Synthetically engineered *Medea* gene drive system in the worldwide crop pest *Drosophila suzukii*

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Synthetic gene drive systems possess enormous potential to replace, alter, or suppress wild populations of significant disease vectors and crop pests; however, their utility in diverse populations remains to be demonstrated. Here, we report the creation of a synthetic *Medea* gene drive system in a major worldwide crop pest, *Drosophila suzukii*. We demonstrate that this drive system, based on an engineered maternal "toxin" coupled with a linked embryonic "antidote," is capable of biasing Mendelian inheritance rates with up to 100% efficiency. However, we find that drive resistance, resulting from naturally occurring genetic variation and associated fitness costs, can be selected for and hinder the spread of such a drive. Despite this, our results suggest that this gene drive could maintain itself at high frequencies in a wild population and spread to fixation if either its fitness costs or toxin resistance were reduced, providing a clear path forward for developing future such systems in this pest.

Medea | gene drive | *Drosophila suzukii*

Spotted wing *Drosophila*, *Drosophila suzukii*, is a major worldwide crop pest of various soft-skinned fruits (1). Unlike other drosophilids that prefer to oviposit on overripe fruits, *D. suzukii* utilizes its serrated ovipositor to lay eggs inside ripening fruits, causing significant crop losses (1–3). Found only in Japan before the 1930s (4), in the last several decades *D. suzukii* has spread invasively to every continent except Antarctica (1, 2). In the United States, for example, *D. suzukii* was initially discovered in Santa Cruz, CA, in 2008 and since then has rapidly invaded many states and is a significant threat to fruit industries across the country (2). For example, between 2009 and 2014, *D. suzukii* caused an estimated $39.8 million in revenue losses for the California raspberry industry alone (5) and is responsible for 20–80% crop losses in other fruit production areas (1, 3, 4, 5). Current methods to control *D. suzukii* rely considerably on the use of broad-spectrum insecticides (e.g., malathion), which have variable efficacy (2), are difficult to use due to timing of fruit infestation (6), and face the risk of *D. suzukii*'s evolving resistance (7). While other forms of control may be possible [e.g., the use of recently identified natural predators (8) or oral delivery of dsRNA by microbes (9)], these approaches have not been widely adopted (10, 11). Therefore, given the rapid worldwide spread and potential economic impact of *D. suzukii*, novel effective control measures are urgently needed.

An alternative approach that would complement existing control methods would be the use of engineered *D. suzukii* as a genetic-based control strategy (12). Use of genetically modified insects for wild population manipulation was first suggested over half a century ago (13–15) and has garnered considerable interest in recent years (16–18). In fact, one method of using genetically modified insects for population control, a system called RIDL (Release of Insects carrying a Dominant Lethal) (19–21), where males carrying a repressible dominant lethal transgene are released to mate with wild females and produce nonviable progeny, has recently been implemented in the field. Although this strategy has been shown to be effective in reducing insect populations (22–24), it requires continuous rearing and ongoing inundative releases of large numbers of individuals, making it rather costly and labor-intensive; furthermore, it has not been developed for *D. suzukii*.

Other proposed methods of using genetically modified organisms for population control rely on engineered gene drive systems that function in a non-Mendelian fashion, allowing the drives to increase in frequency with each generation even without conferring fitness advantages to their host (16, 20, 25). Such methods could be utilized to spread desirable genes through populations or even to suppress target populations (26) and are promising self-sustaining tools for various applications where manipulation of wild populations may be desirable (17, 27). A number of engineered gene drive mechanisms have been proposed (16, 17, 20, 25, 26, 28) and tested in the laboratory (29–33); however, to date, only *Medea* (Maternal Effect Dominant Embryonic Arrest) and an underdominance-based approach have been demonstrated to bring about robust population replacement in WT laboratory populations (34–36). Specifically, *Medea* systems rely on expression of a toxin–antidote combination, such as a microRNA (miRNA) toxin that is expressed during oogenesis in *Medea*-bearing mothers, and a tightly linked antidote expressed early during embryogenesis in *Medea*-bearing progeny (Fig. 1*A*). The toxin is inherited by all progeny from a *Medea*-bearing mother, resulting in miRNA-mediated suppression of an essential embryonic gene that causes disruption of normal development during embryogenesis (Fig. 1 *A*–*C*). Offspring that inherit *Medea* receive a tightly linked antidote, consisting of a zygotically active miRNA-resistant copy of the targeted essential gene, that allows for restoration of normal development (Fig. 1*D*); non-*Medea*-bearing progeny from *Medea*-bearing mothers lack this antidote and perish (Fig. 1*E*). Due to this biased inheritance, *Medea* is predicted to rapidly spread itself, and any linked cargo genes, through a target population (34–36).

Significance

Here we describe a fully functional gene drive system constructed in a major worldwide crop pest, *Drosophila suzukii*. This system is composed of a synthetic *Medea* drive with a maternal miRNA "toxin" and a zygotic "antidote," and we demonstrate that it can bias inheritance with 100% efficiency and can persist in a population given high release frequencies. We discuss how such a system may be used to suppress *D. suzukii* populations or render them harmless to target crops.

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Conflict of interest statement: A.B. and O.S.A. have filed a patent on the Suzukii Medea drive system.

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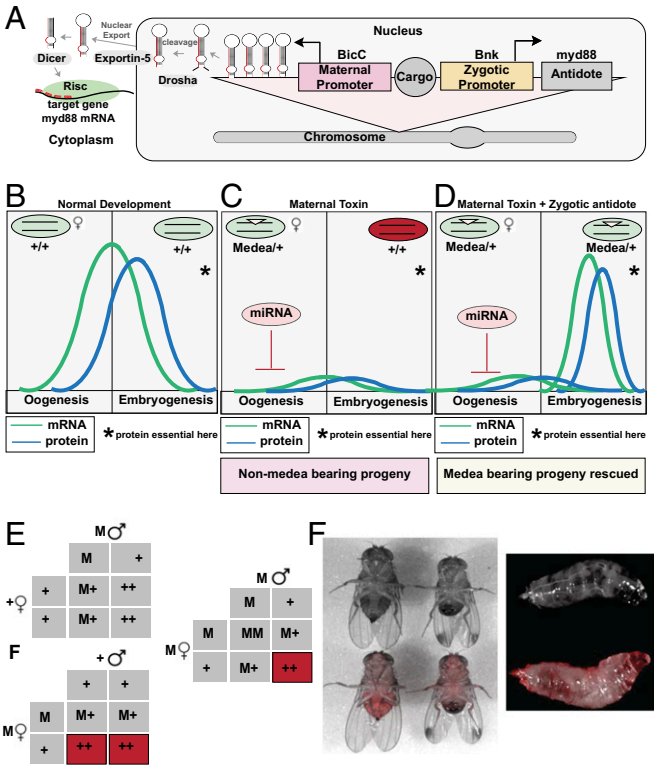

Fig. 1. A synthetic *Medea* system in *D. suzukii*. A *D. suzukii Medea* transgene was generated to comprise an miRNA "toxin" targeting the 5′UTR of *D. suzukii* myd88 expressed under the predicted *D. suzukii* female germline–specific BicC promoter, an "antidote" consisting of *D. suzukii* myd88 coding region driven by the predicted *D. suzukii* early embryo-specific bnk promoter, and two separate transformation markers, eGFP under control of the eye-specific 3xP3 promoter and dsRed under control of the ubiquitous hr5-IE1 promoter (*A*). During normal development maternal myd88 is deposited into the embryo, where it is required for normal development (*B*). The *Medea* miRNA toxin targets myd88 mRNA during oogenesis, preventing proper deposition into the embryo and causing embryonic lethality in progeny that lack the *Medea* system (*C*). In embryos that possess a copy of the *Medea* system, a version of myd88 that is insensitive to the miRNA toxin is expressed during early embryogenesis, rescuing miRNA-induced lethality (*D*). When heterozygous *Medea* males are crossed out to WT females, all progeny survive since the maternal toxin is not expressed; however, when heterozygous *Medea* females are crossed to WT males, 50% of the progeny, the ones that fail to inherit *Medea*, perish. When heterozygous females are crossed to heterozygous males, 75% of the progeny inherit *Medea*, either from the mother or the father, and survive, while those that fail to inherit a *Medea* system perish (*E*). The hr5-IE1 promotes robust expression of dsRed in both *D. suzukii* adults and larvae, allowing for facile identification of *Medea*-bearing individuals (*F*).

For *D. suzukii*, since suppression of the pest population is ultimately desired, a synthetic *Medea* could be used to achieve suppression by spreading a cargo gene proffering susceptibility to a particular chemical, by driving in a conditional lethal gene activated by an environmental cue such as temperature or diapause (36), or by driving a synthetic target site for a population-suppressing homing gene drive system. Although the diapause approach has not been practically demonstrated, mathematical modeling has indicated that this is theoretically possible (36), and the increasing amount of information regarding *D. suzukii* life history (e.g., refs. 37 and 38) may make it more practically feasible. Therefore, given the potential utility of a *Medea* system in *D. suzukii*, we leveraged the limited genetic tools and techniques available in this nonmodel organism, for example the draft genome assembly (39) and transgenesis (40), to engineer a *Medea*-based population control technology. As *D. suzukii* is generally poorly genetically characterized, we had to resolve multiple independent issues to accomplish this feat (e.g., identifying and testing necessary components such as maternal and zygotic promoters and demonstrating the ability to engineer miRNAs that target desired sequences). Notwithstanding, we overcame these challenges and herein describe the successful development of a potent *Medea* system in *D. suzukii*. We demonstrate that this system is capable of drastic biased inheritance to achieve non-Mendelian transmission frequencies of up to 100% in many geographically distinct populations. This represents an example of a synthetic gene drive mechanism developed in a major crop pest.

Results

Generation of a Synthetic *Medea* Gene Drive. To create a synthetic *Medea* gene drive in *D. suzukii*, we engineered a *piggyBac* vector comprising a miRNA toxin coupled with a toxin-resistant antidote, inspired by the architectures used to generate previous *Medea* systems in *Drosophila melanogaster* (36, 34). We designed synthetic miRNAs to target *D. suzukii* myd88, a highly conserved gene shown to be maternally deposited and required for dorsal–ventral patterning in the early embryo in *D. melanogaster* (41). We used the predicted *D. suzukii* female germline-specific bicoid (BicC) promoter to drive expression of a "toxin" consisting of a polycistronic array of four synthetic miRNAs each designed to target the 5′UTR of *D. suzukii* myd88 (Fig. 1*A*). Importantly, to ensure these miRNAs could target the desired sequence, we performed genomic DNA sequencing of the myd88 5′UTR target region in our reference *D. suzukii* strain (collected from Corvallis, OR) and designed the miRNAs against this sequence (Fig. S1). This *Medea* drive also contained an "antidote" consisting of the *D. suzukii* myd88 coding region, insensitive to the miRNAs as it did not contain the miRNA-targeted 5′UTR, driven by the predicted *D. suzukii* early embryo-specific bottleneck (bnk) promoter, and two separate transformation markers: eGFP driven by the eye-specific 3xP3 promoter (42) and dsRed driven by the ubiquitous hr5-IE1 promoter (43).

Characterization of *Medea* Genetic Behavior. Following microinjection of the *Medea* transgene into *D. suzukii* embryos, a single G_1 transformant male was recovered, as identified by ubiquitous hr5-IE1–driven expression of dsRed (Fig. 1*F*), and weak eye-specific 3xP3-driven eGFP. When outcrossed to several WT (non-*Medea*-bearing, +/+) females, this male produced roughly ∼50% *Medea*-bearing and ∼50% WT offspring, as would be expected from standard Mendelian segregation without biased inheritance (Table 1). Resulting heterozygous G_2 *Medea*-bearing progeny were individually outcrossed to WT individuals of the opposite sex to determine inheritance patterns, and these individual outcrosses were continued for six generations (Table 1). Remarkably, until the G_5 generation, all heterozygous *Medea*/+ mothers (n = 91) produced 100% *Medea*-bearing progeny (n = 1,028), while heterozygous *Medea*/+ fathers (n = 16) produced ∼50% *Medea*-bearing progeny (n = 268). While the majority of heterozygous *Medea*/+ G_5 (23/31) and G_6 (16/25) generation females also produced 100% *Medea*-bearing progeny, some heterozygous G_5 (8/31), and G_6 (9/25) females unexpectedly produced a small yet notable number (52/1,219) of WT offspring. Although the exact reason for the difference is unclear, later analysis suggested that resistance to the miRNA toxin might explain this unexpected observation. Notwithstanding, individually these G_5 and G_6 heterozygous *Medea*/+ females displayed significantly biased inheritance rates ranging from 76–96%, with an average rate of 86.4%. Overall, in six generations of individual female outcrosses, the percentage of *Medea*-bearing progeny borne by single heterozygous *Medea*/+ mothers (n = 147) was 97.7% (2,195/2,247; Table 1) as opposed to the 50% that would be expected with standard Mendelian segregation, indicating that the *Medea* drive system is extremely functional at biasing inheritance.

Table 1. *D. suzukii Medea* shows predicted genetic behavior

Generation	Sex (no. crossed)	No. of progeny	Average percent *Medea*, %
G_1	♂ (1)	22	54.5
G_2	♀ (9)	126	100
G_2	♂ (3)	45	43.3
G_3	♀ (32)	299	100
G_3	♂ (12)	201	48.8
G_4	♀ (50)	603	100
G_5	♀ (31)	785	96.8
G_6	♀ (25)	434	93.8
Medea+/total individuals from females (147)		2,195/2,247	97.7

Results of heterozygous *Medea D. suzukii* individual fly outcrosses to WT *D. suzukii*. G1 indicates the offspring from injected G0 individuals, with subsequent numbers (G2–G6) indicating subsequent generations.

***D. suzukii Medea* Exhibits Maternal-Effect Lethality and Zygotic Rescue.** To further characterize the genetics behind the highly biased inheritance patterns described above, additional crosses between individuals of various *Medea* genotypes were performed and confirmed that *Medea* exhibits maternal-effect lethality and zygotic rescue (Table 2). For example, matings between heterozygous *Medea*/+ mothers and WT fathers resulted in 55.63 ± 0.76% total embryo survival with 94.20 ± 1.33% of the progeny being *Medea*-bearing, while matings between heterozygous *Medea*/+ mothers and heterozygous *Medea*/+ fathers yielded 79.11 ± 3.95% total embryo survival with 94.12 ± 0.67% of the progeny being *Medea*-bearing. The higher-than-expected embryo survival is consistent with the observation that not all heterozygous *Medea*/+ mothers give rise to 100% *Medea*-bearing progeny, indicating that not all WT progeny from a heterozygous *Medea*/+ mother perish.

***Medea* Functionality in Geographically Distinct Populations.** To assess whether the *D. suzukii Medea* could function in geographically distinct populations that possibly harbor genetic variability in regions that canonically have less conservation such as the 5′UTR, heterozygous *Medea*/+ flies were tested in eight additional *D. suzukii* strain backgrounds. These strains were collected from various locations around the world, including Mt. Hood, OR; Clayton, WA; Brentwood, CA; Tracy, CA; Watsonville, CA; Oahu, HI; Beltsville, MD; and Ehime, Japan. Interestingly, for three of eight strains the *Medea* inheritance rate from heterozygous *Medea*/+ mothers was 100%, while from five of nine strains the inheritance rate ranged from 87.6 to 99.4%, with an overall transmission rate of 94.2% (Fig. 2). These results strongly demonstrate that the *Medea* drive described here can dominantly bias transmission in diverse *D. suzukii* populations.

Long-Term Population Cage Experiments. The above observations suggested that *D. suzukii Medea* should be able to drive robust population replacement. To test this prediction, we performed several long-term multigenerational population cage experiments specifically challenging the *Medea* drive with a WT strain that harbored preexisting resistance (Corvallis, OR). We set up these population cage studies after maintaining this population for approximately 10 generations; we mated *Medea*-bearing fathers to WT Corvallis, OR strain mothers at three distinct introduction (G_0) frequencies: low frequency (25 heterozygous *Medea*/+ and 25 WT +/+ males mated to 50 WT +/+ virgins, *Medea* allele frequency of ∼12.5% and genotype frequency of ∼25%), medium frequency (50 heterozygous *Medea*/+ males mated to 50 WT +/+ virgins, *Medea* allele frequency of ∼25% and genotype frequency of ∼50%), and high frequency (50 homozygous *Medea*/*Medea* males mated to 50 WT +/+ virgins, *Medea* allele frequency of ∼50% and genotype frequency of ∼50%). These experiments were conducted in separate bottles in biological triplicate for the low- and medium-threshold drives and quadruplicate for the high-threshold drives, producing 10 distinct populations with G_1 *Medea* allele frequencies ranging from ∼12.5–50% and genotype frequencies ranging from ∼25–100%. Altogether, these population cage experiments were followed for nine generations (for lower-allele-frequency populations, as the *Medea* allele disappeared from the population by that time) or 19 generations (for higher-allele-frequency populations), counting the number of *Medea*-bearing adults in each generation to determine the genotype frequency, as described previously (34, 36). Interestingly, the observed changes in *Medea* frequency over time indicated that, for release proportions (defined as the genotype frequency in the G_1 population) of 50% or smaller, the *D. suzukii Medea* drive was unable to drive into the WT population, likely because of selected drive resistance combined with high fitness costs outweighing the effect of drive. However, at higher release proportions of >90%, similar to classical chromosomal rearrangement thresholds (44), the drive largely compensated for the fitness cost, allowing the drive to remain in the population at high frequencies for the duration of the experiment (19 generations; Fig. 3). Although unintended, the self-limiting dynamics of the generated *Medea* system may be useful in achieving a transient population transformation of the type associated with other proposed gene drives (e.g., ref. 45).

Table 2. *D. suzukii Medea* chromosomes show maternal-effect lethality and zygotic rescue

Parental genotype %			Embryo survival, %		Adult transgenic	
♀	♂	Progeny genotype (%)	Predicted	Observed	Predicted	Observed
M/M	M/M	M/M (100%)	100	97.63 ± 0.25	100	100
M/M	+/+	M/+ (100%)	100	95.67 ± 1.87	100	100
+/+	M/M	M/+ (100%)	100	97.07 ± 0.42	100	100
M/+	M/+	M/M (25%) M/+ (50%) +/+ (25%)	75	79.11 ± 3.95	100	94.12 ± 0.67
M/+	+/+	M/+ (50%) +/+ (50%)	50	55.63 ± 0.76	100	94.20 ± 1.33
+/+	+/M	M/+ (50%) +/+ (50%)	100	89.74 ± 1.39	50	48.39 ± 3.22

Crosses between parents of specific genotypes (indicated in the two leftmost columns) were carried out, and progeny survival to crawling first-instar larvae was quantified (% of surviving progeny and SD, third column from right). M indicates *Medea*, + indicates WT, and red text indicates genotypes expected to be inviable. The percentage of transgenic adults resulting from each cross type, together with the SD, was quantified (rightmost column).

Fig. 2. *Medea* functions in diverse populations of *D. suzukii*. Heterozygous *Medea*/+ individuals were crossed with eight geographically distinct *D. suzukii* populations and *Medea* inheritance was measured. Overall, *Medea* biased inheritance with rates ranging from 87.6 to 100%, suggesting that a *Medea* system generated in the laboratory could be utilized to manipulate some, but not all, diverse wild populations of *D. suzukii*. Green stars indicate the collection locations of the flies tested, green pie charts indicate the percentage *Medea* inheritance observed from heterozygous *Medea*/+ females, and shaded areas on the map indicate locations where *D. suzukii* populations have been confirmed. The Corvallis, OR strain was our reference *D. suzukii* strain used to engineer the *Medea*.

Molecular Characterization of Resistance. To understand whether resistance of the target mRNA to the toxin played a role in observed *Medea* inheritance rates of <100%, we performed genomic DNA sequencing of the myd88 5′UTR miRNA target region from randomly selected *Medea*/+ and +/+ progeny from generation 19 of the highest-threshold drive experiments described above to determine whether the miRNA target sites contained any mutations compared with our reference strain (against which the miRNAs were designed). Genomic sequence analysis revealed that, out of four miRNA target sites, one or two sites were perfectly conserved in *Medea*/+ individuals (site 4 or sites 1 and 4, depending on the individual), while only one (site 4) was perfectly conserved in +/+ individuals (Fig. S1). Additionally, for sites that had mutations, some of the mutation types were found in both *Medea*/+ and +/+ flies (an A → T mutation at position 7 of site 1, a C → A mutation at position 3 of site 2, and an A → T mutation at position 6 of site 3), while one mutation (an addition of an A after position 15 for site 1) was only found in +/+ flies, which may be indicative of its role in creating resistance.

To further this analysis, we also sequenced +/+ individuals from all of the geographically distinct populations tested for *Medea* functionality (shown in Fig. 2) and discovered a similar trend. In particular, only one of the four miRNA target sites was perfectly conserved (4), two others (2 and 3) had the same mutations in all strains (and the same mutations found in the *Medea*/+ and +/+ individuals described above), and a third site (1) had variable mutations (either deletion of the A at position 15 or an A → T mutation at position 7) that may correlate with *Medea* efficiency, since all strains with the deletion showed inheritance rates of <100%, while all strains with the A → T mutation had inheritance rates of 100%. Together, these observations indicate that the nature of mutations differed between genetic backgrounds with different observed *Medea* inheritance rates, and that mutations in the 3′ end of target site 1 (in the approximate region of the miRNA seed sequence) in particular seemed to correlate with inheritance rates of <100%. This suggests that the efficiency of the miRNA "toxin" is likely influenced by resistance alleles, which reduce *Medea* transmission; the exact effect of these alleles is worth further investigation, given the ability of such alleles to potentially block diverse types of drive systems in the wild.

Mathematical Modeling. To characterize the population dynamics observed in the above cage experiments, we fitted a mathematical model to the observed data in which the *Medea* drive had an associated fitness cost in heterozygotes and homozygotes and there was a *Medea*-resistant allele present in the population that reduced toxin efficiency. For the fitted model, the *Medea* drive was estimated to have a toxin efficiency of 93% in individuals homozygous for the resistant allele [95% credible interval (CrI): 90–95%] and was assumed to have a toxin efficiency of 100% in individuals lacking the resistant allele. The *Medea* drive was estimated to confer a large fitness cost on its host—28% in heterozygotes (95% CrI: 27–30%) and 65% in homozygotes (95% CrI: 62–67%)—and the resistant allele was estimated to have an initial allele frequency of 78% in the population (95% CrI: 57–97%).

Predictive mathematical modeling based on these parameter estimates suggests that the *Medea* drive would spread to fixation in the absence of toxin resistance if released above a threshold frequency of 79% (Fig. S2*A*). Spread to fixation would also be expected if the fitness costs of the generated *Medea* drive were halved (Fig. S2*C*), even if all individuals in the population were homozygous for the *Medea*-resistant allele (Fig. S2*D*), provided the drive was released above a threshold frequency of ∼25–27%. Consistent with the experimental results (Fig. 3), a *Medea* drive with a large fitness cost in a *Medea*-resistant population is expected to be maintained at high frequencies through its drive; however, its eventual elimination is inevitable unless supplemental releases are carried out. However, for high release frequencies (90–95%), the drive may be maintained at high frequencies (>75%) for ∼20 generations (Fig. S2*B*), which likely exceeds the duration required for agricultural impact. Of note, the ability of the drive to counteract large fitness costs is significant, as demonstrated by comparison with nondriving alleles with analogous fitness costs that rapidly decline in frequency following a 95% release (black lines in Fig. S2 *A* and *C*).

Prior mathematical modeling demonstrated the potential for *Medea* to induce population suppression by spreading a conditional lethal gene into a population (36). In Fig. S3, we illustrate

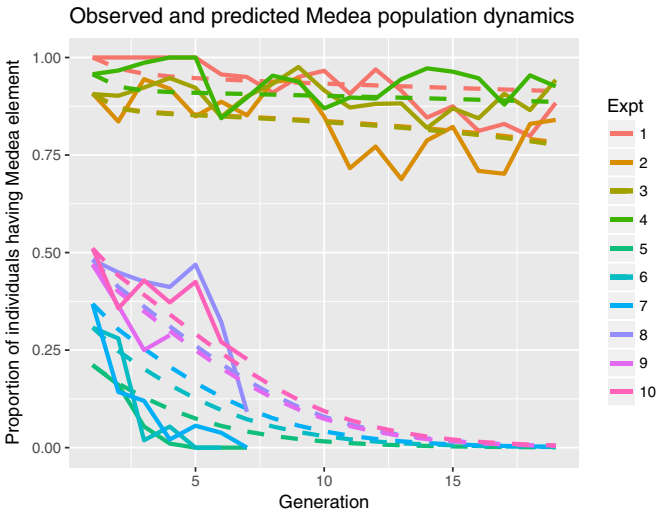

Fig. 3. Observed and predicted dynamics of the *D. suzukii Medea* drive system. Population cage experiments were set up by mating WT (+/+) and heterozygous *Medea* (*Medea*/+) or homozygous *Medea* males (*Medea*/*Medea*) with WT (+/+) females, producing a frequency of heterozygotes (*Medea*/+) in the first generation of 25–100%. Population counts were monitored over 19 generations. Results from these experiments are shown as solid lines, with fitted model predictions shown as dashed lines. Observed data are consistent with a toxin efficiency of 100% in *Medea*-susceptible mothers, 93% in *Medea*-resistant mothers (95% CrI: 90–95%), a heterozygote fitness cost of 28% (95% CrI: 27–30%), a homozygote fitness cost of 65% (95% CrI: 62–67%), and an initial resistant allele population frequency of 78% (95% CrI: 57–97%). For high initial heterozygote frequencies (90–100%), the drive is capable of manipulating inheritance in its favor to maintain its presence at high population frequencies, despite a fitness cost. For lower initial heterozygote frequencies (∼50% or less), the drive is eliminated from the population.

that a *Medea* drive may also be used to drive a synthetic target site for a population-suppressing homing gene drive system. If the *Medea* drive has negligible fitness costs, then the WT allele can be driven out of a population. The *Medea* allele then acts as a target site for a population-suppressing homing construct, which can cause a population crash provided that it carries the zygotic antidote to the *Medea* toxin in its cargo (Fig. S3*D*). Such a two-phased approach may be preferred over the use of a homing-based drive by itself due to the invasiveness of homing-based gene drive systems (46); however, it should be noted that the WT allele acts as a homing-resistant allele following release of the homing construct and hence must first be eliminated entirely. Such approaches require further modeling in finite, structured populations.

Discussion

This study represents a comprehensive characterization of a fully functional *Medea*-based gene drive being challenged with preexisting resistance in long-term, multigenerational population cage experiments (19 generations). The synthetic *Medea* drive described here showed maximal levels of biased inheritance, up to 100% in some populations, but <100% inheritance bias in other populations, and <100% inheritance bias in later generation outcrosses performed with the original population. Although it is not entirely clear why <100% inheritance bias appeared in the patterns observed (e.g., in G_5 and G_6, but not previous generation outcrosses), we hypothesized that, in general, this difference in biased inheritance rates could be attributed to the presence of resistance arising from naturally occurring genetic variation that rendered certain embryos immune to the miRNA toxin. This hypothesis is supported by the sequencing data, as many of the sequenced miRNA target sites contained mutations that likely affected miRNA function and lowered toxin efficiency. Although we did not attempt to measure individual miRNA efficiency, it is possible that not all of the miRNAs are effective at target gene knockdown, and that particular target site mutations reduce toxin efficiency significantly enough to allow survival of a few WT individuals. This is further supported by sequencing data collected from the eight distinct geographic populations, which suggest that certain target site mutations may be correlated with incomplete *Medea*-biased inheritance patterns, although such patterns may also be explained by variable penetrance, and warrants further investigation, as it may have practical implications for the ability to use multiplexing to overcome drive resistance in general (47).

The above observations highlight the importance of resistance as a possible impediment to the use of many kinds of gene drives, including toxin–antidote drive systems, in the field (17, 25, 27). Multiple recent studies have highlighted resistance as a major obstacle to gene drive utility, mostly in the context of homing-based CRISPR/Cas9 drives (47–51). Although a *Medea* drive system may be less prone to resistance-associated spread impediment because, unlike homing-based drive, its mechanism of action is not likely to generate resistant alleles (17, 47), it will face preexisting resistant alleles given the natural genetic diversity found in wild populations. Furthermore, such mutations would be expected to face strong positive selection in the presence of the drive and increase in frequency over time, which would likely expand their effect and mitigate drive spread. Therefore, any meaningful attempt at generating a *Medea*-based gene drive system capable of manipulating diverse wild populations must plan for, and mitigate the effects associated with, both resistant alleles and fitness costs, which may be achieved in several ways.

To reduce the chances of resistance acting as an impediment to spread, sequencing-based characterization of naturally occurring genetic variation in geographically distinct target populations can be used to help guide selection of target sites that are well conserved across all populations in which the drive is intended to function. Additionally, miRNA target site selection could be limited to the coding DNA sequence regions of a genome, which tend to be strongly conserved, as opposed to regions such as the 5′UTR, which canonically have higher tolerance for sequence variation. Finally, the choice of multiple target sites that have been independently validated to achieve knockdown and the creation of a polycistronic "toxin," perhaps consisting of eight miRNAs as opposed to the currently utilized four, may ensure that toxin efficiency is maximally high and unlikely to be deactivated by a single target site mutation. To decrease fitness costs, verification that the "antidote" components (e.g., the recoded targeted gene and promoter used to express said gene) function efficiently enough to restore WT function can ensure that an imperfect antidote does not impose fitness costs. Additionally, reducing the expression of the marker gene to a specific tissue type will likely reduce some of any possible fitness costs associated with high ubiquitous overexpression of an exogenous gene.

Moreover, modeling results suggest that a *Medea* drive having a high fitness cost and high (though imperfect) toxin efficiency may be capable of maintaining itself in a population for a period of several years following a series of large-scale releases of homozygous males. Either decreasing the fitness cost of the drive or minimizing resistance to the toxin is expected to enable the drive to spread to fixation above a release threshold of ∼25–79% (the lower bound corresponds to halved fitness costs). While the stated release thresholds are high, they may be achievable given multiple successive releases and are well below releases associated with the successful sterile insect technique for the Mediterranean fruit fly (52). This may be desirable for biosafety considerations, as *Medea* drive with significant fitness costs is less likely to spread widely (52). *Medea* also has the added benefit that, if fitness costs decline over time, its drive is frequency-dependent and hence large, and intentional releases are more likely to lead to spread than small, unintentional ones (45). That said, the potential and implications of fitness costs' evolving should be further investigated.

Materials and Methods

Construct Assembly. To generate plasmid OA-961B, components were cloned into the *piggyBac* plasmid pBac[3xP3-EGFP afm] (53) using Gibson assembly/EA cloning (54). Specifically, the predicted *D. suzukii bottleneck* (bnk) promoter was amplified from *D. suzukii* genomic DNA using primers 961B.5 and 961B.6, the predicted *D. suzukii* myd88 coding region was amplified from *D. suzukii* genomic DNA using primers 961B.3 and 961B.4, and the SV40 3′UTR fragment was amplified from template pWalium20-10XUAS-3XFLAG-dCas9-VPR (Addgene plasmid 78897) using primers 961B.1 and 961B.2. The pBac [3xP3-EGFP afm] plasmid was digested with AscI and FseI, and the above three fragments were cloned in via EA cloning. The resulting plasmid was then digested with PmeI, and the following fragments were cloned in via EA cloning: the predicted *D. suzukii* Bicaudal-C (BicC) promoter region amplified with primers 961B.7 and 961B.8 from *D. suzukii* genomic DNA, the SV40 3′ UTR fragment amplified with primers 961B.9 and 961B.10 from template pWalium20-10XUAS-3XFLAG-dCas9-VPR (Addgene plasmid 78897), the hr5-IE1 promoter region (43) amplified from vector pIEx-4 (Novagen plasmid 71235-3) using primers 961B.11 and 961B.12, and the dsRed-SV40 3′UTR fragment amplified from template pScarlessHD-DsRed (Addgene plasmid 64703) with primers 961B.13 and 961B.14. Assembled miRNAs were then subcloned into final plasmid OA-961B using PacI and FseI. A list of primer sequences used in the above construct assembly can be found in Table S3; the *D. suzukii* myd88 coding region sequence, bnk promoter region sequence, and BicC promoter region sequence can be found in Table S1. The *D. suzukii* myd88, BicC, and bnk gene orthologs were identified using the Augustus gene prediction tool (55). The full sequence of final plasmid OA-961B is available on Addgene (plasmid 104967).

Fly Culture and Strains. *D. suzukii* WT flies from Corvallis, OR, were a kind gift of P. Shearer, Oregon State University, Corvallis, OR and were maintained under standard conditions described in detail in *Supporting Information*, which also describes the specific schemes utilized to assess *Medea* genetic behavior. To prevent any unintentional release of the *D. suzukii Medea*-bearing flies into the environment we have undertaken stringent precautions including the following measures: (*i*) shatterproof polypropylene plastic vials were used, (*ii*) boxes with triple-contained flies were kept in locked facilities at all times in an institutional biosafety committee-approved BSL2 insectary, and (*iii*) only a single highly expert investigator handled the

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flies. We reasoned that these precautions are sufficient given that spread of this *Medea* drive requires a significant introduction threshold.

Mathematical Modeling. Model fitting was carried out using Bayesian Markov chain Monte Carlo (MCMC) methods in which parameters describing the population dynamics of the *Medea* drive were estimated, including 95% CrIs. Estimated parameters include fitness costs associated with being heterozygous, s_{Het}, or homozygous, s_{Hom}, for the *Medea* drive, and the reduced maternal toxin efficiency associated with the *Medea*-resistant allele, e_R, present in the population at a given initial frequency, p_R. Prior information on the parameter e_R was inferred from G_5 and G_6 outcrosses in which heterozygous *Medea* females were mated with WT males and the proportion of WT offspring was nonzero. A simplified version of the fitted model was used to infer the expected dynamics of the generated *Medea* drive and one with its fitness costs halved in both a fully *Medea*-susceptible population and a fully *Medea*-resistant population. It was also extended to model the use of *Medea* to introduce a target site for a population-suppressing homing construct. The modeling framework is described in *Supporting Information*.

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