



Consequences of resistance evolution in a Cas9-based sex conversion-suppression gene drive for insect pest management

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The use of a site-specific homing-based gene drive for insect pest control has long been discussed, but the easy design of such systems has become possible only with the recent establishment of CRISPR/Cas9 technology. In this respect, novel targets for insect pest management are provided by new discoveries regarding sex determination. Here, we present a model for a suppression gene drive designed to cause an all-male population collapse in an agricultural pest insect. To evaluate the molecular details of such a sex conversion-based suppression gene drive experimentally, we implemented this strategy in *Drosophila melanogaster* to serve as a safe model organism. We generated a Cas9-based homing gene-drive element targeting the *transformer* gene and showed its high efficiency for sex conversion from females to males. However, non-homologous end joining increased the rate of mutagenesis at the target site, which resulted in the emergence of drive-resistant alleles and therefore curbed the gene drive. This confirms previous studies that simple homing CRISPR/Cas9 gene-drive designs will be ineffective. Nevertheless, by performing population dynamics simulations using the parameters we obtained in *D. melanogaster* and by adjusting the model for the agricultural pest *Ceratitis capitata*, we were able to identify adequate modifications that could be successfully applied for the management of wild Mediterranean fruit fly populations using our proposed sex conversion-based suppression gene-drive strategy.

homing endonuclease | integrated pest management | molecular entomology | sex reversal | Tephritid fruit flies

The use of CRISPR-Cas9 systems as a homing-based gene-drive tool to alter the genotype of insect populations has theoretically (1–5) and practically (6–8) been shown to be feasible. These systems can potentially allow the spread of any desired trait in a wild population of target species even if the desired phenotype imposes a fitness cost (2, 4, 5, 8). Therefore, the spread of lethality or sterility traits that could result in suppression and eventually collapse of the target population should be possible. This has recently attracted special attention in pest and disease vector control (1, 3, 6–8). However, the effort had focused mainly on disease-vector mosquitoes such as *Anopheles* (7, 8). In homing CRISPR/Cas9 gene-drive (HCGD) systems, a CRISPR/Cas9 homing element (CHE) composed of at least the Cas9 endonuclease-coding sequence and a guide RNA (gRNA) is integrated in the host genome at the gRNA target site. In the heterozygous state, Cas9 introduces an RNA-guided double-strand break in the wild-type allele (similar to homing endonucleases) which then will be repaired either by homology-directed repair (HDR) or error-prone mechanisms such as nonhomologous end joining (NHEJ). In the former case, the CHE allele serves as the repair template and is copied into the homologous chromosome. Directing this process to the germline will result in super-Mendelian inheritance driving the CHE and any accompanying genes into

the population. Therefore, the highly customizable nature of CRISPR/Cas9 allows simple design of HCGDs to drive any desired trait, even those resulting in sterility, into wild populations as long as the cost of this phenotype does not surpass a certain threshold (1, 4).

In a recent study, Hammond et al. (8) identified a set of genes whose knockout resulted in female-specific sterility in *Anopheles*. However, they found that only one of these genes could be used as a target for HCGDs to achieve an efficient drive of female-specific sterility into the population. The remaining sterility genes imposed a very strong cost on the carriers that eventually resulted in the elimination of the drive allele from the population. As predicted by mathematical population genetics models, the spread of female-specific sterility traits in a population using HCGDs should eventually result in a population collapse and local or global elimination of the target species (1, 8). Another proposed strategy to achieve this goal is to design drive elements that alter the population's sex ratio toward males. Surprisingly, such gene-drive elements have naturally been observed in some organisms. In *Aedes aegypti*, for example, a type of drive element

Significance

Resistance evolution caused by CRISPR/Cas9 gene-drive systems has a major impact on both the future scientific design of such gene-drive systems and on the politics of regulating experimentation and use of such systems. In our study, we show that in-frame drive-resistant alleles can be produced readily and inherently in a suppression gene-drive system. The rate at which such alleles emerge will determine the maximum size of the population that could be targeted for collapse and elimination. Here, we provide a potential target site and the modeling framework for implementation and optimization of a suppression gene-drive strategy to control Mediterranean fruit fly populations.

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known as a “Killer-Y chromosome” is able to shatter the X chromosome during spermatogenesis, and therefore all offspring of mosquitoes carrying such a chromosome will be male. To replicate this phenomenon, Galizi et al. (9) employed a specific homing endonuclease, I-PpoI, to specifically shatter the X chromosome during spermatogenesis of *Anopheles gambiae*. By generating transgenic males carrying an engineered version of such a homing endonuclease gene (HEG) on somatic chromosomes, they have shown that at high initial load frequencies these flies will result in population collapse in cage experiments. They proposed that integration of such a HEG on the Y chromosome could be an effective gene-drive strategy for population control of *An. gambiae*. The distortion of the sex ratio using an X chromosome-specific CRISPR/Cas9 system has also been shown to be successful in *An. gambiae* (10).

Here, we propose an independent approach that converts female individuals into fertile males by disturbing the developmental sex-determination pathways, which distorts the sex ratio without adverse effects on the reproductive success of carrier males. A prime target gene to achieve this goal is *transformer* (*tra*). *tra* plays a pivotal role in female sex determination in different insect orders, including Diptera (11). In a devastating agricultural fruit pest, the Mediterranean fruit fly, *Ceratitis capitata* (“medfly”), *tra*-knockdown XY males develop normally, while XX individuals develop as fertile males (12). Therefore, *C. capitata* XX males carrying a CHE-targeted *tra* locus could further spread the CHE to all their progeny (Fig. 1A), resulting in an effective gene drive without any direct effect on the fecundity of individuals carrying the drive element. This in theory could lead to an all-male population collapse that can be used for controlling the wild population of this aggressive pest.

Because of the strict guidelines on gene-drive experiments and to adhere to recommendations of scientific communities (13–15), we decided to test this gene-drive strategy first using *Drosophila melanogaster* as a model organism. In *D. melanogaster*, *tra*-mutant XX individuals develop into infertile pseudomales (16), not giving rise to further progeny (Fig. 1B). Since the cost of this infertility is significantly higher than the threshold tolerated by gene-drive systems (1, 4), a CHE targeting the *tra* locus in *D. melanogaster*, despite its ability to show super-Mendelian inheritance in individual crosses, is not able to drive into a population (Fig. 1C and D). This biological confinement allows us to employ *D. melanogaster* as a safe model organism for studying the limitations of our suggested suppression gene-drive systems at the molecular level in the laboratory and thereby experimentally identify parameters that might need to be adjusted to achieve an efficient suppression gene-drive system in *C. capitata*.

In our study, we found that targeting *tra* works as an efficient means of sex conversion in *D. melanogaster*. However, the early onset of the formation of in-frame drive-resistant alleles compromises drive efficiency. Based on our observations, we simulated the use of a *tra*-based suppression gene-drive system for control of *C. capitata* populations and showed that HCGD systems employing multiple gRNAs that target the *tra* locus can serve as an effective pest-control strategy for *C. capitata*.

Results

Design of a *tra*-Based Sex Conversion-Suppression Gene-Drive System.

The proposed CHE is composed of a *spCas9*-coding sequence under the control of a suitable promoter, as explained below, a gRNA targeting the first exon of *tra* under the control of a *Pol III* promoter, and a fluorescent marker to identify the genomic integration (*SI Appendix*, Fig. S1C). The activity of this CHE unit will be similar to that of homing endonucleases and would be able to perform homing into the wild-type *tra* allele. For our *tra*-targeting CHE to drive in a population, it is essential that Cas9 is expressed in the germ cells to promote homing into the wild-type *tra* allele by HDR. To achieve sex conversion, however, *tra* needs to be inactivated in the somatic cells of XX individuals. Thus two

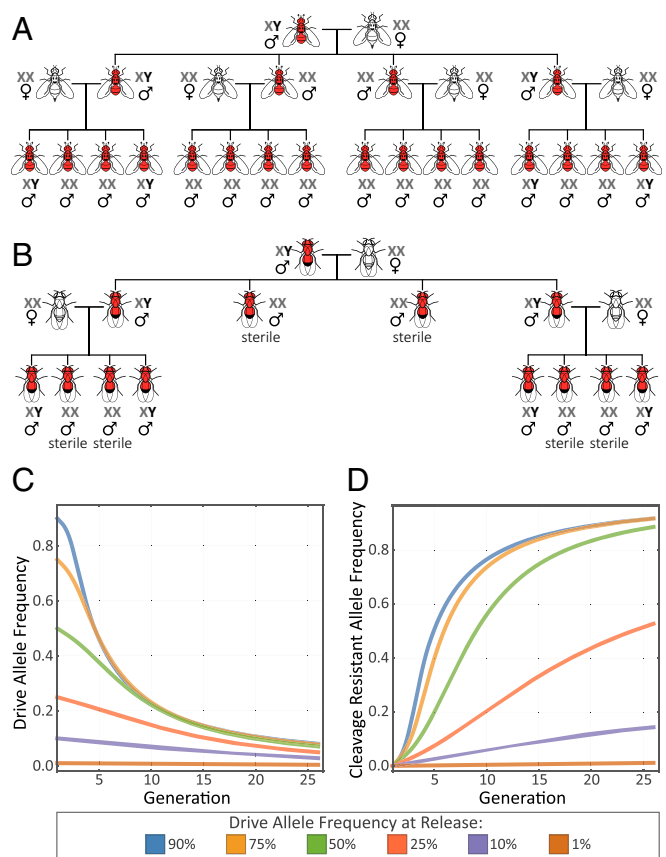


Fig. 1. Insect suppression gene drive based on forced all-male offspring. (A) In *C. capitata*, a *tra*-targeting CHE with both germline and somatic expression causes super-Mendelian inheritance of the red fluorescence-marked CHE-null allele but also results in the transformation of XX individuals into males and in theory leads to a subsequent collapse of the population. (B) In *D. melanogaster*, homing into *tra* in somatic cells transforms XX individuals into sterile pseudomales, which halts the spread of the selfish element but allowed us to safely study the dynamics and molecular consequences of using CHEs in a suppression gene-drive system. (C) Predicted transience of a Cas9-based homing construct targeting the *tra* locus in *D. melanogaster*. Predictions are based on the introduction of *tra*^{CHÉ/+} males at frequencies of 1–90% into a population of otherwise wild-type males and females in equal proportion. We assume a Cas9-mediated cleavage efficiency of 100%, a probability of accurate HDR following cleavage of 90%, one-third of drive-resistant alleles (NHEJ products) being in-frame indels, and no fitness cost associated with in-frame drive-resistant alleles. A construct having these parameter values and released in the form of *tra*^{CHÉ/+}; XY males is expected to be reduced to an allele frequency of less than 10% within ~25 generations with a trajectory tending toward elimination regardless of the introduction frequency. (D) At high release frequencies, the presence of the drive allele results in the generation and establishment of cleavage-resistant alleles in the population. At low release frequencies, which may occur because of accidental escapes, the drive allele will be eliminated early, and the cleavage-resistance alleles will appear at only negligible frequencies. This indicates that *D. melanogaster* is a safe model organism for the evaluation of a *tra*-based suppression gene drive causing sex conversion.

scenarios in XX individuals heterozygous for the drive allele are plausible: (i) Cas9 protein is expressed only in a fraction of the cells, and its activity results in the development of mosaic intersex individuals or (ii) Cas9 is expressed in all somatic cells and uniformly destroys the wild-type *tra* allele, resulting in the development of XX males. In *C. capitata*, the latter will result in development of fertile XX males (12), which can further spread the drive allele into the population (Fig. 1A). It is important to note that it is irrelevant whether the mutation of the wild-type *tra* allele in the somatic cells is based on HDR or NHEJ as long

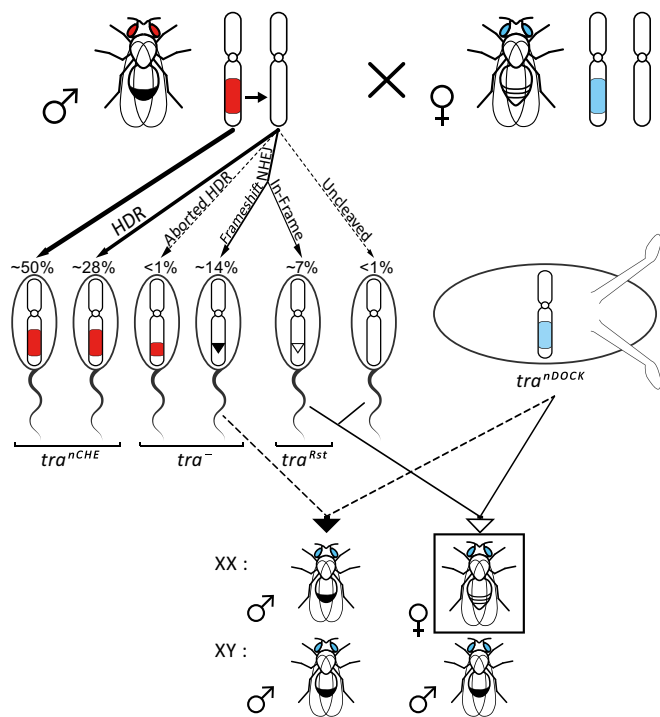


Fig. 2. CHE targeting of the homologous gene locus. By analyzing the ECFP/non-DsRed progeny of $tra^{nCHE/+}$ males and $tra^{nDOCK/+}$ virgins, we focused on non-HDR targeting events at a single tra locus. Estimation of each genotype frequency based on the observed efficiency values is indicated above sperm illustrations. Molecular analysis of the CHE target site in F₁ female progeny (boxed) identified independent NHEJ-derived in-frame indels that resulted in drive-resistant functional alleles (SI Appendix, Fig. S5A).

offspring from this cross revealed a large deletion in the CHE as the result of an aborted HDR event (SI Appendix, Fig. S4A). The other crosses, however, showed an efficient super-Mendelian inheritance, indicating the presence of an active CHE in the mother, which could be a sign of the presence of a tra^{Rst} allele in the mother. Sequencing the tra locus of these mothers confirmed the presence of in-frame indel mutations in the recognition site of the gRNA (similar to sequences in SI Appendix, Fig. S4B). By crossing such females carrying an active CHE with tra^{nCHE} males, we were able to obtain homozygous tra^{nCHE}/tra^{nCHE} males. When these homozygous males were crossed with w^- virgins, all offspring were either male or intersex (SI Appendix, Fig. S3A), which further confirmed the high sex-conversion efficiency of this tra -targeting CHE.

To further evaluate the drive-resistant allele hypothesis and to estimate the rate at which resistant alleles may emerge from NHEJ events, we crossed heterozygous virgins carrying the docking-null allele ($tra^{nDOCK/+}$) with heterozygous ($tra^{nCHE/+}$) driver males (Fig. 2). By looking at the progeny that carry the tra^{nDOCK} allele (marked by ECFP fluorescence) but lack a tra^{nCHE} allele (DsRed fluorescence), we confined our analysis to situations of non-HDR at the paternal wild-type tra allele. Sequencing the tra allele in non-DsRed, ECFP females of the first generation resulted in the discovery of various independent in-frame indel mutations (SI Appendix, Fig. S5A). This suggests that drive-resistant alleles, tra^{Rst} , are readily created as a result of NHEJ in heterozygous males that carry the CHE allele. To determine the frequency at which these tra^{Rst} alleles are generated, we crossed four heterozygous ($tra^{nCHE/+}$) males individually to $tra^{nDOCK/+}$ virgins and sequenced all progeny that showed only an ECFP fluorescence. We identified in-frame indels (tra^{Rst}) in up to 10% of all progeny, representing about one-third of all NHEJ events. The relative high emergence rate of such tra^{Rst}

alleles in the F₁ progeny demonstrates the rapid evolution of resistance as a direct consequence of an active homing CHE (SI Appendix, Fig. S5B) and confirms similar results from other groups (21, 30).

Resistant Allele Dynamics and Spread. To estimate the dynamics of resistance allele emergence and spread in a population, we crossed (in five replicates each) w^- virgins with four different ratios of heterozygous $tra^{nCHE}/+$ males to w^- males and followed the progeny for up to 15 generations. Thereby we documented the sex ratios as well as the spread of the DsRed-marked tra^{nCHE} allele, whose presence in females indicates potential drive-resistant tra^{Rst} alleles (Fig. 3 and SI Appendix, Figs. S6–S8). The ratio of such DsRed-positive females increased progressively over the generations, corresponding to the expected selective increase of resistance allele frequency. To characterize the molecular basis of the resistance to HCGD, we sequenced the tra locus from DsRed-fluorescent females from all the experimental settings at generation F₆ and observed a diverse set of in-frame mutations representing drive-resistant tra^{Rst} alleles (SI Appendix, Fig. S4B). We also selected one setting for a molecular time-course analysis (setting D, replicate 4, at generations F₁, F₂, F₆, and F₁₃) and found that such mutations were heritable (SI Appendix, Fig. S5C). The diversity of these in-frame indels across experimental settings and generations shows that these tra^{Rst} alleles are constantly created, independently of each other, at the site of cleavage. Interestingly, we already had observed DsRed-fluorescent females at the F₁ generation of this replicate. These possessed a wild-type tra allele (SI Appendix, Fig. S5C) but contained a large deletion in the *Cas9* gene of the tra^{nCHE} allele, which was likely the result of a rare, aborted HDR event (similar to that in SI Appendix, Fig. S4A). Following the populations to

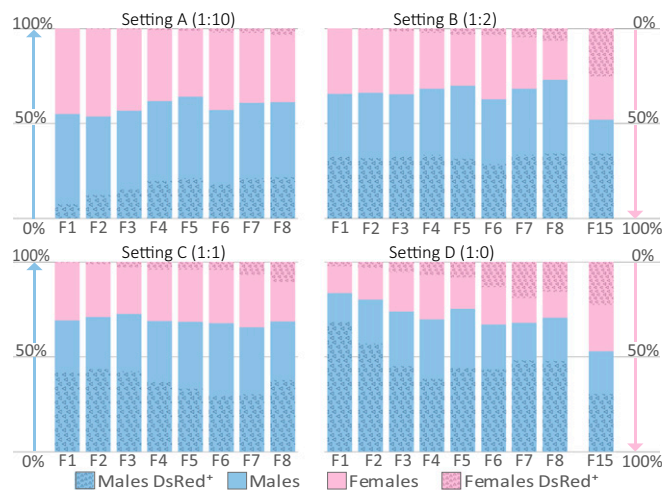


Fig. 3. Dynamics of sex ratio and indicated resistance allele spread in population experiments. w^- virgins were crossed with various ratios of CHE ($tra^{nCHE}/+$) and wild-type (w^-) males (settings A–D). For each setting five replicates were carried out (SI Appendix, Fig. S6). Progeny were screened for sex and the presence of the DsRed eye marker for up to 15 generations. In setting D, where only tra^{nCHE} males were used, a sex ratio of over 80% males was achieved within one generation, indicating the collapse potential of this forced male offspring-only system. In XX embryos, tra^{nCHE} attacks the wild-type tra locus in somatic cells, resulting in intersex individuals (SI Appendix, Fig. S2). Thus, only females carrying nonfunctional defective tra^{nCHE} or drive-resistant functional tra^{Rst} alleles can show the DsRed marker. Therefore, the DsRed marker serves as an indicator for the presence of the tra^{Rst} allele in females, and the rise in the percentage of DsRed females indicates the spread of resistance in the population. Screening the F₁₅ progeny in settings B and D showed that the populations adapted to the presence of the tra^{nCHE} homing allele with the female sex ratio returning to about 50% (SI Appendix, Fig. S6).

generation F_{15} , we found an almost regular 1:1 ratio of males to females in all the replicates (Fig. 3 and *SI Appendix*, Fig. S8), independent of the original frequency of tra^{nCHE} allele inoculation (settings B and D).

A tra -Based Suppression Gene-Drive System for *C. capitata*. Having shown the capability of our proposed CHE in inducing sex conversion in *D. melanogaster* and after identifying potential weaknesses of the system due to resistance evolution, we simulated the outcome of using our proposed method as a pest-control strategy in *C. capitata* (Fig. 4). Our population dynamics simulation results indicate that the evolution of in-frame drive-resistant alleles at rates that we observed in *D. melanogaster* would indeed impede a population collapse in *C. capitata* (Fig. 4A), even if multiple releases were implemented in quick succession (Fig. 4C). To tackle the issue of in-frame drive-resistant alleles (these are problematic because they prevent homing while still allowing tra expression and hence are not removed due to a selective advantage), we considered the use of multiple gRNAs to target the tra gene to reduce the proportion of resistant alleles that are in-frame. Using multiple gRNAs may not have a drastic effect on the overall NHEJ rate but will reduce the in-frame resistant allele formation rate exponentially with each additional gRNA, as each new target site would have to obtain an in-frame mutation that does not affect the function of the protein (22, 31). Our simulation study predicts that by using multiple gRNAs, and thereby reducing the generation rate of in-frame resistance alleles by at least two orders of magnitude, the effectiveness of the system is greatly improved. A single release is still not sufficient to achieve a population collapse in *C. capitata* (Fig. 4B); however, three releases in quick succession are sufficient (Fig. 4D).

The above-mentioned simulations are for the scenario in which $tra^{nCHE}/+$; XX individuals are infertile intersexes; however, we also explored the case in which these individuals are fertile males (Fig. 4 E and F). In *D. melanogaster*, we observed that heterozygous ($tra^{nCHE}/+$) XX individuals develop into mosaic intersex individuals (*SI Appendix*, Fig. S3A). This is likely because the ectopic expression of *Cas9* under the control of the *Rcd-1r* promoter in only a proportion of the cells results in a mosaic phenotype. Since the intersex-based infertility of tra^{nCHE} XX individuals places a fitness load on the system and reduces the drive (by preventing its occurrence in XX individuals), we propose the use of an early embryonic promoter, such as *Sry- α* , for the expression of *Cas9*. Expression from *Sry- α* in germ cells will allow gene drive to occur, and the early blastoderm expression guarantees a uniform destruction of the wild-type tra allele in all cells of the embryo at a very early stage; therefore heterozygous ($tra^{nCHE}/+$) XX individuals could develop into fertile medfly males. This in turn reduces the fitness load associated with the drive allele and increases the drive (by allowing super-Mendelian inheritance of the drive allele to occur in $tra^{nCHE}/+$; XX individuals). Our simulation shows that enabling the fertility of heterozygous ($tra^{nCHE}/+$) XX individuals does enhance the effectiveness of the system in collapsing a *C. capitata* population following a single release, provided that the in-frame drive-resistant allele generation rate is reduced by using multiple gRNAs (Fig. 4 E and F). The tolerable generation rate of in-frame resistant alleles depends on the size of the targeted medfly population. Our simulations predict the extent to which this rate must be reduced to achieve a population collapse as a function of population size (*SI Appendix*, Fig. S9).

Discussion

Our mathematical modeling has shown that two main factors—the formation rate of the in-frame resistance allele and the fitness of heterozygous ($tra^{nCHE}/+$) sex-converted XX individuals—can have a significant effect on the expected outcome of a release in the wild. When heterozygous XX individuals are infertile, our model predicts that a population collapse can be achieved only if multiple

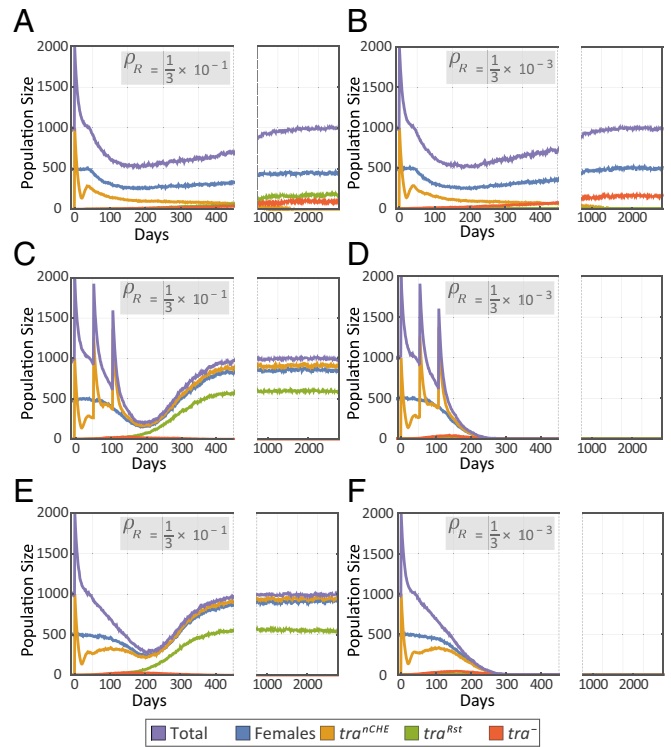


Fig. 4. Predicted dynamics of a Cas9-based homing system targeting the tra locus in *C. capitata*. Predictions are based on the population genetics model depicted in *SI Appendix*, Fig. S10 combined with the population dynamics model depicted in *SI Appendix*, Fig. S11 in which the life cycle of *C. capitata* is divided into four stages, egg, larva, pupa, and adult, with density-dependent mortality occurring at the larval stage (*SI Appendix*, Table S1). Homing occurs only in $tra^{nCHE}/+$ heterozygotes, where “+” represents the wild-type allele and “ tra^{nCHE} ” represents the intact drive allele. We assume a Cas9-mediated cleavage efficiency of 100% and a probability of accurate HDR following cleavage of 90% [NHEJ rate (δ) = 0.1]. By default, in-frame drive-resistant alleles (tra^{rst}) account for one-third of generated resistant alleles, although this proportion may be reduced through gRNA multiplexing. The remaining cleavage-resistant alleles are out-of-frame or other mutations that result in a tra^- -null allele. The equilibrium population size of *C. capitata* is 1,000. Releases consist of 1,000 tra^{nCHE} ; XY males at a single time or at intervals. In A–D, the scenario in which tra^{nCHE} ; XX individuals are infertile intersexes is considered. (A) For a homing efficiency of 90% and an in-frame resistant allele generation rate ($\rho_R = \delta\theta$, where δ is the NHEJ rate and θ is the fraction of NHEJs that produce in-frame indels) of one-third of 10%, a single release of 1,000 tra^{nCHE} ; XY males results in temporary population suppression, halving the adult population size, with the population rebounding over a period of several years. (B) Decreasing the in-frame drive-resistant allele generation rate, ρ_R , by two orders of magnitude to 1/300 of 10%, and hence increasing the out-of-frame resistant allele generation rate, $\rho_B = \delta(1 - \theta)$, to \sim 10%, the population suppression is still only moderate and transient. (C) If three releases of 1,000 tra^{nCHE} ; XY males are carried out in succession, the extent of population suppression is much greater (>75% suppression); however at a ρ_R of one-third of 10%, the population still rebounds over a period of several years with an increase in the frequency of tra^{rst} alleles. (D) If three consecutive releases are carried out for a construct with the decreased in-frame drive-resistant allele generation rate, population elimination can be achieved within \sim 1 year after the last release. (E and F) The scenario in which tra^{nCHE} ; XX individuals are fertile males is considered. For a homing efficiency of 90% and an in-frame resistant-allele generation rate, ρ_R , of one-third of 10%, a single release of 1,000 tra^{nCHE} ; XY males results in temporary population suppression, as in-frame drive-resistant alleles become prevalent, preventing population elimination (E). However, if the in-frame resistant-allele generation rate, ρ_R , is reduced by two orders of magnitude, to 1/300 of 10%, the emergence of in-frame drive-resistant alleles is unlikely, and the population can be eliminated following a single release of 1,000 tra^{nCHE} ; XY males (F).

inundative releases of the driver males are performed (Fig. 4D). While this limitation could potentially be overcome by using an early embryonic stage promoter such as *Sry-α* (Fig. 4F), this may not be desirable to ensure the local treatment of an insect pest population without the potential concern about the elimination of an entire species. Similar considerations have also been brought forward by Prowse et al. (32) with respect to fighting invasive vertebrate species.

In addition, we have shown that in our system it is the generation rate of in-frame drive-resistant alleles, rather than the overall NHEJ rate, that has a significant impact on the outcome of release scenarios. This is of significant importance for species such as *D. melanogaster* (and possibly for related pest species such as *Drosophila suzuki*) that might seem semirefractory toward homing-based gene-drive strategies (27), as it indicates that future designs may not necessarily require an extremely high homing rate but that only lowering the formation rate of the in-frame resistance allele and thus employing multiple gRNAs (22, 31) might be sufficient for an efficient suppression gene-drive strategy in such species.

Our results support the idea that using a CHE to target genes that are essential for female-specific development in insects, such as *tra*, can effectively result in a gender-biased population, finally resulting in a population collapse. This provides a basis for further development of similar suppression gene-drive strategies to introduce a gender bias in wild populations of insect pests such as the medfly or disease vectors. If such a gender bias can be sustained long enough, species-specific elimination of the target species can be achieved. If the HCGDs explored here were applied to efficient pest-control management, the strategy in which

tra^{nCHE/+}; XX individuals are infertile intersexes is safer, because it requires multiple releases to achieve population collapse and hence will cause a population collapse only where these releases are carried out.

Overall, we provide here an example, an implementation strategy, and the mathematical modeling required for the design and optimization of a homing-based sex conversion-suppression gene-drive approach for local or global species-specific elimination of insect pest or disease vector species. Moreover, we show that only lowering the formation rate of in-frame drive-resistant alleles by employing multiple gRNAs may be sufficient to achieve an effective suppression gene-drive outcome, which has important implications for the design of such systems in species that exhibit a low homing rate in their germ cells.

Materials and Methods

Detailed methods on cloning, transgenesis, screening, molecular analysis, stock keeping of *D. melanogaster* strains, population modeling, and simulations can be found in *SI Appendix, SI Materials and Methods*.

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