

Review



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Control of malaria-transmitting
mosquitoes using gene drives

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Gene drives are selfish genetic elements that can be re-designed to invade a population and they hold tremendous potential for the control of mosquitoes that transmit disease. Much progress has been made recently in demonstrating proof of principle for gene drives able to suppress populations of malarial mosquitoes, or to make them refractory to the *Plasmodium* parasites they transmit. This has been achieved using CRISPR-based gene drives. In this article, I will discuss the relative merits of this type of gene drive, as well as barriers to its technical development and to its deployment in the field as malaria control.

This article is part of the theme issue 'Novel control strategies for mosquito-borne diseases'.

1. Introduction

Malaria is a disease transmitted exclusively through the bite of an infected female mosquito. As she takes a bloodmeal, an infected mosquito can release a few *Plasmodium* parasites into the peripheral circulation of the human host and these are sufficient to establish infection, allowing other mosquitoes to take up parasites in subsequent bites and complete the cycle. Of the 3500 or so mosquito species that exist though, only those within the *Anopheles* genus are actually capable of transmitting human malaria. Among these, about 40 species are capable of transmitting malaria at a level of major concern to public health [1,2]. Looking in more detail at sub-Saharan Africa, where the majority of the malaria burden falls, an even more restricted set of *Anopheles* species are responsible for vast proportion of disease transmission. This means that vector control—reducing the numbers of mosquito vectors in a given area—can focus on a small number of species yet still achieve large gains in reducing malaria transmission.

The use of vector control in reducing malaria transmission is best illustrated by the demonstration that the halving of malaria-related deaths, from close to a million per year in 2000 to approximately 450 000 in 2015, is largely attributable to the use of insecticide-treated bednets and indoor residual spraying of insecticide, which kill mosquitoes as they look to bite or when they rest on treated walls after biting, respectively [3]. Despite these large gains, progress in this area is stalling, largely owing to the widespread emergence of insecticide resistance [4]. In addition to the search for novel insecticide classes and formulations, as well as improvements to their deployment, there is a need for additional innovation in vector control. One such innovation is the idea of genetic control—the deliberate introduction of genetic traits into a population that will affect its ability to transmit disease, either by suppressing the population or by affecting the intrinsic capacity of the insect to host the disease agent.

2. Genetic control

Genetic control of insects involves the deliberate release of individuals containing some desirable (for the purposes of control) genetic trait in order to introduce this trait into the extant population via mating. Because mating is a very species-specific process and since the process relies on released individuals seeking out the target population this type of approach can be very targeted in

its effect. The most widely used form of genetic control is the sterile insect technique, first proposed in the 1950s [5]. The technique relies on the mass rearing of large numbers of sterile males which outnumber the wild population locally and compete with wild-type males for female mates. It has been used successfully to control a wide range of agricultural pests as well as urban populations of *Aedes aegypti* mosquitoes that serve as vectors of the Dengue and yellow fever viruses [6–8]. Because the technique generally entails mass rearing on an industrial scale and constant release, it requires sustained investment and is best suited to the localized control of relatively contained populations, where problematic immigration of mated females from outside the release area is less likely.

The possibility of making transgenic mosquitoes has opened up new possibilities for genetic control. Indeed there are already examples of modified versions of the sterile insect technique, which rely on sterility being conferred by a transgene, having been deployed in the field successfully to suppress urban mosquito populations [9–12]. What is more, the availability of an assembled genome for many of the major malaria vectors, coupled with an ability to manipulate these genomes through modern genome editing tools, offers the possibility of identifying the genetic determinants of key traits such as reproduction, host-seeking behaviour and parasite susceptibility. This, in turn, offers the possibility to modify these traits in wild populations, but one crucial question remains: how do you spread these traits in a population? The solution to this is not immediately trivial—any of these traits are unlikely to confer a significant fitness benefit and therefore will not be selected, meaning that their frequency in a given population will not increase over time. In fact, many of these engineered traits may even incur a negative fitness effect, upon the mosquito at least.

In the case of sub-Saharan Africa, where the malaria burden is highest, malaria vectors are rurally distributed across large geographical ranges and it may not be feasible to release sufficient numbers of mosquitoes in order to reach an appreciable frequency in the target control area, at scale [13]—even for an introduced trait that were neutral to the mosquito one would have to release, *at minimum*, a quantity of mosquitoes far in excess of the wild-type into *every* village population. This means that without some other force to push these traits through a population they would be rapidly lost, either owing to selection or genetic drift, or both. Such a force can be provided by a ‘gene drive’, which describes any genetic element that is able to bias its inheritance among offspring. Often, the strength of the bias in transmission is sufficiently extreme that the gene drive element can increase in frequency even if each individual element imposes a fitness cost on the host. For this reason, gene drives have been proposed as a form of genetic control that could introduce traits into a population over a rapid timeframe, even starting from a very low release frequency [14].

3. Gene drives in *Anopheles* mosquitoes

In looking to build synthetic versions of gene drives, inspiration has been taken from naturally occurring examples of this phenomenon. These can include sex distorter genes or chromosomes, genetic underdominance, toxin : antitoxin systems and

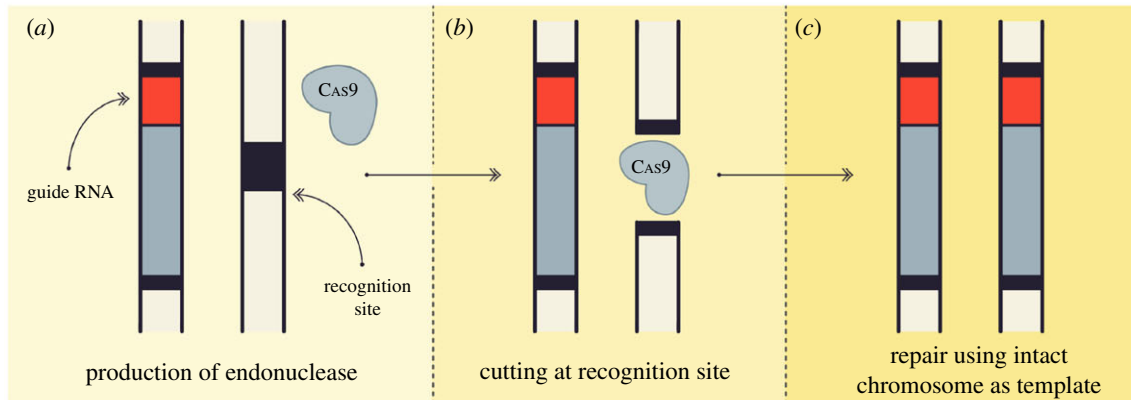
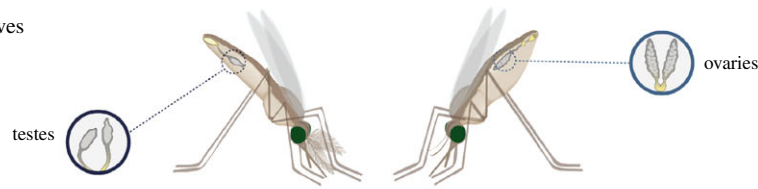
other selfish genetic elements that make extra copies of themselves in the germline [14–21].

In malaria-carrying mosquitoes, one such example that has proved the most fruitful to date is that of the homing endonuclease genes (HEGs) [14], that will form the bulk of the discussion here, although many of the considerations arising can be applied more broadly to gene drives in general.

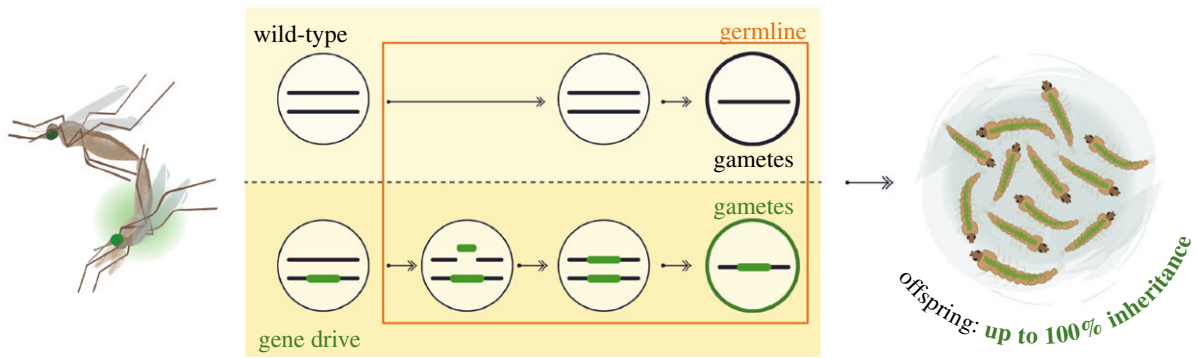
HEGs encode only a DNA endonuclease, which recognizes and cuts a unique DNA sequence. New endonuclease technologies such as CRISPR-Cas9, which are easier to re-programme to recognize a sequence of choice, have been repurposed to mimic the action of an HEG. Ordinarily an HEG resides on a chromosome within its own recognition sequence but, in a diploid organism, when this chromosome is paired with a chromosome that is void of the HEG and contains only the intact recognition sequence, that chromosome is cut, causing a double-stranded break in the DNA (figure 1, top). In repair of the broken strand, the loose DNA strands are resected slightly and anneal to the intact chromosome at regions of homology. The host cell DNA repair machinery uses this chromosome as a template on which to synthesize new DNA to ‘fill’ the gap, in a process called homology-directed repair. In so doing it leads to ‘homing’ of the HEG and a small amount of surrounding sequence so that it is now duplicated on both chromosomes. For homing to lead to a biased inheritance of the HEG it must occur in the cells that contribute to the next generation (the ‘germline’ in multicellular organisms)—if it happens efficiently in the cells that become gametes, then an individual heterozygous for the HEG will produce a majority of gametes with a copy of the HEG and its associated trait, compared to the 50% that would be expected to receive it through normal Mendelian segregation (figure 1, middle). Therefore, the HEG can rapidly increase in frequency each generation.

In both *Anopheles gambiae* and *Anopheles stephensi* mosquitoes, CRISPR-based HEGs have been shown to show biased inheritance rates of close to 100% [22–24]. Broadly, in order to couple a trait of interest to the gene drive, two main approaches are available: the gene drive knocks out some essential gene in the mosquito; the gene drive is tightly linked to some desirable effector gene (a cargo) (figure 1, bottom panel). In the former case, the presence of the gene drive should cause a recessive phenotype, effectively acting as a sort of genomic parasite—as the gene drive spreads through the population heterozygous individuals are fully viable, meaning they continue to carry and transmit the gene drive, whereas individuals homozygous for the gene drive are non-viable, leading to a reduction in the reproductive capacity of the population. Examples of this type of drive have targeted genes essential for female viability and have been shown in the laboratory to have a strong suppressive effect on populations [24–26]. Examples of the latter type of drive with a cargo focus on incorporating anti-*Plasmodium* effectors have similarly been shown capable of spread in laboratory populations of mosquitoes [23,27]. The type of cargo has so far taken the form of single-chain antibody fragments but could feasibly incorporate other broad-acting anti-microbial peptides that show strong levels of inhibition of parasite strains in the laboratory [28,29].

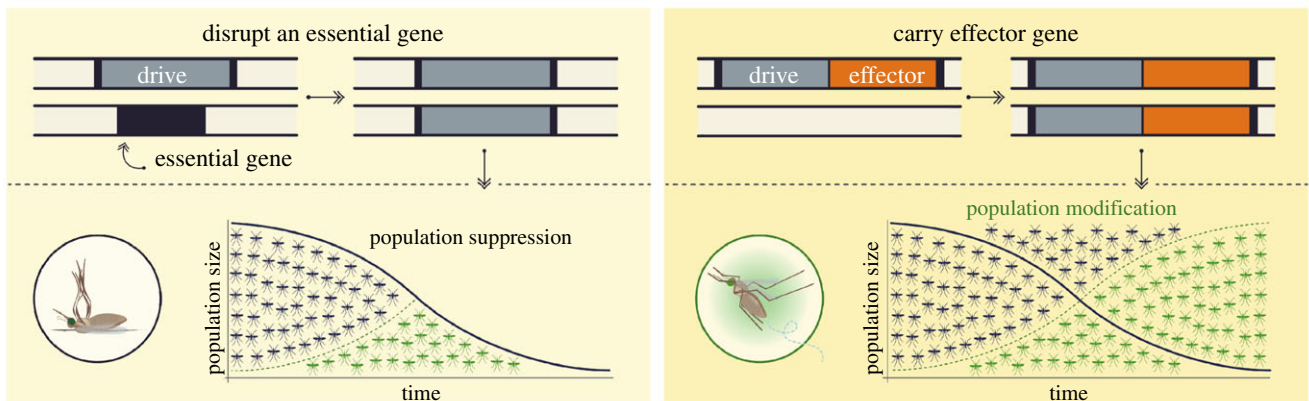
One interesting possibility, yet to be realized, is the option of a hybrid of the two approaches whereby, rather than carrying an anti-*Plasmodium* gene as cargo, the gene drive instead disrupts a gene encoding for a mosquito ligand that is essential for the parasite to complete its life cycle in the mosquito.



1 copying



2 gene drive inheritance



3 application

Figure 1. Mode of inheritance of CRISPR-based gene drives and their potential applications. Panel 1: the gene drive cassette consists of a source of Cas9 under a germline-specific promoter and a guide RNA designed to target the Cas9 to recognize and cut the target site on a wild-type chromosome. Because the gene drive is initially inserted within its own recognition site the chromosome containing the gene drive is immune from cleavage. Thus in an individual heterozygous for the gene drive, the wild-type chromosome is cut. The homology-directed repair pathway, invoked by the host cell, causes the broken ends of the cleaved chromosome to invade the intact homologous chromosome and pair at regions of homology before then using templated DNA synthesis along the intervening sequence, followed by resolution of the two chromosomes once this is complete. This 'homing' process leads to a duplication of the gene drive so that it is now on both chromosomes. Panel 2: if the homing process happens in the germline prior to gamete formation then a higher proportion of the gametes from heterozygous individual will contain a copy of the gene drive than the 50% that would be expected if normal Mendelian inheritance applied. In *Anopheles* mosquitoes, the homing process can be very efficient and in some cases the rate of inheritance of a synthetic homing gene drive can approach 100%. Homing can occur in the germline of both sexes. Panel 3: left panel: population-wide knockout of some essential gene that produces a desirable phenotype (from a malaria control perspective) in individuals homozygous for the gene drive, such as female infertility, by virtue of the gene drive disrupting the coding sequence of the target gene. Importantly for this approach to work most effectively, 'carrier' individuals heterozygous for the drive should be fully viable. right panel: population-wide spread of an effector gene as 'cargo'. Gene drives that interfere with mosquito reproduction or viability aim to eliminate or suppress the population to levels that do not support disease transmission (left panel). Alternatively, gene drives engineered to directly impair vector competence can invade the vector population, transforming it over time to become one that is refractory to malaria transmission (right panel).

4. Limitations and challenges of gene drive technology for malaria control

Gene drives that are designed to spread and impose a suppressive effect on either the mosquito or the parasite it transmits are expected eventually, like any intervention, to select for resistance in each organism, respectively. In order to mitigate against resistance, the usual rules apply: target sites and processes that are strongly constrained and target them at multiple points. This is perhaps best illustrated by the selection of resistant target site variants that occurred in the face of the first generation of homing-based gene drives that did not prioritize functional constraint in the choice of target site [27,30]. The resistant alleles that were selected both prevented cutting by the nuclease in the gene drive and conferred a positive fitness effect relative to the gene drive allele. By contrast, a gene drive designed to incapacitate a gene essential for female sexual development, targeting a highly constrained site, led to suppression of a laboratory population and failed to select for resistance [26]. The best indicator of functional constraint is sequence conservation over evolutionary time and in this aspect the availability of genomes from across the *Anopheles* genus and the resequencing of wild-caught *A. gambiae* and *Anopheles coluzzii* are an invaluable resource, allowing the identification of ultraconserved sites that are targetable by CRISPR-based gene drives [31–33]. As further contingency several guide RNAs can be included in the same gene drive construct in order to target multiple sites in the same target gene, where only one need be active in order for the gene drive to function [20,22,34].

If targeting the parasite directly, effector genes that form part of the gene drive cargo must be equally robust to parasite resistance. Again, contingency planning is available here by targeting the parasite at different stages throughout its life cycle in the mosquito, and choosing to interrupt parasite-specific pathways, or epitopes (if using single-chain antibodies) that are highly constrained [35,36].

5. Containment of gene drives

There are many flavours of gene drive potentially available but for the CRISPR-based homing gene drives so far developed in *Anopheles* mosquitoes, one of the most attractive and potent features of gene drives—their ability to spread from a very low initial frequency—is also one that causes concern. For example: accidental releases are hard to contain; a phased pathway of local test trials is challenging for a technology so invasive; issues of governance arise where the intervention spreads outwith the original release area, or across new territories. It is beyond the scope of this technical article to address these in detail but these concerns have prompted interest in alternative gene drive systems that are less invasive or require a threshold of release frequency to be superseded in order for the drive to invade the population [20,37,38]. As an intermediate, ‘split’ gene drive systems, where the source of Cas9 is uncoupled from the element that is copied through homing, have also been proposed [39–41] and may allow useful optimization of the relevant components of a fully invasive gene drive prior to deployment.

An alternative strategy to deliberately limit the spread of a gene drive is to target it to an allele that is private and locally fixed in a particular sub-population [42,43]. Such private alleles might exist owing to a recent selection that acted directly on the allele itself, or owing to a selective sweep that happened to

encompass the allele, or owing to a founder effect that fixed the allele by chance. It is important to distinguish between these scenarios because each would imply a differing level of functional constraint at the target site, and therefore a different propensity to tolerate drive-resistant alleles. For example, an allele that is fixed in population ‘by accident’ is likely to be much more tolerant of mutations that render it resistant to a gene drive than a private allele that was the direct object of selection and therefore likely under some level of functional constraint.

6. Transferring gene drives to other *Anopheles* mosquitoes

Assuming its target site is conserved, a gene drive built in *A. gambiae* would be expected to introgress into *A. coluzzii*, and vice versa, since the level of reproductive isolation between these two species of the wider *A. gambiae* species complex is incomplete [44,45]. Indeed a precedent for a similar type of event is the recent adaptive introgression of insecticide-resistant alleles between these two species [46,47]. Even if hybridization between the two genomes occurs only rarely, as long as it happens at some appreciable frequency, CRISPR-based gene drives would be expected to complete their introgression into the new genome very rapidly, since in the act of homing from one chromosome to the other only the gene drive and a tiny amount of its original neighbouring genomic sequence are copied (figure 1, top).

The extent of reproductive isolation between *Anopheles arabiensis*, another significant malaria vector in the species complex, and *A. gambiae* is probably more extreme and although introgression can be forced in the laboratory, the extent to which it occurs in the wild is unclear.

Supposing one were to build a gene drive de novo, for example to target a different *Anopheles* species that was the dominant vector species locally, the following would be prerequisites: an ability to edit the genome initially in order to introduce the gene drive cassette; promoter sequences that can direct Cas9 expression in the germline; a propensity for Cas9-induced cleavage to lead to homing of the gene drive allele (rather than repair through pathways that do not depend on homology); and high-quality genomic data in order to allow optimal choice of conserved target sites. For the prospects of making a transgenic organism, the universality of Cas9 genome editing as a tool is a boon and has been used to introduce germline modifications in a diverse range of malaria vectors including *Anopheles funestus* and *Anopheles albimanus* [48], meaning that it should be possible to build and test gene drives across a wider range of *Anopheles* species. Moreover, a recently developed technique that obviates the need to develop an embryo injection protocol for each species could also widen the scope of vectors to be targeted, providing it can be adapted to introduce genetic constructs rather than just induce small deletions, as is currently the case [49].

Assuming technical barriers to creating a gene drive in a mosquito species can be overcome, the optimal parameters for its performance, and the feasibility of its success in malaria control, will be exquisitely dependent on the particular ecology and life-history traits of the species at hand [50].

7. Summary

Targeting the mosquito vector in order to interrupt transmission has been the mainstay of successful malaria control

programs over the years. Gene drives represent a powerful tool to achieve this in a targeted way that is species-specific, requires minimal infrastructure and is self-sustaining. Moreover, if successful, the benefits of this type of intervention would be available to all, regardless of differential access to healthcare.

Gene drives able to crash mosquito populations or to spread anti-parasite effectors to fixation have been shown to work in the laboratory. Following on from this promise, future steps will need to consider how to test these at scale and test their robustness and efficacy in larger population sizes under more realistic ecological settings. Because gene drives still represent a relatively novel technology, with unique characteristics, the pathway to regulating their use and obtaining consensus on whether and how to use them is challenging.

There has been a concerted effort by scientists working on gene drives to develop both a pathway to their development [35,51–53] and to establish minimum efficacy and safety criteria [54]. It is encouraging also to see a similar weight of attention given to the process of public engagement and

knowledge transfer around gene drives [55–57]. This is especially important for gene drive mosquitoes, given that they are designed to be invasive and that they are genetically modified, both features that generate a certain sensitivity among the public.

Notwithstanding the transformative potential of gene drive technology it is unlikely that it, or any other novel malaria control tool for that matter, will be a silver bullet, given the proven resilience of both the parasite and the vector in the past. It is therefore essential that there is a combination of approaches, applied in a coordinated fashion to achieve maximal synergy between them, in order to achieve the goal of malaria elimination.

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