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Engineering Genetic Biocontrol Strategies for Large-scale Insect Population Control

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Biology

by

Junru Liu 刘俊儒

Committee in charge:

Professor Omar S. Akbari, Chair
Professor Kimberly Cooper
Professor Prashant Mali
Professor Michael Perry

2024

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University of California San Diego
2024

DEDICATION

I dedicate this dissertation to my beloved family and friends, whose unwavering support and encouragement have played an integral role in shaping me into who I am today. This is dedicated to my beloved mother, 明华 (**Minghua**), who embodies strength, exudes emotional stability, and possesses an insatiable thirst for knowledge. Her example has taught me how to conduct myself as a person and guided me toward success. This is dedicated to my partner, **Ranger**, whose love and companionship have taught me the true meaning of patience. This is dedicated to my two cats, **Noboru** and **Mizuki**, the cutest cats ever, providing me with the best emotional support I could ever ask for. This is dedicated to my grandpa, 永福(**Yongfu**), who taught me the meaning of family. It's a long stream of love, support, and presence, where nobody needs to say anything, but just being there is enough. There are so many words I haven't said to you, but I'm glad you can see me whenever you want to now. Please visit me anytime, anywhere grandpa. And to all the family members I have in both China and the United States, I can't be any luckier to have such a supportive family that makes me feel at home no matter where I am. Your endless encouragement, love, and belief in my abilities have been the foundation of my strength and determination. You all have contributed to my journey and success. I am forever grateful for your presence in my life.

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pillars of support. My achievements stand as a testament not only to my own efforts but also to the faith and encouragement you have bestowed upon me. I see myself a very lucky person, being able to meet all these awesome people along my journey.

EPIGRAPH

「放下助人情节，尊重他人命运。」

“Respect the fate of others and do not interfere with their karma.”

TABLE OF CONTENTS

DISSERTATION APPROVAL PAGE	iii
DEDICATION	iv
EPIGRAPH	vi
LIST OF FIGURES	x
LIST OF TABLES	xii
ACKNOWLEDGMENTS	xiii
VITA	xv
ABSTRACT OF THE DISSERTATION	xvii
Chapter 1: Precision guided Sterile males suppress populations of an invasive crop pest.....	1
1.1 Abstract.....	1
1.2 Introduction.....	1
1.3 Results.....	3
1.3.1 CRISPR-mediated disruption of essential genes	3
1.3.2 Development of pgSIT strains	6
1.3.3 pgSIT males are competitive	7
1.3.4 Repeated releases of pgSIT males suppress and eliminate wt populations	8
1.4 Discussion.....	10
1.5 Material and Methods	14
1.5.1 Selection of gRNA target sites	14
1.5.2 Construct design and assembly.....	14
1.5.3 Fly rearing and transgenesis	15
1.5.4 Fluorescent selection of trans-heterozygous pgSIT flies.....	15
1.5.5 Genotyping loci targeted with gRNAs	16
1.5.6 Assessment of pgSIT male sterility.	16
1.5.7 Mating competition assays	17
1.5.8 Longevity assays.....	18
1.5.9 Population suppression experiments.....	18
1.5.10 Model fitting for population suppression experiments	19
1.5.11 Statistical analysis.....	20
1.6 Data Availability	21
1.7 Ethical conduct of research.....	21
1.8 Acknowledgements.....	22
1.9 Figures	23
1.10 Supplemental Figures	28
1.11 Tables.....	35
1.12 References.....	36
Chapter 2: A Fluorescent Sex-Sorting Technique for Insects with the Demonstration in <i>Drosophila melanogaster</i>	42
2.1 Abstract.....	42
2.2 Introduction.....	42
2.3 Results.....	44

2.3.1 Development of female-specific expression of the fluorescent protein	44
2.3.2 <i>DmtraF</i> , <i>DstraF</i> , and <i>CctraF</i> resulted in female-specific fluorescence	45
2.3.3 Confirmation of sex-specific fluorescence at multiple developmental stages.....	46
2.3.4 Assessing the fitness of the sex-sorting strains.....	47
2.4 Discussion.....	48
2.5 Materials and Methods	52
2.5.1 Molecular cloning.....	52
2.5.2 Reverse transcription PCR (RT-PCR) of the female-specific splicing transcripts.....	52
2.5.3 Rearing and fly transgenesis.....	52
2.5.4 Genetics and sex selection	53
2.5.5 Fitness estimation	53
2.6 Statistical analysis.....	53
2.7 Data availability	54
2.8 Acknowledgments	54
2.9 Figures	55
2.10 Supplemental Figures	60
2.11 Supplementary Tables	62
2.12 References.....	64
Chapter 3 Fluorescent based sex-separation technique in <i>Drosophila suzukii</i>	69
3.1 Abstract.....	69
3.2 Introduction.....	69
3.3 Result	71
3.3.1 All three experimental introns result in female-specific dsRed expression in <i>D. suzukii</i>	71
3.3.2 <i>CcTraF</i> , <i>DmTraF</i> , and <i>DsTraF</i> allow for sex sorting as early as L2 instar	72
3.3.3 Fitness of fluorescent SEPARATOR strains	72
3.4 Discussion.....	73
3.5 Methods	75
3.5.1 Molecular constructs design and assembly.....	75
3.5.2 Rearing and fly transgenesis.....	76
3.5.3 Reverse transcription PCR (RT-PCR) of the female-specific splicing transcripts.....	76
3.5.4 Genetics and sex selection	76
3.5.5 Fitness estimation	77
3.5.6 Statistical analysis.....	77
3.6 Data availability.....	77
3.7 Acknowledgments	77
3.8 Funding.....	77
3.9 Author contributions.....	78
3.10 Competing interests	78
3.11 Reference	78
3.12 Acknowledgement	81
3.13 Figures	82
3.14 Supplymentary Tables	84
Chapter 4 Inducing Female-Specific Lethality in <i>Drosophila</i> through Mitochondrial DNA Fragmentation	85

4.1 Abstract.....	85
4.2 Introduction.....	85
4.3 Results.....	88
4.3.1 MLS-PacI resulted in complete lethality in females.....	88
4.3.2 NLS-PacI and PacI without localization signal	90
4.3.3 Implementing a Tetracycline-Off System for Repressible Female-Specific Lethality.....	90
4.3.4 Inducible female-specific lethality in <i>Drosophila suzukii</i>	91
4.4 Discussion.....	93
4.5 Methods	96
4.5.1 Molecular cloning.....	96
4.5.2 Fly transgenesis and genetics.....	96
4.5.3 Embryo hatching rate and larvae to adult survival rate	97
4.5.4 Survival curves for Longevity estimates for the MitoFail flies	97
4.5.5 Statistical analysis.....	97
4.6 Data availability.....	97
4.7 Acknowledgments	97
4.8 Author contributions	98
4.9 Competing interests	98
4.10 Reference	98
4.11 Acknowledgement	101
4.12 Figures	102
4.13 Supplymentary figures.....	105

LIST OF FIGURES

Figure 1.1: Comparison of Sterile Insect Technique (SIT) and precision-guided Sterile Insect Technique (pgSIT).....	23
Figure 1.2: Assessment of Cas9/gRNA-mediated disruption of individual target genes.	24
Figure 1.3: Genetic characterization of three pgSIT systems.	25
Figure 1.4: Characterization of pgSIT males mating competitiveness and longevity.	26
Figure 1.5: Repeated releases of pgSIT males suppress <i>D. suzukii</i> populations.	27
Figure S1.1: Schematic maps of gRNA constructs and gRNA target sites used in the study.	28
Figure S1.2: Cas9/gRNA-mediated mutagenesis of <i>D. suzukii</i> <i>Sxl</i> induce female lethality or transformation into intersex.....	29
Figure S1.3: Cas9/gRNA-mediated mutagenesis of targeted sequences in <i>D. suzukii</i> <i>dsx</i> (A), <i>tra</i> (B), and <i>βTub</i> (C).	30
Figure S1.4: <i>D. suzukii</i> intersex phenotypes induced by Cas9/gRNA-mediated of <i>dsx</i> and <i>tra</i> . nitals and ovipositors.....	31
Figure S1.5: Genetic characterization of two X-linked triple gRNA strains.....	32
Figure S1.6: Genital structures of intersex, female, and male <i>D. suzukii</i>	33
Figure S1.7: The simultaneous disruption of two genes by triple gRNA constructs.....	34
Figure S1.7: The simultaneous disruption of two genes by triple gRNA constructs.....	34
Figure 2.1: Sex-sorter cassette in <i>Drosophila</i>	55
Figure 2.2 Expression of Opie2- <i>TraF</i> -dsRed or Hr5ie1- <i>TraF</i> -eGFP transgenes in <i>D. melanogaster</i> can be observed in different developmental stages (a) L1-L3 larval stage, (b) pupal stage, (c) adult under white light, Red Fluorescent Protein (RFP) and Green Fluorescent Protein (GFP) filters.	56
Figure 2.3: The <i>traF</i> introns splicing patterns in <i>D. melanogaster</i>	57
Figure 2.4: Female selection efficiency at different life stages in <i>D. melanogaster</i> for all six sex-sorter cassettes that exhibited female-specific fluorescence.....	58
Figure 2.5: Fitness cost of all eight sex-sorting cassettes was accessed through two parameters: (a) egg-hatching rate and (b) survival rate to adulthood.	59
Figure S2.1 (a) Sex-determination pathways of Drosophilidae and Tephritidae. (b) Alternative Splicing of <i>Cctra</i> in <i>D. melanogaster</i> and <i>C. capitata</i>	60
Figure S2.2 Splicing sites of the transformer from <i>D. melanogaster</i> , <i>D. suzukii</i> , <i>C. capitata</i> , and <i>A. ludens</i> in <i>D. melanogaster</i>	61
Figure 3.1: (A) Schematic of the SEPARATOR constructs developed and tested for the study, along with the corresponding fluorescence. (B) Female selection efficiency at different developmental stages for all three constructs that exhibit female-specific dsRed.	82
Figure 3.2: The expression of Opie2- <i>TraF</i> -dsRed in <i>D. suzukii</i>	83
Figure 4.1: (A) Schematic of MLS-PacI protein import into the mitochondria (B) PacI endonuclease cutting site sequence (C) PacI recognition sites on <i>Drosophila melanogaster</i> and <i>Drosophila suzukii</i> mitochondrial DNA.	102
Figure 4.2: (A) Female-specific toxin or gene and localization sequence (n=3, N =). A schematic representation of (B) the complete MitoFail cassette and (C) the Tetracycline-off inducible system.	103
Figure 4.3: (A) Embryo hatching rate and (B) Survival to adulthood for both wt and MitoFail flies with the presence and absence of doxycycline. Longevity (C) when Doxycycline is not present and (D) when Doxycycline is present.....	104

Figure S4.1: Genetic constructs and the respective transgenic lines developed in this study	105
Figure S4.2: Adult females or dead female pupae or unaffected male.....	106
Figure S4.3: (A) 1180 control cross and (B) MitoFail circuits at 18, 21 and 26°C. 107	

LIST OF TABLES

Table 1.1 Fertility of pgSIT (trans-heterozygous) males that inherited paternal <i>vasCas9</i> , measured by the hatching rate of sired eggs	35
Table S2.1: Primer sequences used in this study	62
Table S2.2: Sex-specific fluorescence sorting at multiple developmental stages.	63
Table S3.1: Primer sequences used in this study	84

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Chapter 1, in full, is a reprint of the material as it appears in GEN Biotechnology. Nikolay P Kandul¹, Junru Liu¹, Anna Buchman¹, Isaiah C Shriner, Rodrigo M Corder, Natalie Warsinger-Pepe, Ting Yang, Amarish K Yadav, Maxwell J Scott, John M Marshall, Omar S Akbari. Precision guided Sterile males suppress populations of an invasive crop pest. (2022) [¹ Equal contribution]. The dissertation/thesis author was the co-first-author and primary investigator of this paper.

Chapter 2, in full, is a reprint of the material as it appears in GEN Biotechnology. Junru Liu, Danny Rayes, and Omar S. Akbari. A Fluorescent Sex-Sorting Technique for Insects with the Demonstration in *Drosophila melanogaster*. (2024). The dissertation/thesis author was the first author and primary investigator of this paper.

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Chapter 4, in full, is a copy of the material that is currently being prepared for the publication. Kandul, Nikolay*; Liu, Junru*; Rayes, Danny; Yang, Minzhe; Akbari, Omar S. “Inducing Female-Specific Lethality in *Drosophila* through Mitochondrial DNA Fragmentation.” The dissertation author was the primary researcher and author of this material.

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“Next-generation genetic sexing strain establishment in the agricultural pest *Ceratitis capitata*” Scientific Reports. (2023).

“Precision guided Sterile males suppress populations of an invasive crop pest” GEN Biotechnology. (2022).

“Temperature-inducible precision-guided sterile insect technique” The CRISPR Journal. (2021).

“Development of a rapid and sensitive CasRx-based diagnostic assay for SARS-CoV-2” ACS sensors. (2021).

“Genetically Encoded CRISPR Components Yield Efficient Gene Editing in the Invasive Pest *Drosophila suzukii*” The CRISPR Journal. (2021).

“Suppressing mosquito populations with precision guided sterile males” Nature communications. (2021).

“Engineered reproductively isolated species drive reversible population replacement” Nature communications. (2021).

“A confinable home-and-rescue gene drive for population modification” Elife. (2021).

“A drug-inducible sex-separation technique for insects” Nature communications. (2020).

“Assessment of a split homing based gene drive for efficient knockout of multiple genes” G3: Genes, Genomes, Genetics. (2020).

“Transforming insect population control with precision guided sterile males with demonstration in flies”
Nature communications. (2019).

FIELDS OF STUDY

Major Field: Molecular and Genetic Engineering

Studies in Genetic Modification

Professor Omar Sultan Akbari

ABSTRACT OF THE DISSERTATION

Engineering Genetic Biocontrol Strategies for Large-scale Insect Population Control

by

Junru Liu 刘俊儒

Doctor of Philosophy in Biology

University of California San Diego, 2024

Professor Omar S. Akbari, Chair

The management of crop pests and invasive species presents a significant global challenge in food production, exacerbated by climate change and increased species mobility. Traditional non-specific pesticides, utilized for centuries, now pose environmental risks and encounter diminishing efficacy against evolving insect resistance. Consequently, there is a pressing need for safer, species-specific alternatives.

In addressing this need, the first aim of my thesis was to implement the precision-guided sterile insect technique (**pgSIT**) for combatting the prevalent crop pest, *Drosophila suzukii*. With **pgSIT**, only sterile *Drosophila suzukii* males emerge from the population, which can be released into the wild to compete with the wild-type males for population suppression purposes. Our findings underscored the

potential of using pgSIT as a safe, economically viable, and scalable approach to managing *Drosophila suzukii* populations.

Additionally, to enhance the efficiency of existing mass-rearing strategies, my secondary aim was to develop a precise, rapid sex-sorting method using genetic techniques that can be used in a wide range of insect species. Leveraging fluorescence, I developed a sex-sorting technique, **SEPARATOR**, based on the transformer (tra) intron. This technique demonstrated efficacy across two insect species: *Drosophila melanogaster*, and *Drosophila suzukii*. The SEPARATOR allows female- or male-specific selection at different developmental stage using fluorescence markers, which opens up the sexing window and enable automatic sex-sorting, thereby allowing mass rearing of the genetically engineered insects.

Finally, the last aim of my thesis was to develop an easily transferable and resistant-proof female-killing biocontrol technology. With this aim, we developed **MitoFail** (Mitochondrial female-associated lethality). This technique leverages the universally active restriction endonucleases shredding mitochondrial DNA molecules (mtDNA) only in females. By combining this with an inducible system, we were able to control female lethality with either temperature or doxycycline, providing a robust and adaptable biocontrol strategy.

Chapter 1: Precision guided Sterile males suppress populations of an invasive crop pest

1.1 Abstract

The *Drosophila suzukii* invasion of western countries has created an immense agricultural and economic threat to crop production. Despite many attempts to suppress its population, *D. suzukii* continues to destroy soft-flesh fruits. The precision guided sterile insect technique (pgSIT) utilizes the accuracy of programmable CRISPR gene targeting to generate sterilized males that can be deployed to suppress populations. Here, we generate pgSIT in *D. suzukii* and empirically and mathematically demonstrate that sterilized males are fit, competitive, and can eliminate populations of *D. suzukii*. Altogether, we describe an efficient way to generate sterile *D. suzukii* for release and safe effective population suppression.

1.2 Introduction

Drosophila suzukii is a crop pest that has invaded much of the world creating significant agricultural and economic damage to soft and stone fruit production.¹⁻⁸ They oviposit their eggs deep within the soft flesh of ripening fruit,⁹ which leads to the ultimate destruction of the crop.^{10,11} It has been found to damage cherries, raspberries, blackberries, blueberries, strawberries, peaches, plums, pluots, nectarines, grapes,¹² olives,⁸ tomatoes, kiwis, figs, and apples, for example.¹¹ In 2008 in California, Oregon, and Washington state, a *D. suzukii* crop infestation resulted in an estimated half billion dollars worth of damage.⁴ This agricultural and economic burden has prompted significant attempts to suppress populations of *D. suzukii*. Pesticides have been predominantly used for the control of *D. suzukii*, however there is increasing evidence that they are becoming resistant to pesticides presenting the potential of widespread resistance.^{13,14} This, combined with the environmental impact of using non-specific broad-spectrum insecticides, underscores the necessity for more targeted environmentally-friendly approaches. Combining the use of pesticides with other control methods, like mass trapping, sanitation, crop rotation, overhead sprinkler irrigation, exclusion netting, cold treatment of fruits post-harvest, behavioral modifiers, and biological control technologies has been suggested to increase the success of population control.¹⁵⁻¹⁸

Many biological control methods have been experimentally tested in *D. suzukii*. For example, multiple natural enemies (i.e. pathogens, predators, or parasitoids) of *D. suzukii* have been identified and characterized.^{19–21} However, *D. suzukii* larvae can evade or become resistant to parasitoid wasps due to a high hemocyte load,^{22–26} suggesting that natural enemies may be ineffective to control *D. suzukii*. Interspecies competition between *D. suzukii* and *D. melanogaster* has been shown to decrease *D. suzukii* survival in the laboratory setting,²⁷ but given that *D. melanogaster* oviposits on rotting fruit while *D. suzukii* females prefer non-rotting fruit,^{9,10} implies that this approach may be ineffective in the field. *Wolbachia*-induced incompatible insect technique (IIT) has been shown to be effective in *D. suzukii* with specific *Wolbachia* strains, however the risk of accidental release of *Wolbachia*-infected females remains unavoidable or would require an additional fitness-compromising irradiation step to sterilize females prior to release.^{28,29} Multiple conditional female lethality (FL) strategies using a tetracycline-off system have also been developed.^{30–32} FL systems are effective at killing females and are predicted to be effective at population suppression,³³ however FL systems for wild population control depend on releasing fertile transgenic males, posing a challenge for regulatory/social acceptance. Additionally, leaky FL toxins in males and the requirement for rearing the parents on tetracycline may have contributed to the observation that resulting males had reduced mating competitiveness.³¹ Finally, a *D. suzukii* gene drive system was developed for population modification, however risk of gene drive resistance, unknown ecological vulnerabilities, and obtaining regulatory/social acceptance remain critical challenges for strategies leveraging self-propagating genetic systems.^{34,35}

The sterile insect technique (SIT) has been used for over half a century to successfully suppress and even eliminate wild pest populations through repeated releases of sterilized males that sexually compete against wild males for mating with wild females.^{36,37} To utilize SIT efficiently, an ideal solution would allow for (i) mass rearing, (ii) efficient selection against females, (iii) male sterility, (iv) fit and sexually competitive released males, and (v) cost-effective logistics and deployment of sterilized males (Fig. 1A).^{37,38} Traditional SIT programs utilize DNA-damaging agents, such as ionizing radiation, to generate sterilized

males, thereby significantly reducing the overall fitness and mating competitiveness of released males and therefore, requiring large release ratios to achieve suppression of local populations.^{38–41} To overcome the limitation of using DNA-damaging agents, we previously developed a next-generation SIT based approach, termed precision guided SIT (pgSIT). This approach relies on programmably inducing specific mutations that result in female lethality and male sterility utilizing CRISPR/Cas9 technology⁴² (Fig. 1B). We demonstrated this technology in both *D. melanogaster*⁴² and *Aedes aegypti*⁴³. Here we engineer a *D. sukukii* pgSIT system that can be used to generate competitive sterile males. We demonstrate empirically and mathematically that repeated releases of pgSIT sterile males can result in suppression and elimination of populations. Together, pgSIT reveals itself as a useful and effective tool for control of *D. sukukii* pest populations, which in the future could be adapted to control wild populations of this global pest.

1.3 Results

1.3.1 CRISPR-mediated disruption of essential genes

To engineer pgSIT in *D. sukukii*, we utilized two types of transgenic strains including a genetically encoded Cas9 strain, supporting robust germline and somatic Cas9 expression, and a guide RNA (gRNA) strain expressing multiple CRISPR gRNAs targeting genes essential for both female viability and male fertility (Fig. 1B).^{42,43} *Sex lethal (Sxl)*, *doublesex (dsx)*, and *transformer (tra)* are sex determination genes essential for female-specific development in *D. melanogaster* and their disruption results in female lethality or transformation into intersex.^{42,44–46} The disruption of *βTubulin 85D (βTub)* arrests spermatid elongation and sperm mobility resulting in male-specific sterility,⁴⁷ together making these genes excellent targets for pgSIT. In *D. sukukii*, we previously generated four dsRed-tagged strains in which the Cas9 endonuclease is expressed under different promoters: *vasa-Cas9 (vasCas9)*, *BicC-Cas9 (BicC.Cas9)*, *nanos-Cas9 (nosCas9)*, and *Ubiq-Cas9 (UbiCas9)*⁴⁸. Prior data determined that *nosCas9* induced the least F₁ phenotypic disruption in combination with gRNAs targeting the *white* or *yellow* loci and that *UbiCas9* incurred some fitness costs to its carriers. Based on these results, we utilized *vasCas9* and *BicC.Cas9* strains for assessment of gRNAs in this study. Four gRNA plasmids were engineered to induce Cas9/gRNA-mediated disruption of *Sxl*, *dsx*, *tra*, and *βTub*: *gRNA^{Sxl}*, *gRNA^{dsx}*, *gRNA^{tra}*, and *gRNA^{βTub}* (Supplementary Fig. S1). To track transgenes,

gRNA^{dsx}, *gRNA^{tra}*, and *gRNA^{βTub}* plasmids harbored *Opie2-mVenus*, a yellow fluorescent protein (YFP), while the *gRNA^{Sxl}* plasmid harbored a red fluorescent protein (RFP), *Ubiq-dsRed* (Supplementary Fig. S1). To generate transgenic strains, each gRNA plasmid was injected into embryos of *D. suzukii* harboring the *Hsp70Bb-piggyBac* transposase.⁴⁹ One or two homozygous stocks were established for each plasmid including *gRNA^{Sxl}.S1*, *gRNA^{Sxl}.S2*, *gRNA^{dsx}*, *gRNA^{tra}.S1*, *gRNA^{tra}.S2*, *gRNA^{βTub}.S1*, and *gRNA^{βTub}.S2*.

We assessed the efficiency of each gRNA strain at directing programmable Cas9/gRNA-mediated gene disruption (Supplementary Fig. S1), by genetically crossing each gRNA strain to either homozygous *vasCas9* or *BicC.Cas9* strains, and scoring resulting sex ratios and fertility of F₁ trans-heterozygous progeny. Reciprocal crosses were conducted to explore the effect of Cas9 carryover, i.e. maternal Cas9 protein deposition into embryos, on gene disruption in the subsequent generation (Fig. 2A-B)⁵⁰. The *gRNA^{Sxl}.S1* and *gRNA^{Sxl}.S2* harbored a single gRNA targeting *Sxl* (*gRNA^{Sxl#1}*, Supplementary Fig. S1) and induced either female lethality, or transformed females into sterile intersexes, in combination with either *vasCas9* or *BicC.Cas9* strains (Fig. 2C, Supplementary Data File). Notably, the direction of the genetic cross with *gRNA^{Sxl}* affected the phenotype penetrance independently of the Cas9 strain. For example, maternal *Cas9* resulted in nearly complete female lethality, while paternal *Cas9* resulted in a weaker effect, where some females survived and others perished or were transformed into sterile intersexes (Fig 2C, Supplementary Fig. S2A, Supplementary Data File). Sanger sequencing of the *Sxl* gRNA target confirmed that the gRNA DNA target site was mutated (Supplementary Fig. S2B). The *gRNA^{dsx}* strain expresses two separate gRNAs targeting *dsx*, both of which direct Cas9/gRNA-mediated disruption as evidenced by Sanger DNA sequencing (Supplementary Fig. S3A) and induced transformation of most trans-heterozygous females into sterile intersexes (Supplementary Fig. S4A). Maternal *Cas9* induced significantly higher intersex frequencies by *gRNA^{dsx}* with *BicC.Cas9* (49.1±14.6% ♀ with vs 0±0% ♀ without Cas9 carryover, $p > 0.0001$, two-sided student *t*-test with equal variance), and with *vasCas9* (52.5±7.5% ♀ with vs 36.8±13.3% ♀ without Cas9 carryover, $p = 0.031$; Fig. 2C).

The *gRNA^{tra}* plasmid harbors two separate gRNAs targeting *tra*, however only one *tra* gRNA, *gRNA^{tra#1}* (Fig. S1), induced mutations at the *tra* target site as evidenced by Sanger DNA sequencing (Supplementary Fig. S3B). Using this plasmid, two *gRNA^{tra}* strains (S1 and S2) were generated that each revealed different intersex transformation efficiencies (Fig. 2C, Supplementary Fig. S4B) irrespective of the Cas9 strain they were crossed with. The majority of generated *gRNA^{tra}.S1*;+ *Cas9*/+ females survived and were fertile (Fig. 2C, Supplementary Data File), while a small fraction of trans-heterozygous females were transformed into sterile intersexes with either maternal or paternal *vasCas9* ($6.7 \pm 3.8\%$ ♂ and $1.5 \pm 3.2\%$ ♂, respectively) and *BicC.Cas9* ($3.4 \pm 5.1\%$ ♂ and $0 \pm 0\%$ ♂, respectively). *gRNA^{tra}.S2* induced a more efficient transformation of females into sterile intersexes with both *vasCas9* and *BicC.Cas9* strains. We observed that the majority of the *gRNA^{tra}.S2*/+; *vasCas9*/+ females were transformed into sterile intersexes with either maternal or paternal *Cas9* ($43.1 \pm 6.9\%$ ♂ vs $35.2 \pm 6.8\%$ ♂, respectively, $p = 0.100$; Fig. 2C). Interestingly, *BicC.Cas9* induced transformation of *gRNA^{tra}.S2*/+; *BicC.Cas9*/+ females into sterile intersexes only when *BicC.Cas9* was maternally inherited, and the trans-heterozygous females harboring paternal *BicC.Cas9* were not transformed, and were fertile ($0.28 \pm 0.7\%$ ♀ vs $52.7 \pm 3.9\%$ ♀, respectively, $p < 0.0001$; Fig. 2C, Supplementary Data File). This discrepancy in *tra* transgenic strains to induce similar rates of female transformation into intersex flies remains unknown but likely due to differences in transgene integration sites. Two independent insertion strains for *gRNA^{βTub}* (S1 and S2), did not induce complete male sterility in combination with either *vasCas9* or *BicC.Cas9* (Supplementary Data File) despite mediating mutations at one target site (*gRNA^{βTub#1}*; Supplementary Fig. S3C). In sum, these results suggest that (i) *Sxl* is the most efficient gene target to eliminate females, (ii) *vasCas9* provides consistent gene disruption whether inherited maternally or paternally as compared to *BicC.Cas9*, and (iii) additional gRNAs targeting *βTub* need to be generated and tested to induce robust male-specific sterility phenotypes.

1.3.2 Development of pgSIT strains

After assessing the disruption of several genes individually, we assembled gRNA plasmids for disruption of *Sxl* and *βTub* simultaneously. Three additional gRNAs (*gRNA^{βTub#3}*, *gRNA^{βTub#4}*, *gRNA^{βTub#5}*, Supplementary Fig. S1) each targeting different sites in *βTub*, in an attempt to achieve higher rates of sterility than those with *gRNA^{βTub#1}* and *gRNA^{βTub#2}*, were designed. Each triple gRNA plasmid combined the two gRNAs targeting *Sxl* (one was tested above and another was tested in *D. melanogaster*⁴² and had 100% complementary to *D. suzukii* *sxl* target) with one of three newly designed gRNA targeting *βTub* (either *gRNA^{βTub#3}*, *gRNA^{βTub#4}*, or *gRNA^{βTub#5}*), generating three unique triple gRNA plasmids: *gRNA^{Sxl,βTub#3}*, *gRNA^{Sxl,βTub#4}*, and *gRNA^{Sxl,βTub#5}* (Supplementary Fig. S1). The triple *gRNA^{Sxl,βTub}* plasmids also encoded *Opie2-mVenus* to facilitate genotyping in the presence of the *dsRed*-tagged *vasCas9* transgene. A total of 6 independent transgenic strains were established: zero *gRNA^{Sxl,βTub#3}*, four *gRNA^{Sxl,βTub#4}* (S1-S4), and two *gRNA^{Sxl,βTub#5}* (S1-S2) strains. Unfortunately, homozygous *gRNA^{Sxl,βTub#4}*.S4 induced fitness costs and was excluded from further analysis. Moreover, we determined that the *gRNA^{Sxl,βTub#4}* and *gRNA^{Sxl,βTub#5}* constructs were each integrated onto the X chromosome in two separate strains, *gRNA^{Sxl,βTub#4}*.S3 and *gRNA^{Sxl,βTub#5}*.S2, as they displayed X-linked genetic inheritance patterns (Supplementary Fig. S5A-B). Since, *D. suzukii* fathers transmit their X chromosome only to daughters, crossing female *vasCas9* with males harboring an X-linked *gRNA^{Sxl,βTub}* results in *vasCas9*/+ fertile F₁ males and limits application of the X-linked *gRNA^{Sxl,βTub}* strains (Supplementary Fig. S5B). Notwithstanding, both X-linked *gRNA^{Sxl,βTub#4}*.S3 and *gRNA^{Sxl,βTub#5}*.S2 strains induced complete female lethality and male sterility with paternally inherited *vasCas9* (Supplementary Fig. S5A) and could thus be used to generate pgSIT males, however autosomal transgenic gRNA lines would provide more versatility as they can be used to generate pgSIT males when inherited either maternally or paternally.

We next analyzed the three generated autosomal strains, *gRNA^{Sxl,βTub#4}*.S1, *gRNA^{Sxl,βTub#4}*.S2, and *gRNA^{Sxl,βTub#5}*.S1, and determined that (i) all *vasCas9*/+; *gRNA^{Sxl,βTub}*/+ females generated by both reciprocal genetic crosses perished or were transformed into sterile intersexes (n=286), (ii) all the trans-heterozygous males that inherited maternal *vasCas9* were sterile (n=1274), and (iii) one *gRNA^{Sxl,βTub}* strain

(*gRNA^{Sxl,βTub#4}.S2*) produced sterile males with paternally inherited *vasCas9* (Fig. 3). Fertility of F₁ trans-heterozygous males produced by *gRNA^{Sxl,βTub#4}.S1* and *gRNA^{Sxl,βTub#5}.S1* with paternal *vasCas9* was significantly diminished (8.0±17.9% and 5.0±11.2%, respectively, vs 100.0±0% fertility of control males, $p < 0.0001$ and 0.0001 , respectively, two-sided Pearson's chi-squared tests) (Fig. 3). Maternal Cas9 resulted in significantly higher female lethality of *vasCas9/+; gRNA^{Sxl,βTub#4}.S1/+* (0.5±0.8% maternal Cas9 ♀ vs 14.3±4.3% paternal Cas9 ♀, $p = 0.0017$, two-sided student *t*-test with equal variance) and *vasCas9/+; gRNA^{Sxl,βTub#5}.S1/+* (1.6±2.8% maternal Cas9 ♀ vs 7.3±2.3% paternal Cas9 ♀, $p = 0.0082$; Fig. 3). Notably, relatively high numbers of *vasCas9/+; gRNA^{Sxl,βTub#4}.S2/+* intersexes emerged (85 maternal Cas9 ♀ and 144 paternal Cas9 ♀; Fig. 3), and maternal Cas9 did not significantly affect the female lethality (16.9±4.7% vs 21.2±6.6%, respectively; $p = 0.2257$; Fig. 3, Supplementary Data File). Unsurprisingly, many emerged F₁ intersex flies were unfit and died soon after emerging or in a next few days (Fig. 3, Supplementary Fig. S6). To quantify the fertility of pgSIT males that inherited paternal *vasCas9*, we crossed batches of 5 pgSIT males to 20 wt virgin females and quantified the percentage of eggs laid and eggs hatched. Not a single egg laid by females mated with either *gRNA^{Sxl,βTub#4}.S1/+; vasCas9/+* (952 eggs) or *gRNA^{Sxl,βTub#5}.S1/+; vasCas9/+* males (321 eggs) hatched (Table 1). Five eggs hatched out of 518 eggs laid by females mated with *gRNA^{Sxl,βTub#4}.S2/+; vasCas9/+* males (0.9% egg hatching rate, Table 1), suggesting that male fertility was very strongly suppressed. Finally, the induced pgSIT phenotypes, i.e. female lethality or intersex transformation and male sterility, correlated with mutations at *Sxl* and *βTub* target loci induced by Cas9/gRNA complexes in trans-heterozygous flies (Supplementary Fig. S7). Taken together, these data indicated that multiple effective pgSIT strains were generated and these could be used to suppress populations.

1.3.3 pgSIT males are competitive

To evaluate the competitiveness of pgSIT males (Cas9/+; gRNA/+) against wt males, we assessed the ability of pgSIT males to secure mating with wt females in the presence of wt males and longevity of

pgSIT males as compared to *wt* males. We opted to evaluate the *gRNA*^{*Sxl,βTub#4*}.S1 as this line directed robust pgSIT phenotypes (Fig. 3) and did not exhibit observable fitness costs. Ten *wt* virgin females were cohabitated with either *wt* males, or pgSIT males, or an equal mix of both *wt* and pgSIT males for the period of 12 hours, then males were removed and female egg laying and hatching rates were quantified (Fig. 4A). Combining pgSIT males with *wt* males did not significantly alter the egg lay rate as compared to when only *wt* males were introduced (Fig. 4B). Therefore, mating success of the pgSIT males could be determined by the decrease of egg hatching rates. Combining pgSIT males with *wt* males indeed significantly decreased the egg hatching rate from 85.9±6.2% (with *wt* males alone) to 62.0±13.7% ($p = 0.019$, a two-sided *t*-test with equal variance; Fig. 4C) indicating that sterile pgSIT males successfully mated with *wt* females in the presence of *wt* males and were 56% as competitive as *wt* males (Supplementary Data File). Notably, *wt* females mated with *wt* males laid significantly more eggs than females mated with pgSIT only males: 61.2±15.1% and 67.9±39.6% vs 13.7±2.8%, respectively ($p > 0.001$ and $p = 0.034$; Fig. 4B). As expected, no hatched eggs were found when introducing only pgSIT males with *wt* females as these males are sterile.

We next quantified the longevity of pgSIT males relative to that of *wt* males. Two types of pgSIT males were generated by a genetic cross of the *gRNA*^{*Sxl,βTub#4*}.S1 and the *vasCas9* strains in both reciprocal directions. The survival curves showing average survival percentage and 95% confidence intervals (±CI) are depicted in Fig. 4D. The median survival of *wt* males was significantly longer than that of the pgSIT males harboring paternal or maternal *vasCas9*: 53 days vs 31 or 41 days ($p < 0.0001$ or $p < 0.001$, respectively, Gehan-Breslow-Wilcoxon test). The observed difference between the longevity of pgSIT males with paternal or maternal *vasCas9* was not statistically significant ($p = 0.1015$, Gehan-Breslow-Wilcoxon test; Fig. 4D). In sum, our data indicate that the pgSIT males longevity is 77% of the longevity observed in *wt* males.

1.3.4 Repeated releases of pgSIT males suppress and eliminate *wt* populations

The efficacy of pgSIT-mediated population suppression was assessed by performing discrete generation population experiments. For controls, we seeded a 2:1 ratio (*vasCas9/vasCas9* males to *wt* males)

per population each generation. Using this ratio, we expect to produce progeny made of $\frac{2}{3}$ *vasCas9/+* flies and $\frac{1}{3}$ *wt* flies (i.e., 2:1 ratio). However, we found that a significantly smaller fraction of *vasCas9/+* flies were produced than expected ($p < 0.0001$, a two-sided t test with equal variance; Fig. 5A) suggesting that competitiveness and/or fecundity of *vasCas9/vasCas9* males were lower than of *wt* males. Total progeny numbers produced by control populations at each subsequent generation after generation 0 were not significantly different, 474 ± 54 flies ($P = 0.4047$, ANOVA total progeny number by generation, Fig. 5B), and each *wt* female in average produced 11.84 ± 1.36 progeny flies (Supplementary Data file). For experimental populations, which were set up with *wt* and pgSIT (i.e., *vasCas9/+; gRNA^{Sxl,βTub#4}.S1/+*) males, we did not score any progeny harboring either dsRed or mVenus indicating that pgSIT males were indeed sterile (Supplementary Data file).. Release ratios (pgSIT:wt) of 3:1 and 5:1 showed significant decrease in progeny after generation #1 ($p = 0.0004$ or $p = 0.0003$, respectively; a two-sided t test with equal variance) and release ratio of 2:1 showed significant decrease in progeny after generation #2 ($P = 0.0025$; Fig. 5B). The higher release ratio of pgSIT to *wt* males induced a more rapid population collapse/decline (pgSIT:wt): 5:1 test populations collapsed by generation #5; 3:1 test populations collapsed by generation #7; and 2:1 test populations declined to 30% of the control population (Fig. 5B). The fact that the pgSIT males did not sire any progeny, while the total numbers of progeny declined each generation for test populations (Fig. 5B) strongly indicates that pgSIT males secured mating with *wt* females and caused population suppression. Taken together, these data indicate that releases of pgSIT males can be an effective measure for population suppression and elimination.

1.3.5 Mathematical simulations of pgSIT and estimation of male mating competitiveness

A deterministic mathematical model of pgSIT population dynamics was developed to characterize the discrete generation population experiments and determine parameter values related to the population suppression experiments. The best-fitting model outputs and parameter estimates are shown in Fig. 5C and Figs. 5D-F, respectively. Model fitting was performed considering normal prior distributions defined as follows: for the number of progeny per female, we estimated as 11.84 ± 1.36 (mean $\pm$ one standard deviation,

SD) (Supplementary Data File) from the control population bottles by dividing progeny numbers at each generation by 40 females seeded per bottles; for male mating competitiveness, we assumed a normal prior distribution with 0.56 ± 0.16 (Supplementary Data File); and lastly, for female remating probability, we assumed a normal prior distribution with mean 0.18 (informed by Lanouette et al, 2020⁵¹) and used an arbitrary SD of 0.08 which represents a 95% CI between 0.03 to 0.35.

The experimental data were consistent with generated pgSIT males being 108% (95% credible interval (CrI): 90–138%) as competitive as *wt* males and a mating event between a *wt* female and *wt* male producing an average of 26 (95% CrI: 24–27) progeny flies. We additionally estimated that the probability of re-mating is 18% (95% CrI: 4–37).

These results suggest that: for the 2:1 male pgSIT:wt ratio, 36% (95% CrI: 27–47%) of mating events are with *wt* males and produce progeny; for the 3:1 ratio, 27% (95% CrI: 20–37%) produce progeny, and for the 5:1 ratio, 18% (95% CrI: 13–26%) produce progeny (Fig. 5C). A stochastic implementation of the model (50 simulations for each male pgSIT:wt ratio) was implemented to capture the variability in the data (Fig. 5C). This suggests that, in the 2:1 male pgSIT:wt ratio experiment, the simulations display an overall tendency to reduce the population after each generation; however, the population may occasionally increase due to stochasticity. Notwithstanding, the 5:1 male pgSIT:wt ratio experiment is less sensitive to the stochasticity implemented in the model and, in this case, simulations suggest that the population is likely to be suppressed after each generation until it is eliminated.

1.4 Discussion

Here we describe the development of pgSIT in *D. sukukii* and demonstrate effectiveness for suppression of *D. sukukii* populations in the laboratory. We demonstrate that the generated sterile pgSIT males are sexually competitive with *wt* males and can rapidly suppress discrete populations at release ratios comparable to a *D. sukukii* conditional female-lethality strain³⁰ and lower than the ratios recommended for conventional SIT (i.e. at the minimum or higher than 10:1 ratio of SIT males to *wt* males)^{52–54}(Fig. 5B). Our

findings suggest that the *D. suzukii* pgSIT system may be an efficacious biological control method for suppressing *D. suzukii* infestations and should be scaled and tested in the field going forward.

Remarkably, in some strains our pgSIT technique produces up to 100% sterile males with all females killed or transformed into intersex flies (Fig. 3). Two engineered *D. suzukii* transgenic gRNA strains ($gRNA^{Sxl,\beta Tub\#4}.S1$ and $gRNA^{Sxl,\beta Tub\#5}.S1$) whose males, when crossed to female *vasCas9* *D. suzukii*, produced 100% sterile males (Fig. 3). All females that emerged had been transformed into sterile intersex flies. Despite these notable results, it should be said that not all strains resulted in perfect penetrance. For example in some strains, rates of female lethality, and/or transformation, as well as male sterility were imperfect and may depend on multiple factors, including maternal deposition of Cas9 and other unexplored factors including transgene insertion location and/or gRNA target sequences (Fig. 3). Given these results, it may be possible to further improve the system by targeting additional genes. For example, in addition to *βTub* , simultaneous disruption of additional genes essential for male fertility, for example male fertility factors (*kl-1*, *kl-3*⁵⁵, *kl-5*), or *zpg* (*zero population growth*), may further improve the robustness and penetrance (i.e. for strains that resulted in less than 100% sterility) of induced male sterility. Additionally, the disruption of *βTub* results in immobile sperms⁵⁶ that may not reach the female's spermatheca. Although we observed complete sterility and population suppression when targeting *βTub* , perhaps targeting paternal embryonic lethality genes that do not affect sperm ability to swim or fuse with the gamete, but instead are essential for early embryonic development, such as *sneaky* (*snky*)⁵⁷, *Heterochromatin Protein 1E* (*HP1E*)⁵⁸, *misfire* (*mfr*)⁵⁹ or *K81*⁶⁰ could be better future gene targets for pgSIT systems in insect species with polyandrous females. Finally, the *D. suzukii* U6:3 endogenous promoter and terminator, instead of the *D. melanogaster* U6:3 promoter, could increase the expression level of gRNA, resulting in further improvement of Cas9/gRNA-mediated disruption.^{61,62}

The pgSIT approach is expected to generate competitive pgSIT males ready for releases (Fig. 1B), because only two genes are specifically knocked out in F₁ pgSIT progeny. However, according to the decrease in egg hatching rate, the generated *D. suzukii* pgSIT males were only 55.6±31.8% as competitive as *wt* males (Fig. 4D). This value was likely underestimated because the *wt* females mated with only pgSIT

males laid significantly fewer eggs than females mated with only *wt* males suggesting that fewer eggs were also laid by females mated with both *wt* and pgSIT males vs those mated only with *wt* males (172 ± 145 vs 404 ± 235 or 364 ± 90) resulting in the overestimation of egg hatching rate (Fig. 4C). Our data on discrete generation control populations indicated that *D. sukuzii* *vasCas9/vasCas9* males were less competitive and/or fertile than *wt* males (Fig. 5A), suggesting that one *vasCas9* allele may contribute to fitness costs of pgSIT males. Nevertheless, the population suppression experiments indicated that pgSIT males were quite competitive against *wt* males. In fact, a rapid collapse of the populations was observed after 5-7 generations at 3:1 or 5:1 ratios of pgSIT males to *wt* males (Fig. 5B). The population suppression was achieved in spite of *D. sukuzii* females having ample opportunities to re-mate with *wt* males^{51,63,64} during a two-week period until parent flies were discarded. Furthermore, the Bayesian MCMC analysis fitted to the population suppression data inferred the posterior distribution of the pgSIT male competitiveness to approximate 100% (Fig. 5F). Taken together, these data suggest that the pgSIT males did not suffer large fitness costs and were able to compete against *wt* males for mating with *wt* females resulting in a decrease in the total population fecundity resulting in a rapid population collapse. Going forward, it remains to be determined if reducing female fecundity in wild populations will have the same direct impact on population size as we saw in our cage experiments.

Traditional SIT and *Wolbachia* IIT each require manual adult sex sorting methods whereby each individual male sorted is handled and manually released. With pgSIT, sex sorting is also required but at the parental generation (Fig. 1). This provides a unique advantage as each female sorted can give rise to up to 300 progeny,⁶⁴⁻⁶⁶ half of which will be sterilized males. Therefore, with pgSIT each female sorted can result in a release of up to 150 sterilized males that can be deployed at any life stage including as eggs. This results in a potential 150x improvement to scalability as compared to traditional SIT based approaches. Moreover, the direct deployment of eggs/larvae into the environment can help reduce logistical costs in addition to preserving the fitness of the released animals as they will not need to be directly handled. These males could emerge genetically sex-sorted and sterilized directly into the environment and immediately begin their search for wild females. Finally, given that pgSIT requires a genetic cross between two inbred strains that

are raised in complete separation, recessive deleterious mutations that may accumulate in the parental strains will be unlikely to be problematic in the released F1 hybrid pgSIT males, which may even display a degree of heterosis making them even more competitive.

As compared to gene drive approaches, pgSIT is much more robust when it comes to resistant alleles. Firstly, genetic variation or naturally existing mutations in the target genes that may be present in the wild will not affect the pgSIT approach as the released males are sterile and should not produce progeny. Additionally, if in the factory a rare resistant allele harboring animal survived, it would be released among pgSIT sterilized males, as they would all hatch together and be released en masse. So more than likely, this animal would not pass along these mutations. That said, even if it were to pass along resistant alleles in the target genes - these alleles would likely result in fitness costs and also they would not affect the pgSIT approach as we are releasing dead-end sterile males and no resistant allele can result in viable progeny being produced.

In the described binary two-locus pgSIT system for control of *D. sukukii*, Cas9 and gRNA transgenic strains are maintained separately and crossed together to generate trans-heterozygous mutant pgSIT progeny, requiring homozygous strains to be mass reared separately without contamination, sex-sorted, and crossed to generate releasable progeny.^{42,43} While we do believe this feat is possible to achieve at scale with sophisticated robotics, to further simplify the logistics of production of pgSIT males in *D. sukukii* going forward, a one-locus pgSIT genetic cassette could be engineered. For example, the innovative Temperature Inducible pgSIT (TI-pgSIT) system⁶⁷ could be generated to induce female lethality and male sterility in the subsequent generation while only requiring maintenance of one strain without the need for setting up genetic crosses. In order to achieve an effective TI-pgSIT system in *D. sukukii*, temperature inducible promoters will need to be functionally tested in this organism. Finally, to facilitate global logistics and deployment of pgSIT males, a method to store *D. sukukii* eggs and/or embryos, similar to cryopreservation of *D. melanogaster* embryos,⁶⁸ would expand global logistics and save costs for deployment of pgSIT solutions for insect control.⁴³

1.5 Material and Methods

1.5.1 Selection of gRNA target sites

To induce Cas9/gRNA-mediated female-specific lethality and male sterility, we targeted female-specific exons of sex-determination genes including either *sex lethal* (*Sxl*, DS10_00005289), or *transformer* (*tra*, DS10_00010087), or *doublesex* (*dsx*, DS10_00011892), and both exons of the male-specific β *Tubulin* 85D (β *Tub*, DS10_00012485). CHOPCHOP v2⁶⁹ was used for choosing gRNA target sites from selected sequences in the *Drosophila suzukii* genome (<http://spottedwingflybase.org>)⁷⁰ to minimize off-target activity. Sequences of gRNA target sites are listed in Supplementary Fig. S1.

1.5.2 Construct design and assembly

Genetic constructs were built into *piggyBac* transformation plasmids using the Gibson enzymatic assembly method.⁷¹ The *D. melanogaster* U6-3 promoter was used to direct the constitutive transcription of gRNAs. To terminate the gRNA transcription, we included the *D. melanogaster* U6.3 3'-UTR fragment, directly after the gRNA scaffold. Genscript (<https://www.genscript.com/>) was used to synthesize a Genepart containing the U6-3 promoter, gRNA scaffold and U6-3 terminator (1056A.2). Each gRNA transcription unit was cloned as two fragments (i.e., U6-3 promoter and *scaffold-3'UTR*), which overlapped at the gRNA sequence ± 10 bases at each side, into a single-cut and dephosphorylated *piggyBac* plasmid. The $gRNA^{Sxl}$ unit was added to the *piggyBac* harboring the *Ubiquitin-dsRed-p10* marker. To assemble the $gRNA^{tra}$, $gRNA^{dsx}$, and $gRNA^{\beta Tub}$ plasmids, we first generated a *piggyBac* plasmid carrying the *Opie2-mVenus-SV40* transformation marker by cloning three fragments: the *Opie2* promoter; the IDT GeneBlock (www.idtdna.com) of *mVenus*'s CDS, a yellow fluorescent protein derived from *Aequorea victoria*;⁷² and 3'UTR SV40. Then, we added pairs of $gRNA^{tra}$, $gRNA^{dsx}$, and $gRNA^{\beta Tub}$ units one at a time on each side of the *Opie2-mVenus-SV40* marker. Three triple $gRNA^{Sxl, \beta Tub}$ plasmids were assembled following the same strategy: the $gRNA^{Sxl\#1}$ and $gRNA^{Sxl\#2}$ units were added at each side of *Opie2-mVenus-SV40*, and then an additional $gRNA^{\beta Tub}$ unit was added directly downstream from the 5' *pBac transposon* sequence. Importantly, $gRNA^{Sxl\#1}$ was previously tested for activity in *D. suzukii*,⁷³ and $gRNA^{Sxl\#2}$ was tested in *D. melanogaster*.⁴²

and targets a sequence in *D. melanogaster* that is 100% complementary to the corresponding nucleotide DNA sequence in *D. suzukii*. The schematic map of each plasmid assembled in the study is presented in Supplementary Fig. S1. The plasmids DNA and complete sequences can be downloaded from Addgene ID # (183132, 183133, 183134, 183136, 183137, 183138, 185614).

1.5.3 Fly rearing and transgenesis

D. suzukii flies were maintained in an ACL2 insectary as approved by the UCSD biosafety committee and handled by limited expert investigators to prevent any unintended releases. The flies were maintained on a cornmeal-yeast-agar diet at 21°C with a 12 h/12 h light/dark cycle, and all genetic crosses were performed under these conditions. The *D. suzukii* wild-type (wt) strains used in the study originated from Corvallis, Oregon.³⁴ Embryo injections were carried out at Rainbow Transgenic Flies, Inc. (www.rainbowgene.com). Plasmids diluted in water to 250µg/1µL were injected into freshly collected embryos of the *D. suzukii* harboring *Hsp70Bb-piggyBac* transposase.⁴⁹ G₀ adults were outcrossed to *D. suzukii* wt flies and their G₁ progeny were screened for transgenesis using the presence of fluorescent markers with a Leica M165FC fluorescent stereomicroscope. Generated transgenic *D. suzukii* strains expressing independent transgenic insertions were repeatedly introgressed over multiple generations to generate stable homozygous stocks. Briefly, three virgin females and two/three males, each harboring the brightest markers, were selected among each generation progeny and repeatedly intercrossed over at least 7 generations before the strain was expanded and fluorescence of each fly was confirmed. If any flies without a fluorescent marker were observed, the introgression was repeated.

1.5.4 Fluorescent selection of trans-heterozygous pgSIT flies

To assess the disruption efficiency of gRNA targeted genes, we set up reciprocal genetic crosses between Cas9 and gRNA strains and generated F₁ trans-heterozygous progeny. All genetic crosses were performed in fly vials using groups of seven to ten flies of each sex and repeated at least three times using different parent flies. The generated F₁ flies were examined and sex sorted using a Leica M165FC

stereomicroscope. We used the ET mCHER filter (Leica #10450195) to score the *dsRed* transgene in *Cas9* and *gRNA^{Sxl}* strains and the ET GFP (Leica #10447408) for the *mVenus* and *eGFP* transgenes in the other *gRNA* strains. The GFP3/mCH double-pass filter (Leica #10450203) was applied to expedite fluorescent genotyping of trans-heterozygous flies. Sample sizes and numbers of biological replicates can be found in the Supplementary Data File.

1.5.5 Genotyping loci targeted with gRNAs

Separate gRNAs targeting nearly identical sequences can direct different cleavage efficiencies by the same *Cas9* transgene.^{42,43,74} Therefore, we examined the molecular changes at target sites for each gRNA sequence tested, irrespective of successful induction of the pgSIT phenotype. The *Sxl*, *tra*, *dsx*, and *βTub* targeted loci were PCR amplified from groups of flies and sequenced in both directions using the Sanger sequencing at Retrogene, Inc (www.retrogen.net). The primer sequences are presented in Supplementary Table S1. Sequence reads were aligned against the corresponding reference sequences in SnapGene® 4 and sequence chromatograms were examined. When a PCR amplicon mixture contains indels localized at the *Cas9*/gRNA cut site and *wt* alleles, Sanger sequencing reads show consensus chromatograms and will thus have unambiguous single chromatogram peaks upstream and downstream the *Cas9*/gRNA cut site, after which the chromatogram quality typically deteriorates (Supplementary Figs. S2B, S3, S7).

1.5.6 Assessment of pgSIT male sterility.

Unlike *D. melanogaster*, single-pair crosses of *D. sukii* are inefficient and frequently result in no progeny and dead P₀. Therefore, we tested the sterility of F₁ trans-heterozygous pgSIT males in bulk by crossing 10-20 males to 15-20 virgin *wt* females per vial. Males were flipped to new vials 2-3 times every 4-5 days and were paired with new virgin *wt* females. Presence of F₂ larvae was recorded up to 20-25 days after initial pairing. If no larvae were observed after 20-25 days, the group of pgSIT males were scored as sterile. However, if F₂ larval activity was observed, F₂ progeny were reared and examined for fluorescence at the larval and adult stages to verify the genotype of the F₁ males. We therefore referred to a group of

pgSIT males as fertile only after both *gRNA^{Sxl,βTub}* and *vasCas9* transgenes (i.e. as *mVenus* and *dsRed*) were identified among the F₂ progeny. Consequently, a single fertile male in a cross can result in the overestimation of male fertility.

1.5.7 Mating competition assays

To assess the competitiveness of pgSIT males, we evaluated their ability to court and mate with *wt* females in the presence of *wt* males. The *vasCas9* virgin females and *gRNA^{Sxl,βTub#4}.S1* males were used to generate the pgSIT males for the competition assay. pgSIT or *wt* males and virgin females were aged for 4-5 days prior to the competition assay. The pgSIT and *wt* males were aged separately to prevent mixing of their cuticular hydrocarbons to accurately model female choice. Since single pairs of *D. suzukii* do not consistently produce sufficient numbers of progeny per vial for progeny viability, we used the previously described mating competition assay with ten females.^{42,75} The mating assay with multiple females does not measure the male competitiveness *sensu stricto*, however the mating assays with single and multiple females were shown to infer the same conclusion.⁶³ Groups of 10 virgin females were confined with 10 *wt* males alone, 5 *wt* males alone, 5 *wt* and 5 pgSIT males, or 10 pgSIT males alone per vial for 12 hours in the dark. After 12 hours of mating, the females were transferred into small embryo collection cages (Genesee Scientific 59–100) with grape juice agar plates and the percentage of hatched eggs laid over 20 days was calculated. Numbers of laid eggs were converted into percentages by normalizing to the highest number of laid eggs, i.e. 595 eggs. Mated *Drosophila* females are non-receptive to re-mating for 12-24 hours,^{76–78} therefore a decrease in percentage of hatched eggs indicates the ability of a sterile pgSIT male to successfully mate with females in the presence of a *wt* male providing a readout of the competitiveness of pgSIT males. The relative competitiveness of pgSIT males was estimated by subtracting the observed decrease of the hatched egg percentage found for the females courted by both 5 *wt* and 5 pgSIT males for each replicate from the mean hatched egg percentage found for the females courted by only 5 *wt* males (i.e. 86%, Fig. 4C), and normalizing the remainder over the expected decrease under perfect competitiveness (i.e. 86/2).

1.5.8 Longevity assays

To compare differences in lifespan between pgSIT and *wt* males, we performed longevity assays comparing *wt* males to pgSIT males with either maternally or paternally inherited Cas9. 10 age-matched males of a single genotype were allowed to age in vials. Ten replicates per genotype were used to estimate survival curves. Males were transferred to new vials every 3 days and the number of dead flies per vial were recorded. Survival curves were graphed and compared in Prism 9 for macOS by GraphPad Software LLC.

1.5.9 Population suppression experiments

We used discrete generation population experiments (in rearing bottles) to assess the efficacy of pgSIT-mediated population suppression. A similar experimental design was previously used to demonstrate population modification by a gene drive^{34,79} and population suppression by a repressible female-specific lethal.³⁰ The trans-heterozygous *vasCas9/+; gRNA^{Sxl,βTub#4}.S1/+* males, i.e. the pgSIT males, were released by mixing them with *wt* males at specific ratios while populations were seeded. To establish the fixed size populations of *D. suzukii*, we seeded groups of forty 4-5-day-old virgin *wt* females in 0.3 L plastic bottles (VWR Drosophila Bottle 75813–110). The mixture of 4-5-day-old 40 *wt* and 80 *vasCas9* (1:2 ratio) males was added to control bottles. For test bottles, we added 40 *wt* and 80 *pgSIT* (1:2 ratio) or 40 *wt* and 120 *pgSIT* (1:3 ratio) or 40 *wt* and 200 *pgSIT* (1:5 ratio) males per bottle. After 4 days at 21 °C, parent flies were transferred into fresh bottles and the first bottles were discarded. In 5-7 days, depending on the food condition, the parent flies were transferred again into the fresh bottles before being removed permanently in another 5 days. After a total of 18-25 days, progeny adults that emerged from the second and third bottles were collected, sexed, sorted for the presence or absence of the fluorescent protein marker, and then counted.

The control bottles were set with the same number of flies, 40 *wt* pairs and 80 *vasCas9* males, per bottle each generation. For the test bottles, the number of virgin *wt* females per bottle was based on the average number of females produced in the previous generation in the test bottles normalized that in the control bottles. The number of added females was calculated according to the formula $N = 40 \times (T/C)$, where

N is the number of females added to the current generation of test bottles, T is the average number of females produced from the test bottles in the previous generation and C is the average number of females produced from the control bottles in the previous generation. To keep the release ratio of pgSIT males constant, N *wt* males mixed with $2N$ or $3N$ or $5N$ *pgSIT* males were added to the corresponding test bottles. The experiment continued until no females were produced from the test bottles with the 1:3 *pgSIT* male release ratio.

1.5.10 Model fitting for population suppression experiments

We designed a deterministic model to describe the discrete generation population dynamics and fitted the output from the model to experimental data. In designing the model, we assumed that *wt* female flies: (i) mate at least once per generation; (ii) might re-mate up to 3 times, and; (iii) only produce progeny in the case of at least one mating with a *wt* male. At each generation i ($i = 1, \dots, 7$, with $i = 0$ representing the initial condition), a total of K_i *wt* flies (we assume an equal ratio of males and females) are produced according to the equation,

$$K_i = \left(40 \cdot \frac{K_{i-1}}{2C_i}\right) \cdot \alpha \cdot p(r, c). \#1$$

The expression $\left(40 \cdot \frac{K_{i-1}}{2C_i}\right)$ captures the dynamics of the number of *wt* females being added to bottles at each generation as described in the subsection “Population suppression experiments,” where C_i is the average number of females produced from the control bottles at generation i ; α represents the number of progeny produced by a mating between *wt* female and *wt* male flies; and $p(r, c)$ represents the probability that a *wt* female mates at least once with a *wt* male fly according to the experiment *pgSIT*:*wt* male ratio, r (2, 3 or 5), and the relative mating competitiveness of *pgSIT* males relative to *wt* males, c . We assume that female flies mate up to 4 times and define $p(r)$ as follows,

$$p(r, c) = p_{wt}(r, c) + \sum_{n=1}^3 (1 - p_{wt}(r, c))^n \cdot (p_{rm})^n \cdot p_{wt}(r, c), \#2$$

where $p_{wt}(r, c)$ is the probability of mating between a *wt* female fly and a *wt* male fly according to the ratio r and the relative mating competitiveness c , and p_{rm} is the probability of the female fly re-mating. To account for differences in mating competition between *wt* and *pgSIT* males, we define $p_{wt}(r, c)$ as,

$$p_{wt}(r, c) = \frac{1}{1 + r \cdot c}. \#3$$

We used Bayesian Markov chain Monte Carlo (MCMC) methods to estimate parameters α , c and p_{rm} and their respective 95% credible intervals assuming normal prior distributions for all three parameters. The log-likelihood of observing the population bottle experiment data was calculated assuming that the total number of flies at each generation ($i = 0, \dots, 7$) and in each experiment (male *pgSIT*:*wt* ratios r of 2:1, 3:1 and 5:1) follows a Poisson distribution with model-predicted mean estimated according to the mathematical model in Equations 1-3 and sampled parameter values. The MCMC sampling procedure was used to calculate posterior parameter distributions and was run for 10^6 iterations.

A stochastic version of the fitted model was implemented to capture the variability in the experimental data. At each generation ($i = 1, \dots, 7$) each *wt* female fly might mate or not with a *wt* male fly according to a random sample from a binomial distribution with probability $p(r, c)$ according to the experiment ratio r and the relative mating competitiveness c .

1.5.11 Statistical analysis

Statistical analysis was performed in JMP Pro 16 by SAS Institute Inc. and Prism v9 for macOS by GraphPad Software, LLC. Three to seven biological replicates were used to generate statistical means for comparisons. P values were calculated for a two-sample two-sided Student's t test with equal variance. One-way ANOVA was used to assess the significance of interactions between the laid egg rates or egg hatching

rate and the experimental setting, and between the progeny number and the generation. To test the significance of male fertility, Pearson's chi-squared tests for contingency tables were used to calculate two-sided *P* values. To assess the significance of lifespan for pgSIT males vs *wt* males, we used the Gehan-Breslow-Wilcoxon test, which does not require a consistent death rate throughout all time points. All plots were constructed using Prism 9 for macOS by GraphPad Software, LLC.

1.6 Data Availability

All plasmids and annotated DNA sequence maps are available at www.addgene.com under accession numbers: # 183132, 183133, 183134, 183136, 183137, 183138, and 185614. The Fly strains developed here will be made available upon request. A Supplementary Data File is provided.

1.7 Ethical conduct of research

All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as recommended by the National Institutes of Health and approved by the UCSD Institutional Animal Care and Use Committee (IACUC, Animal Use Protocol #S17187) and UCSD Biological Use Authorization (BUA #R2401).

1.8 Acknowledgements

This chapter, in full, is a reprint of the material as it appears in GEN Biotechnology. Nikolay P Kandul¹, Junru Liu¹, Anna Buchman¹, Isaiah C Shriner, Rodrigo M Corder, Natalie Warsinger-Pepe, Ting Yang, Amarish K Yadav, Maxwell J Scott, John M Marshall, Omar S Akbari. Precision guided Sterile males suppress populations of an invasive crop pest. (2022) [¹ Equal contribution]. The dissertation/thesis author was the co-first-author and primary investigator of this paper.

1.9 Figures

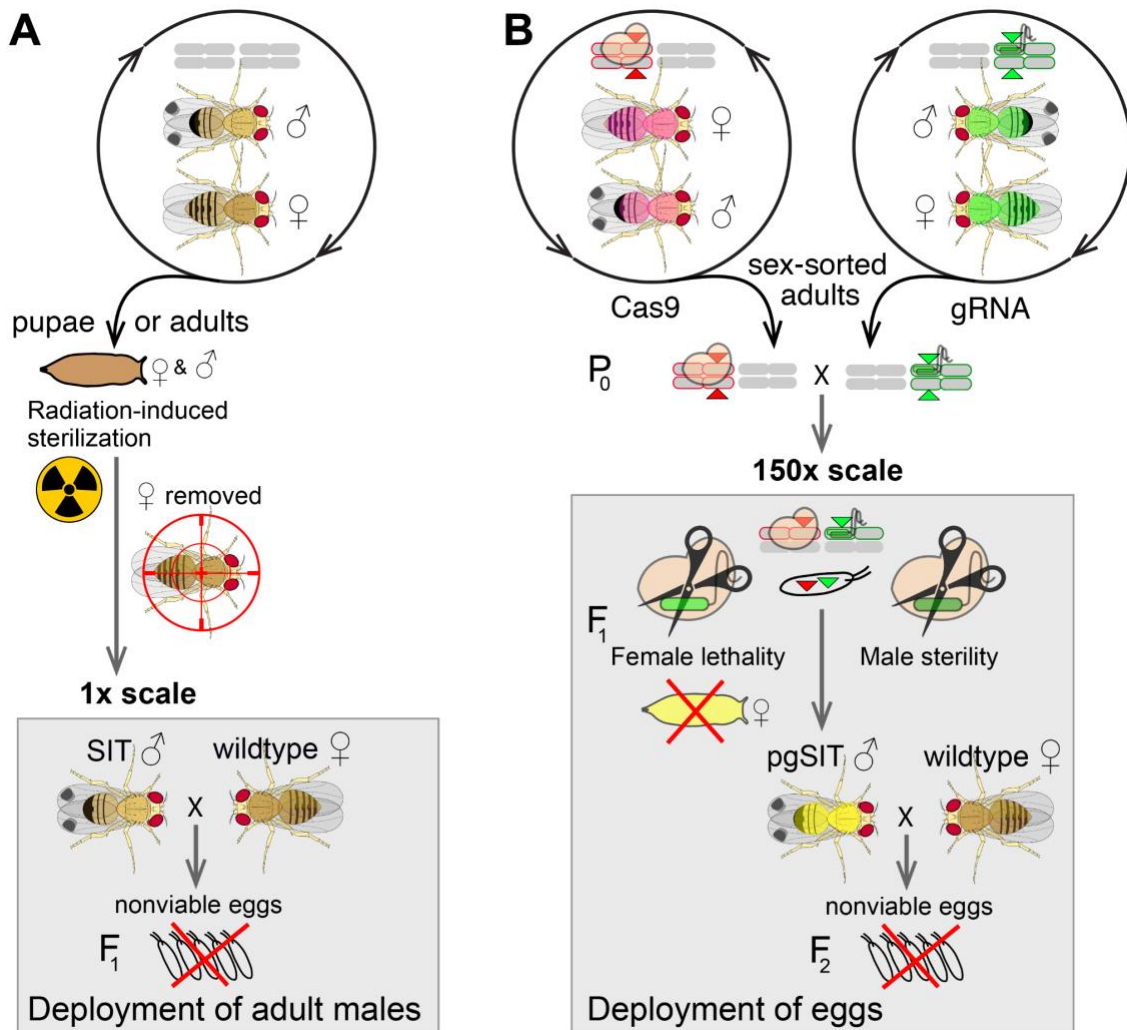


Figure 1.1: Comparison of Sterile Insect Technique (SIT) and precision-guided Sterile Insect Technique (pgSIT). (A) Classical SIT relies on ionizing radiation to sterilize pupae or adult flies, then females are discarded and adult males are deployed. As a result, every male released in the environment is reared, sterilized, and sex-sorted at a 1x production scale. (B) In pgSIT, a genetic cross between parental strains harboring Cas9 and guide RNAs (gRNAs) targeting genes essential for female viability and male fertility results in Cas9/gRNA-mediated disruption of the targeted genes leading to female lethality (aka. sex-sorting) and male sterility in the F₁ progeny. Each parental female sex sorted can produce up to 300 progeny and 50% of those will be sterilized males resulting in 150 sterile males produced for each sorted female. This results in a potential scaling benefit of 150x. Additionally, traditional SIT requires the handling and release of adult males resulting in reduced fitness costs due to manual manipulations. With pgSIT, eggs can be directly deployed and hatched in the environment thereby eliminating the need to manually manipulate the sterilized males, resulting in preservation of their fitness.

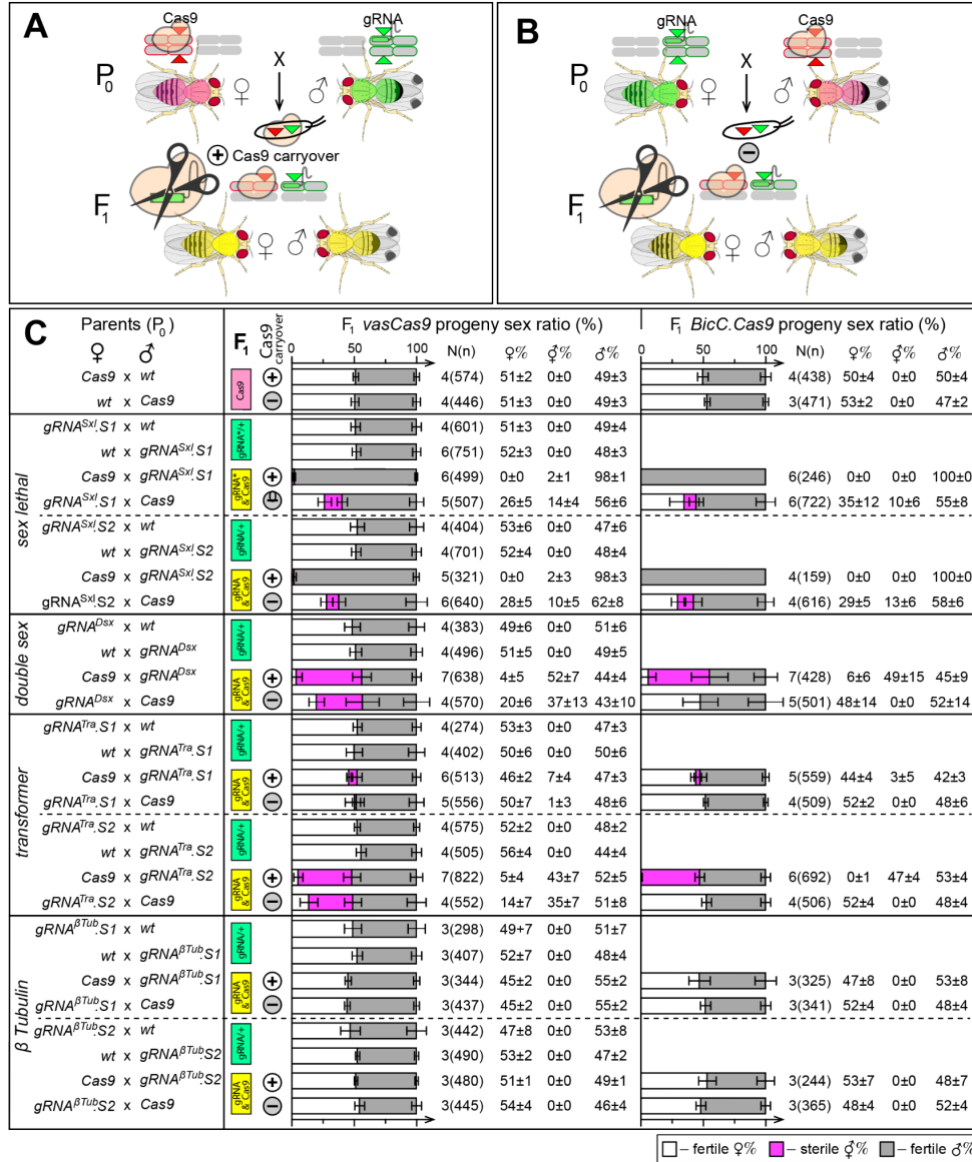


Figure 1.2: Assessment of Cas9/gRNA-mediated disruption of individual target genes. Different gRNA strains expressing one or two gRNA targeting *Sex lethal* (*Sxl*), *doublesex* (*dsx*), *transformer* (*tra*), or *βTubulin85D* (*βTub*) (Supplementary Fig. S1) were genetically assessed in combination with either *vasCas9* or *BicC.Cas9*. Each tested gRNA strain was genetically crossed to Cas9 and wt strains in both reciprocal directions to generate F₁ heterozygous (*Cas9*/+ or *gRNA*/+) and trans-heterozygous (*gRNA* & *Cas9*) progeny. Maternal inheritance of Cas9 (A) results in deposition of Cas9 protein into embryos, i.e. Cas9 carryover (+); while paternal inheritance of Cas9 (B) does not (-). Sex ratios of the progeny of the crosses indicated on the left side of panel C. The sex of eclosed adults was scored as female (♀), intersex (♂), or male (♂). Bars indicate the mean frequency and one standard deviation (±SD), as well as fertility for each observed sex. *N* is the number of biological replicates and *n* is the total number of scored flies. F₁ counts and fertility data can be found in the Supplementary Data File.

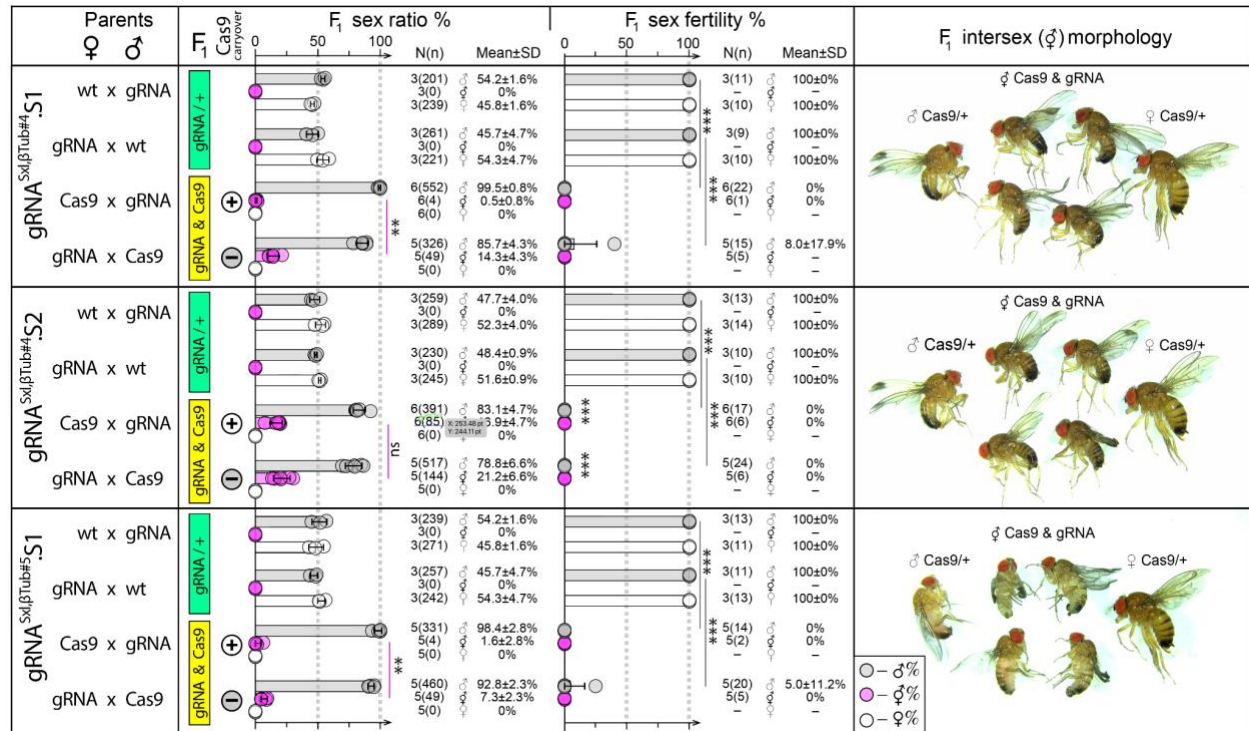


Figure 1.3: Genetic characterization of three pgSIT systems. Three autosomal *gRNA^{Sxl,βTub}* strains expressing two gRNAs targeting *Sxl* and one gRNA targeting *βTub* were assessed by genetically crossing each strain with the *vasCas9* and *wt* strains. The direction of a genetic cross is indicated on the left panel. Maternal inheritance of *Cas9* results in deposition of *Cas9* protein into embryos, i.e. *Cas9* carryover (+); while paternal inheritance of *Cas9* does not (–). The sex and fertility percentages of eclosed F₁ heterozygous (*gRNA*+) and trans-heterozygous (*gRNA* & *Cas9*) flies are reported as two separate bar graphs. The sex of eclosed adults was scored as female (♀), intersex (♂), or male (♂). Bars indicate the mean frequency and one standard deviation (±SD). *N* is the number of biological replicates and *n* is the total number of scored flies. Both *N* and *n* numbers are reported for each scored sex. Images of four representative intersexes compared to both male and female flies are presented for each triple gRNA strain in the right panel. Statistical significance in sex ratio was estimated using a two-sided *t* test with equal variance. Pearson’s two-sided chi-square tests for contingency tables were used to assess the difference in male sterility ($p \leq 0.05^{ns}$; $p < 0.001^{***}$). Source data is provided as a Supplementary Data File.

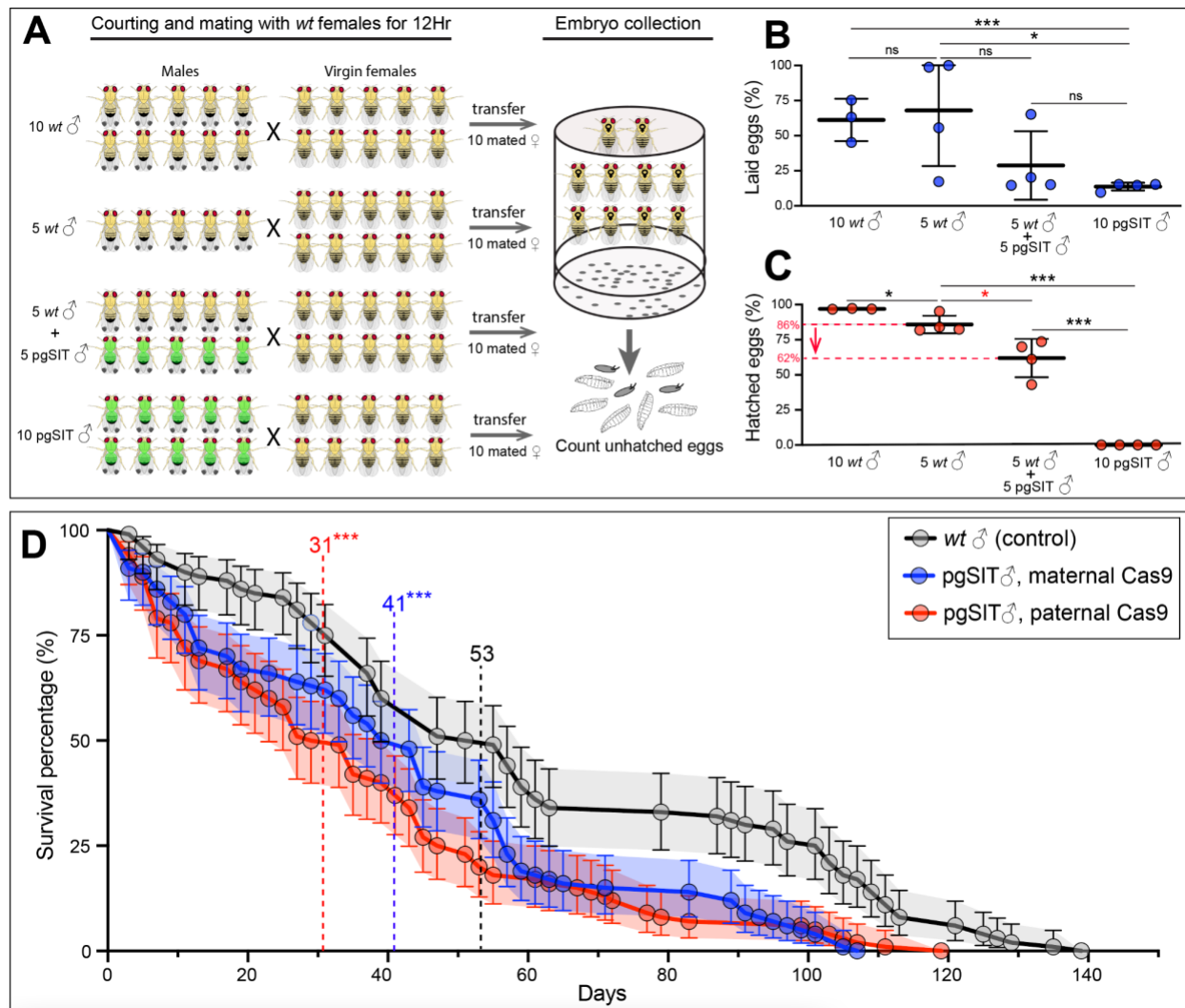


Figure 1.4: Characterization of pgSIT males mating competitiveness and longevity. (A) Schematic of mating assay. Graphs indicate percentages of laid (B) and hatched (C) eggs as well as the mean and one Standard Deviation ($\pm$ SD) for each group. Red arrow depicts the significant decrease in egg hatching rate in the experimental group (5pgSIT males + 5 wt males). Statistical significance was estimated using a two-sided t test with equal variance. (D) Survival curves of average survival percentage and 95% confidence intervals ($\pm$ CI). wt males (gray points), pgSIT males with paternal Cas9 (red points) and pgSIT males with maternal Cas9 (blue points). Statistical significance in survival was estimated with the Gehan-Breslow-Wilcoxon test. ($p \leq 0.05^{ns}$; $p < 0.05^*$; $p < 0.001^{***}$) Source data is provided as a Supplementary Data File.

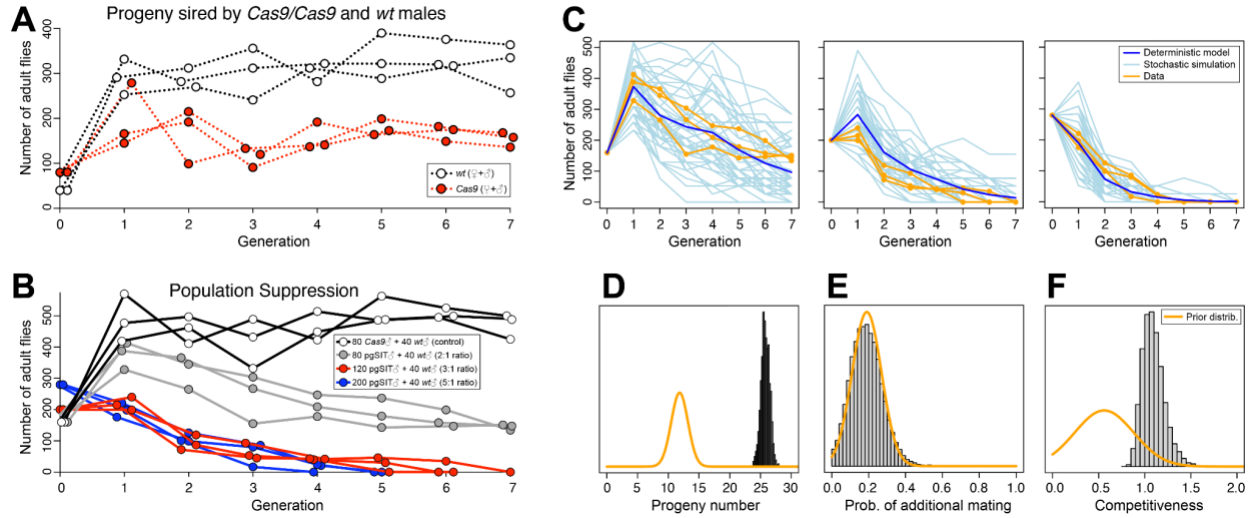


Figure 1.5: Repeated releases of pgSIT males suppress *D. suzukii* populations. Discrete generation population bottles were seeded with 40 wt virgin females. For triplicate control populations, 80 *vasCas9/vasCas9* males and 40 wt males (2:1, Cas9 males : wt males) were released. (A) Generated progeny flies were scored for the *vasCas9* fluorescent marker, dsRed. The *vasCas9/vasCas9* males sired a smaller fraction of progeny than that expected for the 2:1 release ratio. (B) To assess the ability of pgSIT males to suppress populations, specific ratios of pgSIT males : wt males (2:1, 3:1, and 5:1) were released in replicates to experimental populations and total progeny numbers were scored at each generation and compared to that for control populations. (C) Deterministic (dark blue line) and 50 stochastic (light blue lines) simulations were performed corresponding to population experiments with 2:1, 3:1 and 5:1 ratios of pgSIT:wt males. A Bayesian Markov chain Monte Carlo (MCMC) procedure was used to infer the parameters related with the population dynamics in lab experiments. (D) A normal prior distribution (yellow curve) for the progeny number per single wt female (parameter α) was 11.8 ± 1.4 flies (control in B). A posterior distribution (histogram) for the number of progeny produced by each mating between a wt female and wt male was inferred to be 26 (95% CrI: 24–27). (E) A normal prior distribution (yellow curve) for additional mating (re-mating; (parameter p_{rm}) for females was 0.19 ± 0.08 . A posterior distribution (histogram) for additional mating was inferred to be 18% (95% CrI: 4–37%). (F) A normal prior distribution (yellow curve) for relative mating competitiveness of pgSIT males (parameter c) was chosen based on laboratory data with a mean of 0.56 ± 0.16 (control in B). The Bayesian MCMC analysis utilized the population experiment data depicted in Fig. 5C and inferred a posterior distribution (histogram) for the relative mating competitiveness of 108% (95%CrI: 90–138%).

1.10 Supplemental Figures

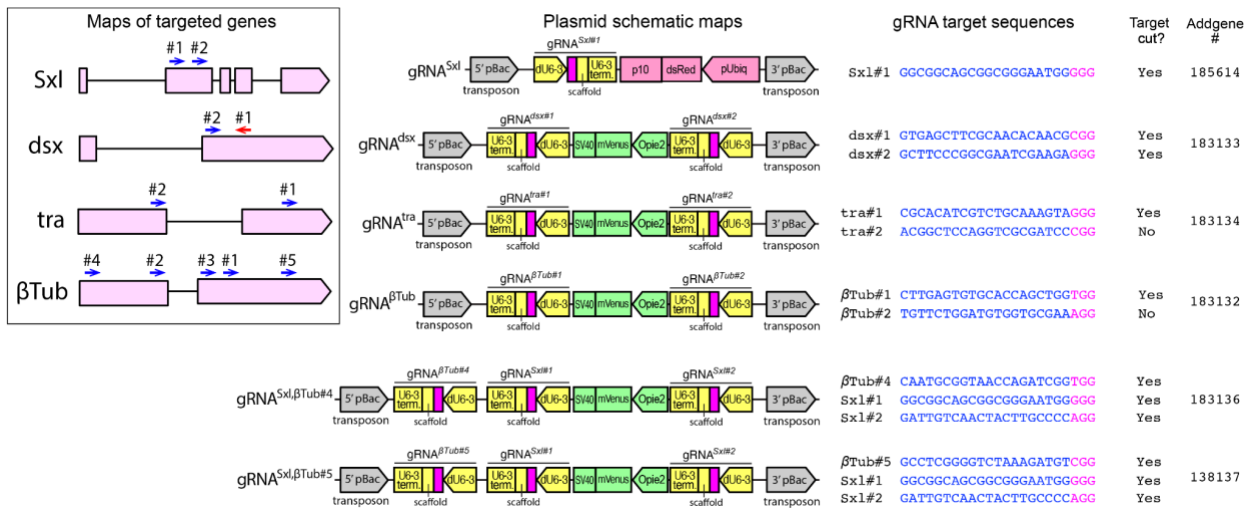
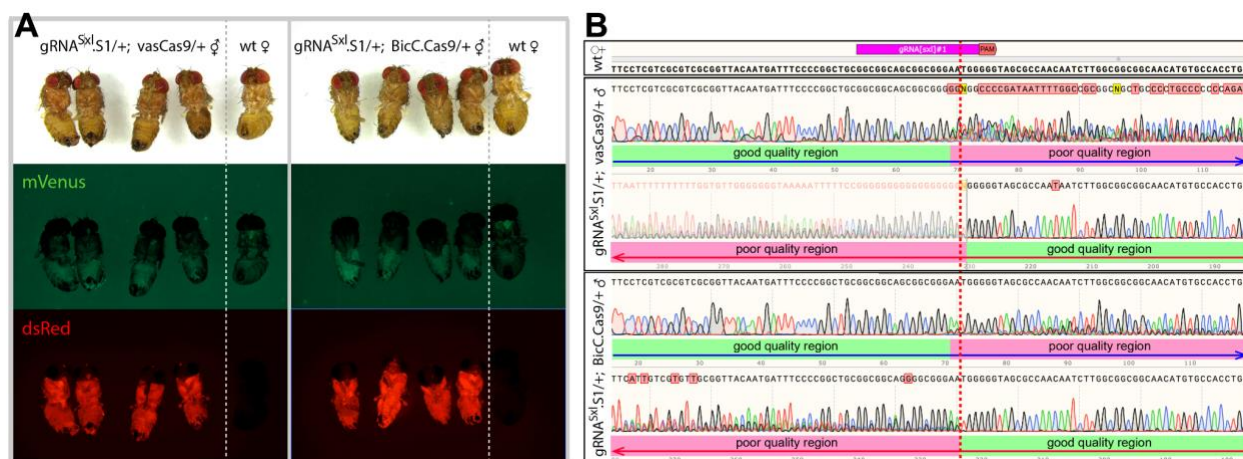


Figure S1.1: Schematic maps of gRNA constructs and gRNA target sites used in the study. Four separate *D. suzukii* genes were targeted for Cas9/gRNA-mediated mutagenesis by gRNA constructs harboring one or multiple gRNAs. Relative locations and orientation of gRNA target sequences (blue and red arrows) inside coding sequence of four genes – Sex lethal (Sxl, S10_00011892), double sex (dsx, DS10_00011892), transformer (tra, DS10_00010087), or β Tubulin 85D (β Tub, DS10_00012485) – are indicated in the box. Sequences of tested gRNAs and their cleavage ability as assessed using Sanger sequencing are displayed to the right of each gRNA construct. Each tested construct was deposited at Addgene.org under the corresponding accession number.



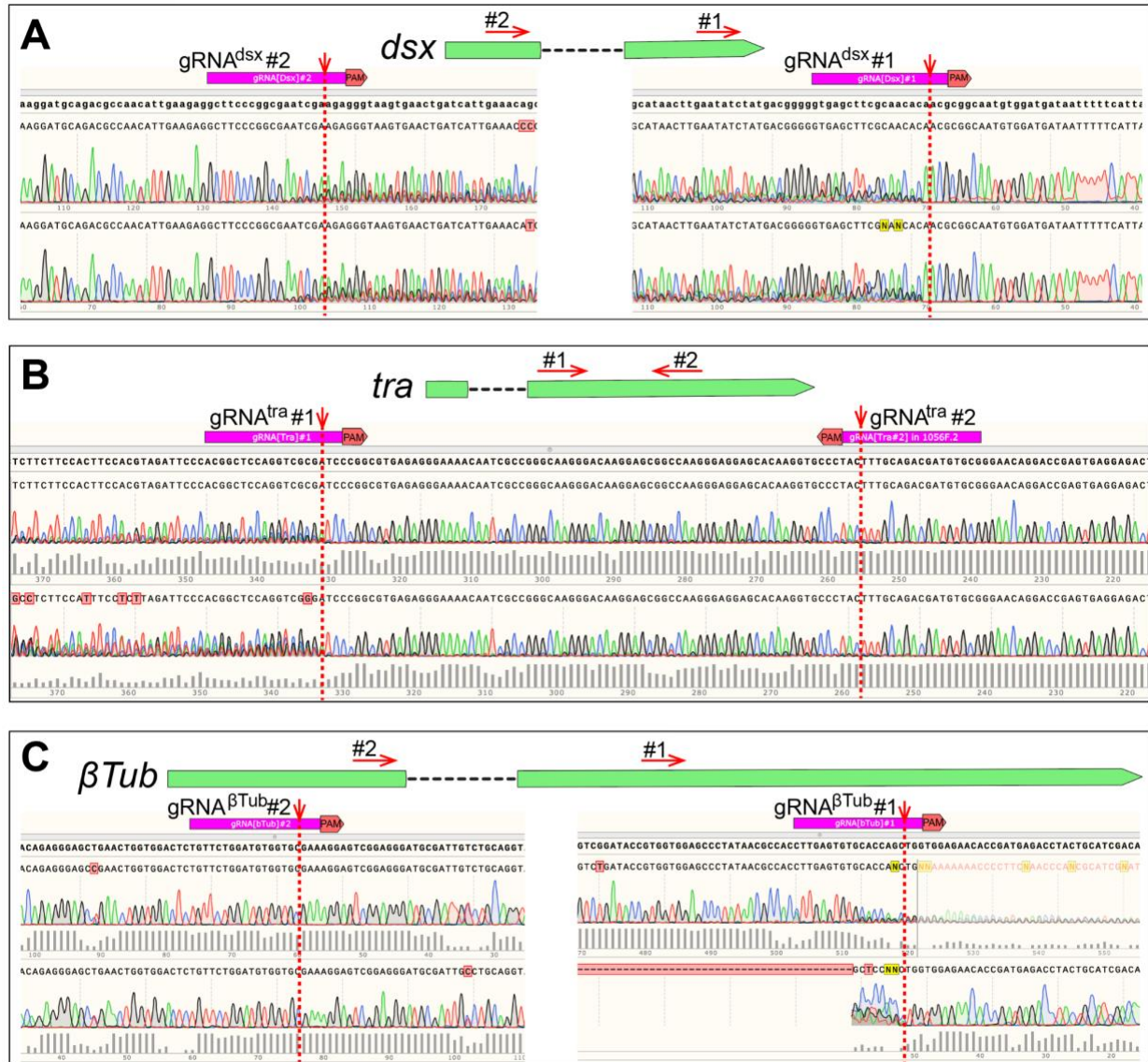


Figure S1.3: Cas9/gRNA9-mediated mutagenesis of targeted sequences in *D. sukuzii* *dsx* (A), *tra* (B), and *βTub* (C). Both gRNAs targeting two separate sequence sites in *dsx*, gRNA^{dsx}#1 and gRNA^{dsx}#2 resulted in mutations at their respective target sites (red dotted line) (A). Sanger sequencing indicating one out of two tested gRNAs targeting *tra*, gRNA^{tra}#1 (B), and *βTub*, gRNA^{βTub}#1 (C) generated mutations at their target sites.

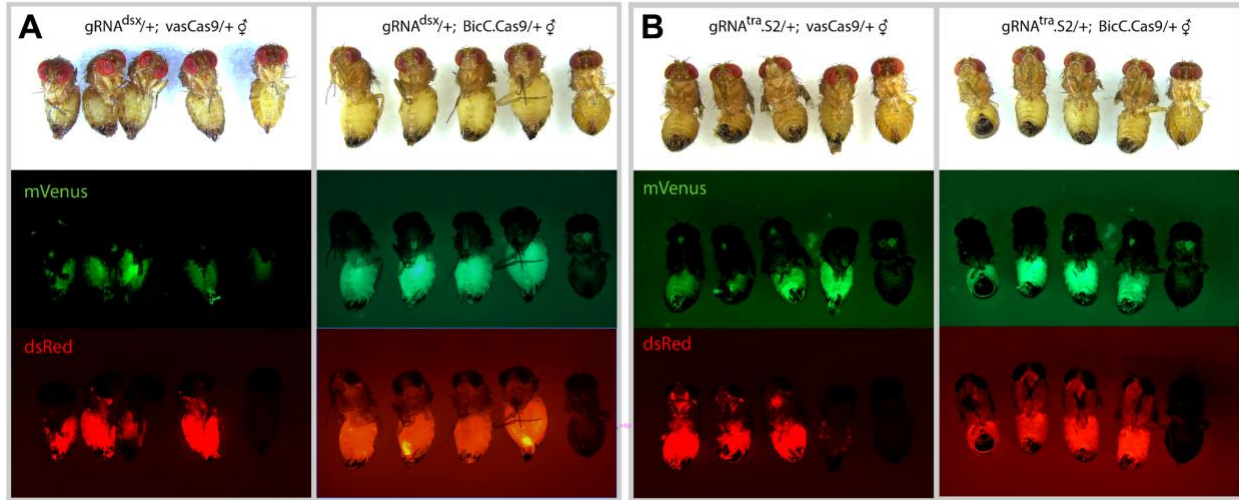


Figure S1.4: *D. suzukii* intersex phenotypes induced by Cas9/gRNA-mediated of *dsx* and *tra*. Representative images of intersexes generated with *vasCas9* or *BicC.Cas9* in combination with *gRNA^{dsx}* (A) or *gRNA^{tra}.S2* (B) revealing malformed genitals and ovipositors. mVenus (gRNA tag) shown in green and dsRed (Cas9 tag) shown in red.

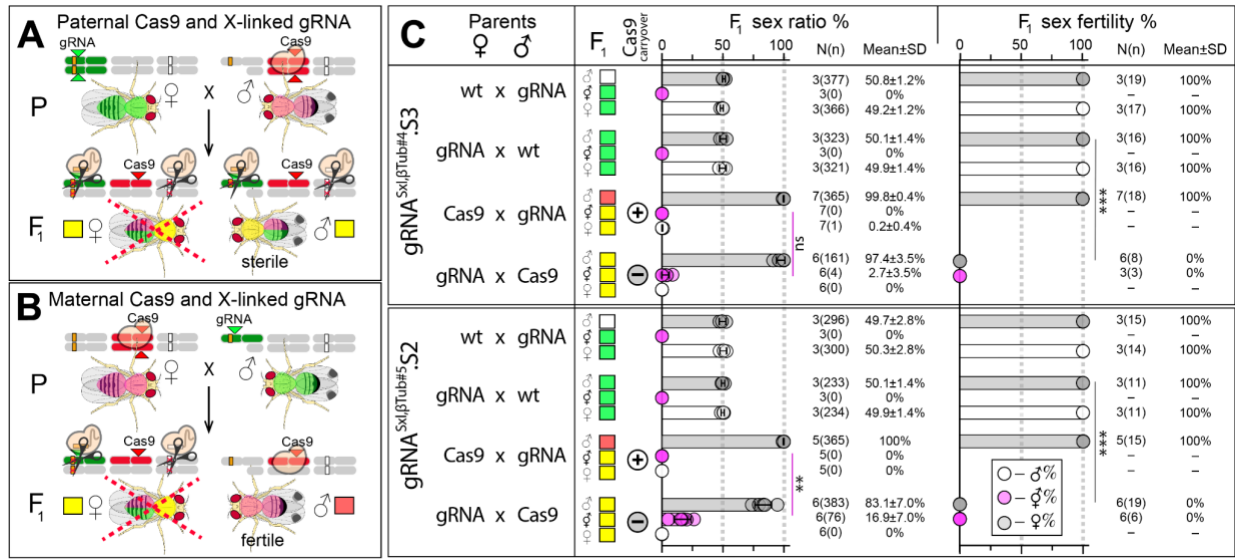


Figure S1.5: Genetic characterization of two X-linked triple gRNA strains. The transgene inheritance of *gRNA^{Sxl,βTub#4}.S3* and *gRNA^{Sxl,βTub#5}.S2* followed an X-linked inheritance pattern. The females of both homozygous strains when crossed with homozygous Cas9 strain pass their transgenes to both progeny sexes (A), while the male parents pass the *gRNA^{Sxl,βTub}* transgenes only to female progeny (B). (C) Both *gRNA^{Sxl,βTub#4}.S3* and *gRNA^{Sxl,βTub#5}.S2* were genetically crossed to the *vasCas9* and *wt* strains in reciprocal directions, then the produced F₁ progeny were scored and their fertility was assessed. The direction of a genetic cross is indicated in the left panel. Maternal inheritance of Cas9 results in deposition of Cas9 protein into embryos, i.e. Cas9 carryover (+); while paternal inheritance of Cas9 does not (–). The sex and fertility percentages of eclosed F₁ heterozygous (*gRNA*+) and trans-heterozygous (*gRNA* & *Cas9*) flies are reported as two separate bar graphs. The sex of eclosed adults was scored as female (♀), intersex (♂), or male (♂). Bars indicate the mean frequency and one standard deviation (±SD). *N* is the number of biological replicates and *n* is the total number of scored flies, and both *N* and *n* numbers are reported for each sex. Source data is provided as a Supplementary Data file. Statistical significance in sex frequency was estimated using a two-sided *t* test with equal variance. Pearson’s chi-square tests for contingency tables were used to assess the difference in male sterility ($p \leq 0.05^{ns}$; $p < 0.01^{**}$; $p < 0.001^{***}$).

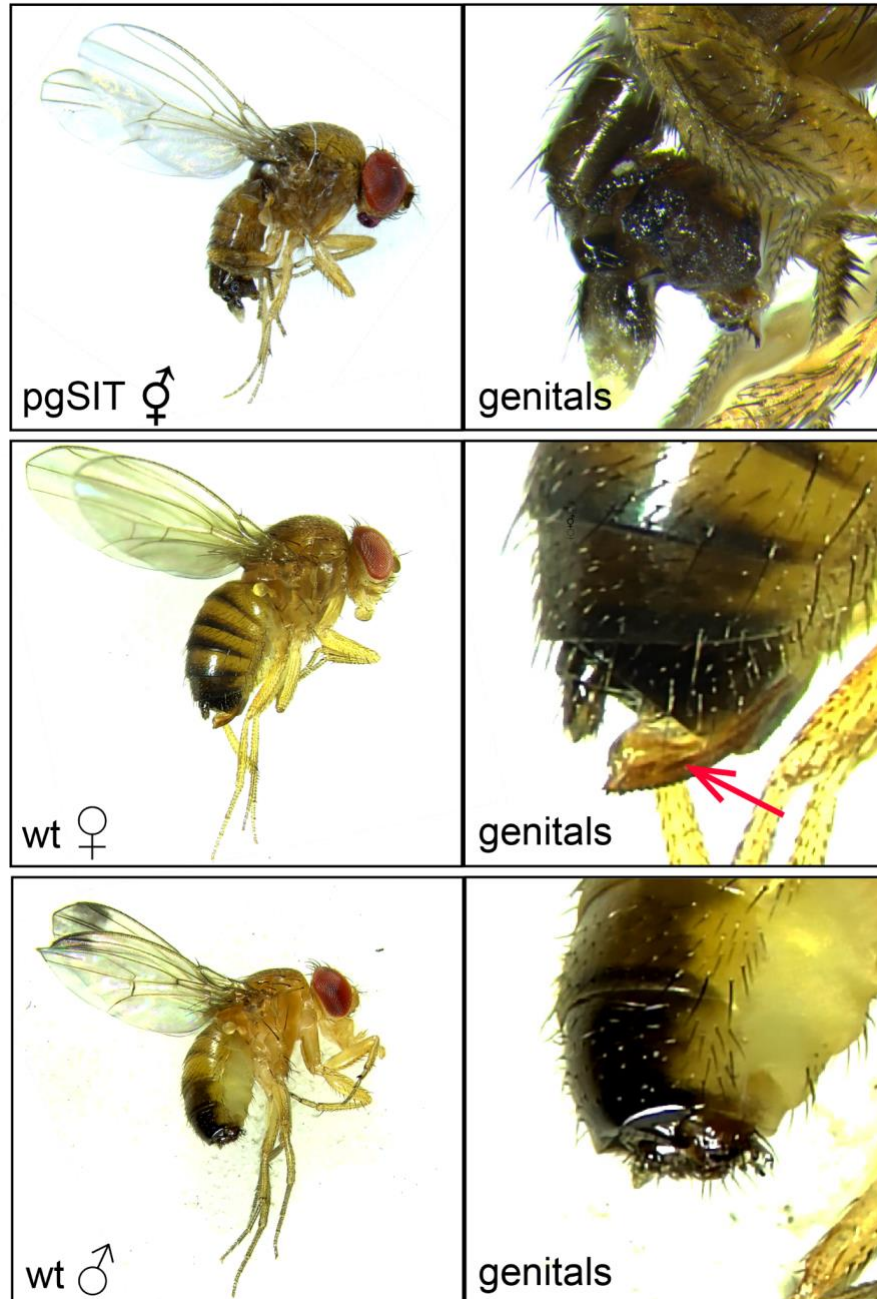


Figure S1.6: Genital structures of intersex, female, and male *D. sukuii*. Left panel (top to bottom): pgSIT *vasCas9*/+; *gRNA^{Sxl,βTub#4}.S1*/+ intersex (paternal Cas9), female, male, all imaged at the same scale. Right panel: corresponding images of genital structures at a higher magnification. The serrated ovipositor (red arrow) indicated in the wt female genital image was completely absent in the intersex genital.

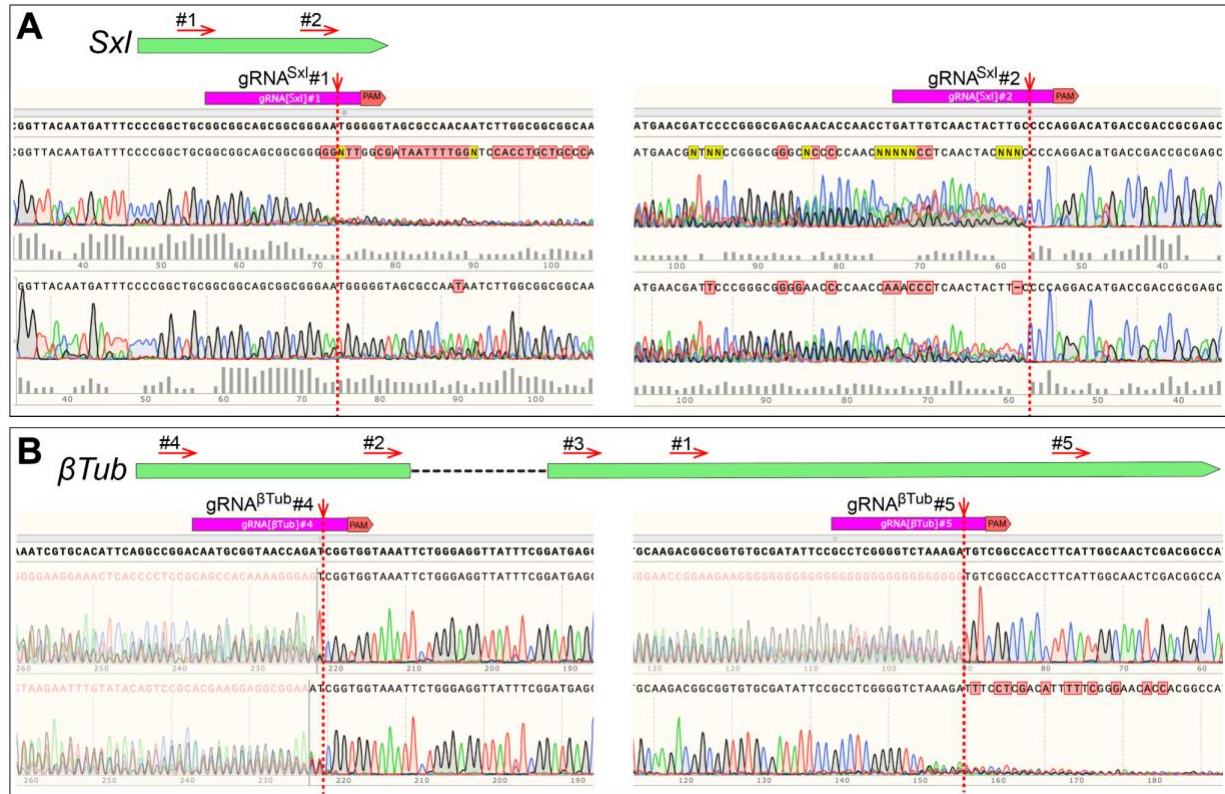


Figure S1.7: The simultaneous disruption of two genes by triple gRNA constructs. Each gRNA harbored in the triple gRNA constructs were able to guide cleavage by Cas9. Both *Sxl* (A) and *βTub* (B) genes were simultaneously mutagenized in *vasCas9/+; gRNA^{Sxl,βTub}#4.S1/+* and *vasCas9/+; gRNA^{Sxl,βTub}#5.S1/+* pgSIT males. Sanger sequencing indicated that the target sites for the corresponding gRNAs – *gRNA^{Sxl}#1*, *gRNA^{Sxl}#2*, *gRNA^{βTub}#4*, and *gRNA^{βTub}#5* – were mutagenized site-specifically at the cleavage site (red dotted line).

1.11 Tables

Table 1.1 Fertility of pgSIT (trans-heterozygous) males that inherited paternal *vasCas9*, measured by the hatching rate of sired eggs

<i>gRNA strain</i>	<i>Groups of 5 ♂ and 20 virgin ♀</i>	<i>Total number of laid eggs</i>	<i>Total number of hatched eggs</i>	<i>Hatching rate</i>
<i>gRNA^{Sxl,βTub#4}.S1</i>	5	952	0	0%
<i>gRNA^{Sxl,βTub#4}.S2</i>	3	518	5	0.9%
<i>gRNA^{Sxl,βTub#5}.S1</i>	3	321	0	0%

Groups of 5 pgSIT males (♂) were mated with 20 virgin wt females (♀), then laid eggs were scored every 12 hours and their hatching rates were assessed.

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Chapter 2: A Fluorescent Sex-Sorting Technique for Insects with the Demonstration in *Drosophila melanogaster*

2.1 Abstract

Recent advances in insect genetic engineering offer alternative genetic biocontrol solutions to control populations of pests and disease vectors. While success has been achieved, sex-sorting remains problematic for scaling many genetic biocontrol interventions. Here, we describe the development of a genetically stable sex-sorting technique for female and male selection with a proof-of-concept in *D. melanogaster* termed SEPARATOR (Sexing Element Produced by Alternative RNA-splicing of A Transgenic Observable Reporter). This elegant approach utilizes dominantly expressed fluorescent proteins and differentially spliced introns to ensure sex-specific expression. The system has the potential for adaptability to various insect species and application for high-throughput insect sex-sorting.

2.2 Introduction

Genetic biocontrol methods are effective and sustainable alternatives to traditional insecticide-based approaches for suppressing pest and vector populations.^{1,2} Sterile insect technique (SIT), for example, is a genetic biocontrol technology that has successfully eradicated screwworms in the United States.^{3,4} This technology suppresses populations through the frequent releases of sterile males, as female insects are often responsible for destroying agricultural resources or pathogen transmission.⁵ While it's feasible to release sterile females alongside sterile males in specific programs, such as the New World screwworm, this approach often leads to substantial increases in rearing costs and a reduction in overall efficiency. Sterile females may still contribute to damage through oviposition, and there remains the possibility of sterile females mating with sterile males.⁶

On the other hand, although SIT has demonstrated effectiveness in certain species, it demands the exclusive release of sterile males in cases in the Tsetse fly or mosquitoes where females transmit human pathogens.⁶⁻⁸ This indeed remains a grand challenge for many genetic biocontrol technologies. What is

critically needed is a genetically stable technology that can be rapidly developed in many species to enable rapid and accurate sex sorting.

The primary method for sex sorting in the lab, which relied on manual sorting based on sexual dimorphisms, is labor-intensive and severely limited by throughput and scalability.^{9,10} More recent approaches utilize machine learning to automate sex sorting.^{11,12} However, these still rely on natural sexual dimorphisms, making them challenging to adapt to other species.

Several Genetic Sex Separation (GSS) lines were established, enabling sorting based on pupal color, temperature sensitivity (*tsl*), or chemical sensitivity.^{9,13–15} These approaches have demonstrated their effectiveness and scalability in the Mediterranean fruit fly (Medfly). Specifically, using pupal color as a selection marker, generated through Y-translocation, enabled efficient separation of mutant females from wild-type males using comparatively inexpensive rice sorters.^{16–18} However, the generation of GSS involves irradiation-induced translocation of a conditional lethal gene, such as the gene conferring resistance to the insecticide dieldrin (*Rdl*), to the Y chromosome.¹⁹ As a result, only males carrying the translocated gene on their Y chromosome can survive when exposed to the insecticide. This GSS method has success in specific insect species, such as the Mediterranean fruit fly. However, as a whole, the development of this method poses significant challenges.

A scalable sex-sorting method is essential to ensure cost-effective and scalable genetic technologies are available for the control of a variety of insect species. Incorporating fluorescent sex-specific markers facilitates the visual identification and separation of males and females with a Complex Parametric Analyzer and Sorter (COPAS, Union Biometrica) instrument. The COPAS has been used to sort *D. melanogaster* embryos and larvae based on fluorescence as well as transgenic *Aedes aegypti* and *Anopheles gambiae* larvae.^{10,20} The COPAS instrument was used to fully automate the sorting process, achieving the efficiency, speed, and accuracy needed to scale genetic technologies.¹⁰

Alternative splicing, a crucial post-transcriptional process in gene expression, enhances protein diversity from a single gene. We leverage sex-specific alternative splicing of the sex-determination gene *transformer* (*tra*) to achieve female-specific expression. *TraF* from four separate species were examined,

with two species belonging to the Drosophilidae family (*Drosophila melanogaster* and *Drosophila suzukii*) and the other two belonging to the Tephritidae family (*Ceratitis capitata* and *Anastrepha ludens*). While the alternative splicing of *tra* remains conserved, *tra* regulations use different mechanisms between Drosophilidae and Tephritidae. In Drosophilidae, *tra* is regulated by the presence of the *Sex-lethal* (*Sxl*) gene. On the other hand, in Tephritidae, *tra* is autoregulated in females and is turned off by the presence of *Maleness-on-the-Y* (*MoY*) (**Supplementary Fig. S1a**).^{21–23}

Here, we describe the proof of principle for a sex-sorting technique termed SEPARATOR (Sexing Element Produced by Alternative RNA-splicing of A Transgenic Observable Reporter) that utilizes fluorescent proteins and sex-specific introns of the sex-determination gene *transformer* (*tra*) to ensure sex-specific expression.^{24,25} Female-specific expression is achieved by disrupting the coding sequences (CDS) of a fluorescent reporter with female-specific *transformer* introns (*traF*). When *traF* from *D. melanogaster*, *D. suzukii*, and *C. capitata* is inserted into the CDS of fluorescence protein, we achieved 100% female-specific fluorescence. Notably, *C. capitata traF* permits the selection of females as early as L1 instar larval stage. However, *A. ludens traF* resulted in dsRed expression in both sexes in *D. melanogaster* flies, indicating that *A. ludens traF* is not sex-specific when transcribed in *D. melanogaster*.

This SEPARATOR technique is a valuable method for insect sex-sorting as it (1) exploits highly conserved sex-specific splicing mechanisms, making it widely transferable to different insect species; (2) allows 100% positive selection of either females or males based on fluorescence instead of morphological differences, providing better accuracy; (3) permits sex-sorting during different life stages and as early as L1 instar stage; (4) can be combined with existing genetic control methods and a COPAS machine for precise high-throughput screening.

2.3 Results

2.3.1 Development of female-specific expression of the fluorescent protein

To engineer the female-specific expression of a reporter for positive selection of females, we exploited the sex-specific alternative splicing of a conserved sex-determination gene. In *D. melanogaster*,

the *transformer* (*tra*) intron between exons 1 and 2 is spliced out in females, resulting in a functional *tra* protein. In males, alternative *tra* splicing results in a premature stop codon that terminates the *tra* protein.²⁶ This female-specific alternative splicing mechanism occurs not only in *Drosophila* but also in *Ceratitis* and *Anastrepha*, suggesting that it is highly conserved in Dipterans (**Fig. 1a, Supplementary Figs. s1 and s2**).²⁷ Consequently, the *traF* from *D. melanogaster*, *D. suzukii*, *C. capitata*, or *A. ludens* were inserted into the fluorescent protein coding sequences to test for female-specific fluorescent protein expression (**Fig. 1b**).

We generated two sets of dual fluorescent marker constructs encoding a fluorescent marker for both sexes and a female-specific fluorescent marker (**Fig. 1c**). The constructs were cloned into a plasmid containing an attP recombination site and a *piggyBac* (PB) transposable element. Set 1 constructs have an enhanced Green Fluorescent Protein (eGFP) fluorescence expressed under a ubiquitous promoter *Hr5Ie1* (*Hr5Ie1*-eGFP) as the selectable marker for the transgene. To promote constitutive expression of female-specific fluorescent proteins, we used another ubiquitous promoter *Opie2* to express dsRed and inserted *traF* immediately downstream of the translational start codon (ATG, adenine-thymine-guanine) of dsRed (*Opie2*-ATG-*traF*-dsRed). Constructs in set 2 have the opposite marker configuration, with *traF* inserted downstream of the ATG translational start codon of eGFP under promoter *Hr5Ie1* (*Hr5Ie1*-ATG-*traF*-eGFP) for the female-specific fluorescent expression and *Opie2*-dsRed as the selectable marker for the transgene. In total, nine constructs were created: a control construct (795G) and eight experimental constructs, with four constructs in each set (**Fig. 1d**).

2.3.2 *DmtraF*, *DstraF*, and *CctraF* resulted in female-specific fluorescence

The transgene integration site can impact gene expression, so we opted to integrate all nine constructs into the same site through phiC31 *attP* integration on the second chromosome (BDSC #25709). Nine homozygous transgenic strains were established. Six constructs that harbor *DmtraF*, *DstraF*, and *CctraF* resulted in female-specific fluorescence (795H,I,J,L,M, and N, **Fig. 2**). These results indicate that inserting the *traF* into the coding sequence of the fluorescence proteins can result in female-specific fluorescence. However, for constructs harboring *AltraF* (795K and O), both females and males exhibited

the intended female-specific fluorescence (**Fig. 2**). This result suggests that *AltraF* is spliced out in both females and males rather than in a female-specific manner.

Adult flies were collected for RT-PCR analysis to validate the alternative splicing variants and obtain the fluorescent protein transcripts. Primers were designed to anneal to the 5' untranslated regions (UTR) region at the 3' end of the *Opie2* promoter, and the 3' end of the dsRed sequence (**Supplementary Table S1**). Multiple bands were obtained from the RT-PCR samples for *CctraF* males, and both sexes of *DmtraF* and *DstraF* (**Fig. 4a and 4b**). Sequencing of these bands indicates that *CctraF*, *DmtraF*, and *DstraF* resulted in functional dsRed expression explicitly in females, while *AltraF* had dsRed expression in both males and females (**Fig. 4c**). In *Cctra-dsRed* male, a band that is very similar in size from the *Cctra-dsRed* female-specific product was observed. The sequencing result from that particular band indicates that *Cctra-dsRed* male produced a male-specific splicing product with the M1 intron that is only 40bp larger than the female-specific product. Therefore, the two bands look similar in size on the gel, while the sequencing results indicate otherwise (**Fig. 4c & Fig. s2**).

The molecular results obtained from RT-PCR analysis were consistent with our observations in the flies, confirming that *CctraF*, *DmtraF*, and *DstraF* exhibited female-specific splicing in *D. melanogaster*. Another interesting observation was that the splicing of *Cctra* male products (*CctraM*) differs in *D. melanogaster* and *C. capitata* (**Fig 4 & Fig. s1b**). The M2 exon is skipped in both *CctraM1* and *CctraM2* in *D. melanogaster* but preserved in *C. capitata*. Nevertheless, the outcome demonstrates the feasibility of this fluorescent sex-sorting approach, as these female-specific splicing events allow for the positive selection of either sex.

2.3.3 Confirmation of sex-specific fluorescence at multiple developmental stages

Next, we evaluated the intensity and sex specificity of the fluorescence over multiple life stages. The six constructs (795H, I, J, L, M, and N) that exhibited female-specific fluorescent expression were evaluated. Female-specific fluorescence was observed as early as in the first instar larvae (L1) life stage in both *CctraF* transgenic lines: 795H and 795L (**Fig 2a, Fig 3, Supplementary Table 2**). Female-specific

fluorescence was also observed in the third instar larvae (L3) of *DmtraF* 795I and *CctraF* 795L, and the pupal stage for *DmtraF* 795M and *DstraF* 795N. Despite the identical introns in the *DmtraF* 795I and *DmtraF* 795M and the *CctraF* 795L and *DstraF* 795N strains, female-specific fluorescence was detected earlier in strains with the female-specific dsRed marker. This result is presumably due to the deeper tissue penetrance and lower auto-fluorescence of red fluorescent protein (RFP).²⁸ Notably, the intensity of the female-specific fluorescence varies among introns. The *CctraF* exhibits the highest brightness, followed in order of brightness by *DmtraF* and *DstraF* (**Fig. 2**). This is unexpected as *CctraF* is an exogenous/non-native intron for *D. melanogaster*, potentially hindering successful intron recognition and splicing efficiency.

2.3.4 Assessing the fitness of the sex-sorting strains

Strain fitness is essential for scalability. Fluorescent proteins have documented fitness costs to genetically engineered organisms, but we expected that including *traF* in their coding sequences would minimally affect the fitness of the sex-sorting strain. We, therefore, compared the egg hatching and larval to adult survival rate of all eight homozygous sex-sorting strains to a control strain (795G) containing fluorescent reporters lacking *traF* introns. Our finding indicates that there is a significantly higher hatching rate in flies harboring *Opie2-ATG-traF-dsRed* constructs when compared to 795G control (**Fig. 5a**). This effect could possibly be attributed to the fitness cost associated with dsRed functioning as a tetramer.²⁹ With the inclusion of *traF* introns, the expression of dsRed occurs at a reduced level. As a consequence, the fitness cost is diminished, which, in turn, leads to a higher hatching rate.

We observed lower larvae to adult survival only in the *CctraF-dsRed* 795H strain ($p < 0.05$, Student's t-test with equal variance, **Fig. 5b**). These results suggest that the *traF* intron does not impose substantial fitness costs on the strain, making them suitable for potential large-scale insect population control projects. Similar fitness was observed from egg hatching for constructs with *DmtraF* and *AltraF*, this corresponds to the dsRed expression level.

2.4 Discussion

In this study, we engineered a novel sex-sorting method, SEPARATOR, that integrates a female-specific intron into the coding sequencing of a fluorescent reporter to generate an efficient female-specific marker. This innovative technique uses splicing mechanisms conserved across dipteran species and has even been successfully engineered in other dipteran species.²⁵ We examined the splicing patterns of four *traF* introns, *DmtraF*, *DstraF*, *CctraF*, and *AltraF*, and found that three of them (*DmtraF*, *DstraF*, and *CctraF*) had 100% female-specific splicing. Among the six constructs tested for female-specific fluorescence expression in *D. melanogaster*, those carrying the *CctraF* introns (795H and 795L) exhibited expression at the earliest life stage and the brightest expression. These strains can be used for sex-sorting as early as the L1 stage and throughout the entire life cycle. This observation aligns with previous studies demonstrating the functional conservation of *CctraF* and its applicability in various insect species.^{30–32}

It is worth noting the significant size difference between the *traF* introns in *Drosophilidae* (*DmtraF* and *DstraF*) and Tephritidae (*CctraF* and *AltraF*). The *traF* introns in *Drosophilidae* species are approximately 200 bp in length, whereas in Tephritidae species, they are approximately ten times longer. Although *AltraF* is not female-specifically spliced, it is spliced out entirely in both females and males, thus resulting in functional fluorescent expression in both sexes. This result could be attributed not only to differences in the sex-determination pathway but also to species-specific variations in splicing signals, given that *AltraF* is an intron foreign to *D. melanogaster*.

According to the RT-PCR result, *D. melanogaster* could recognize the splicing site for *CctraM1*, but not that of *CctraM2*. In *A. ludens*, the splicing of *traM* (*AltraM*) results in the production of five isoforms (Fig. 1a). It is possible that the splice sites of *AltraM*, or Tephritidae species in general, may not be accurately recognized in *Drosophila*. These size differences may also impact intron splicing efficiency and, consequently, the dual sex expression of the *AltraF* fluorescent reporter. Larger introns, for example, possess more heterogeneous nuclear ribonucleoproteins (hnRNP) and serine/arginine-rich (SR) protein recognition sites, leading to higher splicing efficiency.^{33,34} However, further testing in Tephritidae species like *C. capitata* or *A. ludens* may better elucidate the capabilities of *AltraF* intron for SEPARATOR systems.

The *CctraF* introns, on the other hand, generate efficient and easily screenable female-specific fluorescent phenotypes. This efficiency may be due to the simplified splicing of *CctraF*, which does not depend on the presence of the *sex-lethal (Sxl)* protein required in *Drosophila* for *tra* transcript splicing.^{30,35–38} In *C. capitata*, the *tra* gene is autoregulated and continuously expressed from the embryo to adulthood, allowing for the consistent and stable splicing of *CctraF*.^{30,39} Therefore, *CctraF* may be a versatile tool for sex-specific expression in a wide range of insect species.

SEPARATOR has several potential advantages over traditional sexing methods. These include its potential multiple species adaptability, positive selection based on dominant fluorescence for both sexes, sex differentiation during early stages of development, genetic stability, and minimal impact on fitness. All of these characteristics improve the potential scalability and timing of sex sorting. The fluorescent sex-sorting cassettes were engineered to be highly transferable to numerous insect species. They utilize a PB transposable element, which has high transgenesis efficiency in many insect species.⁴⁰ The two constitutive baculovirus promoters for fluorescent protein expression, *Opie2* and *Hr5Ie1*, have been shown to facilitate high expression in various insects throughout development.^{41–43} Furthermore, dsRed and eGFP are the two most commonly used fluorescent proteins and can penetrate most insect tissues.⁴⁴ Female-specific alternative splicing of *tra* intron is also highly conserved across insect species.^{38,45} Given these adaptable features, we have recently developed SEPARATOR strains in both mosquitoes and Tephritid fruit flies.^{24,25}

The SEPARATOR technology enables the positive selection of females or males in all post-embryonic developmental stages.²⁵ Sex sorting at earlier developmental stages would be advantageous for large-scale insect population control projects, as it would extend the sex sorting timeline, facilitate the release of earlier life stages, and may reduce the costs of large-scale releases.⁴⁶ Early-stage sex sorting in the larval phase can indeed offer significant advantages in the context of Sterile Insect Technique (SIT) programs. This is primarily because the larval diet constitutes a substantial cost factor in such programs. By efficiently eliminating females at an early developmental stage, substantial cost savings can be achieved.

Our approach simplifies manual sex sorting, but the primary benefit is to large-scale sex-sorting applications. The SEPARATOR can be combined with existing genetic control methods and a COPAS

machine for precise and high-throughput screening. Although the COPAS machine has demonstrated its ability to sort 1st instar *D. melanogaster* larvae, it remains uncertain whether the female-specific fluorescence will be sufficiently robust for detection.⁴⁷ This uncertainty arises because autofluorescence can be strong in many species. A point worth emphasizing is that *DmtraF* or *DstraF* should primarily be applied in species that utilize *Sxl* as the master switch gene for somatic sex determination. This is because the alternative splicing of *DmtraF* or *DstraF* relies on *Sxl* to splice out properly in females. Considering all these factors, *CctraF* appears to be a more suitable option as it produces the strongest female-specific fluorescence and the earliest expression. Additionally, it is essential to highlight that the splicing of *CctraF* may vary across different species. Therefore, examining the sequencing results of *CctraF* splicing in the specific species is crucial.

Furthermore, enhancing the accuracy of COPAS sorting can be achieved by incorporating distinct fluorescence markers for both females and males. We have previously demonstrated that the male-specific intron in *double-sex (dsxM)* in *D. melanogaster* can be inserted into the coding sequence of an antibiotic resistance gene to confer male-specific antibiotic resistance.⁴⁸ Given the conservation of male-specific *dsx* introns across many insects, a similar strategy can be employed to achieve male-specific fluorescence as we have done to develop SEPARATOR in mosquitoes.^{25,38,45} Alternative methods that have been proposed for achieving male-specific fluorescence include the use of a male-specific promoter (e.g. β 2-tubulin promoter) or the use of a Y-linked transgene.^{16,17,49–51} Nevertheless, it's essential to choose a promoter or intron that can be expressed throughout most of the life cycle and, ideally, throughout the entire body for the technology to be effective.

Our system may potentially be vulnerable to undesired genetic events, including loss-of-function (LOF), gain-of-function (GOF), or genetic exchanges like chromosomal translocation and recombination, ultimately resulting in the loss of fluorescence. Fluorescence can potentially serve as a reliable indicator, as any mutation affecting fluorescence could result in its loss and be subject to selection. Nevertheless, mutations affecting fluorescence or splicing specificity should be further investigated in a large-scale rearing setting.

The GSS used for many current SIT applications relies on reciprocal chromosomal translocations between the Y-chromosome and a region of the autosome containing a selectable marker. These GSS methods have been designed for male-only release programs, where females possess distinct phenotypes or markers that facilitate easy identification and separation.⁵² Incorporating the D53 inversion has been an effective method for increasing the GSS stability. This D53 inversion, located on autosome 5 and carrying the *wp* and *tsl* markers, helps maintain strain stability by preventing recombination within the inverted region.^{23,53,54} Recombinations in this area result in unbalanced gametes and, consequently, lethality. While having reciprocal chromosomes is the same as having a balancer for *D. melanogaster*, which increases the stability of GSS. Traditional GSS methods were historically discovered by chance, focusing on one species at a time, making them tailored to specific species. Studies on the multi-generation of GSS lines have revealed that chromosomal recombination can still occur at a frequency of approximately 0.07%, leading to the breakdown of the sexing system.⁵⁵ Therefore, it is essential always to utilize a safeguard system similar to the Filter Rearing System (FRS). The FRS was explicitly designed to prevent genetic issues, such as genetic recombination, within the Medfly GSS program.^{56,57} The FRS is accomplished by maintaining a small colony and regularly sorting to ensure that no recombination occurs. Additionally, to enhance stability, another potential solution is to generate new translocations with a breakpoint located in close proximity to the sexing genes.⁵⁵

Although the main emphasis of this work has sex-sorting applications, SEPARATOR can be used to study fly development. Differentiation between female and male adult flies is easily accomplished under the microscope, but accurate larvae sex-sorting can be challenging, particularly when they are embedded within the food medium. The major distinguishing factor between female and male larvae is the presence or absence of gonads, which is not easily visible.⁵⁸ With the *CcraF* 795H or 795L sex-sorter cassettes, the fluorescent protein markers can be easily observed as early as first instar larvae, even in the food medium and with minimal autofluorescence. The ability to accurately sex-sort larvae at early developmental stages provides a valuable tool for tracking the sex of flies and could potentially facilitate the study of sex-specific developmental processes in flies.

2.5 Materials and Methods

2.5.1 Molecular cloning

All genetic constructs were produced utilizing the Gibson enzymatic assembly. The construct 795G was created using a pre-existing plasmid containing *piggyBac*, attB-docking sites, and an *Opie2* promoter regulating dsRed. This plasmid was subsequently linearized with XhoI and NotI enzymes. The *Hr5Ie1* promoter, along with eGFP were cloned into the linearized plasmid to make 795G, which serves as the control plasmid. To generate female-specific dsRed (795H-K), the plasmid 795G was linearized with AvrII and BamHI to allow insertion of introns into dsRed. Alternatively, to generate female-specific eGFP (795L-O), 795G was linearized using MluI and BsrGI to insert introns into eGFP. The *traF* introns from *D. melanogaster*, *D. suzukii*, *C. capitata*, or *A. ludens* were amplified from their respective genomic DNA using the primers listed in the Supplementary Table 1.

2.5.2 Reverse transcription PCR (RT-PCR) of the female-specific splicing transcripts

To access the splicing transcripts of four *traF* introns, we screened for female- and male-specific dsRed mRNA. Total RNA of ten virgin females or males from w-, 795G, H, I, J, and K were extracted using the miRNeasy Tissue/Cells Advanced Kits (Qiagen). DNase treatment is done using the TURBO™ DNA-free (Invitrogen), and followed by the cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™). The genomic DNA (gDNA) was amplified using primers 795.s2F and 795.s2R, and the cDNA was amplified using primers 795.s3F and 795.s1R (Supplementary Table 1). The gDNA samples were run on 1% TAE (Tris-acetate-EDTA) agarose gel, and the cDNA samples were run on a 2% TAE agarose gel.

2.5.3 Rearing and fly transgenesis.

Transgenic flies were maintained under standard conditions at 25°C with a 12H/12H light/dark cycle and fed on the Old Bloomington Molasses Recipe. Embryonic injections were performed in the lab

following the standard injection protocol. Plasmids diluted to 300-350ng/μL in water were inserted at P{CaryP}attP40 on the 2nd chromosome (Bloomington #25709). Recovered transgenic lines were balanced on the 2nd chromosome using a single chromosome balancer line w¹¹¹⁸; CyO/sna[Sco]. Multiple independent lines were obtained for each plasmid and tested for sex-specific fluorescence. We used homozygous transgenic lines containing two copies of the transgene to assess the sex-sorting efficiency. Sex-sorting lines with *CctraF* introns 795H and 795L are deposited at the Bloomington Drosophila Stock Center (BDSC# pending).

2.5.4 Genetics and sex selection

To assess the fluorescent sex selection efficiency, we crossed ten virgin females to ten males in a fly vial. The parental flies were flipped into a fresh vial every 12hrs, and the numbers of the laid embryos were scored. After hatching, the larvae or pupa were scored and transferred to different vials based on their fluorescent markers. The sex and the fluorescent markers of the adult offsprings were recorded after eclosion. Flies were scored using a Leica M165FC fluorescent stereomicroscope. Images were taken using a View4K camera. Each genetic cross was set up five times using different parental flies.

2.5.5 Fitness estimation

The fitness of the sex-sorting strains is assessed based on two parameters: the rate of egg-hatching (from embryos to larvae) and the rate of adult survival (from larvae to adult). To evaluate the egg-hatching rate, flies are allowed to lay embryos in fly vials for a duration of 24 hrs, and the number of eggs laid in each vial is recorded. After 24 hrs of egg laying, the number of larvae is recorded. To assess the adult survival rate, the number of both female and male adult flies that successfully eclosed is recorded.

2.6 Statistical analysis

Statistical analysis was performed in Prism9 by GraphPad Software, LLC. Three to five biological replicates were used to generate statistical means for comparisons.

2.7 Data availability

Complete sequence maps and plasmids are deposited at Addgene.org (#205481-205489). Transgenic lines 795H and 795L have been made available for order from Bloomington Drosophila stock center. The other transgenic lines are available upon request to O.S.A.

2.8 Acknowledgments

Chapter 2.1, in full, is a reprint of the material as it appears in GEN Biotechnology. Junru Liu, Danny Rayes, and Omar S. Akbari. A Fluorescent Sex-Sorting Technique for Insects with the Demonstration in *Drosophila melanogaster*. (2024) The dissertation/thesis author was the first author and primary investigator of this paper.

2.9 Figures

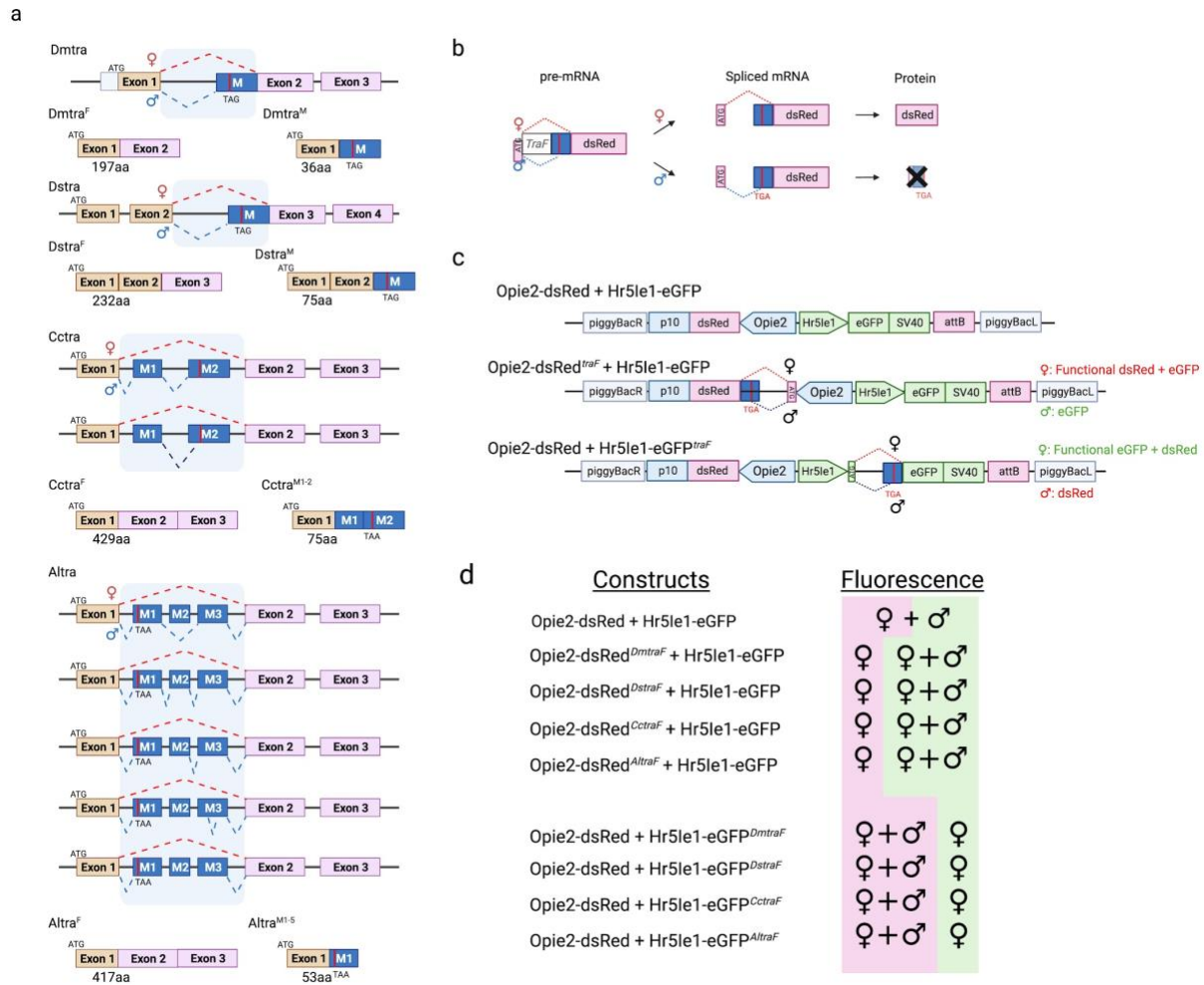


Figure 2.1: Sex-sorter cassette in *Drosophila*. (a) Sex-specific alternative splicing and the resulting protein of the *transformer* (*tra*) in *D. melanogaster*, *D. suzukii*, *C. capitata*, and *A. ludens*. (b) Splicing of the female-specific *transformer* (*TraF*) intron should result in functional dsRed protein in females but not in males. (c) Schematic of the sex-sorter constructs engineered and tested in the study. *TraF* introns from *D. melanogaster*, *D. suzukii*, *C. capitata*, and *A. ludens* are inserted into the coding sequence of either dsRed or enhanced Green Fluorescent Protein (eGFP) after the translational start codon (ATG). (d) Fluorescence expression of females and males carrying the respective constructs.

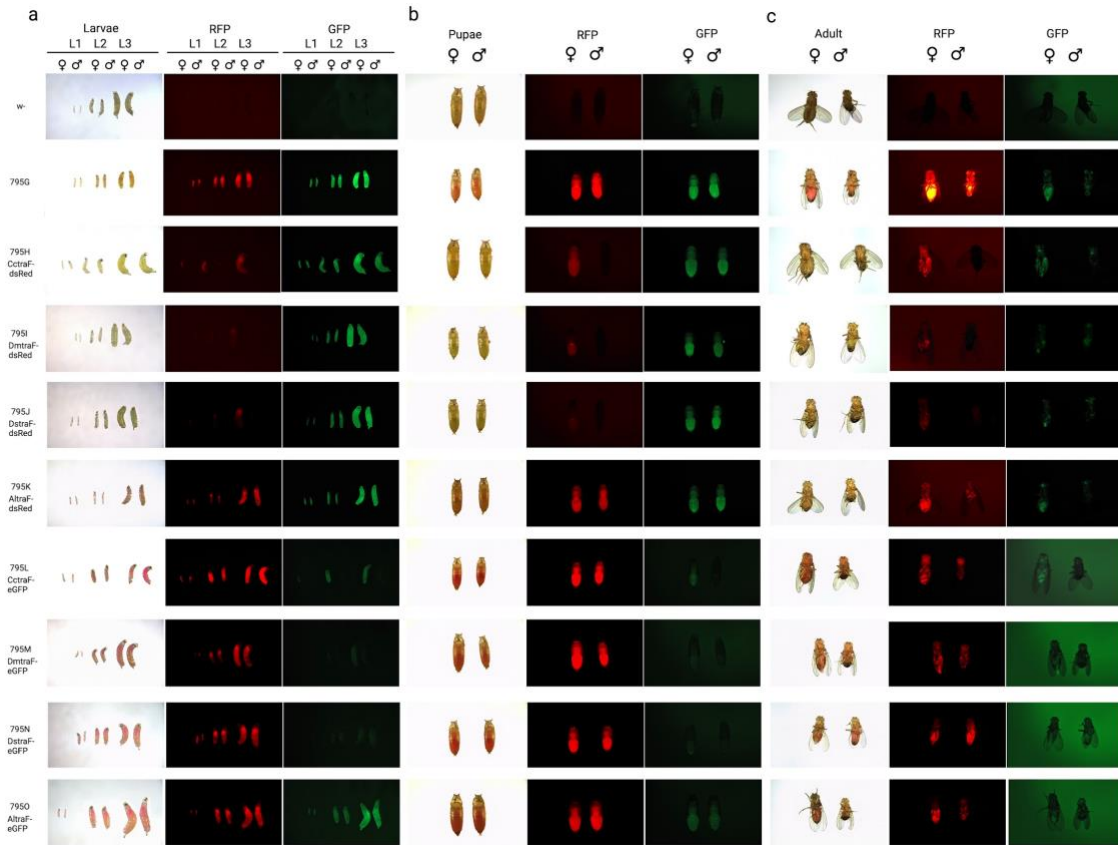


Figure 2.2: Expression of Opie2-TraF-dsRed or Hr5ie1-TraF-eGFP transgenes in *D. melanogaster* can be observed in different developmental stages (a) L1-L3 larval stage, (b) pupal stage, (c) adult under white light, Red Fluorescent Protein (RFP) and Green Fluorescent Protein (GFP) filters.

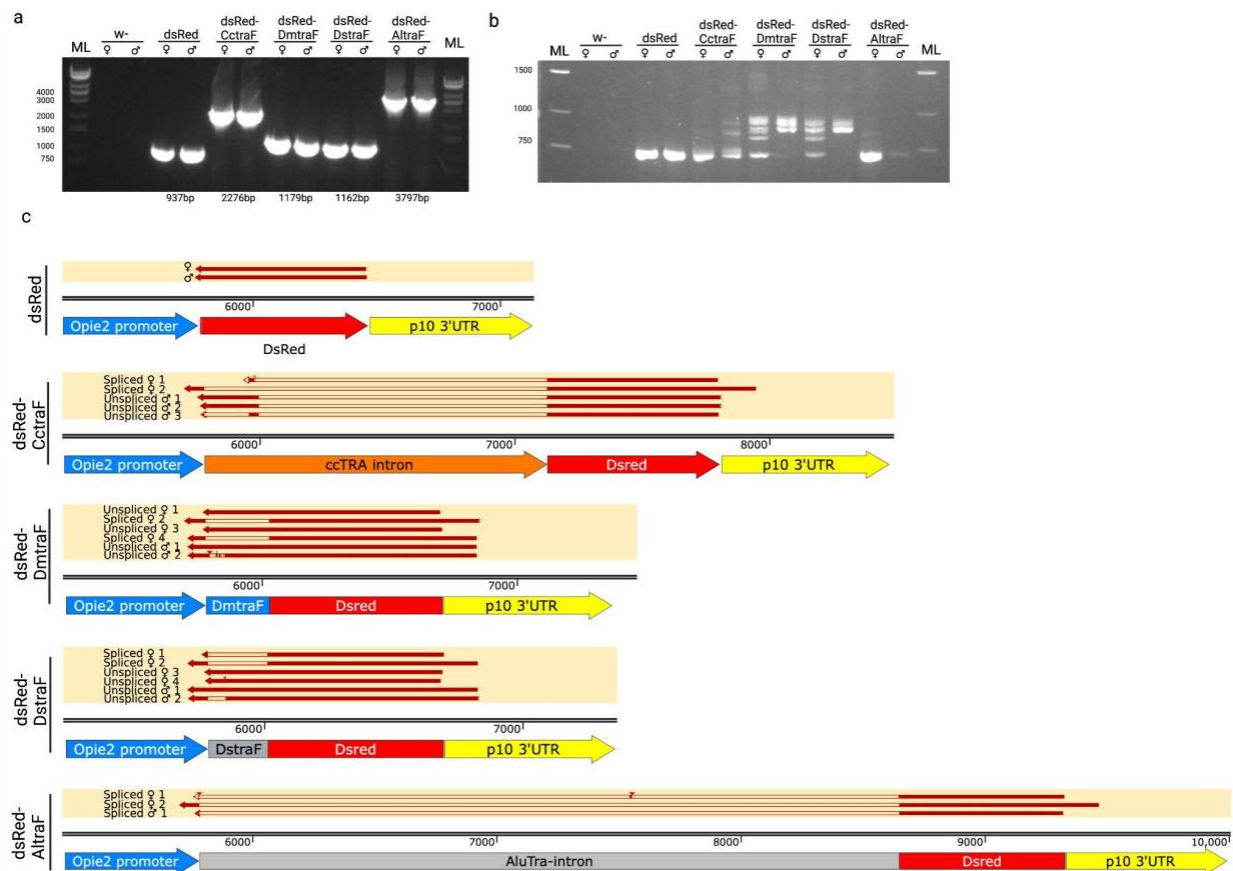


Figure 2.3: The *traF* introns splicing patterns in *D. melanogaster*. Gel electrophoresis images show (a) the genomic DNA PCR for dsRed-*traF* (b) the cDNA for the dsRed-*traF*. ML: molecular ladder. (c) sequencing results of the cDNA from each band.

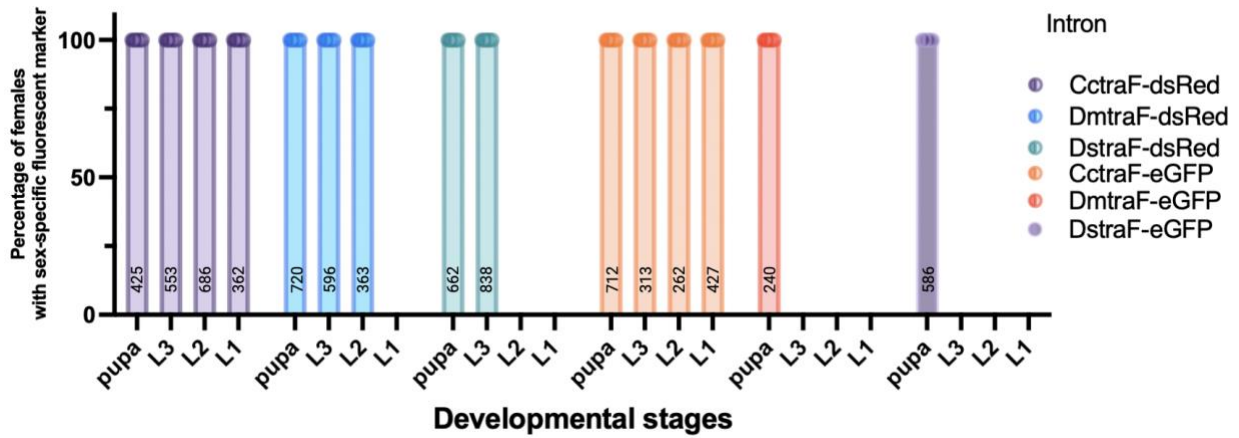


Figure 2.4: Female selection efficiency at different life stages in *D. melanogaster* for all six sex-sorter cassettes that exhibited female-specific fluorescence. The number of scored flies is indicated for each bar. The sex-sorting process achieved 100% accuracy, as no males were detected from either larvae or pupae that were sorted based on sex-specific DsRed or eGFP fluorescence.

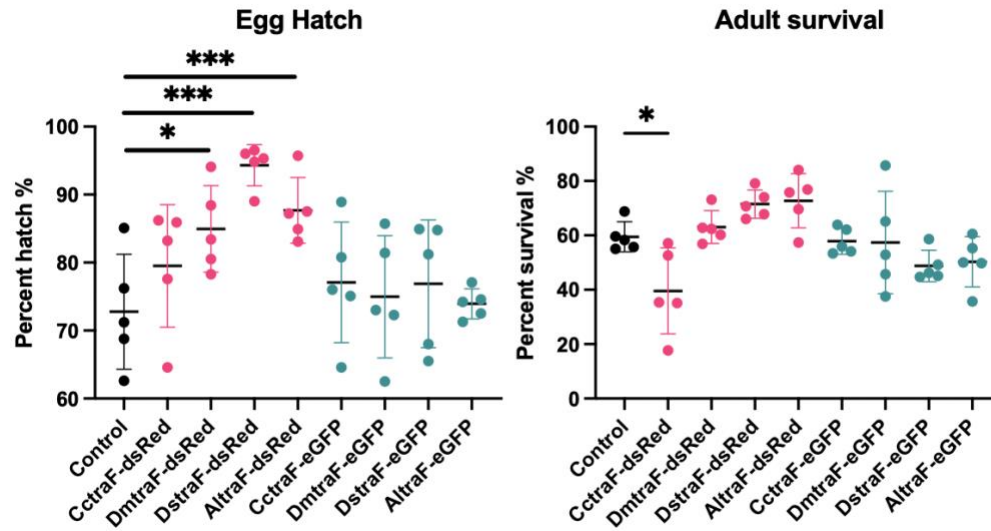


Figure 2.5: Fitness cost of all eight sex-sorting cassettes was accessed through two parameters: (a) egg-hatching rate and (b) survival rate to adulthood. Fitness cost was observed in *CctraF*-dsRed strain in the parameter of survival rate to adulthood (* $p < 0.05$, * $p < 0.001$, Student's t-test with equal variance.)**

2.10 Supplemental Figures

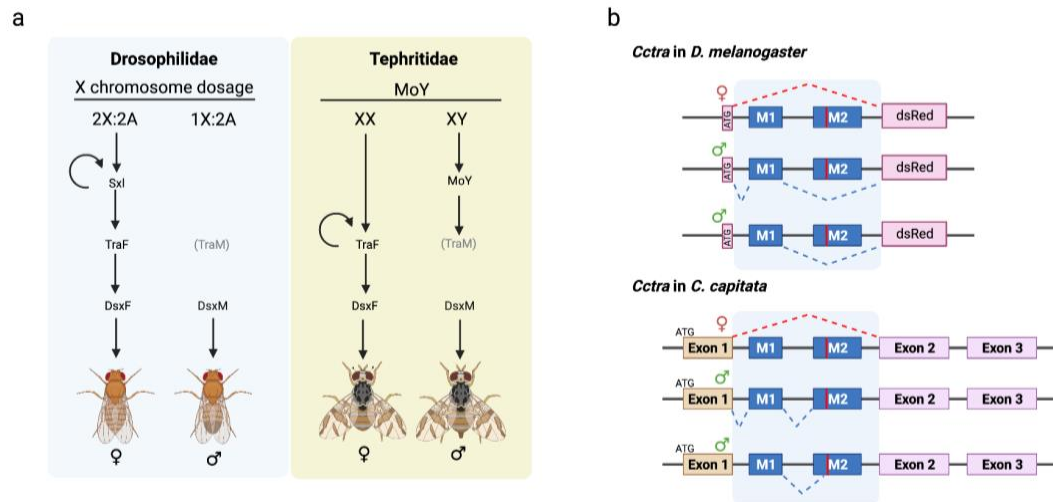


Figure S2.1: (a) Sex-determination pathways of Drosophilidae and Tephritidae. (b) Alternative Splicing of *Cctra* in *D. melanogaster* and *C. capitata*.

CotraF ATG|GTAATTTTAAAGCATATTTTTTCTTT...CTAATCCATACATCTATGGTATCTATAG|GTGCGCTCCTCCAAG

CotraM1 ATG|GTAATTTTAAAGCA...CTCTCACCTGGAAAA|GCAACCAACTACAATCAACATTCTATGGGATAATCGACAA|GTGAGTAAATTATA...CTATGGTATCTATAG|GTGCGCTCCTCCAAG

M1 intron

CotraM2 ATGGTAATTTTAAAGCA...TGGGATAATCGACAA|GTGAGTAAATTATA...CTATGGTATCTATAG|GTGCGCTCCTCCAAG

DmtraF ATG|GGTACCCACTATATA...ACTCTTTTCACATAG|GTGCGCTCCTCCAAG

DmtraM ATG|GTACCCACTATATAG...TTOTTTTTTTCTAG|TGTATATTGTGTGA

DstraF ATG|GTATATATACACATA...TTCCACTTCCACGTA|GTGCGCTCCTCCAAG

DstraM ATG|GGTATATATACACAT...GTTGTTTTTTTCTA|TGCCAAATTGTGTG

AltraF ATG|GTAATTGTAACCTAC...TTCATGCGCTTCAAG|GTGCGCATGGAGGGC

AltraM ATG|GTAATTGTAACCTAC...TTCATGCGCTTCAAG|GTGCGCATGGAGGGC

Figure S2.2: Splicing sites of the transformer from *D. melanogaster*, *D. suzukii*, *C. capitata*, and *A. ludens* in *D. melanogaster*.

2.11 Supplementary Tables

Table S2.1: Primer sequences used in this study.

		Primer name	Primer sequence (5'-3')
genotyping introns	DstraF	1122.S1F	cggctagggttaaacgagtagtgtccacctc
		1122.S2R	GTAATTTACTAACGTAGAAATCCTGTGCTGGCAC
	CctraF	825A	AAAAATAAGGTCAGCAGCCATGAC
		825B	GTCATGGCTGCTGACCTTATTTT
		825C	AGACATTCCAATTCAGTTAACAAATTAAT
		825D	ATTAATTTGTTAACTTGAATTTGGAATGTCT
	AltraF	1168-Tra-gDNA-R1	CTTGCTCTGGAGTCCGTTAAA
		1168-Tra-gDNA-F2	CCTGAGCAGATAACCCTCTTTC
		1168-Tra-gDNA-F3	GGTCGTTTCGGTAACGTAGAA
		1168-Tra-gDNA-R2	GTCATCAGTTGTCGGCATTAC
Cloning primers		795G2.c1R	ccgcaacctgtctctgggGGATCCatgggtcgctctccaagaacgtcatcaag
		795K	acgccatccaacgcgcgcgcaacctgtctctgggATGgtaattttaaagcatatttttc
		795I	acgccatccaacgcgcgcgcaacctgtctctgggATGgtaattttaaagcatatttttc
		1122J.c1F	aattcaacgcacattattcgtgaggtaccgcgccatactcgttggctcccccac
		795L.c1F	ggccgactgttttctgtatccgtcaccaaacgcgtttttgcattaacattgtatgtcggc
		795L.c2R	tcaaagaaaaaatatgcttttaaaattacCATtttgTATTgtcacttgggtgttcacga
		795L.c3F	tcgtgaacaaccaagtgacAATAcaaaATGgtaattttaaagcatattttttctt
		795L.c4R	ACCCCGGTGAACAGCTCCTCGCCCTTGCTctctatagataccatagatgtatggattagat
		795L.c5F	atactaataccatacatctatgtatctatagAGCAAGGGCGAGGAGCTGTTACCCGGGGT
		795M.c2R	ATCAGATCGGTTTACTATATAGTGGGTACCATtttgTATTgtcacttgggtgttcacga
		795M.c3F	tcgtgaacaaccaagtgacAATAcaaaATGGTACCCACTATATAGTATAACCGATCTGAT
		795M.c4R	CACCCCGGTGAACAGCTCCTCGCCCTTGCTCTATGTGAAAAGAGTGTGCGGTAGTCAAT
		795M.c5F	ATTGACTAACCACACTCTTTTACATAGAGCAAGGGCGAGGAGCTGTTACCCGGGGTG
		795N.c2R	tctgatccgatcgaatatgtgtatatataCATtttgTATTgtcacttgggtgttcacga
		795N.c3F	tcgtgaacaaccaagtgacAATAcaaaATGgtatatatacacatattcgcggtacaga
		795N.c4R	CCCCGGTGAACAGCTCCTCGCCCTTGCTctctacgtggaagtggagaagaggtgttaacac
		795N.c5F	gtgattaacacctcttctccactccacgtagAGCAAGGGCGAGGAGCTGTTACCCGGGG
		795O.c2R	ttcataaaaaaataggtttacaattacCATtttgTATTgtcacttgggtgttcacga
		795O.c3F	tcgtgaacaaccaagtgacAATAcaaaATGgtaattglaacctacattttttttatgaa
		795O.c4R	CACCCCGGTGAACAGCTCCTCGCCCTTGCTctgtgggcaagatgtttttatattagta
		795O.c5F	tactaatataaaaaatcatcgtgcccacagAGCAAGGGCGAGGAGCTGTTACCCGGGGTG
DsRed splicing PCR		795.s2F	CAATTGTGGCGTTTACAGCATTTGTTATACACAGAACTC
		795.s2R	gaacagcatctgttacagcgacacaacatg
		795.s3F	CGATTATCCTAGGctacaggaacaggtg
		795.s1R	catccaacgcgcgcgcaacctgtc

Table S2.2: Sex-specific fluorescence sorting at multiple developmental stages.

Total embryos #		Total flies #		egg#	larval	hatching rate	total emerg + std	pupal	sex from adult sex	sex + std	pupal R+G	♀	♂	%♀	%♂	emergent adult	♀	♂	%♀	%♂	emergent adult	♀	♂	%♀	%♂	wt	♀	♂	emergent adult	♀	♂		
w-	160	156	97.5%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%	159	78.4%
w-	159	156	98.1%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%	157	78.0%
w-	203	194	95.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%	193	96.6%
w-	202	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%	200	99.0%
w-	918	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%	762	83%
F950.1	118	128	76.2%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%	118	58.3%
F950.2	47	40	85.1%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%	34	59.6%
F950.3	111	79	71.2%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%	63	56.8%
F950.4	110	72	62.4%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%	66	59.7%
F950.5	621	306	49.3%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%	185	29.9%
F950.1	180	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%	189	105.0%
F950.2	170	113	66.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%	34	19.4%
F950.3	203	176	86.7%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%	158	77.8%
F950.4	232	193	83.2%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%	143	61.6%
F950.5	1940	426	22%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%	143	7.4%
F950.1	193	144	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%	143	74.6%
F950.2	210	191	90.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%	172	81.9%
F950.3	211	176	83.4%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%	158	74.7%
F950.4	290	227	78.3%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%	223	76.9%
F950.5	1136	720	63.4%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%	246	21.7%
F950.1	173	164	94.8%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%	162	93.7%
F950.2	148	141	95.3%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%	138	93.2%
F950.3	188	180	95.8%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%
F950.4	210	201	95.7%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%	198	94.3%
F950.5	933	682	73.1%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%	202	21.8%
F950.1	296	244	82.4%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%	225	75.9%
F950.2	212	180	84.9%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%	172	81.4%
F950.3	188	180	95.8%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%	179	95.2%
F950.4	207	172	83.1%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%	160	77.3%
F950.5	1060	766	72.3%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%	187	17.6%
F950.1	383	254	66.3%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%	235	61.4%
F950.2	214	173	80.8%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%	160	74.9%
F950.3	244	217	89.3%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%	173	70.9%
F950.4	209	197	94.3%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%	187	90.0%
F950.5	1339	712	53.2%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%	119	8.9%
F950.1	102	83	81.4%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%	61	59.8%
F950.2	83	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%	48	57.8%
F950.3	84	48	57.1%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%	44	52.4%
F950.4	132	118	89.4%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%	104	78.9%
F950.5	500	240	48%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%	109	21.8%
F950.1	181	147	81.2%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%	121	67.3%
F950.2	187	167	89.3%	117	62.6%	117	62.6%	117	62.6%	117																							

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Chapter 3 Fluorescent based sex-separation technique in *Drosophila suzukii*

3.1 Abstract

Insect population biocontrol methods such as the sterile insect technique (SIT) or the incompatible insect technique (IIT) represent highly promising alternatives to traditional pesticide or insect trap applications. One significant challenge when deploying insect population biocontrol strategies in the field is an efficient separation of female and male insects. Having previously characterized the SEPARATOR (Sexing Element Produced by Alternative RNA-splicing of A Transgenic Observable Reporter), a fluorescence-based sex-sorting technique in *Drosophila melanogaster* and *Ceratitis capitata*, we are currently exploring its potential applicability in *Drosophila suzukii*, a prominent pest species that poses a threat to numerous berry crops. In this study, we show three SEPARATOR constructs that allow the selection of females or males as early as the L2 larval stage.

3.2 Introduction

Spotted wing drosophila, *Drosophila suzukii* (*D. suzukii*), has recently emerged as one of the foremost agricultural pests internationally, spreading from East Asia to North America and Europe with notable speed.¹ *D. suzukii* poses a unique issue due to its preference for laying eggs in fresh fruit rather than decaying fruit like most of its close relatives.²⁻⁴ *D. suzukii* females possess a serrated ovipositor to pierce and lay eggs in the fruit which the larvae consume *in situ*..^{2,5} Furthermore, the damage done to fruit solely by oviposition provides an opportunity for secondary infection of bacteria, fungi, and yeasts. The fast generation time of *D. suzukii* also contributes to its rapid spread, resulting in severe economic losses and increased usage of pesticides like organophosphates, pyrethroids, and spinosyns.⁶⁻⁹

Other biocontrol methods, such as sterile insect technique (SIT) or incompatible insect technique (IIT) have been proposed as a more sustainable and environmentally friendly alternatives to pesticide usage. In either case, lab strains must be mass-reared, sterilized through irradiation for SIT, or induced cytoplasmic incompatibility for IIT, and continuously released into the wild to compete and suppress pest

populations.¹⁰ Traditionally, for SIT in species like the New World Screwworm, sterile males and females have been released alongside each other, as no sorting mechanism exists to separate the sexes.^{11,12} However, the release of solely males has been demonstrated to be more cost-effective.¹³ Sterile females can still induce crop damage through oviposition and reduce the efficiency of population suppression by mating with sterile males.¹⁴ Crucially, some techniques like IIT demand the stringent release of only males for the program's success.¹⁵

As it currently stands, sex sorting is primarily performed manually, making it time-consuming, labor-intensive, and prone to human error.^{11,16} Machine learning has been implemented in certain species, such as *Aedes albopictus* and *Aedes aegypti*, to automate sex sorting, but these methods still depend on natural sexual dimorphisms, which provides challenges when attempting to adapt the technology to other pest species.¹⁷ The high throughput of sex-sorting required to implement these population suppression mechanisms at scale demands a highly accurate, efficient, and ubiquitous alternative to the current methods.

Genetic Sex Separation (GSS) lines have been developed in various species to allow sorting based on temperature sensitivity, chemical sensitivity, or pupal color rather than sexual dimorphism.^{16,18–20} Pupal color has been established as feasible regarding functionality and economic effectiveness in Mediterranean fruit flies (Medfly). Pupal color is employed as a selection marker produced by Y-linked translocation to permit efficient separation of mutant females utilizing inexpensive rice sorters.^{21–23} However, engineering GSS necessitates translocation of a conditional lethal gene through irradiation. For example, translocating the gene provides resistance to insecticide dieldrin (Rdl) onto the Y chromosome, which only confers resistance to males.²⁴ Additionally, Y-linked GSSs' can result in partial sterility which complicates rearing logistics, and genetic instability associated with recombination and phenotype loss not to mention reduced fitness and limitation due to the need of well-defined X and Y chromosomes.^{25,26} While Y-linked GSS have proven effective in particular species, a sorting mechanism that can be easily implemented in many species would provide invaluable utility.

Consequently, the genetic instability of Y-linked GSS has led to the advent of several Y-independent GSS in a variety of species.^{27–29} Most of these novel GSSs take advantage of sex-specific alternative splicing of the highly conserved sex determination gene *transformer (tra)*.^{30,31 31,32}

In this study, we demonstrate Sexing Elements Produced by Alternative RNA-splicing of a Transgenic Observable Reporter (SEPARATOR), which was previously characterized in *Drosophila melanogaster*, *Ceratitis capitata*, and *Aedes aegypti*, to be functional in *D. suzukii* as well.^{33–35}

The SEPARATOR exploits alternative splicing, which is ordinarily utilized as a posttranscriptional modification to increase protein diversity encoded by a single gene to allow sex-specific expression of a fluorescent marker. Our plasmid expression cassette's primary feature is a fluorescent protein with a sex-specific intron inserted into the coding sequence, allowing only one sex to express fluorescence after sex-specific splicing. We utilize the female-specific *transformer* intron (*TraF*) from three different species, *D. suzukii*, *D. melanogaster*, and *C. capitata*, which we have previously characterized in *D. melanogaster*, to demonstrate the feasibility of our system in *D. suzukii*.³⁴

SEPARATOR provides numerous advantages such as (1) high transferability due to usage of highly conserved elements; (2) 100% positive selection of females based on fluorescence instead of morphology for higher accuracy; (3) allows sex-sorting at several life stages as early as L2 instar; (4) potential for combination with existing biocontrol methods and automated sorting machines.

3.3 Result

3.3.1 All three experimental introns result in female-specific dsRed expression in *D. suzukii*

Three constructs (795H, I, and J) that exhibit female-specific dsRed in *D. melanogaster* were assessed in *D. suzukii* alongside a control construct (795G) that expresses both dsRed and eGFP constitutively (**Fig 1A**). Multiple individual insertion lines were obtained for each construct. Only one strain exhibiting the most robust *pHr5ie-eGFP* expression for each construct was kept for further testing. All three constructs harboring *DmtraF*, *DstraF*, and *CctraF* introns resulted in female-specific dsRed in *D. suzukii* (**Fig 2**). This observation aligns with our results in *D. melanogaster*.³⁴ While all the constructs

were created through random *piggyBac* integration, it is essential to note that a direct comparison of the transgene expression may not be feasible.

Nevertheless, 795G consistently exhibits the highest dsRed expression. This is attributed to the fact that dsRed is constitutively driven by the *Opie2* promoter. *CcTraF*-dsRed resulted in the highest female-specific dsRed expression in adults among all the introns.

To verify the female-specific splicing molecularly, pupae were collected for RT-PCR analysis (Fig 1D). Primers were designed to align to the 5' and ~400 bp into the 3' region of the dsRed (Supplementary Table S1). The resulting bands were consistent with the phenotype of the flies, with females possessing a ~600 bp band from DsRed and males possessing larger bands due to incomplete splicing of *TraF*. Sequencing results confirmed the accuracy of the bands (Fig S1).

3.3.2 *CcTraF*, *DmTraF*, and *DsTraF* allow for sex sorting as early as L2 instar

We evaluated the sex-specificity of the fluorescence and when fluorescence was first visible across multiple life stages of all three strains (795H, I, and J). In all three lines, dsRed was observable as early as L2 instar with varying levels of fluorescent intensity (**Fig 2**). We attribute differences in fluorescent expression between lines to a combination of intron size, random insertion site, and differences in sex-determination pathways between *Ceratitis* and *Drosophila*. Sex is determined by the ratio of sex chromosomes to autosomes (X:A) in *Drosophila*.³⁶ If the X:A ratio is 1, it triggers the *sex-lethal* (*sxl*) protein production, subsequently producing the *transformer* (*tra*) protein. Conversely, in *Ceratitis*, *tra* undergoes autoregulation in XX females and is suppressed in males by *maleness-on-the-Y* (*MoY*).³⁷ *CcTraF* also resulted in the brightest dsRed expression, followed by *DmTraF* and *DsTraF*. We found this same unexpected result when testing this system in *D. melanogaster*.³⁴

3.3.3 Fitness of fluorescent SEPARATOR strains

Strain fitness is one of the essential concerns when engineering transgenic lines, especially when including a component known to have a fitness cost, such as the fluorescent proteins employed in our cassette. We estimated that including *TraF* within the coding sequence of DsRed would mitigate the

associated fitness cost to a level not significantly different from the *wild type*. Consequently, we measured egg hatching and larval to adult survival rates in three strains harboring *C. capitata*, *D. suzukii*, and *D. melanogaster* *TraF* intron to a constitutively expressed line (795G) and wild-type (Fig 1E & F). The embryo hatching and larval-adult survival rates were not significantly altered compared to 795G and *wt*.

3.4 Discussion

In this study, we demonstrated the functionality of our previously engineered sex-sorting method, SEPARATOR, in the widespread agricultural pest *Drosophila suzukii*. SEPARATOR takes advantage of sex-specific alternative splicing of a highly conserved sex determination gene, *transformer*,³⁸ to allow for the female-specific expression of *DsRed*. Males and females constitutively express eGFP, while only females can fully splice the *traF* intron out to restore functional *DsRed* translation. *TraF* introns from three species, *CctraF*, *DmtraF*, and *DstraF*, were assessed in *D. suzukii*, demonstrating 100% accuracy. We opted to employ *PiggyBac*-mediated transformation in the creation of lines due to its ubiquitous functionality across both model and non-model insect species.³⁹ Due to the stochastic nature of *PiggyBac*-mediated transgenesis, we assessed several strains per construct to compensate for the insertion site-dependent effect on construct expression. All three strains exhibit fluorescent expression as early as the L2 instar and continuously at every subsequent life stage. Therefore, our results demonstrate the system's feasibility in potentially utilizing large-scale, high-throughput automated sex sorting apparatuses. Systems such as IIT could greatly benefit from an automatable, highly accurate sex sorting such as this due to its strict prerequisite for male-only releases lest the system fail.^{15,40} SEPARATOR's permission of sex sorting as early as the L2 instar also signifies pronounced economic savings in systems that may not require sex sorting to function, such as SIT. While release of sterile males and females still produces a suppressive effect on populations targeted by SIT^{11,12} removing sterile females in the larval stage reduces rearing costs, improves suppression efficiency through prevention of sterile females mating with sterile males, and may be necessary if used in disease vector species where females are often solely responsible for biting and disease transmission.^{14,41,42}

In combination with existing biocontrol methods, SEPARATOR can potentially be utilized in conjunction with a Complex Parametric Analyzer and Sorter (COPAS, Union Biometrica) instrument. COPAS has already demonstrated success in sorting *D. melanogaster* embryos and larvae, as well as *Aedes aegypti* and *Anopheles gambiae* larvae via fluorescence.^{11,43} The COPAS machine fully automated the sorting process, allowing efficiency, speed, and precision impossible in manual sorting.¹¹ Although COPAS has been utilized to sort based on fluorescence, it is unknown whether female-specific fluorescence will be sufficient for this process due to varying levels of autofluorescence seen in our results. Importantly, it should be noted that *DmtraF* and *DstraF* should be employed primarily in species utilizing *Sxl* as a master gene for somatic sex determination. Alternative splicing in Diptera relies on *Sxl* for proper female splicing patterns.

Moreover, SEPARATOR does not require antibiotics for line maintenance or sex selection, demonstrating the cost efficiency of our system.^{27,29,44,45}

As previously mentioned, SEPARATOR may be susceptible to random genetic events resulting in loss-of-function, gain-of-function, or translocation and recombination, leading to loss of fluorescence.³⁴ However, loss of fluorescence may not be remarkably detrimental to our system, as any mutations or events leading to loss of fluorescence will also be selected against in sorting. Even so, precaution and wariness to such events should be exercised, particularly at larger scales. Safeguards utilized in classic GSSs such as the Filter Rearing System(FRS) should be put into place to protect against recombination.⁴⁶ The FRS was specifically designed for the Mediterranean fruit fly GSS program and functions by maintaining a small, regularly sorted colony on the side that can be quickly amplified and replaced in case of system breakdown.

One exciting advantage of our system is the high potential for transfer and use in multiple species. Traditionally, GSSs necessitate the presence of an observable or conditional lethal mutation and a wild-type allele capable of being linked to the male-determining region of the chromosome.^{47,48} While some X-linked GSSs have been engineered,⁴⁷ the requirement of a visible single copy allele restricts most GSSs to use within the species in which they are developed.^{26,49} Conversely, SEPARATOR's usage of

tra, a highly conserved gene, and a portion of the sex determination pathway in many Dipteran species, has allowed us to comfortably expand SEPARATOR into four different species and two genera with minimal modifications to our cassette and strategy.^{33,34,50–52} Our proof of concept utilizing our cassette's *attP* integration capability in *D. melanogaster*, and successful transfer into *C. capitata*, and now *D. suzukii* through random *piggyBac* integration exemplifies the versatility of our construct. Site-specific integration provides a streamlined, invariable method of sex-sorting in species compatible with *attP* integration. The inclusion of a *piggyBac* transposon further permits the usage of our system in species that lack landing sites for site-specific integration. The robustness of the cassette's functionality and transferability to a wide variety of species makes it well suited for employment in species that are more poorly characterized to allow for easier and expedited sex sorting.

Fluorescent intensity varied widely between strains and constructs, which may be owed to insertion site impacting the degree to which the construct is expressed or variable splicing efficiency of *tra* introns depending on sequence differences among splice sites. As a consequence of random insertion, it is not possible to make an accurate comparison between lines.

3.5 Methods

3.5.1 Molecular constructs design and assembly

We used the previously described plasmids harboring various *traF* introns inside the dsRed coding sequence and the *Hr5ie1-eGFP* markers to generate the SEPARATOR strains: *Opie2-dsRed*, *Opie2-CctraF-dsRed*, *Opie2-DmtraF-dsRed*, and *Opie2-DstraF-dsRed* (Addgene ##205481-205484).³⁴ All plasmids' *TraF* introns were PCR amplified from the genomic DNA of their respective species. Restriction endonucleases *AvrII* and *BamHI* were used to linearize the plasmids before insertion of the *TraF* intron into the coding sequence of DsRed immediately downstream of the ATG start codon.

3.5.2 Rearing and fly transgenesis

Transgenic flies were maintained under standard conditions at 21°C with a 12H/12H light/dark cycle and were fed on a cornmeal-yeast-agar diet in an ACL2 insectary at UCSD. Embryonic injections were performed following standard injection protocols in lab. Plasmids were diluted to 300-350ng/μL in water and then injected into the *D. suzukii* embryos that harbored the Hsp70Bb-piggyBacTransposase.⁵³ G0 adults emerged from the injection were outcrossed to *D. suzukii* wt flies. Homozygous transgenic lines were used to assess the sex-sorting efficiency.

3.5.3 Reverse transcription PCR (RT-PCR) of the female-specific splicing transcripts

To analyze the splicing transcripts of the three *traF* introns, dsRNA mRNA specific for females and males was screened. Total RNA was extracted from ten female or male pupae from wild-type 795G, H, I, and J strains using the miRNeasy Tissue/Cells Advanced Kits (Qiagen). Subsequently, DNase treatment was performed using the TURBO™ DNA-free kit (Invitrogen). The cDNA synthesis was conducted with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™). Genomic DNA (gDNA) was amplified using 795.s2F and 795.s2R, while cDNA was amplified using primers 795.s3F and 795.s1R (Supplementary Table 1). The gDNA samples were analyzed on a 1% TAE agarose gel, and the cDNA samples were analyzed on a 2% TAE agarose gel.

3.5.4 Genetics and sex selection

To evaluate the effectiveness of the fluorescent sex selection, we paired ten virgin females with ten males in a fly embryo collection chamber. New grape plates were replaced every 12 hours, and the numbers of the laid embryos were recorded and transferred to fresh fly vials. After hatching, the larvae or pupae were sorted into vials based on their fluorescent markers. The sex and fluorescent markers of the adult offspring were documented after they were eclosed. Flies were examined using a Leica M165FC fluorescent

stereomicroscope, and images were captured using a View4K camera. Each genetic cross was done four times with different parental flies.

3.5.5 Fitness estimation

Two parameters were used to assess the fitness of the sex-sorting strains: the egg-hatching rate (from embryos to larvae), and the adult survival rate (from larvae to adult stage). To measure the egg-hatching rate, flies were allowed to lay embryos in the fly embryo collection chamber for 24 hours, embryos were collected and placed onto black filter paper atop the larval diet. The number of unhatched eggs were recorded after 24 hours and subtracted from the total to achieve the hatching rate. To evaluate the adult survival rate, the number of both female and male adult flies that successfully emerged is recorded.

3.5.6 Statistical analysis

Statistical analysis was performed in Prism9 by GraphPad Software, LLC. Three to five biological replicates were used to generate statistical means for comparisons.

3.6 Data availability

Complete sequence maps and plasmids are deposited at Addgene.org (#205481-205484). Transgenic lines are available upon request to O.S.A.

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opinions, and/or findings expressed are those of the authors and should not be interpreted as representing the official views or policies of the U.S. government. Figures were created with BioRender.com.

3.9 Author contributions

J.L. conceived and designed the experiments. J.L. and D.R. performed molecular and genetic experiments. All authors contributed to the writing, analyzed the data, and approved the final manuscript.

3.10 Competing interests

O.S.A. is a founder of Agragene, Inc. and Synvect, Inc. with equity interest. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. All other authors declare no competing interests.

3.11 Reference

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3.12 Acknowledgement

Chapter 3, in full, is a copy of the material that is currently being prepared for the publication.

Liu, Junru*; Rayes, Danny*; Yang, Minzhe; Akbari, Omar S. “Fluorescent sex-separation technique in

Drosophila suzukii.”The dissertation/thesis author was the first author and primary investigator of this paper.

3.13 Figures

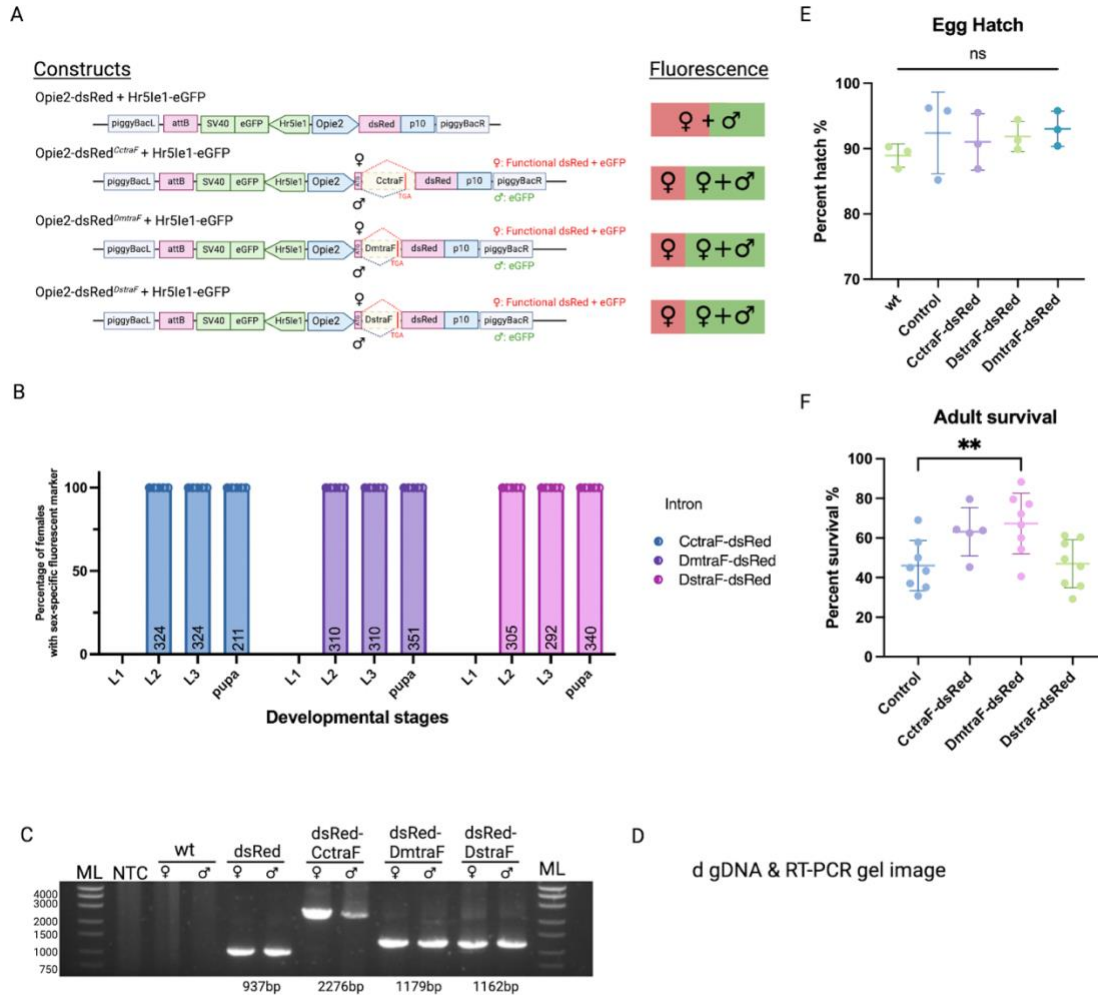


Figure 3.1: (A) Schematic of the SEPARATOR constructs developed and tested for the study, along with the corresponding fluorescence. (B) Female selection efficiency at different developmental stages for all three constructs that exhibit female-specific dsRed. The number of scored flies is indicated in each bar. All three SEPARATOR constructs achieved 100% accuracy at sex separation; no males were detected with female-specific dsRed. Gel electrophoresis images show (C) the genomic DNA PCR for dsRed-*traF*, (D) the RT-qPCR for the dsRed-*traF*. The fitness of the SEPARATOR is measured through (E) Embryo hatching rate and (F) survival rate from larvae to adults. No fitness cost was observed (** $p=0.0099$, One-Way ANOVA multiple comparisons).

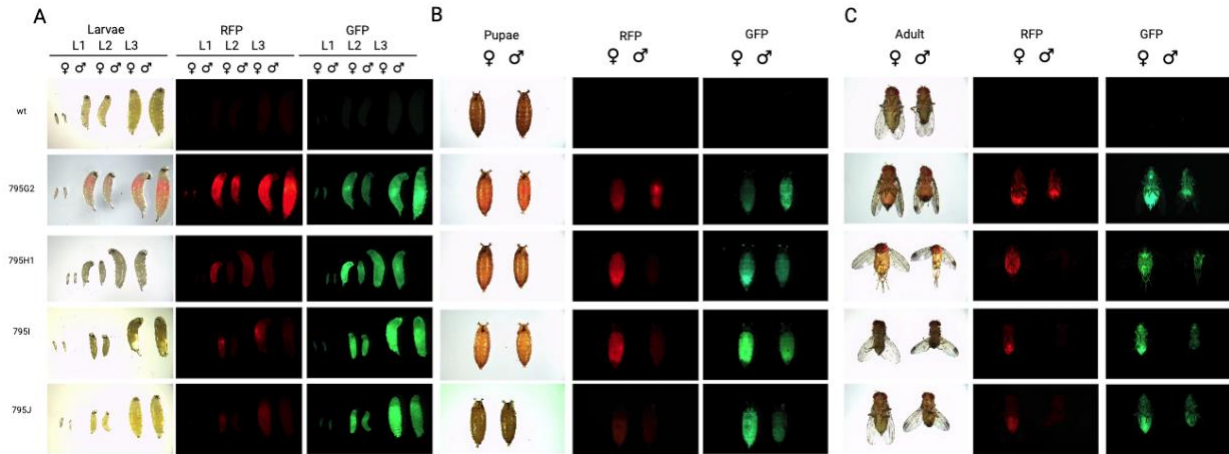


Figure 3.2: The expression of Opie2-TraF-dsRed in *D. suzukii* in (A) L1-L3 larval (B) pupal (C) adult stages for *CctraF*, *DmtraF* and *DstraF* under white light, RFP, and GFP filters.

3.14 Supplementary Tables

Table S3.1: Primer sequences used in this study.

		Primer name	Primer seequence (5'-3')
genotypeing introns	DstraF	1122.S1F	cggctaggttaaacgagtagtgtccacctc
		1122.S2R	GTAATTTACTAACGTAGAAATCCTGTGCTGGCAC
	CctraF	825A	AAAAATAAGGTCAGCAGCCATGAC
		825B	GTCATGGCTGCTGACCTTATTTTT
		825C	AGACATTCCAAATTCAAGTTAACAAATTAAT
		825D	ATTAATTTGTAACTTGAATTTGGAATGTCT
Cloning primers		795G2.c1R	ccgcaacctgtctctgggtGGATCCatgggtgcgctcctccaagaacgtcatcaag
		795K	acgccatccaaccgcccgcgcaacctgtctctgggtATGgtaattttaaaagcatattttttc
		795I	acgccatccaaccgcccgcgcaacctgtctctgggtATGgtaattttaaaagcatattttttc
		1122J.c1F	aattcaacgcacacttattacgtgaggtaccgcgccatactcgggtggcctcccccac
genomic DNA PCR		795.s2F	CAATTGTGGCGTTTACAGCATTTGTTATACACACAGAACTC
		795.s2R	gaacagcatctgttacagcgacacaacatg
RT-PCR		795.s3F	CGATTCATCCTAGGctacaggaacaggtg
		795.s1R	catccaaccgcccgcgcaacctgtc

Chapter 4 Inducing Female-Specific Lethality in *Drosophila* through Mitochondrial DNA Fragmentation

4.1 Abstract

Traditional methods for animal control are often expensive to implement on a large scale. Additionally, novel genetic approaches have their limitations, primarily in their ability to target different animal species beyond those for which they were initially developed. Consequently, there is a pressing need for an environmentally friendly, cost-effective, and adaptable approach to animal control. Here, we introduce a novel genetic circuit termed **MitoFail** (Mitochondrial female-associated lethality), designed for universally applicable female-specific repressible lethality in animals. This genetic circuit takes advantage of the universally active restriction endonucleases that shred mitochondrial DNA molecules (mtDNA), the female-specific intron, and the tetracycline-repressible system. The essential nature of mtDNA in animal viability, combined with its vulnerability to programmable mitochondrial-imported restriction endonucleases, supports the broad adaptability of MitoFail across diverse animal species. With female-specific introns, MitoFail will only be active in females, allowing it to be passed down and spread by competitive males, thereby suppressing the targeted population by reducing females in the population. MitoFail offers multiple advantages over current genetic insect control methods and holds promise as a valuable addition to the genetic toolkit for controlling diverse animal species.

4.2 Introduction

Insect control has traditionally relied on pesticides. However, the consistent application of pesticides has had adverse consequences, leading to the development of resistance to these chemicals in targeted species and harming off-target insect pollinators.¹⁻⁴ Furthermore, the effects of a warming and wet climate have expanded the range of many insect species, exacerbating the burden of pests and vectors. These developments have sparked increased interest in both traditional and innovative biological approaches to insect control.

One such approach is the Sterile Insect Technique (SIT), which offers a species-specific and environmentally friendly method for insect control. SIT involves the mass release of irradiated males, which then reduces the population of wild insects through sterile matings. While SIT has been successfully employed to control certain insect species, its application can be extended to many insect species where females mate only once in their lifetime.^{5,6} However, it is important to note that SIT is a costly and challenging approach that requires large-scale and repeated release to compensate for the reduced competitiveness of irradiated males. Females are essential for colony maintenance and propagation. Nevertheless, complete female removal has been a challenge for mass-rearing procedures.

Alternative genetic approaches have recently been developed to address the limitations of traditional SIT. Many of these genetic methods for insect control are based on the tetracycline-repressible (Tet-off) expression system.⁷ This system relies on two components: a Tetracycline-controlled Transactivator (tTA) and a Tetracycline Response Element (TRE).⁸ The tTA consists of a tetracycline repressor (tetR) and a C-terminal domain of virion protein 16 (VP16). When tetracycline is absent, tTA binds to TRE, thereby enhancing the expression of the gene of interest. When tetracycline or its analog doxycycline is present, it binds to tetR, preventing the binding of tTA to the TRE. Consequently, gene expression is reduced. To induce repressible lethality in insects, researchers have employed tTA to either transactivate an apoptosis effector gene or auto-activate itself, resulting in rampant transcriptional activation.⁹⁻¹⁴ To mitigate leakiness in transcriptional activation and reduce fitness cost in males, a female-specific intron was inserted into the coding sequence (CDS) of the tTA protein,^{12,13} but not into effectors' CDSs.^{9,10} Nevertheless, the precise titration between good repression and complete activation of the current Tet-off systems and reliance on species-specific components have complicated the adaptation of these circuits to other insect species.

The mitochondrial DNA (mtDNA) genome is a small circular molecule located within mitochondria. In each animal cell, numerous mitochondria are present, each containing hundreds of mtDNA molecules. While mtDNA undergoes relatively rapid mutations and evolution at the DNA sequence level, all animal mtDNA genomes share identical genes that are crucial for cellular respiration.

These include two ribosomal RNAs, 22 transfer RNAs, and 13 protein-coding subunits of the Electron Transport Chain (ETC).^{15,16} Multiple cellular pathways exist to ensure the quality control of multiple mtDNA molecules, and failures in these processes can lead to organismal disease, aging, and, ultimately, death.^{17,18}

Mitochondrial-targeted Restriction Endonuclease (mtREs) have emerged as powerful tools for inducing targeted genetic modification in the broader context of manipulating mitochondrial genomes. The mtREs, have been utilized to study mitochondrial genome manipulation for its ability to cleave and generate double-stranded breaks in animal mtDNA.^{19–21} The cut and linearized mtDNA molecules are unstable and quickly digested by exonuclease inside mitochondria.²²

The mitochondrial localization sequence (MLS), also known as the mitochondrial targeting sequence or mitochondrial transit peptide, is a specific sequence of amino acids that directs the protein to be transported into mitochondria.²³ The mitochondrial localization sequence (MLS), typically located at the protein's N-terminus, can be fused with a protein of interest to direct the protein into the mitochondria.^{24,25}

With single-cutter mtREs, scientists have achieved precise cleavage of all mtDNA molecules containing a single recognition site in *Drosophila melanogaster* *mtDNA*^{wt}.^{20,26} This enables the selection of mtDNA molecules harboring preexisting or induced mutation at the endonuclease recognition sites, rendering them resistant to cleavage by the applied mtREs. In contrast, double-cutter mtREs, possessing two recognition sites on *mtDNA*^{wt}, have been employed to induce specific large deletions in mtDNA molecules (*mtDNA*^Δ). These induced deletions coexist in heteroplasmy with *mtDNA*^{wt} molecules, facilitating the study of pathways involved in mtDNA quality control in post-mitotic cells.^{18,27}

With the intention of organismic lethality, we employed a multiple-cutter mtRE capable of robustly fragmenting the *mtDNA*^{wt}. This approach transforms mtREs into sequence-specific “toxin,” leading to organisms' lethality. In this study, PacI endonuclease was selected for our MitoFail cassette for several reasons: firstly, its recognition site is prevalent in the AT-rich insect mtDNA, with *D. melanogaster* and *D. suzukii* mtDNA harboring 21 and 12 recognition sites, respectively (Fig 1C).

Additionally, PacI is one of the smallest endonucleases, comprising only 142 amino acids, facilitating its efficient incorporation into the genome.

Here, we describe a genetic circuit that supports a repressible female-specific lethality across diverse insect species, referred to as Mitochondrial Female-associated lethality or MitoFail. Mitochondria and mitochondrial DNA (mtDNA) genomes are essential for cellular respiration and conserved across diverse animal species. MitoFail's universal action is achieved by targeting restriction endonucleases into mitochondria and mtDNA genomes during an entire insect development resulting in the lethality. To limit the lethality and the circuit-related fitness costs to the female sex, the female-specific intron is inserted into coding sequences of both trans-activator (aka. tetR) and effector (aka. restriction endonucleases). To achieve cross-species portability, MitoFail contains protein-coding sequences, promoters, introns, and 3'UTR sequences known to be active across diverse insect species. The DNA sequence specificity of diverse restriction endonucleases and the universal requirement of mtDNA quality control for animal viability support the precision, versatility, robustness, and engineering flexibility of MitoFail that are unattainable by current approaches for animal control.

4.3 Results

4.3.1 MLS-PacI resulted in complete lethality in females

To achieve complete elimination of mtDNA in females, an endonuclease capable of making multiple cuts within the mtDNA is necessary. In this study, the PacI endonuclease was chosen due to its recognition sites being commonly found in the AT-rich regions of mtDNA (**Fig.1**). The mtDNA of *Drosophila melanogaster* and *Drosophila suzukii* harbors 21 and 12 PacI recognition sites, respectively (**Fig.1C**). To express PacI in the mitochondrial matrix, a mitochondrial localization sequence (MLS) was fused to the N-terminus of PacI coding sequence (*MLS-PacI*). With MLS, a precursor protein containing the MLS together with PacI will be first produced in the cytosol (**Fig1A**). The MLS is recognized by the receptor on the outer mitochondrial membrane, facilitating translocation into the mitochondrial matrix. Upon entry, the MLS is cleaved by the mitochondrial processing peptidase (MPP), resulting in the

production of the mature PacI protein, which becomes activated and capable of cleaving mtDNA within the mitochondrial nucleoids.^{23,28}

To assess the potential role of mtREs as a lethality-inducing toxin, we conducted a comparative analysis of *MLS-PacI* with *D. melanogaster head involuted defective (Hid)*, an apoptosis activator. Previously, overexpression of *Hid^{Ala5}*, a Ras pathway-insensitive variant of *Hid*, has been used to induce lethality in various insects.^{9,10,29–31} To restrict the expression of functional proteins exclusively to females, we inserted *Ceratitis capitata transformer* female-specific intron (*CctraF*) into the coding sequence of *mtPacI* or *Hid^{Ala5}*. The *CctraF* intron undergoes complete splicing exclusively in female mRNA, whereas it results in a male-specific exon with a termination signal in male mRNA, blocking the translation of proteins containing *CctraF* in males.^{32–36}

To enhance expression, we utilized the *Opie2* promoter to drive the transcription of *MLS-PacI-CctraF* (*MLSPacI*♀) and *Hid^{Ala5}-CctraF* (*Hid^{Ala5}* ♀), with *p10 3'UTR* employed for transcription termination. Both *Opie2* and *p10*, originally derived from baculovirus, are known for providing robust and constitutive expression in various insect species.^{37–39} In light of these, two constructs were generated: *Opie2-MLS-PacI*♀-*p10* (1155F) and *Opie2-HidAla5*♀-*p10* (1155C) (**Fig.s1**). For better comparison, both constructs were injected into the same site (R8621) through phiC31 site-specific integration.⁴⁰

Complete female lethality was induced by *mtPacI*♀. No females carrying a single copy of *Opie2-MLS-PacI*♀-*p10* (1155F) ever eclosed, with the latest observed time of female death occurring during the late pupal stage (**Fig 2 & s2a**). To maintain the *Opie2-MLS-PacI*♀-*p10* strain, hemizygous males were continuously bred with wild-type (wt) females over ten generations, and no hemizygous female was observed.

Concurrently, adult females with *Opie2-Hid^{Ala5}*♀-*p10* (1155C) were able to survive and remain fertile, allowing for the establishment of a homozygous *Opie2-Hid^{Ala5}*♀-*p10* (1155C) strain. However, it is worth noting that both hemizygous and homozygous *Opie2-Hid^{Ala5}*♀-*p10* females exhibited a

deformed wing phenotype (**Fig.s2b**). In summary, *mtPacI*[♀] resulted in 100% female-specific lethality in *D. melanogaster*, while *Hid*^{Ala5} ♀ did not.

4.3.2 NLS-PacI and PacI without localization signal

To compare the Mitochondrial Matrix Localization Sequence (MLS) with the Nuclear Localization Signal (NLS), we constructed a plasmid containing *Opie2-NLS-PacI*[♀]-*p10* (1155D, NLS-PacI[♀], **Fig.s1**). Consequently, the NLS-PacI[♀] was inserted into the same insertion site (R8621) as MLS-PacI[♀]. As anticipated, no female progeny were observed in the strain harboring NLS-PacI[♀] (**Fig.2A**). To visualize the developmental stages at which lethality occurs for both MLS-PacI[♀] and NLS-PacI[♀], we crossed MLS-PacI[♀]/cyo and NLS-PacI[♀]/cyo with 795H/cyo (*Opie2-CcraF-dsRed*), respectively, a strain expressing a female-specific fluorescent dsRed marker which we had previously characterized.^{35,36} Female lethality was observed throughout all developmental stages, with the latest stage being the late pupal stage (**Fig.s2A & C**).

4.3.3 Implementing a Tetracycline-Off System for Repressible Female-Specific Lethality

To create a repressible female-specific lethality system, *mtPacI*[♀] was placed under the control of the *tetracycline-off* expression system. Two promoters, *D. melanogaster vasa* (*Dm_vas*) or *Opie2*, were utilized to drive the expression of *tTA* (tetracycline TransActivator), and the *D. melanogaster Hsp70* 3'UTR was used to terminate the *tTA* transcription. To further minimize the potential fitness cost resulting from any leaky expression of *tTA* in females, an additional *CcraF* was inserted into the coding sequence of the *tTA* (*tTA*[♀], **Fig.2B & s1**). With this design, the expression of *tTA*[♀] and *mtPacI*[♀] proteins is exclusively limited to females. In the absence of tetracycline, the *tTA*[♀] protein binds to the *TetO*, initiating the transcription of *mtPacI*, resulting in female lethality. Conversely, in the presence of tetracycline, the *tTA* protein binds to tetracycline itself, preventing the transcription of *mtPacI*[♀], thereby allowing females to survive. (**Fig.2C**).

All three complete cassettes (1180B, D, and L) were injected into the same site (R8621) through *phiC31* site-specific integration in *D. melanogaster*. Death was observed during the late pupal stage when flies were reared on regular food (Dox-) (Fig sX). As expected, for all three construct designs, *D. melanogaster* females only survived when the food was supplemented with 10µg/mL Doxycycline (Dox+) at 21°C. Notably, at 21°C, no homozygous females survived on Doxycycline, indicating that two copies of the transgene are too toxic for females with 10µg/mL Doxycycline (Fig X). To address this issue, a lower rearing temperature was tested.

In the attempt to lower the rearing temperature to 18°C while using the same 10µg/mL Doxycycline concentration, homozygous females for all three constructs were obtained, and homozygous strains were established. However, a small number of females harboring one copy of transgene were able to survive on Dox- food at 18°C for 1180B and D. This could be attributed to the reduced cutting efficiency of mtREs at lower temperatures, allowing females with one copy of the transgene to withstand the Dox- condition. Nonetheless, we succeeded in establishing and maintaining a homozygous transgenic strain on 10µg/mL Dox+ food at 18°C.

The homozygous strain was further tested at 26°C, which is considered the optimal temperature for rearing *D. melanogaster*. No females survived at 26°C when provided with Dox+ food, while males remained unaffected. This observation is consistent with our previous finding at 18°C, where the cutting efficiency of mtREs showed a positive correlation with temperature.

4.3.4 Inducible female-specific lethality in *Drosophila suzukii*

All three complete cassette constructs (1180B, D, and L) were injected through *piggyBac* random integration in *D. suzukii*. Multiple independent insertion lines were obtained for each line. Different results were obtained in *D. suzukii* from *D. melanogaster*. Specifically, none of the 1180B strains in *D. suzukii* exhibited female lethality when reared on Dox- food, and only one out of seven strains for 1180D in *D. suzukii* resulted in female lethality when reared on Dox- food.

Conversely, eight independent insertion strains from the 1180L strains exhibited female-specific lethal phenotypes (Fig. **SX** provides a visual representation of 1180B and 1180L results in DS). Several potential explanations exist for this pronounced difference in lethality between 1180B/D and 1180L strains. Firstly, it could be attributed to differences in insertion sites. In *D. melanogaster*, all the constructs were site-specifically inserted into a strong expression site R8621. In contrast, in *D. suzukii*, all the constructs were randomly inserted into the genome. In the case of random insertion, it is possible that a strain that resulted in female lethality was never obtained. Secondly, the 1180B/D constructs utilize the *pDm_vas* to drive the transcription of the *tetR* gene, while the 1180L construct utilizes the *pOpie2*. Our previous work has demonstrated that the *Dm_vas* promoter can effectively induce robust gene expression in *D. suzukii*.^{41,42} However, it is possible that the expression pattern driven by *Dm_vas* is more restricted to the germline in *D. suzukii*, compared to *pOpie2*, which offers a stronger and ubiquitous expression. Consequently, the expression of *tetR* is higher under *pOpie2* than *pDm_vas* in *D. suzukii*, resulting in female lethality in all the randomly inserted strains.

4.3.5 Fitness of DS tet-off males is not compromised

To evaluate the fitness of the MitoFail *D.suzukii* flies, we compared the embryo hatching rate and larval to adult survival rate to that of wild-type. Our results indicate that there is no significant differences in egg hatching or the larval to adult rate between our *MitoFail* flies and the *wt* whether doxycycline is present or not (**Fig. 3A&B**). We then quantified their longevity relative to the wild-type (*wt*) flies. The survival curves showing average survival percentage with the 95% confidence intervals ($\pm$ CI) are shown in Figure 3C & 3D. In the presence of doxycycline, MitoFail females (37 days) showed no significant differences in median survival compared to *wt* females (33 days) ($p=0.2478$, Gehan-Breslow-Wilcoxon test). However, without doxycycline, MitoFail females (18 days) had significantly shorter median survival than *wt* females (68 days) ($p < 0.0001$, Gehan-Breslow-Wilcoxon test). Conversely, without doxycycline, there was no significant difference in median survival between MitoFail males (60 days) and WT males (60.5 days) ($p = 0.3740$, Gehan-Breslow-Wilcoxon test). Yet, in the presence of doxycycline,

MitoFail males (50 days) exhibited significantly longer median survival compared to WT males (22 days) ($p < 0.0001$, Gehan-Breslow-Wilcoxon test).

4.4 Discussion

The MitoFail genetic circuit possesses several advantageous features that render it highly customizable and adaptable for population control across a wide range of animals, extending beyond insects. One key factor contributing to its versatility is the remarkable conservation of animal mitochondrial DNA (mtDNA). The mtDNA of various animal species contains identical genes that are crucial for the Electron Transport Chain of Oxidative Phosphorylation and exhibit a high degree of amino acid sequence similarity.⁴³ Moreover, animal mtDNA genomes are relatively short, with sizes ranging from 16.3kb in *Mus musculus* to 19.5kb in *Drosophila melanogaster*, and they are typically straightforward and cost-effective to sequence.^{43–46}

Another significant advantage is that a highly conserved mitochondrial import machinery operates across diverse animal species. Consequently, identical mitochondrial matrix localization sequence (MLS) tags remain active not only in insects but also in mammals.^{24,28} This universality of the MLS enhances the applicability and adaptability of the MitoFail circuit for population control strategies in a wide spectrum of animals. Animal mtDNA genomes are localized inside the mitochondrial matrix, making them highly susceptible to restriction endonucleases (REs). By targeting specific mtDNA sequences, we have the flexibility to choose mtREs that can either be broad-spectrum, capable of cutting mtDNAs across different species, such as mtPacI for AT-rich mtDNA sequences found in many insects, or species-specific, tailors to cut only a particular mtDNA genome with an unusual recognition site in specific species. This adaptability allows mtREs to potentially work for nearly every animal and be selected as generalists or specialists, making them ideal universal “toxins” for population control strategies.

PacI primarily cleaves at AT-rich regions, which include the origin of the mtDNA replication and regulatory elements essential for both mtDNA replication and gene expression.^{47,48} Consequently, using the PacI ensures the destruction of existing mtDNA and disrupts mtDNA replication.

The fusion of a mitochondrial localization sequence (MLS) to PacI ensures its targeting to mitochondria, together with female-specific *transformer* (*traF*) intron, achieving the degradation of mtDNA and subsequent female lethality. The system's adaptability is further highlighted by its successful integration and function in *Drosophila suzukii*, albeit with some variation in effectiveness due to differences in promoter activity and insertion sites.

The tetracycline-repressible (Tet-off) system adds additional layers of control, allowing for inducible lethality. This feature is particularly valuable for fine-tuning the system's activation and minimizing potential fitness costs. The Tet-off expression system has been designed to both repress and transactivate, using di-repression, the expression of an effector gene in diverse animal groups, from mammals to insects. Our findings indicate that the presence of doxycycline can effectively minimize lethality, ensuring the survival of females when necessary, which is crucial for maintaining a large, healthy stock for mass-rearing purposes. Without the presence of doxycycline, our MitoFail females do not survive long; this provides another layer of control in which potential escapes will have a low chance of survival in the wild. The specificity or generality of a MitoFail genetic circuit can be further tuned by using regulatory DNA sequences of promoters, introns, and 3'UTR that are known to be active across diverse taxa or species-specific. Furthermore, the temperature sensitivity of the Hsp70 promoter ensures that increasing the temperatures enhance PacI activity, leading to increased female lethality, offering additional control over the system's activation and prevention of female survival in the wild.

To limit MitoFail activity and prevent any leakage of "toxicity" induced by mtREs in females, we used a female-specific intron. However, the female-specificity of *MitoFail* can also be achieved with female-specific promoters. In fact, the female-specific promoters that are active only in female-reproductive tissues can also be used for the expression of *MitoFail*. This *MitoFail* will induce female sterility and cause suppression of a targeted animal species by reducing its female reproductive output.

This strategy of reducing the female reproductive output with *MitoFail* may work well for rodent species. The overarching objective is to ensure that *MitoFail* males maintain their fitness, longevity, and competitiveness to facilitate the effective spread and mating of as many wt females as possible.

Given that mitochondria and mtDNA genomes are strictly inherited maternally in the majority of animal species, it is relatively easy to rectify any unintended or adverse effects resulting from an ineffective *MitoFail* system. This can be achieved through a genetic cross in which healthy wt females are employed to reintroduce robust mtDNA genomes into the offspring, effectively reversing any negative outcomes.

The *MitoFail* genetic circuit offers many potential features that simplify its engineering and adaptation for insect and other animal species control. Its fundamental mechanism, which involves the cleavage of mtDNA molecules by endonucleases like restriction enzymes to induce female-specific lethality or sterility, provides many opportunities for customization and offers the potential for nearly universal functionality. *MitoFail* is poised to become a valuable addition to the genetic tools available for managing pests, disease vectors, and invasive animal species.

In terms of fitness, our study shows that the MitoFail system does not compromise the fitness of *D. sukukii* males, as evidenced by comparing the hatching rate, larval survival, and longevity to wild-type. This highlights the system's potential for practical application without adversely affecting the non-target male population.

While the MitoFail system presents some advantages, it is not without limitations. One primary concern is the reliance on the tetracycline-repressible system. MitoFail necessitates the continuous presence of tetracycline or its analogs to repress lethality during laboratory maintenance and propagation; this dependency could pose challenges for field applications; while our system does not require tetracycline when releasing, a large amount of tetracycline will still be used in the mass-rearing factory. Moreover, the random insertion of the transgene could lead to variable expression patterns and efficacy, as observed in our *D. sukukii* experiments. This variability could require further optimization and testing

to ensure consistent performance in different species. Overall, MitoFail offers a promising addition to the genetic toolkit for insect control, providing a cost-effective and adaptable solution.

4.5 Methods

4.5.1 Molecular cloning

All plasmids were cloned using the Gibson enzymatic assembly. A plasmid containing *attP* site and *piggyBac* was used as a backbone. DNA fragments were PCR amplified using available plasmids in the lab using Q5 High-Fidelity 2X Master Mix (New England Biolabs). Primers used for cloning are listed in Supplementary Table 1.

4.5.2 Fly transgenesis and genetics

D. melanogaster flies were maintained with a 12H/12H light/dark cycle and fed on the Old Bloomington Molasses Recipe. Embryonic injections were performed by Rainbow Transgenic Flies, Inc. (www.rainbowgene.com). We used ϕ C31 Integrase-Mediated integration to insert the constructs either on the second chromosome (BDSC #8621) or the third chromosome (BDSC #8622). Recovered transgenic lines were balanced on the 2nd chromosome using a single chromosome balancer line w¹¹¹⁸; CyO/sna[*Sco*] or balanced on the 3rd chromosome using the double balancer (BDSC #3704). Multiple independent lines were obtained and tested for each plasmid. *D. suzukii* flies were maintained in an ACL2 insectary, with the procedures being in accordance with the approvals granted by the UCSD biosafety committee. Embryonic injections were performed in the lab following the strand injection protocol. Plasmids diluted to 300-350ng/ μ L in water were injected into the *D. suzukii* strain harboring the *Hsp70Bb-piggyBac* transposase.⁴⁹ G0 adults were outcrossed to *D. suzukii* wt flies, and the resulting G1 progeny were screened for transgenesis with a Leica M165FC fluorescent stereo microscope. Generated transgenic *D. suzukii* strains were repeatedly intercrossed over multiple generations to generate homozygous strains.

4.5.3 Embryo hatching rate and larvae to adult survival rate

To evaluate the egg hatching rate, flies are allowed to lay embryos in the fly food with or without doxycycline for 24h. The number of embryos in each vial is recorded. After 24h of embryo laying, the number of larvae is recorded. To evaluate the larvae to adult survival rate, the sex of the adults that successfully eclosed are recorded after eclosion.

4.5.4 Survival curves for Longevity estimates for the MitoFail flies

To compare the differences in survival between the MitoFail flies and the wt flies, we estimated their average longevities both in the presence of doxycycline and absence of doxycycline. Ten replicates for each group were used to estimate the survival curves. Flies were age-matched and aged in batches of 10 flies per vial. Flies were transferred to new vials every 3 days, and the number of dead flies per vial was recorded. Prism 9 was used for graphing the survival curve and for statistical analysis.

4.5.5 Statistical analysis

Statistical analysis was performed in Prism9 by GraphPad Software, LLC. Three to five biological replicates were used to generate statistical means for comparisons.

4.6 Data availability

Complete sequence maps and plasmids are deposited at Addgene.org (#218839-218928). Transgenic lines have been made available for order from Bloomington Drosophila stock center. The other transgenic lines are available upon request to O.S.A.

4.7 Acknowledgments

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be interpreted as representing the official views or policies of the U.S. government. Figures were created with BioRender.com.

4.8 Author contributions

N.P.K and J.L conceived and designed the experiments. J.L, D.R and M.Z performed molecular and genetic experiments. All authors contributed to the writing, analyzed the data, and approved the final manuscript.

4.9 Competing interests

O.S.A is a founder of Agragene, Inc. and Synvect, Inc. with equity interest. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. All other authors declare no competing interests.

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50. Adapted from “Protein Import into the Mitochondria,” created by Alberts et al using BioRender.com (2022). Retrieved from <https://app.biorender.com/biorender-templates/figures/all/t-641dbea8a88e45783ffa6f35-protein-import-into-the-mitochondria>

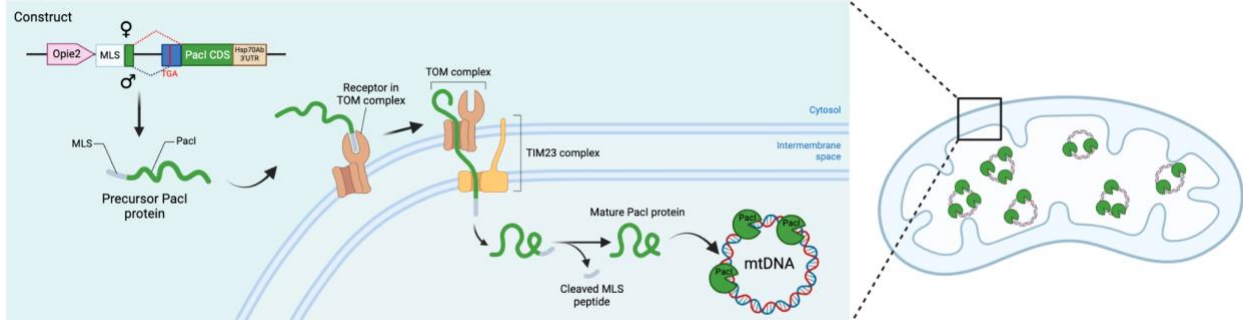
4.11 Acknowledgement

Chapter 4, in full, is a copy of the material that is currently being prepared for the publication.

Kandul, Nikolay*; Liu, Junru*; Rayes, Danny; Yang, Minzhe; Akbari, Omar S. “Inducing Female-Specific Lethality in *Drosophila* through Mitochondrial DNA Fragmentation.” The dissertation author was the primary researcher and author of this material.

4.12 Figures

A



B

PacI cutting site

5' ... TTAAT | TAA ... 3'

3' ... AAT | TAATT ... 5'

C

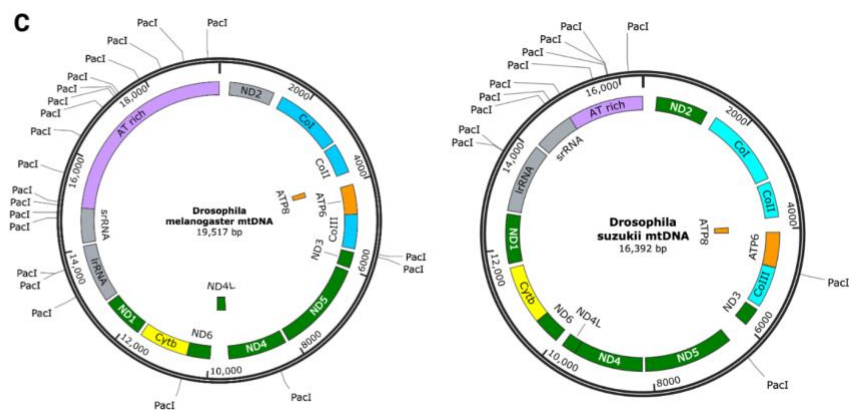


Figure 4.1: (A) Schematic of MLS-PacI protein import into the mitochondria (B) PacI endonuclease cutting site sequence (C) PacI recognition sites on *Drosophila melanogaster* and *Drosophila suzukii* mitochondrial DNA.

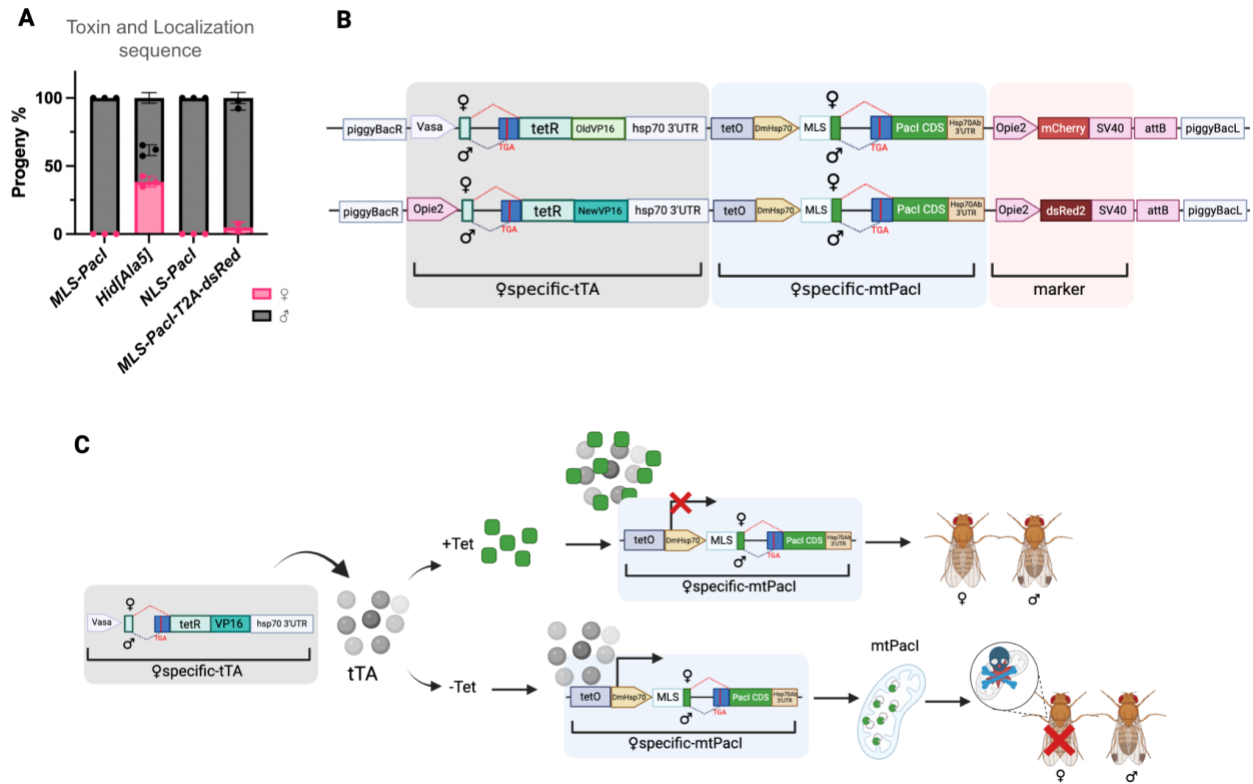


Figure 4.2: (A) Female-specific toxin or gene and localization sequence (n=3, N =). A schematic representation of (B) the complete MitoFail cassette and (C) the Tetracycline-off inducible system.

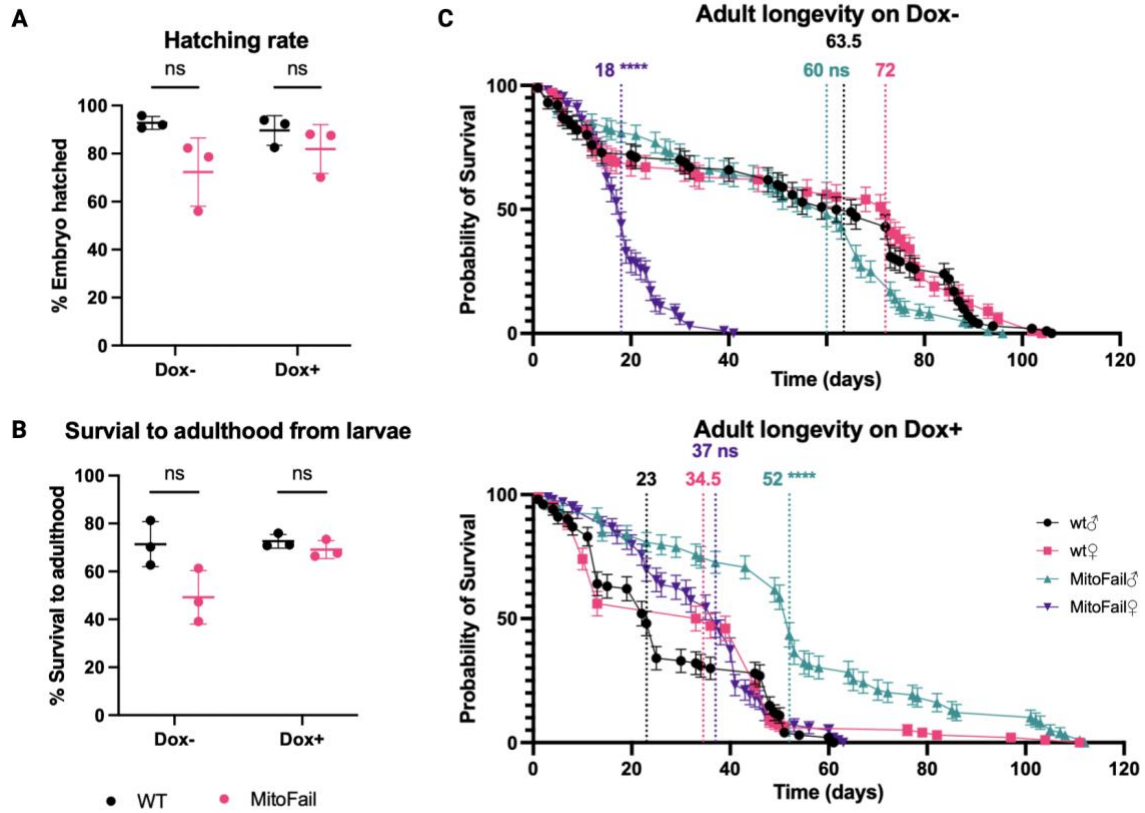


Figure 4.3: (A) Embryo hatching rate and (B) Survival to adulthood for both *wt* and *MitoFail* flies with the presence and absence of doxycycline. Longevity (C) when Doxycycline is not present and (D) when Doxycycline is present.

4.13 Supplementary figures

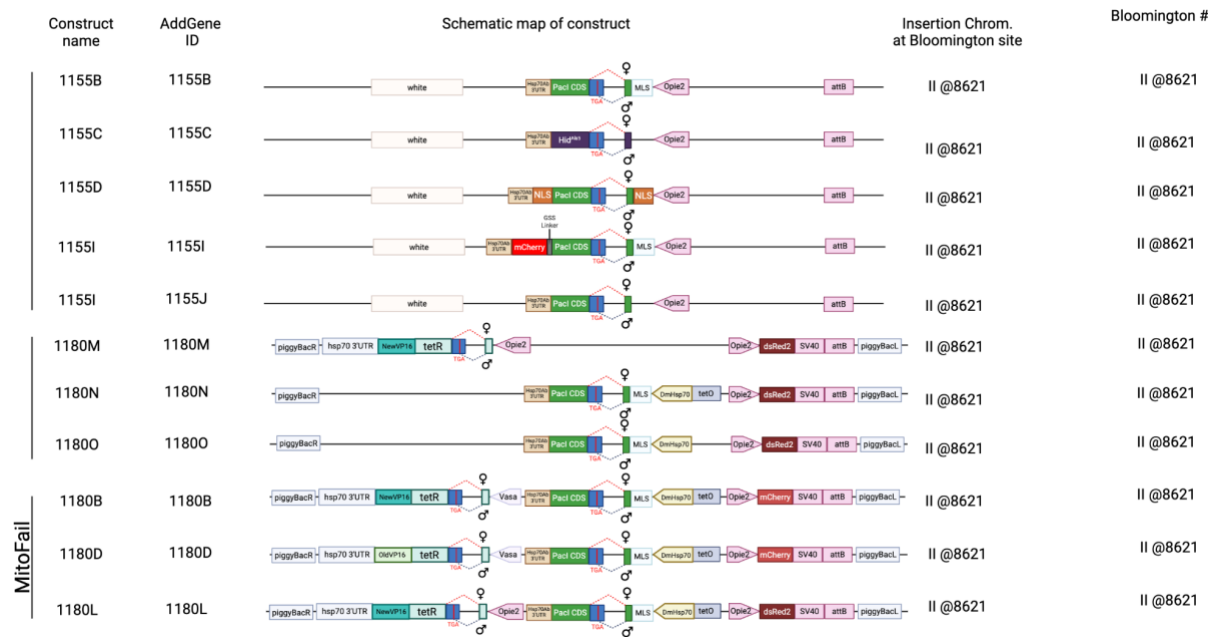


Figure S4.1: Genetic constructs and the respective transgenic lines developed in this study. All the constructs engineered in the study have been submitted to Addgene.org. Fly stocks are upon request to the lab. (MLS: Mitochondria localization signal; NLS: Nuclear localization signals; GS linker: $(GGGGS)_3$).

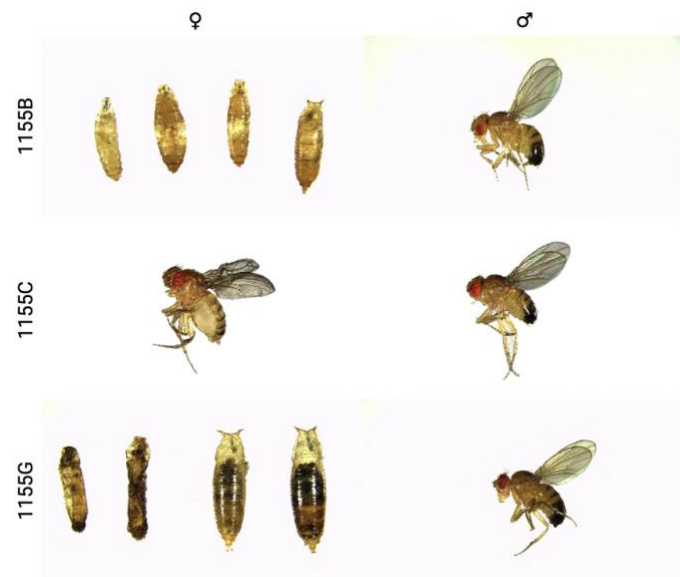


Figure S4.2: Adult females or dead female pupae or unaffected male.

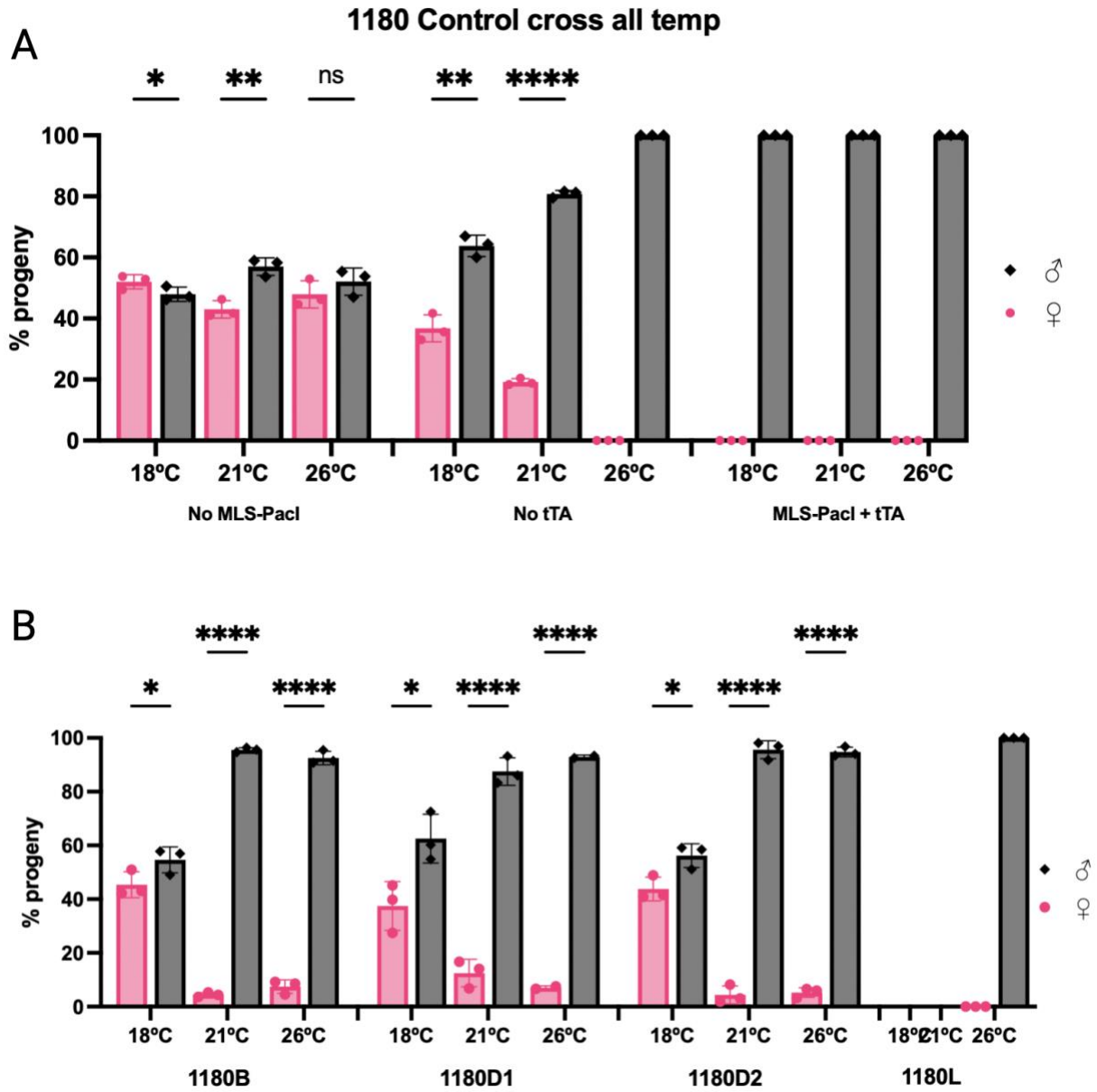


Figure S4.3: (A) 1180 control cross and (B) MitoFail circuits at 18, 21 and 26°C.