

# Gene Drive and Symbiont Technologies for Control of Mosquito-Borne Diseases

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## Keywords

population modification, population suppression, *Wolbachia*, paratransgenesis, toxin–antidote, modeling

## Abstract

Mosquito-borne diseases, such as dengue and malaria, pose a significant burden to global health. Current control strategies with insecticides are only moderately effective. Scalable solutions are needed to reduce the transmission risk of these diseases. Symbionts and genome engineering-based mosquito control strategies have been proposed to address these problems. Bacterial, fungal, and viral symbionts affect mosquito reproduction, reduce mosquito lifespan, and block pathogen transmission. Field tests of endosymbiont *Wolbachia*-based methods have yielded promising results, but there are hurdles to overcome due to the large-scale rearing and accurate sex sorting required for *Wolbachia*-based suppression approaches and the ecological impediments to *Wolbachia* invasion in replacement approaches. Genome engineering-based methods, in which mosquitoes are genetically altered for the modification or suppression of wild populations, offer an additional approach for control of mosquito-borne diseases. In particular, the use of gene drive alleles that bias inheritance in their favor is a potentially powerful approach. Several drives are frequency dependent, potentially giving them broadly similar population dynamics to *Wolbachia*. However, public

acceptance and the behavior of released drives in natural mosquito populations remain challenges. We summarize the latest developments and discuss the knowledge gaps in both symbiont- and gene drive-based methods.

## 1. INTRODUCTION

Dengue fever, yellow fever, malaria, and other diseases transmitted by arthropods are global public health problems (134). Mosquitoes are the major disease vectors worldwide, transmitting a variety of pathogens that impact humans and livestock. The main disease-transmitting mosquitoes are *Anopheles*, *Aedes*, and *Culex* species. Currently, the control of these diseases mainly relies on suppressing vector populations through chemical insecticides (35). However, the rise of insecticide resistance hinders the efficacy of this approach (35), while excessive use of pesticides also causes environmental pollution and damages nontarget organisms. In recent decades, additional issues have arisen due to several factors, including changes in mosquito ranges and/or vector competence (7, 17, 35). New tools to reduce mosquito-borne disease prevalence are needed. Two novel control strategies, symbiont- and genetic-based methods, have the potential to produce high vector-killing or pathogen-inhibition efficiency.

### *Wolbachia*:

a cytoplasmically inherited bacterial genus that is widespread in arthropods and nematodes

### Cytoplasmic incompatibility (CI):

sperm-egg incompatibility where a male infected with *Wolbachia* mates with uninfected females or females infected with an incompatible *Wolbachia* strain

### Population suppression:

a strategy to reduce or eliminate a target population

### Population replacement:

a strategy to modify a target population, such as generating a modified mosquito population with a reduced capacity for pathogen transmission

## 2. SYMBIONTS AND CONTROL OF MOSQUITO-BORNE DISEASES

Mosquitoes harbor a dynamic and diverse microbiome (140), which is mainly influenced by the habitats of the aquatic and terrestrial life cycle stages. The microorganisms of mosquitoes mainly congregate in the midgut and other organs, such as the reproductive tract and salivary glands (140). Some microbes can impact the host's susceptibility to pathogens, and progress has been made in controlling mosquito-borne disease using both natural and engineered symbiotic microbes (Figure 1).

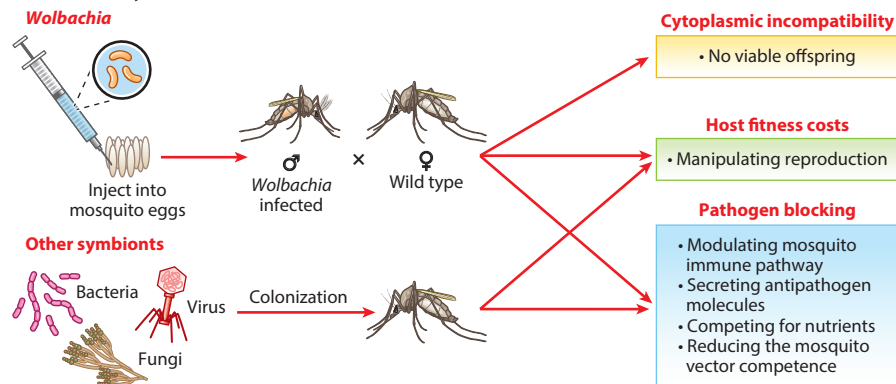
### 2.1. Symbiotic *Wolbachia* and Other Bacteria

The symbiotic bacterium *Wolbachia*, a natural obligate intracellular microbe that infects arthropods, has long been considered as a candidate for disease suppression. Strategies rely on two crucial *Wolbachia* traits, cytoplasmic incompatibility (CI) and pathogen blocking. CI is induced when *Wolbachia*-infected male mosquitoes eliminate or reduce offspring produced by females lacking the same *Wolbachia* strain (10). The CI is characterized by delayed or defective paternal chromatin, which may be lost during the first mitosis or undergo additional division, leading to the formation of haploid or aneuploid embryos, respectively (119).

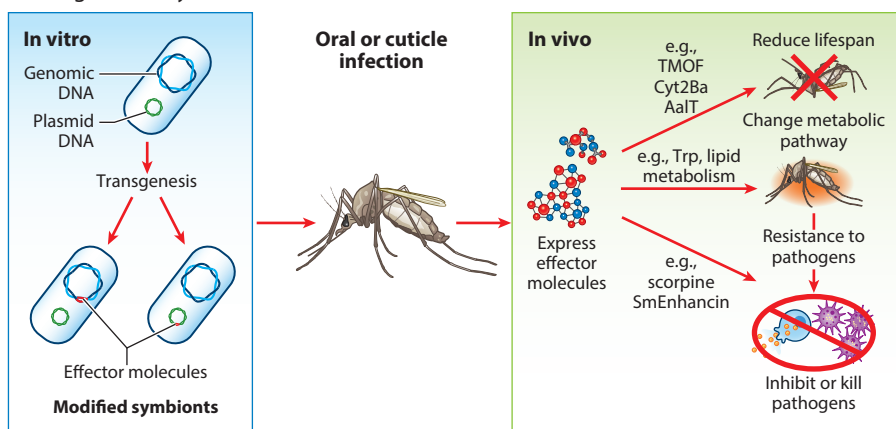
*Wolbachia*-induced CI following repeated release of infected males (but not females) can lead to a sharp decrease in the size of the target wild mosquito population, thereby reducing the potential for disease transmission. This population suppression approach requires ongoing releases of *Wolbachia*-infected males, often across large areas (10).

A different strategy, population replacement, aims to introduce a novel *Wolbachia* infection with pathogen-blocking properties into a population and allow it to spread to fixation (thus the use of the term replacement). The novel infection represents a deliberate introduction from a different species (i.e., a transinfection) aiming to induce CI and pathogen blocking. Both male and female *Wolbachia*-infected mosquitoes are released. Males reduce the target population through CI, while females introduce the *Wolbachia* (88). Once *Wolbachia* reaches a threshold frequency in a population, it starts to spread by itself because females with *Wolbachia* do not exhibit CI when mating with infected males, providing them with a fitness advantage over wild females from the

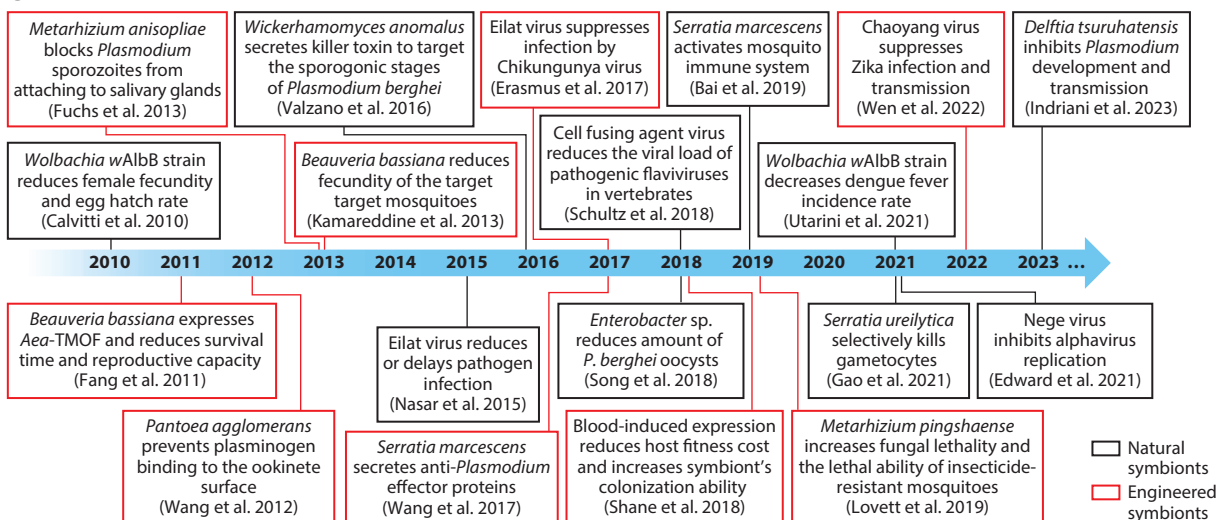
### a Natural symbionts



### b Engineered symbionts



### c



(Caption appears on following page)

Using symbionts as a novel mosquito control strategy. (a) Natural symbionts block pathogen transmission and suppress mosquito population size through three effects: causing cytoplasmic incompatibility, regulating host fitness costs, and blocking pathogens. (b) Engineering symbionts to block pathogen transmission: First, researchers select the target strain and modify it in vitro; second, mosquitoes are fed engineered symbionts or infected with them directly; and third, the modified strains secrete effector molecules that shorten the mosquito life cycle or regulate the host metabolism to enhance pathogen resistance. (c) Selected recent milestones in research on symbiont technologies to control mosquitoes.

target population. The releases of transinfected *Aedes aegypti* with the *Wolbachia* infection *wMel* in Indonesia (122) and with the *wAlbB* infection in Malaysia (57) illustrate how the approach can provide strong reductions in dengue fever (>70%) when *Wolbachia* is at a high frequency, although it can be challenging to reach and maintain this stable state (see below).

Across different tissues of mosquitoes, including the midgut, salivary glands, and reproductive organs, other natural symbionts, apart from *Wolbachia*, can directly or indirectly impact the physiology of mosquitoes or mosquito-borne pathogens. Firstly, gut microbiota can modulate the infection of pathogens through direct inhibition. The gut bacterium *Serratia ureilytica* secretes a specific lipase, AmLip, that selectively kills gametocytes without causing apparent changes in mosquito biology (43). Another natural symbiotic bacterium, *Delftia tsurubatensis* TC1, can inhibit early stages of *Plasmodium* development and subsequent transmission by the *Anopheles* mosquito via secretion of a hydrophobic molecule, harmone (60). Second, the gut microbiota can activate the immune pathways of host mosquitoes, like the Toll, immune deficiency (IMD), and JAK-STAT signaling pathways, through the recognition of molecular markers and thus reduce the abundance of pathogens after blood feeding (94). For instance, *Serratia marcescens* isolated from field-caught female *Anopheles sinensis* activates the mosquito immune system IMD pathway via the transcription factor Relish 2 and inhibits *Plasmodium* (5). Finally, the host insect gut microbiome forms a physical barrier that can block pathogen infection. For *Anopheles stephensi* mosquitoes, antibiotic treatment increases the amount of *Plasmodium berghei* oocysts in the gut, which decreases the rate of reintroduction of an *Enterobacter* sp. from antibiotic-resistant mosquitoes (116). Challenges remain to be solved before this information can be translated into applications. Caution is required because some gut symbionts may enhance the susceptibility of mosquitoes to pathogen transmission (128, 135).

The mosquito gut symbiotic bacteria *Pantoea agglomerans* and *S. marcescens* are modified to secrete salivary gland and midgut polypeptide 1 or phospholipase-A2 targeting *Plasmodium falciparum*, separately blocking the transmission of malaria (129, 130). However, engineered symbiotic bacteria that consistently express effectors have increased fitness cost (113). To address this, the symbiont *Asaia* of *Anopheles* mosquitoes has been engineered to induce expression of the protein scorpine only after blood feeding (113). The blood-induced expression not only reduces the fitness cost of the engineered symbiotic bacterium, but also increases its colonization ability in the midgut of mosquitoes. Engineered symbiotic bacteria for mosquito-borne disease control are mostly based on plasmids that express effector molecules (113, 129, 130), which can be transferred horizontally to other mosquito-associated or environmental bacteria; the consequences of such transformations are unknown.

## 2.2. Natural and Engineered Fungal Associates

The fungal community associated with mosquitoes and the microbes that they transmit is shaped by multiple factors, including environmental conditions, vector species, and feeding behavior (118). Fungi can play several roles in reducing mosquito disease vectoring. First, some fungi reduce mosquito vector competence. For example, the fungus *Wickerhamomyces anomalus* secretes

## PARATRANGENESIS

Paratransgenesis is a technique that utilizes engineered microorganisms to produce effectors that either enhance the ability of mosquitoes to resist or reduce pathogens or kill the mosquitoes; it has been widely used in *Anopheles* mosquitoes. It requires, first, genetically modifying microorganisms to produce effectors that can target pathogens or mosquitoes directly. The modified microorganisms are then reintroduced into the host to block or kill the pathogens or mosquito. Paratransgenesis has been successful in reducing mosquitoes' ability to transmit pathogens such as malaria parasites.

a killer toxin that can target the sporogonic stages of *P. berghei* in vitro (123) (see the sidebar titled Paratransgenesis). Second, fungi are the most common pathogens of mosquitoes, and entomopathogenic fungi (EPFs) often regulate mosquito populations (126). The most widely used and studied EPFs to control adult mosquitoes belong to *Beauveria* and *Metarhizium*, and there is a lot of research about their infection biology (72, 98).

EPFs infect insects via cuticle penetration. After depleting host nutrients and killing insects, the fungus transitions to filamentous growth and produces conidia on the carcass surface (126). Generally, engineering of EPFs focuses on improving resistance to abiotic stress and increasing specific virulence to insects (126). Modified *Beauveria bassiana* that express the insecticidal protein from *Bacillus thuringiensis* toxin Cyt2Ba not only increase virulence against larval and adult *Aedes* mosquitoes, but also decrease fecundity of the infected mosquitoes (26). Engineered *Metarhizium pingshaense* (Mp-Hybrid strain) expressing an insect-specific toxin show increased lethality, while field experiments indicate that the Mp-Hybrid strain kills the *Anopheles* population faster than the wild-type strain (72). Modified *B. bassiana* with two insect peptides that disrupt the host's normal endocrine or neurological balance reduce the survival time and reproductive capacity of adult *Ae. aegypti* (37), while in another study, expression of the *Ae. aegypti* trypsin-modulating oostatic factor inhibited adult and larval food digestion rates of *Anopheles* (61). In addition to modifying fungal virulence to kill mosquitoes directly, it is possible to express effector molecules directly acting on pathogens. Recombined *Metarhizium anisopliae* expressing a peptide that blocks sporozoite attachment to salivary glands in *Anopheles gambiae* profoundly impact mosquito mortality, blood-feeding activity, and the prevalence of *Plasmodium* sporozoites (38). These developments point to the feasibility of developing mycoinsecticides for the control of mosquito-borne diseases, although the effectiveness and safety of engineered EPFs in field applications remain to be tested (38, 126).

### 2.3. Symbiotic Insect-Specific Viruses

Mosquitoes harbor diverse insect-specific viruses (ISVs). These can induce reinfection exclusion or homologous interference, where prior viral infection reduces or prevents subsequent infection by closely related viruses, including viruses vectored by mosquitoes (34, 78, 112). Cell fusing agent viruses identified in *Ae. aegypti* were the first reported ISVs (117). With advances in molecular tools and high-throughput sequencing, numerous other ISVs have been discovered in mosquitoes (114, 136). For example, Nege viruses can inhibit alphavirus replication when coinfecting with alphaviruses in mosquito cells (34), while cell fusing agent viruses can reduce the viral load of pathogenic flaviviruses such as Zika virus (112). These findings are based on mosquito cell models, and further work is needed on mosquitoes themselves; one report indicates that Eilat virus reduces or delays infection of pathogenic arbovirus such as Chikungunya fever virus in *Ae. aegypti* mosquitoes (78). However, the impact is not long-lasting or complete, suggesting that the viral effects would need to be optimized to significantly restrict arbovirus transmission in vivo (78).

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**Entomopathogenic fungi (EPFs):** fungi that can secrete insect toxin proteins to kill pests for population suppression

**Insect-specific viruses (ISVs):** viruses that exclusively infect insect cells without replicating in vertebrate cells

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**Gene drive:** a genetic element that can bias inheritance in its favor

Mosquito viruses can be genetically modified to produce chimeric antigens for the prevention of arbovirus infections in vertebrate animals. For example, a chimeric vaccine based on Eilat virus could induce both neutralizing antibodies and T cell responses in a mouse model, effectively suppressing infection with Chikungunya fever virus, which belongs to the same genus (*Alphavirus*) as Eilat virus (35). Similarly, a vaccine constructed by replacing the glycoprotein of the Chaoyang virus with the protein of the Zika virus can effectively suppress Zika infection and transmission (133). These viruses can only replicate in mosquito cells, providing a significant level of safety when used as a vaccine-based platform. Live-attenuated vaccines offer strong immunogenicity but carry the risk of incomplete inactivation. Conversely, inactivated vaccines prioritize safety at the expense of reduced immunogenicity. Insect virus vaccines, due to their inability to replicate in vertebrate species, can be uniquely designed to balance both immunogenicity and safety. This offers a novel and promising strategy for the prevention and control of mosquito-borne diseases.

### 3. GENE DRIVE

Gene drive alleles can bias their inheritance to spread through populations (53, 125, 127). They can be broadly classified into modification drives and suppression drives. The former can carry cargo genes to prevent disease transmission (2, 39, 100, 131) or make some other type of desired modification (31, 45), while the latter can reduce the size of vector populations.

Gene drives can additionally be classified by their outcomes and dynamics in several ways. The broadest involves limitations in how the drive might spread (see the sidebar titled Level of Confinement). Some drives are unconfined, while others are confined based on an introduction threshold (**Figure 2a**), the necessary frequency at which the drive must be introduced to spread successfully (otherwise, it will be eliminated). Examples of unconfined drives include homing drives (19, 63), which copy themselves, allowing rapid increases even with low starting frequencies. *Wolbachia* has dynamics similar to those of a confined drive (58, 59). Underdominance systems represent gene drives that have an introduction threshold even with perfect performance, while other types of confined drives and *Wolbachia* only have a nonzero threshold based on an

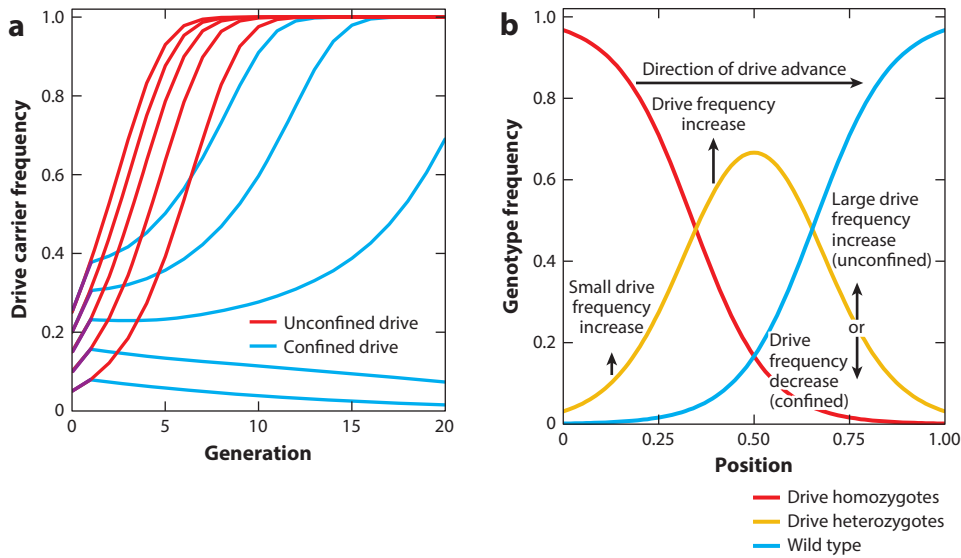
#### LEVEL OF CONFINEMENT

A critical parameter of a self-sustaining gene drive is its introduction threshold. This refers to the necessary release frequency in a panmictic population for the drive to further increase in frequency. If released below this level, the drive will be removed from the population. Confined drives also have a migration threshold, the level needed between two connected populations for a drive to spread from one to another. Unconfined gene drives such as homing drives will usually have an introduction threshold of zero and can thus spread between populations with any level of migration.

Many types of gene drives lack an introduction threshold in ideal form but gain one if there is any fitness cost. These gene drives can potentially spread widely, although their rate of spread tends to be extremely low at low frequency.

Underdominance drives will have a nonzero introduction threshold even in ideal form. This makes them more confined, although they also require higher release sizes for success. In spatially continuous environments, drives with an introduction threshold below 50% tend to be able to spread through well-connected areas, forming a wave of advance. If their introduction threshold is above 50%, they cannot spread well to new areas and may have difficulty persisting.





**Figure 2**

Gene drive properties. (a) The chart shows two drive types, released as homozygotes in a deterministic, discrete generation model. For the homing modification unconfined drive, the initial release frequency does not substantially affect the population dynamics. The CRISPR toxin–antidote confined drive (which has similar properties to *Wolbachia*) spreads rapidly at higher initial frequency and slowly at lower initial frequency. When the frequency is below the drive’s introduction threshold, the drive will decline. (b) In spatial models, gene drives tend to form waves of advance. The drive increases in frequency most quickly where there are more drive heterozygotes, and these regions are essential for confined drives and *Wolbachia*. Drive alleles may actually be lost at the front of the wave, where the local drive frequency is below the introduction threshold, although more drive alleles are dispersing into this area from the middle of the wave. For unconfined drives, the drive frequency will increase in this area even without dispersal, accounting for their greater rate of advance.

imperfection such as a fitness cost (**Figure 2b**). Gene drives in either category can also be self-limiting. These drives can spread rapidly initially but eventually will usually lose their power to spread (often because a critical allele disappears from the population) and decline in frequency toward zero.

Depending on the scenario, certain types of gene drives may be preferred. Modification drives generally have specific cargos that prevent disease transmission (11) or confer some other desired effect, such as pesticide sensitivity (47). However, cargo or other effector methods may not always be available, while suppression could remain a preferred outcome if the target is a pest species. The required level of confinement can also vary based on the regulatory environment, international cooperation, and the target species. For example, support may be available for utilizing an unconfined drive on an international vector species, but it may be preferred to target certain invasive species with a suppression drive that will not significantly spread to the species’ native range.

No gene drives have been released in wild populations to date. Several have been demonstrated in various species of mosquitoes, while other systems have been constructed in flies or other organisms (127). Some designs remain plausible but have only been modeled (127). In this section, we describe several gene drives, focusing on confined but self-sustaining systems, which are most similar to the dynamics of *Wolbachia*.

### 3.1. Unconfined Gene Drive Options

Many unconfined drive variants are of interest for comparisons. Early research into transposons was promising, but ultimately, high-efficiency variants could not be constructed (73, 86). Another early system designed for population suppression was based around X-chromosome shredding. If the drive is located in the Y chromosome, then eggs would mostly be fertilized by drive sperm, allowing drive spread and eventually population suppression via biasing of the sex ratio. However, while X shredders have been demonstrated in mosquitoes (40, 41), there have been no reports of X shredders being successfully inserted onto the Y chromosome, which would be needed for this system to be a gene drive.

The most successful unconfined drives to date have been homing drives (53, 125, 127). These operate by using a nuclease (CRISPR based in all recent studies) to cleave a wild-type allele at the same locus as the drive on the homologous chromosome. Homology-directed repair then copies the drive allele. When this happens in germline cells, inheritance will be biased toward the drive allele. However, mutations from end-joining repair can form resistance alleles, which are DNA sequences not recognized by the drive's guide RNA (gRNA) (18, 42, 48). Addressing these alleles, particularly functional resistance alleles (where the sequence change does not affect target gene function) in suppression drives, represents a major challenge. One strategy is to utilize a germline-specific Cas9 promoter that avoids maternal deposition of Cas9 into embryos, an important source of resistance alleles (12, 33, 49).

By targeting an essential gene, one can remove nonfunctional resistance alleles from the population. Multiplexed gRNAs can ensure that resistance alleles are nonfunctional, although this strategy has limits (22, 62). To avoid removal of the drive in modification drives, the drive must have a recorded rescue version of the target gene, which has been successfully demonstrated in flies (19) and mosquitoes (3). Homing suppression drives do not need a rescue element but also have higher performance requirements for success (22, 27, 137), although new designs can somewhat ease this performance requirement for the drive conversion rate (36). Suppression success has been achieved in *An. gambiae* (63) and *Drosophila melanogaster* (33) in laboratory settings but has not been attempted in other species.

It may be possible to confine a homing drive by using gRNAs with sequences fixed in only the target population (87), but such an approach is difficult considering the need for closely spaced gRNA targets (22). Another flexible method to confine homing drives (thus allowing confined suppression) is to use a tethered drive. This involves placing one component of the homing drive, such as Cas9, into a confined drive, thus preventing the homing drive from spreading beyond where the confined drive spreads (28, 76).

### 3.2. Self-Limiting Gene Drive Options

A self-limiting gene drive is designed to first increase in frequency in a population but eventually decline to zero. One example is the split homing drive, where Cas9 or the gRNAs are placed at a different genomic locus from the driving element (14, 30). Over time, this supporting element will decrease in frequency due to fitness costs or other effects, and the drive will subsequently lose its ability to bias inheritance. Daisy chain drives are similar but will last longer because they link several driving elements, each dependent on the next, with only the last initially following Mendelian inheritance (124). Killer-rescue systems are also self-limiting: The killer will cause lethality if the rescue is not present and thus will be removed from the population while boosting the rescue frequency (132). While potentially useful, self-limiting drive systems can be difficult to control and may require high release sizes (29).



### 3.3. CRISPR-Based Toxin–Antidote Drives

Recent advances in developing frequency-dependent gene drives based on CRISPR allow flexible engineering of systems with varying introduction thresholds. The simplest of these is similar to a homing modification rescue drive targeting a haplosufficient but essential gene. It provides a recoded copy of the target gene, allowing drive alleles to remain viable. However, drive conversion does not take place. CRISPR cleavage only forms nonfunctional resistance alleles. Because these are removed from the population in homozygotes, drive frequency increases in a frequency-dependent manner. Successful experimental demonstrations of this method in fruit flies have placed the drive in the target gene (17) or at a distant location (82), and follow-up studies have shown that one drive can be efficiently replaced with a similar system (83). These systems are fairly strong and lack an introduction threshold unless there are fitness costs. They could thus spread widely in well-connected regions but still be unable to spread between isolated populations. Self-limiting variants could further reduce invasiveness (84).

Functional resistance alleles can still affect CRISPR toxin–antidote drives, but they can be addressed more easily than in homing drives because higher numbers of multiplexed gRNAs can be used without sacrificing drive efficiency (15). Cas9 promoters can be more flexible as well, making efficient CRISPR toxin–antidote drives potentially easier to construct than other drive types. Indeed, maternal deposition of Cas9 actually improves drive spread rates if the target gene is haplosufficient (15).

Using two drive elements, greater confinement can be achieved. This involves having each drive target the gene that the other rescues (13). Drive organisms are thus often nonviable if they possess only one of the two types of drive alleles, increasing the necessary introduction threshold.

In addition to modification, CRISPR toxin–antidote drives targeting a haplolethal gene (where two functional genes are needed for organism viability) could be configured for population suppression (13, 15). This could be done by either placing the drive allele in a haplosufficient female fertility gene or targeting such a gene with additional gRNAs (without rescue). Such a drive system would have a high suppressive power, but maternal Cas9 deposition would increase its introduction threshold.

While most CRISPR toxin–antidote drives are frequency dependent, both modification and suppression drives based on early gamete or male gamete removal could be constructed as zero-threshold drives (15). Potential target genes with essential expression in haploid gametes are rare in animals but more common in plants, with two studies showing high efficiency in *Arabidopsis* (69, 85).

### 3.4. Cytoplasmic Incompatibility Drives

It is possible to use the same CI mechanism as *Wolbachia* in a gene drive by expressing two genes from the *Wolbachia* bacteriophage (115). However, Mendelian inheritance (as opposed to *Wolbachia* maternal inheritance) of the drive means that, without fitness costs, the introduction threshold is 36.5% (65). This drive may suffer less from fitness costs than *Wolbachia* and could support a variety of cargo genes. Self-limiting variants of this drive demonstrate performance that is nearly as good as that of the complete version (65).

### 3.5. Other Confined Drives

Several other types of drives with an introduction threshold of 50% have been demonstrated. One involves species-like incompatibility, where heterozygotes are nonviable (75). A similar system targets a haplosufficient gene with RNA interference while providing rescue, thus reducing heterozygote fitness (99). Another system involves reciprocal chromosomal translocations, although

these can have fitness costs (71). With less confinement, the *Medea* system kills offspring of *Medea* females that lack a *Medea* allele; thus, there is an introduction threshold only if there are fitness costs (23). However, most of these drive systems are older designs, and high-efficiency versions have not been generated in mosquitoes.

## 4. FROM THE LAB TO THE FIELD

### 4.1. Field Trials with *Wolbachia* Aimed at Suppression and Their Challenges

Field trials have now been undertaken with *Wolbachia* strains aimed at suppression. Strains in *Ae. aegypti* include *wAlbB* (6, 25, 90), and in *Aedes albopictus*, a natural double infection is present (9). Requirements for these release strains are relatively straightforward. Males must have high competitive fitness in the field when compared to wild males, and the strain should be easily cultured in the laboratory and sexed successfully to isolate males. Because there have been issues in accurate sexing and isolation of males, an added requirement is that released males maintain their competitive ability after exposure to a low level of radiation, which is used to ensure that any mistakenly released females are sterile (141), although it is not clear if this is always essential (138). Other useful characteristics include levels of pesticide resistance that match what is found in the field (where releases take place in areas that are regularly fogged by pesticides) and variation in male size, which needs to be carefully considered in automated sexing systems (25) given that small females, which are common in the field, prefer to mate with smaller males (8).

Suppression releases have become increasingly sophisticated in recent years, as effective suppression across large areas requires the production of millions of males and efficient delivery systems. Early releases involved sex sorting using plates that allowed separation of sexes based on pupal size, but this approach is not particularly accurate and requires subsequent sex validation by hand. More recently, approaches based on sieving (90) or on sexing through images of mosquitoes (25) have greatly improved sexing and allowed for high throughput, providing that mosquito larvae fall into a relatively narrow size range. Release methods have also evolved quickly from early containers where release was by hand (56) to automatic releases of mosquitoes by motor vehicle (25).

Accurate sexing is important because the release of only a few infected fertile females could mean that population replacement by *Wolbachia* becomes a possibility. This is because infected females are compatible with both released and wild males. Partial establishment occurred in *Ae. albopictus* releases on the outskirts of Guangzhou (141), as well as releases of *Ae. aegypti* in one area in Singapore (90). With the release of infected females, replacement is particularly easy if populations have already been suppressed through the release of infected males. However, the problem can be countered by releasing irradiated sterile males or males carrying a different *Wolbachia* strain that is bidirectionally incompatible with the original strain (90). The likelihood of replacement occurring can be reduced by decreasing the number of mosquitoes being released as population suppression starts to occur (to reduce the likelihood of very rare females being released) (91).

In the case of *Ae. albopictus*, which is often naturally doubly infected by *Wolbachia*, there is disagreement about the utility of releasing males from strains with a single infection (incompatible with either of the natural *Wolbachia* infections) versus a triple infection where a new strain is added on top of the natural double infection (77). A single infection incompatible with both natural *Wolbachia* strains will often have a relatively higher threshold for invasion than a triple infection (102), although this will depend on the size of any deleterious effects associated with each of the infected strains. In practice, the likelihood of invasion can be minimized by adjusting release numbers with the size of the remaining mosquito population; improving sexing methods; or using irradiated mosquitoes that provide an extra layer of protection, as noted above.

Published suppression releases have mainly taken place in small areas such as suburbs or villages (6, 25, 141). However, these are now being upscaled, particularly in Singapore, where there is a well-publicized program with strong government support (90). Public acceptance of such a program is critical, and this has been achieved in Singapore through close involvement with local communities and the transparent delivery of information and plans (67). Small-scale releases in a village outside Guangzhou were also viewed very positively by the public (141). Although the high abundance of nonbiting males during releases can annoy the public, the Singapore program continues to expand. A randomized controlled trial is now being undertaken in Singapore to document levels of disease suppression and measure entomological endpoints; the level of population suppression required to impact dengue transmission was previously established (89).

#### 4.2. Field Trials with *Wolbachia* Aimed at Replacement and Their Challenges

*Wolbachia* strains used successfully to date for replacement approaches in *Ae. aegypti* include *wMel* (from *D. melanogaster*) (109) and *wAlbB* (from *Ae. albopictus*) (79). There is genetic variation in both of these strains based on their origin and history (74). In particular, the DNA sequence of *Wolbachia* strains can be altered by maintaining them in cell culture through adaptive shifts (74), and their DNA sequences can also vary when they are sourced from different laboratory and field origins (74). In contrast, DNA sequences of the *wMel* and *wAlbB* strains and *Wolbachia* density appear stable across several years following *Wolbachia* introduction into the field or into long-term lab culture (4, 59, 66). The genomes of *Ae. aegypti* hosts also do not show much change following *Wolbachia* invasion (64). There is little evidence for any attenuation of virus blockage following *Wolbachia* introductions (4), and host phenotypes associated with *Wolbachia* may also be relatively stable across decades (106).

Unlike suppression releases, *Wolbachia* releases aimed at replacement depend on production of high-quality females, as well as males, to increase the likelihood of establishment. The genetic background of release stocks should match that of the field population as much as possible, which requires regular backcrossing to field-sourced males and/or sourcing of new culture material from an area where *Wolbachia* has invaded. When stock is reared under control artificial conditions, useful traits linked to (for instance) insecticide resistance (44) and quiescent egg viability (104) can be lost, making release stock less effective. Such traits need to be routinely monitored.

These factors and others related to the environment, transmission dynamics, and operational factors (107) influence the success of replacement strategies under field conditions. Replacement has sometimes been fairly easy, such as in *wMel* releases in Australia and in Yogyakarta in Indonesia (56, 60). In other cases, replacement has not been successful. These cases include the loss of *wMel* in Tri Nguyen in Vietnam (54) and the loss of *wAlbB* in some release areas in Selangor in Malaysia (79). In some cases, releases have resulted in intermediate frequencies of *Wolbachia* (97).

Factors that influence replacement success include the loss of insecticide resistance in release stock because of the high costs of resistant alleles (44) and exposure to high temperatures and environmental antibiotics, which can completely or partly clear *Wolbachia* infections from their hosts and even trigger CI in *Wolbachia* strains with lower infection titers (105). High-temperature effects on *wMel* have been documented in the field (105), although *wAlbB* appears to be more stable (103). Where high temperatures occur in nature, it may be desirable to release *wAlbB* or modified strains of *wMel* (46) that are more tolerant of high temperatures. Many other *Wolbachia* strains related to these infections could be sourced for transinfection, particularly strains from tropical hosts.

Theoretical studies have aimed to understand the impact of different factors on the success of *Wolbachia* replacement, focusing on maternal transmission rates, host fitness effects, loss of infection, and levels of CI (1) and building on original models developed and tested in *Drosophila*

*simulans* (58). Theoretical models have emphasized the importance of density-dependent factors in influencing the rates of *Wolbachia* invasion, as well as spatial release strategies to improve *Wolbachia* invasion rates (51). While these models provide useful insights into the potential impacts of different factors on invasion success, local ecological factors influencing *Wolbachia* dynamics are usually unknown.

A major challenge in making predictions is to understand the nature of mosquito dynamics in local breeding sites, which can vary from deep wells and large water storage containers to shallow pot plant bases and old tires that have collected water. Physical conditions in these breeding sites can vary markedly, as reflected by local temperature variation (101). The extent to which water is replenished in these breeding sites directly impacts the fitness of *wMel* and, particularly, *wAlbB*. In the absence of regular replenishment, mosquito eggs need to persist in a dry quiescent state; this is problematic for *Wolbachia*-infected eggs, which can die earlier than quiescent uninfected eggs, and once females emerge from the eggs, the females from *Wolbachia*-infected eggs are more likely to be sterile (64, 95). Replacement can also be affected by unexpected activity at release sites, such as insecticide fogging killing released mosquitoes or the development of unexpected breeding sites (particularly associated with building construction) near release areas (93). Operational issues include inappropriate handling of release stock, particularly if egg containers, which require careful placement and monitoring, are used for releases (57).

Public engagement is often considered more difficult for replacement than suppression because females with biting potential are deliberately released, and there may be an increase in female mosquito population size during the release period. The benefits of replacement releases therefore need to be carefully conveyed to the public and form a critical component of prerelease activities (108). In practice, increases in mosquito populations during releases are often modest (56) and, over time, are tempered by CI decreasing the reproductive output of wild females. In the case of *wAlbB* releases in Malaysia, successful replacement led to other public benefits such as a reduced need for fogging and other interventions (79), as was also noted in Yogyakarta following the successful introduction of *wMel* (60).

Field trials have been completed to assess the impact of *Wolbachia* on dengue disease incidence. An extensive randomized trial in Yogyakarta, Indonesia, using *wMel* showed an 80% reduction in dengue incidence (60, 122). Trials with *wMel* have also been completed in other areas, including Brazil (32), where lower *Wolbachia* frequencies were realized, but there was still a substantial reduction in dengue incidence of 38%. For *wAlbB* releases around Selangor and Kuala Lumpur, an initial survey of dengue fever incidence indicated a reduction of 39% in release sites with variable *Wolbachia* frequencies (79). Recent operational releases in the same region across more sites now indicate a dengue fever reduction of 62%, with a relatively higher reduction in areas where *Wolbachia* frequencies are high (57). Informal observations of dengue fever incidence in specific areas support the substantial impact that *Wolbachia* invasion can have on disease. In the high-rise Mentari Court area in Selangor, a dengue hotspot, successful invasion by *wAlbB* across five years resulted in >80% reduction in dengue fever incidence (24), while a comparison of two large regions near Yogyakarta, including one invaded by *wMel*, suggested a similar level of reduction (60). These trials and additional studies still to be published indicate that both *wMel* and *wAlbB* can have a substantial impact on dengue incidence if *Wolbachia* reaches mid- to high frequency.

### 4.3. Lessons from Gene Drive Modeling

Because no gene drive releases have taken place, it is necessary to use modeling to understand how gene drives would behave in a natural environment. These models can help uncover basic properties of a gene drive, with simpler models, and ultimate outcomes in real-world situations, with more complex models. We review results from basic models above, but spatial models could

also be useful for many scenarios. When individuals are spread over a continuous, two-dimensional landscape, gene drives tend to form waves of advance, which tend to be faster for drives with lower thresholds (92).

Comparisons to field *Wolbachia* releases could inform modeling of gene drives, at least for modification drives. *Wolbachia* has population dynamics similar to those of toxin–antidote drives, but only those with an intermediate threshold, given *Wolbachia*'s moderate to high fitness costs (50). To date, *Wolbachia* replacement trials have taken place in limited areas with *Ae. aegypti* (55, 110, 111, 120), a species with low dispersal rates, although expansion of *Wolbachia* into non-release areas has been documented and may depend on spatial variation in mosquito density (110, 120, 121). These factors increase the difficulty of making accurate wave advance measurements, but it should be possible to parameterize gene drive models based on these field releases.

For suppression gene drives, there is no direct analogy to existing methods. However, other genetic control systems, such as *Wolbachia* male-only release, release of insects carrying a dominant lethal, and the sterile insect technique (52), or even widespread pesticide use could still inform suppression drive models, especially on density-dependent population responses. Other model data, such as dispersal patterns, do not need to be obtained from suppression studies to be useful. However, additional detail may be needed from specific field sites for models to be sufficiently accurate if complicated outcomes are a possibility (see below).

#### 4.4. Spatial Confinement and Suppression

While panmictic models are essential for understanding the dynamics of gene drive and symbiont spread, spatial models can become particularly important in two situations. The first is assessing gene drive confinement and ideal release characteristics of frequency-dependent drives (and *Wolbachia*). While simple spatial models can determine wave advance speeds, this does not allow full consideration of drive persistence and confinement. Even with a large release size, frequency-dependent drives may fail due to incoming migration of wild-type individuals, requiring careful consideration of appropriate release patterns (20, 65, 120). The structure of the landscape can also confine a gene drive (or protect a weaker one), even within a connected population (20, 120).

Population suppression is another area where spatial models can show substantially different outcomes. Wild-type individuals recolonizing empty areas have higher survival due to reduced density-dependent competition. However, the drive is still present and can move back into the recolonized areas. This has been called chasing and has been seen even for highly efficient homing suppression drives in individual-based models with continuous space (16, 21, 68, 96), networks of linked discrete demes (80, 81), and conceptual spatial models (7). Chasing can be avoided in more recent mosquito-specific models with competition from different species (70). Frequency-dependent suppression drives may suffer from slower wave advance speeds than similar modification drives (92, 139), but at least one form of CRISPR toxin–antidote suppression drive can still function at high efficiency without increased vulnerability to chasing compared to homing drives in continuous space models (142).

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**Sterile insect technique:** releasing males sterilized via radiation or chemical treatment to mate with wild-type females, which then do not produce progeny

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#### SUMMARY POINTS

1. With the increasing burden of mosquito-borne diseases, novel mosquito control tools based on symbionts and genome engineering are critically needed.
2. Various symbiont approaches have shown potential for mosquito-borne disease control in the lab, including engineered microbes.

3. *Wolbachia*-based methods have already performed well in field trials aimed at both suppression of mosquito populations and replacement.
4. High levels of dengue suppression have been achieved, but only when *Wolbachia* frequencies are high.
5. Gene drives that spread through populations are a promising means for mosquito-borne disease control.
6. Frequency-dependent gene drives for modification or suppression could have similar performance to *Wolbachia*.
7. Field experience with *Wolbachia* releases highlights some challenges likely to be encountered with gene drives, some of which can be investigated with modeling.

## FUTURE ISSUES

1. The successful delivery and stable colonization of symbionts in the mosquito is the key to disrupting mosquito physiology to reduce vector competence or display antipathogen effects. We need to develop more efficient delivery systems for symbionts.
2. Current genetically modified entomopathogenic fungi (EPFs) for mosquito control are limited to a few *Metarhizium* and *Beauveria* strains; we need to discover more EPF candidates in the future.
3. Current strategies for suppressing arboviruses using insect-specific viruses (ISVs) primarily focus on alphaviruses. The potential of other viral ISVs to achieve similar outcomes remains to be explored.
4. Although the *wMel* and *wAlbB* strains of *Wolbachia* have performed well in field trials to date, there have been issues in achieving successful and stable invasions at some sites, and it is worth exploring additional variation within these strains or their relatives to build up a bank of potential release strains with desirable properties.
5. Beyond *Wolbachia* symbiont releases, which are now well established, semifield and field trials for long-term stability are necessary before the other novel mosquito control approaches can be converted into wider field applications.
6. Gene drive candidates for field release will likely still need improved efficiency and reduced resistance allele formation.
7. More realistic computational models are necessary to accurately predict field performance for scenarios where confinement or population suppression may influence outcomes.

## DISCLOSURE STATEMENT

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