GEN Biotechnology
Volume 1, Number 4, 2022

DOI: 10.1089/genbio.2022.0019

GEN Biotechnology

RESEARCH ARTICLE

Precision Guided Sterile Males Suppress Populations of an Invasive Crop Pest

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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. 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.

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] *D. suzukii* oviposit their eggs deep within the soft flesh of ripening non-rotting fruit,[9] which leads to the ultimate destruction of the crop.[10,11] *D. suzukii* has been found to damage cherries, raspberries, blackberries, blueberries, strawberries, peaches, plums, pluots, nectarines, grapes,[7] olives,[8] tomatoes, kiwis, figs, and apples, for example.[11] In 2008 in California, Oregon, and Washington state, *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 *D. suzukii* are becoming resistant to pesticides presenting the potential of widespread resistance.[12,13] 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, such as 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.[14–17]

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.[18–20] However, *D. suzukii* larvae can evade or become resistant to parasitoid wasps due to a high hemocyte load,[21–25] suggesting that natural enemies may be ineffective to control *D. suzukii*. Interspecies competition between *D. suzukii* and *Drosophila melanogaster* has been shown to decrease *D. suzukii* survival in the laboratory setting,[26] but given that *D. melanogaster* oviposits on rotting fruit whereas *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 before release.[27,28]

Multiple conditional female lethality (FL) strategies using a tetracycline-off system have also been developed.[29–31] The FL systems are effective at killing females and predicted to be effective at population suppression[32]; however, FL systems for wild population control depend on releasing fertile transgenic males, posing a challenge for regulatory/social acceptance. In addition, leaky FL toxins in males and the requirement for rearing the parents on tetracycline may have contributed to the

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observation that resulting males had reduced competitiveness.[30] Finally, a *D. suzukii* gene drive system was developed for population modification; however, the risk of gene drive resistance, unknown ecological vulnerabilities, and regulatory/social acceptance remain critical challenges for strategies leveraging self-propagating genetic systems.[33,34]

The sterile insect technique (SIT) has been used for more than half a century to suppress wild pest populations through repeated releases of sterilized males that sexually compete against wild males for mating with wild females.[35,36] To utilize SIT efficiently, an ideal solution would allow for (1) mass rearing of insects, (2) efficient genetic selection against females, (3) ensuring male sterility, (4) generation of fit and sexually competitive released males, and (5) cost-effective logistics and deployment of sterilized males (Fig. 1A).[36,37]

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.[35,37–39] We previously developed a next-generation SIT based approach, termed precision guided SIT (pgSIT), to programmably induce specific mutations that result in FL and male sterility utilizing CRISPR/Cas9 technology[40] (Fig. 1B). We demonstrated this technology in both *D. melanogaster*[40] and *Aedes aegypti*.[41] Here, we engineer a *D. suzukii* 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

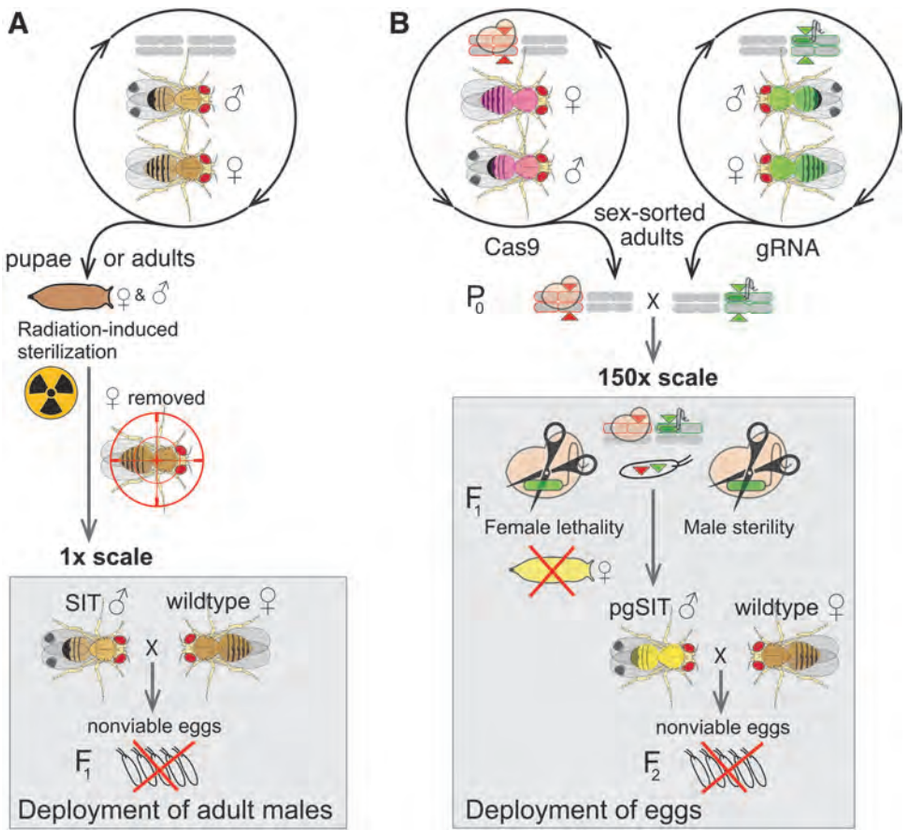

FIG. 1. Comparison of SIT and pgSIT.

(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 1×production scale.

(B) In pgSIT, a genetic cross between parental strains harboring Cas9 and 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 F_1 progeny. Each sex sorted parental female 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 150×. In addition, 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. gRNA, guide RNA; SIT, Sterile Insect Technique; pgSIT, precision-guided Sterile Insect Technique.

a useful and effective tool for control of *D. suzukii* pest populations, which in the future could be adapted to control wild populations of this global pest.

Results

CRISPR-mediated disruption of essential genes

To engineer pgSIT in *D. suzukii*, two types of transgenic strains are required, 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).[40,41] *Sex lethal* (*Sxl*), *doublesex* (*dsx*), and *transformer* (*tra*) are sex determination genes that are essential for female-specific development in *D. melanogaster* and their disruption results in FL or transformation into intersex.[40,42–44]

The disruption of *βTubulin 85D* (*βTub*) arrests spermatid elongation and sperm mobility, resulting in male-specific sterility,[45] together making these genes excellent targets for pgSIT. In *D. suzukii*, 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*).[46] Prior data determined that *nosCas9* induced the least F_1 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 the 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 $gRNA^{\beta Tub}$ (Supplementary Fig. S1). To track transgenes, $gRNA^{dsx}$, $gRNA^{tra}$, and $gRNA^{\beta Tub}$ plasmids harbored *Opie2-mVenus*, a yellow fluorescent protein (YFP), whereas the $gRNA^{Sxl}$ plasmid harbored a red fluorescent protein, *Ubiq-dsRed* (Supplementary Fig. S1). To generate transgenic strains, each gRNA plasmid was injected into embryos of *D. suzukii* harboring the *Hsp70Bb-piggyBac* transposase.[47] One or two homozygous stocks were established for each plasmid, including $gRNA^{Sxl}.S1$, $gRNA^{Sxl}.S2$, $gRNA^{dsx}$, $gRNA^{tra}.S1$, $gRNA^{tra}.S2$, $gRNA^{\beta Tub}.S1$, and $gRNA^{\beta Tub}.S2$.

We assessed the efficiency of each gRNA 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 F_1 trans-heterozygous progeny. Reciprocal crosses were conducted to explore the effect of Cas9 carryover, that is, maternal Cas9 protein deposition into embryos, on gene disruption in the subsequent generation (Fig. 2A, B).[48]

The $gRNA^{Sxl}.S1$ and $gRNA^{Sxl}.S2$ harbored a single gRNA targeting *Sxl* ($gRNA^{Sxl\#1}$; Supplementary Fig. S1) and induced either FL or transformed females into sterile intersexes, in combination with either *vasCas9* or *BicC.Cas9* strains (Fig. 2C; Supplementary Data). 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 FL, whereas 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 and Supplementary Data).

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's *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, $gRNA^{tra\#1}$ (Supplementary 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), whereas a small fraction of trans-heterozygous females were transformed into sterile intersexes with either maternal or paternal *vasCas9* (6.7% ± 3.8% ♂ and 1.5% ± 3.2% ♂, respectively) and *BicC.Cas9* (3.4% ± 5.1% ♂ and 0% ± 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 ± 6.9% ♂ vs. 35.2 ± 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% ± 0.7% ♀ vs. 52.7 ± 3.9% ♀, respectively, $p < 0.0001$; Fig. 2C, Supplementary Data).

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 $gRNA^{\beta Tub}$ (S1 and S2) did not induce complete male sterility in combination with either *vasCas9* or *BicC.Cas9* (Supplementary Data), despite mediating mutations at one target site ($gRNA^{\beta Tub\#1}$; Supplementary Fig. S3C). In sum, these results suggest that (1) *Sxl* is the most efficient gene target to eliminate females, (2) *vasCas9* provides consistent gene disruption whether inherited maternally or paternally as compared with *BicC.Cas9*, and (3) additional gRNAs targeting *βTub* need to be generated and tested to induce robust male-specific sterility phenotypes.

Development of pgSIT strains

After assessing the disruption of several genes individually, we assembled gRNA plasmids for disruption of *Sxl* and *βTub*

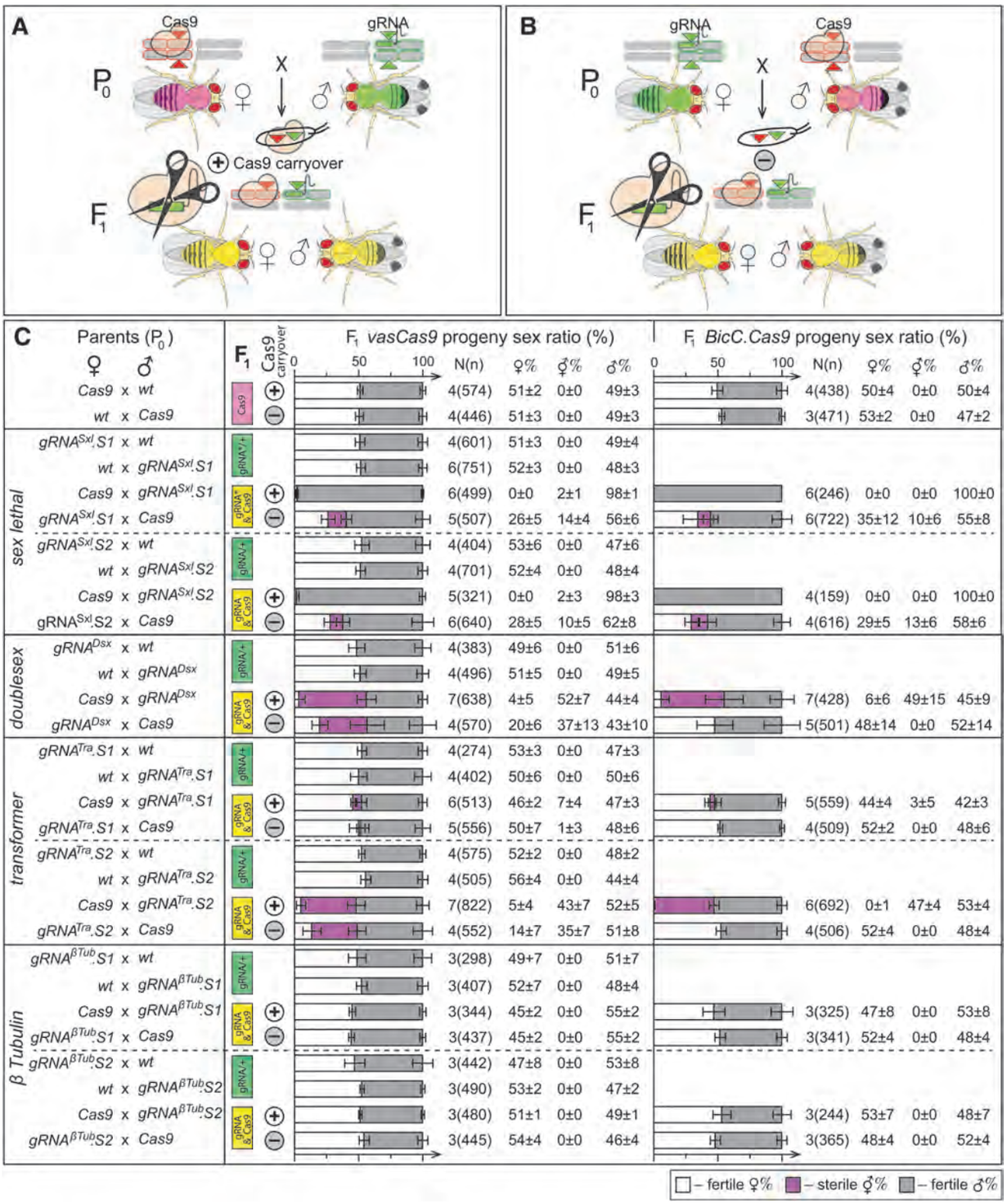

FIG. 2. Assessment of Cas9/gRNA-mediated disruption of individual target genes.

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 F_1 heterozygous (*Cas9/+* or *gRNA/+*) and trans-heterozygous (gRNA and Cas9) progeny. Maternal inheritance of Cas9 **(A)** results in deposition of Cas9 protein into embryos, that is, Cas9 carryover (+); whereas 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 enclosed adults was scored as female (♀), intersex (⚥), or male (♂). Bars indicate the mean frequency and 1 SD (±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. F_1 counts and fertility data can be found in the Supplementary Data. SD, standard deviation.

simultaneously. Three additional gRNAs (*gRNA*$^{\beta Tub\#3}$, *gRNA*$^{\beta Tub\#4}$, *gRNA*$^{\beta Tub\#5}$; Supplementary Fig. S1) each targeting different sites in *βTub*, in an attempt to achieve higher rates of sterility than those with *gRNA*$^{\beta Tub\#1}$ and *gRNA*$^{\beta Tub\#2}$, were designed. Each triple gRNA plasmid combined the two gRNAs targeting *Sxl* (one was tested above, and another was tested in *D. melanogaster*[40] and had 100% complementarity to *D. suzukii sxl* target), with one of three newly designed gRNA targeting *βTub* (either *gRNA*$^{\beta Tub\#3}$, *gRNA*$^{\beta Tub\#4}$, or *gRNA*$^{\beta Tub\#5}$), generating three unique triple gRNA plasmids: *gRNA*$^{Sxl,\beta Tub\#3}$, *gRNA*$^{Sxl,\beta Tub\#4}$, and *gRNA*$^{Sxl,\beta Tub\#5}$ (Supplementary Fig. S1).

The triple *gRNA*$^{Sxl,\beta Tub}$ plasmids also harbored *Opie2-mVenus* to facilitate genotyping in the presence of the *dsRed*-tagged *vasCas9* transgene. A total of six independent transgenic strains were established: zero *gRNA*$^{Sxl,\beta Tub\#3}$, four *gRNA*$^{Sxl,\beta Tub\#4}$(S1–S4), and two *gRNA*$^{Sxl,\beta Tub\#5}$ (S1–S2) strains. Unfortunately, homozygous *gRNA*$^{Sxl,\beta Tub\#4}$.S4 induced fitness costs and was excluded from further analysis. Moreover, we determined that the *gRNA*$^{Sxl,\beta Tub\#4}$ and *gRNA*$^{Sxl,\beta Tub\#5}$ constructs were each integrated onto the X chromosome in two separate strains, *gRNA*$^{Sxl,\beta Tub\#4}$.S3 and *gRNA*$^{Sxl,\beta Tub\#5}$.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*$^{Sxl,\beta Tub}$ results in v*asCas9/+* fertile F_1 males and limits its application of the X-linked *gRNA*$^{Sxl,\beta Tub}$ strains (Supplementary Fig. S5B). Notwithstanding, both X-linked *gRNA*$^{Sxl,\beta Tub\#4}$*.S3* and *gRNA*$^{Sxl,\beta Tub\#5}$*.S2* strains induced complete FL 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 both maternally and paternally.

We next analyzed the three generated autosomal strains, *gRNA*$^{Sxl,\beta Tub\#4}$*.S1*, *gRNA*$^{Sxl,\beta Tub\#4}$*.S2*, and *gRNA*$^{Sxl,\beta Tub\#5}$*.S1,* and determined that (1) all *vasCas9/+*; *gRNA*$^{Sxl,\beta Tub}$*/+* females generated by both reciprocal genetic crosses perished or were transformed into sterile intersexes ($n = 286$), (2) all the trans-heterozygous males that inherited maternal *vasCas9* were sterile ($n = 1274$), and (3) one *gRNA*$^{Sxl,\beta Tub}$ strain (*gRNA*$^{Sxl,\beta Tub\#4}$*.S2*) produced sterile males with paternally inherited *vasCas9* (Fig. 3). Fertility of F_1 trans-heterozygous males produced by *gRNA*$^{Sxl,\beta Tub\#4}$*.S1* and *gRNA*$^{Sxl,\beta Tub\#5}$*.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 FL of *vasCas9/+*; *gRNA*$^{Sxl,\beta Tub\#4}$*.S1/+* (0.5% ± 0.8% maternal *Cas9* ♀ vs. 14.3% ± 4.3% paternal *Cas9* ♀, $p = 0.0017$, two-sided Student's *t*-test with equal variance) and *vasCas9/+*; *gRNA*$^{Sxl,\beta Tub\#5}$*.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*$^{Sxl,\beta Tub\#4}$*.S2/+* intersexes emerged (85 maternal *Cas9* ♀ and 144 paternal *Cas9* ♀; Fig. 3), and maternal *Cas9* did not significantly affect the FL (16.9% ± 4.7% vs. 21.2% ± 6.6%, respectively; $p = 0.2257$; Fig. 3; Supplementary Data). Unsurprisingly, many emerged F_1 intersex flies were unfit and died soon after emerging or in the 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*$^{Sxl,\beta Tub\#4}$*.S1/+*; *vasCas9/+* (952 eggs) or *gRNA*$^{Sxl,\beta Tub\#5}$*.S1/+*; *vasCas9/+* males (321 eggs) hatched (Table 1). Five eggs hatched out of 518 eggs laid by females mated with *gRNA*$^{Sxl,\beta Tub\#4}$*.S2/+; vasCas9/+* males (0.9% egg hatching rate; Table 1), suggesting that male fertility was very strongly suppressed.

Finally, the induced pgSIT phenotypes, that is, FL 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.

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 the longevity of pgSIT males as compared with *wt* males. We opted to evaluate the *gRNA*$^{Sxl,\beta Tub\#4}$*.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 h; 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 with 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). 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*$^{Sxl,\beta Tub\#4}$*.S1* and the *vasCas9* strains in both reciprocal directions. The survival curves showing average survival percentage and 95% confidence intervals (±CI) are depicted in Figure 4D. The median survival of *wt* males was significantly longer than that of the pgSIT males harboring paternal or maternal *vasCas9*: 53 days versus 31 or 41 days ($p < 0.0001$ or

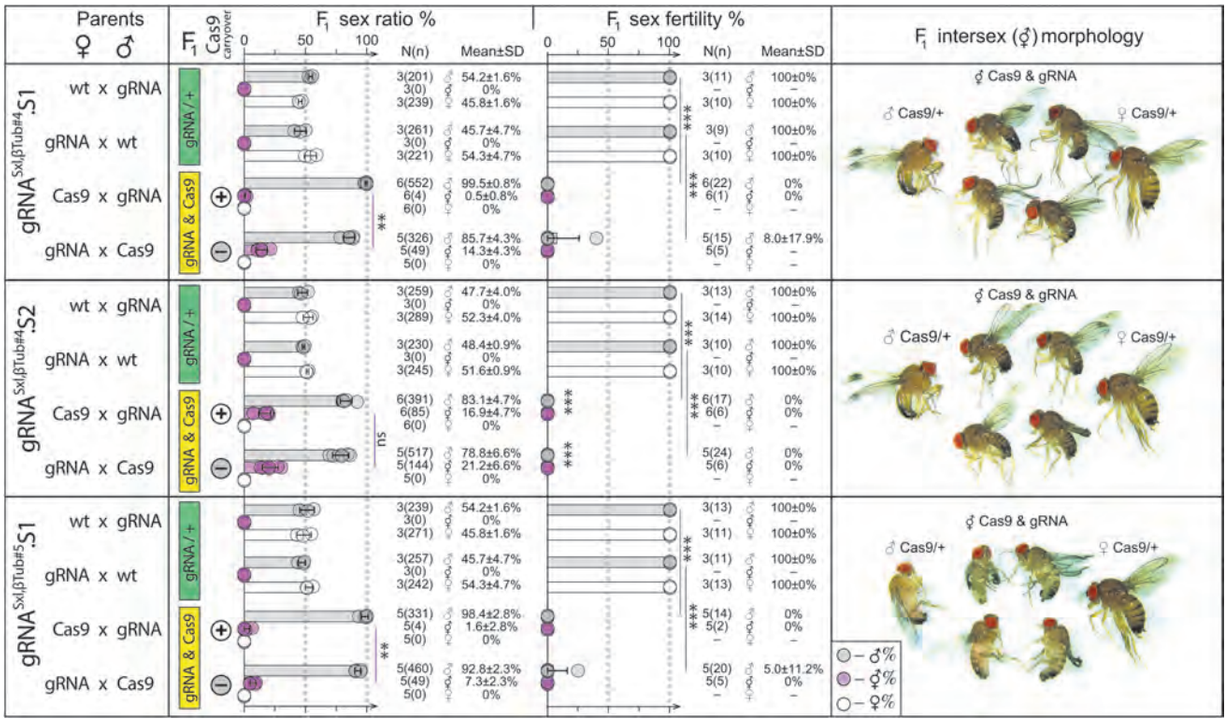

FIG. 3. Genetic characterization of three pgSIT systems.
Three autosomal *gRNA*$^{Sxl,\beta Tub}$ strains expressing two gRNAs targeting *Sxl* and one gRNA targeting *βTub* were assessed by genetically crossing each strain with the *vasCas9* and *wt* strains. The direction of a genetic cross is indicated on the left panel. Maternal inheritance of Cas9 results in the deposition of Cas9 protein into embryos, that is, Cas9 carryover (+); whereas paternal inheritance of Cas9 does not (–). The sex and fertility percentages of enclosed F_1 heterozygous (gRNA/+) and trans-heterozygous (gRNA and Cas9) flies are reported as two separate bar graphs. The sex of enclosed adults was scored as female (♀), intersex (⚥), or male (♂). Bars indicate the mean frequency and 1 SD(±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 with 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 ($^{ns}p \le 0.05$; $^{***}p < 0.001$). Source data are provided as Supplementary Data.

$p < 0.001$, 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.

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 ⅔ *vasCas9/+* flies and ⅓ *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 that of *wt* males.

Total progeny numbers produced by control populations at each subsequent generation after generation 0 were not significantly different, 474 ± 54 flies ($p = 0.4047$, analysis of

Table 1. Fertility of precision guided sterile insect technique (trans-heterozygous) males that inherited paternal *vasCas9*, measured by the hatching rate of sired eggs

gRNA strain	*Groups of 5 ♂ and 20 virgin ♀*	*Total number of laid eggs*	*Total number of hatched eggs*	*Hatching rate (%)*
gRNA$^{Sxl,\beta Tub\#4}$.S1	5	952	0	0
gRNA$^{Sxl,\beta Tub\#4}$.S2	3	518	5	0.9
gRNA$^{Sxl,\beta Tub\#5}$.S1	3	321	0	0

Groups of 5 pgSIT males (♂) were mated with 20 virgin *wt* females (♀), then laid eggs were scored every 12 h and their hatching rates were assessed.
gRNA, guide RNA; pgSIT, precision guided sterile insect technique.

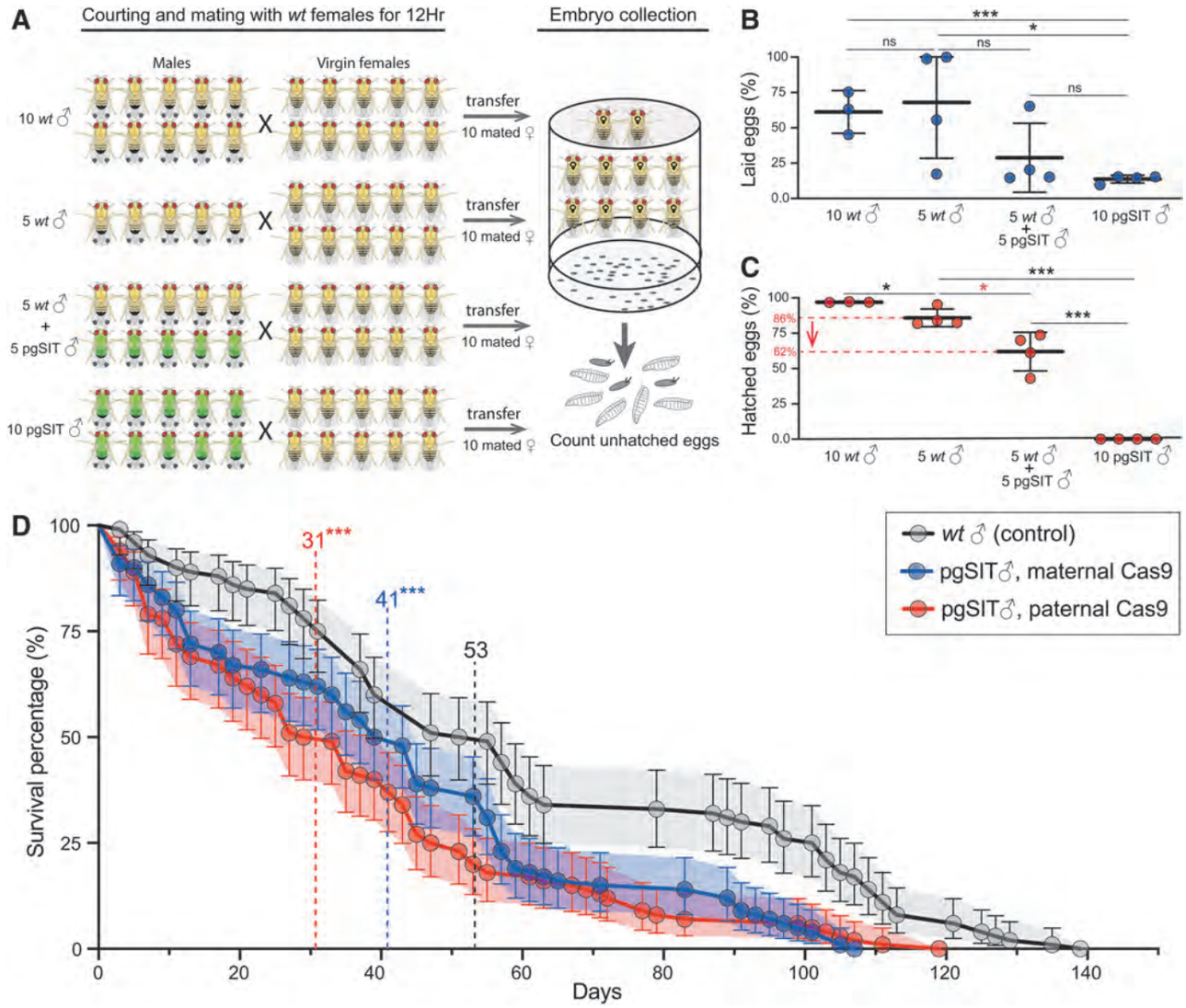

FIG. 4. Characterization of pgSIT males' mating competitiveness and longevity.

(A) Schematic of mating assay. Graphs indicate percentages of laid **(B)** and hatched **(C)** eggs as well as the mean and 1 SD (±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% CIs (±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. ($^{ns}p \leq 0.05$; $^{*}p < 0.05$; $^{***}p < 0.001$). Source data are provided as Supplementary Data. CI, confidence interval.

variance [ANOVA] total progeny number by generation; Fig. 5B), and each *wt* female on average produced 11.84 ± 1.36 progeny flies (Supplementary Data). For experimental populations, which were set up with *wt* and pgSIT (i.e., *vasCas9*/+; $gRNA^{SxI,\beta Tub\#4}$.S1/+) males, we did not score any progeny harboring either dsRed or mVenus, indicating that pgSIT males were, indeed, sterile (Supplementary Data).

The fact that the pgSIT males did not sire any progeny, whereas 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. Release ratios (pgSIT:*wt*) of 3:1 and 5:1 showed a significant decrease in progeny after generation #1 ($p = 0.0004$ or $p = 0.0003$, respectively; a two-sided *t* test with equal

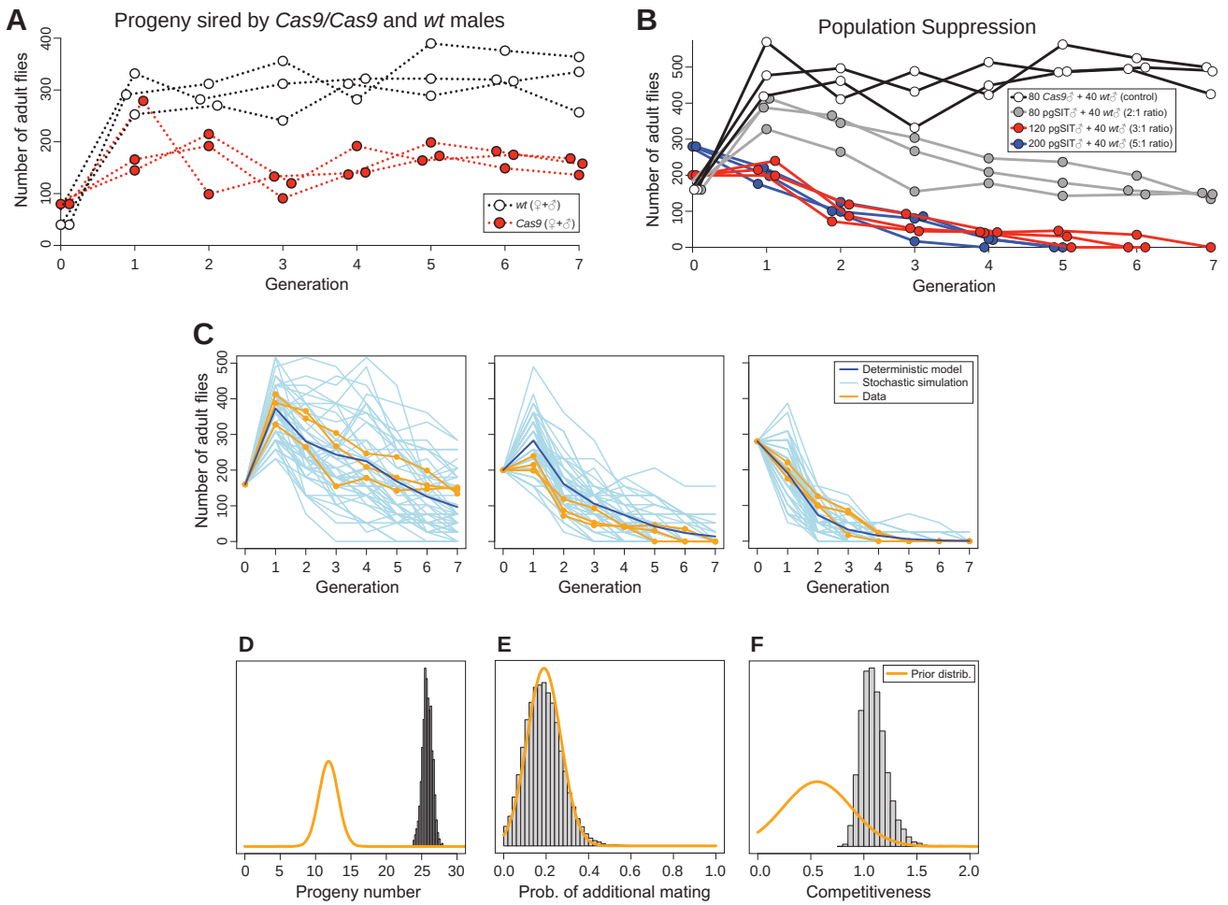

FIG. 5. Repeated releases of pgSIT males suppress *Drosophila suzukii* populations.

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 with 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 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 Figure 5C and inferred a posterior distribution (histogram) for the relative mating competitiveness of 108% (95% CrI: 90–138). MCMC, Markov chain Monte Carlo.

variance) and a release ratio of 2:1 showed a 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). Taken together, these data indicate that releases of pgSIT males can be an effective measure for population suppression and elimination.

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 Figure 5C–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 ± 1 standard deviation [SD]) (Supplementary Data) 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); and lastly, for female remating probability, we assumed a normal prior distribution with mean 0.18 (informed by Lanouette et al[49]) and used an arbitrary SD of 0.08, which represents a 95% CI 0.03–0.35.

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 remating is 18% (95% CrI: 4–37).

These results suggest that: for the 2:1 male pgSIT:*wt* ratio, 36% (95% CrI: 27–47) of females produce progeny, that is, they mate at least once with *wt* males; 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.

Discussion

Here, we describe the development of pgSIT in *D. suzukii* and demonstrate effectiveness for the suppression of *D. suzukii* 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. suzukii* conditional female-lethality strain[29] 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)[50–52] (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 tested in the field.

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. In some strains, rates of FL, 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 sequence (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*,[53] *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 sperms 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*), *Heterochromatin Protein 1E* (*HP1E*), *misfire* (*mfr*), or *K81,* 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.[54,55]

The pgSIT approach is expected to generate competitive pgSIT males ready for releases (Fig. 1B), because only two genes are specifically targeted in F_1 pgSIT progeny. However, according to the decrease in egg hatching rate, the generated *D. suzukii* pgSIT males were only $55.6\% \pm 31.8\%$ as competitive as *wt* males (Fig. 4D). This value was likely an underestimate, 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 versus 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. suzukii 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. suzukii* females having ample opportunities to remate with *wt* males[49,56,57] during a 2-week period until parent flies were discarded. Further, the Bayesian Markov chain Monte Carlo (MCMC) analysis fitted to the population suppression data inferred the posterior distribution of the pgSIT male competitiveness to ~100% (Fig. 5F). Taken together, these data suggest that the pgSIT males, indeed, did not suffer large fitness costs and were able to compete repeatedly against *wt* males for mating with *wt* females and cause 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,[57–59] 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 150× improvement to scalability as compared with 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.

In the described binary two-locus pgSIT system for control of *D. suzukii*, 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.[40,41] Although 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. suzukii* going forward, a one-locus pgSIT genetic cassette could be engineered. For example, the innovative temperature inducible pgSIT (TI-pgSIT) system[60] could be generated to induce FL and male sterility in the subsequent generation while only requiring maintenance of one strain without the need for setting up genetic crosses. To achieve an effective TI-pgSIT system in *D. suzukii*, 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. suzukii* eggs and/or embryos, similar to cryopreservation of *D. melanogaster* embryos[61] would expand global logistics and save costs for deployment of pgSIT solutions for insect control.[41]

The Bigger Picture

Managing crop pests and invasive species poses a significant worldwide burden to food production. Farmers around the globe must continually adapt and apply new measures and techniques to protect their precious output. Climate change and the increased movement of species to new locations due to commerce have also contributed to escalated burdens on farmers to produce sufficient crops to feed the growing world population. Non-species specific pesticides have traditionally been used by humankind for millennia. However, many of these are indeed harmful to the environment and insects have evolved resistance reducing their overall efficacy. Therefore, new safer technologies that are species specific are urgently needed. To fill this void, here we engineer the precision guided sterile insect technique (pgSIT) in a major crop pest, *Drosophila suzukii*, and demonstrate that this technology can provide a safe, and potentially scalable solution for effective population control. Going forward, pgSIT could be adapted to many species, and if proven effective in the wild this could reduce our reliance on harmful pesticides thereby improving environmental health while increasing crop production.

Materials and Methods

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 *βTubulin 85D* (*βTub*, DS10_00012485). CHOPCHOP v2[62] was used for choosing gRNA target sites from selected sequences in the *D. suzukii* genome (http://spottedwingflybase.org)[63] to minimize off-target activity. Sequences of gRNA target sites are listed in Supplementary Figure S1.

Construct design and assembly

Genetic constructs were built into *piggyBac* transformation plasmids using the Gibson enzymatic assembly method.[64] 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 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 *Ubiq-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 of *mVenus*'s CDS, a YFP derived from *Aequorea victoria*[65]; 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*,[66] and $gRNA^{Sxl\#2}$ was tested in *D. melanogaster*,[40] 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 Figure S1. The plasmids DNA and complete sequences can be downloaded from Addgene ID # (183132, 183133, 183134, 183136, 183137, 183138, 185614).

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 12/12 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, OR, USA.[33] Embryo injections were carried out at Rainbow Transgenic Flies, Inc. Plasmids diluted in water to 250 μg/l μL were injected into freshly collected embryos of the *D. suzukii* harboring *Hsp70Bb-piggyBac* transposase.[47]

G_0 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 seven generations before the strain was expanded and the fluorescence of each fly was confirmed. If any flies without a fluorescent marker were observed, the introgression was repeated.

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 F_1 trans-heterozygous progeny. All genetic crosses were performed in fly vials using groups of 7–10 flies of each sex and repeated at least three times using different parent flies. The generated F_1 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.

Genotyping loci targeted with gRNAs

Separate gRNAs targeting nearly identical sequences can direct different cleavage efficiencies by the same Cas9 transgene.[40,41,67] 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. 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, and S7).

Assessment of pgSIT male sterility

Unlike *D. melanogaster*, single-pair crosses of *D. suzukii* are inefficient and frequently result in no progeny and dead P_0. Therefore, we tested the sterility of F_1 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. The presence of F_2 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 F_2 larval activity was observed, F_2 progeny were reared and examined for fluorescence at the larval and adult stages to verify the genotype of the F_1 males.

We, therefore, referred to a group of pgSIT males as fertile only after both $gRNA^{Sxl,\beta Tub}$ and *vasCas9* transgenes (i.e., as *mVenus* and *dsRed*) were identified among the F_2 progeny. Consequently, a single fertile male in a cross can result in the overestimation of male fertility.

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 $gRNA^{Sxl,\beta Tub\#4}$.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 before 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 10 females.[40,68]

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.[56] 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 h in the dark. After 12 h 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, that is, 595 eggs.

Mated *Drosophila* females are non-receptive to re-mating for 12–24 h,[69–71] 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).

Longevity assays

To compare differences in lifespan between pgSIT and *wt* males, we performed longevity assays comparing *wt* males with pgSIT males with either maternally or paternally inherited Cas9. Ten 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.

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[33,72] and population suppression by a repressible female-specific lethal.[29] The trans-heterozygous *vasCas9/+*; *gRNA$^{Sxl,\beta Tub\#4}$.S1/+* males, that is, 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 text bottles normalized than 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.

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: (1) mate at least once per generation; (2) might re-mate up to three times; and (3) only produce progeny in the case of at least one mating with a *wt* male. At each generation i ($i = 1, \ldots, 7$, 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,

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

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 four times and define $p(r, c)$ as follows,

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

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,

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

We used Bayesian MCMC methods to estimate parameters α, c, and p_{rm} and their respective 95% CrIs 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 ($i = 0, \ldots, 7$) 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 ($i = 1, \ldots, 7$), 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.

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 versus *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.

Authors' Contributions

O.S.A., N.P.K., and A.B conceived and designed the experiments. J.L, N.P.K., N.W.-P., I.C.S., and T.Y., performed molecular and genetic experiments. M.J.S. and A.K.Y. provided and carried out an initial assessment of the *gRNA^{Sxl}.S1*, *gRNA^{Sxl}.S2* lines described in this study. J.M.M and R.M.C. performed mathematical modeling. All authors contributed to the writing, analyzed the data, and approved the final article.

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 on request.

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).

Disclaimer

The views, 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.

Author Disclosure Statement

The authors declare the following competing interests. N.P.K. and O.S.A. have submitted a patent application on this technology. O.S.A. is a co-founder of Agragene, Inc., and Synvect, Inc., with an equity interest. N.P.K. is a co-founder of Synvect with an 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 remaining authors declare no competing interests.

Funding Information

This work was supported in part by funding California Cherry Board and Washington Tree Fruit Board (#19-CCB5400-06), Agragene (#200779) awarded to O.S.A., National Institute of Food and Agriculture, U.S. Department of Agriculture Specialty Crops Research Initiative under agreement No. 2015-51181-24252 awarded to M.J.S., and by funds from the Innovative Genomics Institute at UC Berkeley awarded to J.M.M.

Supplementary Material

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References

1. Calabria G, Máca J, Bächli G, et al. First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. J Appl Entomol 2012;136:139–147. doi: 10.1111/j.1439-0418.2010.01583.x.
2. Cini A, Ioriatti C, Anfora G. A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. Bull Insectol 2012;65:149–160.
3. Hauser M. A historic account of the invasion of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in the continental United States, with remarks on their identification. Pest Manag Sci 2011;67:1352–1357. doi: 10.1002/ps.2265.
4. Bolda MP, Goodhue RE, Zalom FG. Spotted wing drosophila: Potential economic impact of a newly established pest. Agric Resour Econ Update 2010;13:5–8.
5. De Ros G, Anfora G, Grassi A, et al. The potential economic impact of *Drosophila suzukii* on small fruits production in Trentino (Italy). IOBC-WPRS Bull 2013;91:317–321.
6. Goodhue RE, Bolda M, Farnsworth D, et al. Spotted wing drosophila infestation of California strawberries and raspberries: Economic analysis of potential revenue losses and control costs. Pest Manag Sci 2011;67:1396–1402. doi: 10.1002/ps.2259.
7. Walsh DB, Bolda MP, Goodhue RE, et al. *Drosophila suzukii* (Diptera: Drosophilidae): Invasive pest of ripening soft fruit expanding its geographic range and damage potential. J Integr Pest Manag 2011;2:G1–G7. DOI: 10.1603/IPM10010.
8. Parchami-Araghi M, Gilasian E, Keyhanian AA. Spotted wing Drosophila, *Drosophila suzukii* (Matsumura) (Dip.: Drosophilidae), an invasive fruit pest new to the Middle East and Iran. Drosoph Inf Serv 2015;98:59–60.
9. Kienzle R, Groß LB, Caughman S, et al. Resource use by individual *Drosophila suzukii* reveals a flexible preference for oviposition into healthy fruits. Sci Rep 2020;10:3132. doi: 10.1038/s41598-020-59595-y.
10. Rombaut A, Guilhot R, Xuéreb A, et al. Invasive *Drosophila suzukii* facilitates *Drosophila melanogaster* infestation and sour rot outbreaks in the vineyards. R Soc Open Sci 2017;4:170117. doi: 10.1098/rsos.170117.
11. Poyet M, Le Roux V, Gibert P, et al. The wide potential trophic niche of the Asiatic fruit fly *Drosophila suzukii*: The key of its invasion success in temperate Europe? PLoS One 2015;10:e0142785. doi: 10.1371/journal.pone.0142785.
12. Gress BE, Zalom FG. Identification and risk assessment of spinosad resistance in a California population of *Drosophila suzukii*. Pest Manag Sci 2019;75:1270–1276. doi: 10.1002/ps.5240.
13. Shaw B, Hemer S, Cannon MFL, et al. Insecticide control of *Drosophila suzukii* in commercial sweet cherry crops under cladding. Insects 2019;10:196; doi: 10.3390/insects10070196.
14. Cock MJW, van Lenteren JC, Brodeur J, et al. Do new access and benefit sharing procedures under the convention on biological diversity threaten the future of biological control? Biocontrol 2010;55:199–218. doi: 10.1007/s10526-009-9234-9.
15. Schöneberg T, Lewis MT, Burrack HJ, et al. Cultural control of *Drosophila suzukii* in small fruit—Current and pending tactics in the U.S. insects 2021;12:172. doi: 10.3390/insects12020172.
16. Rota-Stabelli O, Blaxter M, Anfora G. *Drosophila suzukii*. Curr Biol 2013;23:R8–R9. doi: 10.1016/j.cub.2012.11.021.
17. Tait G, Kaiser C, Rossi Stacconi MV, et al. A food-grade gum as a management tool for *Drosophila suzukii* 2018;71:295–307. ISSN 1721-8861.
18. Kruitwagen A, Beukeboom LW, Wertheim B. Optimization of native biocontrol agents, with parasitoids of the invasive pest *Drosophila suzukii* as an example. Evol Appl 2018;11:1473–1497. doi: 10.1111/eva.12648.
19. Lee JC, Wang X, Daane KM, et al. Biological control of spotted-wing Drosophila (Diptera: Drosophilidae)—Current and pending tactics. J Integr Pest Manag 2019;10:13; doi: 10.1093/jipm/pmz012.
20. Mastore M, Quadroni S, Brivio MF. Susceptibility of *Drosophila suzukii* larvae to the combined administration of the entomopathogens *Bacillus thuringiensis* and *Steinernema carpocapsae*. Sci Rep 2021;11:8149. doi: 10.1038/s41598-021-87469-4.
21. Poyet M, Havard S, Prevost G, et al. Resistance of *Drosophila suzukii* to the larval parasitoids *Leptopilina heterotoma* and *Asobara japonica* is related to haemocyte load. Physiol Entomol 2013;38:45–53. doi: 10.1111/phen.12002.
22. Chabert S, Allemand R, Poyet M, et al. Ability of European parasitoids (Hymenoptera) to control a new invasive Asiatic pest, *Drosophila suzukii*. Biol Control 2012;63:40–47. doi: 10.1016/j.biocontrol.2012.05.005.
23. Daane KM, Wang X-G, Biondi A, et al. First exploration of parasitoids of *Drosophila suzukii* in South Korea as potential classical biological agents. J Pest Sci 2016;89:823–835. doi: 10.1007/s10340-016-0740-0.
24. Miller B, Anfora G, Buffington M, et al. Seasonal occurrence of resident parasitoids associated with *Drosophila suzukii* in two small fruit production regions of Italy and the USA. Bull Insectol 2015;68:255–263.
25. Kacsoh BZ, Schlenke TA. High hemocyte load is associated with increased resistance against parasitoids in *Drosophila suzukii*, a relative of *D. melanogaster*. PLoS One 2012;7:e34721. doi: 10.1371/journal.pone.0034721.

26. Shaw B, Brain P, Wijnen H, et al. Reducing *Drosophila suzukii* emergence through inter-species competition. Pest Manag Sci 2018;74:1466–1471. doi: 10.1002/ps.4836.
27. Cattel J, Nikolouli K, Andrieux T, et al. Back and forth Wolbachia transfers reveal efficient strains to control spotted wing drosophila populations. J Appl Ecol 2018;55:2408–2418. doi: 10.1111/1365-2664.13101.
28. Bourtzis K, Dobson SL, Xi Z, et al. Harnessing mosquito—Wolbachia symbiosis for vector and disease control. Acta Trop 2014;132:S150–S163. doi: 10.1016/j.actatropica.2013.11.004.
29. Li F, Yamamoto A, Belikoff EJ, et al. A conditional female lethal system for genetic suppression of the global fruit crop pest *Drosophila suzukii*. Pest Manag Sci 2021;77:4915–4922. doi: 10.1002/ps.6530.
30. Yamamoto A, Yadav AK, Scott MJ. Evaluation of additional *Drosophila suzukii* male-only strains generated through remobilization of an FL19 transgene. Front Bioeng Biotechnol 2022;10:829620; doi: 10.3389/fbioe.2022.829620.
31. Schetelig MF, Schwirz J, Yan Y. A transgenic female killing system for the genetic control of *Drosophila suzukii*. Sci Rep 2021;11:12938. doi: 10.1038/s41598-021-91938-1.
32. Vella MR, Gould F, Lloyd AL. Mathematical modeling of genetic pest management through female-specific lethality: Is one locus better than two? Evol Appl 2021;14:1612–1622. doi: 10.1111/eva.13228.
33. Buchman A, Marshall JM, Ostrovski D, et al. Synthetically engineered gene drive system in the worldwide crop pest. Proc Natl Acad Sci U S A 2018;115:4725–4730. doi: 10.1073/pnas.1713139115.
34. Lalyer CR, Sigsgaard L, Giese B. Ecological vulnerability analysis for suppression of *Drosophila suzukii* by gene drives. Glob Ecol Conserv 2021;32:e01883. doi: 10.1016/j.gecco.2021.e01883.
35. Knipling EF. Possibilities of insect control or eradication through the use of sexually sterile males. J Econ Entomol 1955;48:459–462. doi: 10.1093/jee/48.4.459.
36. Nikolouli K, Colinet H, Renault D, et al. Sterile insect technique and Wolbachia symbiosis as potential tools for the control of the invasive species *Drosophila suzukii*. J Pest Sci 2018;91:489–503. doi: 10.1007/s10340-017-0944-y.
37. Dyck VA, Hendrichs J, Robinson AS. Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management. Second Edition. CRC Press, Taylor & Francis Group: New York: 2021.
38. Lanouette G, Brodeur J, Fournier F, et al. The sterile insect technique for the management of the spotted wing drosophila, *Drosophila suzukii*: Establishing the optimum irradiation dose. PLoS One 2017;12:e0180821. doi: 10.1371/journal.pone.0180821.
39. Homem R, Mateos-Fierro Z, Jones R, et al. Field suppression of Spotted Wing Drosophila (SWD) (*Drosophila suzukii* Matsumura) using the Sterile Insect Technique (SIT). Insects 2022;13:328. doi: 10.3390/insects13040328.
40. Kandul NP, Liu J, Sanchez C HM, et al. Transforming insect population control with precision guided sterile males with demonstration in flies. Nat Commun 2019;10:84. doi: 10.1038/s41467-018-07964-7.
41. Li M, Yang T, Bui M, et al. Suppressing mosquito populations with precision guided sterile males. Nat Commun 2021;12:5374. doi: 10.1038/s41467-021-25421-w.
42. Slee R, Bownes M. Sex determination in *Drosophila melanogaster*. Quart Rev Biol 1990;65:175–204. doi: 10.1086/416718.
43. Bell LR, Horabin JI, Schedl P, et al. Positive autoregulation of Sex-lethal by alternative splicing maintains the female determined state in Drosophila. Cell 1991;65:229–239. doi: 10.1016/0092-8674(91)90157-t.
44. Boggs RT, Gregor P, Idriss S, et al. Regulation of sexual differentiation in *D. melanogaster* via alternative splicing of RNA from the transformer gene. Cell 1987;50:739–747. doi: 10.1016/0092-8674(87)90332-1.
45. Kemphues KJ, Raff EC, Raff RA, et al. Mutation in a testis-specific β-tubulin in Drosophila: Analysis of its effects on meiosis and map location of the gene. Cell 1980;21:445–451. doi: 10.1016/0092-8674(80)90481-x.
46. Kandul NP, Belikoff EJ, Liu J, et al. Genetically encoded CRISPR components yield efficient gene editing in the invasive pest. CRISPR J 2021;4:739–751. doi: 10.1089/crispr.2021.0032.
47. Chu F, Klobasa W, Grubbs N, et al. Development and use of a piggyBac-based jumpstarter system in *Drosophila suzukii*. Arch Insect Biochem Physiol 2018;97:e21439:1–14; doi: 10.1002/arch.21439.
48. Kandul NP, Liu J, Buchman A, et al. Assessment of a split homing based gene drive for efficient knockout of multiple genes. G3 (Bethesda) 2020;10:827–837; doi: 10.1534/g3.119.400985.
49. Lanouette G, Brodeur J, Fournier F, et al. Effect of irradiation on the mating capacity and competitiveness of *Drosophila suzukii* (Diptera: Drosophilidae) for the development of the sterile insect technique. Can Entomol 2020;152:563–574. doi: 10.4039/tce.2020.38.
50. Oliva CF, Benedict MQ, Collins CM, et al. Sterile Insect Technique (SIT) against species mosquitoes: A roadmap and good practice framework for designing, implementing and evaluating pilot field trials. Insects 2021;12:121; doi: 10.3390/insects12030191.
51. Mastrangelo T, Kovaleski A, Botteon V, et al. Optimization of the sterilizing doses and overflooding ratios for the South American fruit fly. PLoS One 2018;13:e0201026. doi: 10.1371/journal.pone.0201026.
52. Shelly T, McInnis D. Sterile insect technique and control of tephritid fruit flies: Do species with complex courtship require higher overflooding ratios? Ann Entomol Soc Am 2016;109:1–11. doi: 10.1093/aesa/sav101.
53. Yu Z, Ren M, Wang Z, et al. Highly efficient genome modifications mediated by CRISPR/Cas9 in Drosophila. Genetics 2013;195:289–291. doi: 10.1534/genetics.113.153825.
54. Ni X-Y, Lu W-J, Qiao X, et al. Genome editing efficiency of four *Drosophila suzukii* endogenous U6 promoters. Insect Mol Biol 2021;30:420–426. doi: 10.1111/imb.12707.
55. Ahmed HMM, Hildebrand L, Wimmer EA. Improvement and use of CRISPR/Cas9 to engineer a sperm-marking strain for the invasive fruit pest *Drosophila suzukii*. BMC Biotechnol 2019;19:85. doi: 10.1186/s12896-019-0588-5.
56. Upadhyay A, Feltman NR, Sychla A, et al. Genetically engineered insects with sex-selection and genetic incompatibility enable population suppression. ELife 2022;11:e71230; doi: 10.7554/eLife.71230.
57. Clark RD, DiPiero M, Fingerut JT, et al. An Analysis of Female Postcopulatory Behavior in *Drosophila suzukii* and *Drosophila biarmipes*. J Insect Behav 2020;33:193–200. doi: 10.1007/s10905-020-09761-x.
58. Grant BL. Behavioral responses of *Drosophila suzukii* (Diptera: Drosophilidae) to olfactory and visual cues. PhD Thesis; University of Missouri, Columbia, MO. 2019; doi: 10.32469/10355/72218.
59. Wiman NG, Dalton DT, Anfora G, et al. *Drosophila suzukii* population response to environment and management strategies. J Pest Sci 2016;89:653–665. doi: 10.1007/s10340-016-0757-4.
60. Kandul NP, Liu J, Akbari OS. Temperature-inducible precision-guided sterile insect technique. CRISPR J 2021;4:822–835. doi: 10.1089/crispr.2021.0077.
61. Zhan L, Li M-G, Hays T, et al. Cryopreservation method for *Drosophila melanogaster* embryos. Nat Commun 2021;12:2412. doi: 10.1038/s41467-021-22694-z.
62. Labun K, Montague TG, Gagnon JA, et al. CHOPCHOP v2: A web tool for the next generation of CRISPR genome engineering. Nucleic Acids Res 2016;44:W272–W276. doi: 10.1093/nar/gkw398.
63. Chiu JC, Jiang X, Zhao L, et al. Genome of *Drosophila suzukii*, the spotted wing drosophila. G3 2013;3:2257–2271. doi: 10.1534/g3.113.008185.
64. Gibson DG, Young L, Chuang R-Y, et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 2009;6:343–345. doi: 10.1038/nmeth.1318.
65. Kremers G-J, Goedhart J, van Munster EB, et al. Cyan and yellow super fluorescent proteins with improved brightness, protein folding, and FRET Förster radius. Biochemistry 2006;45:6570–6580. doi: 10.1021/bi0516273.
66. Li F, Scott MJ. CRISPR/Cas9-mediated mutagenesis of the white and Sex lethal loci in the invasive pest, *Drosophila suzukii*. Biochem Biophys Res Commun 2016;469:911–916. doi: 10.1016/j.bbrc.2015.12.081.
67. Kandul NP, Liu J, Bennett JB, et al. A confinable home-and-rescue gene drive for population modification. Elife 2021;10:e65939; doi: 10.7554/eLife.65939.
68. Kandul NP, Liu J, Akbari OS. Temperature-inducible precision guided sterile insect technique. CRISPR J 2021;4:822–835; doi: 10.1089/crispr.2021.0077.
69. Chapman T, Bangham J, Vinti G, et al. The sex peptide of Drosophila melanogaster: Female post-mating responses analyzed by using RNA interference. Proc Natl Acad Sci U S A 2003;100:9923–9928. doi: 10.1073/pnas.1631635100.
70. Liu H, Kubli E. Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. Proc Natl Acad Sci U S A 2003;100:9929–9933. doi: 10.1073/pnas.1631700100.
71. Xue L, Noll M. Drosophila female sexual behavior induced by sterile males showing copulation complementation. Proc Natl Acad Sci U S A 2000;97:3272–3275. doi: 10.1073/pnas.97.7.3272.
72. Webster SH, Vella MR, Scott MJ. Development and testing of a novel killer–rescue self-limiting gene drive system in *Drosophila melanogaster*. Proc R Soc B Biol Sci 2020;287:20192994. doi: 10.1098/rspb.2019.2994.

Received: May 12, 2022
Accepted: June 29, 2022
Issue Publication Date: August 18, 2022