Assessing single-locus CRISPR/Cas9-based gene drive variants in the mosquito Aedes aegypti via single-generation crosses and modeling

William Reid, 1,† Adeline E. Williams, 2,3,† Ima Sanchez-Vargas, 2 Jingyi Lin,1 Rucsanda Juncu,1 Ken E. Olson,2 Alexander W.E. Franz 1,∗

1Department of Veterinary Pathobiology, University of Missouri, Columbia, MO 65211, USA.
2Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO 80523, USA
3Present address: Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852, USA.

†These authors contributed equally to this work.

Abstract

The yellow fever mosquito Aedes aegypti is a major vector of arthropod-borne viruses, including dengue, chikungunya, and Zika viruses. A novel approach to mitigate arboviral infections is to generate mosquitoes refractory to infection by overexpressing antiviral effector molecules. Such an approach requires a mechanism to spread these antiviral effectors through a population, for example, by using CRISPR/Cas9-based gene drive systems. Critical to the design of a single-locus autonomous gene drive is that the selected genomic locus is amenable to both gene drive and appropriate expression of the antiviral effector. In our study, we used reverse engineering to target 2 intergenic genomic loci, which had previously shown to be highly permissive for antiviral effector gene expression, and we further investigated the use of 3 promoters (nanos, β2-tubulin, or zpg) for Cas9 expression. We then quantified the accrual of insertions or deletions (indels) after single-generation crossings, measured maternal effects, and assessed fitness costs associated with various transgenic lines to model the rate of gene drive fixation. Overall, MGDrivE modeling suggested that when an autonomous gene drive is placed into an intergenic locus, the gene drive system will eventually be blocked by the accrual of gene drive blocking resistance alleles and ultimately be lost in the population. Moreover, while genomic locus and promoter selection were critically important for the initial establishment of the autonomous gene drive, it was the fitness of the gene drive line that most strongly influenced the persistence of the gene drive in the simulated population. As such, we propose that when autonomous CRISPR/Cas9-based gene drive systems are anchored in an intergenic locus, they temporarily result in a strong population replacement effect, but as gene drive-blocking indels accrue, the gene drive becomes exhausted due to the fixation of CRISPR resistance alleles.

Keywords: gene drive-blocking indels; arbovirus; CRISPR; genomic position effect; population replacement

Introduction

The yellow fever mosquito Aedes aegypti is the principal vector of arthropod-borne viruses (arboviruses) such as dengue, yellow fever, chikungunya, and Zika in tropical regions of the world (Weaver and Vasilakis 2009; Weaver and Reisen 2010; Liu-Helmersson et al. 2019). Presently, the major strategy to control Ae. aegypti populations relies on the use of Bacillus thuringiensis var israelensis (Bti) and chemical insecticides; however, these approaches have led to multiple mechanisms of insecticide resistance, warranting the ongoing quest for alternative methods of control (Manjarres-Suarez and Olvera-Verbel 2013; Liu 2015). Novel approaches, including the incompatible insect technique using the intracellular parasite Wolbachia (Kittayapong et al. 2019; Beebe et al. 2021) and transgenic mosquitoes containing dominant lethal transgenes (RIDL, fsRIDL), have been tested in the field (Carvalho et al. 2015; Waltz 2021). Other promising technologies, including precision-guided sterile insect technique, have been developed for the purpose of localized, confinal management of Ae. aegypti populations (Li et al. 2020, 2021). Another genetic control strategy, termed population replacement, aims at spreading an antipathogen (i.e. antiviral) effector through a targeted mosquito population. Antiviral effectors, specifically those blocking dengue and Zika viruses in Ae. aegypti, have been previously developed and tested (Franz et al. 2006, 2014; Yen et al. 2018; Buchman et al. 2019, 2020; Williams et al. 2020). Linking an autonomous gene drive (GD) system to the antiviral effector could lead to super-Mendelian inheritance of the transgene within the targeted mosquito population (Burt 2003). As a consequence, the proportion of the population harboring the effectors would be increased, resulting in the emergence of mosquitoes refractory to arbovirus transmission. For such a broad-scale population replacement approach, the GD system needs to overcome several limitations, including (1) the
antiviral effector must provide sufficient efficacy to render the targeted population refractory, (2) the inheritance of the GD must be greater than any fitness cost associated with the GD, and (3) the GD must be able to outpace the development of GD-resistant alleles. GD systems utilizing a homing endonuclease gene (HEG) based approach were initially proposed in 2003 and subsequently demonstrated in 2008 in Anopheles gambiae as a fully synthetic homing GD using the DNA cleaving intron-encoded endonuclease I-SceI (Windbichler et al. 2007). The CRISPR/Cas9 system has been modified to allow for HEG-based GDs in Drosophila melanogaster (Gantz and Bier 2015), along with several mosquito species including Anopheles stephensi (Gantz et al. 2015; Adolfi et al. 2020), An. gambiae (Carballar-Lejarrazu et al. 2020), and Ae. aegypti (Li et al. 2020; Verkuilj et al. 2020). The HEG-based GD developed in Ae. aegypti inserted itself into the white gene on chromosome 1 and contained a single-guide RNA (sgRNA) expression cassette that targeted the locus, along with an eGFP eye marker as cargo (Li et al. 2020).

Highly invasive GD systems are thought to carry substantial environmental risks since they are not designed to self-eliminate or be confinable to a region. However, several systems have been developed that would allow for their recall, including systems that destroy or overwrite the GD, and more recently, a "biodegradable" self-eliminating GD system (Wu et al. 2020), with these effectors all being incorporated into the genome in a quasi-random fashion using transposons. While multiple genomic loci have been identified to be suitable for transgene expression in Ae. aegypti, 2 of our previous studies have identified genomic loci that reliably allow for high levels of gene expression in the female midgut following ingestion of a blood meal (Franz et al. 2014; Dong et al. 2017). In these studies, midgut-specific transgenes were placed under control of the carboxypeptidase A promoter and identified 2 genomic loci that exhibited strong transgene expression: "Carb109" (C109) and "TIMP-P4" (T4) (Franz et al. 2014; Dong et al. 2017). Furthermore, the Carb109 locus was found to be highly stable for more than 50 generations for a dengue virus type 2 (DENV2)-targeting inverted-repeat effector, thus representing an ideal genomic locus for the insertion and expression of antiviral effectors. Here, we took a reverse engineering approach to design, build, and test autonomous CRISPR/Cas9-based GD systems that position the GD at intergenic loci known to allow for efficient transgene expression in the midgut and investigated 3 promoters/3'-UTR for the expression of the Cas9 nuclease. Following establishment, we characterized the GD along with fitness parameters of the GD harboring lines and modeled how they would behave as a single release in a confined area. Overall, our study demonstrates the effects of placing an autonomous GD system in an intergenic genomic locus and highlights the many variables that ultimately affect transgenic population fixation in the context of GD performance.

Materials and methods

Generation of cDNA constructs

The constructs used for establishing the GD lines (Fig. 1) were prepared in 3 sequential steps: (1) an "empty" GD destination vector containing the homology arms corresponding to the destination locus (i.e. TIMP-P4 or Carb109), (2) the NLS-Cas9 gene from the pHsp70 Cas9 plasmid (Addgene plasmid # 46294; Gratz et al. 2013) connected to the promoters and 3'-UTRs from the β2tubulin (AAEL019894), nanos (AAEL012107), and imexin-4 (zpg, AAEL006726) genes, respectively, (3) the respective sgRNA targeting either the TIMP-P4 or the Carb109 locus under control of the U6:3 snRNA promoter (AAEL017774).

Construction of "empty" destination vectors for the GD cassettes

The "empty" destination vector for the TIMP-P4 locus (Chr2:32138225) was constructed as previously described for plasmid AaeCFT04 (Supplementary Table 1) with the exception that the eCFP marker was replaced with that of mCherry as a 2-fragment Gibson assembly (Williams et al. 2020). The ORF of mCherry was amplified using primers BR-100 and BR-101, while primers BR-98 and BR-99 were used to amplify the full backbone of the destination vector minus the eCFP marker (Supplementary Table 2). The 2 PCR fragments were then assembled using the HiFi Gibson Assembly kit from NEB (Ipswich, MA) following the manufacturer’s instructions. The "empty" destination vector for the Carb109 locus (3-409699138) was constructed by first amplifying the 459-bp upper homologous arm (genomic positions AaegL5:3-409698681-409699140) using primers BR-368 and BR-122, followed by insertion of the amplicon into pBluescript using KpnI and XhoI. The 541-bp downstream homologous arm (genomic positions AaegL5:3-3-409699139-409699680) was then amplified using primers BR-113 and BR-364, followed by insertion of the amplicon into the previous intermediate plasmid using XhoI and SacII. Finally, the 3xP3-mCherry-SV40 cassette was amplified from the empty TIMP-P4 destination vector using primers BR-54 and BR-55 and cloned into the destination vector using XhoI followed by screening for orientation using primers BR-60 and BR-54.

Construction of the β2tubulinCas9 cassette

The basic components of the β2tubulinCas9 construct were assembled in pUC19 in 3 steps. First, the β2tubulin promoter/5'-UTR (AaeL5.2:326339137–326338047) fragment was amplified from the Ae. aegypti genome (HWE strain) using primers BR-32 and BR-33 and cloned into pUC19 using HindIII and PstI, which introduced an XhoI site upstream of the promoter and an Ncol site at the end of the 5'-UTR of β2tubulin. The 4,272-bp ORF of Cas9 was then amplified from pHsp70 Cas9 plasmid (Addgene plasmid # 46294; Gratz et al. 2013) using primers BR-34 and BR-35 and inserted into the plasmid vector using Ncol and Sall. Finally, the 3'-UTR fragment of β2tubulin (AaeL5.2:326336649–326336454) was amplified using primers BR-28 and BR-29 and inserted into the assembly plasmid vector using SalI and XhoI. The entire cassette was then removable from pUC19 using XhoI and XbaI for cloning...
by yellow-shaded bubbles) were used to drive Cas9 expression in the AeaNosT4GD line. Note: line AeaZpgT4GD has not been generated.

containing the DNA using primers BR-660 and BR-683 and cloned into the vector pHsp70 Cas9 plasmid (Addgene plasmid # 46294; Gratz et al. 2013) was amplified from the Ae. aegypti genome (HWE strain) genomic DNA using primers BR-42 and BR-43 and cloned into the assembly promoter fragment using an XhoI digestion for the plasmid vector. 

Subsequently, the 4,272-bp ORF of Cas9 was amplified from the pHsp70 Cas9 plasmid (Addgene plasmid # 46294; Gratz et al. 2013) using primers BR-99 and BR-41. The PCR product containing the promoter region was digested with XhoI and Sall digest for the PCR product and a PciI/Sall digest for the plasmid vector. Finally, the 594 bp promoter fragment (AaegL5_1:240330–240924) was amplified from the Ae. aegypti genome (HWE strain) genomic DNA using primers BR-42 and BR-43 and cloned into the assembly plasmid vector downstream of the Cas9 ORF using StuI and NheI.

Fig. 1. Schematic representation of the GD constructs used in this study. Word bubbles outside of the plasmid oval represent the combination of cDNA fragments that were switched between the various constructs to generate the transgenic lines tested in this study. Word bubbles that share matching coloration represent the cDNA fragments used for a particular line; for example, for TIMP-P4, all DNA components represented by green-shaded bubbles were contained within the same construct for insertion into the TIMP-P4 locus, while below, the nanos promoter and nanos 3’-UTR (represented by yellow-shaded bubbles) were used to drive Cas9 expression in the AeaNosT4GD line. Note: line AeaZpgT4GD has not been generated.

Construction of the nanosCas9 cassette

The 1,159-bp nanos promoter fragment (AaegL5_1:228706–229865) was amplified from the HWE strain of Ae. aegypti using primers BR-40 and BR-41 and cloned into pBluescript using XhoI followed by screening for orientation using primers BR-99 and BR-41. The 4,272-bp ORF of Cas9 was then amplified from the pHsp70 Cas9 plasmid (Addgene plasmid # 46294; Gratz et al. 2013) using primers BR-34 and BR-35 and inserted into pBluescript downstream of the nanos promoter fragment using a Ncol and Sall digest for the PCR product and a PciI/Sall digest for the plasmid vector. Finally, the 594 bp nanos 3’-UTR fragment (AaegL5_1:240330–240924) was amplified from the Ae. aegypti genome (HWE strain) genomic DNA using primers BR-42 and BR-43 and cloned into the assembly plasmid vector downstream of the Cas9 ORF using StuI and NheI.

Construction of Carb109-zpgCas9GD

The 1,729-bp zpg promoter fragment (AaegL5_2:8486322–84863150) was amplified from genomic DNA of the HWE strain of Ae. aegypti using primers BR-655 and BR-656. The promoter region for the zpg gene in the HWE strain contained a 144-bp deletion (positions AaegL5_2:84863229–848632372), which was 825-bp upstream of the +1 ATG of the zpg ORF. The PCR product containing the promoter region was digested with XhoI and Xhol and cloned into pBluescript. In addition, primer BR-655 was internally tagged and cloned into pBluescript. The promoter region was digested with XhoI/Sall digest for the PCR product and a PciI/Sall digest for the plasmid vector. Finally, the 594 bp promoter fragment (AaegL5_2:84863229–848632372), which was 825-bp upstream of the +1 ATG of the zpg ORF. The PCR product containing the promoter region was digested with XhoI and Xhol and cloned into pBluescript. In addition, primer BR-655 was internally tagged and cloned into pBluescript.

Assembly of the Carb109-3xP3-eCFP eye marker vector

The active U6 promoter from Konet et al. (2007) was synthesized as a gBlock (IIT, Coralville, IA) starting with the promoter region for AAEL017774 (AaegL5_3:382127255–382123154, minus strand) with a G-to-A substitution at the –6 position relative to the TATA box to domesticate a BsiI restriction site and allow for Golden-Gate cloning (Engler et al. 2008). The sgRNA scaffold from Dang et al. (2015) was placed downstream of the promoter and separated by 2 inverted BbsI sites oriented such that the generated 5’ overhang regions were present within the promoter and chIRNA respectively (Gokcezade et al. 2014). Primers tagged with SaclI restriction sites were used to PCR amplify the full gBlock cDNA, which was then cloned into pBluescript using SaclI. Subsequently, the sgRNA programming for all gRNA was prepared by digesting the active Pol III promoter/chIRNA vector with BsiI followed by ligation of the respective adapter primers (BR-360 and BR-361 for the TIMP-P4 locus, BR-362 and BR-363 for the Carb109 locus). Adapters were unphosphorylated and tagged with 5’-AAAT for the protospacer sequence (PAM-sense strand) and 5’-AAAC for the corresponding reverse complement (PAM antisense strand), following the approach outlined in Gokcezade et al. (2014). The respective programmed U6-sgRNA cassettes were then amplified using BR-350 and BR-351 and cloned into the destination vectors using SaclI followed by screening for orientation using the appropriate forward adapter (BR-360 for TIMP-P4 and BR-362 for Carb109) and M13R primers.

Assembly of the pAeT7ku70 plasmid for dsRNA production

A total of 10 fourth instar Ae. aegypti (HWE strain) larvae were collected, flash frozen on dry ice, and then extracted for total RNA using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA).
The T7-tagged PCR product was amplified using HindIII-tagged T7 (BR-80) and cloned into pUC19 using HindIII.

Final assemblies of GD constructs
The final assemblies of the GD constructs were prepared using a 3-step cloning strategy. Cas9 cassettes for the TIMP-P4 or the Carb109 locus were cloned into their respective destination vectors using Xhol/SacII, followed by the addition of the mCherry fluorescent marker under control of the 3xP3 synthetic promoter (Horn et al. 2000) using Xhol, and finally the addition of the appropriate U6 promoter plus sgRNA using SacII. The schematic for our GD constructs is presented in Fig. 1, and the primers used in this study are listed in Supplementary Table 2. The final sequences for the constructs are available at NCBI as outlined in Supplementary Table 1.

Generation of transgenic lines
Mosquito rearing
The HWE strain of Ae. aegypti was used for the generation of all transgenic lines in this study. The larval stages were reared in deionized water at 28°C with a 12:12 (L:D) photoperiod and fed a diet of Tetramin Tropical flakes (Tetramin, Melle, Germany). Pupae were transferred to plastic containers and allowed to emerge in 18 cubic-inch cages and provided with deionized water and raisins as food sources.

CRISPR/Cas9 target site identification
For our initial testing, we first assessed the activities of 6 sgRNAs for activity proximal to the Carb109 genome locus, while the active sgRNA for the TIMP-P4 genome locus was already reported (Williams et al. 2020). To accomplish this, we prepared all injection mix material so that the final concentrations of Cas9-NLS protein was 300 ng/μl and the concentration of sgRNA was 80 ng/μl. Then, 3 sets of approximately 100 preblastoderm embryos were injected with injection mix and incubated overnight (18–24 h) at 27°C before being homogenized and extracted for gDNA into each of 3 pools. The genomic sequence of the Carb109 locus was then PCR amplified using primers BR-60 and BR-65, and the resulting amplicons gel-purified using the Zymo Clean and Concentrator kit (Zymo Research, Irvine, CA). PCR products were subsequently sequenced at the University of Missouri Genomics Technology Core and analyzed visually for trace decay at the predicted CRISPR/Cas9 cleavage site. All products were sequenced in both directions to confirm the correct site of sequence decay.

Harvest and microinjection of embryos
Cages of 7- to 10-day-old mosquitoes were provided with a blood meal (defibrinated sheep blood, Colorado Serum Co., Denver, CO) and maintained at 28°C under a 12:12 (L:D) photoperiod for 4 days prior to injection. Groups of 15–20 hypergravid females were then mouth aspirated into an aluminum foil-wrapped 50 ml conical tube containing two 2.5 cm × 0.5 cm strips of moistened, overlapping Whatman #1 filter paper and allowed to oviposit for 20 min. Early embryos were then aligned over a period of 20–30 min using a fine spotter paint brush such that all posterior ends of the embryos faced the same direction. Aligned embryos were then transferred to double-faced tape (Scotch brand, 3M Columbia, MO) affixed to a plastic coverslip (Thermo Fisher Scientific) and covered with a layer of Halocarbon 27 oil (Sigma-Aldrich, St. Louis, MO) prior to the visible onset of melanization. Embryos were then injected with injection mix using a pulled and beveled micropipette capillary tube (Sutter Instruments, Novato, CA) connected to a Femtojet 5247 air compressor (Eppendorf, Germany) at 650-psi positive pressure and 100-psi backpressure. Immediately following injection, the Halocarbon 27 oil was gently washed from the embryos with deionized water, and the embryos were allowed to age in a humid chamber for a minimum of 2 h prior to transfer to a 500-ml plastic beaker lined with 5–10 layers of moistened Kimwipes. Injected embryos (herein referred to as G0) were then maintained in the humid plastic beaker for 6 days, transferred to a small plastic cup filled with deionized water, and allowed to hatch out. G0 survivors were reared as described above and backcrossed to nontransgenic HWE. For the backcrossing, male G0 individuals were individually provided with 5–10 virgin females and allowed to mate for 3–5 days then pooled into larger cartons using 20 of the smaller cartons for each large carton. Female G0 were collected into large cartons (up to 100 per carton) and provided with half the number of virgin HWE males and allowed to mate for 3–5 days. All cages were then provided with a minimum of 3 blood meals (defibrinated sheep blood, Colorado Serum Co.) and allowed to lay eggs onto filter papers. G1 eggs were allowed to mature for 5 days and were then hatched, reared to fourth instar, and screened for the presence of the fluorescence marker. Positive G1 individuals were subsequently outcrossed to HWE again to establish the transgenic lines. Integration of the transgene was validated by PCR and Sanger sequencing using primers BR-13 and BR-115 for transgenes at the Carb109 locus and primers BR-51 and BR-73 at the TIMP-P4 locus.

Genetic crosses to test for GD activity
An initial pool of OX-0 hemizygous individuals was established for each of the GD lines by outcrossing 40 transgenic males to 200 HWE virgins en masse and allowing them to mate and take a blood meal. The OX-0 eggs were subsequently collected and used as the starting material for each of the drive activity crosses in our study. From the OX-0 pools, a total of 12 hemizygous males were individually cross to 2 virgin HWE females, and 20–25 hemizygous females were crossed to an equal number of male HWE en masse and allowed to mate for 3–5 days. The individual “male founder” containers were each provided with their own blood meals, while the “female founder” container was provided with a common blood meal, followed by the transfer of blood-fed females to individual cartons for egg laying. Following oviposition, egg papers were removed and allowed to develop for a minimum of 5 days prior to hatching individually into 4-oz plastic cups. Larvae were reared to third/fourth instar and scored for the presence of the transgenic marker to determine the level of GD inheritance. Following the scoring of the outcross-1 (OX-1) mosquitoes, 2 pools of crosses representing either a “low” level (i.e. Mendelian-like), or “high” level (i.e. Super-Mendelian-like) were selected from each cross such that an additional 12 female and 12 male hemizygotes could be outcrossed again to HWE for the OX-2 assessment. The selection of the “low” and “high” pool, therefore, did not necessarily represent the lowest and highest levels of GD inheritance, but rather served as representative pools from which to establish the following outcross. From within OX-1, nontransgenic individuals were saved to assess for the presence of GD-blocking indels (GDBI). For the AeaNosC109GD and AeaZpgC109GD lines, all nontransgenic mosquitoes were selected for assessment, while for the AeaNosT4GD and
Aeaβ2tC109<sup>GD</sup> lines, a total of 20 and 10 individuals were selected, respectively. All crosses were duplicated and the data pooled for analysis.

**Statistical analyses**
Comparisons of the OX-1 GD levels for the lines were assessed in R using a Kruskal–Wallis nonparametric ANOVA followed by a Dunn–Bonferroni post hoc means separation procedure to test for significance across all groups and all lines. Comparisons of the OX-2 GD levels, and assessments of GD blocking indels were conducted in R using a Kruskal–Wallis nonparametric ANOVA followed by a Dunn–Bonferroni post hoc means separation procedure to test for significance among individual crosses for all groups within each line. Comparisons of the maternal and paternal contributions were assessed using pairwise T-testing in R following a Shapiro–Wilks test for normality. Deviation from the expected Mendelian inheritance level for the eCFP marker in the paternal contribution testing was conducted manually using the Chi-square analysis.

**Testing for maternal contribution of Cas9-ribonucleoprotein**
Initial crosses to establish trans-heterozygous mosquitoes were conducted using AeaNosC109<sup>GD</sup> females outcrossed to males harboring a 3xP3-eCFP-SV40 fluorescent eye marker at the Carb109 locus (Supplementary Fig. 1). The positionings of the fluorescent markers in both transgenes were in opposite orientation to one another to mitigate the potential for crossing over. Following this cross, fourth instar progeny were screened and selected for the presence of both an mCherry (GD) and an eCFP (null drive) marker. From this cross, 500 females of either the AeaNosC109<sup>GD</sup> or the AeaZpgC109<sup>GD</sup> lines were outcrossed to 200 HWE males. Approximately 1,000 embryos resulting from these crosses were then microinjected with 100 ng/µl of sgRNA targeting the TIMP-P4 locus. Three rounds of injection, each >1,000 embryos were performed for each of the crosses.Embryos were allowed to develop for 1 week prior to hatch, then surviving larvae were reared to L3 and genotyped under a fluorescent microscope for the presence of either the eCFP marker or mCherry marker. The DNA was then individually extracted from each larva and assessed for GDBI at both the Carb109 and TIMP-P4 loci as previously described.

With this assay, any activity at the Carb109 locus in the male (nontransgenic) allele from AeaCFPC109 mosquitoes would indicate a maternal provisioning of both the Cas9 and the sgRNA targeting the Carb109 locus. Since it was possible that the Cas9 protein could be inherited without the sgRNA, we further injected the early embryos with the sgRNA targeting the TIMP-P4 locus. This allowed for us to interrogate whether Cas9 had been supplied maternally (AeaCFPC109 inheriting progeny) or if Cas9 had been supplied maternally and could also be expressed in the early embryo/larva (mCherry GD inheriting).

**Testing for parental effect resulting in GD activity**
To test for a parental effect that results in GD activity, trans-heterozygous crosses were similarly performed as described for the testing for the maternal effect, with the addition that crosses were performed reciprocally to include male trans-heterozygous parents as well. The progeny from these crosses were scored for the numbers of offspring containing either mCherry or an eCFP eye marker to test for the possibility of a fitness cost at the germline level relative to the eCFP (null drive) marker. The female progeny from each of these crosses were saved (within cross) and subsequently mated to an equal number of HWE males and then provided with a single blood meal after which females were separated allowing them to lay eggs individually. Eggs were allowed to mature for at least 5 days and then hatched and scored for the presence of the eCFP marker to determine the level of transgene inheritance.

**Assessment of GDBI**
GDBI were assessed as inheritable refractory indels by analyzing the nucleotide sequence of the alternative inheritable allele in outcrossed hemizygous individuals. For the OX-1 generation of the GD assessment assays, nontransgenic mosquitoes were individually analyzed by PCR using primers BR-20 and BR-23 for the TIMP-P4 locus and BR-60 and BR-65 for the Carb109 locus. PCR products were amplified, purified using the Zymo Clean and Concentrator kit (Zymo Research), and Sanger sequenced. Chromatogram traces were then assessed manually for trace decay at the predicted CRISPR/Cas9 cleavage site. For the assessment of GDBI in the OX-2 generation, all nontransgenic mosquitoes within a cross were pooled and their DNA extracted to obtain an “average” number of GDBI for the nonndriving inheriting mosquitoes. PCR products were then amplified using primers BR-724 and BR-725 for the Carb109 locus and BR-726 and BR-727 for the TIMP-P4 locus to obtain the “round 1” PCR products for illumina whole-amplicon sequencing. The first round PCR was held to 30 cycles. PCR products were subsequently purified using the Zymo Gel Extraction kit (Zymo Research) and adjusted to 1 ng/µl using a Qubit fluorimeter (Thermo Fisher Scientific), and material was subsequently sequenced using paired end reads of 250 bp length (PE250) on an Illumina MiSeq instrument. The resulting reads were trimmed using Cutadapt v1.01 (Martin 2011) and assessed for indels using Crisspresso v2 (Clement et al. 2019). The output files of Crisspresso v2 containing insertions, deletions, and substitutions (assumed to be a composite event of deletion/insertion) were then summed up to generate the value for “indels.”

**Fitness cost studies**
Life parameter data were collected from male hemizygote AeaNosC109<sup>GD</sup> and AeaZpgC109<sup>GD</sup> parentals as well as from HWE for comparison. Each fitness measurement was calculated as an average from a minimum of 100 individual egg papers per mosquito strain ± standard error of the mean. All data were analyzed with 1-way ANOVA, and if a significant difference was found, 1-tailed t-tests were performed comparing HWE and each transgenic line. All mosquitoes were maintained at 28°C with 75–80% relative humidity and a 12 h light/12 h dark cycle. Data are displayed in Supplementary Table 3.

**Fecundity**
Three to five days postemergence, hemizygous males or HWE males were mated en masse with virgin HWE females in ratios of 5 males:1 female using 64-oz cartons (WebstaurantStore, catalog number: 76 9995OU64WB) each containing ~150 mosquitoes. Around 2–3 days later, females were offered an artificial blood meal containing defibrinated sheep blood (Colorado Serum Co.) and 10 mM ATP, after which engorged females were retained. Two to three days later, individual females were placed in 50-ml conical tubes lined with Whatman Grade 1 Qualitative Filter Paper (catalog number: 1001-824) and filled with ~5 ml of tap water. Females were allowed to lay eggs for 1–2 days. Papers were visually inspected for eggs, which were then quantified for fecundity, defined as the number of eggs a female oviposited during 1 gonotrophic cycle. Egg papers were dried for a minimum of 5 days.
Fertility, sex ratios, larva viability, and larva-to-pupa development

Individual egg papers were hatched in ~100 ml of freshly sterilized deionized water using ProPak 1,600 polypropylene clear deli containers (WebstaurantStore) and were offered a ground fish food (Tetramin) slurry ad libitum. Around 2–3 days post-initial hatch, the egg papers were removed from the water and were allowed to dry again overnight. All egg papers were then rehatched in the same containers from which they were initially hatched. Larvae (second to fourth instar) were visually inspected under a fluorescent microscope for expression of the mCherry eye marker. Both positive and negative larvae were counted, but negative larvae were eventually discarded. Fertility was calculated as the total number of larvae/total number of eggs. Female and male pupae were collected every day, up to 14 days post-hatch, and the timepoints of pupation were recorded. Around 150 mosquitoes (either males or females) of each mosquito line were each pooled into small cups and allowed to emerge in 64-oz containers. Sex ratio was defined as the total number of males or females/the total number of pupae; larva-to-pupa development was defined as the average time to pupa development post-egg hatch.

Male competition and adult longevity

Using virgin adults, 50 transgenic and 50 HWE males (age matched) were allowed to mate with 100 HWE females for 2–3 days. Females were then offered an artificial blood meal containing defibrinated sheep blood and 10 mM ATP. Eggs from individual engorged females were obtained as described above for the fecundity assay. Around 5 days later, the egg papers were hatched in sterile water, and larvae were fed ground Tetramin fish food ad libitum. Larvae (second to fourth instars) were visually inspected under a fluorescent microscope for the expression of the mCherry eye marker. Male competition data were calculated as the number of positive G2 larvae/total number of larvae. For the longevity assay, 50 male and 50 female transgenic mosquitoes (5–7 days post-emergence) were maintained in 64-oz containers and offered raisins (as a sugar source) and water. Every day, the number of dead mosquitoes was recorded and longevity was calculated as the average number of days passed when 50% of the male or female mosquitoes had died across cartons.

MGDrvE population modeling

The mosquito GD explorer (MGDrvE v 1.6.0) was used to model the inheritance of the AeaNosC109GD and AeaZpgC109GD lines (Sánchez C et al. 2020). A stable population was set to 10,000 mosquitoes at a 1:1 sex ratio with no migration out of, or into, the main patch. A single release of 2,000 homozygous male mosquitoes (20%) was simulated at day 25 for 100 stochastic samplings for 1,500 days (AeaNosC109GD) and 4,000 days (AeaZpgC109GD). In the MGDrvE package, the cube “Cube-CRISPR2MR.F.R” allows for sex-specific rates of drive and allows for the modeling of 2 types of resistance alleles: those that block the GD, yet cause no fitness cost (R), and those that block the GD but result in a fitness cost (B). Since it was predicted that a GDBI occurring in an intergenic site for the Carb109 locus located 1,214-bp downstream of the mariner/Mos1 insertion site, which is the defining locus in the DENV2-resistant Carb109 line (Franz et al. 2014). Since the Carb109 mariner/Mos1 insertion was determined to be within the 3’ UTR of the polyadenylate-binding protein gene (AAEL010318), we manually selected CRISPR/Cas9 targets as close to, but not within, the AAEL010318 gene locus and assessed for off-targets using any combination of genotypes containing a GD allele and any combination of genotypes without a GD allele to simulation. These values were used to estimate the proportion of a hypothetical linked antiviral effector gene to represent females having a viral refractory phenotype. The parameters used for our model and how they were calculated are provided in Supplementary Table 5. The entomological parameters popGrowth and muAD were left as default, based on literature. tEgg and tPupa were assumed to be 5 and 2, respectively, based on standard laboratory rearing practices and observations. tLarva was calculated as the average number of days of larva-to-pupa development. betaK was calculated as the average fecundity × 4 (the average number of blood meals a female takes during her lifetime)/11 (the average lifespan of a female mosquito in an urban environment) (Otero et al. 2006). The homing rate parameters were determined from Table 3 by adding the marker-positive mosquitoes from the “low” and “high” pools together to obtain the total number of correct homing events. The number of incorrect homing events (i.e. GDBI formation) was estimated by multiplying the total number of marker-negative mosquitoes from the “low” and “high” pools by the fraction of the test OX-1 marker-negative progeny identified to contain GDBI. This was conducted in place of a direct count because a few of the OX-1 marker-negative larva samples did not provide a PCR product that could be sequenced for the assessment of GDBIs. Then, to calculate the parameters cF and cM, the numbers of marker-positive and GDBI-positive mosquitoes were summed and divided by the total N. From these parameters, the proportions that gave rise to correct homing events were determined as chF and chM, with the resistance rate formation parameters cF and cM calculated as 1-chF or 1-chM, respectively. The rate of maternal deposition, df, was calculated as the average rate of GDBI observed for the Carb109 locus for eCFP-inheriting offspring from the trans-heterozygous cross between the GD and the eCFP-blocked GD. For both the AeaNosC109GD and AeaZpgC109GD lines, we then further set the rate of correct maternal effect homing (dhF) to 0 and the rate of incorrect maternal effect homing (dhF) to 1. Larval viability was significantly reduced for the AeaNosC109GD line, which we incorporated into the model under the xif/xM variables by subtracting the average larval viability of the HWE strain from the average larval viability of the AeaNosC109GD line. In the AeaZpgC109GD line, the larval viability was slightly higher than in HWE, but not significantly different. Therefore, we set the parameters xF and xM to NULL for the AeaZpgC109GD line. Finally, according to the approach by Li et al. (2020), we estimated an overall decrease in fertility by 10% for both of our modeled GD lines.

Results

Identification of active target sites and target site polymorphisms

Prior to testing the GD lines, we first identified 2 targets for CRISPR/Cas9 cleavage, which were located in nonprotein encoding regions of the mosquito genome. In our previous work (Williams et al. 2020), we identified a highly active sgRNA for the TIMP-P4 locus (Table 1). In our current work, we identified a novel active CRISPR/Cas9 target site for the Carb109 locus located 1,214-bp downstream of the mariner/Mos1 insertion site, which is the defining locus in the DENV2-resistant Carb109 line (Franz et al. 2014). Since the Carb109 mariner/Mos1 insertion was determined to be within the 3’-UTR of the polyadenylate-binding protein gene (AAEL010318), we manually selected CRISPR/Cas9 targets as close to, but not within, the AAEL010318 gene locus and assessed for off-targets using...
Table 1. Active CRISPR/Cas9 target sites proximal to the original TIMP-P4 and Carb109 mariner/Mos1 insertion loci.

| Locus   | sgRNA number | Genomic locus (distance from original mariner/ Mos1 insertion site) | Activity (# embryo pools/total pools) |
|---------|--------------|---------------------------------------------------------------------|--------------------------------------|
| TIMP-P4 | sgRNA #5*    | 2: 321382225 (623 bp)                                                | Active (3/3)                          |
| Carb109 |              | 2: 3: 409717866 (19,942 bp)                                          | Not active (0/3)                      |
|         |              | 4: 3: 409723215 (25,291 bp)                                          | Not active (0/3)                      |
|         |              | 6: 3: 409699241 (1,317 bp)                                           | Not active (0/3)                      |
|         |              | 15: 3: 40969138 (1,214 bp)                                           | Active (3/3)                          |
|         |              | 21: 3: 409698885 (1,961 bp)                                          | Not active (0/3)                      |
|         |              | 29: 3: 40969526 (1,602 bp)                                           | Not active (0/3)                      |

* Previously reported in Williams et al. (2020).

Establishment of transgenic *Ae. aegypti* lines

A total of 6 transgenic lines (5 of them GD lines and 1 balancer line for Carb109 loci) were established via homologous recombination with embryo injection efforts ranging from 568–1,142 embryos (Table 2). In general, the survival rate of the injected *G. oovo* mosquitoes ranged from a low of 3.9% (AeeaeCFPC109) to a high of 23% (AeNanosC109$^{GD}$). In addition, we found that the nanosCas9-U6sgRNA constructs required no helper Cas9 or helper sgRNA to integrate into the genome. Due to the timing of expression of native nanos, there was coexpression of Cas9 from the injected plasmid, and due to the ubiquitous expression of the AAELO17774 U6 gene, there was coexpression of sgRNA as well. Regardless, after the initial successful injection of the nanosCas9-U6sgRNA construct into the TIMP-P4 locus, helper Cas9 protein was added to all injection mixes, with the omission of the helper sgRNA and continued inclusion of the ku70 dsRNA trigger.

GD inheritance rates over 2 outcrossed generations

To test for the rates of GD inheritance, a starting population was established by outcrossing transgenic males of the GD lines to nontransgenic HWE females to generate hemizygous "OX-0" (outcross 0) mosquitoes. This was performed to ensure that all mosquitoes used for testing were hemizygous. Overall, the strongest GD performance was observed in the *AeNanosC109$^{GD}$* line, where the median inheritance rate among progeny was 70% and 73% for male and female parentals, respectively (Fig. 2 and Supplementary Table 7). Furthermore, the levels of drive ranged from 46% to 49% (no drive, comparable to Mendelian inheritance rates) to super-Mendelian rates, reaching up to 96%. When the same cargo was inserted and retargeted for the TIMP-P4 locus, however, no GD activity was observed with median values for marker inheritance remaining at 49% and 47% for male and female parentals, respectively (Fig. 2 and Supplementary Table 7). Taken together, these results show that nanosCas9 GDs were principally functional in *Ae. aegypti* and overall GD activity was strongly influenced by the GD’s genomic insertion site. Regarding the β2ubulinCas9 controlled GD construct, the median values for marker inheritance demonstrated no drive activity for the *Aeaβ2IC109$^{GD}$*, with overall transgene inheritance rates resembling Mendelian inheritance patterns as they ranged from a low of 43% in male parentals (n = 24/56) to a high of 55% in both male (n = 16/29) and female (n = 11/20) parental groups. Given that GD activity was observed at the Carb109 locus using the nanos promoter, we tested another promoter in the same locus, zero population growth (zpg), which has been shown to have strong GD activity in *An. gambiae* but has not yet been tested in *Ae. aegypti* (Hammond et al. 2016; Kyrou et al. 2018). The median GD inheritance rates for the *Aea0pgC109$^{GD}$* line were 56% and 66% for male and female parentals, respectively (Fig. 2 and Supplementary Table 7). The male transgenic parentals had a significantly lower (P = 0.0258) level of drive when compared to either parental cross of the *AeNanosC109$^{GD}$* line, or to female transgenic parentals of the *Aea0pgC109$^{GD}$* line. These results showed that while the zpg promoter is active in *Ae. aegypti*, there may be a sex-specific effect on Cas9 expression, which was not anticipated for this promoter since the zpg gene (innexin-4) is involved in early germ cell development in both male and female *Drosophila* (Tazuke et al. 2002). Furthermore, given that the median GD inheritance rates for the *Aeaβ2IC109$^{GD}$* line were below 50% for both male and female parentals, it suggested that there was no CRISPR/Cas9 activity in the germline for this line. Recent work by Terradas et al. (2021) indicated that in *An. gambiae*, the β2-tubulin promoter is expressed postmeiotically, which could explain why we did not see GD activity when using this promoter. Therefore, we did not continue the *Aeaβ2IC109$^{GD}$* line for further assessment at the OX-2 level. Meanwhile, although the *AeaNanosT4$^{GD}$* line also did not demonstrate any GD activity, its cargo was identical to that of the *AeaNanosC109$^{GD}$* line, prompting us to continue this line for further assessment at the OX-2 level.

Regarding the OX-2 crosses, we were interested in answering 2 questions: (1) Would the effects of "low" and "high" levels of GD inheritance from the OX-1 crosses be consistent? and (2) Do GDBI accrue differently for female and male transgenic parentals? We assessed for these by first identifying individual crosses from each of the OX-1 groups that represented "low" and "high" levels of drive (Table 3) and subsequently outcrossed transgenic female and males reciprocally to HWE mosquitoes as was performed in OX-1. This resulted in a total of 8 crossing groups within each line to which we applied 3-letter codes that identified the parental (F "female" or M "male"), the grandparental (F "female" or M "male"), and the level of drive observed for the OX-1 pool (L "low" or H "high"). When the progeny (n ranging from 795 to 3,162) for all OX-2 crosses were scored, we again observed Mendelian-like inheritance of the GD cassette for the *AeaNanosT4$^{GD}$* line. However, 1 cross in the FFH group resulted in 90% inheritance (n = 124),
suggesting that while GD activity was inefficient at the TIMP-P4 locus, occasional cases of drive might still occur (Fig. 3 and Supplementary Table 8). The results for the AeaZpgC109 GD line displayed similar levels of drive for all 157 crosses, with median inheritance rates ranging from 52% in the MML group to 59% in the MFL group. Further, among all AeaZpgC109 GD crosses, 5 groups contained individual crosses that demonstrated > 80% rate of GD inheritance. These results suggest that the effect of “low” or “high” level of drive was not inheritable by the OX-2 generation. Finally, for the AeaNosC109GD line, the median GD inheritance rates ranged from 57% in the FML group to 76% in the MFL group (Fig. 3 and Supplementary Table 8). Of particular interest was that in the AeaNosC109GD line, there were outcrosses that occasionally resulted in 100% inheritance of the GD. While these events were rare, they were found across multiple parental and grandparental groups, including the MMH, MFH, FFL, and MFL groups (n = 20, 44, 114, and 137, respectively) (Fig. 3 and Supplementary Table 8). These results highlight that while, overall, the crossing groups within line AeaNosC109 GD line displayed median GD inheritance rates ranging from 57% to 76%, complete drive (100% inheritance) was achievable. Given that the levels of GD in the AeaNosC109 GD line were statistically similar (P > 0.05) for male parentals with any grandparental combinations irrespective of their “low” or “high” level of GD origins, we ruled out the effect of grandparent sex and the possibility that the trait of “low” or “high” GD levels was inheritable.

Accrual of GDBI

To better understand why a broad range in GD activity was observed, we assessed for the occurrence of GDBI among the OX-1 crosses (Fig. 4 and Supplementary Table 9). Overall, the occurrence of GDBI in the OX-1 crosses was most pronounced in the AeaNosC109GD line, which also demonstrated the highest average level of drive (Figs. 2 and 3 and Table 3). Meanwhile, the zpg promoter similarly displayed some GDBI, albeit at a much lower level (7.4% for “low” GD individuals, and 4.3% for “high” GD individuals), and only in pools of offspring originating from females. For both the Aeaβ2tC109GD and AeaNosT4GD lines, no GDBI were

Table 2. Embryo injection efforts to establish the transgenic Aedes aegypti lines used in this study.

| Line name         | Marker | Purpose                        | Embryos injected | Survivors (F:M; %) |
|-------------------|--------|--------------------------------|------------------|-------------------|
| Aeaβ2tT4GD        | 3xP3 mCherry | GD at TIMP-P4 locus           | 1,115            | 72 (31:41; 6.5%)   |
| AeaNosT4GD        | 3xP3 mCherry | GD at TIMP-P4 locus           | 959              | 154 (66:88; 16%)   |
| Aeaβ2tC109GD      | 3xP3 mCherry | GD at Carb109 locus          | 882              | 122 (64:58; 13%)   |
| AeaNosC109GD      | 3xP3 mCherry | GD at Carb109 locus          | 912              | 206 (90:116; 23%)  |
| AeaZpgC109GD      | 3xP3 mCherry | GD at Carb109 locus          | 568              | 45 (17:28; 7.9%)   |
| AeaeCFPC109       | 3xP3 eCFP   | Genetic balancer for Carb109 locus | 1,142          | 44 (24:20; 3.9%)   |
identified in the OX-1 crosses; however, these lines did not demonstrate GD activity and had Mendelian-like inheritance of the marker; thus, it was not expected that GDBI would arise in these lines as there was likely no CRISPR/Cas9 activity. Given that the GDBI identified in the OX-1 crosses were inherited from the original transgenic males, which had been crossed to HWE females in the OX-0 cross, we further assessed the levels of GDBI for the OX-2 crosses to reveal any differences between female and male groups with respect to the number of GDBI that arose in the population (Fig. 4 and Supplementary Table 9). Overall, we identified negligible proportions (<5%) of amplicons containing GDBI in the AeaNosT4GD line, comparable to the single HWE control sample (Fig. 4). This observation was in accordance with the results of the OX-1 GDBI assessment, and again was likely due to the lack of activity of CRISPR/Cas9 when the GD was positioned at the TIMP-P4 locus. Regarding the assessment of the OX-2 GDBI in the AeaNosC109GD line, all crossing groups displayed variable proportions of amplicons containing GDBIs (Supplementary Table 9).

In addition, we observed 9 individual crosses for which the proportion of amplicons containing GDBI was 50% (Fig. 4). A proportion of 50% represents the theoretical maximal proportion of amplicons that can contain indels when considering outcrossed (GD-negative) larvae. Of these 9 crosses, 2 data points were close to 50% (FFL: 54%, MFL: 53%), while the remaining 7 data points ranged from 64% (MMH) to 100% (MML). These elevated proportions of amplicons containing GDBI could be an artifact of PCR chimera. Thus, our analysis of pooled larvae based on high-throughput sequencing was likely overestimating the actual number of GDBI in the sample. One crossing group within the AeaNosC109GD line, MFH, contained very few amplicons showing the presence of indels (median = 14.5%); however, the median proportion of amplicons containing indels was not significantly lower in this group than in the other crossing groups within the AeaNosC109GD line (P = 0.0831). Finally, in the AeaZpgC109GD line, a significantly greater median number of GDBI was observed for the FFL group (median = 2.6%) when compared to the MMH (median = 0.02%) group (Fig. 4 and Supplementary Table 9). In general, all male parental crosses appeared to have fewer GDBI (median range = 0.02%—1.6%) than the female parental crosses (median range = 2.0%—11.9%), regardless of the sex of the grandparental transgenic individual. As explained above, this result

**Table 3.** Testing of nontransgenic offspring from OX-1 individuals to assess for the presence of GDBI among the GD lines.

| Line            | GD parental | GD inheritance rate (% marker inheritance; total number assessed) | Number of GD-negative larvae with indels/total number of GD-negative larvae assessed (%) |
|-----------------|-------------|------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| AeaNosT4GD      | Male Low   | (45.9%; 290) 0/40 (0%)                                           |                                                                                           |
|                 | High       | (58.1%; 136) 0/40 (0)                                           |                                                                                           |
|                 | Female Low | (42.9%; 163) 0/40 (0)                                           |                                                                                           |
|                 | High       | (51.3%; 117) 0/40 (0)                                           |                                                                                           |
| AeaNosC109GD    | Male Low   | (55.3%; 228) 9/54 (16.7)                                        |                                                                                           |
|                 | High       | (86.4%; 214) 7/27 (25.9)                                        |                                                                                           |
|                 | Female Low | (61.5%; 148) 7/46 (15.2)                                        |                                                                                           |
|                 | High       | (89.7%; 136) 7/12 (58.3)                                        |                                                                                           |
| AeaZpgC109GD    | Male Low   | (56.5%; 232) 0/41 (0)                                           |                                                                                           |
|                 | High       | (59.2%; 228) 0/51 (0)                                           |                                                                                           |
|                 | Female Low | (57.1%; 98) 2/27 (7.4)                                          |                                                                                           |
|                 | Male No GD | (50.7%; 138) 0/10 (0)                                           |                                                                                           |
|                 | Female No GD (52.5%; 40) 0/10 (0)                               |                                                                                           |

**Fig. 3.** Frequency of marker gene inheritance in progeny from second-outcrossed (OX-2) individuals (594 groups, average n = 72 ± 2, total n = 43,015) originating from either “high” or “low” parental GD pools harboring the GD at 2 different genomic loci (Carb109 and TIMP-P4) with Cas9 expression under the control of 2 different promoters (nanos or zpg). The bar inside each box plot represents the median value, while the lower and upper borders of the boxes represent Q1 and Q3, respectively. Groups within a GD line that are superseded with the same letter are not statistically different (P > 0.05). The first letter of each cross designation indicates the OX-2 parental transgenic sex and the second letter indicates the OX-1 grandparental transgenic sex, where F = female and M = male. L = low/no drive level in the grandparental generation, H = high drive level in the grandparental generation. Each data point represents the percentage of transgene inheritance resulting from the offspring of the parental crosses where each transgenic female parental was allowed to mate with 2 nontransgenic males, and each transgenic male parental was allowed to mate with 2 nontransgenic females. A minimum of 20 larvae was set for each group in order to be scored.
was unexpected for the zpg promoter (Tazuke et al. 2002). Interestingly, there was no apparent grandparental effect for the zpgCas9 controlled GD. In addition to the abundance of GDBI assessed for the OX-2 cross, we also assessed the size distributions of the resultant insertions/deletions (Supplementary Fig. 2). Overall, deletions were the most abundant type of indel, representing 86%, 94%, and 97% of all indels for the AeaNosT4GD, AeaNosC109GD, and AeaZpgC109GD lines, respectively. Nearly all identified deletions were present within 50 bp of the CRISPR/Cas9 target site, with the largest deletion being 82 bp in the AeaZpgC109GD line. The largest identified insertion was 60 bp in the AeaNosC109GD line, although nearly 100% of the insertions in all lines tested were within 50 bp of the CRISPR/Cas9 target site. Taken together, these results suggest that for the purpose of an autonomous GD system in Ae. aegypti, the homologous region required for strand invasion of the cleaved DNA proximal to the predicted CRISPR/Cas9 target is likely a minimum of 50 bp in size.

**Testing for maternal contribution of CRISPR/Cas9**

Maternal and/or paternal contribution of the CRISPR/Cas9 components leads to extra-germline activity of the Cas9 ribonuclease-protein (RNF) in the early embryo, which could result in the development of GDBI. Notably, this will occur in offspring that do not inherit the GD allele, which in the absence of a donor for HDR would cause GDBI formation. To test for maternal contribution, the paternal allele must be distinctly identifiable from the inherited maternal allele when the GD allele is absent. Previous studies have used recessive phenotypes to identify these effects (Chaverra-Rodriguez et al. 2018; Li et al. 2020; Carballar-Lejarazu et al. 2022), where the maternal allele is balanced over a null-allele for the recessive marker and the female is then outcrossed to a wild-type male. The progeny from this cross is phenotypically wild-type and genotypically heterozygous. In the event of cleavage of the paternal allele, however, the progeny will be biallelic/homozygous and display the recessive phenotype. Since we targeted an intergenic locus, we accomplished the same goal through the development of the AeaeCFPC109 line, which contained a marker-only cargo and no GD construct: a codominant alternate color marker (eCFP). This construct was then integrated into the Ae. aegypti genome through homologous recombination using sgRNA #15 and was therefore positioned at the same (Carb109) locus as the mCherry-marked GD systems. Since the sgRNA #15 target site was interrupted by the 3xP3-eCFP marker, it established a "blocked drive" because the sgRNA #15 protosporas is no longer present in the genome. When the AeaeCFPC109 line is crossed to either the AeaNosC109GD or the AeaZpgC109GD lines, offspring containing both eCFP and mCherry eye markers would harbor 1 allele with an eCFP marker, and the other allele with the mCherry-marked GD. No drive or GDBI activity would occur in the germline as the genomic GD target is blocked by the eCFP marker meaning that the Carb109/sgRNA #15 locus is no longer present (Supplementary Fig. 1). The genotypes of the eggs of trans-heterozygous females resulting from the cross will then consist of either the mCherry-marked GD or the GD-blocking eCFP marker. When these trans-heterozygous females are outcrossed to nontransgenic male HWE mosquitoes, the males will contribute an allele that is targetable by CRISPR/Cas9 in the embryo. This then can allow for GD/cleavage activity to be assessed. Given that CRISPR/Cas9 activity requires both the Cas9 protein and the sgRNA transcript, we further microinjected embryos from the cross with synthetic sgRNA targeting a secondary genomic locus (TIMP-P4 locus). These assays allowed us to reveal any maternal effect in the early embryo and this way provided insight to possible early embryo (extra-germline) activity of CRISPR/Cas9 components. Three sets of approximately 1,000 embryos were injected for each of the HWE outcrossed trans-heterozygotes (WT × [AeaNosC109GD/AeaeCFPC109] and WT × [AeaZpgC109GD/AeaeCFPC109]) and
allowed to develop to third instar to enable visual genotyping of the fluorescent marker (Table 4). DNA was extracted from individually surviving mosquitoes and PCR followed by Sanger sequencing was conducted across both the Carb109 and TIMP-P4 loci to assess for the presence of GDBI at the target loci. We observed for both the AeaNosC109GD and the AeaZpgC109GD lines CRISPR/Cas9 activity in the GD-targeted Carb109 locus and in the TIMP-P4 locus targeted via injected sgRNA. We also found that the survival rate of sgRNA-injected trans-heterozygotes was lower when compared to that of the sgRNA-injected (nontransgenic) HWE strain, which could be due to the concurrent targeting of 2 loci on different chromosomes.

In general, the CRISPR/Cas9 activity observed for maternal deposition (inheriting the eCFP eye marker) was highest in the AeaNosC109GD line, with 40% of the larvae demonstrating Cas9 activity at the TIMP-P4 locus and 19% displaying Cas9 activity at the Carb109 GD locus (Fig. 5). In the AeaZpgC109GD line, 16% and 14% of larvae exhibited Cas9 activity at the TIMP-P4 and Carb109 sites, respectively, although statistically, these values were not significantly different across genomic targets or mosquito lines (P > 0.05) (Fig. 5). We did, however, find that in the AeaNosC109GD line, significantly more indels were detectable at the Carb109 locus when the GD allele was inherited (P = 0.0309). In addition, there were significantly more indels present among GD inheriting progeny from the AeaNosC109GD line than among GD inheriting progeny arising from the AeaZpgC109GD line crosses (P = 0.0051). Taken together, these results suggest a clear and strong maternal inheritance of the CRISPR/Cas9 RNP. Furthermore, early embryonic activity of Cas9 was stronger when additional sgRNA was supplied, either exogenously through microinjection of synthetic TIMP-P4 targeting sgRNA, or endogenously as Pol-III expressed.

### Testing for a parental contribution of CRISPR/Cas9 resulting in GD activity

To test for a parental effect resulting in GD activity, we performed reciprocal trans-heterozygous crosses as outlined above. In addition to female trans-heterozygotes outcrossed to nontransgenic males, we also tested male trans-heterozygotes outcrossed to nontransgenic females (Fig. 6a). In the first outcross (OX-1) of the trans-heterozygotes individuals, we found that the inheritance rates of the eCFP eye marker and the mCherry-marked GD were nearly 50% for both females and males and were not statistically different when tested using pairwise T-testing (P = 0.5911 and P = 0.7587, respectively). When females from the eCFP-inheriting individuals were further outcrossed to nontransgenic (OX-2) to investigate the level of parental GD contribution, the median inheritance rate was 47% for offspring from eCFP expressing females and 49% for offspring from eCFP expressing male parents (Fig. 6b), providing no evidence for a parental effect resulting in GD activity; regardless, a few data points were greater than 50% with 2 points reaching ~80%.

### Table 4. Numbers of embryos injected for maternal contribution testing and resulting genotype frequencies of surviving larvae used for CRISPR/Cas9 activity assessment.

| Cross                  | Rep | Embryos injected | Surviving larvae, n (% of injected) | eCFP, n (% of larvae) | mCherry, n (% of larvae) |
|------------------------|-----|------------------|------------------------------------|----------------------|-------------------------|
| AeaNosC109GD × AeaCFPC109 | 1   | 1,056            | 26 (2.4)                           | 15 (53.8)            | 12 (46.1)               |
|                        | 2   | 1,017            | 33 (3.2)                           | 18 (54.5)            | 15 (45.5)               |
|                        | 3   | 1,170            | 24 (2.0)                           | 14 (58.3)            | 10 (41.7)               |
| AeaZpgC109GD × AeaCFPC109 | 1   | 1,007            | 23 (2.3)                           | 13 (56.5)            | 10 (43.5)               |
|                        | 2   | 1,015            | 26 (2.6)                           | 12 (46.1)            | 14 (53.8)               |
|                        | 3   | 1,004            | 46 (4.6)                           | 22 (47.8)            | 24 (52.2)               |

Based on the experimentally derived data regarding GD inheritance including frequency of GDBI accrual, testing for maternal effect of the CRISPR/Cas9 RNP, and analysis of fitness parameters of the GD harboring mosquito lines (Supplementary Table 3), we modeled the performance of AeaNosC109GD or AeaZpgC109GD in a hypothetical field release scenario for the purpose of population replacement (Fig. 7 and Supplementary Tables 4 and 5). To model the lines, we used the MGDrivE mosquito population modeling package in R (Sánchez et al. 2020) and modeled for a static population of A. aegypti with a stable population size of 10,000 mosquitoes at a sex ratio of 1:1. We allowed for a single release of males at day 25 at a rate of 1/5th of the population (i.e. 2,000 homozygous male mosquitoes) and performed 100 stochastic simulations for each of the transgenic lines until the allelic frequencies of the genotypes stabilized. Given that we targeted an intergenic region for GD insertion, all GDBI were assumed to incur no fitness cost to the mosquito, and we therefore merged the “B” allele from MGDrivE (i.e. a GD resistance allele that results in a fitness cost) with the “R” allele (GD resistance allele with no fitness cost), such that all resistance alleles had the same fitness as the nontransgenic alleles (Supplementary Table 4). Figure 7 displays the various prevalence levels of each genotype in the predicted model for females (all genotypes), males (all genotypes), and females divided between genotypes carrying at least 1 copy of the GD (displayed as “Hypothetical antiviral effector gene” to model a linked antiviral effector) and genotypes not carrying the GD (displayed as “wild-type”). Overall, there was a rapid decline in the number of wild-type mosquitoes for both simulated releases and the rate at which the genotypes stabilized within a population differed between the 2 transgenic lines. In the AeaNosC109GD line, the homozygous wild-type genotype was eliminated by ~400 days postrelease (PR) in the simulated closed system, with the hemizygous GD (no GDBI) and homozygous GD genotypes quickly replacing the homozygous wild-type genotype. By 450–500 days PR, however, the GDBI harboring resistance allele genotype became dominant and rapidly overtook the population eventually causing the GD to fall out of the population and the near entirety of the population to contain GDBI alleles by 1,500 days PR. When a hypothetical antiviral effector linked to the GD was considered, the proportion of female mosquitoes carrying at least 1 copy of the antiviral effector gene peaked at 320–370 days PR with ~80% of females carrying at least 1 copy of the antiviral effector. Any window of antiviral protection eventually causing the GD to fall out of the population and the near entirety of the population to contain GDBI alleles by 1,500 days PR.
Discussion

Previous GD studies in mosquitoes have targeted recessive marker genes that provide for a visual phenotype when both alleles are disrupted. Since we selected intergenic loci, TIMP-P4 and Carb109, any disruption of these loci would not result in...
The spatio-temporal expression of transgenes in *Ae. aegypti* is strongly influenced by the promoter used for expression as well as the genomic transgene insertion site, which can be affected by position effect variegation (Henikoff 1992; Franz et al. 2009). The importance of genomic position for optimal Cas9 activity in *Ae. aegypti* was identified in the work by Li et al. (2017), where the activity of Cas9 expression varied by genomic locus when placed under control of 6 different promoters and integrated into the *Ae. aegypti* genome in a quasi-random fashion using the piggyBac transposable element. The forward approach from Li et al. (2017) allowed for the identification of optimal genomic insertion loci for Cas9 activity in the germline, while in our study, we took a reverse approach by utilizing 2 genomic loci (TIMP-P4 and Carb109) previously identified to be highly permissive to antiviral transgene expression (Franz et al. 2014; Williams et al. 2020). Our reasoning for this was that if the GD cassettes were positioned in a genomic locus that supported a strong expression of antiviral effectors, the entire system could be built and established as a single-locus autonomous GD line. It was unclear, however, if the autonomous GD in an intergenic locus would perform. Our reverse engineering approach demonstrated that genomic loci that are well-suited toward the expression of antiviral effectors may not necessarily be ideal for the functioning of a CRISPR/Cas9 autonomous GD system. Notably, the presence of the GD system at the TIMP-P4 locus resulted in no GD activity and further demonstrated a very low level of inheritable GDBI when assessed via whole-amplicon sequencing. This suggests that the GD components were not efficiently expressed in the germline when positioned at the TIMP-P4 locus although our previous work demonstrated strong expression of transgenes in the adult female midgut at the same locus (Dong et al. 2017; Williams et al. 2020). Meanwhile, a second genomic locus that had also been shown to be ideal for stable antiviral activity (i.e. Carb109; Franz et al. 2014) exhibited high GD activity, however, there was a concomitant level of GDBI detectable that eventually blocked the GD in our population modeling simulation. This demonstrated that while the Carb109 locus supported GD activity by allowing rapid introgression of either GD cassette into the target population, the locus was prone to the generation of heritable GDBI, which over time blocked and eliminated the GD in the population modeling.
exhausting. Our simulation models provided valuable information regarding the dynamics of the GD. For example, the AeaZpgC109GD line provided a wider hypothetical window of protection (females harboring at least 1 copy of the GD and a hypothetical antiviral effector) than the AeaNosC109GD line, although the latter GD line had stronger GD inheritance. The major differences in GD performance and biological parameters between the AeaNosC109GD and AeaZpgC109GD lines were the levels of GD activity, female deposition rate, resistance allele formation rate, and pupation success. To estimate which of these effects most strongly influenced the performance of the GD, we conducted simulations of the AeaNosC109GD line in which maternal deposition rate, resistance allele formation rates, or pupation success parameters were substituted with the respective (experimentally determined) parameters from the AeaZpgC109GD line (Supplementary Fig. 3). However, there was no appreciable difference in overall GD persistence when the levels of GDBI resistance alleles, or the levels of maternal deposition were reduced in the AeaNosC109GD line. Furthermore, Terradas et al. (2021) investigated the deposition of germline expressed genes in the developing oocytes of autonomous Anopheles sp. GD lines, including the AgNosCd1GD line (Carballar-Lejarazu et al. 2020), which used the nanos promoter and 3′UTR for Cas9 expression. In their study, the authors found that nanos driven Cas9 transcripts were indeed expressed in the nurse cells along with native nanos transcripts; however, unlike the native nanos transcripts, the Cas9 transcripts were found at very low levels or were even absent in the developing oocyte (Terradas et al. 2021). In males, by contrast, Cas9 expression controlled by the nanos regulatory elements paralleled that of the native nanos transcript. In regard to the β2-tubulin regulatory elements, it was shown by the same authors that in An. stephensi, β2-tubulin transcripts were male specific. In addition, transcript expression occurred in the late stages of spermatogenesis, which may not be a suitable time window of expression when attempting to use the β2-tubulin promoter for a CRISPR/Cas9-based GD. We speculate that this could be a reason for the lack of GD activity of our Aeaβ2C109GD line. Furthermore, Terradas et al. (2021) investigated the pattern of native zpg expression and found that its expression pattern was similar to that of vasa but at a lower general level. They found that there was expression of zpg throughout premeiotic and meiotic stages in males and high-level zpg expression in the sperm flagella, which could increase the prevalence of GDBI. In our AeaZpgC109GD line, however, we identified a lower level of GDBI for male parentals and a lower level of GD compared to the AeaNosC109GD line. Thus, it is likely that the expression of the Cas9 transcript under control of the zpg promoter does not perfectly parallel that of the native zpg transcript. Furthermore, although the An. gambiae AgNosCd1GD line did not have an appreciable deposition of mRNA transcripts for Cas9 when expressed from the nanos promoter and 3′UTR (Terradas et al. 2021), a recent analysis of GDBI by Carballar-Lejarazu et al. (2022) found that there was indeed a maternal effect of CRISPR/Cas9 activity in the AgNosCd1GD line. Taken together, these results suggest that the maternal contribution could be provisioned to the developing oocyte as residual Cas9 RNP complexes might be expressed during early germline development and further persist in the zygote.

Simulations of the different GD performances and life parameters of the AeaNosC109GD and AeaZpgC109GD lines highlight the importance of fitness and maternal contribution with respect to the accrual of GDBI. Our population modeling revealed that in the absence of any fitness costs directly associated with GDBI, yet there was still a fitness cost associated with the GD as such because an autonomous GD in an intergenic locus will ultimately block itself due to the accrual of GDBI alleles and the drive will be lost. Therefore, an accumulation of GDBI alleles will ultimately exhaust and impair the GD. Given the significance of these observations obtained from our simulated release study, multigenerational cage trials aiming at modeling population replacement under more realistic field conditions (Adolfi et al. 2020; Carballar-Lejarazu et al. 2020) will be imperative in the future to confirm the effects of fitness cost on single-locus GD performance in A. aegypti.

Since we selected intergenic loci, which were assumed to have no effect on fitness if cleaved, the persistence of the autonomous drive system could be strengthened by its placement into a protein-encoding locus as performed for kh in An. stephensi (Gantz et al. 2015; Adolfi et al. 2020). This could also extend the window of protection provided by the antiviral effector. Nevertheless, there is a practical rationale for a nonpersistent GD: the associated antipathogen effector gene could lose its efficacy over time. Such a loss of efficacy could be due to adaptations of the pathogen to evade the effect of the antipathogen effector gene, or spontaneous mutations leading to a loss of function of the antipathogen effector. An autonomous GD system that will ultimately self-block may have several benefits as it could rapidly drive an antipathogen transgene through wild mosquito populations while providing a window of protection from a circulating arbovirus, before dropping out of the population due to the fixation of GDBI alleles.

Data availability

The nucleotide sequences for all our GD constructs listed in Supplementary Table 1 are available at NCBI (ncbi.nlm.nih.gov) under the following accession numbers: construct AeaecCFPT4: accession # MT926371; construct AaeecCFPC109: accession # OL452018; construct AeaNosT4GD: accession # OL452014; construct Aeaβ2C109GD: accession # OL452017; construct AeaNosC109GD: accession # OL452015; construct AeaZpgC109GD: accession # OL452016; construct pAeU6-MT: accession # OL452019; and construct pAeT7ku70dsRNA: accession # OL452021. The code modification to MGDrivE is provided in Supplementary Table 4.

Supplemental material is available at G3 online.

Acknowledgments

The authors would like to thank Drs. Héctor Sánchez Castellanos, Rodrigo Corder, and John Marshall for their guidance and assistance with the use of the MGDrivE simulation for population modeling. The authors thank Drs. Hanno Schmidt and Gregory Lanzaro for kindly sharing the genomic vcf data for the A. aegypti genomes and Susi Bennett for all her mosquito maintenance. The authors also thank the University of Missouri Genomics Technology Core for technical support.
**Funding**

The authors acknowledge the National Institutes of Health—National Institute of Allergy and Infectious Diseases (NIH-NIAID) for the funding of this research work (grant applications: R01 AI130085-01A1, KEO, and R56 AI167980-01, AWEF).

**Author contributions**

WR, AEW, KEO, and AWEF designed research; WR, AEW, JL, IS-V, and RJ performed research; WR contributed new reagents/analytic tools; WR, AEW, KEO, and AWEF analyzed data; and WR, AEW, KEO, and AWEF wrote the article.

**Conflicts of interest**

None declared.

**Literature cited**

Adelman ZN, Jasinskiene N, Onal S, Juhn J, Ashikyan A, Salampessy M, MacCauley T, James AA. Nanos gene control DNA mediates developmentally regulated transposition in the yellow fever mosquito *Aedes aegypti*. Proc Natl Acad Sci U S A. 2007;104(24):9970–9975.

Adolfi A, Gantz VM, Jasinskiene N, Lee H-F, Hwang K, Terradas G, Bulger EA, Ramaiyah A, Bennett JB, Emerson JJ, et al. Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. Nat Commun. 2020;11(1):5553.

Akbari OS, Antoshechkin I, Amrhein H, Williams B, Diloreto R, Sandler J, Hay BA. The developmental transcriptome of the mosquito *Aedes aegypti*: an invasive species and major arbovirus vector. G3 (Bethesda). 2013;3(9):1493–1509.

Basu S, Aryan S, Overcash JM, Samuel GH, Anderson MAE, Dahlern TJ, Myles KM, Adelman ZN. Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. Proc Natl Acad Sci U S A. 2015;112(13):4038–4043.

Beebe NW, Pagendam D, Trewin BJ, Boomer A, Bradford M, Ford A, Liddington C, Bondarenco A, Barro D, Gilchrist PJ, et al. Releasing incompatible males drives strong suppression across populations of wild and Wolbachia-carrying *Aedes aegypti* in Australia. Proc Natl Acad Sci U S A. 2021;118(41):e2106828118.

Buchman A, Gamez S, Li M, Antoshechkin I, Li H-H, Wang H-W, Chen C-H, Klein MJ, Duchemin J-B, Paradkar PN, et al. Engineered resistance to Zika virus in transgenic *Aedes aegypti* expressing a polycistronic cluster of synthetic small RNAs. Proc Natl Acad Sci U S A. 2019;116(9):3656–3661.

Buchman A, Gamez S, Li M, Antoshechkin I, Li H-H, Wang H-W, Chen C-H, Klein MJ, Duchemin J-B, Crowe JE, et al. Broad dengue neutralization in mosquitoes expressing an engineered antibody. PLoS Pathog. 2020;16(1):e1008103.

Burt A. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc Biol Sci. 2003;270(1518):921–928.

Calvo E, Walter M, Adelman ZN, Jimenez A, Onal S, Marinotti O, James AA. Nanos (nos) genes of the vector mosquitoes, *Anopheles gambiae*, *Anopheles stephensi* and *Aedes aegypti*. Insect Biochem Mol Biol. 2005;35(7):789–798.

Carballar-Lejarazu R, Ogagwcu C, Tushar T, Kelsey A, Pham TB, Murphy J, Schmidt H, Lee Y, Lanzaro GC, James AA. Next-generational gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae*. Proc Natl Acad Sci U S A. 2020;117(37):22805–22814.

Carballar-Lejarazu R, Tushar T, Pham TB, James AA. Cas9-mediated maternal effect and derived resistance alleles in a gene-drive strain of the African malaria vector mosquito, *Anopheles gambiae*. Genetics. 2022;7:iya055.

Carvalho DO, McKerny AR, Garziera L, Lacroix R, Donnelly CA, Alphay L, Malavasi A, Capurro ML. Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. PLoS Negl Trop Dis. 2015;9(7):e0003864.

Cenic R, Miura H, Malina A, Robert F, Ethier S, Schmeing TM, Dostie J, Pelletier J. Protoscaler adjacent motif (PAM)-distal sequences engage CRISPR Cas9 DNA target cleavage. PLoS One. 2014;9(10):e105213.

Chae K, Dawson C, Valentin C, Contryeras B, Zapletal J, Myles KM, Adelman ZN. Engineering a self-eliminating transgene in the yellow fever mosquito, *Aedes aegypti*. Proc Natl Acad Sci U S A. 2015;112(13):4038–4043.

Chen C-H, Klein MJ, Duchemin J-B, Paradkar PN, et al. Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency. Genome Biol. 2015;16:280.

Chen C-H, Klein MJ, Duchemin J-B, Crowe JE, et al. Engineered RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. Proc Natl Acad Sci U S A. 2006;103(11):4198–4203.

Chen C-H, Klein MJ, Duchemin J-B, Paradkar PN, et al. Efficient population modification gene-drive rescue system in the malaria mosquito, *Aedes aegypti*. Proc Natl Acad Sci U S A. 2020;117(9):5553.

Clement K, Rees H, Canver MC, Gehrke JM, Farouni R, Hsu JY, Cole MA, Liu DR, Joung K, Bauer DE, et al. CRISPResso2 provides accurate and rapid genome editing sequence analysis. Nat Biotechnol. 2019;37(3):224–226.

Dang Y, Jia G, Choi J, Ma H, Anaya E, Ye C, Shankar P, Wu H. Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency. Genome Biol. 2015;16:280.

Dong S, Balaraman V, Kantor AM, Lin J, Grant DG, Held NL, Franz AWE. Chikungunya virus dissemination from the midgut of *Aedes aegypti* is associated with temporal basal lamina degradation during bloodmeal digestion. PLoS Negl Trop Dis. 2017;11(9):e0005976.

Engler C, Kandzia R, Marillonnet S. A one pot, one step, precision cloning method with high-throughput capability. PLoS One. 2008;3(11):e3647.

Franz AW, Sanchez-Vargas I, Adelman ZN, Blair CD, Beaty BJ, James AA, Olson KE. Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. Proc Natl Acad Sci U S A. 2006;103(11):4198–4203.

Franz AW, Sanchez-Vargas I, Piper J, Smith MR, Khoo CC, James AA, Olson KE. Stability and loss of a virus resistance phenotype over time in transgenic mosquitoes harbouring an antiviral effector gene. Insect Mol Biol. 2009;18(5):661–672.

Franz AWE, Sanchez-Vargas I, Raban RR, Black WI, James AA, Olson KE. Fitness impact and stability of a transgene conferring resistance to Dengue-2 virus following introgression into a genetically diverse *Aedes aegypti* strain. PLoS Negl Trop Dis. 2014;8(5):e2833.

Gantz VM, Bier E. The mutagenic chain reaction: method for converting heterozygous to homozygous mutations. Science. 2015;348(6233):442–444.

Gantz VM, Bier E. The dawn of active genetics. Bioessays. 2016;38(1):50–63.

Gokcezade J, Sienski G, Duchek P. Efficient CRISPR/Cas9 plasmids for transgenic *Drosophila* and *Anopheles gambiae*. PLoS One. 2014;9(11):e105213.

Gratz SJ, Cummings AM, Nguyen JN, Hamm DC, Donohue LK, Harrison MM, Wildonger J, O’Connor-Giles KM. Genome
engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. Genetics. 2013;194(4):1029–1035.

Hammond A, Galizi R, Kryou K, Simoni A, Siniscalchi C, Katsanos D, Gribble M, Baker D, Marois E, Russell S, et al. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito Anopheles gambiae. Nat Biotechnol. 2016;34(1):78–83.

Henkoff S. Position effect and related phenomena. Curr Opin Genet Dev. 1992;2(6):907–912.

Horn C, Jaunich B, Wimmer EA. Highly sensitive, fluorescent transmembrane marker for Drosophila transgenesis. Dev Genes Evol. 2000;210(12):623–629.

Kittayapong P, Ninphomchai S, Limohpasmanee W, Chansang C, Chansang U, Mongkalapong P. Combined sterile insect technique and incompatible insect technique: the first proof-of-concept to suppress Aedes aegypti vector populations in semi-rural settings in Thailand. PLoS Negl Trop Dis. 2019;13(10):e0007771.

Konet DS, Anderson J, Piper J, Akkina R, Suchman E, Carlson J. Short-hairpin RNA expressed from polymerase III promoters mediates RNA interference in mosquito cells. Insect Mol Biol. 2007;16(2):199–206.

Kyrou K, Hammond AM, Galizi R, Kranjc N, Burt A, Beaghton AK, Nolan T, Crisanti A. A CRISPR—Cas9 gene drive targeting double-sex causes complete population suppression in caged Anopheles gambiae mosquitoes. Nat Biotechnol. 2018;36(11):1062–1066.

Labun K, Montague TG, Gagnon JA, Thyme SB, Valen E. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. Nucleic Acids Res. 2016;44(W1):W272–276.

Li M, Bui M, Yang T, Bowman CS, White BJ, Akbari OS. Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, Aedes aegypti. Proc Natl Acad Sci U S A. 2017;114(49):E10540–E10549.

Li M, Yang T, Kandul NP, Bui M, Gamez S, Raban R, Bennett J, Sánchez CHM, Lanzaro GC, Schmidt H, et al. Development of a confinable gene drive system in the human disease vector Aedes aegypti. eLife. 2020;9:e51701.

Li M, Yang T, Kandul NP, Bui M, Gamez S, Raban R, Bennett J, Sánchez CHM, Lanzaro GC, Schmidt H, et al. Development of a confinable gene drive system in the human disease vector Aedes aegypti. eLife. 2020;9:e51701.

Liu N. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. Annu Rev Entomol. 2015;60:537–559.

Liu-Helmersson J, Brännström Å, Sewe MO, Semenza Jc, Rocklov J. Estimating past, present, and 700 future trends in the global distribution and abundance of the arbovirus vector Aedes aegypti under climate change scenarios. Front Public Health. 2019;7:148.

Manjarres-Suarez A, Olivero-Verbel J. Chemical control of Aedes aegypti: a historical perspective. Rev Costarric Salud Publica. 2013;22(1):68–75. [accessed 2022 October 23]. http://www.scielo.sa.cr/scielo.php?script=sci_arttext&pid=S1409-1429201300010012&lng=en&nrm=iso. ISSN 1409–1429.

Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBO j. 2011;17(1):10–12.

Otero M, Solari HG, Schweigmann N. A stochastic population dynamics model for Aedes aegypti: formulation and application to a city with temperate climate. Bull Math Biol. 2006;68(8):1945–1974.

Sánchez HM, Wu SL, Bennett JB, Marshall JM. MGDrivE: a modular simulation framework for the spread of gene drives through spatially explicit mosquito populations. Methods Ecol Evol. 2020;11(2):229–239.

Schmidt H, Collier TC, Hanemaaijer MJ, Houston PD, Lee Y, Lanzaro GC. Abundance of conserved CRISPR–Cas9 target sites within the highly polymorphic genomes of Anopheles and Aedes mosquitoes. Nat Commun. 2020;11(1):1425.

Tazure SI, Schulz C, Gilboa L, Fogarty M, Mahowald AP, Guichet A, Ephrussi A, Wood CG, Lehmann R, Fuller MT. A germline-specific gap junction protein required for survival of differentiating early germ cells. Development. 2002;129(10):2529–2539.

Terradas G, Hermann A, James AA, McGinnis W, Bier E. High-resolution in situ analysis of Cas9 germline transcript distributions in gene-drive Anopheles mosquitoes. G3 (Bethesda). 2021;12(1):jkb369.

Verkuilj SAN, Gonzalez E, Li M, Ang J, Kandul NP, Anderson MAE, Akbari OS, Bonsall MB, Alphey I. A CRISPR endonuclease gene drive reveals two distinct mechanisms of inheritance bias. [accessed 2022 October 23]. bioRxiv 2020.12.15.421271. doi:10.1101/2020.12.15.421271, 2020.

Waltz E. First genetically modified mosquitoes released in the United States. Nature. 2021;593(7858):175–176.

Weaver SC, Vasilakis N. Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infect Genet Evol. 2009;9(4):523–540.

Weaver SC, Reisen WK. Present and future arboviral threats. Antiviral Res. 2010;85(2):328–345.

Williams AE, Franz AWE, Reid WR, Olson KE. Antiviral effectors and gene drive for mosquito population suppression or replacement to mitigate arbovirus transmission by Aedes aegypti. Insects. 2020;11(1):52.

Williams AE, Sanchez-Vargas I, Reid WR, Lin J, Franz AWE, Olson KE. The antiviral small-interfering RNA pathway induces Zika virus resistance in transgenic Aedes aegypti. Viruses. 2020;12(11):1231.

Windbichler N, Papathanos PA, Catteruccia F, Ranson H, Burt A, Crisanti A. Homing endonuclease mediated gene targeting in Anopheles gambiae cells and embryos. Nucleic Acids Res. 2007;35(17):5922–5933.

Wu B, Luo L, Gao XJ. Cas9-triggered chain ablation of Cas9 as a gene drive. Nat Biotechnol. 2016;34(1):78–83.

Yen PS, James AA, Li JC, Chen CH, Failloux AB. Synthetic miRNAs induce dual arboviral-resistance phenotypes in the vector mosquito Aedes aegypti. Commun Biol. 2018;1:11.

Zapletal J, Najmitabrizi N, Erraguntla M, Lawley MA, Myles KM, Adelman ZN. Making gene drive biodegradable. Philos Trans R Soc Lond B: Biol Sci. 2021;376(1818):20190804. [accessed 2022 October 23]. bioRxiv 2020.12.15.421271. doi:10.1101/2020.12.15.421271, 2020.