100 times more efficient. Sex sorting for Gyencider happens in F0 for the Cas9 and gRNA line as opposed to for the released generation, so for 4 mosquitoes sorted, 1 of them is Cas9 female, and each female gives rise to 400-600 eggs, half of which are male. This yields a 1:50 sort: release (400/4/2 = 50).

In order to further optimize our Gynecider and pgSIT systems, we are planning on developing a sex sorting system for the F0 generation by integrating a genetic circuit that uses the alternative sex-splicing mechanism in *Dsx* to fluorescently distinguish males and females (*32*). This will allow the gRNA parent and Cas9 parent to be sex-sorted by COPAS, a modified cell sorter machine which sorts based on transgene color and intensity, which is orders of magnitude faster than manual sorting (*33*, *34*).

Method:

Mosquito rearing and maintenance

A. gambiae was derived from the G3 strain. The mosquitoes were reared in 12h light/ dark cycles at 27°C, in cages (Bugdorm, 24.5 × 24.5 × 24.5 cm). The cages were placed in a transparent plastic bag to maintain humidity and allow the transmittance of light. For every 2 cages, 1 humidity cup was placed on top of the cage to maintain high humidity in the bag. Adults were provided with 0.3 M aqueous sucrose and allowed to feed *ad libitum*, and females were blood fed on anesthetized mice for 2 consecutive days for ~15 min at a time. Males and females were allowed to mate for at least 2 days before blood meal. Egg dishes were provided 2 days post-blood meal. Eggs were allowed to melanize for 2 days before being floated in trays. Roughly ~400 larvaes were reared in food grade plastic trays and fed fish food. For experimental crosses, pupaes were screened and sexed manually under the microscope by sex specific morphology, and allowed to eclose in different cages to ensure female virginity before being crossed together as adults. All the experimental cage rearing procedures were the same as stock cages rearing procedure, unless otherwise noted.

gRNA Design

All target gene reference sequences were extracted from VectorBase. Wild type mosquito genomic DNA was extracted (Qiagen, DNeasy Blood & Tissue Kits, Cat. No. / ID: 69504), and the target genome was validated by PCR and sanger sequencing. All sequence differences between reference genome and sequencing result were noted in our Snapgene file. The gRNAs

were designed 23bp in length, excluding PAM (NGG) sequence. All gRNAs were designed on CRISPOR to minimize off-targeting. All gRNA was ordered as gBlock from IDT.

gFle 7: CGACGGCTCGTTCATCGCTGGGG

gFle 10: ATCGAGCGCGTCGCCTGGTACG

gDsx1: CCCGTCAGAGTGGATGATAA

gDsx2: CCGCTTGACCTGTGTTAAAC

gZpg1: GATCCGATCACGCAGTCGAT

gB2.1: GCTCGATATCGTGCGCAAGG

gB2.2: CCAAATAGGCGCTAAGTTCT

Cloning the gRNA-expressing Plasmid

To generate a gRNA expressing plasmid, we ligated a gRNA-encoding fragment with a plasmid backbone that encoded PiggyBac transposase and Actin-eGFP. We constructed the plasmid backbone with 3 PCRs. First, we PCR-amplified and digested the 1114B plasmid with the restriction enzyme EagI and gel extracted the 8kb fragment encoding Vasa-PiggyBac transposase. Then, we reconstituted the *LacZ* gene and Actin promoter from the 1114B plasmid through PCR. Third, we PCR-amplified eGFP from the 1056H plasmid. We ligated these 3 PCR amplicons (1114B backbone, LacZ and Actin promoter, and eGFP sequence) using a Gibson 1-step assembly to generate the plasmid backbone. This ligation product was transformed into *E. coli* on kanamycin plates. Then, we performed mini prep, colony PCR, mini prep, and maxi prep. To generate the final plasmid, we ligated our synthesized backbone with a gRNA-encoding fragment synthesized by the gBlocks® Gene Fragment service (Integrated DNA Technologies,

Coralville, Iowa) using Gold Gate Ligation. We transformed this ligation mix into E. coli, and verified sequence integrity of the final plasmid with sequencing.

Establishing Transgenic Lines

The pre-established homozygous Cas9 line had a transgene expressing *Vasa-Cas9* and *3xP3-dsRed* integrated in the PhiC 31 insertion site and was previously described in (*35*). To establish gRNA lines, early preblastoderm *A. gambiae* embryos were injected with a solution of piggyBac transposon plasmid and DI water. Out of injected embryos, the surviving larvaes show episomal fluorescence. Fluorescent individuals were separately crossed to 50 wildtype individuals of the opposite sex. The fluorescent offspring from this cross were again individually crossed with wild type mates of the opposite sex to establish our 3 gRNA lines (G, I, J).

Fluorescent Sorting, Sexing, Imaging, and General Cross Setup

A. gambiae were fluorescently sorted, sexed, and imaged using the Leica M165FC fluorescent stereomicroscope with the Leica DMC2900 camera. Fluorescence was visualized using the CFP/YFP/mCherry triple filter. *A. gambiae* were sexed as pupae by differential morphology in their abdominal termini.

Genetic Cross Setup

For all crosses, *A. gambiae* were sexed as pupae, fluorescently sorted, and allowed to emerge as adults in separate cages to ensure female virginity before crossing. For experimental crosses, at least 50 males and females each are used. We waited 2 days before blood feeding to allow mating. Mouse was used for blood feeding. The egg dish was placed in the cage 2 days

blood feeding. Then, the egg dish is in the cage for 4 days to allow mosquitoes to lay eggs and eggs to develop. Larvae rearing and adult rearing are described above. The experimental individual will be analyzed based on what is needed for the experiment.

DNA Extraction and Sequencing Analysis

Mosquito egg, larvae, pupae, or adult is extracted using Qiagen DNeasy Kit (Cat. No. / ID: 69504). The sequencing region is PCR-amplified with the Q5 mastermix. The PCR product was run on a 1% agarose gel to check for correct amplification. Then, the PCR fragments were gel extracted and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat. No./ID: D4007). The PCR product can be directly sent for sequencing or clone into CloneJET PCR Cloning Kit (Catalog number: K1231) then sent for sequencing.

PCR Amplification

NEB Q5® High-Fidelity 2X Master Mix (Cat: M0492S) was used for PCR amplification. All procedures and conditions are based on NEB protocol.

S28 forward primer: ATTGTGCTACATCGCCGA

S28 reverse primer: CTAACGCTCCGGCATACACT

fle forward primer: CGGATACGCTACCAAGACTCAATAC

fle reverse primer: GTCGGTTGTATCGTCGCGT

Y chromosome forward primer: CAAAACGACAGCAGTTCC

Y chromosome reverse primer: TAAACCAAGTCCGTCGCT

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