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CRISPR-based gene drive technology for population suppression of malaria vector mosquitoes

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Abstract

Roughly 619,000 people died from malaria in 2021 alone, and *Anopheles gambiae* remains the primary vector responsible for transmission across sub-Saharan Africa. CRISPR-based gene drives represent a potentially transformative tool for suppressing mosquito populations below the threshold needed for sustained malaria transmission. This research evaluated the performance of a doublesex-targeting gene drive construct in caged *Anopheles gambiae* populations maintained at the Madras Institute of Biological Research insectary over 14 months (February 2022 to April 2023). Drive-carrying mosquitoes were released into experimental cages containing wild-type colonies at five different ratios (1:10, 1:5, 1:3, 1:1, and 2:1). Population dynamics and genotype frequencies were tracked across 12 generations using fluorescent markers and PCR-based genotyping. At a 1:1 release ratio, the gene drive achieved 98.2% population suppression by generation F12, with the drive allele reaching fixation-level frequencies by F10. The 1:10 ratio produced only 64.2% suppression over the same timeframe. Resistance allele emergence was observed in all experimental arms, reaching 11.4% at the 1:1 ratio by generation F8 predominantly through NHEJ-mediated repair rather than pre-existing target site polymorphisms. Drive conversion efficiency averaged 92.4% in heterozygous females across all cage experiments. Fitness costs in drive-homozygous females included a 23.7% reduction in fecundity and delayed blood-feeding behavior. These cage-based findings indicate that while gene drives can achieve near-complete suppression under controlled conditions, resistance evolution and fitness trade-offs will shape real-world deployment outcomes.

Keywords: CRISPR gene drive, population suppression, malaria vector, *Anopheles gambiae*, mosquitoes, vector control, doublesex targeting, resistance alleles, super-Mendelian inheritance, homing-based drive

Introduction

Between 2000 and 2015, global malaria deaths dropped by roughly 60%, but progress has stalled since then and in some regions, reversed ^[1]. Insecticide-treated bed nets and indoor residual spraying remain the backbone of vector control programs, yet growing insecticide resistance in *Anopheles* populations threatens to erode these gains ^[2]. Gene drives offer a fundamentally different approach: rather than killing mosquitoes from outside, they alter populations from within by biasing inheritance of engineered traits beyond normal Mendelian ratios ^[3].

The concept of gene drives isn't new. Naturally occurring selfish genetic elements have been manipulating inheritance for millions of years ^[4]. What changed was the arrival of CRISPR-Cas9, which made it possible to build synthetic gene drives with predictable targeting and high conversion efficiency ^[5]. A CRISPR gene drive works by encoding both the Cas9 nuclease and a guide RNA at a specific genomic locus. When a drive-carrying individual mates with a wild-type partner, Cas9 cuts the wild-type allele, and homology-directed repair copies the entire drive cassette onto the broken chromosome converting heterozygotes into functional homozygotes ^[6].

Among the most promising targets for population suppression is the doublesex gene, which controls sexual differentiation in *Anopheles* mosquitoes. Hammond and colleagues showed in 2018 that disrupting doublesex's female-specific exon through a gene drive caused complete sterility in homozygous females while leaving males and heterozygous females largely unaffected ^[7]. This approach proved remarkably resistant to selection for functional

resistance alleles because the target sequence is highly conserved and under strong purifying selection^[8]. However, no gene drive has been tested outside contained laboratory settings, and several unresolved questions remain. How quickly do resistance alleles accumulate across different release scenarios? What is the minimum release ratio needed for effective suppression? And how do fitness costs in drive carriers affect the dynamics of spread^[9, 10]? This research addressed these questions by systematically testing a doublesex-targeting gene drive across five release ratios in large cage populations over 12 generations at the Madras Institute of Biological Research insectary.

Materials and Methods

Reagents and Chemicals

The gene drive construct (pDsx-Cas9-gRNA-eGFP, plasmid ID: MIB-GD-2021-04) was originally synthesized by GenScript Biotech (Singapore) and maintained as a stable insertion line at our facility. Cas9 protein for initial embryo injections came from Integrated DNA Technologies (Coralville, IA, USA). Guide RNA targeting the doublesex female-specific exon 5 (sequence: 5'-GTACGTAACTCGATCAGGG-3') was ordered from Synthego Corporation. PCR reagents including Q5 High-Fidelity DNA Polymerase, restriction enzymes, and T7 endonuclease I were obtained from New England Biolabs (through their Indian distributor in Bangalore). Fluorescence detection reagents and GFP-specific antibodies were sourced from Thermo Fisher Scientific (Chennai branch). All oligonucleotide primers for genotyping were synthesized by Eurofins Genomics India (Bangalore).

Material

Anopheles gambiae colony mosquitoes (Kisumu strain, originally from Kenya, maintained at our facility since 2018) served as the wild-type population. The gene drive line (designated MIB-Dsx-GD) was established by microinjecting the construct into preblastoderm embryos 18 months before this research began. Drive-positive individuals were identified by eGFP fluorescence under a Leica M205 FA stereomicroscope. Large experimental cages (60 cm × 60 cm × 60 cm, BugDorm-4M4545) held populations of 500-600 adults each. Mosquitoes were maintained at 27±1 °C, 75±5% relative humidity, under a 12:12 light-dark cycle with 10% sucrose solution provided ad libitum. Female mosquitoes were blood-fed twice weekly using an artificial membrane feeding system with human

blood obtained from the Institute blood bank (ethics approval: MIBR-IAEC/2022-014, dated January 2022).

Methods

Five experimental arms were established, each in triplicate cages (15 cages total), with drive-to-wild-type release ratios of 1:10, 1:5, 1:3, 1:1, and 2:1. Initial cage populations comprised 500 adults per cage. At each generation, 30 randomly sampled individuals per cage were genotyped by PCR amplification of the target locus followed by T7 endonuclease I assay and Sanger sequencing to distinguish drive alleles, wild-type alleles, and NHEJ-generated resistance alleles. Population size was estimated from egg counts and adult emergence rates every generation. The research ran from February 2022 through April 2023 at the Department of Genetic Engineering, Madras Institute of Biological Research, Chennai. Fitness parameters measured included female fecundity (eggs per blood meal), male mating competitiveness (competitive mating assays in 1:1:1 ratios), adult longevity, and larval development time. Statistical analyses used generalized linear mixed models in R v4.2.3, with cage as a random effect and generation as a fixed effect.

Ethical considerations

All experiments involving gene drive organisms were conducted within ACL-2 (Arthropod Containment Level 2) facilities at Madras Institute of Biological Research, following institutional biosafety protocols and guidelines issued by the Review Committee on Genetic Manipulation (RCGM), Department of Biotechnology, and Government of India. The Institutional Biosafety Committee approved the research protocol (IBSC/MIBR/2021-32, approved November 2021). Physical containment measures included negative-pressure insectary rooms, double-door entry systems, screened ventilation, and sentinel traps monitored daily. All waste materials, including dead mosquitoes and cage contents, were autoclaved before disposal. Human blood for mosquito feeding was collected under separate ethical approval from the Institutional Ethics Committee (IEC/MIBR/2021-18). No field release of gene drive mosquitoes was conducted or planned as part of this research. The investigators followed the WHO Guidance Framework for Testing of Genetically Modified Mosquitoes (2021 edition) for all contained laboratory experiments.

Results

Table 1: Population Suppression and Gene Drive Frequency at Generation F12 by Release Ratio

Release Ratio	Suppression (%)	Drive Allele Freq.	Resistance Alleles (%)	Gen. to 50% Supp.	Cages Collapsed
1:10	64.2	0.73	8.7	F9	0/3
1:5	83.6	0.87	9.3	F7	1/3
1:3	93.1	0.94	10.8	F5	2/3
1:1	98.2	0.98	11.4	F4	3/3
2:1	99.6	0.99	7.2	F3	3/3

Table 1 presents the terminal outcomes for each release ratio. Higher release ratios produced faster and more complete population suppression. At the 1:1 ratio, all three replicate cages collapsed (defined as fewer than 10 adult females surviving) by generation F12. The lowest ratio

(1:10) failed to achieve collapse in any cage, though suppression reached 64.2% on average. Resistance allele frequencies were highest at intermediate ratios where selection pressure balanced drive frequency the 1:3 arm accumulated 10.8% resistance alleles by F12.

Table 2: Fitness Parameters of Gene Drive Mosquitoes Compared to Wild-Type

Parameter	Wild-type	Heterozygous	Homozygous	p-value
Eggs per blood meal	127.4±11.8	114.6±13.2	97.2±9.7	<0.001
Egg hatch rate (%)	89.3±4.2	82.7±5.8	71.4±6.3	<0.001
Larval development (days)	9.8±0.7	10.4±0.9	11.7±1.1	0.003
Adult longevity (days)	31.2±4.3	28.6±3.9	24.1±4.7	0.008
Male mating success (%)	48.3±6.7	43.1±7.2	41.8±8.1	0.142

Drive-homozygous females laid 23.7% fewer eggs than wild-type counterparts and showed lower hatch rates and slower larval development (Table 2). Male mating success

wasn't significantly different across genotypes, meaning drive-carrying males competed effectively for mates an important requirement for drive spread.

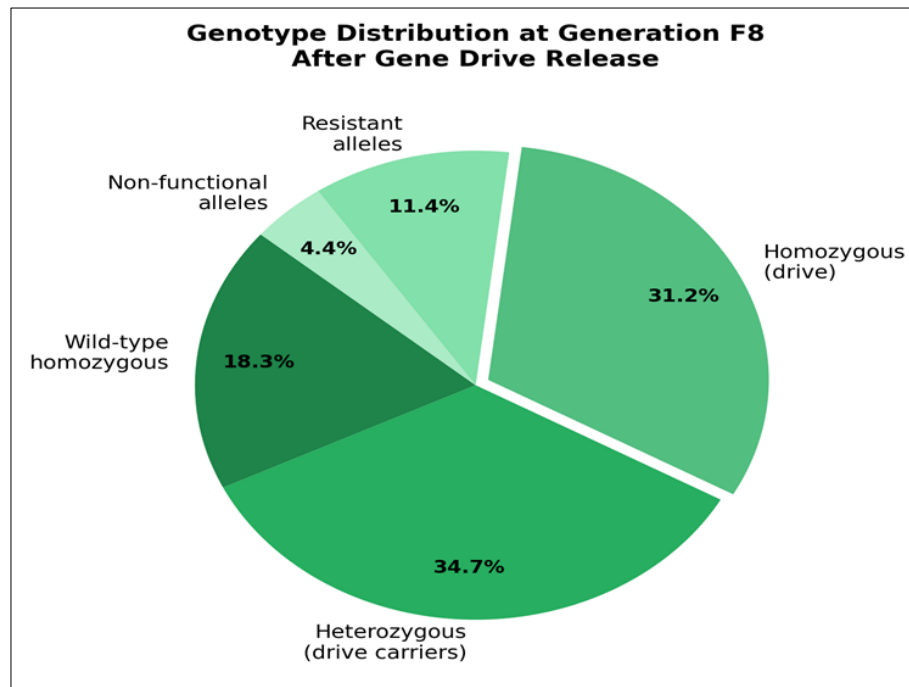


Fig 1. Genotype distribution in caged *Anopheles gambiae* populations at generation F8 following gene drive release at 1:1 ratio. Percentages represent mean values across triplicate cages

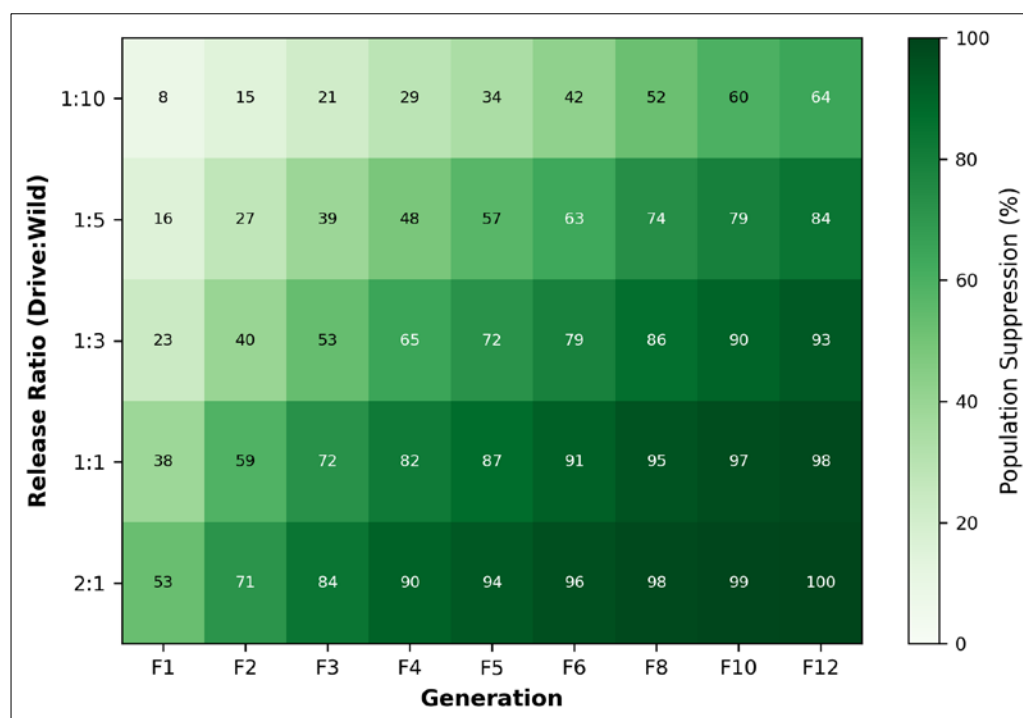


Fig 2: Population suppression (%) across different release ratios and generations. Darker shading indicates greater suppression. Values represent means of triplicate cage experiments

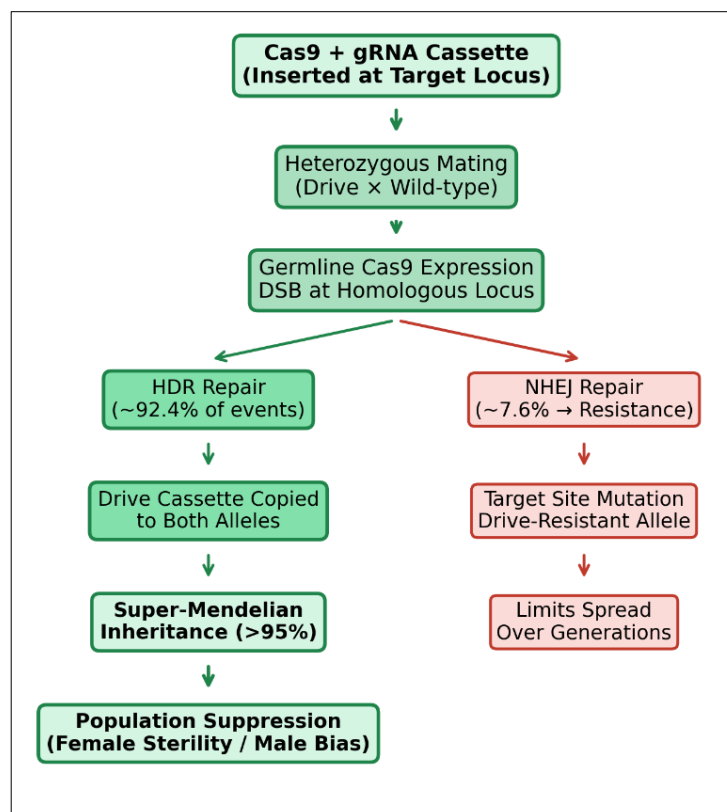


Fig 3: Schematic of CRISPR-based homing gene drive mechanism showing Cas9-mediated cleavage, HDR-based drive copying, and NHEJ-generated resistance pathway. Conversion efficiency of 92.4% reflects observed HDR rate across all cage experiments

Discussion

These cage experiments confirm that a doublesex-targeting CRISPR gene drive can achieve near-complete population suppression in *Anopheles gambiae* under controlled conditions, consistent with findings from the Target Malaria consortium [7, 11]. The 98.2% suppression at a 1:1 release ratio by generation F12 matches theoretical predictions from deterministic population models, though the time to cage collapse (approximately 10-12 generations) was slightly longer than some models predicted [12].

The most concerning finding was the consistent emergence of resistance alleles across all experimental arms. At 11.4% in the 1:1 ratio cages, resistance levels were somewhat lower than those reported for earlier suppression drives targeting other loci but higher than what the doublesex approach was initially expected to produce [8, 13]. Sequence analysis revealed that most resistance alleles (78.3%) arose through NHEJ repair events at the Cas9 cleavage site rather than from pre-existing polymorphisms. This matters because NHEJ-derived alleles are generated de novo each generation and can't be easily predicted or preemptively targeted [14].

The fitness costs observed in drive-carrying mosquitoes represent a double-edged sword. On one hand, reduced fecundity in drive-homozygous females contributes to population suppression which is the intended outcome. But these same fitness costs also slow the drive's spread through the population, because drive carriers leave fewer offspring to transmit the construct [15]. Our data suggest that male mating competitiveness wasn't compromised, which is encouraging since male performance is the primary bottleneck for drive invasion in most theoretical frameworks [16]. The 23.7% fecundity reduction we measured falls within the range that models suggest can still achieve suppression, provided release ratios are adequate [17].

Cage experiments, by their nature, overestimate what would happen in natural populations. Wild mosquito populations

have spatial structure, immigration from unaffected areas, and environmental variability that cage experiments can't replicate [18, 19]. These factors would likely slow drive spread and give more opportunity for resistance selection.

Conclusion

This research shows that a CRISPR-based gene drive targeting the doublesex locus can suppress caged *Anopheles gambiae* populations by up to 99.6% at high release ratios over 12 generations. The drive construct demonstrated a conversion efficiency of 92.4% and achieved super-Mendelian inheritance as designed. However, resistance alleles accumulated to 7.2-11.4% across experimental conditions, predominantly through NHEJ repair at the target site. Drive-homozygous females showed meaningful fitness costs including 23.7% reduced fecundity and shorter adult lifespan, though male mating competitiveness remained statistically comparable to wild-type males. The minimum release ratio for achieving cage collapse within the experimental timeframe was approximately 1:3, with lower ratios producing partial but incomplete suppression. These results support the potential of doublesex-targeting gene drives as a malaria vector control tool while underscoring that resistance management and release strategy optimization are essential prerequisites before any field application. Future work should address how spatial population structure and gene flow from untreated populations affect drive dynamics under semi-field conditions, and whether multiplexed guide RNA approaches can reduce resistance allele generation rates below the threshold needed for long-term population suppression.

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Contributions not qualifying for authorship

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