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16 Population Modification Using Gene Drive for Reduction of Malaria Transmission

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16.1 Introduction

The global incidence of malaria has been reduced in the past 15 years and much of this success results from the application of vector control methods to prevent pathogen transmission (Bhatt *et al*., 2015). However, the decline in incidence has slowed recently, and disease incidence and prevalence remain high (World Health Organization, 2020). Traditional vector control methods, although effective in many regional and local applications, are limited in others and may be complemented by new synthetic biological tools. Transgenic and/or genome-edited mosquitoes can carry special traits for spread into wild populations that are expected to assist in local elimination and contribute ultimately to disease eradication (Carballar-Lejarazú and James, 2017). These traits can be designed to reduce the ability of vector mosquitoes to sustain parasite transmission, or to decrease their lifespan or ability to reproduce, known respectively as population alteration/modification/replacement (henceforth referred to as 'population modification') and population suppression.

The prospect of driving genetic modifications that confer malaria parasite refractory or resistance qualities into mosquito vectors has been captivating scientists for more than 50 years (Curtis, 1968). Naturally-occurring 'selfish' genetic elements were proposed as a way to achieve genetically-engineered populations (Curtis, 1992; Burt, 2003). However, the major technological breakthrough in the field came only recently with the discovery of CRISPR/Cas9-based gene-editing approaches, which offer simplicity and efficiency when compared to other genome-editing tools (Ran *et al.*, 2013). Since then, studies of vector-pathogen biology and malaria immunology combined with the development of molecular tools for manipulating mosquito genomes have been fueling new developments, including mosquitoes that express exogenous or endogenous anti-parasite effectors, or lack important host factors that affect *Plasmodium* transmission (Sreenivasamurthy *et al.*, 2013; Carballar-Lejarazú and James, 2017; Simões, Caragata and Dimopoulos, 2018).

We discuss here the application of some of these studies and how vector manipulation tools such as those derived from CRISPR/Cas9-based gene-drive technologies can be used to modify anopheline

mosquito populations. We examine the core concepts and mechanisms for inducing efficient modifications that have already been identified or that represent promising targets. In the last section, we will discuss how effective these transmission-blocking interventions must be to adequately support malaria elimination efforts.

16.2 Features of Gene-Drive Population Modification systems

Gene-drive systems in mosquitoes utilize CRISPR/Cas9-based technology in which an endonuclease, Cas9, makes a double-strand break in the chromosomal DNA at a specific ‘target’ site directed by a guide RNA (gRNA) (**Figure 1**). Subsequent homology-directed repair and recombination then can be used to insert specific exogenous DNA into the cleavage site. If this exogenous DNA contains the Cas9 endonuclease and gRNA, a self-propagating (autonomous) genetic element will continue to copy itself into any appropriate target site, thus generating gene drive. Non-autonomous systems also can be developed by unlinking the Cas9-encoding gene from the gRNA (Gantz and Bier, 2015). Comprehensive information on insect germline transformation and overall CRISPR/Cas9 knock-in methods are described elsewhere in this book.

Figure 1. Schematic representation of Cas9/gRNA-mediate gene drive. A gene drive element, including the Cas9, a guide RNA targeting the gene of choice and the antimalarial molecules, is inserted at the homologous chromosomal Cas9-cleaved locus, through DNA repair by homologous recombination (HDR). The occurrence of this event in the germline favors the inheritance of the transgenic cassette. The ability to maintain inheritance of transgenically-encoded traits despite potential fitness defects enables driving of otherwise costly beneficial genetic traits through wild populations.

A number of practical considerations guide the design and construction of population modification gene-drive systems (Carballar-Lejarazú and James, 2017). For example, it is important that the components of a synthetic construct be as small in nucleotide length as possible and still allow appropriate function. The smaller size of components reduces the probability of adventitious sequence similarity to other components of the system or the target genome. This reduces the probability of unintended recombination events disrupting the drive system as well the generation of mutations or rearrangement of the host genome.

The locus of the target site specified by the gRNA in the mosquito genome should be well-characterized and conserved across populations of the same species. Characterization includes screening mosquito genomes for naturally-occurring target-site polymorphisms that may halt or

reduce drive dynamics. However, this is a challenge because highly-conserved target sites likely may be so because they are under negative selection and the insertion of a large gene-drive element into them may have a deleterious effect. While this may be a benefit to suppression approaches, a well-designed population modification system will avoid this.

In addition to naturally-occurring variants in the target site, drive-resistant alleles may be generated by non-homologous end joining (NHEJ) events during drive. As targets that are highly-conserved in nature are indicative of strong sequence constraints, a NHEJ resistance allele that still encodes a functional product may be generated mis-repair and increase in frequency (Hammond and Galizi, 2017).

Insertion of exogenous DNA at a target site is potentially mutagenic for the gene in which the site is located, and this could impose a genetic load as a result of the interrupted gene (insertion-site effect) or expression of the inserted DNA (transgene effect). These alterations could make the resulting mosquitoes less competitive with wild-type counterparts.

An example of informed decision during target selection is presented by Carballar-Lejarazú *et al.* (2020) in the development of an efficient gene-drive system in the African malaria vector, *Anopheles gambiae*. Through target-site sequence screening of genomes of natural populations and Cas9/gRNA *in vitro* cleavage assays of polymorphic alleles with potentially critical modifications in the sequence, the authors concluded that naturally-prevalent or NHEJ-induced resistant alleles are not likely to prevent (only delay) delivery of the intended modification (Carballar-Lejarazú *et al.*, 2020). Furthermore, resistant alleles are not expected to rise in frequency if the targeted sequences cannot tolerate disruption (Unckless, Clark and Messer, 2017). Therefore, an alternative strategy uses a gRNA sequence that purposely targets an essential gene and links the antimalarial effector and drive cassette to a sequence that restores the essential function and is resistant to cutting (Beaghton *et al.*, 2017; Noble *et al.*, 2017). This was demonstrated successfully on a recoded gene-drive rescue system for population modification of the Indo-Pakistan malaria vector, *An. stephensi*; individuals carrying NHEJ alleles had a low fitness and were purged quickly from the population (Adolfi *et al.*, 2020).

The rationale for target site selection must be appropriate for the proposed modification strategy. The intended modification might require disruption of a pre-determined target (e.g., insecticide-resistant gene or host-factor for parasite development) or an insertion of a novel anti-pathogen effector. As mentioned, there is a trade-off between the targeting of a conserved sequence with a potential resulting fitness cost versus the insertion of the transmission-blocking gene on a neutral genomic site.

The latter usually exhibits poor sequence conservation and facilitates the emergence of drive resistance because non-functional regions can more easily accommodate cost-free sequence alterations, especially if the gene drive allele carries a fitness cost.

Another concern for modification systems is that mutations can occur in the effector gene(s) rendering them non-functional and leaving a drive-only cargo to be spread through a population, with no effect on disease transmission. This may be challenging to measure considering that the spread of this non-functional replacement could be much slower than that of the initial drive system and fixation of the effector-drive construct. Although the technology performance in the field may be different than that in the laboratory, parameters such as fitness and vector competence of the modified mosquitoes should be tested through multiple generations with appropriate laboratory cage trials (Pham *et al.*, 2019). Furthermore, drive experiments on recently-colonized local populations and field trials in naturally-isolated settings in the wild are relevant to test laboratory predictions and contemplate long-term population dynamics and persistence of the introduced modification (Carballar-Lejarazú and James, 2017; Schmidt *et al.*, 2020). Mathematical modelling can be used to explore and assess the utility of a wide range of scenarios that would be costly, time-consuming or even unfeasible to test experimentally (Robert *et al.*, 2012). Despite the fact that effector or drive loss-of-function mutations may arise beyond our testing abilities, disease protection may nonetheless persist sufficiently long enough to provide a public health benefit (Beaghton *et al.*, 2017).

16.3 Design features of parasite-resistant mosquitoes for population modification

Improved understanding of mosquito-pathogen interactions and developments of immune-related or synthetic-derived antimalarial factors have rapidly advanced prospects for generating refractory mosquito populations (Caragata *et al.*, 2020). Similarly, progress in mosquito genomics has enabled the design and production of engineered genes expressed under the control of specific promoter-regulatory DNA, achieving great parasite blocking performances in laboratory settings, mostly through transposon-based transgenesis (Chen, Mathur and James, 2008; Häcker and Schetelig, 2018). Reviewing the landmarks that built the current knowledge and achievements can enlighten prospective and innovative ideas for future implementation of this technology.

Population-wide genetic modifications benefit if the transformed insects exhibit as low fitness cost as possible. Therefore, the expression of effector genes should be restricted ideally to infection-relevant development phases and tissues in the mosquito. To complete their transmission cycle, *Plasmodium* parasites must traverse three key mosquito compartments, the midgut, the hemocoel, and the

salivary glands, during the course of several days after initial acquisition by the mosquito through an infectious blood meal (**Figure 2**). Different *cis*-acting control DNA sequences that regulate appropriate patterns of expression have been identified in malaria vectors. Mosquito midgut invasion is a crucial step to parasite infection, as this is the first host tissue encountered by the parasites. Therefore, control DNA sequences, particularly those that are blood meal-inducible and regulate midgut-specific expression, confer abundant activation of the effector genes that is synchronized with parasite ingestion. Examples of such control sequences come from genes that encode a zinc carboxypeptidase A1 (*CP*), a late trypsin (*Antryp1*), and the *G12* regulatory elements (Ito *et al.*, 2002; Nolan *et al.*, 2011). The adult peritrophic matrix (*Aper1*) gene promoter has been used to direct constitutive midgut expression, and is relevant to target initial stages of parasite development (Abraham *et al.*, 2005). Approximately one day after blood ingestion, *Plasmodium* parasites cross the midgut as ookinetes, lodge under the basal lamina and develop into oocysts that face the hemocoel. Several thousand sporozoites develop in each oocyte and these are released into the hemolymph in the open body cavity of the insect. The vitellogenin (*Vg*) gene *cis*-acting sequences can be used in anophelines to induce late-digestion and sex-specific expression of desired gene products in the fat body for secretion into the hemolymph to target the sporozoites (Nirmala *et al.*, 2006; Chen *et al.*, 2007). Additionally, effectors under the control of appropriate salivary gland-specific promoter regions can be used, including those from apyrase (*Apy*) and D7-related (*D7r*) genes (Lombardo *et al.*, 2005), and more strikingly, the anopheline antiplatelet gene (*aapp*) gene (Yoshida and Watanabe, 2006; Sumitani *et al.*, 2013). Other control DNAs from the genes encoding a prophenoloxidase (PPO6, hemocyte-specific), actin5C (act5C, expressed-constitutively in the midgut) and lipophorin (Lp, expressed-constitutively in the fat body) may be convenient for alternative transgene expression strategies (Volohonsky *et al.*, 2015). However, one hypothetical set of genes as of yet to be identified are those that are tissue-specific and that respond uniquely to a parasite infection. In principle, if discovered, these would minimize even further potential fitness costs associated with transgene expression.

Currently, genome modifications for engineering refractory *Anopheles* mosquitoes include an induced expression of exogenous or endogenous genes with known antipathogen effects, and/or gene editing of mosquito host factors required for parasite development. Many infection-blocking effectors are classified as exogenous lytic peptides, such as the scorpion venom protein, scorpine (Conde *et al.*, 2000), bee venom phospholipase (*PLA_2*) (Moreira *et al.*, 2002), the antimicrobial peptide mellitin (Carter *et al.*, 2013), sea cucumber hemolytic C-type lectin (CEL-III) (Yoshida *et al.*, 2007), and the synthetic peptides Shiva1 (Yoshida *et al.*, 2001), Vida3 (Arrighi *et al.*, 2002) and TP10 (Arrighi *et al.*,

2008). All have been expressed by transgenes in mosquitoes and successfully suppress *Plasmodium* development (Meredith *et al.*, 2011; Dong, Simões and Dimopoulos, 2020). Another class of exogenous effector molecules is designed to bind the parasites or mosquito tissues, preventing invasion and development following infection. One of first molecules of this type for a malaria parasite blocking strategy, the salivary gland midgut peptide 1 (SM1), was selected from a bacteriophage library (Ghosh, Ribolla and Jacobs-Lorena, 2001; Ito *et al.*, 2002) and binds both the midgut receptor EBP (enolase-binding protein) on the luminal side of the midgut, and the Saglin receptors on the distal lobes of the salivary glands, blocking parasite interaction with these tissues (Ghosh *et al.*, 2009, 2011; Vega-Rodriguez *et al.*, 2014). Furthermore, work leveraged from transmission-blocking vaccines led to the identification and synthesis of potent modified monoclonal antibodies, single-chain fragments (scFv), that are directed specifically against *Plasmodium* parasite antigens (Yoshida *et al.*, 1999). The inaugural work showing the feasibility of using expression systems to limit mosquito vector competence achieved virus-mediated transient expression of an anti-sporozoite scFv, with a resulting reduced salivary gland infection of as much as 99.9% when compared to controls (Capurro *et al.*, 2000). Transgenes expressing 1C3, 4B7, or 2A10 scFvs, the first two of which inhibit ookinete invasion of the midgut and the third sporozoite invasion of salivary glands, resulted in fewer *P. falciparum* oocysts in transgenic *An. stephensi* lines and decreased significantly sporozoite mean intensities of infection in salivary glands (Isaacs *et al.*, 2011). Alternatively, using antibodies against mosquito-specific epitopes (Barreau *et al.*, 1995; Brennan *et al.*, 2000), as well as the transgenic expression of the mouse gene *Bax*, which causes salivary cell death in mosquitoes (Yamamoto *et al.*, 2016), can also inhibit *Plasmodium* progression through mosquitoes. When considering the fitness of these transposon-based effector-expressing transgenic lines, different combinations of molecules and expression systems were shown to have contrasting impacts on mosquito survival and consequent transgene integration into populations. For example, midgut expression of the bee venom PLA_2 or expression of the peptide SM1 driven by the *Vg* promoter were shown to impose a significant fitness load to transgenic mosquitoes (Moreira *et al.*, 2004; Li *et al.*, 2008). However, PLA_2-expressing mosquitoes seem to have an advantage when fed *P. falciparum*-infected blood (Smith *et al.*, 2013) and *CP*-driven expression of SM1 does not impact fitness of transgenic females (Moreira *et al.*, 2004). Besides, presumably due to parasite-target specificity, expression of a dual scFv transgene can completely inhibit *P. falciparum* development without significantly affecting fitness cost of the mosquitoes (Isaacs *et al.*, 2012). Single synthetic peptide (Vida3) expression in the female midgut did not affect fitness parameters of the transgenic population (McArthur, Meredith and Eggleston, 2014), whereas expression of multiple toxins and synthetic molecules with broader activity can exert

undesired impacts on crucial physiological processes or on the gut microbiota (Dong, Simões and Dimopoulos, 2020).

The effectiveness of the endogenous mosquito response against parasites can be boosted by the transcriptional induction of immune effectors or the repression of negative regulators of immunity, as proved over the years through the use of transient reverse genetics (Frolet *et al.*, 2006; Garver, Dong and Dimopoulos, 2009; Clayton *et al.*, 2013; Garver, de Almeida Oliveira and Barillas-Mury, 2013). The yellow fever mosquito, *Aedes aegypti*, was the first engineered genetically-stable transgenic mosquito with an element of systemic immunity (Defensin A) activated through a blood meal-triggered cascade (Kokoza *et al.*, 2000). Since then, successful demonstrations that endogenous immune effectors can be expressed in transgenic *Anopheles* mosquitoes include the overexpression of the NF-κB transcription factor Rel2 (Dong *et al.*, 2011), the antimicrobial peptide cecropin A (CecA) (Kim *et al.*, 2004), as well as the co-expression of these in multi-effector strategies (Isaacs *et al.*, 2012; Dong, Simões and Dimopoulos, 2020), all of which strongly reduce parasite numbers in salivary glands. However, overexpression of the anti-parasitic protein TEP1 in *An. gambiae* does not result in increased resistance to *Plasmodium* (Volohonsky *et al.*, 2017), demonstrating that simply augmenting the level of a given immune factor may not be sufficient to achieve greater resistance levels.

Another class of immune regulators that have significance in pathogen infection in mosquitoes are microRNAs (miRNAs). miRNAs have been shown in *An. gambiae* to function as both immune agonists and antagonists, regulating *Plasmodium* infection (Biryukova, Ye and Levashina, 2014; Dennison, BenMarzouk-Hidalgo and Dimopoulos, 2015). Transgenic depletion of specific miRNAs transcriptionally-induced several immunity genes and increased mosquito refractoriness to *Plasmodium*, with minimal effect on fitness parameters (Dong *et al.*, 2020). In addition, some metabolic interventions, for example, genetic manipulation of the insulin pathway regulator, Akt, elicits mitochondrial dysfunction that enhances parasite killing in the midgut, but also shortens mosquito lifespan (Corby-Harris *et al.*, 2010). In contrast, overexpression of an inhibitor of the same pathway (PTEN) extends mosquito lifespan and increases resistance to *P. falciparum* development, by improving the integrity of the midgut barrier (Hauck *et al.*, 2013). These data demonstrate that while there is promise in immune-modulating intervention strategies, exploring different aspects of vector biology as well as a greater understanding of mosquito-parasite interactions, can be useful to develop efficient and targeted genetic control strategies (Shaw and Catteruccia, 2019; Talyuli *et al.*, 2021).

The association between *Plasmodium* and *Anopheles* species has resulted in some mosquito genes being required for successful parasite infection and development in the vector host (Simões,

Caragata and Dimopoulos, 2018). Key examples include the previously-mentioned Saglin, that promotes salivary gland invasion (Ghosh *et al.*, 2009), as well as *Lp* and *Vg*, which reduce parasite-killing efficiency of TEP1 (Rono *et al.*, 2010). The geographic compatibility of *P. falciparum* strains and *Anopheles* species based on parasite-mosquito receptor ligand-like interactions provides an opportunity for increased specificity in the development of infection blocking strategies (Molina-Cruz *et al.*, 2015, 2020). Although parasite-host interactions can be limited by genetic targeting of host factors, complete disruption of these can be challenging. Inactivation of the fibrinogen-related protein 1 (FREP1) gene via CRISPR/Cas9 gene editing, while suppressing infection with malaria parasites, also results in a wide array of fitness costs for the mosquito (Dong *et al.*, 2018). Yang *et al.* (2020) using a similar CRISPR-related approach, showed a failure to obtain a homozygous knockout mosquito following the complete deletion of the *mosGILT* gene. However, genetic mosaics with reduced mosGILT protein levels showed abnormal ovaries, but importantly, also refractoriness to parasite infection (Yang *et al.*, 2020). Therefore, the exploration of mosquito *Plasmodium* agonists for the development of malaria control strategies based on parasite suppression has lagged behind other approaches, likely because of difficulties of generating benign, fitness-neutral targeting methods. This can be altered by future investigation of gene variants or the development of an efficient conditional knockout of host factors via blood-meal inducible expression of either the Cas9 protein or gRNAs in infection relevant tissues (Xue *et al.*, 2014). Given that *P. falciparum* is a highly polymorphic pathogen (Zhang *et al.*, 2019), whose sequence diversity has been shown to limit effectiveness of single-target blocking strategies (Neafsey *et al.*, 2015), it is important to consider exploring combinations of different effector molecules and also potentially targeting host factors required for propagation (**Figure 2**).

Figure 2. Genetic targets for modification during malaria parasite development in the mosquito host. Plasmodium parasites enter the mosquito midgut lumen through a blood meal. The sporogonic cycle starts with the gametocytes' maturation into female and male gametes (early midgut infection). Upon fertilization, the zygote is formed and develops into an ookinete. Ookinetes cross the peritrophic matrix and traverse the midgut epithelium (midgut invasion) before settling in its basal side, where they develop into oocysts. Oocyst maturation results in the production of thousands of sporozoites that are released into the hemocoel (hemolymph passage), from where they infect the salivary glands (salivary gland invasion). Several gene expression systems, effectors and host factors can interfere with the different mosquito–parasite interactions throughout this cycle. aapp – anopheline antiplatelet gene; act5C – actin 5C; Antryp1 – late trypsin; Aper1 – adult peritrophic matrix gene; Apy – apyrase; CecA – cecropin A; CEL-III – hemolytic C-type lectin; CP – zinc carboxypeptidase A1; CSPBP – CSP-binding protein; D7r – D7-related gene; ESP – epithelial serine protease; FREP1 – fibrinogen-related protein 1; Lp – lipophorin; PLA2 – bee venom phospholipase A2; SGS1 – salivary gland surface protein 1; SM1 – salivary gland midgut peptide 1; Vg – vitellogenin.

16.4 Performance objectives of population modification

The ultimate goal of all genetic control strategies is to reduce the number of infectious mosquitoes below a threshold level so that the probability of transmission falls to a point where the parasite population is too small to maintain the infection cycle. This has been defined as reducing the basic reproductive rate (R_0) of the disease below one ($1>R_0$) (Sinden, 2015). However, assessment of the necessary frequency and efficiency of a genetic modification capable of limiting a mosquito's infectious potential in the field is complicated. Typically, established concepts and metrics for measuring malaria transmission do not explicitly distinguish between light and heavy infections or the likelihood that a deemed infectious mosquito bite actually will result in a blood-stage infection in humans (Smith *et al.*, 2012). This understudied area of malaria biology is of key importance to better appreciate the dynamics of infection in natural settings and predict the impact of genetic interventions (Graumans *et al.*, 2020).

Early clinical and experimental evidence supported the idea that even a mosquito with a strongly lowered parasite burden can be infectious, since the small inoculum of only ten sporozoites is sufficient for infecting humans (Ungureanu *et al.*, 1976) and low-sporozoite numbers were implicated in an avian model of parasite transmission (Jasinskiene *et al.*, 2007). Therefore, the fundamental thinking was that if only one ookinete successfully transposes the midgut barriers, develops into an oocyst and produces thousands of sporozoites, this should be enough to sustain infectiveness (Rosenberg *et al.*, 1990). This notion encouraged transgenic mosquito researchers to take for many years the most stringent endpoint and set a goal for 'zero prevalence' of sporozoites in the salivary glands (Jasinskiene *et al.*, 2007; Isaacs *et al.*, 2012). However, classic reports in malaria epidemiology support the conclusion that the majority of infected mosquito bites may not result in a detectable infection (Davey and Gordon, 1933; Davidson and Draper, 1953; Pull and Grab, 1974). More recently, it was shown with the rodent malaria model that mosquitoes with fewer *P. berghei* sporozoites (≤ ~400/salivary gland) are less infectious (Ito *et al.*, 2002; Churcher *et al.*, 2017). In a novel study, Aleshnick *et al.* (2020) proved experimentally that the relationship between mosquito salivary gland infection load and transmission probability is not linear and indeed must meet a threshold. The chance of infection increases particularly at a range between 10,000-20,000 sporozoites per salivary gland (Aleshnick *et al.*, 2020). However, it is difficult to extrapolate model system transmission data directly to human malaria settings. One of the reasons is because the

protocol used for the assessment of sporozoite infectivity in humans (in controlled malaria infections to evaluate vaccine efficacy) is carried out purposely using heavily-infected mosquitoes, which is not reflective of parasite densities found in nature (Walk *et al.*, 2018). In fact, most laboratory studies that describe efforts for malaria parasite suppression in mosquitoes also overestimate infection levels, likely underestimating the success rate of the resistance achieved. This is despite the fact that the data on the infection intensity in naturally-occurring infected mosquitoes indicate that the majority of them harbor a limited number of parasites (< five oocysts/gut and < 10,000 sporozoites/gland) (Pringle and Avery-Jones, 1966; Beier *et al.*, 1987; Billingsley *et al.*, 1994; Gouagna *et al.*, 2014). In addition, the effectiveness of transmission-blocking vaccine candidates in vertebrates is considered to be regulated tightly by mosquito parasite density, with the antibodies being more efficient at lower forces of infection in the mosquito (Bompard *et al.*, 2017; Churcher *et al.*, 2017). Measurements compatible to a natural infection system, with control parasite levels within the range of those found in wild-caught insects, are needed for genetically-engineered mosquitoes to evaluate accurately and correctly the potential of a given transmission-blocking strategy. It is not yet sufficiently defined whether a 'no sporozoite' phenotype is indeed necessary to significantly impact malaria transmission, therefore interventions that decrease the mean intensity of infection might be as important as those that reduce parasite prevalence in the mosquito (Graumans *et al.*, 2020).

It appears intuitive that the expression of multiple anti-*Plasmodium* transgenes in different mosquito tissues would result in a synergistic effect and potentiate the level of refractoriness to parasite infection. It was observed in mice immunizations that the combination of two partially-effective anti-malarial antibodies do achieve synergy in efficacy upon lower mosquito parasite loads (Sherrard-Smith *et al.*, 2018). This indicates both that a similar improved effect might be expected when combining effectors for mosquito refractoriness, and that interventions aimed to reduce infection intensity can be useful to aid in disease elimination strategies that combine vector modification and host vaccination efforts. Nevertheless, it is important to consider that in genetically-engineered insects, a single transgene that produces polycistronic mRNAs can result in reduced levels of each of the effectors than what could be achieved through single-effector constructs (Daniels *et al.*, 2014). Dong *et al*. (2020) reported that combinations of endogenous and exogenous effectors were able to induce highly potent suppression of parasite load and infection prevalence. However, this is not valid for all the effector combinations, and reproductive fitness and mosquito survival can be impaired significantly in some multi-effector transgenic lines (Dong, Simões and Dimopoulos, 2020). Spatiotemporal expression of multiple antiparasitic effectors targeting different *Plasmodium* stages should still be a goal because it limits the probability of emergence of parasite resistance.

Furthermore, strategic planning for malaria control should include multiple parameters and be adjusted to the level of transmission-blocking efficacy required, given that even short-effect interventions could eliminate *Plasmodium* from vector and host populations in low transmission settings (Blagborough *et al.*, 2013).

Perhaps the most attractive feature of population modification gene-drive systems is the possibility of creating a high-impact, low-cost and sustainable tool for controlling disease transmission (Carballar-Lejarazú and James, 2017). Whether gene drives are predicted to succeed in wild populations depends on two key parameters: the homing efficiency and fitness, meaning the relative fecundity or death rate the drive and its cargo confer on the modified organisms compared to the wild-type counterparts. Therefore, drives are favored by selection if the inheritance bias of the drive exceeds its fitness penalty (Noble *et al.*, 2018). In fact, the first population modification by CRISPR-based gene drive in *Anopheles* achieved a proof-of-concept drive efficacy despite a substantial fitness cost of female mosquitoes homozygous for the drive (Gantz *et al.*, 2015). A carefully considered genome target may prevent an unintended disruption of important or essential genes and associated fitness loads. For this, the primary insertion site can be tested for its impact on fitness, as discussed previously. Furthermore, the effectors produced may exert physiological imbalance, or transgene expression might usurp resources needed for normal survival or reproductive functions (Terenius *et al.*, 2008). A number of life-table parameters must be determined under conditions that better mimic the natural environment of the mosquitoes and the release strategy proposed (Facchinelli *et al.*, 2019; Pham *et al.*, 2019). Given that the higher rate of inheritance associated with effective gene-drive systems render them capable of increasing in frequency up to fixation in the population, the concept of a necessary complete fitness neutrality in modified mosquitoes can be reasonably challenged on an approach for implementation (James *et al.*, 2018).

Finally, it is important to discuss issues that are not yet fully understood or cannot be experimentally predicted in modification efforts. One puzzling example is the ‘suppression escape phenotype’ on modified lines that exhibit an overall strong refractoriness, represented by the few individual mosquitoes that present high levels of parasite infection (Isaacs *et al.*, 2012; Dong, Simões and Dimopoulos, 2020). Phenotypic variability and incomplete penetrance are frequently observed in transgenic animals (DeLoia and Solter, 1990; Pereira *et al.*, 1994; Kearns *et al.*, 2000; Seda *et al.*, 2019). In the case of effector-expressing mosquito populations, environmental and epigenetic factors could contribute to exceptional lowered effector expression or immune-suppressed phenotypes in some individuals. It is also possible that parasite variations (e.g., polymorphisms, developmental deviations) would make them spatiotemporally circumvent the expression of transgenic effectors.

However, it is unclear whether this heterogeneity could lead to a modified dominant pattern of inheritance in the mosquito population or accelerate the emergence of parasite resistance. Certainly, parasite selection or evolution of resistance to transgenic blocking mechanisms are important subjects to address because no efficient animal models exist yet for studying transmission of human malaria parasites. As mentioned above, population genetics mathematical models are needed to demonstrate the future dynamics and nature of the proposed systems and whether they exhibit robustness to imperfect homing, incomplete penetrance and transgene fitness costs, each of which are of practical significance given that real-world components inevitably have such imperfections. Furthermore, it is of paramount importance to address questions not only from the scientific community, but also concerns expressed by the public and the media about the potential ecological, ethical, and social impact of gene-drive modification systems, in order to consider regulatory approval prior to any field trials (Singh, 2019).

16.5 Conclusions

CRISPR/Cas9 systems have revolutionized the ability to produce genetics-based tools to add to the current incomplete toolkit for disease elimination and malaria eradication goals. Among the applications, coupling anti-pathogenic transgenes to gene-drive systems have a strong potential to combat vector-borne diseases, due to their combined ability to spread into natural populations and block pathogen transmission. Population modification strategies should not be seen as a single solution, but as a component of a set of robust new methods, that integrated with current tools, should improve outcomes towards the elimination of malaria. The greatest opportunity for impact on eradication is a better understanding of vector-parasite interactions and transmission features, as well as genetic systems in mosquitoes, that may be used for the development and validation of novel disease control tools.

Malaria control presents variable challenges across its transmission spectrum and strategic planning for elimination should consider a number of factors, with particular emphasis on the transmission-blocking effectiveness required, and the transmission intensity in the targeted area. Regarding the development framework, a target product profile helps researchers identify their specific goals and realistic go/no-go criteria for efficacy of an investigational product before moving further along the testing pathway (James *et al.*, 2018). Evidence of laboratory efficacy, as well as fitness, safety, release strategies, and minimally acceptable performance parameters provide the basis for evaluating novel technologies for field use (Carballar-Lejarazú *et al.*, 2020; Long *et al.*, 2021). The

design and field implementation of a population modification product is likely to be both complex and multifaceted, although current data suggest that we may be closer than we previously thought to the utilization of an effective and safe antimalarial technology that might reverse the current global disease trend.

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Figure 1

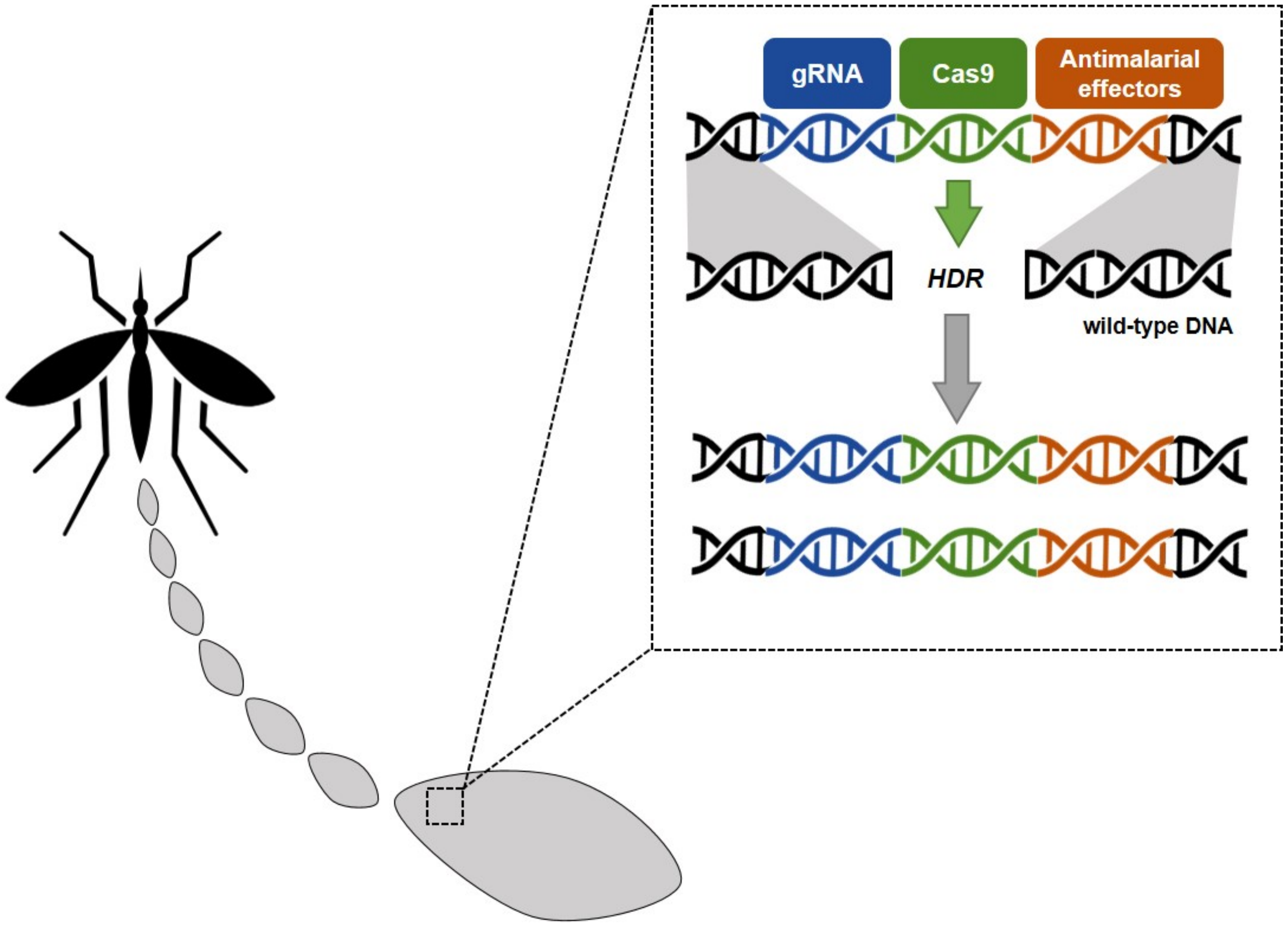

Figure 2