Title

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Journal

BioEssays : news and reviews in molecular, cellular and developmental biology, 43(8)

ISSN

0265-9247

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Publication Date

2021-08-01

DOI

10.1002/bies.202000282

Peer reviewed

Received: 20 October 2020 | Revised: 13 May 2021 | Accepted: 31 May 2021

DOI: 10.1002/bies.202000282

BioEssays

PROBLEMS & PARADIGMS

Prospects & Overviews

Population modification strategies for malaria vector control are uniquely resilient to observed levels of gene drive resistance alleles

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Funding information
Open Philanthropy Project

Abstract

Cas9/guide RNA (gRNA)-based gene drive systems are expected to play a transformative role in malaria elimination efforts., whether through population modification, in which the drive system contains parasite-refractory genes, or population suppression, in which the drive system induces a severe fitness load resulting in population decline or extinction. DNA sequence polymorphisms representing alternate alleles at gRNA target sites may confer a drive-resistant phenotype in individuals carrying them. Modeling predicts that, for observed levels of SGV at potential target sites and observed rates of de novo DRA formation, population modification strategies are uniquely resilient to DRAs. We conclude that gene drives can succeed when fitness costs incurred by drive-carrying mosquitoes are low enough to prevent strong positive selection for DRAs produced de novo or as part of the SGV and that population modification strategies are less prone to failure due to drive resistance.

KEYWORDS
fitness, gene drive, genetically engineered mosquitoes, malaria

INTRODUCTION

Gene drive technologies offer the promise of managing targeted pest species for the benefit of public health, agriculture, and the environment.[1] Gene drives in mosquito vectors of malaria are currently at the forefront in the advancement of this technology. Malaria is one of the most significant causes of human morbidity and mortality globally, with a reported 228 million cases and over 400,000 deaths in 2018 alone.[2] Although precise numbers are lacking[3] the heaviest malaria burden, an estimated 93% of all cases, occur in sub-Saharan Africa.[2] The reasons are manifold and include a constellation of biological, economic, and political factors.[4] A major global initiative to eradicate malaria was rolled out in 2000. This effort led to a significant decrease in the disease, but unfortunately since 2015 this progress has stalled.[5] This alarming trend prompted the WHO Strategic Advisory Group on Malaria Eradication to recommend research and development of a new generation of vector control tools, singling out a need for research "…*to develop new genetic technologies that can alter mosquitoes' ability to transmit the parasite*".[6]

GENETIC CONTROL OF MALARIA VECTORS

The concept of applying genetic-based methods for vector control is not new.[7,8] However, current advances in our understanding of the molecular genetics of mosquito reproduction[9] and immunity[10] sparked renewed interest in genetic control strategies targeting genes related to these functions with the ultimate goal of disrupting malaria parasite transmission. Significant progress toward this goal includes targeting and disrupting genes associated with female fertility,[11] methods for altering sex-ratio,[12] and engineering synthetic genes that encode single chain antibodies or other effectors to destroy mosquito stages of the parasite.[13] These systems have been proposed within two broad strategies, population suppression, with the aim of driving mosquito populations toward local extinction,[14] and population modification,[15] which eliminates the mosquito's ability to transmit malaria by driving a genetic element into the mosquito population that disrupts *Plasmodium* development, but otherwise leaves the mosquito unaffected (also referred to as population replacement).

GENE DRIVE STRATEGIES FOR MALARIA ELIMINATION

Both population suppression and population modification strategies rely on a gene drive system, which on introduction into a wild-type population, rapidly increases toward fixation by altering normal Mendelian inheritance in favor of a transgene.[16] However, existing population modification and suppression strategies fundamentally differ in the way in which they use gene drives. The modification strategy links synthetic anti-parasite effector genes with the drive, using the drive to introduce and spread the anti-parasite genes through a target population. In this specific example, both the effector genes and the gene drive are mostly exogenous. The suppression strategy uses the drive to target an essential gene or chromosome and to disrupt its function. The gene drive is an exogenous component, while the targeted gene or chromosome is endogenous.

The gene-drive systems used most widely in mosquitoes today are based on Cas9/guide RNA technology (designated herein as Cas9-based gene drive [CGD]).[17] These systems include a minimum of three components, the first of which is CRISPR-associated protein 9 (Cas9), a DNA endonuclease that cleaves double-stranded DNA. Another component is the guide RNA (gRNA) which recognizes a specific target DNA sequence 23 base pairs in length and directs Cas9 cleavage at that specific site within the genome, a characteristic referred to as "homing".[18] A third component, ''homology arms'', are sequences of DNA flanking the gRNA-directed cut site used to promote recombination. The normal cell machinery repairs the cut DNA by homology-driven repair (HDR), a process that uses the CGD construct as a template to repair the cut DNA, essentially converting a heterozygote to a homozygote in individuals carrying a CGD and thus altering the normal inheritance to overrepresent the CGD. This "super-Mendelian" inheritance results in a rapid increase in the frequency of the CGD and its associated cargo within the targeted population.

FITNESS CONSIDERATIONS

The introduction of exogenous DNA resulting in a genetically engineered mosquito (GEM) is likely to impose fitness costs on the modified organism.[19] These include loads resulting from the direct impact of the integration (site-specific effects) and those associated with the expression properties of the inserted DNA. Gene-drive systems potentially confer additional effects, including those associated with the gene drive cleavage activities, for example, off-target cleavage and insertion and deletion mutations (indels) generated by the DNA repair mechanics and non-homologous end-joining (NHEJ). Gene drive systems may generate site-specific effects by negatively affecting the function of the gene into which the construct has been inserted, potentially resulting in some maladaptive phenotype. This is an intentional cost for population suppression approaches but could be an unintentional cost in the population modification strategy.

The efficacy of a gene drive depends on (i) the fitness of the GEM relative to wild-type mosquitoes and (ii) its homing efficiency, the probability of successful target site-binding with subsequent DNA cleavage and repair. Fitness of the individuals that carry the CGD is typically defined as the fecundity (male and female competitiveness and female output) and death rate of the transgenic mosquito compared with wild type.[20,21] The drive will continue to increase in frequency (spread) as long the homing efficiency is greater than the fitness cost relative to other genotypes.[18,22] Because the homing efficiencies are so high in mosquito CGDs (> 0.98)[23,24] it is anticipated that they will effectively spread in wild-type populations despite even substantial fitness costs. Reductions in homing efficiency and fitness associated with anomalous cleavage repair, such as NHEJ, do seem to be minimal (occurring at a frequency $\leq 10\%$[21,23]).

LABORATORY EVALUATIONS

Tests conducted thus far support the conclusion that mosquito CGDs possess the capacity to spread and suggest that this approach to malaria control has great potential. A consideration of the expectations and outcomes of these tests serves to illustrate the major difference between the population modification and suppression strategies. An evaluation of a population modification GEM aimed at driving *Plasmodium*-blocking genes toward fixation in a target population of the mosquito *Anopheles gambiae* (a major malaria vector in Africa) was recently completed.[23] This GEM suffered no measurable fitness costs relative to wild type in small cage trials and the CGD homing efficiency was not significantly impacted by issues with DNA cleavage or transgene integration. The observed outcomes of these tests met expectations, that is, the frequency of the transgene reached fixation within 6–10 generations and the mosquito population size remained more or less constant, supporting the hypothesis that these GEMs were equal to wild type in terms of fitness under laboratory conditions.

Population suppression GEMs that act by reducing female fertility have been explored.[11,25] One of the most promising of these

targets a gene known as *doublesex (dsx*) in the insect sex determination pathway.[11] This gene controls differentiation of the sexes by producing alternatively spliced, sex-specific transcripts. Disruption of the intron 4-exon 5 boundary by insertion of a CGD results in complete sterility of females homozygous for the CGD but has no effect on males. Homozygous females also exhibit an intersex phenotype and are unable to take a bloodmeal. Female heterozygotes had a 50% reduction in fecundity, nearly 80% reduction when the drive was inherited through the male parent. Inheritance rates were ~99% and male bias was high (92–84%). Drive is achieved via inheritance through males and heterozygous females. The outcomes from two cage trials met expectations with the construct reaching frequencies of 100% by generation 7 in one trial and generation 11 in the second. The populations went extinct in both trials in the generation following construct fixation. Both trials used an initial introduction of this GEM at 12.5% of the total population.

Other population suppression strategies are based on sex-ratio distortion, wherein the construct results in the production of offspring with a strong bias in favor of males, ultimately resulting in population suppression or even extinction.[12,26,27] A recent approach is the development of a sex-distorter gene drive (SDGD) which acts to both reduce female fertility and bias sex ratio.[14] The sex distorter gene, which encodes the I-PpoI endonuclease,[28] destroys X-chromosomes during spermatogenesis resulting in a male biased sex-ratio. The SDGD is generated by coupling the I-PopI with a CRISPR-based drive inserted into the *dsx* gene.[14] Insertion of the SDGD into *dsx* results in sterility in females but has no effect on male fertility. This GEM will spread rapidly, resulting in a strongly male-biased population and, due to disruption of the *dsx* gene, impair female fertility. This double activity can result in the elimination of the mosquito population into which it is introduced. Females heterozygous for the construct displayed a 30% reduction in the generation of viable offspring compared with wild type, whereas heterozygous males suffered no effects. Inheritance rates were > 96% and male bias was high (92–84%). Drive is achieved via inheritance through males and heterozygous females. The outcomes from cage trials met expectation with the SDGD reaching frequencies of 100% resulting in a male only population and resultant population elimination by generations 9–13 with an initial introduction of this GEM at 25% of the total population.

These studies illustrate the difference in GEM fitness between the two strategies. In the population modification strategy, the GEM is equal or nearly equal to wild-type, whereas GEM females in the suppression strategy suffer a severe fitness cost, a reduction of 100% for CGD homozygotes, and of 30–50% for CGD heterozygotes. To date, these studies have been conducted in laboratory caged populations with long-colonized mosquito strains and have not been evaluated in natural mosquito populations.

All fitness estimates associated with CGD genotypes thus far have been determined from laboratory cage experiments. These may have little relation to the fitness of these genotypes upon introduction into the natural environment. Indeed, it is well known that fitness in nature is difficult to measure[29] being complicated by factors such as age structuring and temporal and spatial environmental dynamics that alter patterns of selection. One additional and critical aspect of measuring the fitness of a particular genotype is that it will vary in different genetic backgrounds. Genome diversity and environmental heterogeneities that exist in nature are impossible to replicate in the laboratory and these almost certainly will have a profound effect on the relative fitness of CGD genotypes and potentially even their ability to function.

APPLICATION OF GENE DRIVES TO NATURAL MOSQUITO POPULATIONS

Natural populations of anopheline mosquitoes are known to be highly polymorphic.[30] This high level of SGV includes base-pair substitutions within gRNA target sites.[31] These may or may not render the gRNA target site uncleavable. Those polymorphisms that result in uncleavable gRNA target sites may lead to drive failure and are referred to as "drive resistance alleles" (DRAs). Many researchers have argued that the presence of DRAs represents a major obstacle to the application of CGD systems.[32,33,34]

The potential for the rapid evolution of drive resistance, either as part of SVG or arising de novo, was recognized and discussed in the earliest theoretical work on population suppression gene drives.[18,22] The emphasis focuses on the importance of making suppression drives as recessive as possible so that the fitness of heterozygotes is as equivalent to wild type as possible.[20,22] These authors also suggest a number of methods to mitigate drive resistance, which include the selection of multiple sites within a targeted gene using multiplexed gRNAs[20,35,36] and the selection of highly conserved genes to avoid sequence variation in SVG.[11,18] The *dsx* gene was thought to be highly conserved,[11] however a deeper examination revealed extensive polymorphisms.[37] Although none of these polymorphisms were in the target site these results do illustrate the need for caution when characterizing a potential target gene as being "highly conserved".

A recent publication presented an assessment of potential DRAs present as part of SGV in natural mosquito populations.[31] They concluded that although potential DRAs are abundant they may occur at frequencies low enough to not represent a major problem for CGDs, provided they are applied as part of a population modification strategy. We explore here how the population modification and population suppression strategies differ in their sensitivity to DRAs and why DRAs may pose a significant hurdle for suppression, but not modification approaches.

SIMULATIONS

CGD gRNA target alleles can be classified into four types: (i) wild-type alleles are cleavable and impose no fitness cost, (ii) transgenic alleles contain an inserted active gene drive system, (iii) functional DRAs that are non-cleavable by CGD system, and (iv) non-functional DRAs. The CGD will spread to high frequency in a target population as long as the

proportion of drive-resistant, functional individuals stays low. Therefore, it becomes important to consider the relative fitness of genotypes including individuals that do or do not carry a DRA and how this will affect the behavior of different CGD systems.

We utilized MGDrivE[38] to simulate a mosquito population under a range of drive element fitness costs and initial DRA frequencies to investigate quantitatively the impact of DRA frequency on GEM population suppression and modification strategies. MGDrivE is a modular simulation framework created specifically to investigate gene drive control in arthropod pests with an emphasis on mosquitoes. It incorporates both a genetic inheritance module and an explicit life-stage module (Figure S1).

We simulated panmictic populations of *An. gambiae* with an equilibrium adult population size of a million individuals to approximate a mosquito population on a small, isolated island, such as those that might be ideal for early GEM field trials.[39] Each simulation starts with a release of 10,000 transgenic males homozygous for the drive allele (~1% of the total population). We assume perfect Cas9-mediated cleavage and allow accurate HDR to vary between 99–100%. When accurate HDR does not occur, 1/6 of the resulting alleles (formed by NHEJ or some other mechanism) are assumed to be functional DRAs, while 5/6 are assumed to be non-functional DRAs. This is based on the calculation that ~1/3 of the resulting alleles will be in-frame and ~1/2 of those will be functional, in approximate agreement with experimental studies where gRNA target sites reside within coding regions.[25] The proportion of functional DRAs may be smaller for CGDs employing other gRNA target sites, such as the *dsx* gene; however, to constrain the dimensionality of our simulations, we chose 1/6 as a set value and varied the total rate of DRA generation to explore low rates of functional DRA generation. The simulations presented here are similar to the stochastic examples of MGDrivE[38] with exact parameter values describing the mosquito life cycle tailored for *An. coluzzii* on the islands of São Tomé and Príncipe – namely an egg stage duration of 3 days, a larval stage duration of 7 days, a pupal stage duration of 2 days, and an adult mosquito mortality rate of 0.15 per day (Table S1).

The key parameters being investigated here are the fitness cost of the CGD (fitness cost) and the frequency of DRAs existing in the population at the time of initial drive element introduction (initial DRA frequency). So that the same parameter values could be used for both the population suppression and modification GEM strategies, fitness cost was applied as a fecundity reduction to females homozygous for the drive allele, with drive heterozygotes suffering 50%, 10%, or 0% the fitness cost applied to drive homozygotes. The resulting homozygote fitness cost values were varied linearly between 0 and 1, with 1 representing a population suppression system and 0 representing an ideal population modification system.

Values for DRA frequencies within SGV were varied on a log-scale between 10^{-8} and $10^{-2.}$ The production of new resistance alleles through NHEJ or other mechanisms has been a key part of previous modeling work on GEM.[35,40] We considered these sources of DRAs in addition to SGV by conducting a set of simulations for three different DRA de novo formation rates – 0, 10^{-5} and 10^{-2} per Cas9-mediated cleavage event – while varying SGV between 10^{-8} and 10^{-2}.

One hundred stochastic simulations were run for each combination of parameters to account for random variations that can influence model outcome for rare events and small population sizes.

SIMULATION RESULTS

To compare simulation results, we use a "window of protection" concept, defined as the number of days in which the number of female mosquitoes without the transgene is below 5% of the total equilibrium population size (for at least 90% of the stochastic repetitions of the simulation).

Time series results showing the numbers of female mosquitoes extending through four years post-release for three different homozygote fitness costs (0.0, 0.5, and 1.0), heterozygote fitness costs that are half the homozygote fitness costs, and three initial DRA frequencies as part of standing variation (10^{-7}, ~10^{-5}, 10^{-2}) are illustrated in Figure 1A. The window of protection also is shown in light blue. A window of protection of roughly four years has been indicated as a minimal acceptable value in target product profiles for a GEM.[15,23,40] These results demonstrate how a population modification strategy using a drive element imposing a minimal fitness cost will spread through the population irrespective of rare DRAs with frequencies of up to 10^{-2} (Figure 1A bottom row). For a drive element imposing an intermediate (50%) homozygote fitness cost, which might correspond to a less-than-ideal modification strategy, a prolonged window of protection (> 2 years) can still be achieved, although this is sensitive to the initial DRA frequency and stochastic effects (Figure 1A middle row). In contrast, suppression strategies (in which females homozygous for the drive allele are completely infertile) impose a strong selective advantage on DRAs, so that DRAs with initial frequencies as low as 10^{-5}, rapidly increase in frequency, causing a population rebound (Figure 1A, top row).

Figure 1B shows how the window of protection varies across a range of parameter combinations for initial DRA frequency and fitness cost. The time series results presented in Figure 1A are points from within the heatmap displayed in Figure 1B. For initial DRA frequencies less than $\sim 5 \times 10^{-7}$, the window of protection exceeds 4 years – likely long enough to interrupt malaria transmission on a small, isolated island given minimal disease importation. Some low frequency DRAs are likely to be lost due to stochastic drift, an effect that depends on effective population size. For initial DRA frequencies greater than $\sim 5 \times 10^{-7}$, both the DRA frequency and fitness cost influence the window of protection. For drive elements that impose a large fitness cost, such as the 100% cost for population suppression elements the window of protection is short-lived (less than one year). As the fitness cost decreases, higher initial DRA frequencies can be tolerated. Notably, for an initial DRA frequency of 1%, a fitness cost of less than 18% can lead to a window of protection of 3–4 years or longer – parameter values that are very achievable for a population modification program.

A design aim of population suppression gene drive systems is to have fitness costs that are as recessive as possible.[18] To reflect the fact that recent population suppression gene drives[11,14] have

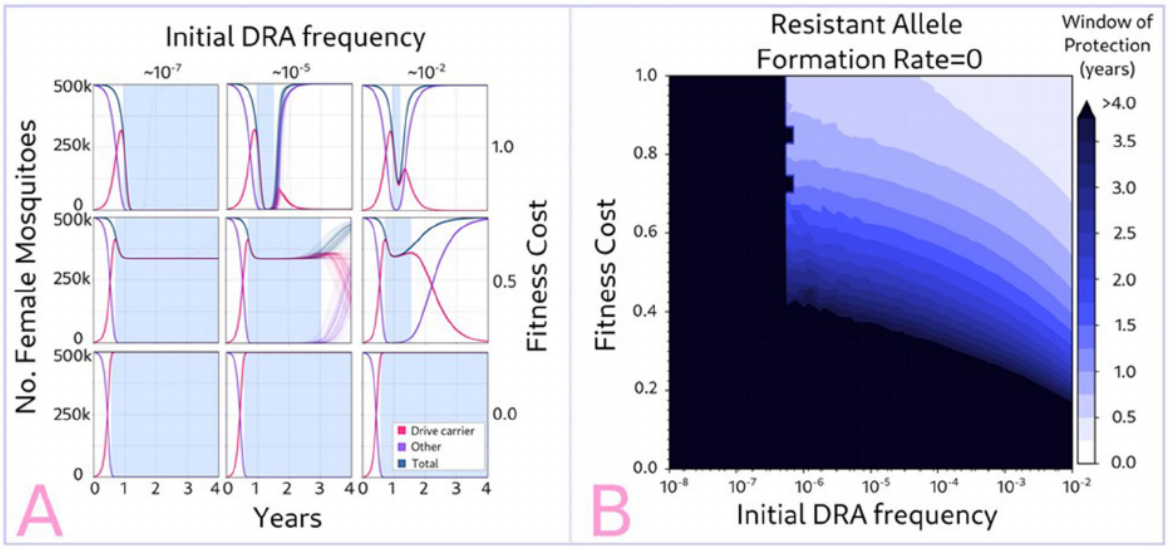

FIGURE 1 Panel A: Expected gene drive population dynamics for different combinations of initial driveresistant allele (DRA) frequencies and GEM fitness costs (manifest as fecundity costs on females homozygous for the drive allele, with half that fitness cost for drive heterozygotes). Red lines represent the number of females having at least one copy of the drive allele over time, purple lines represent the number of females without the drive allele, and blue lines represent the total female mosquito population size. The "window of protection" (cyan shaded region) is the period of time in which the number of mosquitoes capable of *Plasmodium* transmission falls below 5% of the baseline population (of 500,000 females) for 90% or more of the stochastic model repetitions. Panel B: Windows of protection for model runs over a range of GEM fitness costs and initial DRA frequencies when no additional de novo DRA formation occurs. Color denotes the duration of the window of protection for each parameter combination

heterozygote fitness costs < 50%, we conducted a sensitivity analysis on the dominance of the fitness cost, depicted in Figure S2. Results for population suppression gene drives (homozygote fitness cost of 100%) suggest that reducing the dominance of the fitness cost increases the speed at which the drive system spreads into the population; but has little effect on the window of protection conferred by the drive. For a heterozygote fitness cost of 50% (Figure 1A, top row) and an initial DRA frequency of 10^{-7}, the number of wild-type female mosquitoes without the transgene is reduced by 95% within 345 days of the release. For a heterozygote fitness cost of 10% (Figure S2A, top row), this is achieved within 232 days, and for no heterozygote fitness cost (Figure S2C, top row), this is achieved within 217 days. In each case, the window of protection exceeds 4 years for initial DRA frequencies less than $3–5 \times 10^{-7}$ and is less than a year otherwise.

DRAs introduced de novo through NHEJ events, mutation and other mechanisms reduce the parameter ranges that lead to a long window of protection (3–4 years or higher, Figure 2). For DRA generation rates as low as 10^{-5} per Cas9-mediated cleavage event, the change is most visible for initial DRA frequencies of less than 10^{-6}. Here, a homozygote fitness cost of less than 40% is required for the window of protection to exceed 3–4 years, meaning that the population suppression strategy (fitness cost of 100%) is no longer able to achieve an extended window of protection. For higher DRA generation rates, $\sim 10^{-2}$ per Cas9-mediated cleavage event, as measured in several recent laboratory studies,[24,25] the window of protection for the population suppression strategy is less than 200 days for all parameter combinations. However, the population modification strategy can still induce a long window of protection (exceeding 3–4 years), e.g. for an initial DRA frequency of 10^{-2} and a homozygote fitness cost less than ~15%.

CONCLUSIONS AND OUTLOOK

The simulations presented here serve to illustrate that CGD strategies for malaria differ significantly with respect to their sensitivity to DRAs. Conclusions concerning the potential impact of these results can be informed by what we know about the distribution of such DRAs in target mosquito populations. Information derived from a recent bioinformatic study of potential DRAs in this group of mosquitoes is summarized in BOX 1.

The protein-encoding portion of the genome of this mosquito contains an abundance of potential gRNA target sites, however when the presence of potential DRAs is considered the availability of these sites decreases significantly. The simulations presented here indicate that a GEM with a fitness cost of as high as 15% if introduced into a population carrying a DRA at a frequency of 1% will provide a window of protection of at least 3–4 years. A GEM introduced into the same population with a fitness cost of 100% in homozygous females, as would be the case for the population suppression strategy, would be expected to provide a window of protection of less than one year and this GEM would provide a four-year window of protection only if the DRA frequency is $< 5 \times 10^{-7}$. Any allele occurring at such a low population frequency is difficult to accurately measure by sampling. Based on these results it appears that CGD population

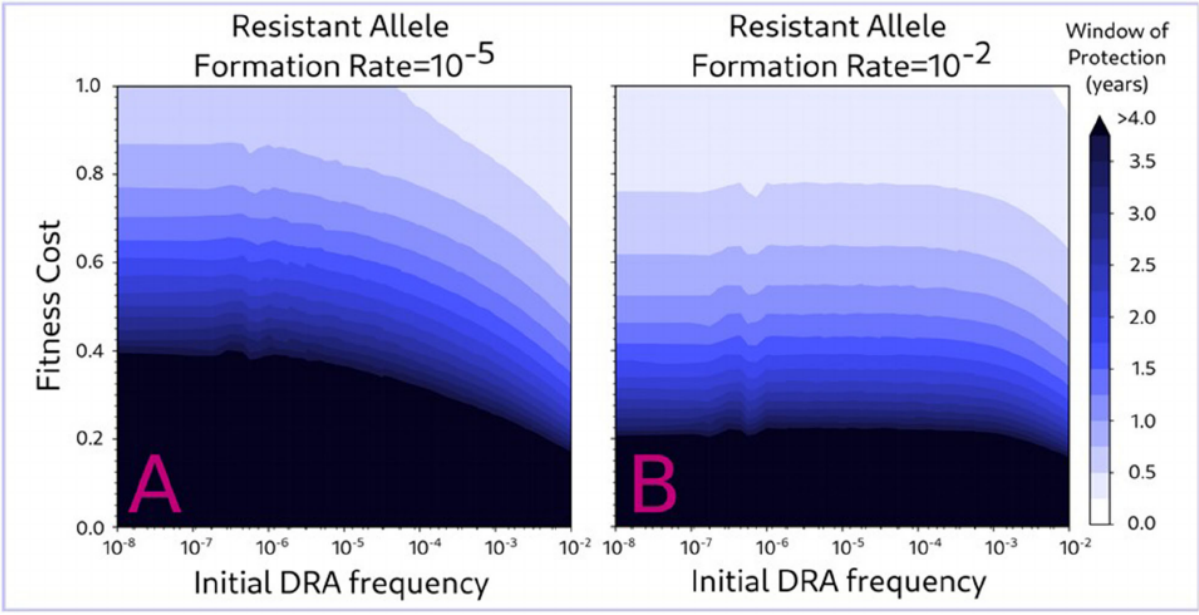

FIGURE 2 Modeled windows of protection for a range of GEM fitness costs and initial DRA frequencies when additional de novo DRA formation occurs (e.g. through non-homologous end-joining events, mutation, etc.). Color denotes the duration of the window of protection for each parameter combination. Panel A. The rate of additional de novo DRA formation is 10-5 per CRISPR-mediated cleavage event. Panel B. The rate of additional de novo DRA formation is 10-5 per CRISPR-mediated cleavage event

Box 1. Key attributes of Cas9/gRNA target sites and presence of drive resistant alleles in natural populations of the malaria mosquito, *Anopheles gambiae* from Schmidt *et al.*, 2020.[31]

- Median number of targetable sites per coding gene in *An. gambiae* genome: 72
- "Optimal" target sites can be defined as those with no DRAs above a predefined frequency D
- The fraction T of optimal target sites among all potential targetable sites varies with D:

$$D = 1\% \rightarrow T = 28.2\% \quad D = 0.15\% \rightarrow T = 6.3\%$$

suppression strategies face serious challenges if not introduced into a population in which the gRNA target site carries essentially no DRA polymorphisms.

This study shows that population modification approaches to vector control are uniquely resilient to DRAs, whether pre-existing, as part of SGV or created de novo. Several assumptions have been made in the modeling portion of this study, most notably that mosquito populations on small islands are panmictic. Modeling to include population sub-structuring would present the opportunity to incorporate spatial structure in the distribution of DRAs, as well as the potential for monitoring efforts to detect and react to population rebounds for the population suppression strategy. It has been suggested that CGD strategies can mitigate issues with DRAs through releases of alternative GEM strains having different gRNA target sites or by using multiplexed gRNAs to target multiple sites within one or several essential genes in the same genome.[35,41] However, identifying mosquito gRNA targets free of DRAs at frequencies low enough to be useful for suppression strategies will likely be problematic.[31]

Issues with population modification strategies, such as the evolution or prior existence of parasite resistance to anti-*Plasmodium* effector gene(s), remain to be explored, but resilience to DRAs is a major strength that this strategy has over population suppression. This must be considered among the set of strengths and weaknesses for each strategy as they advance through the development and deployment pipeline.

ACKNOWLEDGMENTS

This work was supported by grants from the University of California Irvine Malaria Initiative and Open Philanthropy. The authors thank Ana Kormos, Melina Campos and Marc Crepeau for helpful comments to earlier drafts of this paper.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

This paper deals with a perspective on resistance to gene flow. It includes simulations based on published information and does not include new data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Lanzaro, G. C., Sánchez C., H. M., Collier, T. C., Marshall, J. M., & James, A. A. (2021). Population modification strategies for malaria vector control are uniquely resilient to observed levels of gene drive resistance alleles. *BioEssays*, e2000282. https://doi.org/10.1002/bies.202000282