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RESEARCH ARTICLE

Temperature-Inducible Precision-Guided Sterile Insect Technique

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Abstract

Releases of sterile males are the gold standard for many insect population control programs, and precise sex sorting to remove females prior to male releases is essential to the success of these operations. To advance traditional methods for scaling the generation of sterile males, we previously described a CRISPR-mediated precision-guided sterile insect technique (pgSIT), in which Cas9 and gRNA strains are genetically crossed to generate sterile males for mass release. While effective at generating F_1 sterile males, pgSIT requires a genetic cross between the two parental strains, which requires maintenance and sexing of two strains in a factory. Therefore, to advance pgSIT further by removing this crossing step, here we describe a next-generation temperature-inducible pgSIT (TI-pgSIT) technology and demonstrate its proof-of-concept in *Drosophila melanogaster*. Importantly, we were able to develop a true breeding strain for TI-pgSIT that eliminates the requirement for sex sorting—a feature that may help further automate production at scale.

Introduction

Many insect population control approaches require the generation and release of large numbers of sterile males into natural populations. This control strategy was first proposed in 1955, when Edward Knipling proposed releasing sterile males to suppress insect populations—coined the ''sterile insect technique'' (SIT).[1] SIT has since been successfully implemented to suppress wild populations of a variety of insects,[2,3] such as in the eradication of the new world screw-worm fly, *Cochliomyia hominivorax*, in the United States and Mexico.[4] Notwithstanding, Knipling's vision of sexing sterilized insects to remove females prior to release has been challenging to accomplish, even in the screw-worm example, which has limited its implementation to other insects.

Finding better ways to separate insects by sex is necessary, as field trials and models illustrate that releasing only sterile males significantly improves the efficiency of population suppression and can significantly reduce production costs.[1,5] Furthermore, since females are often the sex that transmit pathogens (e.g., mosquitoes), a reliable sexing method to guarantee female elimination prior to release is highly desirable for the implementation of these programs. Other related methods of insect population control, such as the release of insects carrying a dominant lethal[6] and the *Wolbachia*-mediated incompatible insect technique (IIT),[7–9] also require precise sexing methods to avoid female releases. Notably, IIT programs are based on repeated releases of *Wolbachia*-infected males, which are incompatible with wild females that lack the specific *Wolbachia* strain. Even the accidental release of a small fraction of *Wolbachia*-infected fertile females could lead to the wide-scale spread of *Wolbachia,* which would immunize populations against the particular IIT program, underscoring the importance of effective sex separation. However, with a few species-specific exceptions,[10,11] insect sex sorting can be time-consuming, labor intensive, error prone, and species specific.[12–14]

We recently developed an alternative platform for the generation and sex separation of sterile males using the CRISPR-mediated precision-guided SIT (pgSIT) technology.[15,16] This technology mechanistically relies on lethal/sterile mosaicism,[15,16] mediated by the precision and accuracy of CRISPR, to disrupt simultaneously specific genes essential for female viability and male fertility

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during development, ensuring the exclusive production of sterile males. To generate pgSIT sterile males in this system, two homozygous strains are raised that harbor either Cas9 or guide RNAs (gRNAs), which are genetically crossed to produce F_1 sterile male progeny that can be deployed at any life stage for population suppression. To advance this system further and to mitigate the need for the genetic cross, we herein describe a next-generation temperature-inducible pgSIT (TI-pgSIT) technology and demonstrate its proof-of-concept in *Drosophila melanogaster*.

Methods

Assembly of genetic constructs

All genetic constructs generated in this study were engineered using Gibson enzymatic assembly.[17] To assemble *Hsp70Bb-Cas9*^{dsRed} (Supplementary Fig. S1A), the *BicC-Cas9* plasmid[18] was digested with *Not*I and *Pme*I to remove the *BicC* promoter. The 476-base-long fragment encompassing the *Hsp70Bb* promoter and cloning overhangs were polymerase chain reaction (PCR) amplified from the pCaSpeR-hs plasmid (GenBank #U59056.1) using primers 1137.C1F and 1137.C3R and cloned inside the linearized plasmid (Supplementary Table S1). Then, the *Hsp70Bb-Cas9-T2A-eGFP-p10* fragment was subcloned from *Hsp70Bb-Cas9*^{dsRed} into the mini-*white* plasmid with the attB site. The *dgRNA*$^{TraB,\beta Tub}$ plasmid was assembled following the strategy used to build *dgRNA*$^{Sxl,\beta Tub}$ in a previous work[15] (Supplementary Fig. S1B). Briefly, the *U6.3-gRNA*^{TraB} fragment was PCR amplified from the *sgRNA*$^{Tra\text{-}B}$ plasmid using primers 2XgRNA-5F and 2XgRNA-6R and was cloned into the *sgRNA*$^{\beta Tub}$ plasmid (Addgene #112691). To build the *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ and *TI-pgSIT*$^{TraB,\beta Tub,Hsp\text{-}Cas9}$ constructs (Supplementary Fig. S1C), the U6.3 3′-UTR fragment was amplified using primers 1098A.C1F and 1098A.C2R from the pVG185_w2-y1 plasmid (GenBank #MN551090.1),[18] and the *Hsp70Bb-Cas9-T2A-eGFP-10* fragment was amplified using primers 1098A.C3F and 1098A.C6R from the *Hsp70Bb-Cas9* plasmid. Both were cloned into the *dgRNA*$^{Sxl,\beta Tub}$ (Addgene #112692) or *dgRNA*$^{TraB,\beta Tub}$ plasmid, respectively, after linearization at *Xba*I. The gRNA and primer sequences used to assemble the genetic constructs in the study are presented in Supplementary Table S1.

Fly transgenesis

Embryo injections were carried out at Rainbow Transgenic Flies, Inc. (www.rainbowgene.com). We used φC31-mediated integration[19] to insert the *Hsp70Bb-Cas9*^{dsRed} construct at the PBac{y+-attP-3B}KV00033 site on the third chromosome (BDSC #9750) and to insert the *Hsp70Bb-Cas9*^{dsRed} construct at the P{CaryP}attP2 site on the third chromosome (BDSC # 8622). The *dgRNA*$^{TraB,\beta Tub}$ construct was inserted at the P{CaryP}attP1 site on the second chromosome (BDSC # 8621), and the *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ and *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ constructs were inserted at the P{CaryP}attP2 site on the third chromosome (BDSC # 8622). We maintained the embryos injected with the *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ and *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ constructs and any of their progeny starting from the G_1 generation at 18°C. Recovered transgenic lines were balanced on the second and third chromosomes using single-chromosome balancer lines (w^{1118}; *CyO/sna*^{Sco} for II and w^{1118}; *TM3*, *Sb*^{1}*/TM6B*, *Tb*^{1} for III).

Fly maintenance and genetics

Flies were examined, scored, and imaged on a Leica M165FC fluorescent stereo microscope equipped with a Leica DMC2900 camera. We tracked the inheritance of *Hsp70Bb-Cas9*^{dsRed} using the *Opie2-dsRed* genetic marker. The other transgenes were tracked using the mini-*white* marker. All genetic crosses were performed in the *w*– genetic background. Flies harboring both *Hsp70Bb-Cas9* and *dgRNAs* in the same genetic background were maintained at 18°C on a 12 h/12 h light/dark cycle, while the flies harboring either *Hsp70Bb-Cas9* or *dgRNAs* were raised under standard conditions at 26°C. All genetic crosses were performed in fly vials using groups of 7–10 flies of each sex and repeated at least three times with different parent flies. Sample sizes and numbers of biological replicates can be found in Supplementary Data S1–S4.

We first assessed the heat shock–induced activation of *Hsp70Bb-Cas9* by visualizing green fluorescent protein (GFP) fluorescence. The GFP coding sequence was attached to the C-terminal end of the *Streptococcus pyogenes*–derived *Cas9* (*SpCas9*) coding sequence via a self-cleaving T2A peptide and served as a visual indicator of Cas9 expression. The embryos that were laid overnight as well as the larvae, pupae, and adult flies of both *Hsp70Bb-Cas9* and *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ homozygous lines were heat shocked for 2 h at 37°C, and at 6, 15, or 24 h post heat shock, their GFP expression was imaged and compared to that of the non-treated embryos, larvae, pupae, or flies raised at 18°C or 26°C. To assess the inducible expression of *Hsp70Bb-Cas9* directly, we compared the Cas9/dgRNA knockout phenotypes induced by a heat shock to those without the heat shock. We tested three different double guide RNA (dgRNA) (*dgRNA*$^{sxl,\beta Tub}$, *dgRNA*$^{traA,\beta Tub}$, and *dgRNA*$^{traB,\beta Tub}$) lines with the same *Hsp70Bb-Cas9* line as the F_1 transheterozygotes—the classic pgSIT. The homozygous

dgRNA and Cas9 lines were genetically crossed, and their trans-heterozygous embryos were raised at either 18°C or 26°C. Additionally, groups of these embryos underwent various durations of heat shocks at 37°C during the first or second day post oviposition (Fig. 1). For heat-shock treatments, glass vials with staged embryos and/or larvae were incubated in a water bath at 37°C. We tested different temperature conditions to assess the induction levels between the baseline and complete expression of Cas9 for each dgRNA construct: the development at 18°C with no heat shock ($18°C^{NHS}$), a 1 h heat shock at the first instar larval stage ($18°C^{1H\text{-}37°C}$), or a 4 h heat shock at the first instar larval stage ($18°C^{4H\text{-}37°C}$). The development at 26°C was tested with no heat shock ($26°C^{NHS}$) or with a 2 h heat shock at the first instar larval stage ($26°C^{2H\text{-}37°C}$; Fig. 1).

The generated transgenic lines harboring one or two copies of *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ and *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ genetic cassettes were maintained for more than 10 generations at 18°C. To induce the pgSIT phenotypes, staged embryos were generated at 18°C and shifted to 26°C to complete their development. We assessed different temperature conditions to activate the Cas9 expression fully: the development at 18°C with no heat shock ($18°C^{NHS}$) and the development at 26°C with no heat shock ($26°C^{NHS}$), a 1 h heat shock at the first instar larval stage ($26°C^{1H\text{-}37°C}$), or a 2 h heat shock at the first or second larval stages ($26°C^{2H\text{-}37°C}$; Fig. 2). To estimate the efficiency of knockout phenotypes, we scored the sex of emerging adult flies as female, male, or intersex and tested the fertility of generated flies as previously described in Kandul *et al.*[15] Note that the induced male sterility was tested in multiple groups of 7–20 males per group from the same biological sample. A single fertile male would designate an entire sample as fertile. Each experimental test was repeated a minimum of three times for statistical comparisons.

Genotyping loci targeted with gRNAs

We examined the molecular changes that caused female lethality and male sterility following the previously described protocol.[15] Briefly, the *sxl*, *tra*, and *βTub* loci targeted by the gRNAs were PCR amplified from individual flies and were sequenced in both directions using the Sanger method at GeneWiz®. The sequence reads were aligned against the corresponding reference sequences in SnapGene® v4. The primer sequences used for the PCR of the *sxl*, *tra*, and *βTub* loci are presented in Supplementary Table S1. We also sequenced *sxl* and *βTub* loci using DNA extracted from multiple *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ females or males reared at 18°C to assess leaky *Hsp70Bb-Cas9* expression in somatic cells using the Sanger sequencing method.

Reverse transcription quantitative PCR

We used the *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ line to quantify the activation *Hsp70Bb-Cas9* expression. Vials containing staged larvae were maintained at 18°C. Heat-treated vials were incubated in the heat block for 2 h at 37°C and then for 4 h at 26°C. The vials that were not heat treated stayed at 18°C. Larvae were separated from food in water at room temperature. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), quantified using the NanoDrop 2000 (Thermo Fisher Scientific), and then treated with DNAse I (Thermo Fisher Scientific) following the protocol. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with a primer mixture of 1:6 of Oligo $(dT)_{18}$ primer and random hexamer primers. Real-time quantitative PCR was performed using LightCycler® 96 Instrument (Roche). Reverse transcription quantitative PCR (RT-qPCR) quantification of *Hsp70Bb-Cas9* expression was done relative to *RPL32* and *ATPsynCF6*. Reverse-transcribed cDNA samples from replicates that were not heat treated were serially diluted over 50× to build standard curves for each amplified gene fragment and test primer performance (Supplementary Table S1). A 10× dilution of cDNA (middle of the standard curve range) was used for relative quantification of *Hsp70Bb-Cas9* expression. Real-time qPCR reactions (20 μL) contained 4 μL sample, 10 μL SYBR Green Master Mix, 0.8 μL forward primer and 0.8 μL reverse primer, and 4.4 μL ultrapure water. The negative control (20 μL) contained 10 μL SYBR Green Master Mix, 0.8 μL forward primer and 0.8 μL reverse primer, and 8.4 μL ultrapure water. Three technical replicates were run per place for each of four biological replicates. The real-time qPCR data were analyzed in LightCycler® 96 (Roche Applied Science) and exported into a Microsoft Excel datasheet for further analysis. RNA levels were normalized to *RPL32* or *ATPsynCF6* to generate two separate relative quantifications of *Hsp70Bb-Cas9* mRNA after a 2 h heat shock.

Competition assay of TI-pgSIT males

We evaluate the competitiveness of the induced *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ males by their ability to mate with females in the presence of wild-type (wt) males. We previously demonstrated that one fertile male is able to mate at least 9/10 virgin females in 12 h.[15] To increase mating competition, we confined 10 virgin females with 5 wt males alone, 5 wt and 5 TI-pgSIT males, 5 wt and 10 TI-pgSIT males, or 10 TI-pgSIT males alone in a vial for 12 h in the dark. As previously, freshly emerged induced TI-pgSIT and wt males were isolated from females and aged for 4 days before the competition assay to increase the male courtship drive. After 12 h of mating,

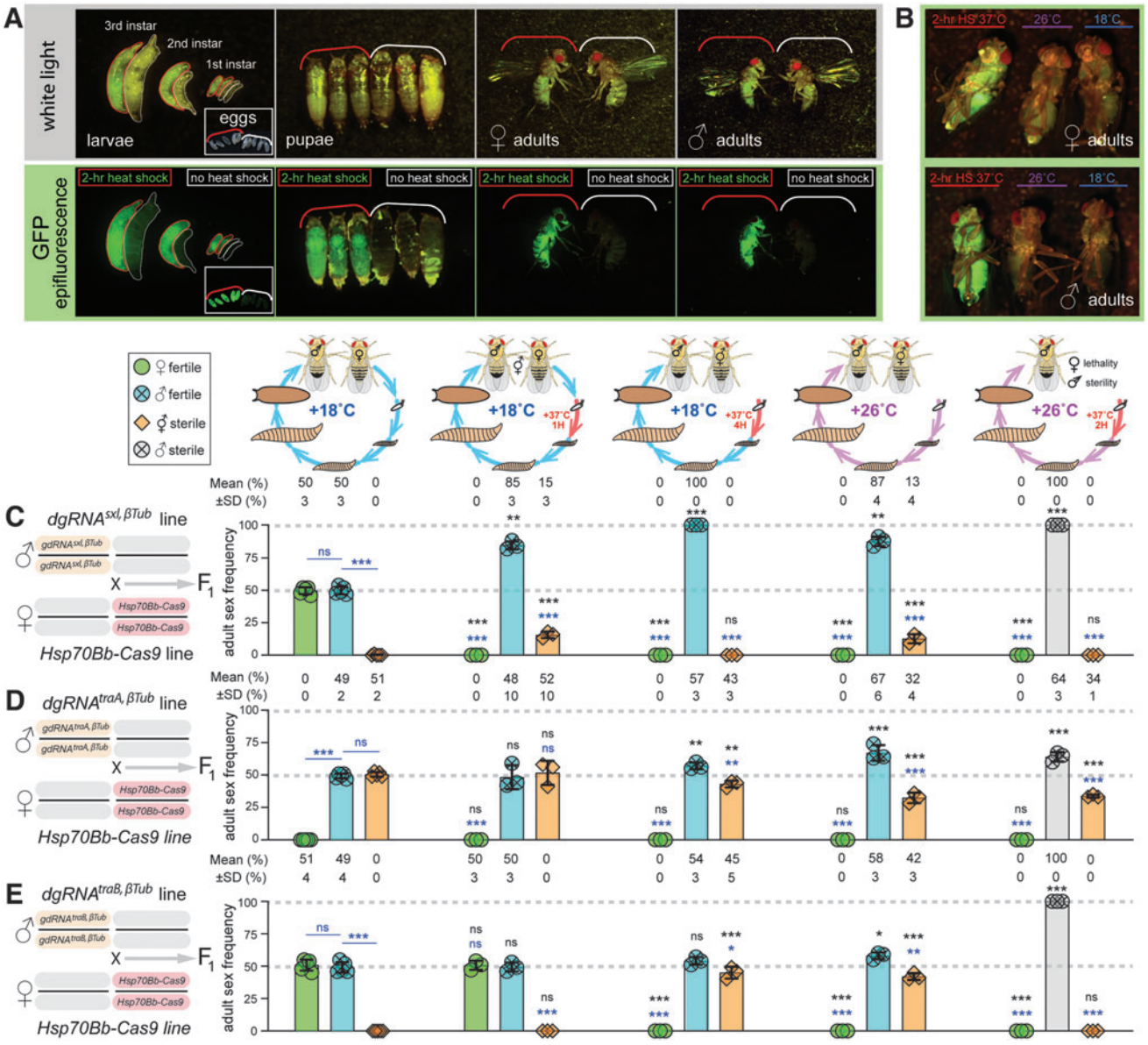


FIG. 1. Assessment of temperature-inducible pgSIT systems. To establish a visual indicator of Cas9 expression, the green fluorescent protein (GFP) coding sequence was attached to the C-terminal end of the *Streptococcus pyogenes*-derived *Cas9* (*Cas9*) coding sequence via a self-cleaving T2A peptide. **(A)** and **(B)** A 2 h heat shock at 37°C activates the expression of *Hsp70Bb-Cas9* at the P{CaryP}attP2 site, as indicated by the GFP expression. **(B)** Raising embryos harboring the *Hsp70Bb-Cas9* to adult flies at 26°C does not activate visible GFP fluorescence in living flies. The baseline and activated expression of *Hsp70Bb-Cas9* was tested in combination with three different *dgRNAs*—**(C)** $dgRNA^{sxl,\beta Tub}$, **(D)** $dgRNA^{traA,\beta Tub}$ and **(E)** $dgRNA^{traB,\beta Tub}$—to assess the feasibility of the temperature-inducible precision guided sterile insect technique (TI-pgSIT) design. The staged trans-heterozygous F_1 embryos generated by reciprocal genetic crosses between homozygous *dgRNAs* and *Hsp70Bb-Cas9* lines were raised at 18°C or 26°C with additional heat shocks at 37°C. The sex and fertility of emerged adult flies were scored and plotted as bar graphs. Since the knockouts of *sxl* and *tra* transform the normal-looking females into intersexes, the emerging F_1 flies were scored as females (♀), males (♂), or intersexes (⚥). The frequency of each sex that emerged under a specific temperature condition was compared to that of the same sex that emerged at 18°C without a heat shock. Additionally, the ♂ frequency was compared to the ♀ and ⚥ frequency for each condition. *Bar plots* show the mean ± standard deviation (*SD*) over at least three biological replicates. The frequency of each sex and its fertility was compared to those of the corresponding sexes reared at 18°C (*in black*). Additionally, the ♂ frequency was compared to the ♀ and ⚥ frequency under each condition (*in blue*). Statistical significance in sex frequency was estimated using a two-sided Student's *t*-test with equal variance. Pearson's chi-square tests for contingency tables were used to assess the difference in male sterility ($p \geq 0.05$, n.s.; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

the females were transferred into small embryo collection cages (Genesee Scientific 59–100) with grape juice agar plates. Grape plates were changed, and laid eggs were counted four times every 12 h for a total of 48 h post mating. Unhatched eggs were scored to assess numbers of hatched eggs. The decrease in female fertility, estimated by the number of unhatched eggs, indicated the ability of a sterile TI-pgSIT male to score successful matings with females in the presence of a wt male, and thus provided a readout of the competitiveness of the induced *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ males.

Statistical analysis

Statistical analyses were performed in JMP v8.0.2 (SAS Institute, Inc). Three to five biological replicates were used to generate statistical means for comparisons. *p*-Values were calculated for a two-sided Student's *t*-test with equal variance for frequency data. To test for significance of male sterilization, Pearson's chi-square tests for contingency tables were used to calculate *p*-values.

Results

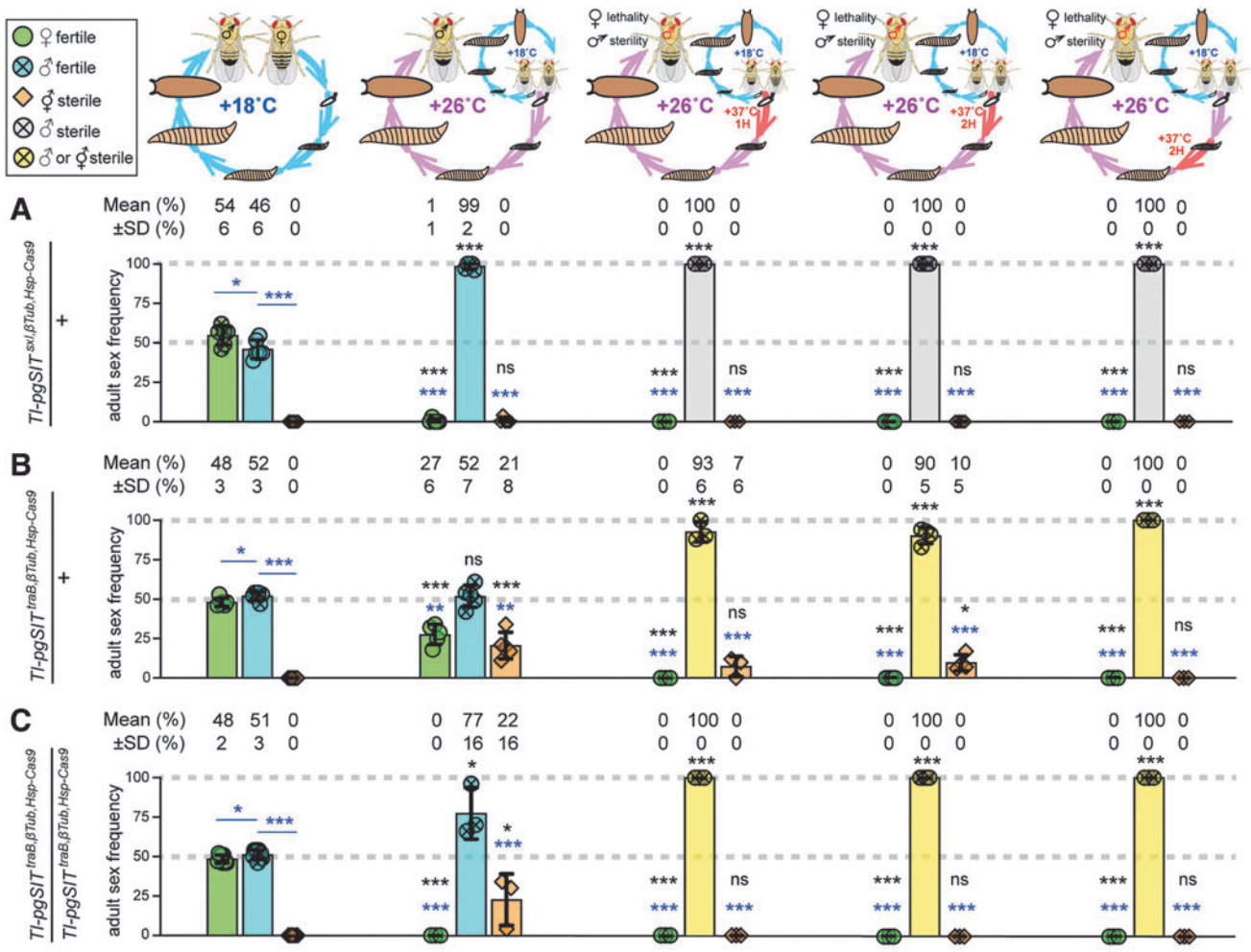
Temperature-inducible Cas9 activation

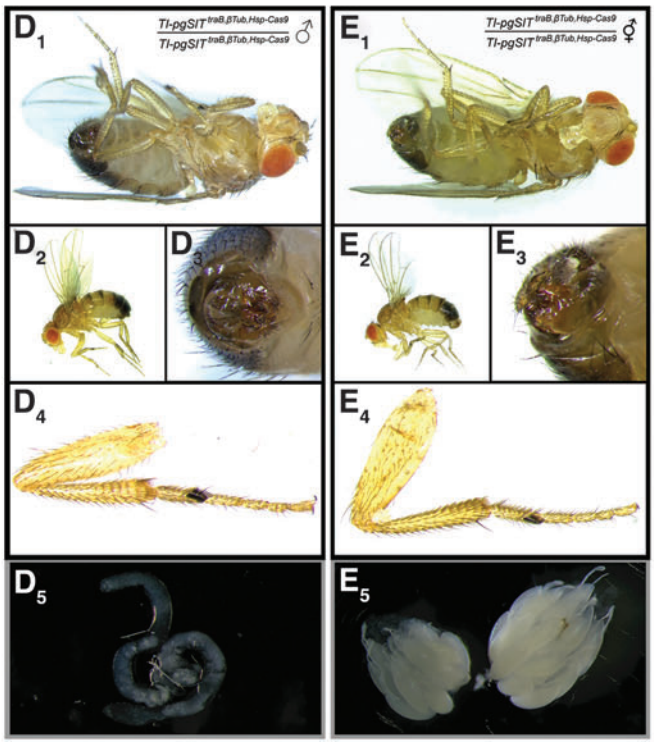
To generate an inducible platform that does not require exposure to radiation/chemicals/antibiotics, which can impact the fitness of released animals,[20–24] we utilized a temperature-inducible activation system. We took advantage of the mechanism controlling the expression of *Hsp70Bb*, from the conserved heat-shock 70 family of proteins, which can be temporarily activated by simply raising temperature to 37°C, a heat shock. When the temperature drops, the expression rapidly returns to pre-shock levels.[25–30] Given this feature, we leveraged the classical Hsp70Bb (Hsp70, Hsp, CG31359) promoter to generate a temperature-inducible Cas9 expression cassette (*Hsp70Bb-Cas9*; Supplementary Fig. S1A). For a visual indicator of promoter activity, we also included a self-cleaving T2A peptide and eGFP coding sequence downstream (3′) from the Hsp-driven Cas9. With this, we established a homozygous transgenic strain of *D. melanogaster*. As the baseline expression of the *Hsp70Bb* promoter at 25°C is well known,[31–33] we wanted to see if it was further reduced at even lower temperatures, and we compared expression at two temperatures: 18°C and 26°C. To assess the activity of *Hsp70Bb-Cas9* visually, we compared GFP fluorescence in *Hsp70Bb-Cas9* embryos, larvae, and adults raised at either 18°C or 26°C with and without a 37°C heat shock during early development (Fig. 1A). Without heat shock, we did not detect visible changes in GFP fluorescence between flies raised at 18°C or 26°C (Fig. 1B). However, the heat-shocked individuals raised either at 18°C or 26°C had significantly brighter GFP fluorescence, indicating that exposure to 37°C induces robust expression (Fig. 1B).

Basal expression of Cas9

To determine the basal activity of *Hsp70Bb-Cas9* genetically at 18°C, we performed a series of genetic crosses

FIG. 2. Elevating the temperature of one-locus TI-pgSIT lines produces desired phenotypes. Assessment of phenotypes upon temperature treatments comparing two single-locus TI-pgSIT cassettes **(A)** *TI-dgRNA*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ and **(B)** and **(C)** *dgRNA*$^{traB,\beta Tub,Hsp\text{-}Cas9}$. At 18°C, transgenic flies harboring one or two copies of the TI-pgSIT cassette produce both females and males at a nearly equal sex ratios and can be pure bred for many generations. The full activation of the TI-pgSIT cassette is achieved by raising the flies at 26°C with an additional heat shock at 37°C during the first days of development. This activating temperature condition induces 100% penetrance of the pgSIT phenotypes, female-specific lethality, and male-specific sterility, and as a result, only sterile males emerge. The sex and fertility of emerged adult flies was scored and plotted as bar graphs. The emerging flies were scored as females (♀), males (♂), or intersexes (⚥). The frequency of each sex that emerged under 18°C treatment was compared to that of the same sex. Additionally, the ♂ frequency was compared to the ♀ and ⚥ frequency under each condition. Bar plots show the mean ± *SD* over at least three biological replicates. The frequency of each sex and its fertility was compared to those of the corresponding sexes reared at 18°C (*in black*). Additionally, the ♂ frequency was compared to the ♀ and ⚥ frequency under each condition (*in blue*). Statistical significance in sex frequency was estimated using a two-sided Student's *t*-test with equal variance. Pearson's chi-square tests for contingency tables were used to assess the difference in male sterility ($p \geq 0.05$, n.s.; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). **(D)** and **(E)** Notably, after close examination of heat-induced *dgRNA*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ males, we inferred that a fraction of flies referred to as males are indeed intersexes. These intersexes have very similar external morphology, including abdomen pigmentation **(E_{1-2})**, genitals **(E_3)**, and sex combs **(E_3)** to that of males **(D_{1-4})**, prohibiting their correct identification. Some older intersexes can be identified when, instead of testes **(D_5)**, they develop ovaries **(E_5)**, which result in abdomen extension (E_2 vs. D_2).


♀ fertile
♂ fertile
♀ sterile
♂ sterile
♂ or ♀ sterile
+18°C
+26°C
♀ lethality
♂ sterile
+37°C 1H
+37°C 2H
A
Mean (%) 54 46 0 1 99 0 0 100 0 0 100 0 0 100 0
±SD (%) 6 6 0 1 2 0 0 0 0 0 0 0 0 0 0
Tl-pgSIT^sxl,βTub,Hsp-Cas9 / +
B
Mean (%) 48 52 0 27 52 21 0 93 7 0 90 10 0 100 0
±SD (%) 3 3 0 6 7 8 0 6 6 0 5 5 0 0 0
Tl-pgSIT^traB,βTub,Hsp-Cas9 / +
C
Mean (%) 48 51 0 0 77 22 0 100 0 0 100 0 0 100 0
±SD (%) 2 3 0 0 16 16 0 0 0 0 0 0 0 0 0
Tl-pgSIT^traB,βTub,Hsp-Cas9 / Tl-pgSIT^traB,βTub,Hsp-Cas9
adult sex frequency
100
75
50
25
0
*
***
ns


D1
Tl-pgSIT^traB,βTub,Hsp-Cas9 / Tl-pgSIT^traB,βTub,Hsp-Cas9 ♂
E1
Tl-pgSIT^traB,βTub,Hsp-Cas9 / Tl-pgSIT^traB,βTub,Hsp-Cas9 ♀
D2
D3
E2
E3
D4
E4
D5
E5

that would enable us to measure leaky expression. We used constitutively expressing dgRNA lines that target essential female viability genes, including sex-determination genes *sex lethal* (*sxl*)[34] or *transformer* (*tra*)[35] in addition to an essential male fertility gene that is active during spermatogenesis, *βTubulin 85D* (*βTub*).[36] To target these genes, we used previously generated lines ($dgRNA^{sxl,\beta Tub}$ and $dgRNA^{traA,\beta Tub}$)[15] and generated a new dgRNA line ($dgRNA^{traB,\beta Tub}$) that targets a unique site in *tra*, each constitutively expressing two gRNAs: one targeting *βTub* and one targeting either *sxl* or *tra* (Supplementary Fig. S1B and Supplementary Table S1). Note that both $gRNA^{traA}$ and $dgRNA^{traB}$ target the same female-specific exon, and their cut sites are only 62 bases apart. We crossed homozygous *dgRNA* males to homozygous *Hsp70Bb-Cas9* females and raised the F_1 progeny at 18°C. The trans-heterozygous F_1 progeny harboring *Hsp70Bb-Cas9* together with either $dgRNA^{sxl,\beta Tub}$ or $dgRNA^{traB,\beta Tub}$ developed into fertile females and males at equal frequencies: 49.8 ± 2.7% female versus 50.1 ± 2.8% male ($p > 0.884$, two-sided Student's *t*-test with equal variance; Fig. 1C and Supplementary Data S1), and 51.0 ± 4.1% female versus 49.0 ± 4.1% male ($p > 0.452$, two-sided Student's *t*-test with equal variance; Fig. 1E and Supplementary Data S1), respectively. Notably, the combination of paternal $dgRNA^{traA,\beta Tub}$ and maternal *Hsp70Bb-Cas9* resulted in complete conversion of females into intersexes (50.7 ± 1.7% intersex vs. 49.3 ± 1.7% male; $p > 0.217$, two-sided Student's *t*-test with equal variance; Fig. 1D and Supplementary Data S1), suggesting some degree of toxicity likely resulting from the leaky basal activity of *Hsp70Bb-Cas9* combined with $dgRNA^{traA,\beta Tub}$. To assess the fertility of the surviving F_1 progeny from these crosses, we intercrossed F_1 flies and generated viable F_2 progeny at 18°C, except from intersex parents, which were sterile. The reciprocal genetic cross of *dgRNA* females to *Hsp70Bb-Cas9* males did not cause significant differences in the corresponding F_1 sex frequencies (Supplementary Fig. S2 and Supplementary Data S1), suggesting that *Hsp70Bb-Cas9* does not induce substantial maternal carryover of Cas9 protein at 18°C. Taken together, these results indicate that the *Hsp70Bb* promoter directed some leaky basal expression sufficient to convert females into intersexes when combined with $dgRNA^{traA,\beta Tub}$. However F_1 trans-heterozygous flies ($dgRNA^{sxl,\beta Tub}$/+; *Hsp70Bb-Cas9*/+ and $dgRNA^{traB,\beta Tub}$/+; *Hsp70Bb-Cas9*/+) developed normally into fertile females and males.

Given that generation rates in *D. melanogaster* are faster at 26°C, we also wanted to test the possibility of raising trans-heterozygous flies at this temperature. Therefore, we raised trans-heterozygous flies ($dgRNA^{sxl,\beta Tub}$/+; *Hsp70Bb-Cas9*/+, $dgRNA^{traA,\beta Tub}$/+; *Hsp70Bb-Cas9*/+, and $dgRNA^{traB,\beta Tub}$/+; *Hsp70Bb-Cas9*/+) at 26°C and scored the sex ratios and fertility of emerging flies. Unexpectedly, we found that progeny from these flies could not be maintained at 26°C, since all F_1 females perished during development or were converted into sterile intersexes in 12.7 ± 3.5% and 41.9 ± 2.5% of cases, respectively (Fig. 1C and D). However, the emerging trans-heterozygous males were fertile, indicating that male sterilization will require additional expression of the CRISPR components (Fig. 1C and D and Supplementary Data S1). Taken together, these data suggest that the system is sufficiently leaky at 26°C to kill female progeny but not leaky enough to sterilize male progeny.

Temperature-inducible phenotypes

To identify the optimal heat-shock conditions required for the complete penetrance of desired TI-pgSIT phenotypes in F_1 progeny (i.e., female lethality and male sterility), we heat shocked (37°C) F_1 progeny raised at either 18°C or 26°C and quantified the sex ratios and fertility of emerging progeny. To determine the optimal heat-shock conditions, we compared several temperature profiles. At 18°C, we compared the development with no heat shock ($18°C^{NHS}$), a 1 h heat shock at the first instar larval stage ($18°C^{1H\text{-}37°C}$), or a 4 h heat shock at the first instar larval stage ($18°C^{4H\text{-}37°C}$). At 26°C, we compared the development with no heat shock ($26°C^{NHS}$) or with a 2 h heat shock at the first instar larval stage ($26°C^{2H\text{-}37°C}$; Fig. 1C–E and Supplementary Data S1). The $18°C^{1H\text{-}37°C}$ condition killed most of the females expressing *sxl* and transformed the surviving $dgRNA^{sxl,\beta Tub}$/+; *Hsp70Bb-Cas9*/+ and $dgRNA^{traA,\beta Tub}$/+; *Hsp70Bb-Cas9*/+ trans-heterozygous females into sterile intersexes (Fig. 1C and D and Supplementary Data S1). However, this condition was insufficient to transform or kill $dgRNA^{traB,\beta Tub}$/+; *Hsp70Bb-Cas9*/+ trans-heterozygous females expressing $U6.3\text{-}gRNA^{traB}$ (Fig. 1E). Interestingly, simply increasing the heat-shock period to 4 h ($18°C^{4H\text{-}37°C}$) completely eliminated the $gRNA^{sxl,\beta Tub}$/+; *Hsp70Bb-Cas9*/+ females (Fig. 1C) and transformed all $gRNA^{traB,\beta Tub}$/+; *Hsp70Bb-Cas9*/+ females into intersexes (Fig. 1E and Supplementary Data S1). Notwithstanding the complete transformation and killing of females observed above, none of the $18°C^{4H\text{-}37°C}$, $18°C^{1H\text{-}37°C}$, or $26°C^{NHS}$ conditions ensured the complete sterility of F_1 trans-heterozygous males (Fig. 1C–E). Given these results, next we raised trans-heterozygous F_1 progeny at 26°C with a 2 h heat shock at the first instar larval stage ($26°C^{2H\text{-}37°C}$), which resulted in the development of sterile males and/or sterile intersexes for each trans-heterozygous combination (Fig. 1C–E and Supplementary

Data S1). Notably, we did not identify *gRNA*$^{traB,\beta Tub}$/+; *Hsp70Bb-Cas9*/+ intersex individuals under the 26°C$^{2H\text{-}37°C}$. Taken together, these results indicate that *Hsp70Bb-Cas9* can direct the temperature-inducible expression of Cas9, which is sufficient to cause the 100% penetrance of the desired TI-pgSIT phenotypes. However, careful titration is necessary to optimize the temperature conditions to achieve the desired phenotypes.

Simplified one-locus TI-pgSIT

Given that both the designed trans-heterozygous combinations generated fertile flies when raised at 18°C and only sterile males when heat shocked (26°C$^{2H\text{-}37°C}$; Fig. 1C–E), next we wanted to explore TI-pgSIT systems that function in *cis* to simplify the approach further. Therefore, we engineered two additional constructs combining *Hsp70Bb-Cas9* and one of two best dgRNA, *gRNA*$^{sxl,\beta Tub}$ and *gRNA*$^{traB,\beta Tub}$, hereafter referred to as *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ and *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$, respectively (Supplementary Fig. 1C). Each *TI-pgSIT* cassette was site-specifically inserted into an attP docking site located on the third chromosome (*P{CaryP}attP2*) using φC31-mediated integration[19] to enable direct comparison between the two systems. We generated both *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ and *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ transgenic lines and maintained these as heterozygous balanced flies for more than 10 generations at 18°C. While we were unable to generate a homozygous line for *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$, we obtained one for *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$.

To assess the baseline expression of the one-locus TI-pgSIT systems at 18°C, we evaluated the female-to-male ratio and fertility in lines harboring a copy of either the *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ or *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ cassette. We found a slightly female-biased ratio for the *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$/+ line maintained at 18°C: 54.5 ± 6.0% female versus 45.5 ± 6.0% male ($p = 0.025$, two-sided Student's *t*-test with equal variance; Fig. 2A and Supplementary Data S2). The *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ line had a slightly male-biased ratio: 47.9 ± 2.8% female versus 52.0 ± 8.3% male for heterozygous flies ($p < 0.030$; two-sided Student's *t*-test with equal variance; Fig. 2B and Supplementary Data S2), and 48.4 ± 2.4% female versus 51.2 ± 2.9% male for homozygous flies ($p < 0.044$, two-sided Student's *t*-test with equal variance; Fig. 2C and Supplementary Data S2). We have maintained both heterozygous *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ and homozygous *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ for nearly 2 years or more than 40 generations and counting at 18°C. Taken together, these experiments indicate that one-locus TI-pgSIT systems can be engineered, expanded, and maintained at 18°C. However, given that we could not generate a homozygous line for *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$, again careful titration of expression is necessary.

Temperature-inducible one-locus TI-pgSIT

Next, we explored the effects of heat shock on the penetrance of desired TI-pgSIT phenotypes. To activate the *Hsp70Bb-Cas9* expression, we collected eggs from one-locus TI-pgSIT flies maintained at 18°C, and we raised the staged eggs at 26°C with or without an additional heat shock at 37°C. We compared several different heat-shock conditions including: the development from embryos to adult flies at 26°C with no heat shock (26°C^{NHS}), with a 1 h heat shock at the first instar larval stage (26°C$^{1H\text{-}37°C}$), or with a 2 h heat shock at the first or second instar larval stages (26°C$^{2H\text{-}37°C}$). For the 26°C^{NHS} condition, when *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$/+ progeny were raised continuously at 26°C, this resulted in the near-complete elimination of females (54.5 ± 6.0% females at 18°C vs. 0.7 ± 1.4% females at 26°C; $p < 0.0001$, two-sided Student's *t*-test with equal variance), with 98.5 ± 2.0% of males emerging. However, not all of these males were sterile (Fig. 2A and Supplementary Data S2). Moreover, raising the flies with one or two copies of the *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ cassette at the 26°C^{NSH} condition affected the sex ratio of the emerging progeny—some or all females, respectively, were transformed into intersexes, though the emerging males were still fertile (Fig. 2B and C and Supplementary Data S2). Nevertheless, an additional 1 h (26°C$^{1H\text{-}37°C}$) or 2 h (26°C$^{2H\text{-}37°C}$) heat shock of the first instar or the second instar larvae harboring either one copy of *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ or two copies of *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ eliminated the females and intersexes and sterilized 100% of the males (Fig. 2A–C and Supplementary Data S2). Taken together, these data indicate that heterozygous as well as homozygous viable strains harboring a one-locus *TI-pgSIT* genetic cassette can be generated and maintained at 18°C, and when progeny from these flies are simply grown at 26°C and heat shocked during early larval development, the desired fully penetrant TI-pgSIT phenotypes can be achieved.

Heat shock induces TI-pgSIT$^{traB,\beta Tub,Hsp\text{-}Cas9}$ *intersex flies*

We previously observed that *tra* knockout (KO) using *Cas9/gRNA*^{traA} induces an incomplete masculinization of *D. melanogaster* females, converting them into intersexes.[15] To explore further what happens with *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ females under the 26°C$^{2H\text{-}37°C}$ conditions, heat-induced homozygous *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ males were thoroughly examined. We noticed that several heat-shocked TI-pgSIT flies developed extended

abdomens. Dissections of their abdomens identified ovaries with oocytes (Fig. $2E_5$). Therefore, we inferred that a fraction of *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ flies, which were raised using the 26°C$^{2H\text{-}37°C}$ conditions, were indeed intersexes. These intersexes, unlike the heat shock–induced *dgRNA*$^{traA,\beta Tub}$/+; *Hsp70Bb-Cas9*/+ intersexes reared under the 26°C$^{2H\text{-}37°C}$ (Fig. 1D) or the *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ intersexes raised under the 26°C without a heat shock (26°C^{NHS}; Fig. 2B and C), are difficult to distinguish from true males. The abdomen pigmentation (Fig. $2E_{1-2}$), external genitals (Fig. $2E_3$), and sex combs (Fig. $2E_3$) of the *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ intersexes reared under 26°C$^{2H\text{-}37°C}$ are nearly identical to those of males (Fig. $2D_{1-4}$), prohibiting their correct identification (Fig. 2B and C). Therefore, to avoid intermixing true males with intersexes, we focused on the *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ line for further experiments, quantifying the basal Cas9 expression and assessing the competitiveness of heat-shocked sterile TI-pgSIT males.

Fitness and basal Cas9 expression

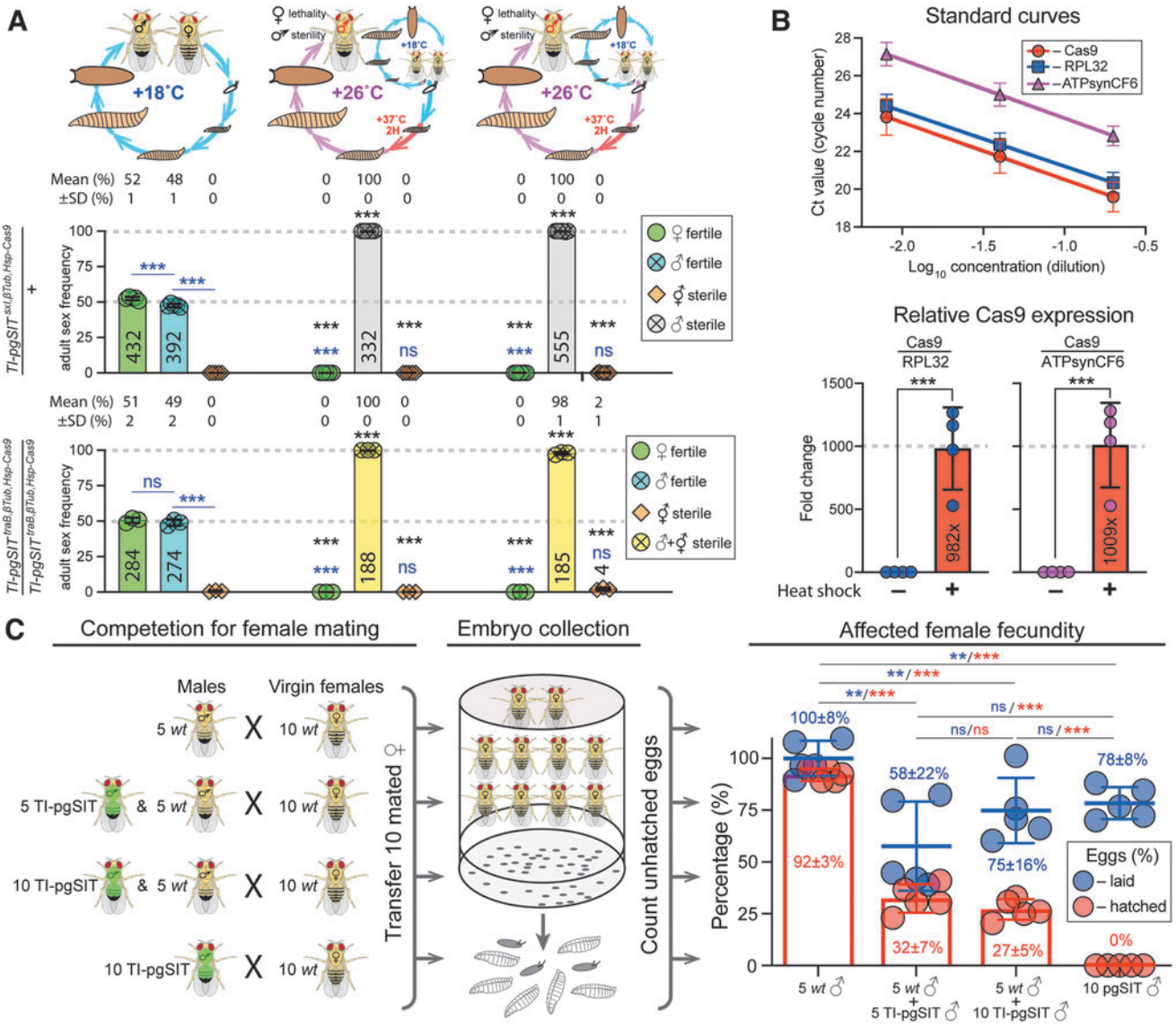
We attempted to establish the homozygous *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ line. However, homozygous females are only partially fertile, and homozygous lineages cannot be maintained. To explore the reasons behind fitness costs of two copies of *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ genetic cassette, we examined both *sxl* and *βTub* target sequences in flies raised at 18°C. Using Sanger sequencing, we found that both target sequences were mutagenized resulting in ambiguous sequence reads downstream from the corresponding gRNA cut site (Supplementary Fig. S3). These sequencing reads indicate that individual flies were likely mosaic for wt and insertion and deletion (indel) alleles at both *sxl* and *βTub* loci. However, it is not clear whether indel alleles were induced in only somatic cells or both somatic and germline cells. If functional indel alleles, which are resistant to Cas9/dgRNA$^{sxl,\beta Tub}$-mediated cleavage, are induced in germline cells, they will be selected and propagated through multiple generations. We examined this possibility by assessing the penetrance of heat-induced pgSIT phenotypes (i.e., female lethality/transformation and male sterility) using both *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$/+ and *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$/*TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ lines after having maintained them for 12 months at 18°C. After heat shocking multiple batches of larvae and analyzing large numbers of flies raised at 26°C, we found that all females either perished or were transformed into intersexes, while all resulting males were sterile (Fig. 3A). For *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$/+, we scored 877 sterile males and a single sterile intersex reared under the 26°C$^{2H\text{-}37°C}$ condition compared to 432 fertile females and 392 fertile males raised at 18°C (Fig. 3A and Supplementary Data S3). For *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$/*TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$, we scored

FIG. 3. Stability and performance of the TI-pgSIT system 12 months after its development. **(A)** Reassessment of *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ and *TI-pgSIT*$^{traB,\beta Tub,Hsp\text{-}Cas9}$ one-locus TI-pgSIT lines 12 months later. The sex and fertility of emerged adult flies was scored and plotted as bar graphs. The emerging flies were scored as females (♀), males (♂), or intersexes (⚥), and numbers of scored flies are indicated for each bar. Eggs were collected at 18°C and 26°C, and emerging larvae were heat shocked at 37°C for 2 h and then reared at 26°C. Bar plots show the mean ± *SD* over at least three biological replicates. The frequency of each sex and its fertility was compared to those of the corresponding sexes reared at 18°C (*in black*). Additionally, the ♂ frequency was compared to the ♀ and ⚥ frequency under each condition (*in blue*). Statistical significance in sex frequency was estimated using a two-sided Student's *t*-test with equal variance. Pearson's chi-square tests for contingency tables were used to assess the difference in male sterility. **(B)** In *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ line, *Cas9* transcription increases nearly 1,000 times after a 2 h heat shock at 37°C. Total RNA was extracted from second instar larvae 4 h after heat shock, and reverse transcription polymerase chain reaction quantification of *Cas9* expression was done relative to *RPL32* and *ATPsynCF6*. **(C)** The heat-induced *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ ♂ successfully compete against wild-type (wt) ♂ to secure matings with wt ♀. The mating success of sterile TI-pgSIT ♂ was evaluated by fecundity decrease (i.e., increase of unhatched egg rate). *D. melanogaster* mated ♀ is resistant to the next mating for 12–24 h.[37–39] Previously, we found that one wt ♂ could fertilize at least 9/10 virgin ♀ during 12 h.[15] To intensify the ♂ competition, we confined 10 wt virgin ♀ with 5 wt ♂ and 5 or 10 TI-pgSIT ♂ for 12 h before removing ♂ and assessing ♀ fecundity. The graph indicates percentages of laid (*blue points*) and hatched (*red points*) eggs for five replicates, as well as a mean ± one (*SD*) for each group. The addition of 5 or 10 sterile TI-pgSIT ♂ to 5 fertile wt ♂ mating with 10 virgin ♀ caused a significant decrease in laid and hatched eggs during 48 h post mating (Supplementary Data S4). Statistical significance was estimated using a two-sided Student's *t*-test with equal variance ($p \geq 0.05$, n.s.; $^{**}p < 0.001$; $^{***}p < 0.001$).

377 sterile males and/or intersexes and no females (Fig. 2E$_{1–6}$) following the heat shock (26°C$^{2H\text{-}37°C}$), while 284 fertile females and 274 fertile males emerged under 18°C (Fig. 3A and Supplementary Data S3). Taken together, these results suggest that a basal Cas9 expression at 18°C induces some indel alleles at *sxl* and *βTub* loci. However, the leaky Cas9 expression is likely limited to somatic cells.

Quantification of temperature-induced Hsp70Bb-Cas9 expression

To assess the extent of temperature-induced Cas9 activation, we quantified changes in the Cas9 mRNA relative to other genes using RT-qPCR. Two separate constitutively expressed genes, *Ribosomal protein L32* (*RPL32*) and *ATP synthase-coupling factor 6* (*ATPsynCF6*), were used for relative quantification of Cas9 expression. We found that a 2 h 37°C heat shock and 4 h 26°C incubation of second instar larvae induced a three-order-magnitude increase (i.e., 1,000-fold) in the level of the *Cas9* mRNA relative to that in the larvae maintained at 18°C (Fig. 3B). Notably, two separate RT-qPCR qualifications based on independent normalizations genes (*RPL32* and *ATPsynCF6*) inferred consistent estimations of the increase in Cas9 expression following the heat shock: 982-fold as Cas9/*RPL32*, and 1,009-fold as Cas9/*ATPsynCF6* (Fig. 3B). Notably, we raised the larvae remaining in the vials and verified that only sterile males emerged from vials raised under the 26°C$^{2H\text{-}37°C}$ condition, while fertile females and males developed in vials maintained at 18°C. Therefore, a single copy of *Hsp70Bb-Cas9* is sufficient to provide a 1,000-fold transcription increase from its basal



expression and induce efficient Cas9/gRNA-mediated mutagenesis, which in turn results in *sxl* and *βTub* knockouts at the organismal level.

Competitiveness of heat-induced TI-pgSIT$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ males

To explore potential fitness costs of activated Cas9 expression, we assessed the competitiveness of heat-induced *TI-pgSIT*$^{sxl,\beta Tub,Hsp\text{-}Cas9}$ males. The short-term resistance of mated *D. melanogaster* females to the next mating lasts for 12–24 h, is caused by accessory gland proteins, and does not require sperm storage and usage.[37–39] We previously found that one wt male could fertilize at least 9/10 wt virgin females during 12 h.[15] Notably, a single pgSIT male generated by crossing *nanos-Cas9* and *dgRNA*$^{sxl,\beta Tub}$ was able to court and secure matings with many wt females in the presence of one wt males.[15] To increase competition further between males, we confined 10 virgin females with five TI-pgSIT or 10 TI-pgSIT males in the presence of five wt males for 12 h in the dark (Fig. 3C) before removing males and scoring the number of laid and hatched eggs during the next 48 h to assess female fertility. Since heat-induced TI-pgSIT males are sterile, eggs laid by the wt females that mated with TI-pgSIT males will not hatch, and a significant decrease in female fertility will indicate that TI-pgSIT males are able to court, mate, and successfully compete with wt males. We found that addition of 5 or 10 TI-pgSIT sterile males to five fertile wt males resulted in a significant decrease in female fertility measured by laid egg numbers ($100.0 \pm 8.5\%$ vs. $57.6 \pm 21.5\%$ or $74.8 \pm 15.8\%$, respectively; $p < 0.003$ and $p < 0.013$, two-sided Student's *t*-test with equal variance, blue points on Fig. 3C) and egg hatching rates ($91.6 \pm 3.5\%$ vs. $32.3 \pm 6.8\%$ or $27.1 \pm 5.0\%$, respectively; $p < 0.0001$, two-sided Student's *t*-test with equal variance; red points on Fig. 3C). To assess the extent of reduction in laid eggs versus hatched eggs, we subtracted percentages of hatched eggs from that of laid eggs for each replicate and compared the differences across groups. The significant excessive reduction in hatching eggs was identified for the wt male group competing with 10 TI-pgSIT males and not with five TI-pgSIT males compared to the group with only five wt males ($47.8 \pm 12.1\%$ or $25.2 \pm 24.5\%$ vs. $10.5 \pm 9.4\%$, respectively; $p = 0.0006$ and $p = 0.2451$, two-sided Student's *t*-test with equal variance; distances between matched pairs of blue and red points on Fig. 3C and Supplementary Data S4). We did not score a single hatched egg out of 1,112 eggs laid by females confined and mated with only 10 TI-pgSIT males (Fig. 3C and Supplementary Data S4), further supporting the induced male sterility of TI-pgSIT males. The mating competition assay indicated that the activated *Hsp70Bb-Cas9* expression did not compromise the fitness of TI-pgSIT males, and they were competitive with wt males at courting and mating with wt females.

Discussion

Here, we provide the proof of concept for a next-generation TI-pgSIT technology. TI-pgSIT addresses two major limitations of the previously described pgSIT.[15,16,40] First, pgSIT relies on the separate inheritance of two required components—Cas9 endonuclease and gRNAs—that are activated in the F_1 progeny when combined by a genetic cross. As a result, two transgenic lines harboring either the Cas9 endonuclease or gRNAs must be maintained separately, which increases the production costs. Second, though the F_1 progeny of pgSIT undergo autonomous sex sorting and sterilization during development, enabling their release at any life stage, the genetic cross leading to the production of these F_1 sterile males requires the precise sex sorting of parental Cas9 and gRNAs strains. Therefore, although pgSIT ensures the release of only sterile males, it still does not eliminate the insect sex-sorting step. Together, these limitations can constrain applications of the original pgSIT technology for insect population control.

The TI-pgSIT system offers possible solutions to these limitations, as instead it relies on a single pure breeding strain, which eliminates the need for maintaining two strains that must be sex sorted and mated in a facility for production of sterile males. One limitation of the TI-pgSIT approach is the heat-shock requirement during F_1 development, which would preclude the release of eggs. This means that the original pgSIT approach may be better suited for insects with a diapause during the egg stage,[15,16] though both the pgSIT and TI-pgSIT approaches will work well for the insects with a pupal diapause. Other than this limitation, the TI-pgSIT approach retains the benefits of the pgSIT technology, such as its non-invasiveness and high efficiency.[15] Also, like the pgSIT approach, TI-pgSIT can in principle be engineered and applied to many insect species with an annotated genome and established transgenesis protocols. It utilizes CRISPR, which works in diverse species from bacteria to humans,[41–43] to disrupt genes that are conserved across insect taxa, such as genes required for sex determination and fertility. To establish TI-pgSIT in other species, a temperature-inducible promoter is needed. The heat-shock 70 proteins have high interspecies conservation in insects and play important roles in helping them survive under stressful conditions. The *D. melanogaster Hsp70Bb* promoter is one of the most studied animal promoters[28,29] and has been widely used for the

heat-inducible expression of transgenes in many insect species for more than 20 years.[44–47] For example, *Hsp70B* promoters demonstrated robust heat-inducible expression of transgenes in the yellow fever mosquito, *Aedes aegypti*,[48] the Mediterranean fruit fly, *Ceratitis capitata*,[49] and the spotted wing drosophila, *Drosophila suzukii*.[50] This promoter should therefore be able to drive the heat-inducible expression of Cas9 in many insect species, especially when lower baseline expression is desirable.[49] Moreover, the *Hsp70Bb* promoter could be ideal for inducing positively activated genetic circuits, as the activation of expression is rapid and does not require chemicals or drugs such as antibiotics, which can affect insect fitness directly[20–22] or indirectly by ablating their microbiomes.[23,24] Unlike common Tet-Off systems with conditional lethal transgenes[6,51,52] that are derepressed by withholding tetracycline, activation of the *Hsp70Bb* promoter is achieved by elevated temperatures. Heat-shock treatments can reduce maintenance costs compared to other inducible systems, as temperature is relatively costless compared to drugs and antibiotics. Notwithstanding these benefits, maintenance of low temperature in a large-scale facility rearing TI-pgSIT strains can also be a substantial fraction of the operating expenses, particularly in a tropical environment.

Even though we demonstrate that *Cas9* expression can be regulated by temperature using the *Hsp70Bb* promoter, the use of this promoter did result in some leaky expression. The leaky baseline expression of the *D. melanogaster Hsp70Bb* promoter in somatic cells at 25°C is well known[31–33] and can be mitigated either by testing multiple genomic integration sites[31] to titrate the leaky expression, or by targeting alternative genes to create a TI-pgSIT system that is robust to the leaky expression. For example, we generated two transgenic lines harboring each TI-pgSIT construct. Flies harboring one or two copies of the *TI-pgSIT$^{traB,\beta Tub,Hsp\text{-}Cas9}$* genetic cassette could be pure bred and maintained at 18°C. However, only one copy of the *TI-pgSIT$^{sxl,\beta Tub,Hsp\text{-}Cas9}$* genetic cassette could be maintained at this temperature, as its homozygous females were sterile at 18°C. Because these two lines are inserted at the same genomic insertion site, it suggests that the target gene is important. Perhaps the regulation of *sxl* is more sensitive to mosaic mutations in somatic cells than that of *tra*, which would not be surprising, as *sxl* is the master gene that controls both female development and X chromosome dosage compensation in *D. melanogaster*, and females homozygous for a loss-of-function mutation died due to the X chromosome hyperactivation.[53] Nevertheless, a multimerized copy of a Polycomb response element (PRE) could be used to attempt to suppress further the leaky *Hsp70Bb-Cas9* expression[54] and facilitate homozygosity of an engineered TI-pgSIT cassette.

It should be noted that Cas9 itself is known to possess temperature-dependent activity. For example, the highest SpCas9 activity was achieved at temperatures >32°C in plant cells[55] and >37°C[55,56] but <42°C[57] in animal cells. Moreover, a heat stress at 37°C was reported to increase the efficiency of SpCas9-mediated mutagenesis up to 100-fold in plants reared at 22°C.[58] These data suggest that the reduced activity of SpCas9 at 18°C may further limit Cas9/gRNA-mediated cleavage in cells, even if the *Hsp70Bb* promoter was a bit leaky, thereby benefiting the maintenance of TI-pgSIT strains. Therefore, the temperature-dependent expression (via *Hsp70Bb*) and temperature-dependent activity of Cas9 endonuclease are likely both important factors contributing to the overall performance of the TI-pgSIT system.

The *Hsp70Bb*-directed expression was reported to be suppressed in germline cells,[59] even in response to heat-shock stimulation.[60] In *D. melanogaster*, the basic promoter of *Hsp70Bb*, which was incorporated in an upstream activation sequence (UASt) in the Gal4/UAS two-component activation system,[61] was shown to be targeted by Piwi-interacting RNAs (piRNAs) in female germline cells, leading to degradation of any mRNA harboring endogenous *Hsp70Bb* gene sequences.[62] We inferred the presence of indel alleles at both *sxl* and *βTub* target sites by Sanger sequencing these loci in *TI-pgSIT$^{sxl,\beta Tub,Hsp\text{-}Cas9}$* flies raised at 18°C. After maintaining *TI-pgSIT$^{sxl,\beta Tub,Hsp\text{-}Cas9}$*/+ and *TI-pgSIT$^{traB,\beta Tub,Hsp\text{-}Cas9}$TI-pgSIT$^{traB,\beta Tub,Hsp\text{-}Cas9}$* lines for 12 months at 18°C, we reconfirmed the complete penetrance of heat-induced pgSIT phenotypes (i.e., female lethality/transformation and male sterility). Taken together, our results are consistent with the absence of basal Cas9 expression in germline cells. The piRNA-mediated degradation of mRNA molecules harboring *Hsp70Bb* sequences in germline cells safeguards against generation and accumulation of mutant alleles. However, the three-order-magnitude activation (i.e., 1,000-fold) of Cas9 expression following the heat shock ensures the complete penetrance of both pgSIT phenotypes at the organismic level without compromising male mating competitiveness.

In summary, here we demonstrate that by using a temperature-inducible CRISPR-based approach, we can maintain a single true breeding strain and induce the production of sterile and competitive males simply by shifting the temperature. This opens an entirely new approach for the generation of sterile males, eliminating the need for sex sorting that is still required by other similar methods. In the future, TI-pgSIT could be adapted to both

agricultural pests and human disease vectors to help increase the production of food and reduce human disease, respectively, thereby eliminating the need for harmful insecticides and revolutionizing insect population control.

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Author Disclosure Statement

N.P.K. and O.S.A filed the provisional U.S. patent application describing this technology. All other authors declare no competing interests.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Table S1
Supplementary Data S1
Supplementary Data S2
Supplementary Data S3
Supplementary Data S4

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