Trends in Genetics

CellPress

Review

Symbionts and gene drive: two strategies to combat vector-borne disease

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Mosquitoes bring global health problems by transmitting parasites and viruses such as malaria and dengue. Unfortunately, current insecticide-based control strategies are only moderately effective because of high cost and resistance. Thus, scalable, sustainable, and cost-effective strategies are needed for mosquito-borne disease control. Symbiont-based and genome engineering-based approaches provide new tools that show promise for meeting these criteria, enabling modification or suppression approaches. Symbiotic bacteria like *Wolbachia* are maternally inherited and manipulate mosquito host reproduction to enhance their vertical transmission. Genome engineering-based gene drive methods, in which mosquitoes are genetically altered to spread drive alleles throughout wild populations, are also proving to be a potentially powerful approach in the laboratory. Here, we review the latest developments in both symbionts and gene drive-based methods. We describe some notable similarities, as well as distinctions and obstacles, relating to these promising technologies.

Highlights

Safe and sustainable approaches for mosquito control are critical due to the global increasing burden of mosquito-transmitted diseases.

Novel control approaches based on symbionts are currently proposed to modify or suppress mosquito populations and *Wolbachia*-based methods have already achieved some success in field trials.

Transgenic mosquitoes carrying gene drives that spread through populations are a promising control approach to block disease transmission or suppress vector species.

Transgenic-based approaches potentially offer more power and flexibility, but symbiont-based approaches are usually more socially accepted and well-developed.

Mosquitoes can be found almost anywhere in the world, but in the tropics and subtropics, half of the world's population is under the threat of mosquito-borne pathogens such as dengue virus (DENV), Zika virus (ZIKV), chikungunya virus (CHIKV), yellow fever, West Nile virus (WNV), malaria, and filarial nematodes [1,2]. For example, DENV incidence has grown over 30-fold in the past 50 years, now reaching about 400 million cases per year [3]. The recent ZIKV outbreak resulted in hundreds of thousands of infections and large-scale social and economic disruption [4]. While malaria cases are falling in southeast Asia, infections are rising in other parts of the world and remain 'unacceptably high' according to the World Health Organization [5].

Re-emergence and expansion of mosquito-borne diseases are due to many factors, including increased urbanization and global travel and trade, climate change, land use pattern changes, and unreliable piped water supply [6]. Current mosquito control strategies, including long-lasting insecticide-treated bed nets, chemical insecticides, and environmental management [7], have been unable to address these diseases due to increasing genetic and behavioral vector resistance to these interventions [8]. In addition, chemical interventions have an unintended effect on important nontarget insects, such as pollinators [9]. Thus, new, more effective control strategies are urgently needed to address mosquito-borne diseases.

In response to this growing need, the number of novel mosquito control technologies have expanded in recent years. Many of these involve the release of mosquitoes that aim to achieve **population suppression** (see Glossary) or **population modification** of wild type mosquitoes. Population suppression strategies aim to reduce or eliminate mosquito populations. Such strategies include **sterile insect technique (SIT)**, **incompatible insect technique (IIT)**, and transgenic-based technologies, where sterile insects mate with wild type insects and reduce

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population sizes in the next generation. **Gene drive** approaches, where alleles can increase in frequency over multiple generations, could potentially suppress populations after a single release or modify mosquitoes to be refractory or resistant to pathogens and prevent pathogen transmission. The release of mosquitoes carrying a symbiont or a gene drive into wild populations can enable the spread of the modification and result in entire populations becoming refractory to a pathogen. In this review, we summarize recent developments in the use of symbiont-infected mosquitoes and transgenic gene drive strategies, focusing on their different varieties and capabilities.

Natural symbionts for mosquito control

The early symbiont-mediated mosquito [10] control was the introduction of nonmodified microorganisms into insects to reduce vector competence (Box 1). ***Wolbachia*** is the most extensively studied system for natural symbiont-based mosquito control. It may be the most common intracellular endosymbiont in arthropods and nematodes, with 60% of all insects harboring *Wolbachia* [11]. *Wolbachia* are transmitted vertically from mother to offspring and can maximize their transmission by manipulating host reproduction through feminization, parthenogenesis, male killing, and/or **cytoplasmic incompatibility (CI)**. CI is induced when *Wolbachia*-infected males mate with uninfected females, which results in nonviable offspring. *Wolbachia* can inhibit or block infection with DENV, yellow fever, ZIKV, other arboviruses, and malaria parasite (Figure 1, Key figure) [12–14]. Transfected or native *Wolbachia* infections have both been used for population suppression strategies [10,15]. Interestingly, some important vector species, such as *Aedes aegypti*, are naturally free of *Wolbachia* [16,17], providing an open niche for infection. While there is conjecture if some of the major *Anopheles* vectors are truly infected [18], recent reports indicate other Anopheline species possess high-density native *Wolbachia* infections [19]. This offers renewed promise for infection of medically relevant *Anopheles* vectors with these native strains that are

Box 1. Using symbionts as novel mosquito control strategies

The increasingly emerging interactions among mosquito hosts, pathogen infection, and symbionts are inspiring the development of new strategies to exploit symbionts for vector-borne disease control [111]. Most importantly, symbiont-based mosquito control shows potential power to minimize the resistance problem and cause minimal side effects to the environment. The application of symbionts in vector control includes: (i) delivering natural symbionts into the mosquito directly to disrupt mosquito physiology to reduce vector competence or display antipathogen effects; (ii) genetic modification of symbionts to express antipathogen effector molecules, then delivering the engineered symbiont into the mosquito so that the mosquito is resistant to pathogen or there is decreased vector competence [112] or vectorial capacity [113] (Figure I).

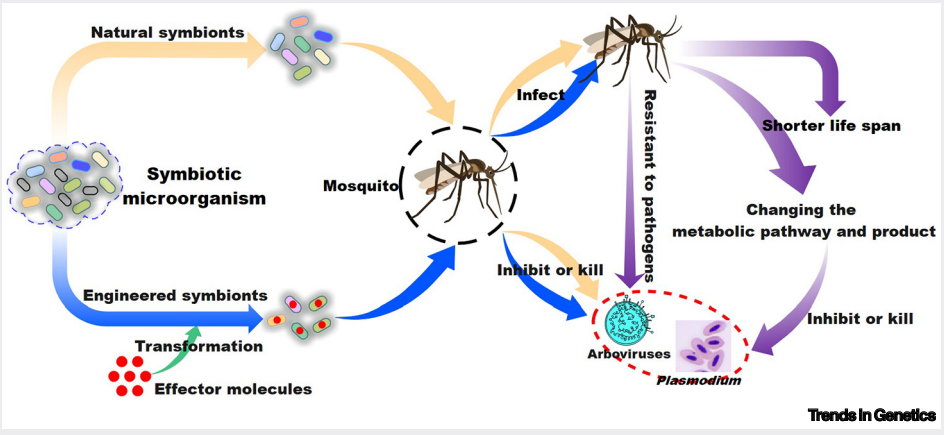


Figure I. Symbionts (natural or engineered) can be used for mosquito control.

Glossary

Cytoplasmic incompatibility (CI): sperm–egg incompatibility preventing uninfected females from producing viable offspring if they mate with a male infected with *Wolbachia*. No offspring are also produced if the female and male have incompatible strains of *Wolbachia*.
Gene drive: a genetic element that can bias inheritance in its favor. This can allow it to quickly increase in frequency in a population. For this to occur, some drives require the presence of a supporting allele or the drive itself to be present above a critical threshold frequency.
Homing drive: a type of gene drive element that works by cutting a specific site in the genome, which is then repaired by homology-directed repair. This results in the drive allele being copied and passed on to offspring at a super-Mendelian rate. These systems are able to rapidly invade populations and can be used for modification or suppression.
Incompatible insect technique (IIT): an insect population reduction or elimination approach that involves release of large numbers of *Wolbachia*-infected males. Uninfected females or those infected with an incompatible *Wolbachia* strain will not produce progeny when mating with released males.
Paratransgenesis: a strategy to genetically engineer symbiotic bacteria to express antipathogen effector molecules.
Population modification: a gene drive strategy to modify a target population by spreading a drive allele, often with a desired cargo gene.
Population suppression: a gene drive strategy to reduce or eliminate a target population, often by disrupting an essential gene.
Resistance allele: changes in the target sequence recognized by the drive nuclease such that it cannot be cleaved. Resistance alleles in a target gene can potentially disrupt or preserve its function.
Split drive: a gene drive, typically CRISPR-based, where essential components are located at separate genomic sites, with at least one component unable to spread. Such drives require higher release frequencies for success, so split drive can thus potentially be used as a safety or confinement mechanism.

adapted to the Anopheline environment. Several reviews have covered recent progress of *Wolbachia*-based mosquito control that exploit the bacterium [20–22].

Besides *Wolbachia*, research in other natural symbionts for mosquito control has made rapid progress recently (Figure 1A,B). *Serratia* Y1 bacteria from field-caught *Anopheles sinensis* can inhibit *Plasmodium berghei* by modulating mosquito immunity genes to inhibit *Plasmodium* development [23]. *Asaia* bacteria can interact with the *Anopheles* mosquito immune system to slow malaria parasite development [24]. Symbionts can not only interact with mosquitoes to interfere with pathogens, but they can also inhibit pathogens directly. For example, *Serratia ureilytica* Su_YN1 directly secretes an antimalarial lipase that kills *Plasmodium* parasites at different stages, effectively preventing parasite infection [25]. The symbiont can also show antipathogen activity by their secondary metabolites [26]. Likewise, *Chromobacterium* inhibits other midgut bacteria growth and displays entomopathogenic activity to mosquito larvae and adults. Romidepsin might be the *Chromobacterium* secondary metabolite responsible for the antiplasmodial activity [27]; the *Chromobacterium* secondary metabolite aminopeptidase interferes with DENV-2 attachment by increasing the degradation of the Flavivirus E protein [28]. Natural symbiotic fungi also show potential for mosquito-borne disease control. *Wickerhamomyces anomalus* is a yeast that secretes a killer toxin protein that shows strong activity against *P. berghei* at different developmental stages [29]. *Beauveria bassiana* induces the Toll/Jak-Stat immune pathways and reduces mosquito vector competence for DENV-2 in *A. aegypti* [30] and *Aedes albopictus* capacity for ZIKV [31].

Beyond bacteria and fungi, insect-specific viruses (ISVs) also can be used to control arboviruses. Cell fusing agent virus (CFAV) is the early recognized ISV from *A. aegypti* cells that can cause cell fusing phenotype in *A. albopictus* cells [32]. Another mosquito ISV, Eilat virus (EILV), can reduce CHIKV titers and delay replication *in vitro*. When *A. aegypti* mosquitoes were infected with EILV, dissemination of CHIKV was delayed by a heterologous interference mechanism [33]. Co-infection of different ISVs also can inhibit arbovirus development. For example, CFAV and Phasi Charoen-like virus co-infection can inhibit the growth of ZIKV and DENV in *A. albopictus* cells [34]. *Negevirus* is another recently discovered ISV [35]. When *Negevirus* infected *A. albopictus* cells, the cells could not be infected with CHIKV and Mayaro viruses [36]. All these results suggest that ISVs can be potential tools to control arboviruses through **superinfection exclusion**, which needs further testing in mosquito population.

Engineered symbionts for mosquito control

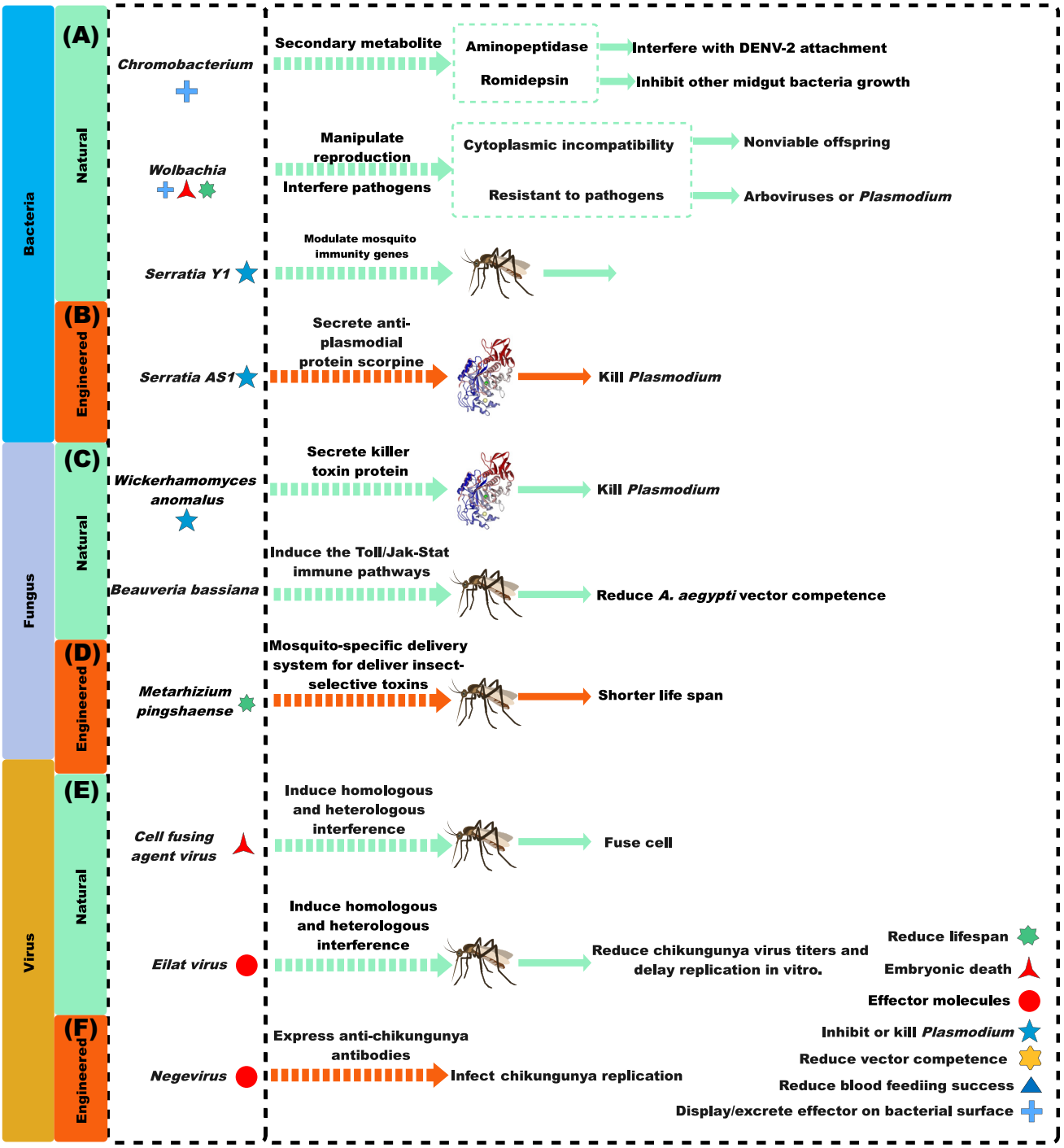
Engineered symbionts producing antipathogen or immunomodulatory effector molecules (termed **paratransgenesis**) is another powerful symbiont-mediated mosquito control approach (Figure 1C,D). After the symbiont is engineered, it is reintroduced into the arthropod host to reduce its vector competence (Box 1). There are some critical requirements for the candidate symbiont. First, the symbiont should be able to stably spread into the population vertically and/or horizontally and maintain in the population long enough to express the effector molecules [37]. Second, the symbiont should be easily culturable and genetically manipulatable, while not reducing the host fitness [38]. Third, the symbiont should express the effector molecules to interfere with the target pathogen [39]. There are several candidates that have shown potential attributes to be a paratransgenesis symbiont.

Serratia (AS1), which was isolated from *Anopheles* ovaries, can be transmitted vertically and horizontally, facilitating its spread into mosquito populations. Furthermore, the genetically engineered AS1 can express anti-*Plasmodium* effector proteins that inhibit *Plasmodium* development in mosquitoes [39]. Together, this suggests that AS1 is a promising candidate for *Plasmodium* control.

Sterile insect technique (SIT): an insect population reduction or elimination approach that sterilizes males via radiation or chemical treatment. When released, sterilized males mate with wild type females, which then do not produce progeny.
Superinfection exclusion: a phenomenon in which an established virus infection prevents a secondary infection with the same or a closely related virus.
Underdominance: the allele with the highest initial frequency will tend to increase in frequency when heterozygotes have a lower fitness than homozygotes. These systems have a high introduction threshold and are likely to be confined to a local area.
***Wolbachia*:** a cytoplasmically inherited rickettsiae bacteria genus that are found in reproductive tissues of a wide range of arthropods and nematodes.
X-shredder: an allele that cleaves the X-chromosome in the male germline at multiple sites simultaneously, biasing the sex ratio toward males. If located on the Y-chromosome, it will increase in frequency, leading to population suppression.

Key figure

Symbiont-based approaches for mosquito control



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Asaia bogorensis is transmitted vertically and can populate the larval and adult gut and reproductive organs of *Anopheles* and *Aedes* mosquitoes [40]. Recently, *Asaia* was successfully engineered to conditionally express the anti-plasmodial protein scorpine, which significantly inhibits the development of malaria parasites, while displaying a reduced fitness cost compared with an *Asaia* stain constitutively expressing the antiplasmodial effector [41]. More recently, *Asaia* was engineered to induce an immune response within *Aedes* and *Anopheles* mosquitoes to control the heartworm parasite *Dirofilaria immitis* [42]. Notably, both engineered AS1 and *Asaia* can be spread into mosquito populations and keep the antipathogen capability in the laboratory or semi-field conditions [39]. Intriguingly, *Wolbachia* and *Asaia* appear antagonistic to one another. *Wolbachia*-infected mosquito showed lower *Asaia* densities compared with their uninfected counterparts, while removing *Asaia* from *Anopheles* mosquitoes enabled vertical transmission of *Wolbachia* [43,44]. Genetically engineered *Metarhizium pingshaense* (Mp-hybrid) infection of *Anopheles coluzzii* had shorter lifespans and reproductive output compared with wild type mosquitoes. Furthermore, Mp-hybrid showed higher virulence and lower inoculum load than wild type fungus in a semi-field trial in Burkina Faso [38]. Finally, modifying the recently discovered ISV, *Negevirus*, to express anti-CHIKV antibodies inhibited CHIKV replication [36]. All these results indicate that engineered bacteria, fungus, and even viruses can be used directly or combined with existing chemical control strategies for mosquito control.

Engineered hosts with genes inducing CI

The genetic basis of CI in *Wolbachia* has recently been identified by compelling evidence that two genes, *cifA* and *cifB*, are involved in induction and rescue. While the specific models and mechanism(s) of CI still remain to be elucidated, it has been shown that expression of these bacterial genes in the host germline can recapitulate the CI phenotype. Expression of *cifA* in females rescues CI, while intriguingly, it appears that coexpression of both *cifA* and *cifB* in males is required to induce this phenotype [45,46]. CI phenotypes can also be replicated with crosses between transgenic insects expressing *cif* genes and insects harboring native *Wolbachia* infection [45]. While most of these studies examining the molecular basis of CI have been accomplished in flies [45–48], CI was also recapitulated by expression of *cif* genes from *w*Pip in *Anopheles* mosquitoes (though here, only *cifB* was needed in males to induce CI) [49], demonstrating these approaches can be transferred to medically important vector species. Further insights into the molecular mechanism(s) underpinning CI will enable evaluation of how these systems will function in the field and how resistance might emerge. Gene drive based on such an approach would likely be more intrinsically confined than *Wolbachia* endosymbionts, but perhaps would also have a less detrimental fitness effect on the insect. Overall, the exploitation of symbiont genes for population modification and suppression is an exciting new avenue to explore for vector control.

Classification of gene drive approaches for pest control

Much progress has been made on gene drive recently, especially with the advent of CRISPR technology. These engineered alleles can bias their inheritance to efficiently spread through a

Figure 1. (A) Natural bacteria-based approaches for mosquito control. *Wolbachia*-infected males can suppress mosquito populations through cytoplasmic incompatibility (CI) effects or *Wolbachia*-infected mosquitoes modify mosquito populations for pathogen resistance. *Serratia* Y1 and *Asaia* induce the mosquito immunity system and slow the malaria parasite development. The secondary metabolites of *Serratia ureilytica* Su_YN1 and *Chromobacterium* can be responsible for antiplasmodial or dengue virus (DENV)-2. (B) Natural fungus-based approaches for mosquito control. *Wickerhamomyces anomalus* can be used against malaria parasite development through secreting toxin protein and *Beauveria bassiana* can be used against DENV-2 and Zika virus (ZIKV) activity through inducing the mosquito immunity system. (C) Natural insect-specific viruses (Eilat virus, cell fusing agent virus, Phasi Charoen-like virus, and *Negevirus*) can inhibit arbovirus development, either alone or in combination. (D) Engineered bacteria-based approaches for mosquito control. Engineered *Serratia* AS1 and *Asaia* can express antiplasmodial effector proteins to inhibit *Plasmodium* development. Engineered *Asaia* can induce mosquito immunity to control parasite *Dirofilaria immitis*. (E) Engineered fungus-based approaches for mosquito control. Engineered *Metarhizium pingshaense*-infected mosquito has shorter life spans and reproductive output than wild type mosquitoes. (F) Engineered virus-based approaches for mosquito control. Engineered *Negevirus* expressed an anti-chikungunya virus (CHIKV) antibody that can inhibit the CHIKV replication.

population of mosquitoes after just a single, modest-size release. However, gene drive systems have encountered an array of technical challenges that must be overcome before they can be used successfully. These are often different for the wide variety of possible drive mechanisms (Figure 2A), which can be generally categorized into those designed to modify populations or suppress them (Box 2). They can also be classified into drive types that are unconfined and those that will be confined to desired target populations (Box 3 and Figure 3). Additionally, gene drives can sometimes have 'self-limiting' mechanisms. If released in a certain frequency range, these will persist only temporarily in a population before being naturally eliminated (Figure 3).

Unconfined gene drives

Homing drives

Homing drives work by encoding an endonuclease, which cleaves a target site in the homologous chromosome and copies itself during homology-directed repair. This process converts wild type alleles into drive alleles in a heterozygote's germline, thereby increasing inheritance of the drive (Figure 2C). Researchers have recently utilized the CRISPR/Cas9 system to develop homing drives with high efficiency in *Drosophila melanogaster* [50–52], *Saccharomyces cerevisiae* yeast [53], *Anopheles* mosquitoes [54,55], and viral populations [56]. CRISPR-based homing drives have also been developed in mice [57], *Aedes* mosquitoes [58], and *Arabidopsis* plants [59], though such drives have yet to reach high efficiency. These drives all use Cas9 or a similar endonuclease directed by a guide RNA (gRNA), allowing for highly flexible targeting of natural genomic sites. Though initially thought to occur in the early embryo [50], subsequent studies showed that homing (also called 'drive conversion') of such drives occurs in the germline [51,60]. A key obstacle to current CRISPR homing drives is their propensity to generate **resistance alleles** after Cas9 cleavage that can prevent the spread of the drive. Multiple studies have characterized these resistance alleles and developed methods to mitigate them (Box 4).

Because homing drives spread rapidly, even with low release sizes, some studies have attempted to develop constructs that could be deployed to limit their spread or revert organisms to a wild type phenotype. One of these can overwrite existing drives in *D. melanogaster* [61], while another method in *Anopheles gambiae* can prevent a suppression drive from eliminating a population by reducing (though not eliminating) the genetic load [62]. Methods for use of chemicals in inducible genetic systems allow for control of Cas9 expression (and thus drive efficiency) [63] or removal of gene drives [64] in flies, allowing laboratory manipulation or potentially even use in wild populations if the inducing chemical can be safely and widely deployed.

Homing modification drives

Several homing drives with features specific to modification systems have been developed. In the first example of homing drives in *Anopheles stephensi*, two large cargo genes expressing antimalarial antibodies were included in a drive targeting *kh* [54]. The drive retained high efficiency, but it also had high embryo resistance allele formation. Coupled with fitness costs due to the drive's target site, functional resistance allele formation prevented success in cage populations [65].

To overcome this issue, a rescue homing drive was designed in flies to target a haplolethal gene [66]. Two functioning copies of such a gene are required for viability, so nonfunctional resistance alleles would be nonviable. Two gRNAs were used to prevent formation of functional resistance alleles. The drive element contained a recoded copy of the target gene that could not be cleaved by gRNAs, 'rescuing' the target gene function. This drive was successful at eliminating resistance alleles and spreading through a cage population. Similar drives targeting haplosufficient genes (for which only a single functioning copy is needed for viability) have been designed in *A. stephensi* [67] and *D. melanogaster* [68,69]. The mosquito study [67] improved on the original design

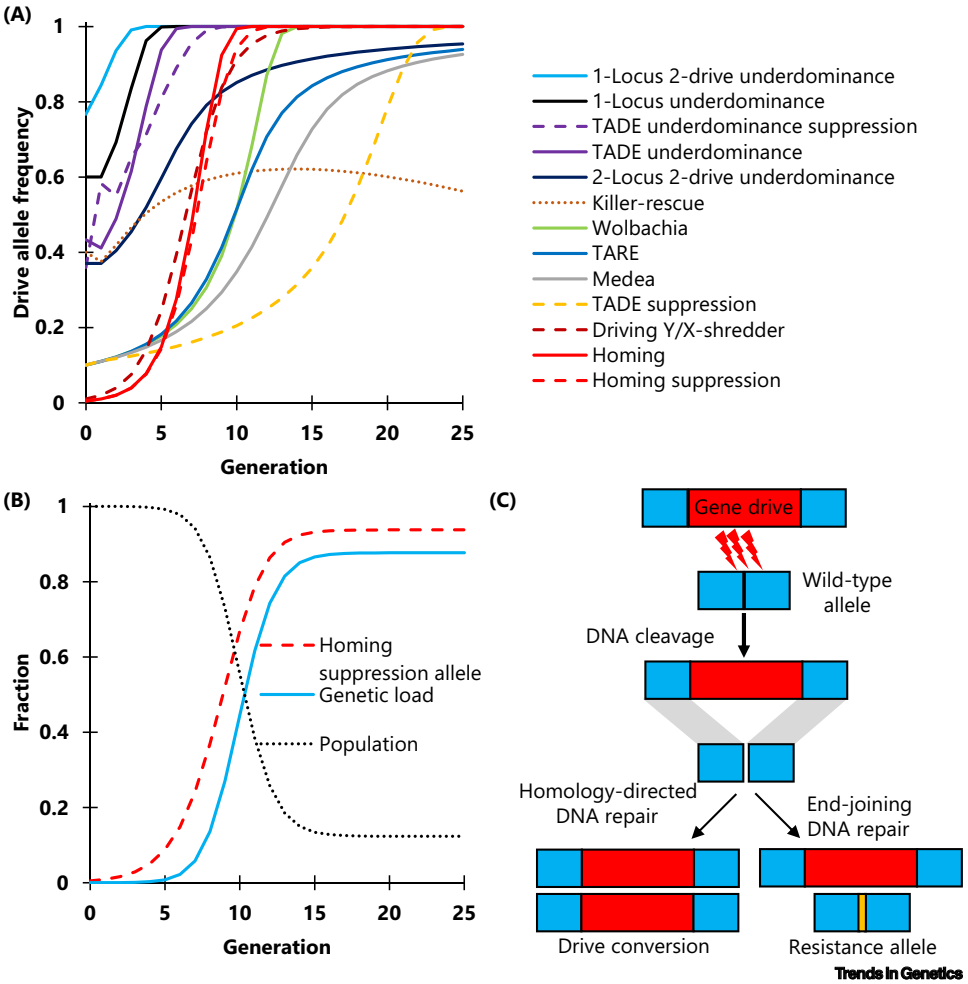

Figure 2. Gene drives dynamics. (A) The allele frequency trajectories are shown for several types of gene drives. Drives are released as driving Y carriers, *Wolbachia*-bearing individuals, homozygotes for the modification underdominance forms, and heterozygotes for others. Each is released 10% above their introduction frequency threshold, except for zero-threshold drives, which have a 1% release, and the killer rescue drive, which has a 40% release to show self-limiting dynamics. All drives have ideal performance except for the killer-rescue drive, which has a fitness of 0.9 in rescue homozygotes. The 2-locus-2-drive underdominance, TARE, and *Medea* carrier frequencies reach 100% well before their allele frequencies. TARE and TADE are CRISPR toxin-antidote drives targeting haplosufficient and haplolethal genes, respectively. (B) A suppression drive is designed to reach a high equilibrium frequency, causing suppression or elimination, depending on the exact genetic load and ecological characteristics of the population. The genetic load refers to the reduction in reproductive capacity caused by the drive. Imperfections in the drive often result in an equilibrium frequency and genetic load of less than 1, such as in this female fertility homing drive with 90% drive conversion efficiency. (C) The best studied gene drive mechanism is the homing drive, which targets a wild type allele with a nuclease (usually Cas9) in germline cells. If the DNA break undergoes homology-directed repair, the drive allele will be copied to the other chromosome. However, if end-joining occurs, the target site may be mutated, forming a resistance allele that cannot be cut by the drive.

specifically by adding a recoded *kh* [54], eliminating the fitness cost of the drive from disrupting this gene. However, all three of these studies saw incomplete success due to functional resistance alleles, likely because they possessed only one gRNA. Comparing these methods, haplolethal gene targeting would eliminate resistance alleles more quickly and reach 100% equilibrium frequency, even with fitness costs. However, targeting a haplosufficient gene would ease construction due to more flexibility in rescue gene expression levels and greater viability in the presence of embryo or somatic Cas9 cleavage.

Box 2. Classification of gene drives by type of outcome

Gene drive-based strategies can be broadly classified by their goal, population modification or suppression, and their level of confinement [114–120]. Population modification strategies usually involve spreading the gene drive throughout the target population while carrying a useful 'cargo', 'payload', or 'effector' gene. It is this cargo gene that has the desired effect, such as blocking transmission of malaria [109,121–125], DENV [126,127], or ZIKV [128]. These cargo genes can work via various mechanisms such as modulation of the immune system or direct targeting of pathogens by RNAi or antibodies. Even an ideal modification drive could eventually fail due to pathogen resistance to the cargo or mutational inactivation of the cargo. This latter issue could be addressed by using a modification drive without a cargo, such as targeting of a host gene essential for transmission of a pathogen [129,130]. Thus far, effectors have usually not been combined with gene drives, with the exception of one early CRISPR homing drive that carried two single-chain antibodies targeting *Plasmodium falciparum* malaria parasites [54,65]. Aside from vectors for disease prevention, modification of populations could be useful in other contexts, such as providing aid to an endangered species (such as by blocking a pathogen) or confining a suppression drive to a target population (see 'tethered drives').

Suppression gene drives are designed to reduce or eliminate a population. This is often accomplished by using the drive to target a haplosufficient but essential gene, spreading a gene drive in heterozygotes while reducing the fertility or viability of homozygous individuals. An alternative mechanism is to bias the sex ratio of the target population, resulting in lower reproductive capacity. In general, suppression drives proceed toward a drive-carrier equilibrium, rather than to fixation as in most modification drive types (see Figure 2B in main text). This equilibrium is characterized by the 'genetic load' of the drive, a measure of its suppressive power that represents the fractional reduction in viable offspring of a given generation compared with the number of offspring if the entire population was wild type (a genetic load of 1 represents no offspring in the next generation, while a genetic load of 0 represents no impact on the number of viable offspring generated). High genetic load values close to 1 will tend to result in population elimination, while lower values will tend to result in an equilibrium population size lower than the original population. The exact size depends on complex population dynamics [131].

Homing suppression drives

Homing drives can be designed for population suppression by targeting an essential but haplosufficient gene (without rescue). In this manner, drive homozygotes are sterile or nonviable, but the drive allele can still spread in heterozygotes. An ideal drive would have a genetic load of 1 (Box 2), but if the drive carries unintended fitness costs or especially has conversion efficiency below 100%, then genetic load would be reduced. Thus far, the most common strategy for population suppression has been targeting female fertility genes, which prevents removal of drive alleles in male homozygotes, while still rapidly suppressing the population by sterilizing female homozygotes.

Box 3. Classification of gene drives by level of confinement

Another important distinction between different types of drives is the level of confinement of the drive. This refers to how easily a gene drive will spread between populations. This can be a critical consideration because sociopolitical and regulatory factors can demand that a drive be confined to a given area, regardless of the drive's application. These issues can stem from public fear of an uncontrolled drive or possibly regulatory issues associated with national borders. Groups involved in initial testing of gene drives in general may also prefer a limited study area. Also of essential importance is that application of various types of drives may only be useful in certain populations of the target species. While prevention of diseases in mosquitoes could be useful in most regions where the species are found, gene drives for agricultural purposes would likely only be useful in the relevant agricultural areas, not extending to natural environments. Similarly, suppression of invasive species for conservation purposes is only desirable in the invasive populations themselves and not in the native range of the target species.

Many gene drives are unconfined or 'global', representing drives that will generally spread from one population to another with even a small number of migrants (see Figure 3 in main text). Confined drives are characterized by 'introduction thresholds', a critical drive frequency above which it will increase to fixation or a high equilibrium (in a deterministic model) and below which it will decline and be eliminated. Closely linked are 'migration thresholds', the level of migration above which the drive will be able to spread from a gene drive population to a wild type population. Below this threshold, some drive migrants may move to the wild type population, but the frequency will generally remain low due to continuous removal of drive alleles (see Figure 3 in main text). Such confined drives are loosely classified as 'regional' drives and 'local' drives. Regional drives lack an introduction threshold (and will thus act as unconfined drives) in the absence of fitness costs, but any fitness cost at all will impose an introduction threshold frequency (*Wolbachia* for population modification would have similar dynamics as a regional drive). Local drives, however, have an introduction threshold frequency even without fitness costs, which should usually make them more stringently confined to target populations, even with higher migration levels. Regional drives will usually be more capable of spreading and have lower required release rates.

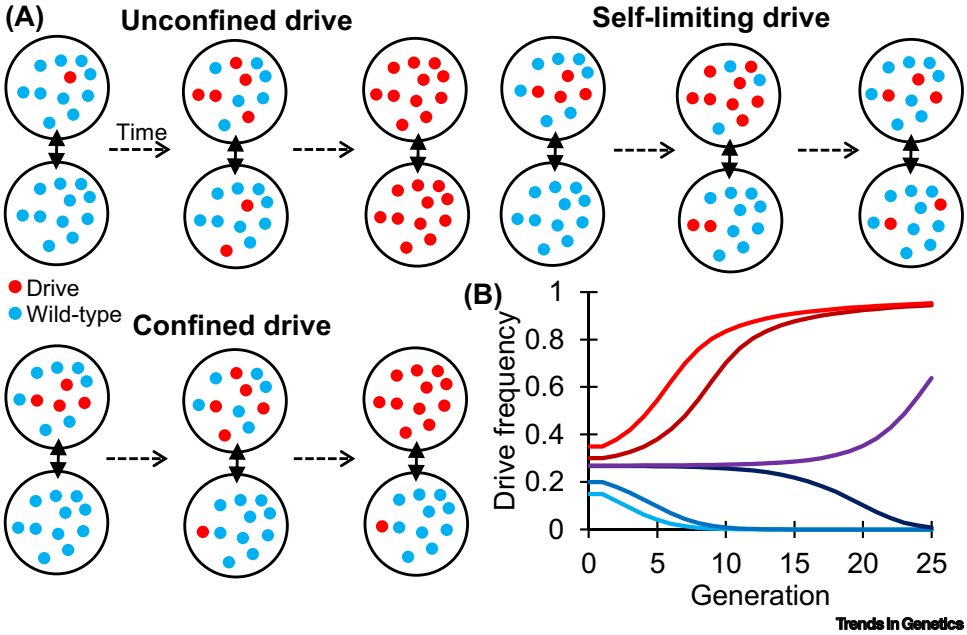

Figure 3. Confinement of gene drives. (A) Different types of gene drives carriers (red) are released into the upper wild type (blue) population, which is connected to another population by migration (unbroken arrows). Unconfined drives can spread rapidly from a small initial release, resulting in spread to both the release and the distant population. Confined drives have a release threshold and the drive can only spread about the threshold, requiring a larger initial release. If the migration rate is low enough, the drive will not spread to the distant population. Self-limiting gene drives can initially spread, but eventually, they disappear from the population if they did not fixate. They can potentially spread to the distant population, but the exact amount depends heavily on release quantity, drive performance, and migration rates. (B) Double drive homozygotes for a 2-locus 2-toxin antidote pair drive with no fitness costs are released at varying frequencies (colored lines). The introduction threshold of 26.9% can be seen, with higher starting frequencies increasing to fixation and lower frequencies declining to elimination.

Box 4. General considerations for resistance alleles in CRISPR gene drives

Resistance alleles can form in the germline after the Cas9 cleavage was repaired via the end-joining pathway instead of via homology-directed repair (see Figure 2C in main text), but also in the early embryo due to the persistence of maternally deposited Cas9 [51,54,60]. They can be classified into two types: those that disrupt the function of the target gene and those that preserve it. Functional resistance alleles that preserve target gene function are rarer, but they are also far more detrimental to the progress of a gene drive [51,70,132]. Recent studies in flies showed that resistance allele formation rates in the early embryo varied substantially among genetically diverse fly lines [51,133].

To minimize resistance alleles, DNA cleavage should occur only in the germline in gametocytes when cells are predisposed to use homology-directed repair instead of end-joining. Avoiding cleavage in embryos due to maternally deposited Cas9 will reduce resistance alleles, and avoiding cutting in somatic cells can reduce drive fitness costs, particularly in suppression drives where wild type alleles are required in somatic cells to maintain high fitness in drive heterozygotes. Reduction of undesired Cas9 activity can be accomplished by using a different promoter for Cas9. In flies, switching from the *vasa* to *nanos* promoter eliminated detectable somatic activity [60], while in mosquitoes, switching from *vasa* to *nanos* [134,135] or *zpg* [135] largely eliminated maternal Cas9 deposition into embryos and substantially reduced somatic cleavage.

Another possible complementary option for reducing resistance alleles is to use multiple gRNAs targeting adjacent sites. In fly trials, this method worked well for increasing drive efficiency with closely spaced target sites [60,132], but not if the target sites were far apart [136]. Modeling based on experimental results indicates that there will be an optimal number of gRNA for maximizing drive conversion due to complexities such as homology end mismatch and Cas9 activity saturation [132]. However, higher numbers of gRNAs are still effective at preventing formation of functional resistance alleles due to the need for specific functional repair at each individual cut site [132]. Multiplex gRNAs have not yet been experimentally tested in mosquitoes.

The first homing suppression drive was developed in *A. gambiae* mosquitoes using the *vasa* promoter, which achieved high drive conversion efficiency [55]. However, *vasa*-Cas9 had high somatic expression, greatly reducing drive/wild type heterozygous female reproduction. High embryo resistance allele formation also quickly resulted in functional resistance alleles in cage studies [70]. To overcome this issue, the *zpg* promoter was used to reduced undesired cleavage in the early embryo and in somatic cells [71]. This was coupled with a highly conserved target site in the *doublesex* gene, preventing functional resistance allele formation and allowing for successful suppression of cage populations. Multiplexed gRNAs were also able to prevent functional resistance allele formation in *D. melanogaster*, but the drive efficiency was too low to suppress cage populations [137].

As the most technically well-developed gene drives in mosquitoes, homing suppression drives are certainly promising, particularly if further improved promoters could be combined with multiplexed gRNAs. However, modeling studies indicate that such drives (like most suppression drive types) may not be able to fully eliminate populations [72–74] and the consequences of species elimination may be unclear, such as replacement of one vector with another.

Sex ratio drives

Drives that cause population suppression via biased sex ratios can be constructed by destroying sex chromosomes in the germline, allowing only gametes with the desired sex to be viable. This has been demonstrated in *A. gambiae* [75,76], *D. melanogaster* [77], and *Ceratitis capitata* [78] with **X-shredders** and *in vitro* with Y-shredders [79]. These shredder alleles cleave repeated regions on the target chromosome, thus overloading DNA repair mechanisms and causing death at the gamete stage, which creates a biased sex ratio.

However, an X-shredding allele alone is not necessarily a gene drive and repeated releases of drive-carrying males would be required to reduce populations. For an X-shredder to increase in frequency, it would need to be located on the Y-chromosome, thus making it a 'driving Y'. By removing the X-chromosome, the driving Y increases the rate at which it is inherited, making it a gene drive. If the shredding efficiency is sufficiently high, the drive can impose a high genetic load. However, engineering the Y-chromosome is difficult, as is expressing genes from the Y-chromosome at high levels. Thus, a driving Y has not yet been developed. To overcome this issue, an X-shredder was linked to the previously constructed homing suppression drive targeting *doublesex* [80]. This allowed the drive to suppress populations by biasing the sex ratio while largely avoiding the moderate somatic costs associated with the earlier drive in female heterozygotes.

Confined gene drives

Locally fixed alleles

One possible way to confine a homing drive to a target population is to use target site sequences that are fixed in the desired population and absent in the nontarget population [81,82]. For suppression drives, the target allele could also be found at moderate frequency in the nontarget population, leading to only temporary suppression effects. However, finding locally fixed target sites in haplosufficient but essential genes, particularly if multiplexed gRNAs are desired, may be difficult [83].

Toxin-antidote drives

Several older forms of toxin-antidote gene drives have been studied, though recent progress has been slow. Chromosomal rearrangements with introduction frequencies of 50% have been engineered in *A. aegypti* [84], *Anopheles* [85–87], and flies [88]; however, difficulty of engineering and often high fitness costs have prevented progress with this method in mosquitoes for the past few decades. The maternal effect dominant embryonic arrest (*Medea*) drive uses an RNAi toxin

and a zygotically expressed rescue element, but it has only been engineered in *Drosophila* [89,90]. Though the normal form only has an introduction threshold if it has a fitness costs, variants with multiple allele types possess an introduction threshold frequency even without fitness costs [91]. Efforts to bring *Medea* to mosquitoes have stalled due to highly specific component expression and target gene requirements.

Other proposed **underdominance** drives based on RNAi target haplosufficient but essential genes with two separate drive alleles, each similarly providing rescue for the target of the other allele [92]. A toxin-antidote drive with a single allele type targeting a haploinsufficient gene was successful in fly cage populations [93]. This design has a high introduction threshold of 50% in the absence of fitness costs in drive homozygotes. More general target gene and promoter requirements of these non-*Medea* designs may make them amenable to engineering in other species. However, effective expression of RNAi from genomic sources is difficult to engineer in mosquitoes.

CRISPR toxin-antidote drives

Functioning similarly to RNAi-based toxin-antidote drives, CRISPR-based systems act as a toxin by targeting essential genes with gRNAs and directly cutting and disrupting them. A recoded version of the target gene, with a native or similar promoter, serves as the antidote element. In most cases, such drives are frequency-dependent, with an introduction threshold if they have any fitness costs [94–96]. Because CRISPR toxin-antidote systems do not require homology-directed repair, they can be constructed more easily than homing or RNAi-based drives. Further, because they are only copied by replication, cargo genes would be more stable than in homing drives due to the lower mutation rate in replication compared with homology-directed repair. Nevertheless, they usually require greater release sizes and act more slowly than homing drives. Additionally, confined toxin-antidote systems can only be used for modification unless a haplolethal gene is targeted [94], which somewhat increases engineering difficulty because many genotypes with disrupted haplolethal target genes are nonviable. It is possible to combine CRISPR toxin-antidote and homing drives to gain some advantages of both systems [97].

Thus far, CRISPR toxin-antidote drives have been constructed at the same site [95] and at a different site [96] from their target gene in *D. melanogaster*. These drives targeted haplosufficient but essential genes, meaning that genotypes were nonviable only when both wild type copies of the target gene were disrupted without the presence of any drive alleles to provide rescue. This allowed for use of promoters with high cleavage activity in the early embryo due to maternally deposited Cas9 and gRNA. Such activity would actually increase the efficiency of these drives, as opposed to homing drives, where undesired resistance alleles are created. Both systems are highly efficient, rapidly spreading though cage populations. The original drive could also be easily replaced by a new drive with a different target gene [98]. These systems are recent and there is every prospect that they can be readily engineered in multiple mosquito species, likely more easily than homing drives due to reduced requirements for germline Cas9 expression and homology-directed repair.

Many simple forms of CRISPR toxin-antidote drives are ‘regional’, where they gain introduction thresholds if the drive has any fitness cost. However, several designs exist for more confined forms that would only spread to a more ‘local’ area because they possess an introduction threshold regardless of fitness cost [99]. These have not yet been experimentally demonstrated, but many could use components of existing drives, making their construction quite feasible. Together, they potentially allow for tailored levels of drive confinement.

Tethered drives

In many cases, unconfined homing drives may not be suitable for a particular situation, such as local suppression of an invasive species. However, confined drives usually have difficulty with population suppression or highly costly cargo genes. In these situations, a tethered drive system could provide the power of a homing drive with the confinement of another system [100]. They can be constructed by developing a homing drive system that lacks an essential component, such as Cas9 or gRNAs. The missing component is provided by a confined drive. This limits the homing drive to only areas where the confined drive can spread. Such tethered homing drives may be particularly compatible with CRISPR toxin-antidote drives that already have Cas9 genes. An experimental demonstration of such a combination was generally successful, with a regional drive providing Cas9 to power tethered homing modification and suppression drives [101]. In principle, tethered drives based on CRISPR toxin-antidote elements could be engineered in any species in which construction of a homing drive is feasible.

Self-limiting gene drives

Killer-rescue drive

The killer-rescue drive is the classic 'self-limiting' drive system consisting of two separate alleles [101]. The 'killer' allele causes nonviability unless the rescue allele is also present. Usually, this means that the killer allele will steadily be removed from the population, but not before driving the rescue allele to high frequency, which occurs due to its advantage over the wild type allele when enough killer alleles are present in the population. The killer allele will be eliminated relatively quickly and the rescue allele will then slowly decline due to fitness costs, meaning that the killer-rescue drive is temporary in nature. This potentially makes it desirable in situations where long-term genetic modification of the population is not desired. A few variants of this system have been successfully constructed in fruit flies [102]. While it is possible to construct in mosquitoes, the higher resource and release requirements needed for such systems to be effective over the wide areas that need protection against certain mosquito diseases may limit prospects for their deployment.

Split drives

Other self-limiting drive systems involve a split driving element and a supporting element. The supporting element cannot increase in frequency and will slowly be removed from the population due to fitness costs. The driving element can increase in frequency when together with the supporting element, where it can take the form of most types of gene drives described earlier. This allows the drive system to initially increase in frequency but eventually decline due to lack of the supporting element. This means that with substantially higher release sizes, **split drives** could possibly be used to temporarily modify mosquito populations. However, populations could still be altered for longer than desired periods of time, depending on release and migration parameters. It would also be difficult to use split drives for population suppression, which requires all drive alleles to operate at high efficiency to achieve high genetic loads.

Thus far, the most common type of split drive is a homing drive lacking Cas9, which is provided by the supporting element. This was first demonstrated in yeast [53] and has since been used extensively in flies [103]. In general, split homing drives usually have similar performance to complete drive systems, making them useful for study in the laboratory without worry that an accidental release would spread the drive to a natural population. In addition to homing drives, the split drive concept has also been applied to CRISPR toxin-antidote drive [104] in fruit flies. Modeling also indicates that a killer-rescue drive could be further limited by using split forms of the system [105].

Daisy drive systems are similar to split drives, except that the supporting element is designed to last longer in the population, allowing for more powerful drives and potentially enabling population suppression. However, this comes at the cost of controllability [92] and increased engineering complexity. In a daisy chain system, the supporting elements consist of a series of split homing drives, with each element providing a component required by the next [13,106]. Because the first element cannot drive, each element will in turn eventually decline after losing its supporting element, making the drive temporary under some circumstances but still potentially propelling the final allele to high frequency.

Comparing symbiont and transgenic approaches

Symbiont and transgene-based tools are two promising tools for mosquito-borne disease control. However, there are advantages and limitations for each approach. Transgenic approaches can target specific pathogens by specific mechanisms, but some symbiont tools are mostly based on the bacterium itself or potentially limited by engineering and expression capabilities of the bacterium that can infect the target mosquitos and inhibit pathogens. *Wolbachia* can block multiple pathogens [13,107,108], but other recently isolated natural symbionts have only been shown to block *Plasmodium* or certain arboviruses [23,25,29]. Recently discovered ISVs can inhibit arbovirus replication and reduce virial titers *in vitro* [34,36], but more work is needed to confirm their *in vivo* effects and potential to spread in populations. Although engineered symbionts can target specific pathogens in principal, successful deployment still depends on the ability of the symbiont to infect and spread in target mosquito populations and the effectiveness of available antipathogen effector molecules. There are already several anti-*Plasmodium* effectors [109] but anti-arbovirus effectors are less common. One advantage of gene drive is the possibility for population suppression after a single modest-sized release, which is particularly important for scenarios where resources are too limited for continued release of *Wolbachia* males. Another important aspect to consider is horizontal transmission. Symbionts can be horizontally transferred to unintended species and insects; this can be problematic, with unintended ecological effects. Conversely, transgenic material is generally transmitted vertically within species, though there is some possibility of a gene drive moving between species that can occasionally form viable hybrids.

Regulatory, ethical, and public acceptance are significantly varied between symbiont and transgenic approaches. Symbiont approaches, especially natural symbionts, are more widely accepted because these symbionts are already present in the environment and *Wolbachia*-based mosquito controls have been implemented in several countries [15,21,110]. For transgenic approaches, testing has been somewhat more limited and the idea of genetic modification often leads to public resistance. These considerations are amplified in gene drives, which have not been tested in the field and which are often designed to modify or suppress populations for long periods of time.

Concluding remarks

Symbiont and transgene-based tools are both innovative approaches that may revolutionize mosquito-borne disease control. Immense progress has been made in gene drive and symbiont-infected mosquitoes, leading to field trials around the world for the latter. However, much work remains to be done (see Outstanding questions). For example, not much is known regarding how the environment affects a symbiont's antipathogen ability. More natural symbiont candidates and transgenes need to be discovered and developed to inhibit DENV, ZIKV, CHIKV, and WNV. Also, we need to develop strategies to control the population if the symbionts lose function in the field. Likewise, additional work on gene drive strategies is required, with only a handful of recent studies overcoming resistance and then only in *Drosophila* and *Anopheles* laboratory populations. Confined

Outstanding questions

If multiple modification and/or suppression options for a particular issue are potentially viable, which would be preferred in different scenarios?

Each species plays some role in ecological networks. Would any ecosystems be substantially disrupted if we eliminate a target species like mosquitoes?

How effectively could a symbiont or gene drive release be reverted, with the population changed back to wild type or with a particular undesired genetic element removed?

What is the ecological effect of releasing symbiont-infected mosquitoes to the field?

How important are environmental factors such as temperature in the success of *Wolbachia* or transgenesis-based approaches?

Can *Wolbachia* place selective pressures on the pathogen to evolve resistance to the bacterium? How does this compare with antipathogen effectors in transgenic mosquitoes?

How difficult will it be to create highly efficient gene drive designs in species other than *Anopheles* and *Drosophila*? Can efficient drives with acceptable levels of confinement also be engineered?

How much certainty is required from modeling studies to move forward on a gene drive release designed to be confined to only a target population?

How quickly can mosquitoes or parasites evolve resistance to suppression gene drives or the effector of a modification drive?

drives should be developed and modeled and social progress must be made to secure public approval for release of a sufficiently effective drive. With more time and effort, symbiont and transgene-based tools will perhaps be integrated with other available approaches to tackle mosquito-borne diseases and even other insect-transmitted diseases of plants and animals.

Acknowledgments

We thank Weihua Li and Xuejiao Xu for useful discussions about the manuscript. G.H.W. is supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant no. XDPB16) and the State Key Laboratory of Integrated Management of Pest Insects and Rodents (Grant No. IPM2117). J.C. is supported by laboratory startup funds from Peking University and the Center for Life Sciences and from a SLS-Qidong Innovation Fund. G.L.H. is supported by the BBSRC (BB/T001240/1 and V011278/1), the UKRI (20197 and 85336), the EPSRC (V043811/1) a Royal Society Wolfson Fellowship (RSWF\R1\180013), and the NIHR (NIHR2000907). G.L.H. is affiliated to the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Emerging and Zoonotic Infections at University of Liverpool in partnership with Public Health England (PHE), in collaboration with Liverpool School of Tropical Medicine and the University of Oxford. G.L.H. is based at LSTM. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, the Department of Health, or Public Health England.

Declaration of interests

The authors have no competing interests to declare.

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