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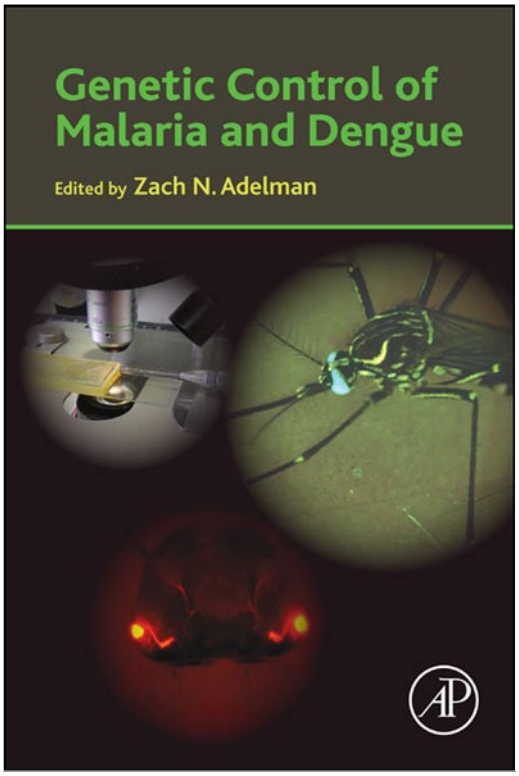

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Chapter 19

Impact of Genetic Modification of Vector Populations on the Malaria Eradication Agenda

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INTRODUCTION

Reports of progress on the global malaria situation are a mixed. The World Health Organization (WHO) has evidence for a continuing reduction in mortality, attributed in part to use of bed nets and combination drug therapies [1,2]. Some 3.3 million lives are estimated to have been saved since 2001. This success supports efforts to increase implementation of existing control measures with the expectation that they will continue to lower malaria incidence. However, many factors threaten these hard-won gains and these include inadequate public health infrastructures, the increasing scale over which previously successful programs must be applied, and insecticide and parasite drug resistance [1,3]. Furthermore, a number of recently recognized challenges have been identified that add to an already complex situation. These include the impact of global warming on mosquito vector distribution and the emergence of additional species of malaria parasites that can infect humans [4–6]. Thus, while there is much to celebrate about the recent reductions, we must continue to apply proven technologies while at the same time develop new disease-control tools.

The renewed call for malaria eradication stimulated cooperative planning among the malaria public health and research communities to develop agendas for reaching this goal [7]. Eradication was defined in the agenda as the reduction of transmission below a threshold level that achieves an impact on the basic reproductive rate (R_o) of the disease such that $R_o < 1$. However, it is more straightforward to express it as the complete absence of parasites in humans so that they are not able to infect mosquito vectors, and the

Genetic Control of Malaria and Dengue.

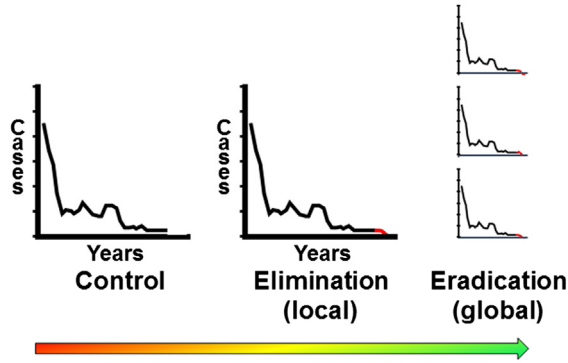

FIGURE 19.1 **Malaria eradication milestones.** Malaria eradication (right) will be achieved through a series of phases that progress (arrow) from control (left) through elimination (center). The *x*- and *y*-axes show numbers of cases and years, respectively, in arbitrary units. The red portion of the curve represents achieving elimination.

complete absence of parasites in mosquitoes so that they cannot infect humans. Recent infections of humans by parasites found previously only in non-human primates requires addressing sources of infections that originate in animal reservoirs [5,6].

Eradication is achieved through the phased operational targets of control, pre-elimination, elimination, and prevention of reintroduction [8] (Figure 19.1). The WHO defines control as less than 5% positive slides in all patients presenting with fever and elimination as no cases of locally acquired malaria for a period of 3 years as a result of deliberate control efforts. Eradication is the global elimination of malaria. This is an ambitious goal and there is a consensus that it is unlikely that any single technology will be sufficient to achieve it [7]. Contributions are needed from diagnostic, therapeutic, and prevention domains and the knowledge from a broad array of scientific disciplines must be recruited to support this effort.

It is important to ask how the goal of eradication informs the research agenda in the many contributing disciplines. This question put explicitly to vector biologists identified a number of critical needs [9]. Existing broadly applicable (insecticide treated nets, indoor residual sprays) and region-specific (environmental modification) vector-targeted prevention tools were sufficient to achieve control and elimination in many regions of the world. It is essential to use these tools where feasible and efficacious. However, there are malaria-endemic areas where it has not been possible to achieve control and elimination. This can be due to the failure to apply the currently available tools because of geographical, political, and economic difficulties, circumstances where these tools were applied but did not work (e.g., insecticide-treated nets do not impede outdoor, day-time feeding mosquitoes), and those situations where the tools

worked previously but are no longer effective (e.g., the emergence of insecticide resistance) or can no longer be delivered because of failed or overwhelmed public health infrastructure. Thus, there is a clear need in the vector biology contributions to malaria eradication for better use of existing control tools and the development of novels ones to complement them.

Two of the major challenges to malaria eradication are the heterogeneity/complexity of transmission dynamics and difficulties in sustaining control efforts [10–12]. This complexity is evident in the vector components by the large number of *Anopheles* mosquito species that have been implicated worldwide in malaria parasite transmission. There are approximately 450 described *Anopheles* species, 68 of which are known to transmit human malaria, and as many as 40 are identified as major vectors [13,14]. Indonesia alone has as many as 24 species involved in regional parasite transmission [15]. Each of these species has its own biology associated with host preferences, feeding behavior (indoor/outdoor, day/night, etc.), mating behavior, breeding-site preferences, and vector competence,[1] all of which affect their vectorial capacity. It is a significant challenge to find a single tool that accommodates all of this diversity, and this supports arguments for having multiple approaches to vector control that can be applied as needed and where effective [9].

Sustainability is a major challenge to all public health efforts and can be destabilized by both success and failure. Successful public health creates the "public health paradox;" when it is working nothing is happening. Specifically, good public health practices are characterized by the lack of disease. It is difficult under these circumstances to continue to devote resources to a problem that is perceived not to exist [8]. Withdrawal of support can lead to disease re-emergence and the ensuing costs of reasserting control are likely to be greater than those incurred by maintaining it [18].

Sustainability of vector control also is challenged by success, but has additional, intrinsic features that lead directly to failure. The most often cited is the development of insecticide resistance, and this has had a major negative impact on maintaining control in many areas of the world [19]. Additionally, migration of infected humans and mosquitoes compromises sustainability; malaria epidemics and focal outbreaks can occur in regions that have achieved elimination through the absence of the parasites but still have local competent vectors [3,20].

The prospects for success in malaria eradication will depend significantly on how well major scientific disciplines can provide tools that can address complexity and sustainability. For example, chemistry coupled with physiological insights can produce new insecticides for the vectors and prophylactic

1. Vector competence is a measurement of the intrinsic ability of the insect to transmit a pathogen and includes genetic components [16]. Vectorial capacity is a measurement of the efficiency of pathogen transmission, and of which vector competence is a parameter [17].

and therapeutic drugs for the parasites; these agents may have a sufficiently broad spectrum of application to be useful in managing complexity. Ecological studies can guide rational, community-wide, environmental management to remove mosquito breeding sites, and behavioral sciences can inform at-risk populations about adopting personal-protection measures (e.g., bed nets and repellents), and these also could have an impact on complexity. Immunology provides tools to probe disease progression and the basis for developing vaccines, and so contributes to sustainability at the individual level. Importantly, new tools being developed in the field of genetics can offer sustainability at a regional level.

Genetic approaches that target mosquito vectors as a means of disease control have been in consideration since the 1940s [21]. Indeed, sterile insect technologies were used to control a vector mosquito in Central America. This success was unsustainable, mostly due to civil unrest, and negative publicity in a separate effort in India decreased enthusiasm for these approaches [22,23]. However, the development of powerful molecular biological tools re-kindled enthusiasm for developing genetic control strategies. Specifically, the ability to genetically engineer specific phenotypes in mosquitoes fostered research to exploit these technologies for malaria control [24]. We anticipate a unique and important role for transgenic mosquitoes in maintaining the sustainable elimination needed for malaria eradication, but this will only be realized by careful and strategic planning.

POPULATION MODIFICATION AS A REGIONAL SOLUTION TO SUSTAINABLE MALARIA ELIMINATION

Genetic strategies for malaria control seek to eliminate vector mosquitoes or reduce their densities below thresholds needed for stable pathogen transmission (population suppression), or make them incapable of transmitting parasites (population replacement/modification) [25–30]. Transgenesis technologies were used to produce mosquito strains that carry genes that result in phenotypes that contribute to both strategies [31–35]. However, long-term, cost-effective, and sustainable malaria elimination requires the development of genetic strategies that are resilient to the immigration of parasite-infected mosquitoes and people, and the lack of such tools represents a significant unmet need in the malaria eradiation agenda. Mosquito strains for population modification carrying genes conferring parasite resistance have the appropriate design features for this purpose [25,33]. Wild, parasite-susceptible mosquitoes invading a region populated by an engineered strain will acquire the parasite resistance genes by mating with the local insects, and persons with parasites moving into the same region will not be able to infect the resident vectors, and therefore not be a source of parasites for infection of other people. Population modification also shares with other genetic control strategies the exploitation of the ability of male

mosquitoes to find females, and this is expected to offer access to vector populations that would be unreachable using conventional tools [36]. Release of a population modification strain alone or in conjunction with other tools should make elimination possible in carefully selected endemic areas. Population modification strategies can be used as early as the control phase of an elimination campaign alongside other measures that will reduce disease incidence. As the efforts progress, this strategy takes on a larger role and ultimately is the mainstay of the prevention of reintroduction phase. As this elimination is achieved, the released modified mosquitoes would facilitate consolidation of this success by allowing resources to be moved to another target region with the confidence that the area just cleared will remain so. Thus, population modification offers a real chance to achieve sustainable elimination and therefore contribute significantly to malaria eradication.

Successful application of a population modification approach will depend on it being effective, that is, it achieves the goal for which it was designed, is not prohibitively more expensive than alternative approaches, and is safe for humans, animals, and the environment. Population modification strains can be generated that meet these requirements. *Anopheles* species have been engineered already with genes whose products disable *Plasmodium falciparum*,[2] and these results support the rationale for continuing to develop this approach [31,32,37]. Furthermore, population modification strains have the best design features and anticipated performance characteristics for sustainable elimination when compared to other approaches. Insecticides in all formulations and applications must be applied routinely and therefore need ongoing cost support. The same is true of proposed genetic population suppression technologies [34]. In addition as noted, the efficacy of insecticides is diminished by the emergence of resistance. Environmental modification often takes a level of infrastructure maintenance that many disease-endemic countries cannot sustain [3,38,39]. Recent work showed that introductions of exogenous symbiotic organisms into mosquitoes may increase their resistance to malaria parasites [40]. However, unlike the published reports of genetically engineered mosquitoes, these organisms have yet to be shown to completely block parasite development.

Cost-effectiveness of genetic approaches has been estimated for population suppression strains for preventing dengue virus transmission, and these are comparable initially to all other strategies [41]. However, the costs are expected to decrease as the approach drives the target population to extinction as the use of male mosquitoes to find residual populations should be less expensive and more effective than using humans to do so. Population modification should provide similar cost benefits with recurring expenses

2. Malaria in humans is caused primarily by four pathogen species, *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. Of these, *P. falciparum* and *P. vivax* are the most significant in terms of morbidity and mortality. Recently, at least two additional *Plasmodium* species have been shown to cause disease in humans [5,6].

limited to surveillance and monitoring, which also are components of all intervention programs [42]. Furthermore, it is reasonable to expect that population modification strategies would decrease significantly the costs associated with the prevention of reintroduction phase of WHO-defined elimination [8]. It is important to acknowledge that both genetic population suppression and modification protocols most likely will only be cost-effective in areas in which a single or small number of vector species are responsible for parasite transmission. Each additional species will add to the costs, and at some point the continuous use of an unsustainable technology (e.g., insecticides) for the duration of the eradication program may be less expensive overall.

The major safety concerns of using of population modifications strains fall generally into the category of perceived risks of off-target effects that would impact other species at and outside the field-trial environment [42,43]. However, transgenic mosquito strains can be engineered with design features that make these hazards sufficiently unlikely so as to not be possible. The inclusion of specific control DNAs (e.g., those that modulate gene expression in response to a blood meal) that function in a narrow range of related species should prevent the genes from being active in beneficial insects, and inundative releases of strains that lack promiscuous (capable of spreading in many species) gene drive systems have little or no probability of moving their genes into non-target species.

The maturation pathway for new products includes distinct discovery, development, and delivery stages, and these are established for drugs, vaccines, and insecticides where rigorous industry-wide standards are used to validate performance and safety features to determine if a candidate product should advance from the laboratory to application. Such a pathway does not exist yet for genetically engineered mosquitoes [8,36]. Furthermore, the financial incentives in these approaches make it unlikely that the formulation and adoption of a consensus pathway will come from commercial interests. Therefore it is incumbent on the vector biologists, end users, and other public health stakeholders to generate such a pathway. A series of researcher-initiated efforts have taken on this challenge and guiding principles have been produced by the WHO and others [8,36]. Included in these principles are recommendations for a series of phases with "go/no-go" criteria for testing genetically engineered mosquitoes (Figure 19.2). These efforts are important but not sufficient for successful testing of a population modification approach. A strategic plan is needed that maximizes the probability of success of the first field trial of this technology while at the same time meets end user concerns about adoption of the approach.

STRATEGIC PLANNING

The primary objective of strategic planning is to ensure that the first field trial of a population modification strategy is an unqualified success. Success

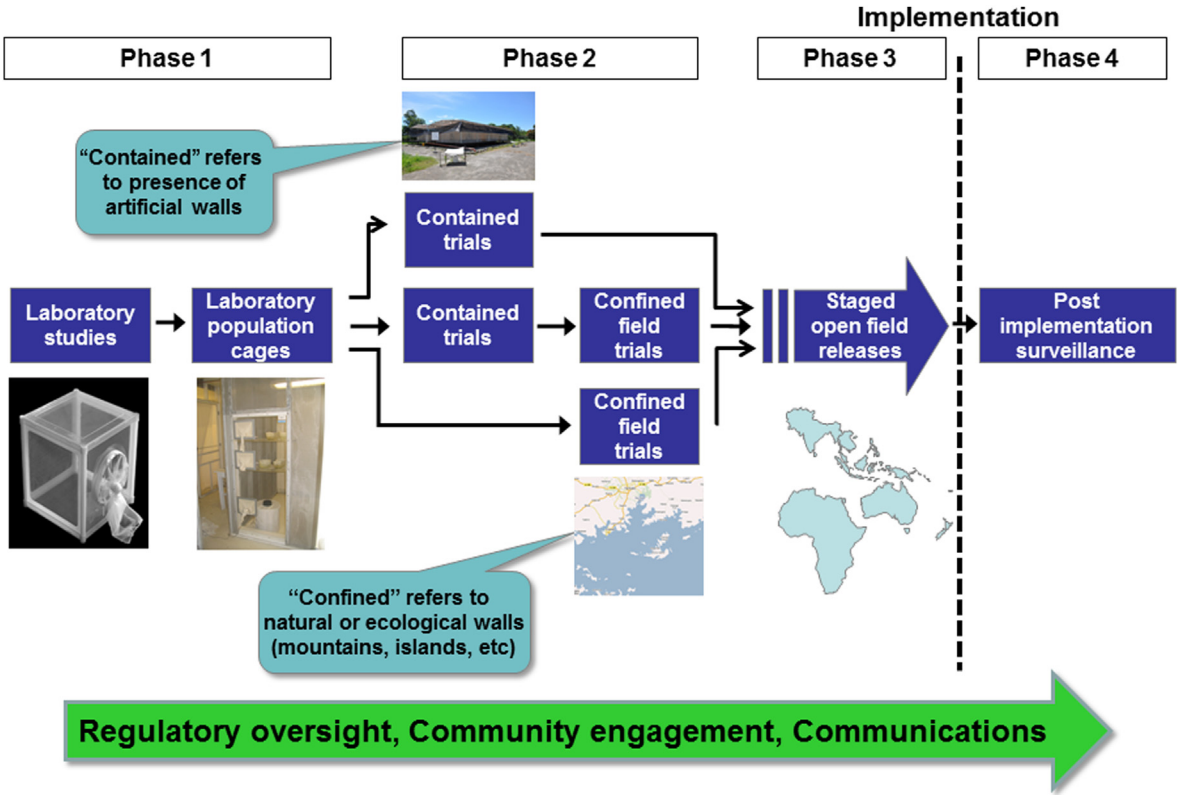

FIGURE 19.2 **Phased testing of genetically engineered mosquitoes.** A proposed scheme for the phased testing of genetically engineered mosquitoes was developed by working groups at the WHO [43]. Phase 1 is carried out entirely in the laboratory and includes the original development of the transgenic strains and small/large cage trials to estimate fitness. Phase 2 takes place in a field setting and may be either contained or confined. Phase 3 is an open field release with either or both entomological and epidemiological end points. Phase 4 is the implementation phase with the intent to achieve a sustained epidemiological impact. Regulatory oversight, community engagement, and communications should initiate early in the program. *Image adapted from Ref. [43].*

is defined here as the stable elimination of malaria at a selected trial site.[3] While this first trial will establish proof-of-principle, it is also expected that the site will remain free of malaria throughout the full duration of any ensuing eradication campaign. Thus, the outcome of the trial is expected to produce a sustained epidemiological impact.

The details of the strategic plan are meant to define major operational objectives and provide tactical guidelines that will ensure a successful trial. This trial will test the scientific features of the genetic strategy and the ability to do so in concordance with un-reproachable community engagement and an informed and transparent regulatory process [44]. Success will depend in part on meeting three operational objectives: (i) the informed selection of an optimum field site, including recruitment of the necessary personnel and resources; (ii) a well-designed and functional population modification strain; and (iii) the development of a detailed trial design and implementation plan for the release protocol.

Field-Site Selection

Site-selection criteria have been elaborated extensively in a number of publications and we highlight those relevant to a first trial of population modification [36,45–47]. Important considerations include local transmission of a single malaria parasite species, the presence of a single vector mosquito species, a limited geographical area, a thorough knowledge of the distribution and population structure of the target mosquito species and malaria epidemiology, local scientific experts with whom to collaborate, and community and government support. Political stability throughout the course of the trial also would be helpful. Importantly, there are no limitations on the persons living or moving through the experimental area.

The requirement for the malaria burden at the field site to result from a single parasite species makes it easier to monitor trial progress. *Plasmodium falciparum* or *Plasmodium vivax* offer the opportunity for the greatest impact on morbidity and mortality. The majority of existing engineered mosquitoes carry parasite resistance genes targeting *P. falciparum* and laboratory-based mosquito challenge assays are available widely for this species, therefore this makes it the best choice for the first trial [31–33,37]. This selection is not meant to diminish the significance of *P. vivax*, but allows immediate planning of trials with genes that have been proven efficacious already in the laboratory. Indeed, anti-pathogen effector genes based on altered mosquito physiology or immune enhancement or symbiont-based modification may have less-specific effects and therefore could be used in a trial targeting

3. The WHO certification of malaria elimination is determined at the country level [8]. We imply here a specific regional elimination that may or may not be countrywide.

multiple parasite species once the approach has been demonstrated to work for a single one.

The different levels of endemicity (hypo-, meso-, hyper-, and holoendemic [48]) are not expected to present a challenge to population modification because modification of the local vector population should suppress all parasite transmission. However, they likely will be variables that influence the amount of time that it takes to first see a positive impact of the technique and should be used as such in models. Furthermore, this technology would not be the first choice for stemming a current malaria epidemic, although once implemented, it should prevent future events.

The choice of a site in which only a single mosquito species is the vector reduces trial complexity. Genetic strategies by definition are restricted to individual interbreeding populations of a species. Complete introgression of the modification gene into a dominant vector (defined as the one contributing the most to the disease incidence in a specific area) would still leave transmission by secondary vectors, and this would prevent the trial from meeting the elimination goal. We anticipate that future applications of population modification technologies can be applied to regions with two or a small number of vectors where cost-benefit analyses provide favorable assessments for engineering strains for each target species.

Vector abundance will affect the speed at which the target population reaches fixation for the anti-pathogen effector gene. While in principle there are no constraints on the target population size, cost and logistical considerations favor a first trial in a region with low vector abundance so that sufficient mosquitoes can be reared and released, and an initial impact observed in the first or second year of a trial.

The initial trial site should maximize the geographical containment of the engineered insects [43,46]. This will assure that the release, monitoring, and surveillance activities take place on a limited scale, and therefore are not overly expensive, and contributes to meeting community engagement and regulatory considerations that we expect to be part of the first trial. This confinement is likely to be less important for future trials should the technology prove effective.

Complete and up-to-date knowledge of the vector ecology and population breeding structure is needed for site selection. This information is also important for designing the release protocol and determining those entomological parameters that can be used to monitor trial progress. Accurate epidemiological data is critical. The defined goal of the trial is to have an impact on incidence, and baseline data are needed to calibrate the success of the releases.

Local scientists, cooperative vector control facilities, and government agencies are vital to the trial process. Local scientists are nationals of the country in which the trials will take place that have the necessary expertise and authority to carry out the experiments. Sites eligible for the trial based on other criteria may lack these scientists, so strategic planning could include

a training component [49]. Furthermore, it is highly likely that the laboratory scientists who developed the trial strains will have to relinquish a substantial level of control over the project as it is taken to the field [44]. Thus, success will depend largely on how well local scientists assume intellectual ownership and responsibility for the trial. This commitment of local scientists to the project goals is a significant outcome of good community engagement practices. In addition, laboratory and other facilities in which the scientists, community, and regulatory authorities have confidence are secure enough to meet the requirements for handling genetically engineered organisms are essential [42,50].

Laboratory successes in genetically engineering mosquitoes stimulated the development of community-engagement objectives and principles [44,47]. A major challenge is to obtain a form of "community consent" that is both meaningful and respects the highest ethical standards. Consent is likely to be more than an arbitrary fraction of a majority vote among community members that allows the trial to proceed. We have argued that certain cultural norms and institutions can serve as ethical surrogates for community consent [44]. These surrogates will be site specific, and what is developed for one place cannot be transferred directly to another. However, while the specifics may vary, the general considerations for developing this consent are common and sets of guidelines can be used [47]. It is worth emphasizing that trust was identified as a critical outcome of a community engagement plan. There are fears that originate from mistrust by the public of the motives of scientists. These include perceptions that the public represents a source of experimental subjects for the scientists, and that the scientists will not do any good for the trial-site community, and may actually cause harm. Here is where community engagement, including recruitment at full partnership of local scientists from the trial country, is essential. Finally, an existing statutory and regulatory structure is needed for providing trial procedure reviews and issuing authorizations to carry out the releases. A number of published documents provide useful considerations for evaluating regulatory structures [36,43,44].

Selection of the Population Modification Strain

The two major criteria for selecting the population modification strain for the trial are that it be effective and safe. Again, a strain will be effective if once released, the parasite resistance gene achieves and remains at a high-enough frequency in the local vector population so as to completely abolish parasite transmission in the target area and result in elimination. Although modeling supports the possibility of a significant effect on pathogen transmission prior to full introgression of the gene [51], it is operationally more straightforward to set complete gene fixation as a goal instead of trying to achieve some predicted sub-complete level. Elimination under these circumstances is

expected to be sustainable in the presence of human and wild, parasite-susceptible mosquito immigration. Safe is defined as no significant off-target effects and no probability of vectoring a new disease agent. The off-target effects are those identified hazards and negative consequences that outweigh the benefit of having sustainably eliminated malaria [43].

We defined the optimal phenotype for a refractory gene to be one that prevents any mosquito-mediated transmission of the human-infectious forms of the parasites [33,37,52]. As far as we can determine, this means that there are *no* sporozoites (malaria parasite forms in mosquitoes that are infectious to humans) in the salivary glands of females. It is encouraging that it has been possible to produce this phenotype using different approaches [32,33].

Past vaccine and drug interventions targeting infectious agents provide many examples where resistance has been selected in pathogens thereby compromising the efficacy of the prevention or therapeutic protocol. We recommend the adoption of a "dual-transgene" approach where the population modification strain carries a compound genetic insertion comprising at least two components that disable the pathogen at different stages of its development [33]. This is functionally analogous to combined drug therapy in which the probability of a pathogen becoming resistant simultaneously to two different modes of drug action is extremely low. We expect this to be a key design feature to prevent emergence of parasite resistance in transgenic mosquitoes and sustain elimination.

The molecular targets of the anti-parasite effector gene may be polymorphic among different populations of the same species and this could affect the efficacy of the resistance phenotype. We chose targets for which there is little known variation [33], but the parasite complexity in the trial site should be characterized prior to the release to establish that it can be incapacitated by the gene products. Monitoring of the parasite population is required to mitigate the introduction of resistant parasite genotypes.

The population modification strain should also meet acceptable standards for fitness. We anticipated that the introduction of any exogenous DNA into a mosquito would necessarily come with a negative fitness cost (genetic load) because wild-type un-engineered mosquitoes have been selected in natural circumstances to be the most fit [27,53]. Any alterations to these genomes would therefore be expected to produce a fitness cost. This assumption did not take into consideration stochastic effects on population structure that could result from adaptive landscapes differing among potential interventions sites, and it is possible that wild vectors in some malaria transmission regions may not be as competitive as laboratory strains derived from them. However, the reduced-fitness rationale was used to support the need for linking gene-drive systems to parasite resistance genes. Laboratory-based empirical efforts to measure fitness produced a full range of results from showing severe negative costs in some strains to others where genes and insertions actually appear to make the transgenic mosquitoes more fit [33,37,54–58]. These results support

the conclusion that engineered strains with no or minimal fitness costs can be obtained by careful selection of an anti-parasite effector gene that has been inserted into a well-characterized site in the genome. Modern genome-editing tools allow the placement of transgenes into specific regions of the host mosquito chromosomes that have been tested previously for, and insulated against, insertion-site effects [33,59].

We argued that adding an exogenous gene to the mosquito genome is likely to minimize fitness impacts when compared to manipulating transcriptional control or product abundance of endogenous genes that are involved in reproductive or immune physiology [60]. However, there are recent results that support the conclusion that targeting specific mosquito genes does not lead *a priori* to a load [61]. However, it is important to determine if laboratory performance is recapitulated in natural mosquito populations. Our experience with a population-suppression strain of the dengue vector mosquito, *Aedes aegypti*, showed that it had excellent performance characteristics in large laboratory cage trials but these could not be matched when the experiment was scaled up to much larger outdoor field cages [62].

Modification strains with some reduced fitness could still have applications in the field. Sterile insects produced for population suppression often are less fit than their wild-type counterparts because of radiation or chemical toxicity [21,63,64]. Insects with mating competitiveness lower than the targeted wild population can be effective in suppressing if released in large enough ratios [64]. Population modification strains may not share this flexibility as they are expected to remain *in situ* in the presence of immigration of wild mosquitoes, but they may be useful if they can persist at a level that is epidemiologically relevant until eradication is achieved. Modeling shows that this is possible if the anti-pathogen effector gene is linked to a chromosomal region containing genes favoring enhanced mating success [51].

An early criterion imposed on field uses of genetically engineered mosquitoes was that they should be done with male-only releases [65]. It was argued that releases of females would not be tolerated because they still could probe and feed, and therefore be a nuisance. However, there seems to be some acceptance for a relaxation of this requirement since recent trials of a *Wolbachia*-infected strain of dengue vectors could only be carried out by releasing females as the symbiont is inherited maternally [66]. Furthermore, modification-based strains rely on leaving an altered mosquito population in place and this will include reproductively active (and therefore feeding) female mosquitoes.

Safety considerations of genetically engineered mosquitoes are mainly issues about the potential for off-target effects [43,67]. These include scenarios where the transgene moves horizontally into a beneficial species in which it has a deleterious effect, potential inhalation/ingestion toxicity of transgene products, and removal of a keystone prey species from an ecological network. Here again, the design features of the strains can include components that mitigate these potential hazards. The specific gene components can be engineered such that

they are functional only in the species to which they are targeted. For example, species-specific DNA control sequences can be used to direct the expression of the effector portion of the transgene, and therefore render the construct inactive if it is in a heterologous species. Inhalation/ingestion hazards also can be addressed by using products that have no inherent toxic or allergenic components, and the design feature can be such that no exogenous proteins are introduced by salivation during feeding. General allergic responses are expected to be no more frequent than human sensitivities to existing mosquito exposures [68]. Furthermore, it should be a strict requirement that all inserted transgenes in the final release strain not contain any bacterial antibiotic- or other chemical-resistance genes. This can be done easily using modern gene-editing technologies.

Population modifications strains also mitigate the issue of removing a species from an ecological network [25]. Although there are many circumstances where specific vector species are invasive and well adapted to highly artificial (not natural), human-generated ecosystems (e.g., large urban areas or agricultural regions in which the landscapes have been reshaped to favor crop production), and where complete removal of that species could be viewed as "bioremediation," there are expressed concerns about the elimination of a vector species from even badly eroded environments [67]. The modification strains leave the resident mosquitoes in place so that extant ecological dynamics remain unchanged.

Another often-expressed concern is that the specific genetic modification can produce a strain that now has the capacity to transmit a new pathogen. Fortunately, the biology of vector−pathogen interactions is complex and this represents a barrier to the transmission of new pathogen species. While there are examples of genetic changes affecting mosquito−arbovirus interactions that increased vector competence [69], these evolutionary events are likely to be rarer for protozoan parasites, including those that cause malaria. A recent cage trial study of a dengue vector in Mexico was granted permission only after laboratory experiments confirmed that the specific strain to be tested could not transmit a number of other viruses that could be expected to be found in the trial site [62]. We agree that it is good policy to test such interactions where there is a reasonable probability that a transgenic mosquito will encounter a known pathogen that it does not transmit and for which there is biological evidence that it could survive in mosquitoes. However, this should preclude unproductive research efforts for those pathogens that have never been found in mosquitoes and whose biology is not compatible with replication in mosquitoes and their cells (e.g., influenza viruses, HIV, hepatitis viruses).

Although there are a number of population modification strains under development, we are most enthusiastic about those based on single-chain antibodies (scFv) [33,37,52,70,71]. Their design features include dual-targeting components to prevent the selection of parasite resistance to the effector

molecules and site-specific integration to mitigate impacts of the expression of exogenous genes on the fitness of the mosquitoes. This latter feature is also significant because it allows the remaking of a specific strain should it be lost or encounter some other difficulty that prevents the use of its original derivation. Efficacy of the scFv-based design was demonstrated in mosquitoes carrying a dual transgene that targeted the developmental stages of *P. falciparum* found in the midgut and salivary glands; no human-infectious forms were seen in the latter organs and no clear effects on fitness were observed [33].

Trial Design and Implementation

Trial implementation requires an organizational structure that maximizes cooperation and communication among all of the participants and elements of the project. The trial will require scientists who are responsible for the production, delivery, and quality control of the release strain, public health officials who will participate in monitoring and surveillance, regulatory personnel to satisfy the demands of the trial statutory conditions, individuals responsible for proactive and reactive communications and community engagement, and an administrative structure that can organize and keep the project on track.

The majority of the discovery phase of the development of population modification technologies takes place in academic settings. This is a direct result of the processes that foster creative innovation in these institutional environments. However, the subsequent stages of product development and delivery require expertise that often is not rewarded in academia, and therefore not present in the skill sets of the research scientists. We propose that a robust trial is best served by not being an academic exercise. This requires that the scientists who conceived and developed the population modification mosquito strains let go of their technology and pass it along to persons with the appropriate expertise for the next steps. This is made somewhat easier for many by the fact that these approaches are being developed for a public health benefit and not for personal or corporate enrichment, and where possible, it would be good to minimize the influence of for-profit agencies on the trials.

Good organizational practices call for the core trial team to be as small as possible while having all of the necessary expertise. Team members should be recruited from local scientists whenever possible. Participants include an on-site operational manager and persons with competency in mosquito molecular biology to ensure quality control of the product, mosquito field biologists to design and monitor release protocols, and modelers to support experimental design and define anticipated outcomes. In addition, an epidemiologist is required to track malaria incidence and prevalence, and persons familiar with regulatory criteria and community-engagement specialist with competency in the local language(s) and English are needed [36].

An explicit decision-making process is essential for the trial. All responsible persons should read and sign off on this process. Furthermore, while there are advantages to running the process as democratically as possible, ultimately there needs to be a single person who is responsible for the entire project. This person in academic settings is the principal investigator, but may have a different designation ("project leader") in a specific trial. This person needs to have the confidence of all project participants and be judged fair and knowledgeable. In addition, this person needs to be a strong advocate for the project goals while at the same time be flexible to circumstances that could demand re-evaluation of the protocols. It is important to have a person different from the project leader be the project manager. The project manager is responsible for ensuring timely coordination and communications among all project participants, and making sure that every component of the project gets what it needs, in the appropriate condition and scale, and on time.

Pre-trial efforts must include acquiring information on mating competitiveness of transgenic males compared to wild type, estimates of local vector population sizes and densities, and the expected level of adult dispersion. These data will help establish baseline entomological data for the subsequent release monitoring [72]. Furthermore, detailed information on malaria epidemiology at the site is needed. There should be enough historical data to define trends in disease incidence and prevalence so that deviations following the trial onset can be measured.

The formal design of the trial will be a challenge. Cluster-randomized trials (CRTs) have gained acceptance as the highest standard for infectious disease interventions, but these are difficult to design and could be prohibitively expensive for a population modification approach [73]. It is highly unlikely that it will be possible to find the multiple, distinct sites within reasonable geographic proximity that share enough of the matching demographic, epidemiological, and vector parameters that are needed to provide the statistical power for CRTs. Therefore, an alternative approach would be to carry out a multi-year study in a region with a previous history of malaria endemicity. Comparisons would be made between the past history of malaria prevalence and incidence at the site and what is observed subsequent to the releases.

While the frequency of the transgene in the mosquito population can be used as a surrogate marker of success, the most important end point is epidemiological. Therefore, conclusions can be confounded by other anti-malaria practices being carried out at the site during the trial, but it would be unethical to halt or withhold any other beneficial control strategy for the sake of the trial. Good trial design and statistical analyses should be able to determine that fraction of reduced incidence that results from the trial intervention. Furthermore, the ultimate outcome is conclusive, as a site that once had malaria now has achieved elimination, and this status is maintained in the relaxation or absence of other anti-malaria practices.

A successful trial requires determining the best developmental stage of the mosquito to be released. The dengue vectors in the genus *Aedes* have the remarkable property that their fertilized eggs (embryos) enter a type of diapause, called estivation, and can be stored in large numbers inactive but alive for several months [74]. These mosquitoes can be released by placing pieces of filter paper carrying as many as several thousand embryos directly into a favorable larval habitat. Unfortunately, this developmental physiology is not present in the Anopheline vectors of malaria, so an alternative stage is required. Larval and pupal stage distribution requires moving large numbers of mosquitoes in volumes of water. Weight considerations likely will make this cost-prohibitive unless the mosquitoes are reared and released locally. Furthermore, it requires placing the mosquitoes at sites in which they can complete their development. Embryo distribution is free of the need for transporting water, but these cannot be stored and also would have to be delivered physically to a place in the environment to complete development.

In the past, adults were distributed because they are have favorable weight-to-number ratios and are capable of immediate dispersal upon release [21,64]. Furthermore, the release of an engineered, blood-fed fertilized female is the numerical equivalent of a delayed release of 100−300 mosquitoes depending on the fecundity and fertility of the strain [75]. Thus, it is best to release mixed populations of transgenic adult males and blood-fed females. However, adult mosquitoes are fragile compared with the sub-adult stages, and there may be significant losses in numbers associated with packaging and transport. Therefore, this favors having a mosquito-rearing facility at or near the trial site. This would have a positive dual purpose of releasing the preferred mosquito stage while at the same time employing local vector control personnel to do the rearing and distribution. This would add significantly to the community engagement needed to engender enthusiasm for the product.

Transgenic mosquitoes are expected to be most competitive if they are similar to the wild populations at the trial site. This implies that it is necessary to put the anti-parasite effector genes into the genetic background of a strain from the trial area. This was a regulatory requirement for large-cage field trial of *Ae. aegypti* in Mexico [44]. However, there may be circumstances where releasing a laboratory-adapted strain is preferable. For example, the laboratory strain may have no or less susceptibility to insecticides than the target population. Furthermore, the laboratory strain may have been tested rigorously for their competence for other pathogens. The decision of the genetic background will be made based on the local regulatory requirements with input from the community in the trial area.

It is important to know if there are seasonal fluctuations in mosquito abundance and density. This knowledge will inform the release periods of the trial protocol to maximize survival of the transgenic adults and their subsequent progeny as well as optimize release ratios. The best time to commence releases would be at the start of the rainy season. This rain would

replenish and create new oviposition and larval rearing habitats allowing the released females to deposit their fertilized eggs. The resident wild population is expected to be at their lowest size at this time, and this should maximize the impact of the released males as they should enjoy a numerical advantage over competing wild males. Habitat flooding early after trial onset could affect the initial stages of releases, so it is important that there be multiple releases over the first few weeks of the season.

Monitoring and surveillance of trial progress and outcomes should involve both entomological and epidemiological parameters. Adult trapping and molecular-detection protocols can be used to monitor the frequency of the anti-parasite gene in the mosquito population, but the capturing methods have to be adapted to the specific habits and distribution of the target species [72]. These data collecting efforts are important as they allow refining predictions of how soon we expect to see an epidemiological impact of the releases.

The specifics of evaluating the epidemiological parameters are adapted from the certification procedures developed for all elimination efforts [8]. These include surveillance mechanisms (active and passive) with full coverage of the target-site areas and reliable laboratory services to diagnose malaria. It is essential that there be full reporting of malaria cases by public and private health services with gold-standard validation of every malaria infection. This will require established and competent health services for detection, treatment, and follow-up of all possible malaria cases.

The length of the trial will be an important consideration. Since the WHO elimination certification requires at least 3 years absence of locally transmitted malaria [8], this should be the minimum trial duration. Hopefully, this will be a period long enough to calibrate stochastic factors (e.g., droughts, human migration, political instability affecting other control practices) that could confound the interpretation of the results. Limitations on the overall length depend mostly on the project showing initial success, financial support, and continued enthusiasm [36]. Two 5-year increments with annual trial review should be sufficient and definitive.

SUMMARY AND CONCLUSIONS

Population modification strategies can play a crucial role in the malaria eradication agenda. They will consolidate elimination gains by providing resistance to parasite and competent vector reintroduction and allow resources to be focused on new sites while at the same time providing confidence that treated areas will remain malaria-free. Strategic planning in three major areas, field-site selection, selection of mosquito strain, and trial design and implementation, is needed to achieve success in the first field trial. Key components of site selection include comprehensive knowledge of vectors and disease epidemiology, and local scientists who can take lead roles in trial

design and implementation, and regulatory and community engagement efforts. The trial site should be geographically limited to ensure the ability to monitor it successfully and to manage costs. The preferred population modification stain should carry multiple genes for parasite resistance to assure mosquitoes have no human-infectious forms of the parasites and no probability of selecting for resistance parasites. The strain has to be sufficiently reproductively competitive to achieve gene fixation and designed such that it can be remade easily if the genes cease to function. Finally, the strain should be designed to incorporate safety features that prevent it from being a hazard to humans and the environment. Trial design and implementation should not be a strictly academic exercise, and should involve a team comprising local scientists, when possible, with specific expertise and an explicit decision-making process. The trial design is likely to be longitudinal and compare before-and-after effects on malaria epidemiology. The trial should be integrated with other malaria control efforts being conducted in the same region and requires a competent and efficient local public health system. Results should be evident and unequivocal in 3–5 years from the initial onset of releases.

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