Field trials of gene drive mosquitoes: Lessons from releases of genetically sterile males and *Wolbachia*-infected mosquitoes

John M. Marshall[1,2], Valeri N. Vasquez[3,4]

[1] Division of Epidemiology & Biostatistics, School of Public Health, University of California, Berkeley, CA 94720, USA
[2] Innovative Genomics Institute, Berkeley, CA 94720, USA
[3] Energy & Resources Group, University of California, Berkeley, CA 94720, USA
[4] Berkeley Institute for Data Science, University of California, Berkeley, CA 94720, USA

Abstract:

The discovery of CRISPR-based gene editing and its application to homing-based gene drive has been greeted with excitement, for its potential to control mosquito-borne diseases on a wide scale, and concern, for the invasiveness and potential irreversibility of a release. At the same time, CRISPR-based gene editing has enabled a range of self-limiting gene drive systems to be engineered with much greater ease, including: i) threshold-dependent systems, which tend to spread only when introduced above a certain threshold population frequency, and ii) temporally self-limiting systems, which display transient drive activity before being eliminated by virtue of a fitness cost. As these CRISPR-based gene drive systems are yet to be field tested, plenty of open questions remain to be addressed, and insights can be gained from precedents set by field trials of other novel genetics-based and biological control systems, such as trials of *Wolbachia*-transfected mosquitoes, intended either for population replacement or suppression, and trials of genetically sterile male mosquitoes, either using the RIDL system (Release of Insects carrying a Dominant Lethal gene) or irradiation. We discuss lessons learned from these field trials, and implications for a phased exploration of gene drive technology, including homing-based gene drive, chromosomal translocations, and split gene drive as a system potentially suitable for an intermediate release.

Introduction:

The discovery of CRISPR and its application as a gene editing tool has enabled gene drive systems to be engineered with much greater ease (Doudna and Charpentier, 2014; Champer *et al.*, 2016). Recent attention has focused on homing-based drive systems and their potential to control mosquito-borne diseases on a wide scale, either by spreading disease-refractory genes (Gantz *et al.*, 2015), or by spreading genes that confer a fitness load or sex bias thereby suppressing mosquito populations (Hammond *et al.*, 2016; Kyrou *et al.*, 2018). However, there is growing awareness of the invasiveness of homing-based drive systems (Noble *et al.*, 2018), and interest in alternatives that could be confined to partially isolated populations and remediated – properties that are well aligned to the conduct of field trials (Marshall and Akbari, 2018).

In addition to homing-based gene drive, the increased ease of gene editing has advanced the entire field of gene drive, including systems that, by design, limit their spread in space and time (Marshall and Akbari, 2018). Such systems would ideally be capable of enacting local population control by: a) effectively spreading into populations to the extent required to achieve the desired epidemiological or ecological effect, and b) being recallable from the environment in the event of unwanted consequences, public disfavor, or the end of a trial period. Two varieties of these systems have been recently engineered: i) threshold-dependent systems that tend to spread when introduced above a certain population frequency (Akbari *et al.*, 2013; Buchman *et al.*, 2018), and ii) temporally self-limiting systems that display transient drive activity before being eliminated by virtue of a fitness cost (Gould *et al.*, 2008; Li *et al.*, 2020).

In this chapter, we discuss considerations for field trials of gene drive systems, with a specific focus on confinement and reversibility criteria, and lessons learned from other genetics-based and biological control systems (Table 1). We pay special attention to reciprocal chromosomal translocations (Buchman *et al.*, 2018), as an example of a threshold-dependent system that is confineable and reversible, and then extend our consideration to CRISPR-based homing gene drive systems, and temporally self-limiting systems, such as split gene drive (Li *et al.*, 2020), which could be used as confineable and reversible intermediate systems in a development pathway of homing-based systems. While these gene drive systems are yet to be trialed in the wild, lessons can be learned from trials of several varieties of sterile male mosquitoes, specifically, those sterilized through radiation (sterile insect technique, SIT), transfection with *Wolbachia* (incompatible insect technique, IIT) (Zheng *et al.*, 2019; Crawford *et al.*, 2020), and release of insects carrying a dominant lethal gene (RIDL) (Harris *et al.*, 2011; Carvalho *et al.*, 2015), as well as releases of *Wolbachia*-infected mosquitoes for population replacement (Hoffmann *et al.*, 2011). We begin by discussing trials of these systems, and discuss threshold-dependent, self-limiting and non-localized gene drive systems in this context.

Lessons from releases of genetically sterile male insects:

Releases of irradiated sterile male insects as a means of population suppression have been discussed since the early 20th century (Klassen and Curtis, 2005), and a transgenic version of this technology was the first transgenic mosquito product to be trialed in the field (Harris *et al.*, 2011). As the first transgenic mosquito release, this intervention has come under high levels of scrutiny, and serves as an important case study for potential releases of gene drive mosquitoes. The traditional SIT approach involves mass rearing insects and applying a carefully calibrated amount of radiation such that their genetic material is mutated to render them sterile while still being able to compete for female mates in the field (Knipling, 1955). Upon release, sterile males (preferably the majority of released insects) seek out wild females, essentially wasting their reproductive potential as the females produce no or significantly less viable offspring. Consecutive releases over a sufficiently wide area result in less productive matings, and a progressive reduction in insect population size over subsequent generations (Hendrichs *et al.*, 2004).

The most widely celebrated application of SIT involved the use of ionizing radiation to eradicate the screw-worm fly, *Cochliomyia hominivorax*, from North America – a program that began in 1957 following successful field trials on the Island of Curacao, and continues to this day to prevent re-invasion of the continent (Wyss, 2000; Klassen and Curtis, 2005). In this intervention, large-scale releases of sterilized insects led the screw-worm fly population in the United States to crash within a decade. Subsequent releases progressively shifted the eradication zone southwards, eventually covering all of North and Central America by 2001 (Robinson, 2002).

The success of the screw-worm SIT project motivated the application of SIT to a range of other insect pest species, including mosquito vectors of disease (Knipling, 1968). Both irradiation and chemosterilization were initially explored for applications to mosquitoes, and in the 1960's and 70's, large SIT field trials were conducted using chemosterilized *Culex quinquefasciatus* in India and *Anopheles alabimanus* in El Salvador (Klassen and Curtis, 2005). The trial in India was halted in the mid-70's, following false accusations that the project was being used to collect data to engage in biological warfare (Nature, 1975), highlighting the importance of effective community and political engagement for international biocontrol programs. Nevertheless, benefits of chemosterilization were demonstrated for this particular intervention due to reduced fitness costs as compared to irradiation.

A significant advancement in SIT technology was ushered in with specific DNA changes introduced by the RIDL construct (Thomas *et al.*, 2000; Alphey, 2002). Insects sterilized through mutagens are subject to a myriad of random genetic mutations, which are invariably associated with significant fitness costs. In theory, releases of insects carrying (in homozygous form) a dominant lethal gene (RIDL) have essentially the same population impact as SIT – i.e. offspring of released males are unviable – although in a more controlled way that has potential for smaller associated fitness costs. Benedict and Robinson (2003) argued that a transgenic version of SIT should be the first application of transgenic mosquitoes in the wild (as it was), both for enhanced efficacy, and for biosafety features – i.e. lethality-inducing transgenes should be quickly eliminated from the environment, causing the intervention to be reversible within a few generations. Quick elimination of transgenes also leads to confinement, since released mosquitoes can only travel so far in a few generations.

Sterile insect approaches based on genetic engineering present more opportunities than those based on mutagenesis, as genes and their associated traits can be modified in a more precise way. The original RIDL strain in *Aedes aegypti*, OX513A, causes lethality in both female and male offspring (bisex-RIDL) (Thomas *et al.*, 2000); however, an alternative construct was engineered soon after that only causes female offspring to be inviable (female-specific RIDL, or fs-RIDL). This allows the population-suppressing trait to persist for a few more generations through the male line, while continuing to suppress the female population, which is effective since only female mosquitoes bite and transmit diseases to humans. Furthermore, the introduced trait is late-acting, affecting the development of wing muscles in adult females (Fu *et al.*, 2010). This has the benefit that viable population reduction is not seen until the adult stage, delaying the reduction in larval density, and hence maintaining high larval mortality rates for longer due to density-dependent competition of larvae in breeding sites (Black *et al.*, 2011).

The first field trials of *Ae. aegypti* mosquitoes having the RIDL construct were conducted using the OX513A strain in Malaysia and the Cayman Islands. In Malaysia, Oxitec Ltd. and the Institute for Medical Research, Malaysia worked closely with the Malaysian Government in conducting a risk assessment. Releases were carried out in an uninhabited area to assess the mortality and dispersal characteristics of released RIDL mosquitoes; however, negative reactions were encountered from non-governmental organizations and the media, preventing a trial from being conducted in an inhabited area where the impact on wild *Ae. aegypti* populations could be assessed (Enserink, 2011).

In the Cayman Islands, Oxitec Ltd. worked with the local Mosquito Research and Control Unit (MRCU), initially conducting smaller releases over the course of four weeks to assess the fitness of genetically modified (GM) sterile males relative to wild males, and subsequently conducting a population suppression field trial over the course of several months, again using the OX513A *Ae. aegypti* strain. In a lab cage study, GM sterile males were found to be more-or-less of equal competitiveness in mating with wild females and the lethality trait was found to be effective in all crosses between GM sterile males and wild females (Harris *et al.*, 2011). Subsequent field releases over a four week period found that GM males successfully mated with wild females in the field, and fertilized their eggs resulting in unviable offspring; however the field competitiveness of the GM males was estimated at ~56% that of wild males, albeit with a very wide 95% confidence interval of 3.2-197% (Harris *et al.*, 2011).

The subsequent suppression field trial in the Cayman Islands was carried out across three contiguous areas on Grand Cayman island (denoted areas A, B and C) over a period of 23 weeks (Harris *et al.*, 2012). The initial goal had been to achieve a 10:1 GM-to-wild male ratio by releasing across all three areas (55 hectares in total); however production limitations led the actual achieved ratio to be significantly less (~2:1 GM-to-wild males), and a subsequent release in areas A and B still only achieved a ratio of ~5:1 GM-to-wild males. The third phase of the release was carried out solely in area A, achieving a release ratio of ~25:1 GM-to-wild males, and demonstrating the benefit of a smaller trial area. Another benefit of the area A release was that area C served as a control, and area B served as a buffer region. Significant population reduction was seen in this phase, with an 80% reduction in the mean ovitrap index in area A relative to areas B and C over the last seven weeks of the release period (Harris *et al.*, 2012).

Releases of GM sterile males in the Cayman Islands faced some controversy (Nightingale, 2010; Enserink, 2010); however, the major criticisms concerned the manner in which information about the trials was disseminated, rather than the conduct of the trials themselves. The releases did abide by national regulations, in particular, a draft biosafety bill that had yet to become law, the MRCU obtained a permit from the Cayman Islands Department of Agriculture, and a risk analysis and environmental impact assessment was carried out. The degree of community engagement was questioned, however, with several groups complaining they had not been given details of the releases in advance (Enserink, 2010).

Subsequent releases in Brazil followed a much more transparent approach. From the outset, a joint project was agreed to, the Projecto Aedes Transgênico (PAT), between the University of São Paulo and Oxitec Ltd. to explore the potential use of GM sterile male *Ae. aegypti* as a form of urban mosquito control in terms of its social, technical and operational dimensions. The project was launched by Moscamed, a Brazilian not-for-profit organization dependent on the Brazilian Ministry of Agriculture. The project enjoyed significant support in its early years as the government and public were aware of dengue outbreaks caused by this mosquito, and governmental support showed that they were being proactive in using the latest technology to control these outbreaks. The PAT worked closely with the Brazilian regulatory system to obtain required permits for field activities, and adopted a vigorous community engagement campaign including school presentations, public events, interviews on TV and radio, house visits, and involvement of the community in trap monitoring and surveillance (de Campos *et al.*, 2017).

The most well documented trial of GM sterile male *Ae. aegypti* in Brazil was carried out in the Itaberaba suburb of the city of Juazerio in Bahia, Brazil. This site had generally low socioeconomic indicators, and relied on stored water to a large extent, providing breeding sites for mosquitoes and leading to relatively high dengue transmission. Similar to the Cayman Islands, the study area was divided into treatment areas A and B and a control area, with treatment eventually being restricted to area A in order to maintain sufficiently high release ratios. A Moscamed mass rearing facility was built specifically for the project, producing millions of GM sterile males over the course of the study. Releases began with a “rangefinder” phase lasting a little over a month, which allowed the release requirements to be calibrated, and estimates of parameters such as male mating competitiveness to be refined. GM male mating competitiveness was estimated to be ~3.1% that of wild males (95% CI: 2.5-3.6%), suggesting that releases for the “suppression” phase would need to be increased nine-fold in order to achieve the target of 50% of mating events involving a GM sterile male (Carvalho *et al.*, 2015).

The GM sterile male field trial in Brazil was successful, achieving a ~95% reduction in mosquito density at the release site, albeit with large release requirements of ~140,000 mosquitoes per week over a 5.5 hectare control site for ~3 months (Carvalho *et al.*, 2015). Enthusiasm for the GM sterile male approach was initially raised when the Zika outbreak began in 2015; however, an unexpected complication arose as untrue claims began to circulate in social media linking the Zika outbreak to past releases of the GM mosquitoes (de Campos *et al.*, 2017). This draws attention to the importance of an enduring community engagement effort as well as political engagement and stakeholder messaging.

While not an invasive technology, these releases of sterile male mosquitoes do provide lessons from which potential field trials of gene drive mosquitoes may learn. Releases of both chemosterilized *C. quinquefasciatus* in India, and of GM sterile *Ae. aegypti* in Brazil, highlight the crucial importance of an effective and sustained community engagement effort. This especially applies to technologies developed in the Global North and applied in the Global South, which provide much potential for community mistrust. Furthermore, releases of GM sterile *Ae. aegypti* in both the Cayman Islands and Brazil highlight the importance of choosing a study site in which the required release sizes can be achieved, and in conducting a rangefinder

release phase to refine release requirements. For threshold-dependent gene drive systems, this will be important to determine release sizes that exceed the threshold, while for non-localized gene drive systems, this will be important to determine release sizes that are expected to demonstrate population control within the timeframe of the trial.

Lessons from the *Wolbachia*-based incompatible insect technique:

A promising alternative to SIT and GM sterile male releases is IIT, in which male mosquitoes are released that are infected with a *Wolbachia* strain absent from the wild population, resulting in sterile matings with wild females that lack the *Wolbachia* strain due to a phenomenon referred to as cytoplasmic incompatibility (CI) (LePage *et al.*, 2017) (Figure 1). This strategy has proceeded with much less resistance than GM approaches in recent years, and serves as a case study for potential releases of novel biological control technologies, particularly regarding the use of factory rearing facilities (Zheng *et al.*, 2019; Crawford *et al.*, 2020).

The first field trial of IIT was conducted in Burma (now Myanmar) in 1967. The technique was seen as an alternative to insecticide-based strategies given growing insecticide resistance among the target species, *Culex pipiens fatigans*, a vector of lymphatic filariasis (LF) which had proliferated in South East Asia at the time (Laven, 1967). Despite successful elimination of the vector species from that trial site, the approach has not been deployed operationally until recently due to concern that accidental releases of *Wolbachia*-infected fertile females could result in the *Wolbachia* strain spreading into the population and preventing further suppression efforts. This is because *Wolbachia* is maternally inherited, and in most cases, the only incompatible crosses are between infected males and uninfected females. In 2009 and 2010, however, subsequent trials were carried out in French Polynesia to suppress populations of *Aedes polynesiensis*, a primary vector of LF in the South Pacific (O'Connor *et al.*, 2012). Results from those field experiments showed that: i) *Wolbachia*-transfected *Ae. polynesiensis* males successfully competed for mates following release, and ii) the trial did not result in population replacement eventuating.

In the last few years, two factory-scale IIT projects have moved forward to achieve community-scale mosquito population suppression: i) an IIT program supplemented with sterilizing irradiation (also termed IIT-SIT) in Guangzhou, China (Zheng *et al.*, 2019), and ii) an IIT program supplemented with factory-scale automation of production and sex-sorting in Fresno, California (Crawford *et al.*, 2020). The two projects represent different approaches to prevent population replacement: i) through greatly reducing the fertility of any *Wolbachia*-infected females that may be accidentally released, and ii) through using automation and machine learning to reduce the number of accidentally-released *Wolbachia*-infected females effectively to zero.

In the IIT-SIT program in Guangzhou, *Aedes albopictus*, the main vector of dengue and other arboviruses in Guangzhou, were generated having an artificial triple *Wolbachia* infection (termed HC), through the addition of the *w*Pip *Wolbachia* strain to the native double infection of the *w*AlbA and *w*AlbB strains of *Wolbachia*. High levels of CI were confirmed, such that matings of HC males with wild females produced no viable offspring, and maternal transmission of the

triple *Wolbachia* infection was confirmed, allowing efficient mass production of HC males. HC males were exposed to low-dose irradiation at the pupal stage to reduce the fecundity of any accidentally released HC females, and semi-field cage studies confirmed that the irradiated HC males effectively competed for mates leading to population suppression, without population replacement occurring due to released HC females. Furthermore, as an additional safety precaution, HC females were shown to be less competent at disease transmission than their wild counterparts (Zheng *et al.*, 2019).

A trial carried out by the Wolbaki Biotech Company in 2016-2017 demonstrated the high degree of population suppression possible when factory rearing of mosquitoes is involved. Irradiated HC males were released on a weekly basis on two riverine islands within the jurisdiction of Guangzhou, with the ratio of released HC to wild males varying between 8.7:1 and 15.8:1 over the 38 week intervention period. Population suppression was highly successful, achieving a >94% reduction in the number of hatched eggs per ovitrap, as compared to control sites, and an 83-94% reduction in the number of wild adult females caught per trap. The success of the program also led to a significant increase in community support, with interviews suggesting 13% of residents were supportive prior to the intervention (notably, with 76% being neutral), and 54% were supportive following the intervention (Zheng *et al.*, 2019).

The IIT program in Fresno, CA showcased the role that large-scale, automated rearing and sex-sorting of mosquitoes can play in increasing the scale of an IIT intervention. In this case, *Ae. aegypti*, the main arboviral vector through much of the Americas, was transfected with the *w*AlbB strain of *Wolbachia*, and sterility of crosses between infected males and wild females was confirmed. An automated larval rearing system was designed that, at maximum capacity, was able to produce almost 3 million pupae per week. A multi-step sex-separation process was then designed that removed 95% of females at the pupal stage, and the remainder at the adult stage based on a machine learning algorithm informed by photographic images as emerging adults walked down a narrow path. Estimates from the operation of this system suggested that a single *Wolbachia*-infected female mosquito would be released for every 900 million males, making the sex-sorting system near-perfect (Crawford *et al.*, 2020).

A trial carried out through a partnership between the Debug Project of Verily Life Sciences, MosquitoMate and the Consolidated Mosquito Abatement District of Fresno County in 2018 demonstrated dramatic population suppression over an area nine times larger than that of the Guangzhou study. A total of more than 14 million *Wolbachia*-infected males were released as part of the study (an average of more than 78,000 per day), which led to a 96% reduction in the wild adult mosquito population; however, despite the large size of the releases, elimination was not achieved, likely due to inward migration of wild mosquitoes from neighboring untreated areas (Crawford *et al.*, 2020). A public information campaign was conducted around the trial; however, formal documentation of this campaign is not yet available. A similar project is currently underway in Singapore through a partnership between Verily Life Sciences and the National Environment Agency of Singapore.

While neither a transgenic nor invasive technology, these IIT releases do provide lessons regarding the scale of releases that can be achieved when investment is made into automated rearing and sex-sorting facilities. Release requirements for low-threshold gene drive mosquitoes will be orders of magnitude lower than those for sterile male releases, and hence a facility capable of producing tens of millions of mosquitoes, such as the one designed by Verily Life Sciences, would be capable of achieving control over a much greater spatial scale than for IIT. The technological capacity for sex-sorting is also encouraging given that male mosquitoes don't bite or transmit diseases to humans, and hence may also be preferable for gene drive mosquito releases. The IIT releases enjoyed much less resistance from communities and regulatory agencies than GM sterile male releases, despite acting through a similar mechanism, highlighting issues that trials of gene drive mosquitoes will likely also face and must invest in.

Lessons from *Wolbachia*-based population replacement:

A second approach to the use of *Wolbachia* to control mosquito-borne disease transmission is to intentionally include *Wolbachia*-infected females in a release. In IIT, care is taken to only release *Wolbachia*-infected males, as CI causes matings between *Wolbachia*-infected males and wild females to be sterile; however CI-induced sterility, combined with the fact that *Wolbachia* is maternally inherited, provides an inheritance bias in favor of *Wolbachia* when *Wolbachia*-infected females are also present (Turelli and Hoffmann, 1991) (Figure 1). For *Wolbachia* strains that also block pathogen transmission, this can be used to drive the pathogen-blocking trait into the mosquito population (Morieria *et al.*, 2009). This strategy has advanced significantly over the last decade (Hoffmann *et al.*, 2011) and like IIT, has faced much less resistance than GM strategies. It serves as an interesting case study for potential releases of transgenic population replacement technologies, as it has faced many of the non-GM issues that future gene drive programs will face.

The first *Wolbachia* population replacement program was carried out by the Eliminate Dengue project (now known as the World Mosquito Program) in the communities of Yorkeys Knob and Gordonvale in Queensland, Australia (Hoffmann *et al.*, 2011). In this program, *Ae. aegypti*, the main vector of dengue and other arboviruses in Queensland, was transfected with the *w*Mel strain of *Wolbachia* from *Drosophila melanogaster*, a strain that has been shown to: i) block dengue transmission, ii) have a small associated fitness cost, and iii) be capable of driving into a small field cage (Walker *et al.*, 2011). *Wolbachia* displays threshold properties in the presence of a fitness cost such that releases above a certain population frequency tend to spread, while releases below that frequency tend to be eliminated. The exact value of the threshold is determined by the point at which the inheritance bias induced by CI outweighs the fitness cost associated with the infection, and has been estimated at ~20-30% for the *Wolbachia* strain used in this release (Hoffmann *et al.*, 2011; Hancock *et al.*, 2019).

The releases in Yorkeys Knob and Gordonvale were a clear success - after 10 weekly releases of 11,000-22,000 *Wolbachia*-infected *Ae. aegypti* per week, the *Wolbachia* infection reached near-fixation in both populations within three months, despite a tropical storm postponing one of the releases in Gordonvale (Hoffmann *et al.*, 2011) (Figure 2). The finer details of this program

provide an excellent example of how gene drive systems may be successfully trialled in the future. To begin, they highlight the importance of a detailed monitoring effort and adaptive release protocol. The releases in Yorkeys Knob and Gordonvale were accompanied by a network of 29 BioGents Sentinel mosquito traps that monitored *Wolbachia* infection frequency at the block-level. Heterogeneity in *Wolbachia* infection frequency was observed, and releases were supplemented in areas where *Wolbachia* frequency was low.

Monitoring for unintended spread outside the study area was also conducted, and this did indeed reveal limited long-distance spread into a neighboring suburb from Yorkeys Knob, and across a freeway from Gordonvale (Hoffmann *et al.*, 2011). Although these migrants were expected to be lost due to being present at subthreshold levels, continued monitoring was important to confirm this. Continued monitoring was also conducted at the trials sites to confirm enduring intervention efficacy, and while the *Wolbachia* infection remained at near-fixation for several years following the release, a low frequency of uninfected mosquitoes has also persisted, likely due to immigration (Hoffmann *et al.*, 2014).

The releases in Yorkeys Knob and Gordonvale also highlight the importance of preparing for unexpected events. In addition to the tropical storm that affected both release sites and postponed one of the releases in Gordonvale, releases in a portion of Yorkeys Knob ceased two-thirds of the way into the intervention following a reported dengue case (Hoffmann *et al.*, 2011). Although this dengue case likely originated elsewhere, a reactive insecticide intervention was carried out in surrounding households in agreement with local disease control protocols. Trials of mosquitoes with gene drive systems should make allowances for events such as these. Encouragingly, the *Wolbachia* infection continued to spread through the Yorkeys Knob *Ae. aegypti* population despite this, and no secondary dengue cases were documented following the reported case.

The Yorkeys Knob and Gordonvale releases provide an example of a successful community and regulatory engagement process. Community engagement was carried out over two years leading up to the releases, and consisted of informal interviews, semi-structured in-depth interviews, qualitative and quantitative surveys, focus groups, historical research, and face-to-face presentations at community meetings (Hoffmann *et al.*, 2011; McNaughton, 2012). Issues explored through these activities included the sociopolitical context, lay knowledge of dengue fever and biological control programs, and the acceptability of the project. Community members did raise concerns about a previous local biological control program - the introduction of the cane toad near Gordonvale in the 1930's. Largely seen as a failed biological control program, this was raised as a cautionary tale indicating the limits of scientific knowledge and the unpredictability of ecological interventions (McNaughton, 2012).

The Queensland releases enjoyed substantial community support, with 85% of respondents viewing *Wolbachia* as an acceptable dengue prevention strategy in a March 2010 telephone survey (ahead of insecticides, at 66% acceptance), and 84% of respondents stating they would support a release that they were informed and updated about, that had regulatory oversight, and that was shown to be safe for people and the environment by a risk assessment carried out by

Australia's Commonwealth Scientific and Industrial Research Organization (CSIRO) (McNaughton, 2012). The releases were ultimately approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA) following a risk assessments by CSIRO (Murphy *et al.*, 2010) and the APVMA with support from the Federal Commonwealth Government's Department of the Environment, Water, Heritage and the Arts (Marshall, 2011). The World Mosquito Program is now exploring application of their technology beyond Australia, with active collaborations throughout Latin America, Asia and Oceania.

In summary, key lessons from the *Wolbachia*-based population replacement strategy include the importance of: i) a detailed monitoring protocol to assess heterogeneity of spread at the field site, ii) an adaptive release scheme to supplement releases in areas of low *Wolbachia* frequency, iii) additional monitoring to assess levels of unintended spread to neighboring areas, and iv) preparing for unexpected events. The fact that *Wolbachia* infection has the potential to persist in the mosquito population for extended periods, and perhaps indefinitely, also emphasizes the need for a long-term, comprehensive and multifaceted community engagement program.

Considerations for trials with reciprocal chromosomal translocations:

Lessons from field trials of *Wolbachia*-based population replacement systems apply most closely to threshold-dependent gene drive systems, which are also expected to spread if released above a certain threshold frequency, and to be eliminated if present below that frequency. One of the first of these systems to be proposed (Serebrovskii, 1940; Curtis, 1968), and perhaps currently one of the most promising (Sánchez *et al.*, 2020), is reciprocal chromosomal translocations. These result from a mutual exchange between terminal segments of two nonhomologous chromosomes and produce a heterozygote reproductive disadvantage because, when translocation heterozygotes mate, several crosses result in unbalanced genotypes and hence unviable offspring. This produces a threshold frequency of 50%, which increases in the presence of a fitness cost (Curtis, 1968). Early attempts to generate translocations through radiation-induced mutagenesis were abandoned due to high associated fitness costs (Laven *et al.*, 1972; Lorimer *et al.*, 1972); however, interest has been reignited as site-specific translocations have recently been generated using CRISPR (Lekomtsev *et al.*, 2016; Jiang *et al.*, 2016), and translocations generated in *D. melanogaster* using endonucleases were recently shown to drive in laboratory experiments with a threshold frequency of ~50% (Buchman *et al.*, 2018).

A recent modeling study suggests that translocations represent one of the best systems to implement in field trials due to their symmetrical threshold properties and strong confinement potential. A key advantage of translocations is that releases required to introduce them into a population are of a similar magnitude to wildtype releases required to eliminate them once they have been introduced (Sánchez *et al.*, 2020). Population replacement and reversion were modeled at the household level in the suburb of Yorkeys Knob, the site of the *Wolbachia* population replacement study, with low levels of migration modeled to the neighboring suburb of Trinity Park in Queensland, Australia (Figure 2). Population replacement could be achieved in

simulations with seven or more weekly releases of 20 *Ae. aegypti* males homozygous for the translocations per household per week (a similar magnitude to that used in the *Wolbachia* population replacement trial at the same site) and for a coverage of 50% of the households in the community. Elimination could be achieved for the same release scheme using wild *Ae. aegypti* mosquitoes.

One benefit of translocations, and other underdominant systems that have a threshold in the absence of a fitness cost, is that their release threshold is more robust than that for *Wolbachia*, which only arises in the presence of a fitness cost. This property leads to translocations being more robustly confineable to a field site than a *Wolbachia* infection, since they are unlikely to exceed the release threshold in a neighboring population purely through migration, even if they spread to near-fixation at the trial site. In the translocations modeling study in Yorkeys Knob and Trinity Park (Sánchez *et al.*, 2020), it was considered unlikely that *Ae. aegypti* mosquitoes would travel from one suburb to another by their own flight, especially in numbers sufficient to exceed the release threshold there, and so “batch migration” was instead considered, in which several mosquitoes are carried, perhaps by a vehicle, from one suburb to another at once. Batch migration events were modeled as occurring between randomly chosen neighborhoods, and the number of daily migration events and effective number of adults carried per event were varied. Results from this modeling study made a strong case for the potential to confine translocations to the release site, as the number of daily migration events required for the translocation to exceed the threshold in the neighboring suburb exceeded those inferred from field data. Specifically, 3-4 daily migration events consisting of batches of 10 adults were required for translocations to spillover to the neighboring suburb in simulations (Sánchez *et al.*, 2020), while field data suggested 1-2 daily migration events consisting of batches of less than 5 adult females (Hoffmann *et al.*, 2011).

Collectively, these modeling results for translocations are encouraging for the potential to conduct field trials of *Ae. aegypti* mosquitoes with translocations because: i) translocations could be introduced on a suburban scale, and remediated through releases of non-disease-transmitting male mosquitoes with release sizes on the scale of what has been previously implemented, and ii) spillover of translocations into neighboring suburbs is unlikely. Lessons for the conduct of field trials with translocations may be drawn from the field trials previously described in this chapter - most importantly, for *Wolbachia*-based population replacement. These lessons highlight the importance of a detailed monitoring effort, including outside the study area, and for an adaptive release protocol that can respond to heterogeneities in spread at the trial site. They also highlight the importance of preparing for unexpected events, and for conducting a long-term and comprehensive community engagement program, given that translocations have the potential to persist in the environment long-term. A comparison of the RIDL and IIT releases suggests that community engagement and regulatory requirements for translocations may be stricter than for those for *Wolbachia* due to the fact that mosquitoes with translocations, generated using CRISPR or other endonucleases, will be considered GM organisms. Finally, regarding the release protocol, including a rangefinder release phase may help to refine fitness cost estimates and release requirements for translocations, as per a lesson from RIDL field trial in Brazil.

Considerations for trials with CRISPR-based gene drive systems:

Finally, lessons from the field trials discussed here have implications for the spectrum of CRISPR-based gene drives, from those that are non-localized to those that are self-limiting. Recent attention has focused on CRISPR-based homing gene drives, for their ability to spread widely and their potential to control vector-borne diseases on a wide scale (Gantz *et al.*, 2015; Kyrou *et al.*, 2018); however, there are also threshold-dependent gene drive systems that can now be engineered using CRISPR, such as chromosomal translocations (Buchman *et al.*, 2018) and various forms of underdominance (Akbari *et al.*, 2013), as well as temporally self-limiting gene drive systems, such as split drive (Li *et al.*, 2020), which display transient drive activity before being eliminated by virtue of a fitness cost. The CRISPR revolution has also enabled gene drive countermeasures to be engineered, such as homing-based drive remediation systems, ERACR (Element for the Reversal of the Autocatalytic Chain Reaction) and e-CHACR (Eracing Construct Hitchhiking on the Autocatalytic Chain Reaction) (Gantz and Bier, 2016).

CRISPR-based homing gene drive systems bias inheritance in their favor by cleaving a highly-specific target sequence in the host genome and copying themselves to the cut chromosome through a mechanism known as homology-directed repair (Gantz and Bier, 2015; Champer *et al.*, 2016). For high homing efficiencies and low-to-moderate fitness costs, these systems are capable of driving into populations from arbitrarily low initial frequencies. This property allows them to spread widely, and hence they are considered “non-localized.” For these gene drive systems, while we may learn from field trials of *Wolbachia*-based population replacement systems, the scale of their potential spread and impact leads to additional and unique challenges that we must carefully consider.

One way to manage the risks associated with the potential wide-scale spread of homing-based gene drive systems is for testing to proceed iteratively through multiple phases, with each phase involving a larger spatial scale and a higher degree of human or environmental exposure (James *et al.*, 2018) (Figure 3). In this phased release pathway, initial studies are to be conducted in contained laboratories and insectaries, where product efficacy and safety is studied. Entering field testing is a big decision, given the anticipated difficulty of remediating a homing-based gene drive system that is capable of spreading widely. Large outdoor cages present one option for moving beyond the laboratory; however, this is not considered essential since some mosquito behaviors, such as mating, and parameters, such as fitness, can only be meaningfully studied in the field. Furthermore, studies in outdoor cages must anticipate the possibility of escape occurring, and hence similar safety and efficacy criteria must be met before either outdoor cage studies of small-scale isolated releases are performed. Initial outdoor testing should be conducted at field sites within which the gene drive system is expected to be contained, for instance, on oceanic islands, following which, open releases would be conducted on iteratively larger spatial scales (James *et al.*, 2018).

Another consideration for trials of non-localized gene drive systems is that regulators are likely to require that a remediation plan be in place prior to field testing (James *et al.*, 2018). The

chosen remediation strategy will depend on a number of factors, including the mode of action of the drive system and the scale and geography of the field site. A default remediation plan would be a large-scale insecticide-based campaign to eliminate the vector population at the field site. This would require an assessment of insecticide-resistance in the local vector population prior to the gene drive trial. Failing this, releases of non-disease-transmitting male mosquitoes carrying a drive-resistant allele that restores the function of the gene targeted by the drive system is an attractive option, especially if the drive-resistant allele is sourced from a wild population.

Gene drive countermeasures such as ERACR and e-CHACR are another option for remediation. The ERACR system consists of a second homing system with a target site corresponding to the original drive system, essentially removing the original system as it homes into it, while utilizing the Cas9 of the original system and thus removing that as well (Gantz and Bier, 2016). The e-CHACR system uses the Cas9 from the original homing system to home into a second site in the genome in addition to the site of the original drive system, thus driving itself into the population while removing the original system and its Cas9 in the process (Gantz and Bier, 2016). Both of these systems hold promise; but they may not be the first choice for remediation efforts as they introduce additional transgenes into populations from which transgenes are trying to be removed.

Another potential phased release pathway is to precede the release of a non-localized gene drive system with a self-limiting one. Ideally, such a release would provide insights into the expected behavior of the non-localized system, and hence there should be strong resemblance between the two systems, to the extent possible. For a CRISPR-based homing gene drive system, one possibility is to begin with a trial of a split drive system, in which the Cas9 and guide RNA components are separated at different loci (Li *et al.*, 2020). In the split drive system, transient drive activity occurs at the guide RNA locus when the Cas9 and guide RNA alleles co-occur in an organism; however, the Cas9 allele is gradually eliminated from the population due to its fitness cost, followed by the guide RNA if it also has a fitness cost. This transient drive activity also leads to spatial confinement, since a gene can only disperse so far in a limited number of generations. Intermediate technologies also exist for other systems. For instance, a driving Y chromosome that spreads by cleaving the X chromosome at multiple sites during spermatogenesis is expected to spread on a wide scale (Galizi *et al.*, 2014); however, if linked to an autosome, it is self-limiting, providing an opportunity for intermediate study in the field.

For self-limiting CRISPR-based gene drive systems that could be used as an intermediate system in a field trial, similar field trial considerations apply as for chromosomal translocations. Namely, the ability to confine the release to the trial site, and to remediate transgenes from the environment as needed, are great strengths. Furthermore, it is important to combine a detailed monitoring effort, both in and outside the trial site, with an adaptive release protocol to respond to heterogeneities in spread, and to make allowances for unexpected events. A rangefinder release phase may help to refine fitness cost estimates and release requirements.

For non-localized CRISPR-based gene drive systems, the potentially wide scale of spread and difficulty of remediation emphasize the need to monitor for the gene drive system both in and

outside the field trial area. Additionally, a rangefinder release phase may help to predict release schemes capable of achieving population control within the desired timeframe. Finally, as the spatial scale of the release grows, lessons may be learned from the experience of the Fresno IIT trial regarding automated rearing and sex-sorting of mosquitoes. Knowledge of the potential scale of mosquito production will allow us to set expectations for wide-scale vector-borne disease control.

As for all of the systems discussed in this chapter, effective community and regulatory engagement is essential prior to field trials of mosquitoes engineered with CRISPR-based gene drive systems; however this is especially important for trials of non-localized gene drive systems. Mosquitoes engineered with these systems are GM organisms capable of spreading widely, potentially across international borders, and are often developed in the Global North for application in the Global South. Their potential to spread across international borders highlights the desirability of a multi-country or regional agreement on their release, especially when a country that shares a border with another is being considered for field trials. Indeed, such agreements may be required by the Cartagena Protocol on Biosafety, which governs the safe transfer, handling and use of GM organisms (referred to as “living modified organisms”in the protocol), including their transboundary spread (Secretariat of the Convention on Biological Diversity, 2000; Marshall, 2010).

Conclusion:

The limitations of traditional insecticide-based strategies to control mosquito populations, and in particular, the widespread emergence of insecticide-resistance, has spurred interest in a variety of novel biological and genetics-based vector control strategies, including SIT, IIT, RIDL, *Wolbachia*-based population replacement, and CRISPR-based gene drive (Benelli *et al.*, 2016). Trials of RIDL, IIT and *Wolbachia* over the last decade provide a series of case studies from which we may learn in preparing for field trials of CRISPR-based gene drive systems (Table 2). There are challenges associated with gene drive technologies - notably, the controversies surrounding GM organisms, and the potential for spread across international borders. However, these challenges are also a reason for promise as half the world’s population is at risk of vector-borne diseases, and genetic engineering provides new opportunities to interfere with pathogen transmission. In learning from recent field trials, we seek to move these technologies forward carefully and responsibly toward the eventual goal of global vector-borne disease control.

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Tables:

Table 1. Genetics-based and biological mosquito control strategies and their potential to be confineable and reversible.

Strategy:	**Variant:**	**Mechanism of action:**	**Confineable:**	**Reversible:**
Sterile insect technique (SIT)	Ionizing radiation or chemosterilization	Offspring of released females and males are unviable	Yes	Yes
Wolbachia	Incompatible insect technique (IIT)	Offspring of released males are unviable	Yes, if no females released	Yes, if no females released
	Population replacement	Spreads through population due to cytoplasmic incompatibility	Yes, for moderate-to-high fitness costs	Possibly, for high fitness costs
Release of insects carrying a dominant lethal gene (RIDL)	Bisex (bi-RIDL)	Both female and male offspring having the RIDL allele are unviable	Yes	Yes
	Female-specific (fs-RIDL)	Only female offspring having the RIDL allele are unviable	Yes	Yes
Chromosomal translocations	CRISPR or other endonucleases	Translocation heterozygotes with unbalanced chromosome sets are unviable, leading to bi-stable dynamics	Yes	Yes
CRISPR-based gene drive	Homing-based drive systems	Bias inheritance by cleaving a target sequence and serving as a template for DNA repair, effectively turning a heterozygote into a homozygote	Potentially, but with difficulty	Potentially, but with difficulty
	Split gene drive systems	Components of drive system are split across two loci, leading to transient drive when they co-occur before being eliminated due to a fitness cost	Yes	Yes

Table 2. Significant field trials of novel biological and genetics-based mosquito control strategies.

Method:	**Species:**	**Location:**	**Year:**	**Outcome:**	**Reference:**
SIT	*Anopheles quadrimaculatus*	Florida, USA	1962	Poor mating competitiveness	Weidhaas *et al.* (1962)
IIT	*Culex pipiens fatigans*	Burma (now Myanmar)	1967	Successful suppression	Laven (1967)
SIT	*Culex quinquefasciatus*	India	1971-1975	Modest suppression	Singh *et al.* (1975)
SIT	*Anopheles albimanus*	El Salvador	1971-1979	Significant suppression	Lowe *et al.* (1980)
RIDL	*Aedes aegypti*	Cayman Islands	2009	Small-scale suppression	Harris *et al.* (2011, 2012)
IIT	*Aedes polynesiensis*	French Polynesia	2009-2010	Demonstration of efficacy	O'Connor *et al.* (2012)
Wolbachia population replacement	*Ae. aegypti*	Queensland, Australia	2011	Successful population replacement	Hoffmann *et al.* (2011)
RIDL	*Ae. aegypti*	Juaziero, Brazil	2012-2013	Community-scale suppression	Carvalho *et al.* (2015)
RIDL	*Ae. aegypti*	Jacobina, Brazil	2013	Suppression & resurgence	Garziera *et al.* (2017)
IIT	*Aedes albopictus*	Kentucky, USA	2014	Significant suppression	Mains *et al.* (2016)
IIT-SIT	*Ae. albopictus*	Guangzhou, China	2016-2018	Community-scale suppression	Zheng *et al.* (2019)
IIT	*Ae. aegypti*	California, USA	2018-2019	Community-scale suppression	Crawford *et al.* (2020)

Figures:

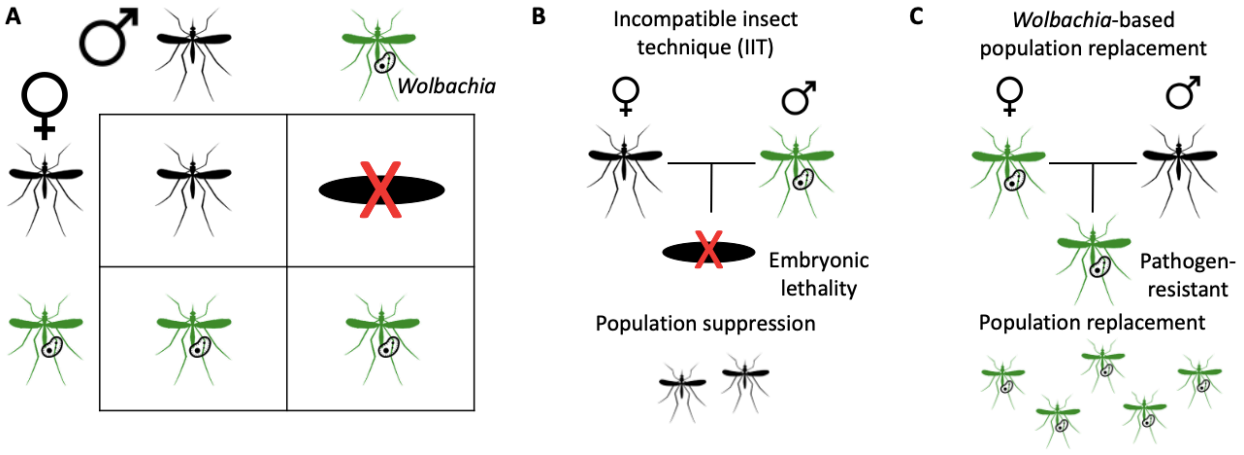

Figure 1. A. Use of *Wolbachia* as a means for both population suppression (incompatible insect technique, IIT) and population replacement hinges on the inheritance pattern in which crosses between *Wolbachia*-infected males and uninfected females produce unviable offspring due to cytoplasmic incompatibility (CI), while crosses involving *Wolbachia*-infected females produce *Wolbachia*-infected offspring due to *Wolbachia* being maternally inherited. **B.** In IIT, *Wolbachia*-infected males are released into a wild population lacking that strain of *Wolbachia*. This leads to population suppression as mating events involving *Wolbachia*-infected males produce no viable offspring. **C.** In *Wolbachia*-based population replacement, *Wolbachia*-infected females are included in the release. This leads to population replacement as CI biases inheritance in favor of *Wolbachia* when *Wolbachia*-infected females are present.

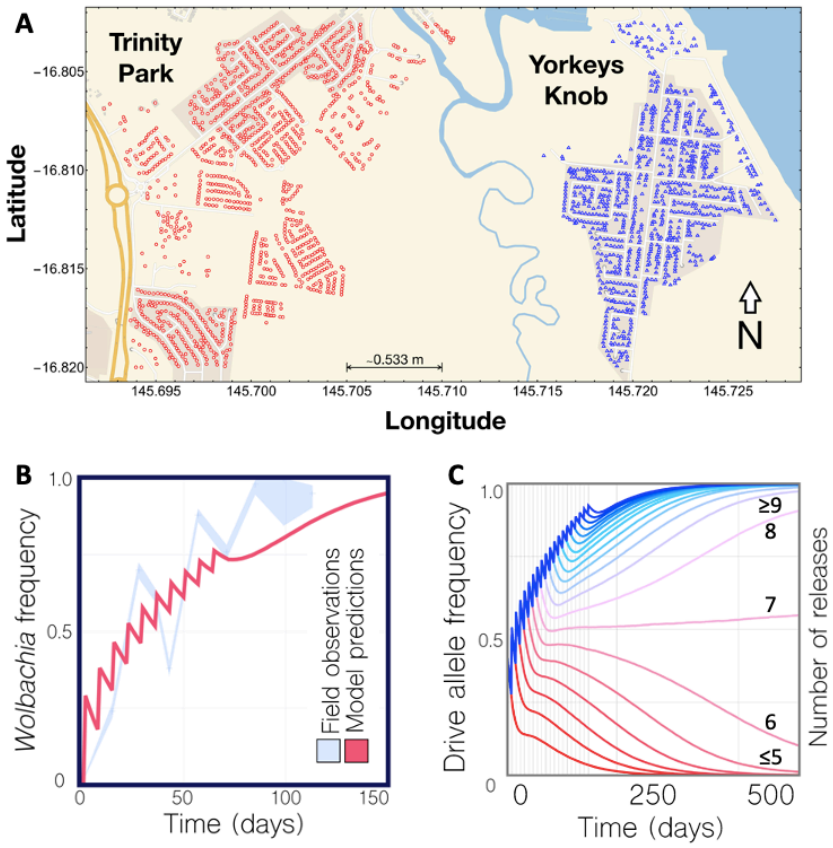

Figure 2. A. Landscape of Yorkeys Knob and Trinity Park in Queensland, Australia where field trials of *Wolbachia*-based population replacement for *Aedes aegypti* were carried out, and where trials of reciprocal chromosomal translocations were simulated. **B.** Blue lines depict data for *Wolbachia* frequency over time from the *Wolbachia* population replacement field trial conducted in Yorkeys Knob in 2011 (Hoffmann *et al.*, 2011), with line thickness representing 95% binomial confidence intervals around observed proportions. Red lines depict simulated data for an analogous release scheme consisting of 20 *Wolbachia*-infected mosquitoes per household at a coverage of 30% over 10 weeks, demonstrating good agreement with field data (Sánchez *et al.*, 2020). **C.** Translocation frequency over time for a given number of weekly releases of 20 adult male *Ae. aegypti* mosquitoes homozygous for the translocation per household with the intent of population replacement in the Yorkeys Knob. Results are depicted for a coverage of 50%, at which seven or more releases result in the translocation being driven into the population (Sánchez *et al.*, 2020). Due to the 50% threshold property of translocations, the same release scheme for wild-types can be used to remediate translocations from the population.

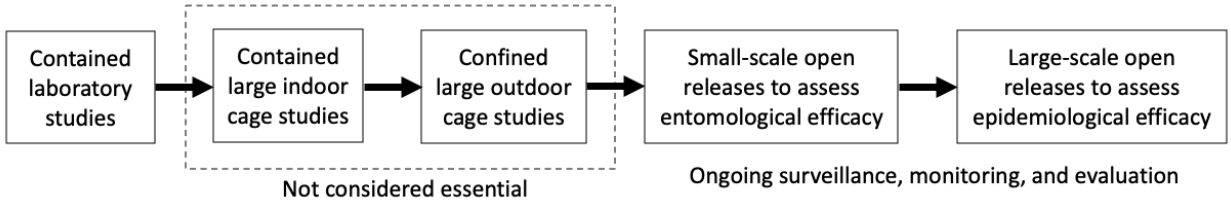

Figure 3. Phased release pathway for CRISPR-based homing gene drive systems.