Chapter

Mosquito Population Modification for Malaria Control

Rebeca Carballar-Lejarazú, Taylor Tushar, Thai Binh Pham and Anthony James

Abstract

Malaria is a mosquito-borne disease that kills millions of people every year. Existing control tools have been insufficient to eliminate the disease in many endemic regions and additional approaches are needed. Novel vector-control strategies using genetic engineering to create malaria-resistant mosquitoes (population modification) can potentially contribute a new set of tools for mosquito control. Here we review the current mosquito control strategies and the development of transgenic mosquitoes expressing anti-parasite effector genes, highlighting the recent improvements in mosquito genome editing with CRISPR-Cas9 as an efficient and adaptable tool for gene-drive systems to effectively spread these genes into mosquito populations.

Keywords: *Anopheles*, mosquito control, genetic manipulation, CRISPR/Cas9

1. Introduction

1.1 Malaria and mosquito control

Mosquitoes in the genus *Anopheles* transmit to humans the *Plasmodium* parasites that cause malaria. Malaria is one of the most devastating mosquito-borne diseases worldwide, affecting more than 225 million people yearly, especially in sub-Saharan Africa and India [1].

Interventions to control anophelines have been ongoing since Sir Ronald Ross's discovery of the complete malaria transmission cycle in the late nineteenth century. The first large-scale vector control interventions in the early twentieth century relied on management and control of anopheline breeding habitats via manipulation of the environment (**Figure 1**) [2]. However, the discovery and subsequent development of dichloro-diphenyl-trichloroethane (DDT) in the early 1940s led to a new era of vector control after successes with the insecticide by the U.S. Army in World War II and various field trials proved its powerful ability to control malaria [3]. The initial successes with DDT were so great that malaria eradication began to appear feasible to some malariologists, and in 1955, the World Health Assembly launched the Global Malaria Eradication Programme (GMEP) with a goal to assist nations in eradicating malaria by providing technical advice and consolidating the resources needed for large-scale eradication campaigns. The World Health Organization (WHO) Expert Committee

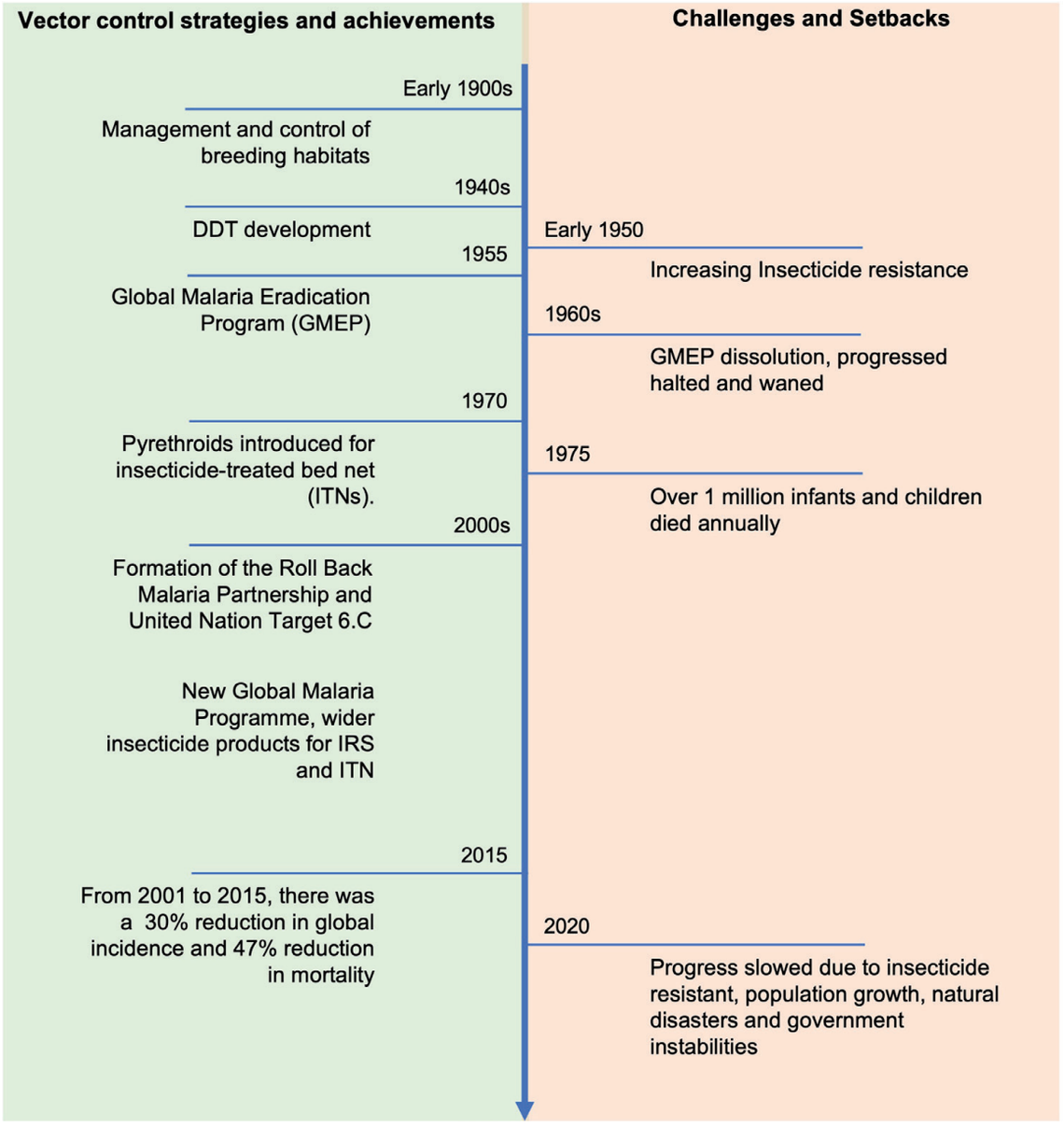

Figure 1.
Timeline of vector-control approaches and outcomes. Important events and timepoints of malaria vector control efforts and progress in the perspective of obstacles and downturns. Although great progress was made through the history of malaria vector control, many natural and artificial challenges have hindered the goal of malaria eradication.

on Malaria was responsible for designing the eradication campaign schedule, which consisted of four distinct phases: preparatory, attack, consolidation, and maintenance. Completion of the eradication schedule was estimated to require 8–10 years [4]. Despite previous observations of insecticide resistance to DDT in Greece in 1951, the attack phase relied almost exclusively on the use of indoor residual spraying (IRS) of this insecticide to reduce adult mosquito populations supplemented by chloroquine to treat infections [5]. Large reductions in malaria case incidence, morbidity and mortality were observed worldwide because of the GMEP campaign and malaria was eliminated in many countries with temperate climates (**Figure 2**) [6–10]. However, progress began to falter by the mid 1960s and some countries participating in the GMEP reverted from the consolidation phase back to the attack phase. Countries such as Sri Lanka, which was an exemplary model for GMEP successes, began to experience

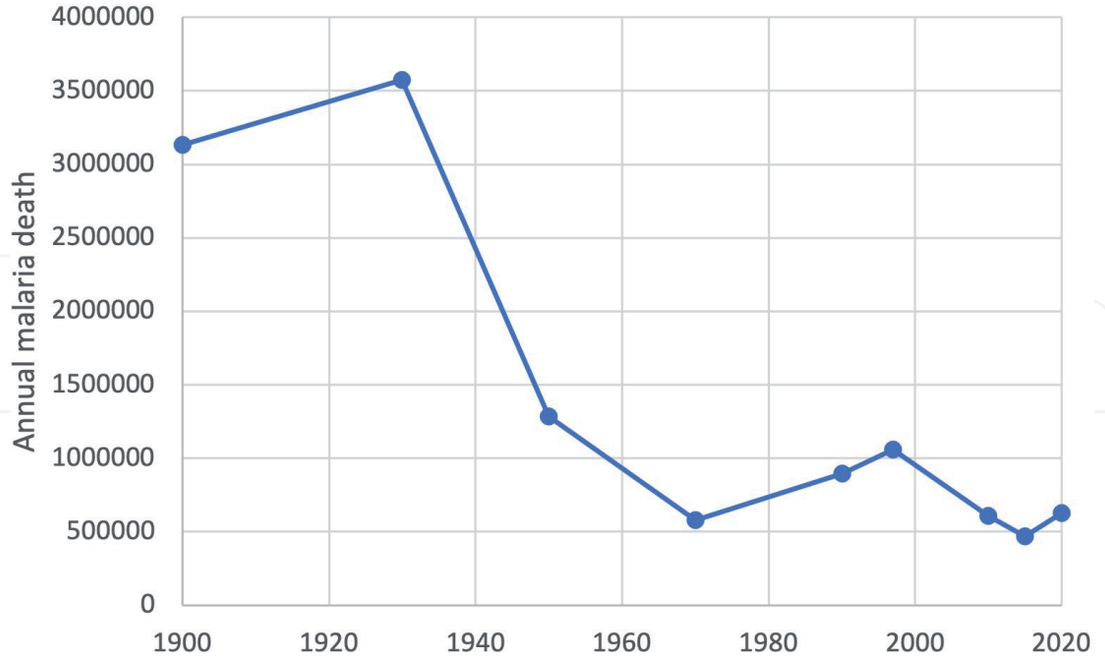

Figure 2.
Global estimated number of malaria deaths. Estimated malaria mortality declined significantly from 1920s to 1970s due to many malaria control efforts countrywide and internationally but slowed from 1970s to 2020. Sources [6–9].

epidemic resurgences of malaria [11]. Additionally, resistance to DDT became widespread throughout the participating countries. By the late 1960s, political and financial support for the GMEP had waned and the aim for eradication within a finite timeline was replaced by the aim of controlling malaria within an indefinite timeline.

Control of malaria after the dissolution of the GMEP devolved to a country-by-country basis. Some nations that had benefited from participation in the GMEP continued to make progress in reducing the burden of malaria. However, most African nations were never included in the GMEP, and without dedicated resources, financial support or personnel trained in vector control techniques, the continent continued to suffer greatly as population growth paralleled an increase in malaria morbidity and mortality. In 1975 the WHO estimated that over one million infants and children were dying annually due to malaria in sub-Saharan Africa [12]. A systemic analysis of global malaria mortality from 1980 to 2010 estimated a peak of malaria deaths occurred in 2004 with over 1.8 million deaths occurring globally [13]. By the beginning of the second millennium, the rapid expansion of disease burden due to the absence of a global strategy and lack of unified political will became soberingly evident in the global malaria mortality rates.

The combination of skyrocketing malaria mortality and philanthropic interests of the world's ultra-wealthy led to a renewed interest and consolidation of financial and political will for advances in malaria control and elimination at the beginning of the second millennium. The formation of the Roll Back Malaria Partnership (RBM) and creation of the United Nations Millennium Development Goals helped to solidify a new global strategy. After years of disparate global malaria control without clearly-defined metrics to track progress, the renewed enthusiasm ushered in a return to specific targets and strategies reminiscent of what was attempted in the 1950s with the GMEP. The new global malaria programme (GMP) had the benefit of additional vector control tools such as a wider variety of insecticide products for IRS and insecticide-treated nets (ITN). The new program also benefited from the historical perspectives of renown

malariologists on the causes leading to the failures of the original eradication effort. The UN development goals included Target 6.C with a stated aim "to have halted by 2015 and begun to reverse the incidence of malaria and other major diseases" (UN Millennium goals [14]) and the RBM created a Global Malaria Action Plan, which outlined an overarching strategy and system of support needed to achieve malaria eradication [15]. The enhanced frameworks for combating malaria also were accompanied by increased funding in the formation of the Global Fund to Fight AIDS, Tuberculosis and Malaria and the US President's Malaria Initiative [16]. The renewed efforts and consolidation of strategies and finances in the early 2000s proved successful, and Target 6.C of the development goals was achieved. There was a 30% reduction in global incidence and 47% decline in mortality due to malaria from 2001 to 2015 [1]. Continuing the momentum of the progress made in the early millennium, WHO member states created and adopted a new global technical strategy (GTS) in 2015 and set an ambitious new target for a 90% reduction in global malaria burden by 2030 [17].

The WHO and RBM developed a new framework of strategies and guidelines to meet the ambitious 2030 targets. The first pillar of the WHO's post-2015 GTS called for expansions of access. Firstly, it called for expanded access to vector control using either IRS or long-lasting insecticide treated nets (LLINs) and secondly, it called for expanded access to chemoprevention and treatment, especially in vulnerable groups such as children and pregnant women. The new guidelines also highlighted the importance of generating entomological and epidemiological surveillance data to guide vector control and disease-treatment efforts and advised that accumulation of these data should be considered an intervention in itself. While supporting elements of the post-2015 GTS encouraged advancements in research and new technology, these were secondary to the ramp-up of coverage using existing vector control and treatment technologies. Unfortunately, despite the restructured objectives and continual commitment to malaria elimination by global parties in 2015, progress in reducing malaria morbidity and mortality has slowed or stalled in many mid- to high-transmission countries. The post-2015 GTS set an interim goal of achieving 40% reductions in malaria case incidence and mortality by 2020, however, the case incidence at that time had only decreased by 3% and mortality decreased by 22% compared to 2015 levels [17].

1.2 Current challenges of vector control

Many factors contribute to the decreased rate of reducing malaria incidence and mortality rates. Population growth in malaria-endemic countries has substantially increased the at-risk population. Initial modeling efforts completed during the creation of the post-2015 GTS predicted that with the existing vector control tools and treatment options available, coverage would have to exceed 80% of high-risk populations to reduce the malaria burden [17]. However, growing populations combined with continuing instabilities of governments, natural disasters, conflicts, and epidemics have hampered the ability to reach this needed intervention coverage. As a result, there has been inadequate access to available vector control interventions. It is estimated that only 46% of the population at risk for malaria is protected by an insecticide-treated net and the percent of at-risk population covered by IRS is only 2.4%, a 2.9% decrease when compared to 2010 coverage [1].

In addition to problems of access, the existing vector control interventions face problems of reduced efficacy due to the widespread emergence of insecticide resistance in the major anopheline vectors. Resistance in the form of either target-site insensitivity or metabolomic changes has been observed for all classes of insecticides

currently being used to treat bed nets or in IRS campaigns [18]. Cuticular or penetration resistance has also been observed [19], which also reduces the impact of bed nets and IRS campaigns. As of 2020, only eight of the 82 malaria-endemic countries reported no resistance to all classes of insecticides. Resistance to pyrethroids, the only insecticide approved to treat bed nets, is widespread and resistance was reported in just under 70% of the locations that performed WHO approved standardized testing [1]. The varied resistance mechanisms and wide geographical spread of resistance imposes a major threat to the objectives of the GTS, yet no vector control products based on a new class of insecticide have been introduced to global markets since pyrethroids were introduced in the 1970s however, several have been re-purposed for their use in bed-nets and IRS and new formulations are under development with the World Health Organization Pesticide Evaluation Scheme [20, 21]. An additional challenge to current vector control tactics is behavioral resistance of the mosquito vectors. The long-term use of ITN and IRS creates a selective pressure that has been shown to result in behavioral and population compositional changes of malaria-vectoring species over time [22]. Changes in *An. gambiae* spp., the primary vector species of sub-Saharan Africa, to bite earlier in the day and outdoors (exophilic) have been observed [23, 24]. Changes in population structure to favor exophilic and day-biting malaria vectors such as *An. funestus* also have been implicated in areas where residual transmission of malaria occurs despite good ITN or IRS coverage [25].

An increase in access to vector control interventions to above 80% coverage of at-risk populations will likely lead to a reduction in case incidence and mortality but may not result in the desired 90% reduction of malaria burdens due to the challenges presented by resistance. With no new classes of insecticide approved for the control of malaria, widespread insecticide resistance and evidence of behavioral changes perpetuating residual transmission, the limitations of the current GTS vector control initiatives are obvious. New tools and technologies are needed urgently to meet the 2030 targets of the GTS. Ideally, novel vector control strategies should be cost-effective and sustainable as well as implementable and maintainable in a variety of regions irrespective of changes in government stability, conflicts or catastrophes. Population modification using genetic techniques to confer parasite refractoriness in mosquitoes is one such novel strategy that could greatly aid in achieving the ambitious goals of the GMP.

2. Population modification for malaria control

2.1 What is population modification?

Population modification is the concept of incorporating genes or genetic elements in vector species that increase their refractoriness to the pathogens they transmit thereby inhibiting transfer of the pathogens to host species (**Figure 3**). Population modification was first described in the contemporary literature using the term 'population replacement' by Christopher Curtis in 1968 [26]. Due to misinterpretations of population replacement and negative connotations of the term 'modification' related to cultural perspectives on genetically-modified organisms (GMOs), a third term, 'population alteration', also was proposed [27]. The early conceptions of population modification were made prior to the discovery and refinement of current gene-drive technologies, however, the original concept as proposed by Curtis suggested the need for a mechanism to elicit fixation of the favorable genes in a population. The advancements and development of genetic-engineering techniques to inhibit *Plasmodium* spp. have occurred in parallel with the development of gene-drive technologies and today proof-of-principle

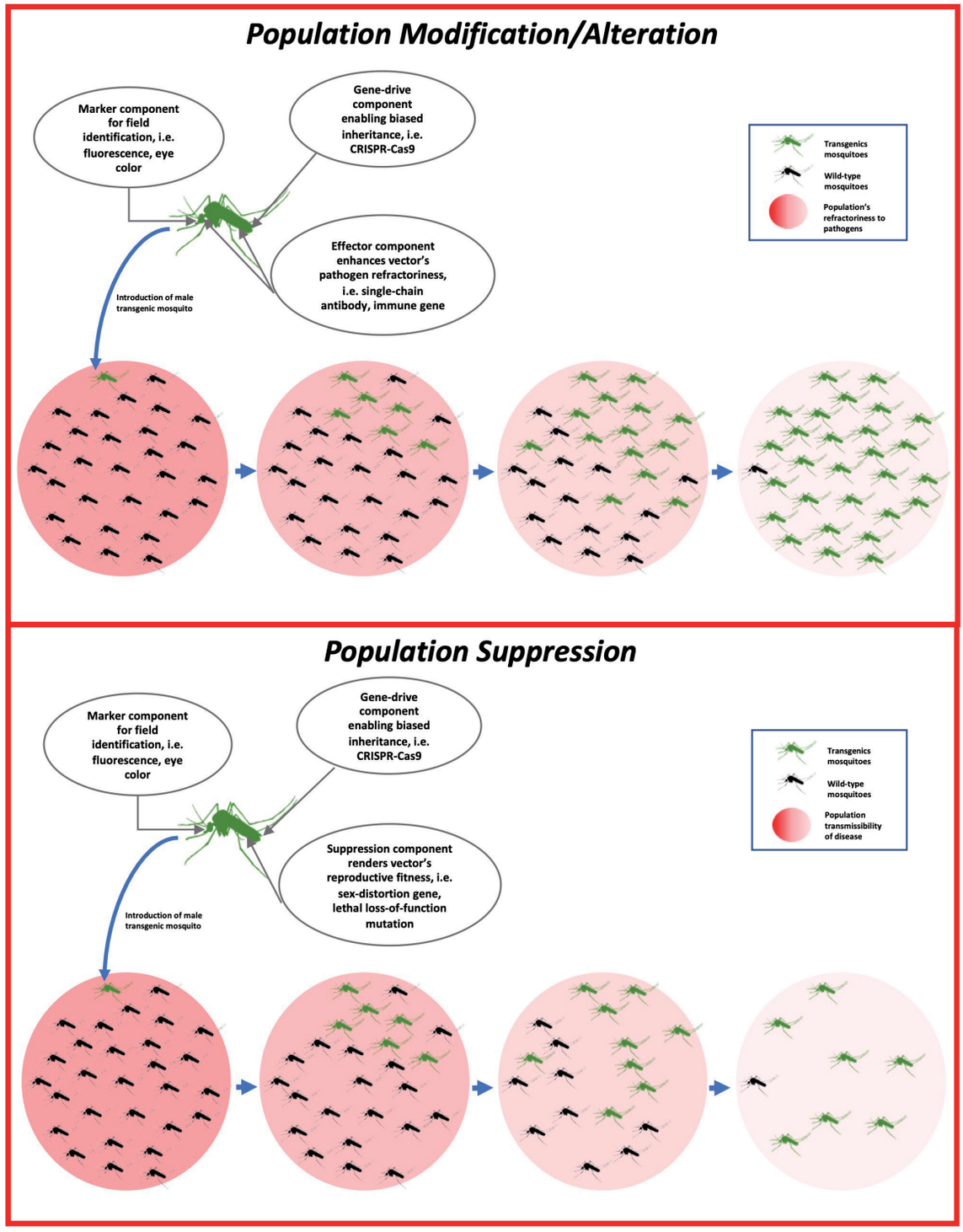

Figure 3.
Outcomes anticipated from genetic control approaches. Vector control strategy utilizes genetic-engineering technology with gene drive via two different approaches, population modification/alteration (top) or population suppression (bottom). In both approaches, the transgenic mosquitoes qualified for releases should carry at least three components: the gene drive system, the marker and the effector or suppression component aiming at reducing the vector competence or the vector population, respectively. The anticipated outcome for the population modification/alteration approach is that the treated population become refractory to pathogen as the effector genes spread into the population; whereas with the population suppression approach the anticipated outcome would be the reduction or elimination of whole population. In both cases, the goal is to break the parasite cycle in the mosquito stages.

concepts for population modification strains exist in both the African malaria vector, *An. gambiae* and the Indo-Pakistani vector, *Anopheles stephensi* (**Table 1**) [28–31].

		AsMCRkh2	Reckh2	AgNosCd-1	AgTP13
Species		*An. stephensi*	*An. stephensi*	*An. gambiae*	*An. gambiae*
Drive system		*vasa*-Cas9	*vasa*-Cas9	*nos*-Cas9	*nos*-Cas9
Target locus		*Kynurenine hydroxylase-white (kh^{w})*	*kh^{w}*	*Cardinal (cd)*	*cd*
Effector		Cp-1C3, Vg-2A10	None	None	Cp-1C3, Vg-2A10
Drive efficiency	Male	~99%	~99%	~99%	~99%
	Female	65–90%	~56%	~95%	~85–96%
	Maternal effect	Significant	Significant	Mild	Mild
Fitness	Male contribution	Comparable with WT male	Comparable with WT male	mild reduction	Moderate reduction
	Fertility and fecundity	Post Blood meal lethality in homozygotes	Comparable with WT females	Comparable with WT females	Comparable with WT females
Small cage trials	Cage trial ratios, gene drive: wild-type males	1:1, 1:3, 1:10	1:1, 1:3, 1:10	1:1, 1:3, 1:10	1:1, 1:3
	Full introduction result	No	>95% introduction for all ratios	Yes	Yes for 1:1 ratio

Cp, carboxypeptidase gene promoter; Vg, vitellogenin gene promoter; 1C3, 2A10: single-chain antibodies; WT: wild-type.

Table 1.
Proof-of-principle gene-drive systems with and without antimalarial effectors in Anopheles mosquitoes for population modification/alteration strategy.

2.2 Population modification vs. population suppression

Population suppression is an alternative strategy to population modification that utilizes genetic-engineering technologies to reduce vector number and therefore reduce pathogen transmission (**Figure 3**). This can be achieved by diminishing the fitness or distorting sex ratios so that the vector populations reduce in number and eventually go extinct locally. Similar to population modification, proof-of-principle concepts also exist for population suppression in *An. gambiae* [32–34]. The advantages and disadvantages of both population modification and suppression drives are described succinctly in a recent review [35]. One advantage of suppression drives is that they create a rapid reduction in vectorial capacity by immediately having suppressive effects on the targeted mosquito population (reductions in entomological inoculation rate and human biting rate) and thus quickly reducing the basic reproductive rate (R0) of malaria. Another advantage is that suppression drives also will reduce transmission of all possible pathogens vectored by the target species. Unanswered questions that give cause for concern are what happens to the empty ecological niche left by the vector species, and will suppression to extremely low population levels allow re-introduction of wildtype mosquitoes that then transmit

the pathogen to a more highly susceptible human population? In contrast, population modification strategies are not likely to have as much of an immediate effect on the vectoral capacity and subsequent R0 as the drive system takes time to introduce the effectors into the population and an R0 < 1 is likely to require a sufficient proportion of the population to carry effector genes [36]. However, population modification strategies do not leave an empty ecological niche and the introduced anti-parasite genes are anticipated to remain stable in a population and this could mitigate the role that re-introduction of infectious wild-type mosquitoes might have in the local population. Population modification is predicted to be sustainable during the control, pre-elimination, elimination, and prevention-of-reintroduction stages of local malaria elimination and thereby provide a cost-effective method for maintaining local elimination [37]. It is expected to be useful in the elimination phase by complementing other strategies that reduce mosquito population sizes. Some potential disadvantages include the potential to select parasites resistant to a single effector mechanism. One strategy to mitigate development of parasite resistance to effectors is by including multiple effector components that target various stages of the parasite development cycle within the mosquito [38]. The effector components used may be exogenous, such as single-chain antibodies (scFvs), endogenous, such as a manipulation of genes associated with mosquito innate immunity or a combination of both [38–41]. A second strategy to mitigate parasite resistance to population modification strains is to reduce the parasite population prior to and during the field release of the modified mosquitoes so that there is less opportunity for resistance to develop due to lower replication rates in the parasite population [42]. Encouragingly, both strategies to mitigate parasite resistance can be combined to provide pathogens with a more insurmountable barrier to developing resistance.

Population modification and population suppression vary in their strengths and weaknesses so a complementary approach that involves the sequential application of both technologies can be proposed (**Figure 4**). This strategy maximizes the benefits of both approaches and lowers their respective hurdles to long-lasting success. The complementary approach includes an initial field release of a population suppression strain that will act to quickly reduce the local population of vectors and their associated population of parasites. When the population structure of native vectors has been sufficiently disrupted by the suppression strategy, the low level of individuals becomes more susceptible to events that may inhibit its ability to persist long term. For example, a re-introduction of wild-type individuals can occur, and these may overwhelm any low levels of remaining drive individuals, or individuals with drive-resistant alleles may build up over time inhibiting future suppression [43]. At this point, when a suppression system has driven the population to levels near extinction, a modification line can be introduced for maximal effect. Allowing a population replacement mosquito line to form the new population of mosquitoes prevents any negative ecological effects that may have occurred due to an empty ecological niche. It also allows the population modification drives to become established in an environment with a minimized risk for resistance to the transgene introduction. The effector genes will be less prone to having pathogen-based resistance develop as the natural pathogen population will have been greatly diminished by the suppression system, and lower pathogen reproduction numbers lower the likelihood of randomly-generated resistance conferring mutations in the pathogens. In the absence of threats from resistance, the only further threat faced by the population modification strain is long-term stability of the effector elements. However, new effector elements can be developed

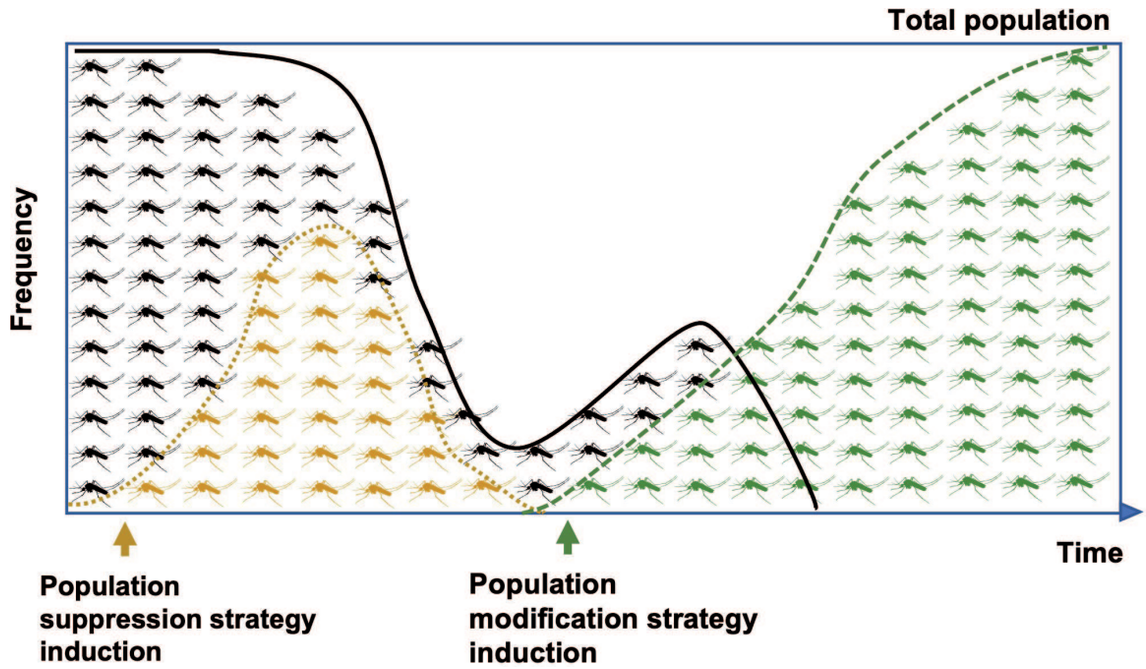

Figure 4.
Vector control with population modification and population suppression complementary approach. Proposed strategy combining sequential releases of mosquitoes with population modification and population suppression drives. The combined approach initiated with releasing population suppression gene-drive mosquitoes, which theoretically reduce the whole mosquito population in the treated area. Follow up with releasing of population modification gene-drive mosquitoes, this strategy ensures avoidance of an empty niche or re-introducing of wild mosquitoes that are susceptible to the malaria parasite. Black: wild-type mosquito; yellow: transgenics mosquitoes with suppression drive; Green: Transgenic mosquitoes with population modification drive.

carefully as the needed window for protection resulting from the complementary approach is likely to be much longer than either approach alone.

3. Engineering refractory mosquitoes

The malaria parasites go through a multi-staged life cycle within their mosquito vectors (**Figure 5**). After the female *Anopheles* mosquito bites an infected human, if ~1000 *Plasmodium* male and female gametocytes are ingested with the blood meal, subsequent fertilization produces as many as 25 diploid zygotes. The zygotes mature to a motile form, the ookinete, that penetrates the mosquito midgut epithelium where only a few (<5) will mature to oocysts. Mitotic and meiotic divisions occur in the oocysts to give rise to several hundred to thousands of haploid sporozoites. The sporozoites (~5000) are released into the hemolymph (the mosquito open circulatory system) within 10–14 days post infection. The sporozoites then travel through the hemolymph to reach and invade the salivary glands and are transmitted as the infectious form of the parasite to a new human host during subsequent bites.

A synthetic approach was used in our laboratory to develop the anti-parasite effector genes and introduce these desired traits into the target genomes to generate the genetically-engineered mosquitoes (GEMs) [37]. This approach has several advantages, for example, the components of a synthetic construct can be relatively small, their functions are more fully known and the site in the mosquito genome where they will be located can be characterized or determined prior to genome integration. A synthetic cassette for population modification has two main components: (1) promoters and (2) antimalarial effector genes.

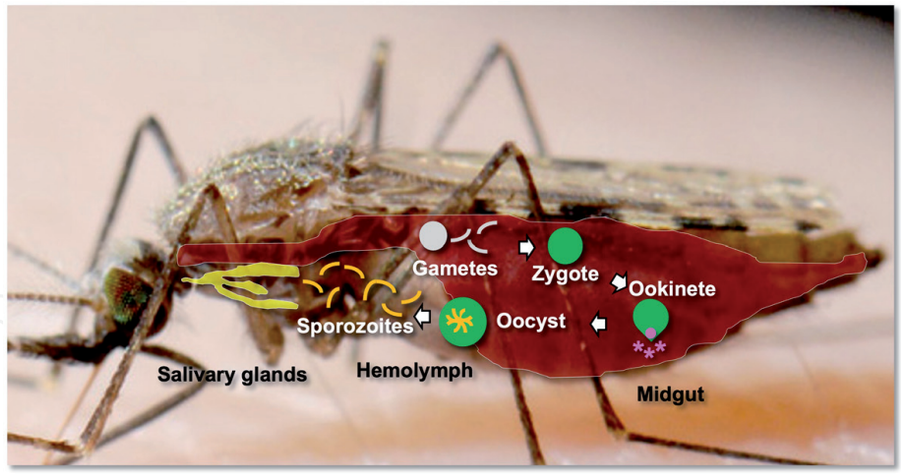

Figure 5.
Malaria developmental pathway and compartments for blocking parasite development. Gametes are ingested with the blood meal. They differentiate, fertile and form a zygote. The zygote develops into a motile form, the ookinete, that then invades the mosquito midgut epithelium. There it develops into an oocyst in which many sporozoites are generated. These burst into the hemolymph and migrate to the salivary glands. From there the sporozoites can be transmitted to a new host during the next blood meal. The midgut compartment allows access to the gametes, zygotes, ookinetes and oocysts. The hemolymph and salivary gland compartments allow access to the sporozoites (image adapted from Isaacs et al. [38]).

3.1 Promoters

Promoters are regulatory DNA sequences that will drive the expression of a transgene (a marker or an antimalarial effector) in mosquitoes. During its development in the mosquito, the malaria parasite occupies three main compartments: midgut lumen, hemocoel and salivary gland lumen (**Figure 5**). Expression of the anti-parasite genes in these compartments is crucial to block their transmission and several tissue-specific promoters have been identified and used in mosquito transgenesis. These include control sequences for a gene encoding a carboxypeptidase, a digestive enzyme, and AgAper1, a peritrophic matrix protein, which are activated in response to a blood meal [44–46]. The vitellogenin-encoding gene promoters drive strong expression in the fat body and hemocoel [47, 48]. A hemocyte-specific hemolectin (hml) gene promoter and three salivary gland-specific promoters, (*Apyrase* [*Apy*], *maltase-like I* [*Mal1*] and *anopheline antiplatelet protein* [*AAPP*] promoter), also have been developed [49–52]. Ubiquitously-expressed gene promoters (*heat-shock protein 70* [*hsp70*], *actin 5C*, and *ubiquitin* and *polyubiquitin*) also could be used to drive expression of the effector genes, however, their generalized expression may impose a higher fitness load in the GEM [53–55]. These gene promoters have been used effectively to drive the expression of genes encoding generally benign fluorescent proteins as dominant markers for transgene presence.

3.2 Antimalarial effector genes

The effector molecules can be classified into four groups depending on their mode of action.

i. Parasite blocking: exogenous molecules that eliminate the parasites such as antimicrobial peptides from the immune system of other insects (gambicin, defensin, cecropin) or other arthropods (scorpine). Natural and synthetic lytic

peptides such as angiotensin II, magainins, Shiva-1, Shiva-3 and gomesin have been used to generate refractory *Plasmodium* mosquitoes [56–62].

ii. Interaction with parasites: single-chain monoclonal antibodies (scFvs) that bind to ookinete or sporozoite surface or secreted proteins, such as m2A10 that targets the *P. falciparum* circumsporozoite protein (CSP), m1C3 that binds to the *P. falciparum* chitinase, scFv 4B7 that binds to *P. falciparum* ookinete surface protein Pfs25, and peptides that inhibits mosquito midgut invasion (EPIP- *Plasmodium* enolase-plasminogen interaction peptide) [38, 39, 63].

iii. Interaction with mosquito tissues: molecules that bind putative mosquito receptors in the midgut or salivary glands blocking the ookinete and sporozoite invasion (for example, SM1) and molecules that can modify the properties of the midgut epithelia (mPLA2- phospholipase A2) [64, 65].

iv. Mosquito immune system: manipulation of mosquito immune-related genes can lead to decreased mosquito vectorial competence. Expression of Akt, a key signaling component in the insulin signaling pathway or overexpression of IMD pathway-mediated transcription factor Rel2 can result in refractoriness to the parasite [66, 67].

The identification and characterization of efficient anti-*Plasmodium* effector genes is essential to generate refractory mosquito phenotypes. Expression of these genes may result in GEMs being less competitive than their wild-type counterparts. Ideally, the effector molecules should interfere with parasite transmission without imposing a fitness cost to the mosquito. Furthermore, these genes clearly impose selection pressures on the parasites and the emergence of parasites resistant to the effector molecules could occur. As discussed previously, this may be mitigated by using combinations of multiple anti-*Plasmodium* effector proteins with different modes of action that can overcome the possibility of parasite resistance. Recently, Dong et al. (2020) showed that it is possible to generate a transgenic line (MultiEff) expressing simultaneously five anti-*Plasmodium* effectors (melittin, TP10, shiva1, EPIP, and scorpine) with a significant parasite-blocking effect at the pre-oocyst stage and low fitness cost [68].

4. Spreading transgenes into mosquito populations

Mobile genetic elements called transposons can spread rapidly through populations despite severe costs to the host [69–72]. Their ability to mobilize (excise and insert) led to their being developed as powerful systems for introducing exogenous DNA into several organisms. The adaptation of the P transposable element for transgenesis of the vinegar fly, *Drosophila melanogaster*, was followed 16 years later by the first reliable system for transforming mosquitoes using the *Hermes* elements in the yellow fever mosquito, *Aedes aegypti* [73, 74]. Shortly after this proof-of-principle in mosquitoes, additional systems based on *Hermes, piggyBac, Minos, and mariner Mos1* were demonstrated in both culicine and anopheline species [75–82]. Unfortunately, while transposons could mediate insertion into these genomes at experimentally-useful frequencies, they were not easily remobilized making them impractical as a basis of a gene-drive system to spread transgenes through a mosquito population [83–85].

Other tools and systems for introducing genes into mosquito genomes include site-specific recombinases. These require the presence of an endogenous nucleotide sequence in the genome that is identical to the recombinase target cleavage site, or a mechanism for introducing such a site (called a docking site; [86]) into that genome. This has been achieved using the previously-described transposons. Two recombinases have been used successfully to generate transgenic mosquitoes, the bacteriophage φC31 integrase and Cre/lox recombinase derived from yeast. Their dependence on a precise site for integration of the desired transgene limits their usefulness as the basis of gene-drive systems for spreading transgenes into populations [82, 87–90].

The application of zinc-finger nuclease (ZFNs) and the transcription activator-like effector nucleases (TALENs) for engineering target-site recognition in mosquitoes introduced a major advance for genetic modification in mosquitoes. However, the high cost and low success rate limited their use [91–93]. The application of homing endonucleases nucleases genes (HEGs) for spreading genes into mosquito populations was proposed in 2003 [94] as useful basis for gene drives and in 2011 a successful HEG-based gene drive in *An. gambiae* was reported [95]. The latter required the fortuitous presence of a nuclease target site in the first chromosome (X) of this species.

A major breakthrough for mosquito transgenesis and gene-drive systems was achieved following the discovery and adaptation of the RNA-guided Cas9 nuclease from the bacterial Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) adaptive immune system [96]. This powerful tool simplified the highly-specific genome editing processes and made possible useful gene-drive systems. The Cas9 endonuclease is directed to its genomic target by a single 20 base-pair guide RNA (gRNA) complementary to its DNA target. This gRNA can be designed to target virtually any locus in a chromosome. CRISPR/Cas9 exploits the natural mechanism of cell repair to precisely insert a synthetic construct through homology-directed repair (HDR), a DNA repair system initiated by a double-strand break made at the site of a target location by the Cas9 nuclease [96]. CRISPR/Cas9 has been shown to be an excellent candidate technology for developing gene drive-based strategies to introduce beneficial genes into mosquito populations [28–30]. The properties of the system bias the inheritance of a desired trait, allowing them to quickly increase in frequency and spread through a mosquito population. CRISPR/Cas9 gene drives can efficiently convert pre-meiotic diploid germline cells in hemizygous mosquitoes (carrying one copy of the drive) into homozygotes carrying two copies [28–30]. Recently, the CRISPR/Cas9 technology has been adapted for the development of gene drives in anopheline mosquitoes and shows great promise for rapid introduction of anti-parasite genes into mosquito populations [28–30, 32].

5. CRISPR/Cas9 gene drives for population modification

The recent adaptation of CRISPR/Cas9-based biology to generate gene drives has been proposed to provide a powerful, inexpensive, and easily-implemented solution for malaria control due to the rapid introduction of the antimalarial genes into mosquito populations [37]. To produce the desired epidemiological outcomes of reduced malaria transmission, the drive system and associated effector components must be introduced quickly and efficiently into wild populations. Rapid introduction requires population modification lines to have high rates of drive allele conversion in the germline so that maximally-biased inheritance is achieved. This will result in a remarkable increase in frequency of the gene-drive system in the following generations.

The first CRISPR/Cas9 gene drive for mosquito population modification was described in 2015 for the Indo-Pakistani vector, *An. stephensi* [28]. The AsMCRkh2 gene-drive synthetic cassette used targets the ommochrome biosynthesis pathway involved in development of mosquito eye-color, specifically the locus that encodes the kynurenine hydroxylase (kynurenine monooxygenase) enzyme (referred here as *kynurenine hydroxylase white* [kh^w]). Mutations in the gene encoding this enzyme have a recessive white-eye phenotype. Drive efficiency in the AsMCRkh2 line was high with ~99% of progeny from both male and female hemizygous parents inheriting a copy of the drive allele [28]. Despite high initial efficiencies from both male and females, follow-up analyses of these lineages uncovered a return to near Mendelian inheritance in the progeny derived from female hemizygous parents. The diminished drive efficiency in female lineages was later attributed to the accumulation of indel alleles in these offspring (Section 6.2). Drive efficiency experiments in a second-generation *An. stephensi* population modification line, Reckh, resembled the observed efficiencies in the AsMCRkh2 line with ~99% of the progeny from hemizygous male parents inheriting a copy of the drive after two generations of outcrossing to wild-type mosquitoes and only ~56% of progeny from hemizygous female parents inheriting a copy of the drive after two generations of outcrosses [30].

A next-generation gene drive system for *An. gambiae* was developed [29]. The resulting strain, AgNosCd-1, targets the *An. gambiae cardinal* gene ortholog, encoding a protein downstream of the *kh* product in the ommochrome pathway. Mosquitoes with two loss-of function (LOF) alleles at this locus have a red-eye phenotype in subadult stages and newly-emerged adults (**Table 1**). AgNosCd-1 has a high drive efficiency in both male and female lineages (maternal/paternal daughters/sons and grand-daughters/grand-sons). AgNosCd-1 hemizygous males can pass the drive system with ~99% efficiency within their lineages and hemizygous females had only a slightly reduced ~95% drive efficiency within their lineages [29]. The individuals not inheriting a copy of the drive were found to have wild-type alleles as opposed to insertion or deletion (indel) alleles indicating that failures of the drive system were more likely due to Cas9/gRNA complexes not performing cleavage as opposed to cleavages that did not result in HDR [97]. Moreover, the AgNosCd-1 drive efficiency achieved a 98–100% inheritance bias in both males and females and full introduction within six to ten generations following single releases of gene drive males in small laboratory cage trials [29]. Drive efficiency experiments in a second-generation AgNosCd-1 population modification line, AgTP13 (AgNosCd-1 background linked to two anti-parasite effector genes), resulted in similar rates of drive efficiency in hemizygous males and females suggesting no impact of the effector load on the ability of the drive system to facilitate accurate HDR in the germline [31].

6. CRISPR/Cas9 considerations

6.1 Fitness impacts

The fitness load in population modification CRISPR/Cas9 drive lines have been assessed on male and female mosquitoes. An ideal CRISPR/Cas9 drive candidate for population modification would have little-to-no fitness effects resulting from the drive system and its corresponding locus, as it is predicted that the effector components are likely to have some effect on overall fitness [98, 99].

One notable example of a fitness cost was observed in the *An. stephensi* AsMCRkh2 gene drive line following disruption of both copies of the *kh*^{w} gene. As described, the enzyme encoded by this gene is responsible for generating the precursors for the formation of eye-pigments, but interestingly it also plays an important role in tryptophan metabolism in adult females following a blood meal [100, 101]. AsMCRkh2 individuals containing two LOF alleles resulting from homozygous or heteroallelic combinations of gene-drive construct insertions or Non-Homologous End Joining (NHEJ) alleles produce a white-eye phenotype and show a high lethality and reduced fecundity following a blood meal. Follow up experiments showed that after achieving fixation in multi-generation cage trial experiments, the populations experienced extinction due to the significant fertility and fecundity load on homozygous AsMCRkh2 females [100]. The AsMCRkh2 prototype was later modified to include a re-coded version of the *kh* locus (Reckh) to reduce the previously observed fitness load on females by restoring the function of the *kh* gene and thereby reversing the eye phenotype to wild-type [30]. Homozygous Reckh female adults show no significant differences in fertility and fecundity in comparison to the hemizygous Reckh or wild-type females. The improvements of female fitness translated to the success of the drive system in multi-generational cage trials with >95% of individuals carrying a copy of the drive at the termination of the experiments [30].

In contrast, AgNosCd-1 individuals do not have reduced fitness in most of the fitness parameters evaluated (fertility, fecundity, longevity, larval and pupal development), but a mild reduction in male mating competitiveness was observed [29]. AgNosCd-1 males are slightly less likely to contribute to the next generation than wild-type males, ~2% less likely for hemizygote males and ~8% for homozygote males. Despite these observed reductions in fitness, the power of the drive system was sufficient to negate the effects in subsequent generations and the AgNosCd-1 line achieved fixation in all multi-generation cage trial experiments at different release ratios of homozygous AgNosCd-1 to wild type males [29]. However, the AgTP13 homozygous males were ~22% less likely to contribute to the next generation than wild-type males in competition experiments and have a significantly reduced median lifespan than the hemizygous AgTP13 or the wild-type males. Despite the increased fitness burden in AgTP13 males, there was no increased fitness load on AgTP13 females [31]. Theoretical modeling supports the conclusion that given an appropriate drive mechanism, a gene-drive system could have a significant fitness cost and still be driven through the population [102, 103].

Ideally, GEMs should have no or minimal fitness costs to avoid reducing the effectiveness of the genetic drive mechanism that is used to introduce the synthetic construct into field mosquito populations and to maximize the likelihood of successfully introducing refractory genes into a wild population [98]. Several factors can impact the fitness, including the possible negative effect of the transgene products, insertional position effects (chromatin rearrangement and/or new regulatory element interactions/pressure), inbreeding, and to "leaky (low level constitutive) promoter expression". GEMS can have different degrees of fitness cost and estimates of transgene fitness costs are essential for modeling and planning release strategies. However, it is clear that a robust drive system can compensate for reduced fitness.

6.2 Maternal effects and resistant alleles

The efficacy of population modification mosquito drive lines may be reduced by the presence of naturally-occurring cleavage-resistant allelic variants of the target site

in wild populations or by such alleles generated through NHEJ during the Cas9/gRNA targeting and DNA repair processes. The latter may result from double-stranded DNA breaks necessary for drive that are occasionally repaired through NHEJ resulting in insertions or deletions at the target site, making them refractory to the drive system. Both the naturally-occurring and induced allelic variants have been called resistance alleles [104–107]. The latter may arise in the germline and be passed on to subsequent generations or may be generated in somatic cells where they give rise to mosaic phenotypes [28–30]. Resistance alleles in the form of naturally-occurring mutations at the target site can be avoided by careful choice of the gene-drive target locus. Resistance alleles occurring because of NHEJ due to undesired Cas9 activity can be controlled by careful choice of the promoter used to induce Cas9 transcription.

Extensive analysis of suitable target loci must be performed prior to the creation of each proof-of-principle modification drive system. Loci must be chosen, in part, based on the minimization of naturally-present single nucleotide polymorphisms (SNPs) and overall conservation of the target site. Several SNPs in the AgNosCd-1 *cardinal* target site were identified after a screening effort of hundreds of diverse *Anopheles gambiae* s.l. sequences [29]. Interestingly, all these major variants still exhibited Cas9/gRNA-mediated cleavage in assays *in vitro*.

The pathways and frequency of resistance allele formation via undesired activity of the drive system was analyzed extensively for the AgNosCd-1 and AsMCRkh2 lines [29, 97]. Exceptional phenotype individuals (mosaics and LOF phenotypes) have been correlated to undesired Cas9 activity and possess indel mutations that would cause LOF in AgNosCd-1 and AsMCRkh2 lines. However, in contrast to the AgNosCd-1 drive system, the mosaic and LOF phenotypes made up the majority of the offspring (>99%) from AsMCRkh2 mothers [28]. The presence of mosaic and LOF phenotypes from female drive parents has been hypothesized to occur due to a maternal effect. The maternal effect is proposed to result from the accumulation of Cas9/gRNA complexes in the cytoplasm of embryos derived from mothers carrying the drive system, which perform cleavage on the paternally-donated allele during embryonic development. The differences in mosaic and LOF phenotypes observed in the progeny from AgNosCd-1 and AsMCRkh2 hemizygote females supports this hypothesis and this affect is higher in females with two copies (homozygous) of the drive system than those with one (hemizygous) [28, 29, 97]. In addition, the frequencies of such events are higher in the AsMCRkh2 line when compared to AgNosCd-1. These differences may result from the difference in the gene promoters used to express the Cas9 nuclease for each drive system, *vasa* for AsMCRkh2 and *nanos* for AgNosCd-1. Follow up studies showed that the transcripts expressed from the *nanos* promoter are more confined to germline cells than those expressed from the *vasa* promoter [108], which likely results in fewer Cas9/gRNA complexes in the cytoplasm of the former embryos.

As described previously, females homozygous for the drive system had a higher rate of resistance allele formation via maternal effect (~57% with mosaic phenotype and ~6% of progeny with LOF phenotype) than hemizygous females (~20% with mosaic phenotype and ~1% of progeny with LOF phenotype) but mosaic individuals were able to bias inheritance of the drive allele and had similar rates of drive efficiency when compared to AgNosCd-1 hemizygotes with wild-type eye phenotypes suggesting that the indels were primary somatic [29].

Suppression gene drive systems are much less flexible to drive-resistant alleles than population modification gene drive systems. Population modification mosquito lines can tolerate higher rates of drive-resistant alleles than population suppression

mosquitoes, however, the former are still susceptible to instability and inability to achieve fixation in a population due to resistance alleles, especially if the drive system and respective cargo are associated with a significant fitness load [109]. Recent work suggests that suppression drive systems that incur a 100% fitness cost (death of females) would require a very low frequency of drive resistant alleles $<5 \times 10^{-7}$ in order to provide a 4–5-year window of protection, as opposed to population modification systems, which would provide a 4–5-year window of protection at a resistance allele frequency of 1%, given that fitness costs of the population modification strain are below 15% [109].

Multiplexed gene drives using additional gRNA target sites are expected to substantially decrease the likelihood of gene-drive resistant allele formation [110]. Practical ways to multiplex Cas9-based gene drives have been demonstrated using post-transcriptional processing of several gRNAs expressed from a single promoter, but these have not yet been applied to mosquitoes [110–113].

6.3 Off-targets

The utility of CRISPR/Cas9 gene-drive systems may be affected by sequence similarity among gRNAs target and off-target sites in the mosquito genomes. Potential off-target sites can be predicted *in silico* by computational algorithms and then confirmed *in vivo* by deep-sequence screening of indels or SNPs by PCR-based assays. The possible impact of unwanted mutations linked to a drive system are higher since the arising mutations will have the potential to persist within the populations. Off-target mutations also can induce a potential fitness load. Efforts to detect Cas9 off-targets in *An. gambiae* gene drive mosquitoes found very few following sequencing of large number of samples containing putative target variants [29]. The detected indels neither increased in frequency nor were detected through multiple generations in long-term cage trials (indicating that they were not heritable) and did not significantly differ in number from variants observed in wild-type individuals [29]. New approaches to increase Cas9 specificity are being developed in other organisms and include the use of highly-specific Cas9 mutant enzymes together with the constant updating of computational algorithms to better predict the possible off-targets, but their applications for gene drive mosquitoes remain unclear [114–119].

6.4 Deployment challenges

The discovery, development, and deployment of CRISPR/Cas9 technologies is challenging due to the lack of an accepted pathway to move them from the laboratory to the field. The WHO released in 2014 the Guidance Framework for testing genetically modified (GM) mosquitoes (WHO Guidance Framework) describing a phased testing pathway and best practices to evaluate GEMs proposed as public health tools [120]. The Framework proposes a pathway to move from physically-confined studies in the laboratory/insectary (Phase 1) to a small-scale confined field-testing (Phase 2) that will lead to a staged open release trial (Phase 3). After successful completion of Phase 3, the national authorities in a malaria-endemic country will be responsible for determining if the tested GEMs can be included as part of their malaria control program and further deployment of the technology (Phase 4) [120]. However, pathways for moving gene-drive population modification mosquitoes to the field will be defined simultaneously with the laboratory work progress. As more CRISPR/Cas9 population modification gene-drive systems and strains are developed, new knowledge is being

generated about the impact of introduced anti-parasite genes on the mosquitoes that carry them. Insight into genetic loads and their effects on fitness, generation of drive-resistant individuals as well as selection of resistant parasites and long-term stability of the system will emerge from these studies. The new empirical data generated is critical in the development of a phased pathway for further development and deployment. In 2018, James et al. published a series of recommendations that attempt to envision the development pathway for gene drive mosquitoes (from discovery to deployment) and to inform decision-making by regulators and policymakers [121]. They recognized that it is important to examine both the benefits and risks of this approach. Risk assessment will provide guidance on decision-making and information for the regulatory applications as well as for the development of mitigation plans, while cost-benefit analyses will compare the projected or estimated costs and benefits associated to the intervention. It also was recommended that these analyses be done by external third-party organizations or institutions with no interests in the success of the product and the outcomes of these analyses be made publicly available.

Any decision made to release gene-drive mosquitoes must be made on a case-by-case basis following a comprehensive environmental risk assessment [122], moreover, gene-drive population modification mosquitoes must meet the established Target Product Profile (TPP) criteria of safety and efficacy. A comprehensive draft TPP for gene-drive population modification mosquitoes was published providing the basis for evaluation of whether gene-drive mosquitoes should be made available for use [37]. Population modification TPPs will need to meet the efficacy and safety standards as well as the demands of different regulatory and social contexts. In addition, viable models for the inclusion of end-user and stakeholder involvement and control are absolutely needed before any such system can be brought to the field. We have favored the relationship-based model (RBM), which gives stakeholders and community key roles at the center of the decision-making processes [123]. It is important that open dialog and relationships with the scientists developing the technologies be established and that appropriate capacity-building take place to empower the communities affected by malaria to make informed decisions about the risk and use of the new technologies.

7. Conclusions

Population modification genetic control focuses on targeting the mosquito vector to interrupt the malaria transmission by introducing effector genes into the mosquito genome with the purpose of generating parasite-refractory mosquitoes.

Advances in gene-editing technologies using CRISPR/Cas9 gene drives have made available new possibilities for an efficient introduction of the desired genetic traits into mosquito populations. Gene drives represent a powerful tool to achieve genome editing in a species-specific targeted way with minimal infrastructure, are predicted to be self-sustaining and able to spread anti-parasite effectors to fixation.

Gene-drive systems for population modification of anopheline vector species to prevent transmission of parasites may play a future role in the malaria eradication agenda. Future steps will need to consider how to evaluate gene drives at large scale and evaluate their efficacy and robustness under more realistic ecological settings.

Challenges to such technologies are being addressed by scientists and regulators by development of pathways for their deployment and establishing acceptable efficacy and safety criteria. Importantly, the knowledge transfer process is being addressed

in new models for public engagement that will further development, testing and eventual deployment of gene drives for malaria control.

Acknowledgements

Funding was provided by the University of California Irvine Malaria Initiative and 'anonymous donor'. AAJ is a Donald Bren Professor at the University of California, Irvine.

Conflict of interest

The authors declare no conflict of interest.

Author details

Rebeca Carballar-Lejarazú[1*,†], Taylor Tushar[1†], Thai Binh Pham[2] and Anthony James[1,2]

1 Department of Microbiology and Molecular Genetics, University of California, Irvine, CA, USA

2 Department of Molecular Biology and Biochemistry, University of California, Irvine, CA, USA

*Address all correspondence to: rcarball@uci.edu

† These authors contribute equally to the preparation of this chapter.

References

[1] World Health Organization. World Malaria Report 2020: 20 Years of Global Progress and Challenges. Geneva: World Health Organization; 2020. Available from: https://www.who.int/publications/i/item/9789240015791

[2] Keiser J, De Castro MC, Maltese MF, Bos R, Tanner M, Singer BH, et al. Effect of irrigation and large dams on the burden of malaria on a global and regional scale. The American Journal of Tropical Medicine and Hygiene. 2005;**72**(4):392-406

[3] Pampana E. A Textbook of Malaria Eradication. 2nd ed. London, New York, Toronto: OXFORD University Press; 1969

[4] Nájera JA, González-Silva M, Alonso PL. Some lessons for the future from the Global Malaria Eradication Programme (1955-1969). PLoS Medicine. 2011;**8**(1):e1000412. DOI: 10.1371/journal.pmed.1000412

[5] Li XH, Kondrashin A, Greenwood B, Lindblade K, Loku Galappaththy G, Alonso P. A historical review of WHO certification of malaria elimination. Trends in Parasitology. 2019;**35**(2):163-171. DOI: 10.1016/j.pt.2018.11.011. Epub 2019 Jan 10

[6] Carter R, Mendis KN. Evolutionary and historical aspects of the burden of malaria. Clinical Microbiology Reviews. 2002;**15**(4):564-594. DOI: 10.1128/CMR.15.4.564-594.2002

[7] World Health Organization. World Malaria Report 2012. Geneva: World Health Organization; 2012. Available from: https://www.who.int/malaria/publications/world_malaria_report_2012/wmr2012_full_report.pdf

[8] World Health Organization. World Malaria Report 2021. Geneva: World Health Organization; 2021. Available from: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021

[9] World Health Organization. The Global Health Observatory, Estimated Number of Malaria Death [Internet]. World Health Organization; 2022. Available from: https://www.who.int/data/gho/data/indicators/indicator-details/GHO/estimated-number-of-malaria-deaths

[10] Ghebreyesus TA. The malaria eradication challenge. Lancet. 2019;**394**(10203):990-991. DOI: 10.1016/S0140-6736(19)31951-8

[11] Nasir SMI, Amarasekara S, Wickremasinghe R, Fernando D, Udagama P. Prevention of re-establishment of malaria: Historical perspective and future prospects. Malaria Journal. 2020;**19**(1):452. DOI: 10.1186/s12936-020-03527-8

[12] World Health Organization. Information on the world malaria situation. In: Weekly Epidemiological Record. Vol. 52(07). Geneva: World Health Organization; 1977. p. 65. Available from: https://apps.who.int/iris/handle/10665/221226

[13] Murray CJL, Rosenfeld L, Lim S, Andrews K, Foreman K, Haring D, et al. Global malaria mortality between 1980 and 2010: A systematic analysis. The Lancet. 2012;**379**(9814):413-431. DOI: 10.1016/S0140-6736(12)60034-8

[14] Way C. The Millennium Development Goals Report. New York, United Nations: 2015. Available from: https://www.

un.org/millenniumgoals/2015_MDG_Report/pdf/MDG%202015%20rev%20(July%201).pdf

[15] Roll Back Malaria. Global Malaria Action Plan For a Malaria-free World. 2008. Available from: https://www.unhcr.org/4afac5629.pdf

[16] Newby G, Bennett A, Larson E, Cotter C, Shretta R, Phillips AA, et al. The path to eradication: A progress report on the malaria-eliminating countries. Lancet. 2016;**387**(10029):1775-1784. DOI: 10.1016/S0140-6736(16)00230-0

[17] World Health Organization. Global Technical Strategy for Malaria 2016-2030, 2021 Update. Geneva: World Health Organization; 2021. Available from: https://www.who.int/publications/i/item/9789240031357

[18] Sougoufara S, Sokhna C, Diagne N, Doucouré S, Sembène PM, Harry M. The implementation of long-lasting insecticidal bed nets has differential effects on the genetic structure of the African malaria vectors in the *Anopheles gambiae* complex in Dielmo, Senegal. Malaria Journal. 2017;**16**(1):337. DOI: 10.1186/s12936-017-1992-8

[19] Riveron J, Tchouakui M, Mugenzi L, Menze B, Chiang MC, Wondji C. Insecticide resistance in malaria vectors: An update at a global scale. In: Manguin S, Dev V, editors. Toward Malaria Elimination—A Leap Forward [Internet]. London: IntechOpen; 2018. DOI: 10.5772/intechopen.78375. Available from: https://www.intechopen.com/chapters/62169

[20] Kupfershmidt K. Pick your poison. Science. 2016;**354**(6309):171-173. DOI: 10.1126/science.354.6309.171

[21] World Health Organization. Report of the twentieth WHOPES working group meeting, WHO/HQ, Geneva, 20-24 March 2017: Review of Interceptor G2LN, DawaPlus 3.0 LN, DawaPlus 4.0 LN, SumiLarv 2 MR, Chlorfenapyr 240 SC. Geneva: World Health Organization; 2017. Available from: https://www.who.int/publications/i/item/who-htm-ntd-whopes-2017.04

[22] Sougoufara S, Ottih EC, Tripet F. The need for new vector control approaches targeting outdoor biting Anopheline malaria vector communities. Parasites & Vectors. 2020;**13**(1):295. DOI: 10.1186/s13071-020-04170-7

[23] Wamae PM, Githeko AK, Otieno GO, Kabiru EW, Duombia SO. Early biting of the *Anopheles gambiae s.s*. and its challenges to vector control using insecticide treated nets in western Kenya highlands. Acta Tropica. 2015;**150**:136-142. DOI: 10.1016/j.actatropica.2015.07.008

[24] Meyer Steiger DB, Ritchie SA, Laurance SG. Mosquito communities and disease risk influenced by land use change and seasonality in the Australian tropics. Parasites & Vectors. 2016;**9**(1):387. DOI: 10.1186/s13071-016-1675-2

[25] Russell TL, Govella NJ, Azizi S, Drakeley CJ, Kachur SP, Killeen GF. Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. Malaria Journal. 2011;**10**:80. DOI: 10.1186/1475-2875-10-80

[26] Curtis CF. Possible use of translocations to fix desirable genes in insect pest populations. Nature. 1968;**218**(5139):368-369. DOI: 10.1038/218368a0

[27] Roberts A, Andrade PP, Okumu F, Quemada H, Savadogo M, Singh JA, et al. Results from the workshop "problem formulation for the use of gene drive in mosquitoes". The American Journal of Tropical Medicine and Hygiene. 2017;**96**(3):530-533. DOI: 10.4269/ajtmh.16-0726

[28] Gantz VM, Jasinskiene N, Tatarenkova O, Fazekas A, Macias VM, Bier E, et al. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. Proceedings of the National Academy of Sciences of the United States of America. 2015;**112**:E6736-E6743. DOI: 10.1073/pnas.1521077112

[29] Carballar-Lejarazú R, Ogaugwu C, Tushar T, Kelsey A, Pham TB, Murphy J, et al. Next-generation gene drive for population modification of the malaria vector mosquito, *Anopheles gambiae*. Proceedings of the National Academy of Sciences of the United States of America. 2020;**117**(37):22805-22814. DOI: 10.1073/pnas.2010214117

[30] Adolfi A, Gantz VM, Jasinskiene N, Lee HF, Hwang K, Terradas G, et al. Efficient population modification gene-drive rescue system in the malaria mosquito *Anopheles stephensi*. Nature Communications. 2020;**11**(1):5553. DOI: 10.1038/s41467-020-19426-0

[31] Carballar-Lejarazú R, Dong YM, Pham TB, Tushar T, Marshall J, Dimopoulos G, et al. An autonomous gene-drive strain for population modification of the African malaria mosquito, *Anopheles gambiae* (in preparation)

[32] Hammond A, Galizi R, Kyrou K, Simoni A, Siniscalchi C, Katsanos D, et al. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. Nature Biotechnology. 2016;**34**(1):78-83. DOI: 10.1038/nbt.3439

[33] Kyrou K, Hammond AM, Galizi R, Kranjc N, Burt A, Beaghton AK, et al. A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes. Nature Biotechnology. 2018;**36**(11):1062-1066. DOI: 10.1038/nbt.4245

[34] Simoni A, Hammond AM, Beaghton AK, Galizi R, Taxiarchi C, Kyrou K, et al. A male-biased sex-distorter gene drive for the human malaria vector *Anopheles gambiae*. Nature Biotechnology. 2020;**38**(9):1054-1060. DOI: 10.1038/s41587-020-0508-1

[35] Bier E. Gene drives gaining speed. Nature Reviews. Genetics. 2022;**23**:5-22. DOI: 10.1038/s41576-021-00386-0

[36] Eckhoff PA, Wenger EA, Godfray HC, Burt A. Impact of mosquito gene drive on malaria elimination in a computational model with explicit spatial and temporal dynamics. Proceedings of the National Academy of Sciences of the United States of America. 2017;**114**(2):E255-E264. DOI: 10.1073/pnas.1611064114

[37] Carballar-Lejarazú R, James AA. P opulation modification of Anopheline species to control malaria transmission. Pathogens and Global Health. 2017;**111**(8):424-435. DOI: 10.1080/20477724.2018.1427192

[38] Isaacs AT, Jasinskiene N, Tretiakov M, Thiery I, Zettor A, Bourgouin C, et al. Transgenic *Anopheles stephensi* coexpressing single-chain antibodies resist *plasmodium* falciparum development. Proceedings

of the National Academy of Sciences of the United States of America. 2012;**109**(28):E1922-E1930. DOI: 10.1073/pnas.1207738109

[39] Isaacs AT, Li F, Jasinskiene N, Chen X, Nirmala X, Marinotti O, et al. Engineered resistance to *plasmodium* falciparum development in transgenic *Anopheles stephensi*. PLoS Pathogens. 2011;7(4):e1002017. DOI: 10.1371/journal.ppat.1002017

[40] de Lara CM, Coleman J, Beerntsen BT, Myles KM, Olson KE, Rocha E, et al. Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in *Plasmodium gallinaceum*-infected *Aedes aegypti*. The American Journal of Tropical Medicine and Hygiene. 2000;**62**(4):427-433. DOI: 10.4269/ajtmh.2000.62.427

[41] Cirimotich CM, Dong Y, Garver LS, Sim S, Dimopoulos G. Mosquito immune defenses against *Plasmodium* infection. Developmental and Comparative Immunology. 2010;**34**(4):387-395. DOI: 10.1016/j.dci.2009.12.005

[42] Marshall JM, Raban RR, Kandul NP, Edula JR, León TM, Akbari OS. Winning the tug-of-war between effector gene design and pathogen evolution in vector population replacement strategies. Frontiers in Genetics. 2019;**10**:1072. DOI: 10.3389/fgene.2019.01072

[43] Godfray HCJ, North A, Burt A. How driving endonuclease genes can be used to combat pests and disease vectors. BMC Biology. 2017;**15**(1):81. DOI: 10.1186/s12915-017-0420-4

[44] Moreira LA, Edwards MJ, Adhami F, Jasinskiene N, James AA, Jacobs-Lorena M. Robust gut-specific gene expression in transgenic *Aedes aegypti* mosquitoes. Proceedings of the National Academy of Sciences of the United States of America. 2000;**97**(20):10895-10898. DOI: 10.1073/pnas.97.20.10895

[45] Ito J, Ghosh A, Moreira LA, Wimmer EA, Jacobs-Lorena M. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. Nature. 2002;**417**(6887):452-455. DOI: 10.1038/417452a

[46] Abraham EG, Donnelly-Doman M, Fujioka H, Ghosh A, Moreira L, Jacobs-Lorena M. Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with AgAper1 regulatory elements. Insect Molecular Biology. 2005;**14**(3):271-279. DOI: 10.1111/j.1365-2583.2004.00557.x

[47] Kokoza V, Ahmed A, Cho WL, Jasinskiene N, James AA, Raikhel A. Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. Proceedings of the National Academy of Sciences of the United States of America. 2000;**97**(16):9144-9149. DOI: 10.1073/pnas.160258197

[48] Chen X, Zhang Y, Zheng X, Wang CM. Robust and regulatory expression of defensin A gene driven by vitellogenin promoter in transgenic *Anopheles stephensi*. Chinese Science Bulletin. 2007;**52**:1964-1969. DOI: 10.1007/s11434-007-0292-z

[49] Pondeville E, Puchot N, Parvy JP, Carissimo G, Poidevin M, Waterhouse RM, et al. Hemocyte-targeted gene expression in the female malaria mosquito using the hemolectin promoter from drosophila. Insect Biochemistry and Molecular Biology. 2020;**120**:103339. DOI: 10.1016/j.ibmb.2020.103339

[50] Coates CJ, Jasinskiene N, Pott GB, James AA. Promoter-directed expression

of recombinant fire-fly luciferase in the salivary glands of Hermes-transformed *Aedes aegypti*. Gene. 1999;**226**(2):317-325. DOI: 10.1016/s0378-1119(98)00557-5

[51] Lombardo F, Nolan T, Lycett G, Lanfrancotti A, Stich N, Catteruccia F, et al. An *Anopheles gambiae* salivary gland promoter analysis in *Drosophila melanogaster* and *Anopheles stephensi*. Insect Molecular Biology. 2005;**14**(2):207-216. DOI: 10.1111/j.1365-2583.2004.00549.x

[52] Yoshida S, Watanabe H. Robust salivary gland-specific transgene expression in *Anopheles stephensi* mosquito. Insect Molecular Biology. 2006;**15**(4):403-410. DOI: 10.1111/j.1365-2583.2006.00645.x

[53] Carpenetti TL, Aryan A, Myles KM, Adelman ZN. Robust heat-inducible gene expression by two endogenous hsp70-derived promoters in transgenic *Aedes aegypti*. Insect Molecular Biology. 2012;**21**(1):97-106. DOI: 10.1111/j.1365-2583.2011.01116.x

[54] Pinkerton AC, Michel K, O'Brochta DA, Atkinson PW. Green fluorescent protein as a genetic marker in transgenic *Aedes aegypti*. Insect Molecular Biology. 2000;**9**(1):1-10. DOI: 10.1046/j.1365-2583.2000.00133.x

[55] Anderson MA, Gross TL, Myles KM, Adelman ZN. Validation of novel promoter sequences derived from two endogenous ubiquitin genes in transgenic *Aedes aegypti*. Insect Molecular Biology. 2010;**19**(4):441-449. DOI: 10.1111/j.1365-2583.2010.01005.x

[56] Kokoza V, Ahmed A, Woon Shin S, Okafor N, Zou Z, Raikhel AS. Blocking of *Plasmodium* transmission by cooperative action of Cecropin A and Defensin A in transgenic *Aedes aegypti* mosquitoes. Proceedings of the National Academy of Sciences of the United States of America. 2010;**107**(18):8111-8116. DOI: 10.1073/pnas.1003056107

[57] Vizioli J, Bulet P, Hoffmann JA, Kafatos FC, Müller HM, Dimopoulos G. Gambicin: A novel immune responsive antimicrobial peptide from the malaria vector *Anopheles gambiae*. Proceedings of the National Academy of Sciences of the United States of America. 2001;**98**(22):12630-12635. DOI: 10.1073/pnas.221466798

[58] Conde R, Zamudio FZ, Rodríguez MH, Possani LD. Scorpine, an anti-malaria and anti-bacterial agent purified from scorpion venom. FEBS Letters. 2000;**471**(2-3):165-168. DOI: 10.1016/s0014-5793(00)01384-3

[59] Maciel C, de Oliveira Junior VX, Fázio MA, Nacif-Pimenta R, Miranda A, Pimenta PF, et al. Anti-*Plasmodium* activity of angiotensin II and related synthetic peptides. PLoS One. 2008;**3**(9):e3296. DOI: 10.1371/journal.pone.0003296

[60] Gwadz RW, Kaslow D, Lee JY, Maloy WL, Zasloff M, Miller LH. Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. Infection and Immunity. 1989;**57**(9):2628-2633. DOI: 10.1128/iai.57.9.2628-2633.1989

[61] Possani LD, Zurita M, Delepierre M, Hernández FH, Rodríguez MH. From noxiustoxin to Shiva-3, a peptide toxic to the sporogonic development of *Plasmodium berghei*. Toxicon. 1998;**36**(11):1683-1692. DOI: 10.1016/s0041-0101(98)00161-5

[62] Moreira CK, Rodrigues FG, Ghosh A, Varotti Fde P, Miranda A, Daffre S, et al. Effect of the antimicrobial peptide gomesin against different life stages of *Plasmodium* spp. Experimental

Parasitology. 2007;**116**(4):346-353. DOI: 10.1016/j.exppara.2007.01.022

[63] Ghosh AK, Coppens I, Gårdsvoll H, Ploug M, Jacobs-Lorena M. *Plasmodium* ookinetes coopt mammalian plasminogen to invade the mosquito midgut. Proceedings of the National Academy of Sciences of the United States of America. 2011;**108**(41):17153-17158. DOI: 10.1073/pnas.1103657108

[64] Ghosh AK, Ribolla PE, Jacobs-Lorena M. Targeting *Plasmodium* ligands on mosquito salivary glands and midgut with a phage display peptide library. Proceedings of the National Academy of Sciences of the United States of America. 2001;**98**(23):13278-13281. DOI: 10.1073/pnas.241491198

[65] Moreira LA, Ito J, Ghosh A, Devenport M, Zieler H, Abraham EG, et al. Bee venom phospholipase inhibits malaria parasite development in transgenic mosquitoes. The Journal of Biological Chemistry. 2002;**277**(43):40839-40843. DOI: 10.1074/jbc.M206647200

[66] Corby-Harris V, Drexler A, Watkins de Jong L, Antonova Y, Pakpour N, Ziegler R, et al. Activation of Akt signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in *Anopheles stephensi* mosquitoes. PLoS Pathogens. 2010;**6**(7):e1001003. DOI: 10.1371/journal.ppat.1001003

[67] Dong Y, Das S, Cirimotich C, Souza-Neto JA, McLean KJ, Dimopoulos G. Engineered anopheles immunity to *Plasmodium* infection. PLoS Pathogens. 2011;**7**(12):e1002458. DOI: 10.1371/journal.ppat.1002458

[68] Dong Y, Simões ML, Dimopoulos G. Versatile transgenic multistage effector-gene combinations for *Plasmodium* falciparum suppression in *Anopheles*. Science Advances. 2020;**6**(20):eaay5898. DOI: 10.1126/sciadv.aay5898

[69] McClintock B. In: Moore JA, editor. The Discovery and Characterization of Transposable Elements. The C ollected Papers of Barbara McClintock. New York: Garland Publishing; 1987. pp. vii-xi

[70] Engels WR. Invasions of P elements. Genetics. 1997;**145**(1):11-15. DOI: 10.1093/genetics/145.1.11

[71] Charlesworth B, Sniegowski P, Stephan W. The evolutionary dynamics of repetitive DNA in eukaryotes. Nature. 1994;**371**:215-220. DOI: 10.1038/371215a0

[72] Mackay T. Transposable element-induced fitness mutations in *Drosophila melanogaster*. Genetical Research. 1986;**48**(2):77-87

[73] Rubin GM, Spradling AC. Genetic transformation of *Drosophila* with transposable element vectors. Science. 1982;**218**(4570):348-353. DOI: 10.1126/science.6289436

[74] Jasinskiene N, Coates CJ, Benedict MQ, Cornel AJ, Rafferty CS, James AA, et al. Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the Hermes element from the housefly. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**(7):3743-3747. DOI: 10.1073/pnas.95.7.3743

[75] Allen ML, O'Brochta DA, Atkinson PW, Levesque CS. Stable, germ-line transformation of *Culex quinquefasciatus* (Diptera: Culicidae). Journal of Medical Entomology. 2001;**38**(5):701-710. DOI: 10.1603/0022-2585-38.5.701

[76] Grossman GL, Rafferty CS, Clayton JR, Stevens TK, Mukabayire O,

Benedict MQ. Germline transformation of the malaria vector, *Anopheles gambiae*, with the *piggyBac* transposable element. Insect Molecular Biology. 2001;**10**(6):597-604. DOI: 10.1046/j.0962-1075.2001.00299.x

[77] Rodrigues FG, Oliveira SB, Rocha BC, Moreira LA. Germline transformation of *Aedes fluviatilis* (Diptera:Culicidae) with the *piggyBac* transposable element. Memórias do Instituto Oswaldo Cruz. 2006;**101**(7):755-757. DOI: 10.1590/s0074-02762006000700008

[78] Nolan T, Bower TM, Brown AE, Crisanti A, Catteruccia F. *piggyBac*-mediated germline transformation of the malaria mosquito *Anopheles stephensi* using the red fluorescent protein dsRED as a selectable marker. The Journal of Biological Chemistry. 2002;**277**(11):8759-8762. DOI: 10.1074/jbc.C100766200

[79] Perera OP, Harrell RA II, Handler AM. Germ-line transformation of the South American malaria vector, Anopheles albimanus, with a *piggyBac*/EGFP transposon vector is routine and highly efficient. Insect Molecular Biology. 2002;**11**(4):291-297. DOI: 10.1046/j.1365-2583.2002.00336.x

[80] Catteruccia F, Nolan T, Loukeris TG, Blass C, Savakis C, Kafatos FC, et al. Stable germline transformation of the malaria mosquito *Anopheles stephensi*. Nature. 2000;**405**(6789):959-962. DOI: 10.1038/35016096

[81] Coates CJ, Jasinskiene N, Miyashiro L, James AA. Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**(7):3748-3751. DOI: 10.1073/pnas.95.7.3748

[82] Labbé GM, Nimmo DD, Alphey L. *piggyBac*- and *PhiC31*-mediated genetic transformation of the Asian tiger mosquito, *Aedes albopictus* (Skuse). PLOS Neglected Tropical Diseases. 2010;**4**(8):e788. DOI: 10.1371/journal.pntd.0000788

[83] Kidwell MG, Ribeiro JM. Can transposable elements be used to drive disease refractoriness genes into vector populations? Parasitology Today. 1992;**8**(10):325-329. DOI: 10.1016/0169-4758(92)90065-a

[84] O'Brochta DA, Sethuraman N, Wilson R, Hice RH, Pinkerton AC, Levesque CS, et al. Gene vector and transposable element behavior in mosquitoes. The Journal of Experimental Biology. 2003;**206**(Pt 21):3823-3834. DOI: 10.1242/jeb.00638

[85] Macias VM, Jimenez AJ, Burini-Kojin B, Pledger D, Jasinskiene N, Phong CH, et al. Nanos-driven expression of *piggyBac* transposase induces mobilization of a synthetic autonomous transposon in the malaria vector mosquito, *Anopheles stephensi*. Insect Biochemistry and Molecular Biology. 2017;**87**:81-89. DOI: 10.1016/j.ibmb.2017.06.014

[86] Morris AC, Schaub TL, James AA. FLP-mediated recombination in the vector mosquito, *Aedes aegypti*. Nucleic Acids Research. 1991;**19**(21):5895-5900. DOI: 10.1093/nar/19.21.5895

[87] Nimmo DD, Alphey L, Meredith JM, Eggleston P. High efficiency site-specific genetic engineering of the mosquito genome. Insect Molecular Biology. 2006;**15**(2):129-136. DOI: 10.1111/j.1365-2583.2006.00615.x

[88] Meredith JM, Basu S, Nimmo DD, Larget-Thiery I, Warr EL, Underhill A, et al. Site-specific integration and expression of an anti-malarial gene in transgenic

Anopheles gambiae significantly reduces *Plasmodium* infections. PLoS One. 2011;**6**(1):e14587. DOI: 10.1371/journal.pone.0014587

[89] Volohonsky G, Terenzi O, Soichot J, Naujoks DA, Nolan T, Windbichler N, et al. Tools for *Anopheles gambiae* Transgenesis. G3 (Bethesda). 2015;**5**(6):1151-1163. DOI: 10.1534/g3.115.016808

[90] Jasinskiene N, Coates CJ, Ashikyan A, James AA. High efficiency, site-specific excision of a marker gene by the phage P1 cre-loxP system in the yellow fever mosquito, *Aedes aegypti*. Nucleic Acids Research. 2003;**31**(22):e147. DOI: 10.1093/nar/gng148

[91] Aryan A, Anderson MA, Myles KM, Adelman ZN. Germline excision of transgenes in *Aedes aegypti* by homing endonucleases. Scientific Reports. 2013;**3**:1603. DOI: 10.1038/srep01603

[92] Smidler AL, Terenzi O, Soichot J, Levashina EA, Marois E. Targeted mutagenesis in the malaria mosquito using TALE nucleases. PLoS One. 2013;**8**(8):e74511. DOI: 10.1371/journal.pone.0074511

[93] DeGennaro M, McBride CS, Seeholzer L, Nakagawa T, Dennis EJ, Goldman C, et al. *Orco* mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. Nature. 2013;**498**(7455):487-491. DOI: 10.1038/nature12206

[94] Burt A. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proceedings of the Biological Society. 2003;**270**(1518):921-928

[95] Windbichler N, Menichelli M, Papathanos PA, Thyme SB, Li H, Ulge UY, et al. A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. Nature. 2011;**473**(7346):212-215. DOI: 10.1038/nature09937

[96] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;**337**(6096):816-821. DOI: 10.1126/science.1225829

[97] Carballar-Lejarazú R, Tushar T, Pham TB, James AA. Cas9-mediated maternal-effect and derived resistance alleles in a gene-drive strain of the African malaria vector mosquito, Anopheles gambiae. Genetics. 2022 Apr 7:iyac055. DOI: 10.1093/genetics/iyac055. Epub ahead of print. PMID: 35389492

[98] Marrelli MT, Moreira CK, Kelly D, Alphey L, Jacobs-Lorena M. Mosquito transgenesis: What is the fitness cost? Trends in Parasitology. 2006;**22**(5):197-202. DOI: 10.1016/j.pt.2006.03.004

[99] Riehle MA, Srinivasan P, Moreira CK, Jacobs-Lorena M. Towards genetic manipulation of wild mosquito populations to combat malaria: Advances and challenges. The Journal of Experimental Biology. 2003;**206**(Pt 21):3809-3816. DOI: 10.1242/jeb.00609

[100] Pham TB, Phong CH, Bennett JB, Hwang K, Jasinskiene N, Parker K, et al. Experimental population modification of the malaria vector mosquito, *Anopheles stephensi*. PLOS Genetics. 2019;**15**:e1008440. DOI: 10.1371/journal.pgen.1008440

[101] Bottino-Rojas V, Ferreira-Almeida I, Nunes R, Feng XC, Pham TB, Kelsey A, et al. Beyond the eye: Kynurenine pathway impairment causes midgut homeostasis dysfunction and survival and reproductive costs in blood-feeding mosquitoes. Insect Biochemistry

and Molecular Biology. 2022;**142**:103720. DOI: 10.1016/j.ibmb.2022.103720

[102] Ribeiro JM, Kidwell MG. Transposable elements as population drive mechanisms: Specification of critical parameter values. Journal of Medical Entomology. 1994;**31**(1):10-16. DOI: 10.1093/jmedent/31.1.10

[103] Boëte C, Koella JC. A theoretical approach to predicting the success of genetic manipulation of malaria mosquitoes in malaria control. Malaria Journal. 2002;**1**:3. DOI: 10.1186/1475-2875-1-3

[104] Champer J, Reeves R, Oh SY, Liu C, Liu J, Clark AG, et al. Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations. PLoS Genetics. 2017;**13**(7):e1006796. DOI: 10.1371/journal.pgen.1006796

[105] Unckless RL, Clark AG, Messer PW. Evolution of resistance against CRISPR/Cas9 gene drive. Genetics. 2017;**205**(2):827-841. DOI: 10.1534/genetics.116.197285

[106] Drury DW, Dapper AL, Siniard DJ, Zentner GE, Wade MJ. CRISPR/Cas9 gene drives in genetically variable and nonrandomly mating wild populations. Science Advances. 2017;**3**(5):e1601910. DOI: 10.1126/sciadv.1601910

[107] Noble C, Olejarz J, Esvelt KM, Church GM, Nowak MA. Evolutionary dynamics of CRISPR gene drives. Science Advances. 2017;**3**(4):e1601964. DOI: 10.1126/sciadv.1601964

[108] Terradas G, Hermann A, James AA, McGinnis W, Bier E. High-resolution in situ analysis of Cas9 germline transcript distributions in gene-drive Anopheles mosquitoes. G3 (Bethesda). 2021:jkab369. DOI: 10.1093/g3journal/jkab369

[109] Lanzaro GC, Sánchez CHM, Collier TC, Marshall JM, James AA. Population modification strategies for malaria vector control are uniquely resilient to observed levels of gene drive resistance alleles. Bioessays. 2021;**43**(8):e2000282. DOI: 10.1002/bies.202000282

[110] Marshall JM, Buchman A, Sánchez CHM, Akbari OS. Overcoming evolved resistance to population-suppressing homing-based gene drives. Scientific Reports. 2017;**7**(1):3776. DOI: 10.1038/s41598-017-02744-7

[111] Gao Y, Zhao Y. Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR-mediated genome editing. Journal of Integrative Plant Biology. 2014;**56**(4):343-349. DOI: 10.1111/jipb.12152

[112] Port F, Bullock SL. Augmenting CRISPR applications in *Drosophila* with tRNA-flanked sgRNAs. Nature Methods. 2016;**13**(10):852-854. DOI: 10.1038/nmeth.3972

[113] Champer J, Liu J, Oh SY, Reeves R, Luthra A, Oakes N, et al. Reducing resistance allele formation in CRISPR gene drive. Proceedings of the National Academy of Sciences of the United States of America. 2018;**115**(21):5522-5527. DOI: 10.1073/pnas.1720354115

[114] Zetsche B, Gootenberg JS, AbudayyehOO,SlaymakerIM,MakarovaKS, Essletzbichler P, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. 2015;**163**(3):759-771. DOI: 10.1016/j.cell.2015.09.038

[115] Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature. 2016;**529**(7587):490-495. DOI: 10.1038/nature16526

[116] Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. Science. 2016;**351**(6268):84-88. DOI: 10.1126/science.aad5227

[117] Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell. 2013;**154**(6):1380-1389. DOI: 10.1016/j.cell.2013.08.021

[118] Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA, Liu DR. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nature Biotechnology. 2013;**31**(9):839-843. DOI: 10.1038/nbt.2673

[119] Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nature Biotechnology. 2014;**32**(3):279-284. DOI: 10.1038/nbt.2808

[120] World Health Organization. Guidance Framework for Testing Genetically Modified Mosquitoes. 2nd ed. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO, 2014. Available from: Guidance framework for testing of genetically modified mosquitoes, second edition (who.int)

[121] James S, Collins FH, Welkhoff PA, Emerson C, Godfray HCJ, Gottlieb M, et al. Pathway to deployment of gene drive mosquitoes as a potential biocontrol tool for elimination of malaria in sub-Saharan Africa: Recommendations of a scientific working group. American Journal of Tropical Medicine and Hygiene. 2018;**98**(6_Suppl):1-49. DOI: 10.4269/ajtmh.18-0083

[122] Australian Academy of Science. Synthetic Gene Drives in Australia: Implications of Emerging Technologies. Australian Academy of Science; 2017. Available from: https://www.science.org.au/files/userfiles/support/documents/gene-drives-discussion-paper-june2017.pdf

[123] Kormos A, Lanzaro G, Bier E, Dimopoulos G, Marshall J, Pinto J, et al. Application of the relationship-based model to community and regulatory engagement for field trials of genetically-engineered mosquitoes for malaria control. The American Journal of Tropical Medicine and Hygiene. 2021;**104**:805-811