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# Gene Drives

## Experience with gene drive systems that may inform an environmental risk assessment

perseus report

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**Experience with gene drive systems that may inform an environmental risk assessment**

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On the cover: *Aedes aegypti* mosquito taking a blood meal on a human skin.


## Disclaimer

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# Foreword

Gene drives are genetic elements that are inherited more frequently than expected. Usually, there is a 50% chance that a genetic element is present in the offspring of sexually reproducing organisms. Gene drives, however, have a mechanism that increases the frequency of their inheritance.

Several organisms are known to possess gene drives. The potential application of these natural gene drive systems to suppress populations of vectors of diseases (insects) has been studied in field trials since the sixties.

Lately, there is an increased interest in gene drives because of the possibilities the recently discovered CRISPR gene editing technique offers to develop synthetic gene drives. These synthetic CRISPR gene drives are presented as efficient tools to spread traits through a population: an individual carrying a CRISPR gene drive will produce offspring that all carry the gene drive.

CRISPR gene drives have many potential applications and provide for example great opportunities to suppress or alter insect populations that transmit diseases or reduce yields. There are, however, concerns about their safety. One concern is that the escape of a gene drive containing organism could alter or even eradicate natural populations. It has, however, been questioned whether traits with a negative impact on the organism will be able to spread efficiently through populations.

To obtain information on the behaviour of gene drives in previous field trials, COGEM has commissioned a research project. Perseus's assiduous search for information resulted in an elaborate report on natural gene drives and provides an overview of field trials that were carried out in the past. The advisory committee is pleased with the result and expects it to be a useful source for further reference.

Dr. Willem Jan de Kogel
Chair of the Advisory Committee.

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# Summary

Gene drives are genetic mechanisms that allow for a trait to be propagated throughout a population beyond Mendelian inheritance. Active in sexually-reproducing species, they are powerful tools to “drive” a gene, *i.e.* increase its frequency, independent of external selection pressure. They have been proposed as offering solutions for many challenges in public health, agriculture, conservation and others. They have inspired researchers to use gene drives to combat diseases transmitted by insects such as malaria, dengue and Zika.

For decennia attempts have been made to use or modify naturally occurring gene drive mechanisms. Yet, natural gene drives have their limitations. Transposable element-based drives turned out to be not efficient enough. Moreover, they cannot be directed. Translocation drives are hard to establish and suffer from a high fitness cost. Others are only active in specific species (*e.g.* meiotic drive, MEDEA).

In recent years advances in genetics have allowed for co-opting natural gene drive systems and the development of synthetic gene drive systems. Especially, since the discovery of the gene editing capabilities of the CRISPR/Cas9 system and the ability to use it as a gene drive system, interest for gene drives has increased as the technology dramatically enhances the abilities to engineer gene drives. CRISPR/Cas-based drives turn out to be very efficient, at least in the laboratory. At the same time concerns were raised regarding the safety of their use.

This study was set up to gain insight in experience with gene drive systems, both natural and synthetic, that have been explored until today. In order to inform the risk assessment, this study was aimed at making an inventory of experience on the behaviour of gene drives, with a particular interest for environmental effects from releases of organism containing gene drives.

Gene drives may be used in two ways:

- Suppression drive:
  Used to suppress populations of human and animal disease vectors, to control agricultural invertebrate pests such as fruit flies, moth pests, thrips and mites, and to eliminate invasive species.
- Replacement drive (also termed modification drive):
  Used to provide an extra trait to the target population, *e.g.* to block pathogen development, or to enforce populations, *e.g.* in endangered species or crop and livestock breeding.

Until now research on synthetic gene drives in most of the cases was limited to lab experiments and modelling. Field experience comes from field cage experiments and releases almost exclusively with mosquitoes (except for the Australian sheep blowfly, *Lucilia cuprina*). Also, the current knowledge on risk management issues associated with gene drives is largely based on work in insects, especially mosquitoes. The behaviour of synthetic gene drives in other species than insects is hardly studied.

The most extensive research has been conducted on a system with the endosymbiont *Wolbachia* that does not involve the use of GMOs as commonly defined. Besides, this system is not always considered to be a true gene drive system. The recent developments on CRISPR/Cas are still too young to have experience beyond the lab.

While field experience remains limited, most reports on gene drive applications focus on the efficacy of the system and influencing factors, not on ecological effects. The most advanced programme is the release of *Wolbachia*-infected *Aedes aegypti* in Australia and other parts of the world to fight mosquito-vectored human diseases, now known as the World Mosquito Program.

In this study, different issues that have been discussed and/or reported, were organised based on:

- Effect on behaviour of the gene drive-bearing organism, covering off-target modifications, interaction with the host genome, modified susceptibility of the host, stability of the gene drive system and horizontal gene transfer;
- Effect on biodiversity; including effects on target organism(s), on non-target organisms, on higher trophic levels and on alternative protection mechanisms;

- Resistance development, both to the gene drive system and to the effector function;
- Malicious intent;
- Effects beyond the target geographical area, with a focus on dispersal.

In trying to assess the effect of gene drives on human health and the environment several factors need to be discerned. Each application needs to be assessed case-by-case, as for any environmental risk assessment. Some effects are specific for the species, others may be related to the way that the gene drive system is introduced (*e.g.* potential for off-target effects). Some effects are not unique to gene drives but also applicable for any approach with the same aim (*e.g.* suppression drives vs. chemical control agents). Eliminating a population, independent of the deployed method, is expected to have potential effects on other species with trophic bonds, pollination requirements, host-pathogen relations, etc.

The environmental impact of an organism carrying a gene drive system is determined by the type of gene drive system on the one hand and the effector, the trait or payload gene(s) that it carries, on the other hand. In some cases, these two factors are combined: if the effect of a CRISPR/Cas-based gene drive is induced by the location where the insertion has occurred, the gene drive directly induces the effector. However, in other cases, the CRISPR/Cas-based gene drive will be linked with a payload gene(s) that induces the effect. *Wolbachia* steers the inheritance mechanism of its host and induces resistance against dengue virus.

Different models were designed to assess the behaviour of gene drive-hosting organisms in terms of efficacy, stability, reversibility, etc. Most of them could not be validated with real world observations. Models are, however, useful in visualising the effect of changing parameters.

A major concern when releasing an organism carrying a gene drive is that a process is initiated that irreversibly results in the suppression or the replacement of all wild-type alleles and/or individuals. However, there are different indications that suggest that this is unlikely or that this can be prevented:

- Many factors determine the “success” of a drive: the biology of the host organism, population dynamics, the drive’s efficacy, its fitness cost to the host. Each factor may function as a brake;
- Development/presence of resistance against the gene drive can reduce or reverse the effect (in particular when the payload gene has a high fitness cost);
- Some gene drives can be removed by natural inflow and/or re-introducing wild-type individuals (in particular high threshold drives);
- Strategies for reversing gene drives by synthetic reversal drives to limit their activity have been proposed.

Indeed, the type of gene drive and the accompanying fitness cost will determine how large the invading population needs to be relative to the target population (low level vs. high level threshold). In the experiments carried out so far the threshold is rather high leading to the hypothesis that an escape of only a few individuals will not result in a successful invasion. The same is true for potential transfer of drive elements to non-intended populations (compatible species, populations outside the target area).

Resistance towards the gene drive is important especially for homing endonuclease and miRNA/shRNA-based methods. Both are extremely sensitive to mutations or genetic variability in their recognition sites; this will likely affect the spread of drives based on such mechanisms, at least for simple designs. Already in the first laboratory experiments these phenomena were observed. Moreover, gene drives that bring a fitness cost are expected to accelerate resistance development. The concern that potent gene drives, once released, would potentially act globally, must therefore be nuanced; they might spread through different populations but not necessarily achieving very high frequencies in each.

So far the focus in experiments and theoretical considerations was on safety design and management rather than risk assessment. Only in two cases a formal risk assessment was conducted prior to the release of mosquitoes equipped with a gene drive: *Wolbachia*-infected *Aedes aegypti* in Australia and in Vietnam. These replacement drives were evaluated to represent a negligible risk.

The results of field trials performed thus far demonstrated a varying degree of “success” of the gene drives. The drive-bearing organisms did not disperse beyond the target population. None of these trials revealed any negative impact on human health and environment.

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# Samenvatting


Gene drives zijn genetische elementen die het mogelijk maken dat een kenmerk toeneemt in een populatie buiten de overervingswetten van Mendel om. Het mechanisme werkt in soorten die zich seksueel voortplanten en is een krachtig instrument om een gen te “drijven”, d.w.z. te doen toenemen in frequentie, zelfs onafhankelijk van externe selectiedruk. Deze techniek werd voorgesteld om problemen aan te pakken in de gezondheidszorg, landbouw, natuurbehoud en andere. Wetenschappers vonden er een manier in om ziekten overgedragen door insecten zoals malaria, knokkelkoorts en Zika, te bestrijden.

Decennialang al zijn er pogingen ondernomen om natuurlijk voorkomende gene drives te gebruiken of aan te passen. Natuurlijke gene drives hebben echter een aantal beperkingen. Zo blijken gene drives gebaseerd op springende genen (“transposable elements”) niet efficiënt genoeg te zijn en kunnen ze niet worden gestuurd. Translocatiedrives zijn moeilijk te maken en brengen een hoge fitnesskost met zich mee. Andere zijn alleen in specifieke soorten actief (bv. “meiotic drive”, MEDEA).

Recent hebben de vorderingen in de genetica geleid tot een coöptatie van natuurlijk gene drivesystemen en de ontwikkeling van synthetische systemen. Meer bepaald heeft de ontdekking van het CRISPR/Cas9 systeem met zijn mogelijkheid tot gene-editing en toepassing als gene drivesysteem, de interesse voor gene drives doen opleven omdat deze technologie de mogelijkheden om gene drives te construeren enorm verruimt. Gene drives gebaseerd op CRISPR/Cas blijken zeer efficiënt te zijn, toch op laboratoriumschaal. Tegelijkertijd worden er bezorgdheden geuit over het veilig gebruik ervan .

Deze studie is opgezet om inzicht te verschaffen in ervaring met gene drivesystemen die tot hiertoe zijn onderzocht, zowel natuurlijke als artificiële. Om de risicoanalyse te ondersteunen had dit onderzoek tot doel de ervaringen te inventariseren over het gedrag van gene drives, meer specifiek de milieueffecten als gevolg van de doelbewuste introductie in het milieu van organismen voorzien van een gene drive.

Gene drives kunnen op twee manieren worden gebruikt:

- Suppressiedrive
  Gebruikt om populaties te onderdrukken van menselijk en dierlijke ziektevectoren, om ziekteverwekkers onder de invertebrata zoals fruitvliegen, motten, trips en mijten in de landbouw te bestrijden, en om invasieve soorten te elimineren.
- Vervangingsdrive (ook wel wijzigingsdrive genoemd)
  Gebruikt om een extra eigenschap aan de doelpopulatie toe te voegen, bv. om een pathogeen te stoppen, of om een populatie te versterken, bv. bij bedreigde soorten of bij de veredeling van gewassen en landbouwhuisdieren.

Tot hiertoe bleef onderzoek aan synthetische gene drives in de meeste gevallen beperkt tot laboratoriumproeven en modelering. Veldervaring werd opgedaan bij experimenten met veldkooien en veldproeven, maar bijna uitsluitend met muggen (met uitzondering van de Australian sheep blowfly, *Lucilia cuprina*). Daarnaast is de actuele kennis van risicobeheerskwesties i.v.m. gene drives voornamelijk gebaseerd op werk met insecten, vooral muggen. Het gedrag van gene drives in andere soorten dan insecten is nauwelijks bestudeerd.

Het meest uitgebreide onderzoek werd uitgevoerd op een systeem met de endosymbiont *Wolbachia* dat geen genetische modificatie behelst in de gebruikelijke zin van het woord. Daarenboven wordt het zelfs niet altijd beschouwd als een echt gene drivesysteem. De ontwikkelingen bij CRISPR/Cas zijn nog te recent voor ervaringen buiten het laboratorium.

Terwijl veldervaring beperkt blijft, bespreken de meeste rapporten over gene drivetoepassingen de efficiëntie van het systeem en factoren die dat beïnvloeden, maar geven deze geen informatie over milieueffecten. Het meest geavanceerde programma is dat van de introductie van *Wolbachia*-geïnfecteerde *Aedes aegypti* in Australië en andere delen van de wereld ter bestrijding van menselijke ziekten die worden overgedragen door muggen. Dit programma staat bekend als het “World Mosquito Program”.

In deze studie werden de verschillende kwesties die werden besproken of gerapporteerd, georganiseerd volgens:

- De effecten op het gedrag van de organismen met een gene drive, onder meer niet-bedoelde wijzigingen, interactie met het gastheergenoom, gewijzigde gevoeligheid van de gastheer, stabiliteit van het gene drivesysteem en horizontale genoverdracht;
- De effecten op biodiversiteit, onder meer effecten op doelorganisme(n), op niet-doelorganismen, op hogere trofische niveaus en op alternatieve beschermingsmechanismen;
- Resistentieontwikkeling, zowel tegenover het gene drivesysteem als de effectorfunctie;
- Kwaadaardig opzet;
- De effecten voorbij het bedoelde geografisch gebied met een nadruk op verspreiding.

Bij de inschatting van gene drive-effecten op de menselijk gezondheid en het milieu moeten verscheidene factoren worden onderscheiden. Elke toepassing moet geval-per-geval worden beoordeeld, zoals voor elke milieurisicobeoordeling. Sommige effecten zijn specifiek voor de soort, andere kunnen verband houden met de wijze van introductie van de gene drive (bv. mogelijke niet-bedoelde effecten). Sommige effecten zijn niet uniek voor gene drives, maar ook toepasselijk op elk systeem met het zelfde oogmerk (bv. suppressiedrives versus chemische bestrijdingsmiddelen). Bij het elimineren van een populatie worden, ongeacht de gebruikte methode, mogelijke effecten verwacht op soorten binnen de voedselketen, op bestuivingsvereisten, op de relatie tussen gastheer en pathogeen, enz.

Het milieueffect van een organisme met een gene drive wordt bepaald door het gene drivesysteem enerzijds en de effector, het kenmerk of het meegevoerde gen (payload gene), anderzijds. In sommige gevallen zijn beide gecombineerd: als het effect van een CRISPR/Cas-gene drive wordt geïnduceerd door de positie van de insertie, is de gene drive ook de effector. In andere gevallen zal de CRISPR/Cas-gene drive worden gekoppeld aan een gen dat het effect induceert. *Wolbachia* stuurt het overervingsmechanisme in de gastheer aan en maakt de gastheer resistent tegen het knokkelkoortsvirus.

Verschillende modellen werden ontworpen om het gedrag van een organisme met een gene drive in termen van efficiëntie, stabiliteit, omkeerbaarheid enz. te beoordelen. De meeste konden niet worden gevalideerd met observaties in het veld. Modellen zijn echter nuttig om effecten van veranderende parameters zichtbaar te maken.

Een belangrijke bezorgdheid bij het introduceren van een organisme met een gene drive is dat er een proces wordt geïnitieerd met onomkeerbare gevolgen bij de eliminatie of vervanging van alle wildtype allelen en/of individuen. Echter zijn er verscheidene indicaties die suggereren dat dit onwaarschijnlijk is of dat dit kan worden tegengegaan:

- Vele factoren bepalen het succes van een drive: de biologie van het gastheerorganisme, populatiedynamiek, de efficiëntie van de drive, de fitnesskost aan de gastheer. Elk van deze factoren kan optreden als een rem;
- Ontwikkeling of het bestaan van resistentie tegen de gene drive kan het effect reduceren of omkeren (in het bijzonder wanneer het meegevoerde gen een hoge fitnesskost heeft);
- Sommige gene drives kunnen worden verwijderd door immigratie en/of herintroductie van wildtype individuen (meer bepaald bij “high threshold” drives);
- Strategieën om de activiteit van gene drives om te keren door gebruik van omkeerdrives werden voorgesteld.

Immers, het type gene drive en de bijbehorende fitnesskost zullen bepalen hoe groot de invasieve populatie ten opzichte van de doelpopulatie moet zijn (“low level” versus “high level threshold”). In de experimenten die tot nu toe werden gedaan was de drempelwaarde eerder hoog. Dit leidt tot de hypothese dat het ontsnappen van slechts enkele individuen niet zal resulteren in een succesvolle introductie. Hetzelfde geldt voor de mogelijke overdracht van drives naar niet-doelpopulaties (compatibele soorten, soorten buiten het doelgebied).

Resistentie tegen een gene drive is een belangrijk element vooral voor “homing” endonucleasen en systemen gebaseerd op miRNA/shRNA. Beide zijn zeer gevoelig voor mutatie of genetische variabiliteit in hun herkenningssequenties; dit zal waarschijnlijk zijn effect hebben op de verspreiding van dergelijke drives, tenminste bij de eenvoudige modellen. Al bij de eerste laboratoriumexperimenten werden deze

fenomenen waargenomen. Meer nog, gene drives met een fitnesskost versnellen naar verwachting resistentieontwikkeling. De bezorgdheid dat krachtige gene drives, eens losgelaten, mogelijk ongebreideld actief zijn, moet daarom worden genuanceerd; ze verspreiden zich mogelijkerwijs in verschillende populaties, maar leiden niet noodzakelijk tot hoge frequenties.

Tot nu toe lag de nadruk in de experimenten en theoretische overwegingen op de veiligheid en de beheeraspecten van het ontwerp, eerder dan op de risicoanalyse. Slechts in twee gevallen werd er een formele risicobeoordeling uitgevoerd vooraleer muggen voorzien van een gene drive werden losgelaten: *Wolbachia*-geïnfecteerde *Aedes aegypti* in Australië en in Vietnam. Het risico van deze vervangingsdrives werd verwaarloosbaar bevonden.

De resultaten van de tot nu toe uitgevoerde veldexperimenten duiden op een wisselend succes van de gene drives. De organismen uitgerust met een gene drive verspreidden zich niet buiten de doelpopulatie. In geen van deze proeven werd enig negatief effect op de gezondheid van de mens en het milieu gevonden.

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# Abbreviations

| | |
|---|---|
| Ab10 | Abnormal 10 chromosome of maize |
| Cas | CRISPR associated protein (endonuclease) |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme-Linked Immuno Sorbent Assay |
| GM | Genetically modified |
| GMO | Genetically modified organism |
| HDR | Homology-directed repair |
| HEG | Homing endonuclease gene |
| MEDEA | Maternal-effect dominant embryonic arrest |
| miRNA | microRNA |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| SD | Segregation Distorter autosomal gene complex |
| siRNA | Small interfering RNA |
| shRNA | Short hairpin RNA or small hairpin RNA |
| SIT | Sterile insect technique |
| IIT | Incompatible insect technique |
| TALEN | Transcription activator-like effector nuclease |
| ZFN | Zinc finger nuclease |

# Glossary

| | |
|---|---|
| Chromosome translocation | Chromosome abnormality caused by rearrangement of parts of chromosomes between non-homologous chromosomes. |
| Cytoplasmic incompatibility | Reproductive incompatibility between individuals of the same species determined by cytoplasmic factors. As a result sperm and eggs of incompatible individuals are unable to form viable offspring. It occurs chiefly in insects as well as some other arthropods. The effect arises from changes in the gamete cells caused by intracellular bacteria. The most studied and apparently most common of these endosymbionts is *Wolbachia pipientis*. |
| Endonuclease | Enzyme that cleaves the phosphodiester bond within a polynucleotide chain. |
| Gene drive | System of biased inheritance in which the ability of a genetic element to pass from a parent to its offspring through sexual reproduction is enhanced independently from selection pressure.[1] |
| Genetically modified organism (GMO) | Used in this report when referring to an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination, as defined in EU Directive 2001/18/EC[2]. |
| Haplo-insufficiency | Refers to a mechanism where a diploid organism has lost one copy of a gene and is left with a single functional copy of that gene that is not sufficient to establish the wild-type phenotype. |
| Haplotype | Group of alleles in an organism that are inherited together from a single parent. |
| Homing | The process by which an endonuclease cleaves a specific DNA target sequence and copies itself, or 'homes', into this target sequence. Homing utilises the cell's homology-directed repair machinery, which relies on sequences that flank the endonuclease and that are homologous to either side of the target sequence. |
| Homology directed repair | Pathway that repairs double-strand breaks in DNA using a homologous sequence to guide repair. |
| Micro-homology-mediated end joining | Pathway that repairs double-strand breaks in DNA using 5–25 base pair micro-homologous sequences on either site of the break during the alignment of broken ends before joining, thereby resulting in deletions flanking the original break. |
| Non-homologous end joining | Pathway that repairs double-strand breaks in DNA by direct ligation without the need for a homologous template. |
| Payload gene | Gene that can be linked to a gene drive to spread a desirable trait throughout a population. |
| Release threshold | The number of individuals with a gene drive to be released relative to the target population that is needed for the gene drive to spread into the population. |
| RNAi | RNA interference is a biological process in which RNA molecules inhibit gene expression or translation, by neutralising targeted mRNA molecules. Two types of RNAi molecules are known: microRNA (miRNA) and small interfering RNA (siRNA). |

[1] This is a commonly used definition of the term and is used in this report. Other definitions exist and their use may impact the scope of the covered systems. *E.g.* "artificial selfish DNA elements" exclude naturally-occurring systems.

[2] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal L 106, 17/04/2001, p.1-39.

| | |
|---|---|
| Transposable element or Transposon | Genetic element that can insert itself into different locations in a genome. |
| Underdominance | Heterozygote inferiority; refers to a situation whereby a heterozygous individual is less fit than homozygous individuals. |

# 1 Introduction

The inheritance of genes usually follows Mendel's laws, *i.e.* each of a parent's two copies of a gene has a 50% chance to end up in each of the progeny of a sexual cross (Figure 1). However, gene drive systems (or gene drives) have a mechanism to increase the frequency of a gene in the offspring beyond Mendelian frequencies.

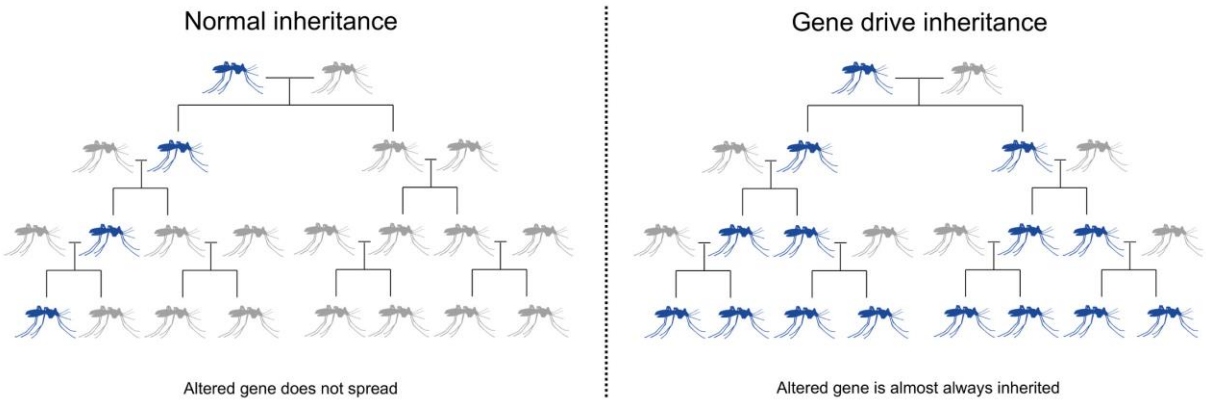


**Figure 1:** Comparison between Mendelian inheritance and an idealised gene drive inheritance

A wide variety of gene drives appears in nature, such as transposable elements, homing endonucleases, and meiotic drives (Burt and Trivers, 2008). They have been studied since the 1920s. These naturally occurring systems are more commonly called selfish DNA systems. They have in common that they can spread through a population, not necessarily providing a fitness advantage to the organisms carrying them. Also, artificial gene drives have been developed.

Applications of gene drives are reported since the early 1970s (Curtis and Adak, 1974; Foster et al., 1972; Laven and Jost, 1971). Most of them have been modelled and/or studied in the lab, and occasionally under semi-field conditions (Curtis, 1976). Some of the manmade gene drives, *e.g.* X-ray induced translocations, and cytoplasmic incompatibility have been studied and trialled in the field (Curtis et al., 1982; Laven et al., 1972; Lorimer et al., 1976; Vogt et al., 1985). They all target mosquito species as a way to combat mosquito-borne diseases. Recently, research has been accelerated by the discovery of the CRISPR/Cas gene editing system and its potential application as a gene drive (Gantz and Bier, 2015).

Gene drives are only working in sexually reproducing organisms. This is because gene drives propagate by replacing other alleles and alleles are mixed at each generation. Bacteria and viruses are excluded as they reproduce asexually always passing 100% of their genome to the next generation. In nature the possibilities in plants are limited to dioecious and self-incompatible systems.

Gene drives could be used to promote the preferential inheritance of a trait beneficial for humans, thereby increasing its prevalence in a population. They have been proposed as offering solutions for many challenges in public health, agriculture, conservation and others (Esvelt et al., 2014). Among these, multiple research groups are working to control mosquito-borne diseases, such as malaria, dengue, Chikungunya and Zika, using gene drives that either suppress the mosquito population or replace it by a less harmful variant (Hammond et al., 2016; Walker et al., 2011; Windbichler et al., 2007).

Gene drives may be used in two ways. They can be classified either as a suppression drive or as a replacement drive, also termed modification drive.

A suppression drive may be targeted to a gene that, when disrupted, results in recessive lethality or sterility. Or it may introduce genes *e.g.* to reduce lifespan or to bias sex ratios. Suppression drives may be used to suppress populations of human and animal disease vectors, to control agricultural invertebrate pests such as fruit flies, moth pests, thrips and mites, and to eliminate invasive species.

In a replacement or modification drive the system includes one or more genes (payload genes) that provide an extra trait to the target population, *e.g.* to block pathogen development, or to block the ability of the target organism to act as a vector for pathogens. Also traits advantageous to the target population may be considered, *e.g.* providing aid for endangered species by spreading pathogen-resistance payload genes through exposed populations (conservation) (Champer et al., 2016; Esvelt et al., 2014). The system may even be used to reverse the development of resistance to insecticides and herbicides (Esvelt et al., 2014) and in livestock breeding programmes (Gonen et al., 2017).

Reports of existing gene drive systems have shown mixed success in driving a desired trait. Systems that theoretically drive a gene to a 100% prevalence in a population as well as self-limiting systems are described. The CRISPR/Cas system promises to be very efficient in its capacity for spreading through a population. Although the technology may bring many benefits, the potential risks need to be evaluated as well. Unintended effects may disrupt populations and species. Gene flow to other species, change in fitness, effects on non-target species and the subsequent ecological effects may hinder a safe application of the technology.

In order to inform the risk assessment, this study was aimed at making an inventory of experience on the behaviour of gene drives, with a particular interest for environmental effects from open releases of organisms containing gene drives. Although the scope of the study was not limited to insects and it was the intention to obtain an understanding of the effects of gene drives in a broader array of organisms, the majority of the reported applications concern insects and reports that were found on open field releases almost only concern mosquitoes.

# 2 Purpose of the study

This study was set up to gain insight in the gene drive systems that have been explored until today. The study aims to answer an array of questions in order to better understand the potential consequences for human health and the environment of gene drive use. Off-target effects (within the organism) and effects on non-targets organisms *e.g.* horizontal gene transfer, hybridisation between related species, and broader ecological effects are reviewed.

The reseach questions are:

- Which organisms with some sort of gene drive (GM or natural) have been reported?
- Which organisms with gene drive systems have been intentionally introduced into the environment?
    - What were the results of these field releases?
    - Where environmental consequences observed?
    - Was resistance to the gene drive observed?
    - How were the risks evaluated?
- Can lessons from previous gene drive studies be drawn that can inform the risk assessment?

# 3 Methods

Searches were performed on the Web of Science and PubMed databases in December 2017.

The search terms that were used (including Boolean operators etc.), are:
“gene drive”
“meiotic drive”
“Wolbachia”
“Cytoplasmic incompatibility”
“MEDEA”
“homing endonuclease”
“selfish gene”
“mutagenic chain reaction”
“translocation”
“underdominance”
“biased sex ratio”
“segregation distorter”
“transmission ratio distorter”

The search was limited to articles available in English. Manual searches through reference lists of the articles were also performed to identify additional studies. Also review articles were included. The collection of information was concluded in May 2018.

The study was further supported by scientific guidance and critical review from Prof. Dr. Luke Alphey (Pirbright Institute, UK), who acted as external expert. In addition, the Advisory Committee provided useful suggestions on studies and publications.

# 4 What are gene drives?

Normal Mendelian inheritance describes that after sexual reproduction a gene ends up in about 50% of the progeny. Therefore, the frequency of a neutral gene in future generations will be similar to the frequency of that gene in the parents' generation. With gene drives (also called 'selfish' genes) the chance for the offspring to inherit a gene from a parent is higher than 50%, even in the absence of selective pressure. As a result the frequency of a gene will increase in the population over time, even without conferring a fitness advantage upon its host (Figure 1). The rate of preferential inheritance, or "drive" depends on the type of gene drive, the species, and environmental conditions.

Gene drives can be defined as (National Academies of Sciences Engineering and Medicine, 2016):

> **"systems of biased inheritance in which the ability of a genetic element to pass from a parent to its offspring through sexual reproduction is enhanced and this independently from selection pressure."**

Thus, the result of a gene drive is the preferential increase of a specific genotype, the genetic makeup of an organism that determines a specific phenotype (trait), from one generation to the next, and potentially throughout the population. These systems encompass the requisite molecular elements and events necessary for biased inheritance to occur. Because inheritance is biased in their favour, the genetic elements encompassed by gene drives are often called selfish genes or selfish genetic elements. Examples of selfish genetic elements include genes or their fragments, all or parts of chromosomes, or noncoding DNA (Burt and Trivers, 2008).

In this report the term gene drive is taken broadly: not only genetic elements such as genes, gene clusters, all or parts of chromosomes, or noncoding DNA are meant, but also the genome of endosymbionts that as such are part of an organism.

Gene drives are present in nature in a variety of organisms and no single molecular mechanism underlies all gene drives. Early observations were done in mice (Chesley and Dunn, 1936) and fruit flies (Dunn, 1953; Gershenson, 1928; Sandler et al., 1959). In recent years advances in genetics have allowed for co-opting natural gene drive systems and the development of synthetic gene drive systems. Most proposed engineered gene drives are based on naturally existing 'selfish' genetic elements (Champer et al., 2016). Examples are synthetic drives based on transposase activity and the MEDEA principle, meiotic drive synthetic systems, etc.

Gene drives may be used in two ways (Champer et al., 2016; National Academies of Sciences Engineering and Medicine, 2016); as a system for:

- Population suppression
  the spread of a genetic element that causes the number of individuals in a population to decrease (suppression drive); and
- Population replacement
  the spread of a genetic element or modification to a natural gene through a population (modification drive).

Gene drives generally move in one direction, *i.e.* driving into a population until fixation (unidirectional). Or they can be bidirectional, *i.e.* moving away from an unstable equilibrium in either direction, all wild-type or all gene drive hosting).

In the subsequent paragraphs the most important gene drive systems are briefly described.

## 4.1 Wolbachia

### 4.1.1 Mode of action

*Wolbachia* are maternally inherited, cytoplasmic, obligate intracellular bacterial endosymbionts. The bacteria inhabit invertebrate organisms and are naturally found in more than 50% of all arthropod species and are present in filarial nematodes (Serbus et al., 2008; Shropshire et al., 2018). There is only one species, *Wolbachia pipientis*, with several strains, which vary considerably in host distribution and effects on their hosts (Murphy et al., 2010). It infects reproductive tissues and manipulates the host reproductive cycle to increase its spread. Inheritance is usually strictly maternal – similar to inheritance of mitochondria, all offspring of a *Wolbachia*-infected female are normally infected, whereas offspring of an infected male are not (Figure 2).

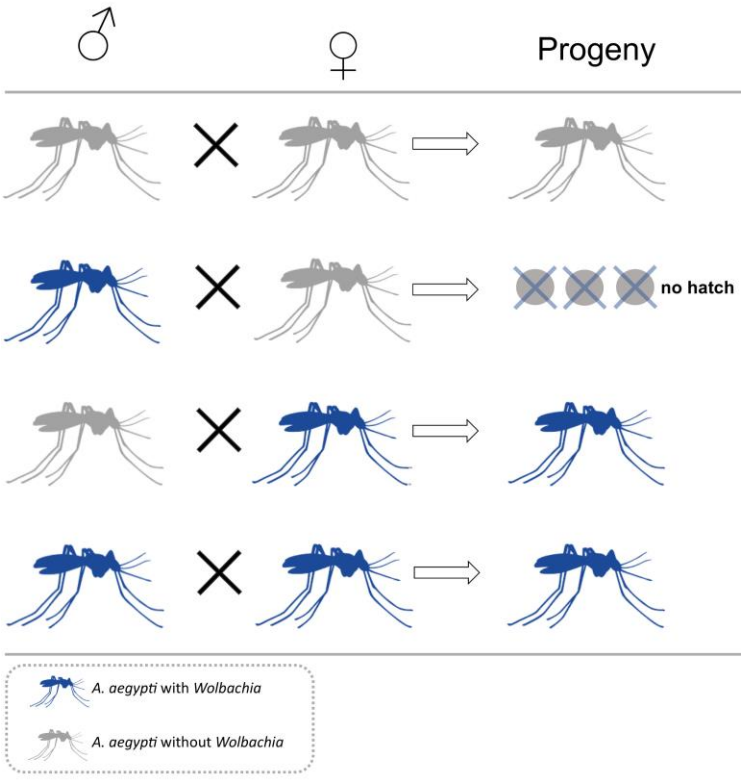


**Figure 2:** Schematic representation of the cytoplasmic incompatibility phenotype induced by *Wolbachia* in *Aedes aegypti* mosquitoes

The strategies employed by *Wolbachia* for disturbing reproduction include selective male killing, parthenogenesis, feminisation of genetically male embryos and cytoplasmic incompatibility. In the latter scenario *Wolbachia*-infected males do not produce viable offspring when mating with either uninfected females or females infected with a different *Wolbachia* strain (Figure 2). Such a strategy releasing only *Wolbachia*-infected males may be used for population suppression (incompatible insect technique, IIT) and is then not a gene drive system. In contrast, infected females can mate with either infected or uninfected males and produce almost 100% infected viable progeny. Therefore, after a release of *Wolbachia*-infected males and females, *Wolbachia*-infected females have a reproductive advantage relative to uninfected females allowing infection to spread rapidly through host populations to a high frequency in spite of fitness costs (*e.g.* relative fecundity, longevity, death rate). The cytoplasmic incompatibility may be unidirectional, promoting the expansion of only one subpopulation composed of *Wolbachia*-infected mosquitoes (Sinkins and Gould, 2006). Alternatively, it may be bidirectional when it results in the development of subpopulations, each infected with one of two or more opposing *Wolbachia* strains.

The molecular basis of cytoplasmic incompatibility was recently discovered (LePage et al., 2017; Lindsey et al., 2018). Two genes, *cifA* and *cifB*, in the *Wolbachia* strain that infects *Drosophila melanogaster* (*w*Mel) appear almost certainly to be responsible. They are not *Wolbachia* genes but reside in the eukaryotic association module of prophage WO. The paternal genes induce embryonic arrest and death, if not rescued by the maternal *cifA* gene (Shropshire et al., 2018). For the *w*Pip strain homologues *cidA* and *cidB* it was shown that *cidB* encodes a deubiquitylating enzyme and *cidA* encodes a protein that binds that enzyme (Beckmann et al., 2017).

Because cytoplasmic incompatibility is a form of reproductive manipulation, some authors refer to *Wolbachia* as a reproductive parasite (Burt and Trivers, 2008; Woolfit et al., 2013). Some other members of the Rickettsiales show similar effects, while *Wolbachia* is the best known species (Perlman et al., 2006).

**Insert 1**
**Is *Wolbachia* a gene drive system?**

*Wolbachia* are obligate intracellular endosymbiotic bacteria belonging to the order Rickettsiales. Its complete genome is residing in the cytoplasm and is therefore inherited maternally similar to mitochondria. The *Wolbachia* genome normally does not integrate into the host's chromosomes as in the strict sense of a gene drive, although integration of parts of their genome has been described (Klasson et al., 2009). Therefore, they do not inherit as nuclear genes, nor are they related to Mendelian inheritance. In that sense they are not gene drive systems.

Nevertheless as the net effect is the rapid increase in the number of infected individuals in a population, other authors consider the *Wolbachia* system to be a gene drive system driving a whole genome into a population. The driving element consists in the fact that all egg cells inherit the endosymbiont and therefore are passed to the next generation beyond Mendelian frequencies as opposed to a normal nuclear gene.

Others refer to it as a microbial biopesticide (Macias et al., 2017b).

### 4.1.2 Potential use

*Wolbachia* may be used to combat virus diseases transmitted by arthropods by increasing the arthropods' resistance to viruses and/or altering their reproductive capacities (Kamtchum-Tatuene et al., 2017). *Wolbachia* has been proposed as a tool in the fight against infections by arthropod-borne viruses (arboviruses), such as dengue, Chikungunya, Japanese encephalitis virus, West Nile virus and Zika virus. The induction of resistance to arboviruses was first demonstrated in naturally infected *Drosophila melanogaster* (Hedges et al., 2008; Teixeira et al., 2008). The biological mechanism of arbovirus resistance incurred by *Wolbachia* infection is largely unknown. It may work via competition for resources, preactivation of the immune system, induction of the phenoloxidase cascade and induction of microRNA-dependent immune pathways (Johnson, 2015).

The bacterium may also have a life-shortening effect on the host. This is interesting since most pathogens have to undergo a significant period of development in their insect vector before they can be transmitted to a new host (McMeniman et al., 2009).

Unlike some of the techniques discussed below the traits that can be driven using *Wolbachia* are limited: currently the scope is limited to refractoriness to certain pathogens that is already associated with *Wolbachia* infection. Genetic modification of *Wolbachia* to further optimise the drive system and actively combat arbovirus infections is currently not possible (Macias et al., 2017b).

### 4.1.3 Experience

*Wolbachia* is not naturally present in *Anopheles* spp. or *Aedes aegypti*, the vector for malaria and several arboviruses resp. In experiments the *Wolbachia* strains *w*MelPop and *w*Mel that occur naturally in *Drosophila melanogaster* were stably introduced in *Aedes aegypti* (McMeniman et al., 2009; Walker et al., 2011). The resulting mosquito strains showed reduced longevity and cytoplasmic incompatibility. *w*MelPop infection also substantially reduces dengue, Chikungunya, and *Plasmodium* load in *Aedes aegypti* (Moreira et al., 2009a). The same is true for *w*Mel and dengue (Walker et al., 2011). In a semi-field facility (cages) *Wolbachia w*Mel showed a higher invasion potential compared to *w*MelPop (Walker et al., 2011). This was due to the higher fitness cost of *w*MelPop.

The first field trials to reduce dengue transmission by *Aedes aegypti* using *Wolbachia w*Mel strain started in 2011 in northern Queensland, Australia (Hoffmann et al., 2011; Schmidt et al., 2017). Here the release of *Wolbachia*-infected females and males is being trialled for population replacement (see 5.3.1.1).

Release of *Wolbachia*-infected males (without releasing infected females) may be used for population suppression as these infected males are effectively sterile with wild females that are not infected with the same type of *Wolbachia* (IIT). In 1967 it was shown that a *Culex pipiens* population could be eradicated using *Wolbachia* (Laven, 1967).

### 4.1.4 Characteristics

- *Wolbachia* is restricted to merely arthropods;
- Currently no ability to genetically engineer these intracellular bacteria;
- High drive efficiency (*i.e.* high rate of progression in a population);
- *Wolbachia*-infected mosquitoes were applied in the field.

## 4.2 Transposable elements (transposons)

### 4.2.1 Mode of action

Since the discovery by Barbara McClintock, transposable elements have been studied and used as a general method to genetically modify a multitude of organisms. In its simplest form a transposon consists of a gene encoding a transposase and inverted repeats at the ends. The transposase recognises specific DNA sequences at or near the ends of the element, cuts the element out of the genome, and inserts it somewhere else. Several mechanisms exist in which the copy number of the transposon within an individual may increase (Burt and Trivers, 2008). *E.g.* the *P*-element is excised at a point in the cell cycle between DNA replication and mitosis, creating a double-strand gap. It then inserts itself somewhere else. The cell's repair system fills the gap using homologous end joining taking the sister chromatid as a template. In case the sister chromatid also contains the *P*-element, the *P*-element is copied. If not, the transposon is just excised. Most of the time the new insertions are deleterious, yet the prevalence of *P*-element in wild populations of *Drosophila melanogaster* has increased dramatically since its natural introduction from *Drosophila willistoni* in the middle of the 20$^{th}$ century. Transposable elements are present in a variety of species including humans.

### 4.2.2 Potential use

Transposable elements were considered as an option for substituting mosquito populations by ones that are resistant to a pathogen or hinder its development. This can be accomplished by introducing a payload gene to the element that affects the pathogen that the mosquito is vectoring.

### 4.2.3 Experience

The *P*-element was the first to be used to introduce foreign DNA sequences in an insect, in *Drosophila melanogaster* (Spradling and Rubin, 1982). It was at that time seen as an efficient DNA-mediated gene transfer system in *Drosophila*. Meister and Grigliatti (Meister and Grigliatti, 1993)

first showed that a *P*-element transposon could rapidly spread a specific gene into an experimental *Drosophila melanogaster* population. However, the *P*-element is only active in *Drosophila* and closely related genera. Later, other transposable elements were identified as active in mosquitoes (Table 1) and may potentially serve as a gene drive.

**Table 1:** Examples of transposable elements in Diptera

| Transposable element(s) | Insect (year of publication) | Reference |
|---|---|---|
| *P*-element | *Drosophila melanogaster* (1982) | Spradling and Rubin, 1982 |
| *Hermes* elements | *Aedes aegypti* (1998)<br>*Culex quinquefasciatus* (2001) | Macias et al., 2017b |
| *mos1/mariner* element | *Aedes aegypti* (1998) | |
| *piggyBac* element | *Anopheles stephensi* (1999)<br>*Anopheles gambiae* (2000)<br>*Culex quinquefasciatus* (2001)<br>*Anopheles albimanus* (2002)<br>*Aedes fluviatilis* (2006)<br>*Aedes albopictus* (2010) | |

Recently it was demonstrated that a synthetic *piggyBac* element-carrying a payload gene can be mobilised in *Anopheles stephensi.* However, remobilisation from one chromosome to another in order to function as a drive occurred at only very low frequencies (one every 23 generations) (Macias et al., 2017a).

### 4.2.4 Characteristics

- The use of transposable elements as a gene drive has several disadvantages:
  - transposition rates are often too low (low drive efficiency), their integration sites are unpredictable, and they have proven to be difficult to mobilise after integration, at least in mosquitoes (Champer et al., 2016; Macias et al., 2017a).
  - the drive would likely incur a fitness cost as a result of continued re-mobilisation into essential sites in the host genome (Macias et al., 2017b).
  - once introduced in a population a transposon-based gene drive cannot be reversed and there is the possibility of losing the link between a transposon and the payload gene (Marshall, 2008).
- Transposon-based gene drives were not tested beyond the lab.

## 4.3 Underdominance

Underdominance is described as negative heterosis, where heterozygotes are less fit than homozygotes. Underdominance can be achieved using translocations. Other systems (cytoplasmic incompatibility, synthetic underdominance) are also considered to be examples of underdominance, although the early developmental stages at which the effects occur and the high efficiency would not fit within the concept of (negative) heterosis (extreme underdominance).

When introduced in a population of wild types by regular releases, the population will evolve either into all wild-types or all gene drive-bearing individuals (bidirectional system, *i.e.* moving away from an unstable equilibrium in either direction). At a certain frequency (the release threshold) the introduced system will dominate and become fixed.

### 4.3.1 Mode of action

**Translocations**

A translocation is a chromosomal rearrangement between non-homologous chromosomes. If the exchange of chromosomal parts is reciprocal, the bearer of the balanced translocation does not

experience any harm as all gene functions are still present. However, his gametes will have an unbalanced gene set, resulting in abnormal progeny or lethality in half of the progeny of a cross of heterozygotes. The homozygotic translocation-bearing individuals and homozygote wild-type individuals are not affected. Also other types of rearrangements may be used (*e.g.* duplication, deletion, inversion).

**Cytoplasmic incompatibility**
In a population with two *Wolbachia* strains mating between individuals with a different strain will result in no progeny. The embryos are arrested in their development (delayed histone deposition, improper chromosome condensation and cell division abnormalities (Lindsey et al., 2018; Serbus et al., 2008)). This may be regarded as an extreme form of reduced fitness, as the heterozygous individual will not even develop.

Cytoplasmic incompatibility can also be accomplished using different chromosomal and cytoplasmic backgrounds.

**Synthetic underdominance (toxin–antidote system)**
More recently a synthetic underdominance system has been proposed and modelled, using two gene constructs (Davis et al., 2001) (Figure 3). Each construct on itself is lethal (or brings a fitness cost) to its owner unless the other is present to suppress the effect. The constructs may be present on one locus in a homologous pair of chromosomes or on different chromosomes (two-locus). Whenever the toxin is to induce lethality, the effect is comparable with cytoplasmic incompatibility. Individuals who carry copies of both constructs are viable. The heterozygous progeny from a cross between an engineered and wild type individual is not. Depending on the position of the insert (single-locus vs. two-locus, autosomal vs. X chromosome) the release threshold for the drive to replace a population differs. Less effort is required to ensure a successful introgression of transgenes when they are carried at unlinked loci. Once introgression has progressed beyond a critical stage, it will continue even if releases are discontinued. This system could be a general method of introducing refractory genes *e.g.* to pathogens vectored by mosquitoes.

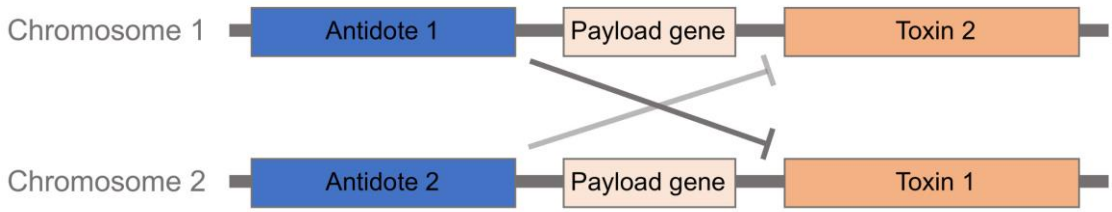


**Figure 3:** Schematic representation of a two-locus toxin-antidote system (redrawn after Champer et al., 2016)

### 4.3.2 Potential use

All these systems are threshold-dependent gene drives that act locally and can be removed through dilution of the population with wild-type individuals (Altrock et al., 2010). Several situations have been modelled using different values for fitness cost and migration from neighbouring populations (Buchman et al., 2016). High threshold gene drives are likely to be most effective in replacing populations in isolated conditions.

### 4.3.3 Experience

**Translocations**
Curtis proposed to use translocations to fix genes in a population (Curtis, 1968). Earlier, a similar idea was projected by Serebrovskii in 1940 (translated in English in 1969 (Serebrovskii, 1969)). This followed from the observation that translocation heterozygotes (T/+) are usually semi-sterile

because some of their gametes do not receive a full chromosomal complement, but translocation homozygotes (T/T), if viable, are usually fully fertile.

To that end translocations were artificially induced via X-rays in *Culex pipiens* (Laven and Jost, 1971) and *Aedes aegypti* (Lorimer et al., 1972). Translocations in *Culex pipiens* have proven in laboratory experiments (Laven, 1969) and in the field (Laven et al., 1972) to reduce a population to a very low level. However, X-ray damage in other regions of the genome may induce a fitness cost.

Cage experiments in *Drosophila* did not succeed in transforming a wild-type population (Robinson and Curtis, 1973). The failure was attributed to a reduced viability of the translocation homozygotes when competing with wild-type. No translocations became established in a field trial with *Aedes aegypti* (Lorimer et al., 1976).

This lower fitness as compared to the wild-type counterparts and the difficulty to obtain viable translocation-bearing individuals made that the approach was abandoned at that time. With present day engineering techniques, several other methods to generate translocations have been used (Buchman et al., 2016) and references therein). Reciprocal translocations were induced in *Drosophila* using a HEG (see 4.6) (Buchman et al., 2016). Lab drive experiments (14 generations) showed that populations can be replaced but only at high introduction frequencies (80%, 70%, 60%). Replacement went slower than expected due to a lower fitness compared to wild-type, the reason why not being clear.

Foster and colleagues discussed the linking of translocations and mosquito genotypes that are *e.g.* refractory to the pest or are more susceptible to insecticides (Foster et al., 1972).

**Cytoplasmic incompatibility**
Bidirectional cytoplasmic incompatibility in *Culex fatigans* was tested using strains with the cytoplasm of either Delhi or Paris (Curtis and Adak, 1974). Depending on the initial frequencies one or the other was completely replaced in 2-4 generations. A cage experiment with *Anopheles gambiae* demonstrated that it was possible to introduce a strain partially refractory to the parasite *Plasmodium yoelii nigeriensis* in a susceptible population (Graves and Curtis, 1982). The susceptibility of the population went from 100% to a stable 50% although the refractory strain had a much lower fitness. A field experiment was performed for *Culex quinquefasciatus* (Curtis et al., 1982) (see 5.2.2).

**Synthetic underdominance (toxin–antidote system)**
An engineered underdominance system was created in *Drosophila melanogaster* using a toxin–antitoxin mechanism (Figure 3) (Akbari et al., 2013). Two constructs were used, each consisting of a maternally expressed toxin (multimers of miRNAs that act to suppress the corresponding gene via a mechanism of RNA interference (RNAi)) and a zygotically expressed antidote (resistant mRNAs). Another set-up in *Drosophila melanogaster* introduces gene constructs on different chromosomes, one having RpL14.dsRNA targeting RNA interference to a haplo-insufficient gene RpL14 and the other an RNAi insensitive RpL14 to rescue (Reeves et al., 2014). Both approaches were successfully tested in lab experiments.

The development in *Drosophila* of a “synthetic species” that is still able to mate wild wild-type species but results in lethality in the offspring (Moreno, 2012) may be regarded as a special case of engineered underdominance.

### 4.3.4 Characteristics

Underdominance systems:

- require a high introduction threshold to spread through a population;
- have a low drive efficiency (translocations);
- they are likely to be confined to a local area;
- can be removed by mass introduction of wild-type organisms;
- have been used successfully in the lab (synthetic underdominance)
- have been used successfully in the field (translocations and cytoplasmic incompatibility).

## 4.4 Meiotic Drive

### 4.4.1 Mode of action

In a meiotic drive the transmission of certain alleles is biased during meiosis, leading to increased frequencies of those alleles in the gametes, and therefore in the offspring. A well-studied meiotic drive is the Segregation Distorter (SD) autosomal gene complex in *Drosophila melanogaster* (Sandler et al., 1959). If present in the male, wild-type sperm does not complete development and only SD complex containing sperm survives. However, the frequency in the natural population remains very low for reasons that are not well understood (National Academies of Sciences Engineering and Medicine, 2016).

Sex-linked meiotic drives work through altering the sex ratios of offspring of affected individuals. The first descriptions of a natural system were mentioned in *Drosophila obscura* (Gershenson, 1928), *Drosophila pseudoobscura* and other *Drosophila* species (Sturtevant and Dobzhansky, 1936). In these species the X chromosome was favoured in male spermatogenesis over the Y chromosome. The drive's action is tempered by autosomal drive suppressors (Atlan et al., 2003). Natural meiotic drives have also been found in mosquitoes: in *Aedes aegypti* (Hickey and Craig, 1966a, b) and in *Culex pipiens* (Sweeny and Barr, 1978) with an increased proportion of males. This drive only works when located close to the male determining factor M of the Y chromosome (Hickey and Craig, 1966a). It preferentially breaks the X chromosome during meiosis.

Certain tailless alleles in mice (*Mus musculus*) are lethal in homozygous condition (t-haplotype) (Sandler and Novitski, 1957). Yet the frequency in a population is higher than expected. When present in the heterozygous (Tt) condition in the male, the wild-type sperm show motility defects and are functionally inactive, so more than 90% of the progeny receive the t-haplotype (National Academies of Sciences Engineering and Medicine, 2016). However, several mechanisms, such as the presence of recessive lethal mutations within the t-haplotype, suppressor genes, multiple matings, etc. keep the frequency in the natural population low (Ardlie, 1998; Safronova and Chubykin, 2013; Silver, 1993).

In plants the Abnormal 10 (Ab10) chromosome of maize (*Zea mays* ssp. *mays*) is known to function as a meiotic drive (Rhoades, 1942). Ab10 affects the segregation of chromosome 10 and other chromosomes if they contain chromosomal knobs. Knobs consist of thousands to millions of tandem 180- and 350-bp repeats (Buckler et al., 1999). They have regions with sequence similarity to maize centromeres.

In female meiosis 4 haploid meiotic products are produced in a row and only the one nearest the base of the ovary develops into the female gametophyte, while the other 3 degenerate (Figure 4). In heterozygotes, normal and knob-bearing chromosomes pair up at meiosis, and typically 1 crossover occurs between the centromere and the knob (Burt and Trivers, 2008). At the first meiotic division, chromatids with and without a knob go to each pole. Because the knob acts as a centromere, the chromatids carrying the knobs get to the poles first. As a result, after the second meiotic division, they tend to be found in the 2 outside meiotic products, one of which forms the megaspore. In male meiosis all 4 meiotic products are viable and therefore no accumulation of knobbed chromosomes occurs.

This neocentromeric activity is observed only when Ab10 is present and thousands of these repeats form a knob. It was hypothesised that the Ab10 drive is responsible for reshaping genetic diversity throughout the maize genome. Somehow the prevalence of Ab10 in natural population is low. The exact reason is not known: it may be gametic selection against Ab10 pollen or late replication of the knobs relative to other chromosomal regions causing a longer synthesis phase of the cell cycle and mitotic abnormalities (Buckler et al., 1999; Burt and Trivers, 2008).

More examples can be found in nature: in insects (several diptera, lepidoptera and others), mammals (*Rodentia*, *e.g.* lemmings, voles, and African pygmy mouse), plants (*e.g.* monkeyflower)

and fungi (*e.g. Neurospora* spp.) (Burt and Trivers, 2008; Fredga et al., 1977; Gileva, 1987; Helleu et al., 2015; Kozielska et al., 2009; Lyttle, 1991; Owen, 1974; Turner and Perkins, 1979).

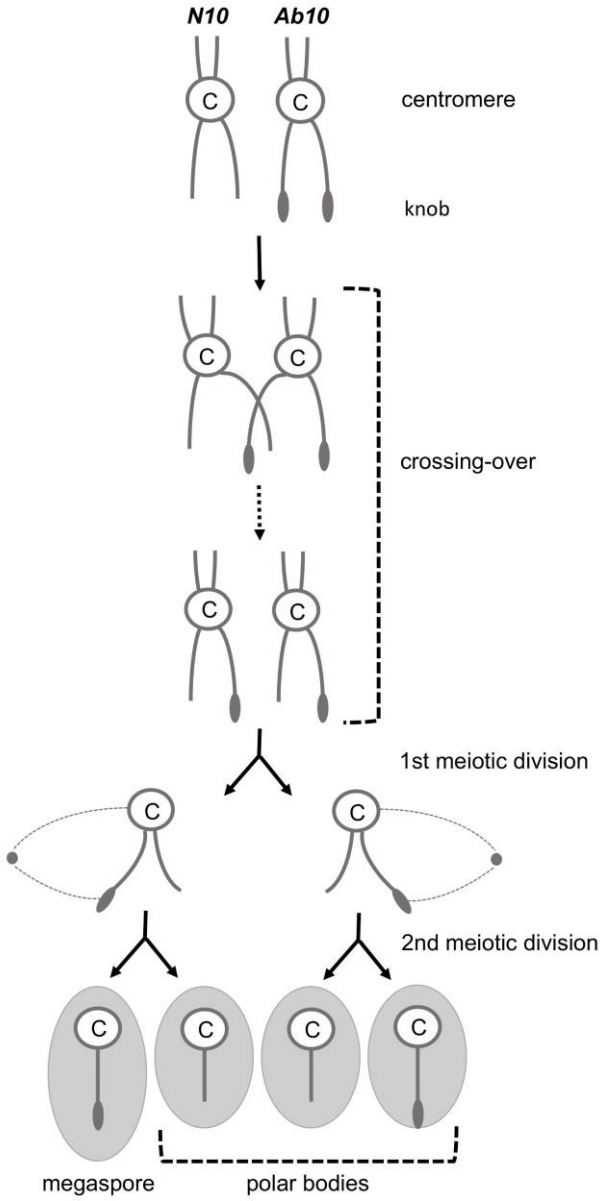


**Figure 4:** Meiotic drive of a chromosomal knob in maize (redrawn after Burt and Austin, 2008)

### 4.4.2 Potential use

Sex-linked meiotic drive may be used to distort the sex ratio towards males to gradually drive a population to extinction. It may be applied in insect pest populations or vector organisms.

### 4.4.3 Experience

Several researchers started work to manipulate natural systems or engineer new systems. Only 2 examples are mentioned here (laboratory experiments).

Wood and colleagues managed to introduce the red eye marker into a laboratory cage population of *Aedes aegypti* using a sex-linked meiotic drive (Wood et al., 1977).

One engineered meiotic drive-based system has been based on an endonuclease that targets and cuts several locations on the X chromosome during spermatogenesis (X-shredder) (Galizi et al., 2014). In this study a modified version of the homing endonuclease *I-PpoI* gene driven by a spermatogenesis-specific promoter was transferred to *Anopheles gambiae*. The progeny of a cross of such an engineered male with wild-type females contains up to 97.4% males. To be functional as a drive the gene should be located at the Y chromosome. All males in the progeny will then carry the X-shredder. However, this system is only functional in species containing the repeated *I-PpoI* target sequences exclusively on the X chromosome. Building on this work, a similar X-shredder has been built with CRISPR-Cas9 (Galizi et al., 2016).

### 4.4.4 Characteristics

- Meiotic drive systems are present in nature in a multitude of organisms;
- Some sort of resistance or control mechanism keeps the drive allele frequency down in nature;
- The mechanisms of the system have been studied extensively for many years;
- Engineered systems are studied in the lab;
- Have a moderate drive efficiency;
- May be applied as suppression drive.

## 4.5 Maternal-effect dominant embryonic arrest

### 4.5.1 Mode of action

Maternal-effect dominant embryonic arrest (MEDEA) was first described in the flour beetle (*Tribolium castaneum*) (Beeman et al., 1992). The M factor (MEDEA factor) on a chromosome confers maternal-effect lethality to all progeny that do not have a copy of the factor inherited either paternally or maternally. The speed of the spread of the M factor in a population is dependent on the degree of maternal effect lethality, the fecundity fitness cost to females carrying the M factor, the degree of dominance of the fecundity fitness cost in females, and the mode of population regulation (*e.g.* density regulation) (Wade and Beeman, 1994). The molecular mechanism is not yet unravelled, but Lorenzen and colleagues found that an insertion of a very large, composite transposon is tightly associated with M1 activity and that it is the probable cause of the maternally controlled selfish behaviour of this locus (Lorenzen et al., 2008). The system seems to be restricted to the *Tribolium* genus.

Another maternal-effect selfish gene system was discovered in *Mus musculus*: the severe combined anaemia and thrombocytopenia (scat) (Peters and Barker, 1993).

### 4.5.2 Potential use

The system, especially the synthetic versions (see below), may be used to replace a population. Synthetic MEDEA systems might also include a payload gene.

### 4.5.3 Experience

The first engineered MEDEA gene drive system was based on an RNA interference (RNAi)-based toxin–antidote combination (Chen et al., 2007) (Figure 5). Maternally inherited *myd88* is required for dorsal-ventral pattern formation in early embryo development. Two microRNAs (miRNAs) were designed to silence expression of the *myd88*. They are expressed during meiosis and take effect during embryonic development. The antidote is a miRNA–insensitive *myd88* transgene and is expressed in the zygote. The gene construct was introduced in *Drosophila melanogaster* (Chen et al., 2007) and *Drosophila suzukii* (4 microRNAs) (Buchman et al., 2018).

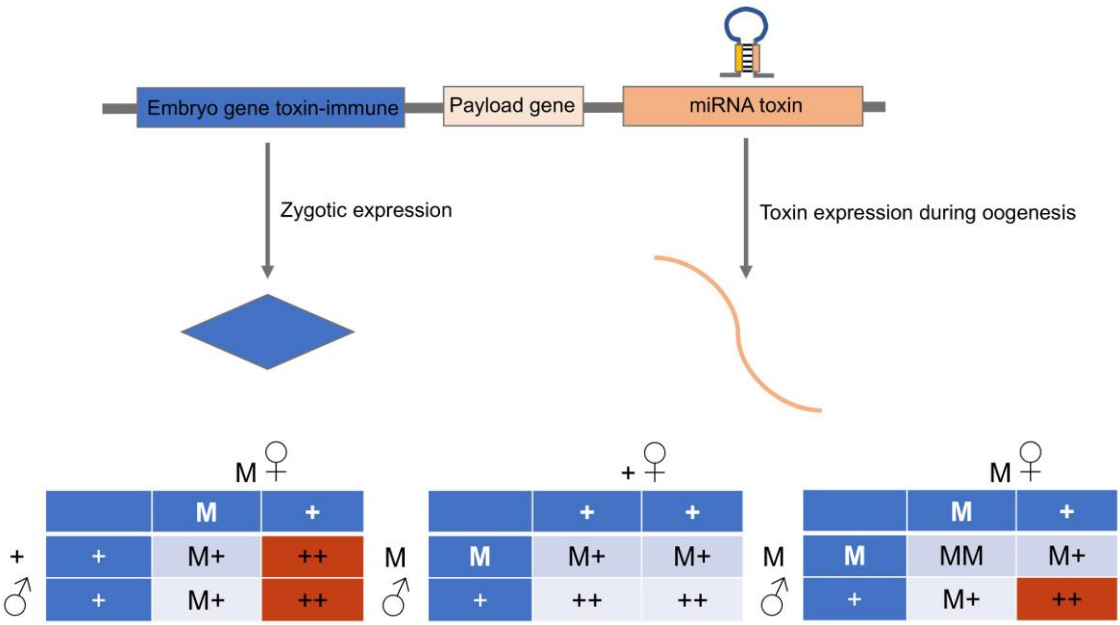


**Figure 5:** General picture of an engineered, RNAi-based MEDEA including a payload gene. In the crossing schemes a red background means lethal; M: MEDEA-bearing; +: wild-type. In the middle scheme all progeny is viable since with a wild-type female no toxin is produced (redrawn after Champer et al., 2016)

Akbari et al. based their system on the silencing of the genes for discontinuous actin hexagons (*dah*, CG6157) and O-fucosyltransferase 1 (*o-fut1*, also known as neurotic, CG12366) (Akbari et al., 2014). Absence of the first gene product is lethal in early embryos, absence of the second leads to the production of excess neurons at the expense of embryonic epidermis. Loss of maternally-provided DAH or O-FUT1 cannot be rescued through inheritance of a wild-type copy of the gene from the father. The genes were silenced using two synthetic miRNAs. The antidote encodes a version of either *dah* or *o-fut1* resistant to silencing. This system was developed in *Drosophila melanogaster*.

Despite considerable effort, functional synthetic MEDEA elements have not yet been developed in a mosquito species.

### 4.5.4 Characteristics

- MEDEA is naturally present in several species;
- Natural MEDEA has a moderate drive efficiency;
- MEDEA-inspired gene drive systems may be used to rapidly drive payload genes throughout wild populations (population replacement);
- No applications yet beyond the lab.

## 4.6 Homing endonuclease genes

### 4.6.1 Mode of action

Burt first proposed to use homing endonuclease genes (HEGs) as gene drives (Burt, 2003). These endonucleases are able to selectively disrupt specific gene sequences (target sequence), combined with a rapid spread in the population. Homing can be defined as:

> *The process by which an endonuclease cleaves a specific DNA target sequence and copies itself, or 'homes', into this target sequence. Homing utilizes the cell's homology-directed repair (HDR) machinery, which relies on sequences that flank the endonuclease and that are homologous to either side of the target sequence. The ultimate result of 'homing' is to generate an exact copy of the endonuclease in the target sequence* (Champer et al., 2016).

Double stranded DNA breaks activate highly conserved cellular repair mechanisms. The DNA ends can be reattached together primarily by either non-homologous end joining or by homology-directed repair. If the chromosome homologous to the one that is cut contains the HEG it can be included as a template to fill the gap. The latter is accomplished because the endonuclease gene has sequences on either side that are homologous to the target sequence. The phenomenon transforms a heterozygote organism into a homozygote for the gene drive (Figure 6). If this successfully happens anywhere in the lineage of the cells that will form the germline, the frequency of the changed allele in the progeny will be higher than expected according to Mendelian rules. Non-homologous end joining may induce resistance to the HEG, as the repair mechanism often joins incorrectly thereby changing the HEG target site.

### 4.6.2 Potential use

Despite 15 years since being first proposed, their use has been limited by the availability of sequence specific endonucleases. For the system to spread effectively, the HEG must cut a target site that will be found throughout the target population. However, the site must be relatively unique in the organism's genome as otherwise severe fitness costs will results from DNA cuts throughout the genome. Finding a meganuclease (restriction enzymes with large recognition sequences) that matches these requirements proved difficult as is reengineering the recognition sequence of previously discovered ones.

### 4.6.3 Experience

Proof-of-concept experiments were performed by Windbichler and colleagues in *Anopheles gambiae* cells and embryos (Windbichler et al., 2007) and by Chan et al. in *Drosophila melanogaster* (Chan et al., 2011). They used a meganuclease and studied its homing into a synthetic target sequence not normally present in that organism's genome. In *Drosophila* homing rates of 2-74% are observed depending on the timing of I-*Sce*I expression (promoter choice) (Chan et al., 2011). In a later publication Windbichler et al. demonstrated the system in receptive mosquito cage populations, validating mathematical models for the transmission dynamics of HEGs (Windbichler et al., 2011). In these experiments with *Anopheles gambiae* the homing rate of I-SceI was found to be 56%.

Since then, easier to reengineer site specific DNA binding proteins have been discovered and developed. Simoni et al. showed that also transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) could be used as gene drives, with homing frequencies of 34% and 49% to available target loci, respectively, in *Drosophila melanogaster* (Simoni et al., 2014). These endonucleases are easier to engineer, but their repetitive structure has a negative effect on their genetic stability. Incomplete cutting is another obstacle for fast driving. More recently, a new class of easily to reprogram site specific endonucleases have been discovered providing the most effective HEG drive systems to date (see 4.7).

### 4.6.4 Characteristics

- The first proposed HEGs have limited applicability as a gene drive because of their specificity.
- Other nucleases may take over to overcome these limitations.
- They possess high rates of drive and can be exploited for both population suppression and replacement.
- Proven effectivity in lab cage populations.

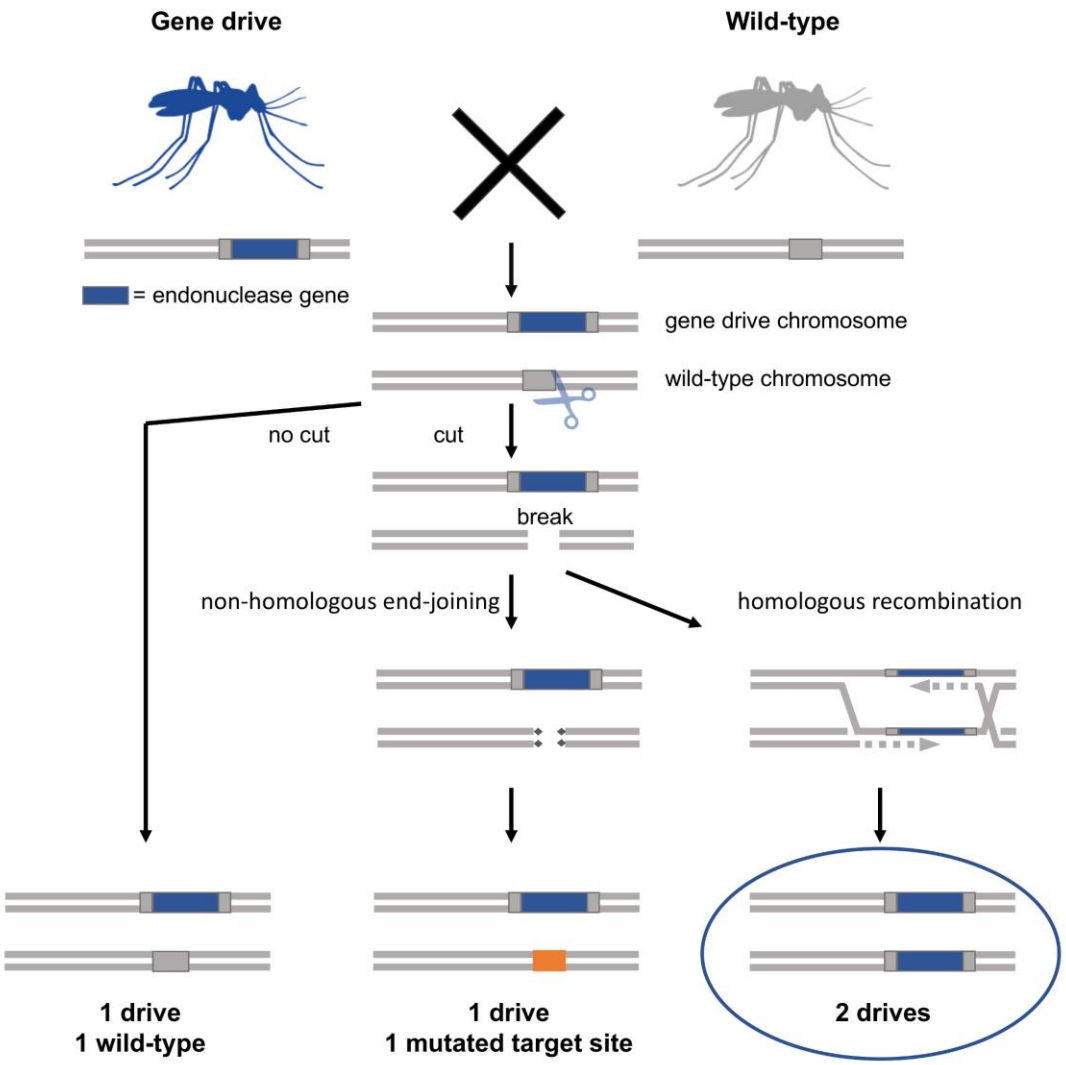


**Figure 6:** The spread of homing endonuclease gene drives (redrawn after Esvelt et al., 2014)

## 4.7 CRISPR/Cas gene drives

### 4.7.1 Mode of action

The CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR associated protein) system that is now widely used as a gene editing system, can be used to create a homing endonuclease drive system (Figure 7). The advantage of CRISPR-Cas over other known nucleases is that it uses an easily reengineerable guide RNA to find its target. Unlike meganucleases, ZFNs, or TALENs, not the sequence of the endonuclease gene, but the guide RNA needs to be adapted to the desired target site. The basic design of a CRISPR/Cas gene drive consists of the CRISPR endonuclease gene, one or more guide RNA sequences and depending on the application a payload gene. The system is often introduced on a plasmid with on either side of the drive cassette sequences homologous to the target site in order to induce homing.

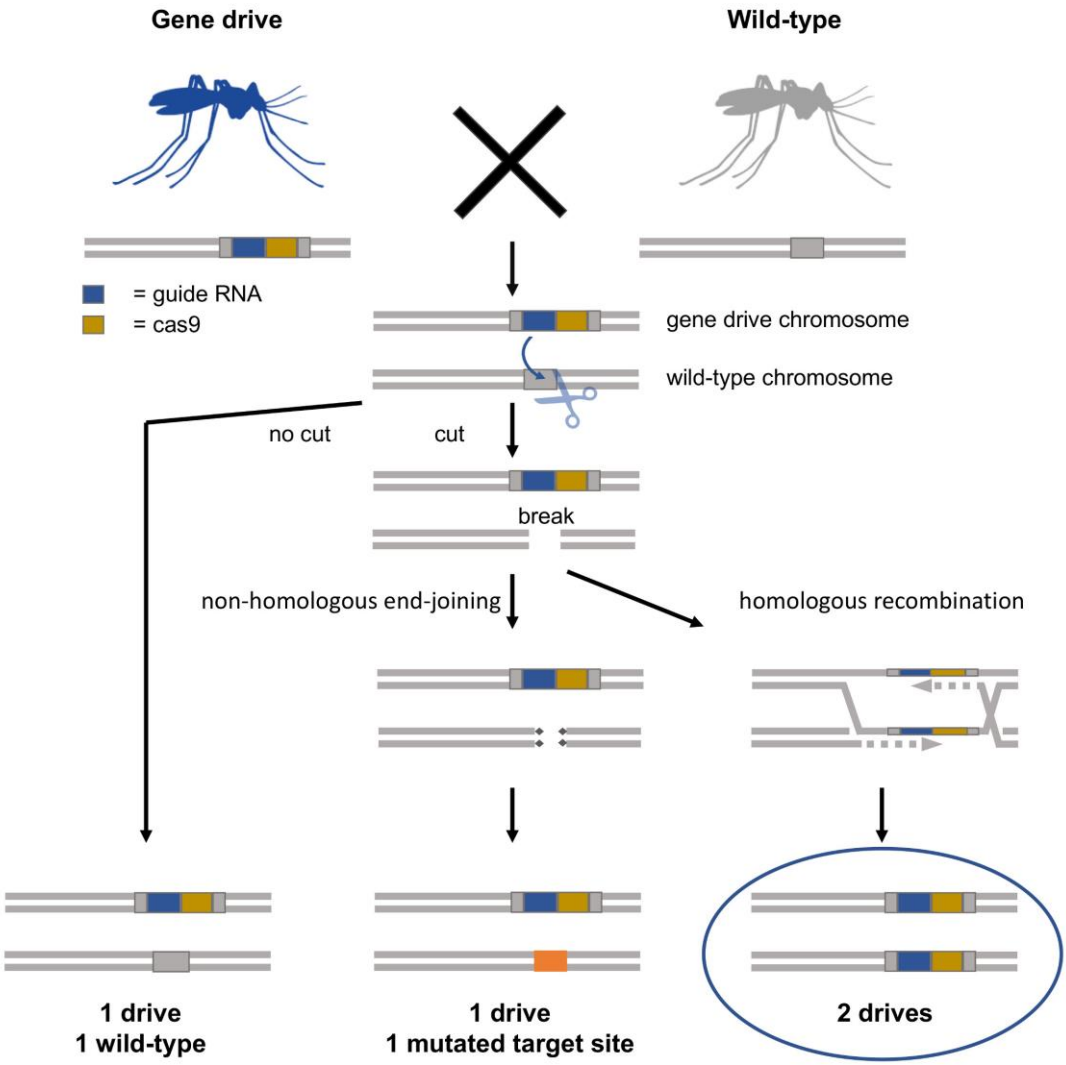


**Figure 7:** The spread of a CRISPR/Cas gene drive (adapted from Esvelt et al., 2014)

### 4.7.2 Potential use

CRISPR/Cas gene drives like some of the drives discussed above can be either used to suppress or replace a population they are introduced into depending on the payload gene. Suppression may also result from a guide RNA targeting an essential gene. Generally, gene-driven modifications to a population are not expected to be permanent. Even if harmful traits can be spread into the population, selection for more fit alleles will occur (Esvelt et al., 2014). This, however, may be long enough to interrupt transmission cycles of certain pathogens or cause a population crash.

## Insert 2
## Can CRISPR/Cas used for gene editing inadvertently lead to a gene drive system?

To make edits to a particular DNA sequence both the sequences for Cas9 and guide RNA are delivered to a cell. This can be done on a plasmid, but guide RNA can also be delivered as such. The guide RNA targets the Cas9 endonuclease to the site to be edited (Figure 8). Cas9 will cut both strands of DNA, creating a blunt-ended double-strand break. When a repair template is supplied containing the desired changes and homology to the sequences on either side of the break, the cell may use homologous recombination to repair the break by copying the repair template into the chromosome. If not, the break will be repaired by non-homologous end-joining, resulting in a mutated gene.

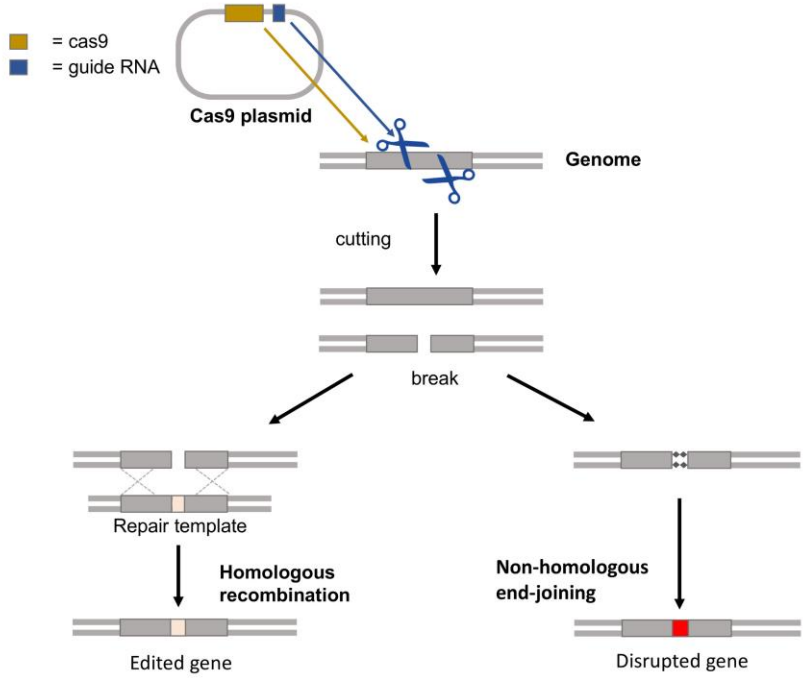


**Figure 8:** Gene editing by CRISPR/Cas9 (adapted from Esvelt et al., 2014)

In this scenario the cassette encoding Cas9 and the guide RNA will not be incorporated in the genome as they lack sequence homology with the target gene on either side. The plasmid will be cleared. The editing event happens only once and will be passed on to the daughter cells. If editing occurs in the germline, the edited gene will inherit in a Mendelian fashion.

However, placing the genes that encode Cas9 and a guide RNA into the template used for homologous recombination generates a gene drive (Figure 7). In other words, it is a requirement that on both sides of the CRISPR/Cas9 cassette sequences are present that are homologous to both sides of the target site. Accidentally introducing a gene drive system using the CRISPR/Cas9 editing system is therefore highly unlikely. The odds that plasmids with the CRISPR/Cas9 cassette that are offered to a cell also contain sequences homologous to the target gene to allow homologous recombination are very small. Transfecting plasmids usually only results in transient expression without integration. However, if plasmids become integrated into the genome (*e.g.* especially using the *Agrobacterium tumefaciens* delivery system in plants), the probability that they integrated near the target site is very small. Moreover, if the guide RNA is provided separately, the unlikely integration of *cas9* in the target site makes it an incomplete system.

### 4.7.3 Experience

Esvelt and colleagues first suggested the use of CRISPR/Cas9 as a gene drive mechanism (Esvelt et al., 2014). The proof came in the next year for yeast (DiCarlo et al., 2015) and fruit fly (Gantz and Bier, 2015). In addition, CRISPR gene drives have been introduced in *Anopheles gambiae* (Hammond et al., 2016), *Anopheles stephensi* (Gantz et al., 2015) and *Candida albicans* (Shapiro et al., 2018). Homing rates in these studies are often very high (greater than 95%) and maintained over several generations (4 for *Anopheles stephensi* (Gantz et al., 2015) and 5 for *Anopheles gambiae* (Hammond et al., 2016)).

As with other HEGs, the targeted locus may develop alleles resistant to the endonuclease *e.g.* due to insertions or deletions at the target sequence in non-homologous end joining. In addition, standing variation in a population may make some individuals resistant. With the CRISPR/Cas system this may be overcome by inserting multiple guide RNAs targeting multiple adjacent sites (Esvelt et al., 2014; Marshall et al., 2017; Prowse et al., 2017). The probability that all sites develop resistance is likely very low. Efficient multiplexing of guide RNAs is technically difficult, but proof-of-principle experiments such as ribozyme flanked guide RNAs have been demonstrated in *Drosophila melanogaster* (Marshall et al., 2017). Other endonucleases, such as the recently described Cpf1-family proteins (Zetsche et al., 2015) may offer a simplified system in multiplexing guide RNAs as they can use a single, one promoter-RNA construct consisting of small RNAs separated by spacers that is processed into single hairpin structures (Zetsche et al., 2017).

Other methods to reduce the development of resistant alleles may include careful selection of target sequences to reduce the frequency of micro-homology-mediated end joining, targeting essential genes where resistance mutations would give a large fitness cost, expression of the endonuclease only in the germ line during meiosis to increase the rate of homology-directed repair, blockage of ligase IV and other components required for non-homologous end joining and activation of homologous repair genes (Champer et al., 2016; Esvelt et al., 2014).

Daisy-chain gene drives were proposed as a method of confining gene drive systems to local populations (Figure 9) (Noble et al., 2016). A daisy drive consists of a series of unlinked drive elements, in which each element drives the next in the chain. The one at the bottom of the chain cannot drive and will be lost over time due to natural selection. The next element in the series will then stop driving and will be lost as well. This continues until all elements are lost. The element at the top may carry a payload gene. The capacity to spread is limited by the successive loss of non-driving elements. Modelling the systems reveals that the longer the chain, the stronger the drive. Only very low release frequencies are necessary.

### 4.7.4 Characteristics

- Highly efficient, synthetic drive system with a low release threshold.
- Easy to engineer because the target site is recognised by the guide RNA and not by the endonuclease as with other HEGs.
- Can be used as suppression or replacement drive.
- Resistant alleles may be present or develop in a population. However, strategies are proposed to circumvent or retard resistance development.
- Only lab experiments performed.

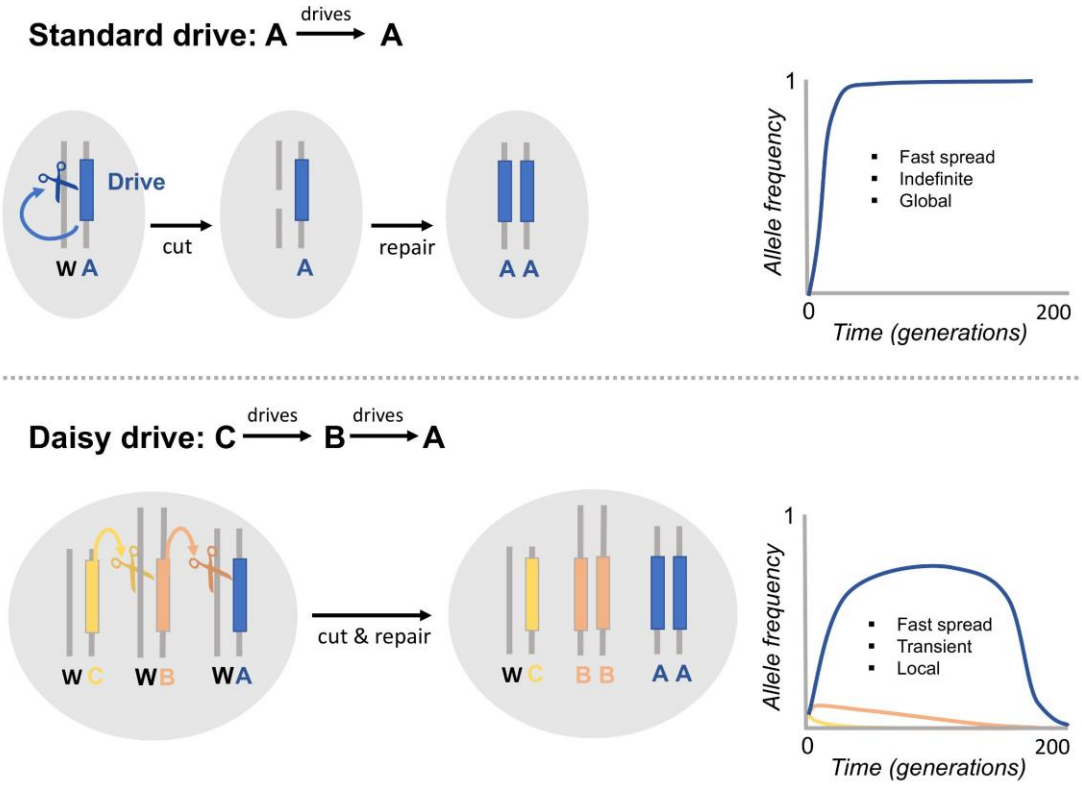


**Figure 9:** **a**, Standard CRISPR gene drives distort inheritance in a self-sustaining manner by converting wild-type (W) alleles to drive alleles in heterozygous germline cells. **b**, A "daisy drive" system consists of a linear chain of serially dependent, unlinked drive elements; in this example, A, B, and C are on separate chromosomes. Elements at the base of the chain cannot drive and are successively lost over time via natural selection, limiting overall spread (redrawn after Noble et al., 2016)

## 4.8 Additional considerations

In comparing the characteristics of the previously reported gene drive systems, it is possible to categorise them in relation to application for either suppression use and/or replacement use (Table 2) (Champer et al., 2016). In this table only ideal-case scenarios are compared to emphasise intrinsic differences of the various types of drives. However, characteristics are variable and depend on a range of factors (*e.g.* ecology of the target species, population distribution, movement patterns, fitness costs, payload characteristics, …). Consequently, the same drive system can be used for different uses. Chromosomal rearrangement can be used also for short-term population suppression. Also, male-killing strains of *Wolbachia* may be applicable for population suppression. Many systems are not in principle locally confined, but high fitness costs may accomplish that, as was seen in field releases of *Aedes aegypti* infected with different strains of *Wolbachia* (see 5.3.1.1). In that case they may be removable as well, if large numbers of wild-type organisms are introduced in the population.

**Table 2**: Summary of characteristics of several (engineered) gene drive systems (adapted from Champer et al., 2016).

| | ***Wolbachia*** | **Transposable elements** | **Underdominance** | **Meiotic drive** | **MEDEA** | **Homing-based drive (including CRISPR/Cas)** |
|---|---|---|---|---|---|---|
| **Type** | Replacement (Suppression) | Replacement | Replacement | Suppression | Replacement | Replacement Suppression |
| **Rate of spread** | Moderate | Slow | Slow | Moderate | Moderate | Fast |
| **Locally confined?** | No, if low fitness cost[1] | No | Yes | No | No, if low fitness cost[1] | No |
| **Resistance allele generation rate** | Unknown | Low | Moderate | Low | Low | High |
| **Reversible?*** | No | Yes | Yes | Yes | Yes | Yes |
| **Removable with wild type?**** | No, if low fitness cost[1] | No[2] | Yes | No[2] | No, if low fitness cost[1] | No[2] |
| **Usability?** | +++ | + | ++ | ++ | ++ | +++ |
| **Prevalence in natural populations** | 0-100% arthropod populations | Widespread (incl. humans) | Not applicable | 1-5% (*Drosophila*) 10-20% (mice) 14% (*Zea* spp.) 80% (*Gibberella*) | 0-100% (*Tribolium castaneum*) | Not applicable |

[1] High fitness costs may make these systems locally confined and removable with the release of large numbers of wild-type organisms.

[2] Suppression types that proceed to fixation and eliminate a population will remove the gene drive system, allowing replacement with wild-type organisms.

* Reversibility is the ability to replace an existing gene drive system with another gene drive. Only ideal-case scenarios are compared to emphasise intrinsic differences of the various types of drives. However, characteristics are variable and depend on a range of factors

** Removability is the ability to completely remove a gene drive system from a population via the release of wild-type organisms.

Bidirectional drives, like the underdominance drives, have a high release threshold (*e.g.* up to and above 50% of the total number in the wild population). Released at numbers below the threshold will be selected out of the population over time while those released in quantities above the threshold will eventually be propagated throughout the population (Marshall and Hay, 2012) (see also experimental results in 5.1 and 5.2). On the other hand, this type of drives are attractive because they induce local rather than global replacement, and transgenes can be eliminated through dilution of the population with wild-type individuals (Akbari et al., 2013). It must be noted that only when wild-type individuals with the same genetic background as the original population are used to remove the gene drive, the original situation may be restored.

The life stage at which the gene conversion takes place (meiosis or embryo), also determines the "success" of a gene drive (de Jong, 2017). In a gametic drive (*e.g.* a CRISPR/Cas drive driven by a promoter restricted to meiosis) the conversion takes place during meiosis. The parent organism is heterozygous for the drive but produces more than 50% drive-containing gametes. In a zygotic drive (*e.g.* MEDEA) the conversion occurs after fertilisation. The resulting organism is homozygous for the drive in (almost) all its tissues. Modelling revealed that the chance of gene drives increasing until fixation is much higher in systems with a drive active at meiosis (de Jong, 2017). Still according to this modelling, the gametic drive will also spread easier and faster. This effect may reduce the probability of resistance development.

Some drives can be reversed, meaning that their activity can be stopped by introducing another drive. Approaches on how to achieve this have been suggested. In CRISPR/Cas systems this may be accomplished by targeting the reversal drive to replace the first drive (Champer et al., 2016). An RNA-guided endonuclease X-shredder (meiotic drive) can be reversed likewise using an X chromosome containing multiple guide RNAs targeting the guide RNAs of the original X-shredder. These X-chromosome-localised guide RNAs would be activated before the guide RNAs on the Y chromosome, resulting in removal of the guide RNAs on the Y chromosome before the X chromosome is shredded. A CRISPR/Cas gene drive may also reverse other gene drive systems, *e.g.* targeting the transposase gene in transposons, the toxin gene in MEDEA, etc. However, reversing does not mean that the genetic elements are removed from the population, only their activity.

The time necessary to spread to all members of a population depends on the number of drive-carrying individuals that are released relative to the total population, the generation time of the organism, the efficiency of the drive, the impact of the drive on individual fitness, and the dynamics of mating and gene flow in the population. In general it will take at least several dozen generations (Esvelt et al., 2014). For example the driving efficiency in HEGs is on average 56%, whereas for CRISPR/Cas9 it is situated between 90 and 100%, and for transposons it is very low (<0.05%) (Macias et al., 2017b). A perfect homing system (100% homing, no resistance, no fitness cost) will double its inheritance rate from heterozygotes. Initially, after introduction of a low number of drive-bearing individuals in the target population, it will double its allele frequency each host generation. Then, as allele frequency increases, the rate of spread will reduce as a higher proportion of elements are in homozygous individuals, in which they cannot home. Even if the presence of a drive reduces the fitness, it may still spread into the population. After some time the population will reach an equilibrium. In its simplest form the relationship between fitness cost and homing rate can be formalised as follows (Godfray et al., 2017):

$$q = e/s$$

Where q is the equilibrium frequency for the gene drive allele, e is the homing frequency and s is the fitness reduction. This applies with abstraction from resistance development. When the fitness effects are greater than the homing frequency ($s > e$) there will be a (stable) polymorphism with both alleles (with and without the gene drive) present, but when the reverse is true ($s < e$, implying $q > 1$, which is impossible) the gene drive will increase to fixation and the wild-type allele will disappear. For population suppression s will be high, and low for population replacement.

Summarising the main shortcomings, natural meiotic drives were not efficient enough (Lyttle, 1977, 1979, 1981), underdominance systems based on translocations suffer from high fitness costs (Curtis, 1968; Foster et al., 1972; Laven et al., 1972; Robinson, 1976). The MEDEA system, originally described in *Tribolium* (Beeman et al., 1992) and engineered to be applied in *Drosophila* (Akbari et al., 2014; Buchman et al., 2018; Chen et al., 2007), has proven difficult to apply in mosquitoes (Champer et al., 2017b). The same is true for transposable element-based drives. All these systems were tried in insects. Other species are being addressed as well. In yeast the CRISPR/Cas9 methods proved to be successful in the lab (DiCarlo et al., 2015; Shapiro et al., 2018). For mice it is too early to report on lab results either using the t-haplotype or CRISPR/Cas9 (Thomas; Threadgill, pers. comm.).

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# 5 Studies on gene drive systems

Table 3 lists a variety of organisms and gene drive systems that have been described or developed. The list is not exhaustive, but rather provides an impression on different options that have been evaluated. Most of the earlier studies aimed at gaining insight in the working mechanism of the gene drive. Others tried to apply them in selected organisms.

**Table 3:** Selected examples of organisms and gene drive mechanisms (D: description of a natural gene drive; L: lab experiments to test gene drive function; Determination of the GMO-status is according to the definitions in EU legislation[3])

| Species | Mechanism | | GMO? | Reference |
|---|---|---|---|---|
| **Micro-organisms** | | | | |
| *Candida albicans* | CRISPR-Cas9 | L | Yes | (Shapiro et al., 2018) |
| *Gibberella moniliforme (Fusarium moniliforme)* | Meiotic drive / Spore killer | D | No | (Kathariou and Spieth, 1982) |
| *Neurospora sitophila* and *N. intermedia* | Meiotic drive / Spore killer | D | No | (Turner and Perkins, 1979) |
| *Saccharomyces cerevisiae* | CRISPR-Cas9 | L | Yes | (DiCarlo et al., 2015) |
| **Plants** | | | | |
| *Nicotiana tabacum* | Meiotic drive / Pollen killer | D | No | (Cameron and Moav, 1957) |
| *Oryza sativa* | Meiotic drive / Pollen killer | D | No | (Sano, 1990) |
| *Silene latifolia* | Meiotic drive | D | No | (Taylor and Ingvarsson, 2003) review article |
| *Zea mays* | Meiotic drive (Ab10 chromosome) | D | No | (Rhoades, 1942) |
| **Insects** | | | | |
| *Acraea quirina* | Meiotic drive | D | No | (Owen, 1974) |
| *Aedes aegypti* | Meiotic drive | D | No | (Hickey and Craig, 1966a, b) |
| | *Wolbachia* | L | No | (McMeniman et al., 2009; Walker et al., 2011) |
| | Underdominance (translocations) | L | No | (Suguna and Curtis, 1974) |
| *Aedes albopictus* | *Wolbachia* | D | No | (Sinkins et al., 1995) |
| *Aedes fluviatilis* | Transposable element (*piggyBac*) | L | Yes | (Rodrigues et al., 2006) |
| *Aedes polynesiensis* | *Wolbachia* | L | No | (Brelsfoard et al., 2008) |
| *Anopheles gambiae* | Underdominance | L | No | (Graves and Curtis, 1982) |
| | Meiotic drive (X-shredder) | L | Yes | (Galizi et al., 2014) |
| | Homing endonuclease (*I-SceI*) | L | Yes | (Windbichler et al., 2011) |
| | CRISPR-Cas9 | L | Yes | (Hammond et al., 2016) |
| *Anopheles stephensi* | CRISPR-Cas9 | L | Yes | (Gantz et al., 2015) |
| | *Wolbachia* | L | No | (Bian et al., 2013) |

[3] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal L 106, 17/04/2001, p.1-39.

| Species | Mechanism | | GMO? | Reference |
|---|---|---|---|---|
| *Ceratitis capitata* | Meiotic drive | D | No | (Shahjahan et al., 2006) |
| *Culex pipiens* | *Wolbachia* | D | No | (Hertig and Wolbach, 1924) |
| | Underdominance (translocations) | L | No | (Laven and Jost, 1971) |
| | Meiotic drive | D | No | (Sweeny and Barr, 1978) |
| *Culex quinquefasciatus (Culex fatigans)* | Underdominance (cytoplasmic incompatibility with and without translocation) | L | No | (Curtis and Adak, 1974) |
| | Underdominance (cytoplasmic incompatibility, translocation) | L | No | (Krishnamurthy and Laven, 1976) |
| | *Wolbachia* | L | No | (Atyame et al., 2011) |
| *Culex tarsalis* | *Wolbachia* | L | No | (Dodson et al., 2014) |
| *Drosophila melanogaster* | Underdominance (translocations) | L | No | (Foster et al., 1972) |
| | Underdominance (translocations) | L | Yes | (Buchman et al., 2016) |
| | Transposable element (P-element) | L | Yes | (Spradling and Rubin, 1982) (Meister and Grigliatti, 1993) |
| | Meiotic drive (Segregation Distorter) | D | No | (Sandler et al., 1959) |
| | *Wolbachia* | D | No | (Zhou et al., 1998) |
| | MEDEA | L | Yes | (Chen et al., 2007) |
| | Underdominance (toxin–antidote) | L | Yes | (Akbari et al., 2013) (Reeves et al., 2014) |
| | Homing endonuclease (*I-SceI*) | L | Yes | (Chan et al., 2011) |
| | Homing endonuclease (ZFNs and TALENs) | L | Yes | (Simoni et al., 2014) |
| | CRISPR-Cas9 | L | Yes | (Gantz and Bier, 2015) |
| *Drosophila obscura* | Meiotic drive | D | No | Gershenson, 1928 |
| *Drosophila pseudoobscura* and others | Meiotic drive | D | No | (Sturtevant and Dobzhansky, 1936) |
| *Drosophila simulans* | *Wolbachia* | D | No | (Turelli and Hoffmann, 1991) |
| | Meiotic drive | D | No | (Faulhaber, 1967) |
| *Drosophila suzukii* | MEDEA (toxin–antidote) | L | Yes | (Buchman et al., 2017; Buchman et al., 2018) |
| *Monomorium pharaonis* | *Wolbachia* | L | No | (Pontieri et al., 2017) |
| *Tribolium castaneum* | MEDEA | D | No | (Beeman et al., 1992) |
| **Mammals** | | | | |
| *Mus musculus* | Meiotic drive (t-haplotype) | D | No | (Sandler and Novitski, 1957) |
| | Combined anaemia and thrombocytopenia (scat) | D | No | (Peters and Barker, 1993) |
| | Meiotic drive (t-haplotype + *sry*) | L | Yes | David Threadgill, Texas A&M University (pers. comm.) |

| Species | Mechanism | | GMO? | Reference |
|---|---|---|---|---|
| | CRISPR-Cas9 | L | Yes | Paul Thomas, University of Adelaide (pers. comm.) |
| *Myopus schisticolor* | Meiotic drive | D | No | (Fredga et al., 1977) |
| *Dicrostonyx torquatus* | Meiotic drive | D | No | (Gileva, 1987) |

## 5.1 Studies in contained environment

Field cage experiments (Table 4) were started for *Culex quinquefasciatus* (*Culex fatigans*) testing the gene drive properties of bidirectional cytoplasmic incompatibility (Curtis, 1976). A strain with Paris cytoplasm and carrying a male-linked translocation causing partial sterility was introduced daily in a Delhi origin population (underdominance). This allowed for generations to overlap. This bidirectional gene drive system is expected to evolve to the cytoplasmic type that was given an initial high frequency, *i.e.* the Paris type in this case. In the first experiment the releases of the Paris type (male/female = 1:1) continued for 32 days. After 4 more months of breeding the original population was replaced. Because of partial compatibility of males of the Delhi population with Paris females, "recombinant" males with Paris cytoplasm and no translocation were produced. The second experiment tried to avoid this. Here males and already mated females were introduced at a ratio of 10:1 for 92 days and finally completely replaced the Delhi type. A third experiment where immigration of Delhi types was allowed, an equilibrium was expected, but a gradual increase of the Paris cytoplasm non-translocated type occurred. This was due to cytoplasmic incompatibility that is partly lost in aged and repeatedly mated males of the immigrating Delhi types. The non-translocated Paris type gradually increased under the pressure of natural selection.

A *Wolbachia* strain *w*Mel originating from *Drosophila melanogaster* was injected in *Aedes aegypti* embryos (Walker et al., 2011). Three independent, stably infected lines were obtained. One line was further investigated and was found to induce strong cytoplasmic incompatibility. Potential fitness effects (fecundity, egg viability, larval development time, longevity of adults) were found to be non-existing, except for a 10% life-shortening effect. On the contrary, another strain, the *w*MelPop strain (see Table 5), induced a fecundity cost of 56% and a lower egg viability and lifespan compared to wild type. A semi-field cage invasion experiment was set up in Cairns, Queensland, Australia. A starting frequency of 0.65 was chosen with additional near-weekly supplementary additions of *Wolbachia*-infected mosquitoes. Experiments lasted 80 to 90 days. The *w*MelPop-infected mosquitoes reach fixation later than the *w*Mel-infected *Aedes aegypti* due to the lower fitness (80% and 100% after 80 days resp.). Predation leads to a quicker fixation.

Modelling has been performed to study the effect of accidental releases from outdoor cages into the environment (Marshall, 2009). The probability of losing the gene drive depends not only on the number of escapes, but also on the type of driving mechanism, the organism itself and the fitness cost. Resistance development was not calculated. Engineered underdominance constructs almost certainly go extinct. HEGs and meiotic drives have the highest probability to establish. Current CRISPR gene drive systems are likely to be highly invasive in wild populations (Noble et al., 2017).

## 5.2 Field studies

Only very few systems were tested in the field (Table 4). They are discussed in detail below. Sofar no GM organisms containing a gene drive have been introduced in the environment.

The field releases mostly concern mosquitoes that are targeted because of their function as a vector for human diseases. *Aedes aegypti* can spread dengue fever, Chikungunya, Zika fever, Mayaro and yellow fever viruses. *Aedes polynesiensis* is a vector of dengue, Ross River virus, lymphatic filariasis, and a probable vector of Zika virus. *Culex pipiens* is a vector for diseases including Japanese encephalitis, West Nile virus, and Usutu virus. *Culex quinquefasciatus* vectors *Wuchereria bancrofti*, avian malaria, and arboviruses including St. Louis encephalitis virus, Western equine encephalitis virus, Zika virus and West Nile virus.

The Australian sheep blowfly, *Lucilia cuprina*, is the source of a parasitic infestation of the skin of sheep by fly larvae.

**Table 4:** Gene drive organisms introduced into the environment. (F: open field release; (F): semi-field/cage; Determination of the GMO-status is according to the definitions in EU legislation[4]).

| Species | Mechanism | | GMO? | Reference |
|---|---|---|---|---|
| *Aedes aegypti* | Underdominance (translocations) | F | No | (Lorimer et al., 1976) |
| | *Wolbachia w*Mel and *w*MelPop-CLA | (F) | No | (Walker et al., 2011) |
| | *Wolbachia w*Mel | F | No | (Hoffmann et al., 2011) |
| | *Wolbachia w*MelPop-CLA | F | No | (Yeap et al., 2014) |
| | *Wolbachia w*MelPop-PGYP | F | No | (Nguyen et al., 2015) |
| *Aedes polynesiensis* | *Wolbachia* | F | No | (O'Connor et al., 2012) |
| *Culex pipiens* | Underdominance (translocations) | F | No | (Laven et al., 1972) |
| *Culex quinquefasciatus (Culex fatigans)* | Underdominance (cytoplasmic incompatibility, translocation) | (F) | No | (Curtis, 1976) |
| | Underdominance (cytoplasmic incompatibility, translocation) | F | No | (Curtis et al., 1982) |
| *Lucilia cuprina* | Underdominance (translocation) | F | No | (Foster et al., 1985; Vogt et al., 1985) |

### 5.2.1 1970 *Culex pipiens* - France

In 1970 a field trial was performed in Notre Dame near Montpellier, France with *Culex pipiens* carrying a gene drive based on a translocation (Laven et al., 1971, 1972). Males heterozygous for a sex-linked translocation that showed 50% sterility were released in a large closed well for two months (August – September). The ratio of released gene drive males to wild-type males went up to 5:1. Egg rafts were inspected for semi-sterility. The number increased to 95% at the end of the release period. The daily production of adults went down in the same period from 20.000 to 100. This is about 10% of the normal production for that time of the year. The next year the population remained very low. 89% of the egg rafts showed translocation sterility. This means that although a high frequency of translocations was present, the population did not go extinct. Next to the gene drive the authors presume that the small population size was also due to a higher predator and parasite pressure compared to the previous year.

### 5.2.2 1973 *Culex quinquefasciatus* - India

In India field trials with *Culex quinquefasciatus* were conducted near New Delhi in 1973 (Curtis et al., 1982). A strain was developed with Paris cytoplasm and Indian chromosomes (Delhi origin) inducing cytoplasmic incompatibility, including a male-linked translocation, inducing partial sterility. Two villages were chosen, one was surrounded by a 3 km wide breeding-free zone established using larvicides, the other was located within that zone. Two other villages treated the same way served as controls. Before the release the number of inseminated females was reduced by fogging

[4] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal L 106, 17/04/2001, p.1-39.

an insecticide. In the laboratory the modified strain behaved the same as the Delhi strain except that pupal yield relative to first instars did not exceed 80% compared to 90% of the Delhi strain. The competitiveness was less with young males but slightly better for more mature males than the control strain (field cage tests). 5.000 to 40.000 males were released per day per village for 2 to 3 months. The proportion of released males to wild-type males was about 90% (hand catch evaluation). The percentage incompatibility rapidly rose, then plateaued and eventually declined (egg raft evaluation). The maximum number was 68%. The level of sterility in crosses decreased from 71% to 65%. In the control villages the mosquito densities increased according to the normal seasonal trend, whereas in the release villages they gradually decreased. Later on, populations decreased at the same rate in all villages.

In this experiment only partial suppression of the population was achieved. The reason may be found in the immigration of already inseminated females from beyond the breeding-free zone. Likewise, marked release males were recaptured up to 11 km from the release site.

### 5.2.3 1974 *Aedes aegypti* - Kenya

In 1974 a sex-linked translocation homozygous *Aedes aegypti* stock was released in 3 rural villages near Mombasa, Kenya (Lorimer et al., 1976). In one release site the resident population was suppressed before the start of the experiment. Mosquitoes were released as pupae - 500 daily against a resident population of about 800 to 950 – for 9 weeks. This initial experiment used a red eye marker closely linked to the translocation. In a second experiment adults with wild-type eyes were released in the same ratio for 10 weeks. In none of the villages the translocation bearing *Aedes aegypti* became established, even not in the village with a suppressed mosquito population. Reasons for the failure were: low fertility, long larval development time, low larval and adult survival and low mating competitiveness. Individuals with the wild-type eye performed better than the red-eyed ones. An important factor may have been the origin of the stock: an *Aedes aegypti* population from New Delhi, India, that lived in a habitat very different from the one in Kenya.

In a review by Asman et al. trials with several techniques to control mosquitoes, a.o. translocations in *Aedes aegypti*, were discussed (Asman et al., 1981). Many failed because the strains showed reduced fertility, viability and could not compete with the wild-type population.

### 5.2.4 1976 and 1978 *Lucilia cuprina* – Australia

Partially sterile males of the Australian sheep blowfly (*Lucilia cuprina*) with sex-linked translocations were used (Foster et al., 1985). These males have normal eyes but carry recessive eye colour mutations. They transmit the translocations to their sons and the mutations to their daughters. Females when homozygous for both eye colour mutations are fully viable in laboratory culture but inviable in the field. When wild-type females mate with gene drive males they will be reduced in fertility (because of the translocations) and in their offspring the mutations will induce genetic death. Field trials were performed in 2 locations over 2 seasons in New South Wales (Vogt et al., 1985). The first trial covered an isolated area of 260 $km^2$, the second 300 $km^2$. Mature male and female larvae were released weekly by plane for 20 months (1$^{st}$ trial) and 8 months (2$^{nd}$ trial). The trials failed for several reasons. The necessary ratio of released males over field-reared males (10:1) was not reached most of the time. On top of that survival rates of released larvae was low, especially in the summer, compared to the survival of the field population, probably due to high soil temperatures at the time of the flights. Also, the mating competitiveness of the released males was low (1/3 of that of field males). Their field-reared, translocation-bearing sons were as competitive as wild-type males (Foster et al., 1985). The levels of deleterious mutations and translocations in the trials were too low to cause a measurable effect on population size. Also, fly movement between the release area and adjacent areas was low, as shown in the 2$^{nd}$ trial without natural barriers.

### 5.2.5 2009 *Aedes polynesiensis* – French Polynesia

A field trial was started with *Wolbachia*-transfected *Aedes polynesiensis* in Toamaro, French Polynesia in 2009 (O'Connor et al., 2012). The *Wolbachia* type originated from *Aedes riversi*. Although this research was based on IIT as opposed to gene drive, the competitiveness and potential for horizontal transfer of *Wolbachia* could be examined. Male infected mosquitoes were

released weekly for 30 weeks. Under these conditions males were not able to transmit *Wolbachia* to conspecific (belonging to the same species) and congeneric (belonging to the same genus) females. It was demonstrated that male *Aedes polynesiensis* carrying *Wolbachia* were competitive mates.

## 5.3 Large scale initiatives

### 5.3.1 World Mosquito Program

*Wolbachia* trials and environmental releases are and have been performed in the framework of the Eliminate Dengue Program, now known as the World Mosquito Program[5], a not-for-profit initiative that works to protect the global community from mosquito-borne diseases. It first concentrated on dengue, but enlarged the scope to other mosquito-borne diseases.

The strategy in these *Aedes aegypti* trials is based on the life-shortening effect and the inhibition of viral replication of the particular strain (McMeniman et al., 2009; Walker et al., 2011).
Several *Wolbachia* strains were developed (Table 5). It was observed that strains that better block dengue transmission also confer greater fitness costs to the mosquito host. For successful invasion a *Wolbachia* strain is needed that balances these two effects. The *w*MelPop-CLA strain strongly blocks dengue virus proliferation, but induces a high fitness cost, whereas the *w*Mel strain is milder. Strain variability is presumably due to variation in the densities and tissue distributions of *Wolbachia* (Walker et al., 2011). A high density of *Wolbachia* in hosts (next to tissue distribution) may increase viral blockage, but decreases host fitness (Hoffmann et al., 2015).

**Table 5:** *Wolbachia* strains used in the framework of the World Mosquito Program (Woolfit et al., 2013).

| ***Wolbachia* strain name** | **Description** |
|---|---|
| *w*Mel | The *Wolbachia* strain endogenous to *Drosophila melanogaster.* |
| *w*MelPop | A *Wolbachia* strain identified during a survey of lab lines of *Drosophila melanogaster.* This pathogenic strain overreplicates in host cells, causing cellular damage and reducing lifespan by approximately one-half in *Drosophila melanogaster* and similarly in *Aedes aegypti.* |
| *w*MelPop-CLA | Derivative of *w*MelPop, that was purified from *Drosophila melanogaster*, transinfected into an *Aedes albopictus*-derived cell line and subsequently into an *Aedes aegypti*-derived cell line. It was serially passaged for approximately 3½ years before being transferred to *Aedes aegypti.* It has reduced pathogenesis: it grows to a lower density and causes a reduced degree of life shortening compared to *w*MelPop. |
| *w*MelPop-PGYP | Derivative of *w*MelPop-CLA. It was purified from *Aedes aegypti* 4 years after transinfection with *w*MelPop-CLA. |

Fretiu and colleagues demonstrated that the *Wolbachia*–infected, field collected *Aedes aegypti*, when challenged with the dengue virus, showed limited rates of body infection, viral replication and dissemination to the salvary glands compared to uninfected controls (Frentiu et al., 2014). A recent study confirms that *w*Mel lowers dengue virus transmission potential and lengthens the extrinsic incubation period (Carrington et al., 2018). It also demonstrates that the difference in susceptibility of field-reared *w*Mel-infected *Aedes aegypti* (with a Ho Chi Minh City background) with wild-type

[5] http://www.eliminatedengue.com/program

mosquitoes was even greater compared to laboratory-reared mosquitoes, when feeding on hospitalised Vietnamese dengue patients. The field-reared *w*Mel-infected mosquitoes were collected as eggs in Tri Nguyen village, central Vietnam, where *w*Mel has been established since 2014[6]. Laboratory-based studies using arbovirus-spiked human blood, have reported that *w*Mel-infected mosquitoes are almost entirely refractory to dengue infection. However, this study shows that feeding on blood of dengue patients infects *w*Mel-infected mosquitoes, sometimes including infectious virus in the saliva.

#### 5.3.1.1 2011 Aedes aegypti - Australia

Field releases were conducted with *Wolbachia* ***w*MelPop-CLA**-infected *Aedes aegypti* in Machans Beach near Cairns in northern Queensland, Australia in 2012[7] (Yeap et al., 2014). Earlier studies showed that *w*MelPop-CLA has deleterious effects on its host including reduced viability of mosquitoes in quiescent eggs, reduced fecundity, reduced ability to blood-feed, and altered development (Yeap et al., 2011). Around 10 females were released weekly per house over a 13-week period. In this experiment this mosquito strain was not able to replace the natural population, although it persisted for several months. Nevertheless, fitness could be studied for mosquitoes reared under field conditions. The lower wing/thorax ratio of released mosquitoes suggests that dispersal ability may be limited influencing host-seeking and oviposition site seeking. Subsequent generations of infected mosquitoes reared in the field had similar size, wing/thorax ratio and shape to uninfected mosquitoes. However, the wing size of the *w*MelPop-CLA-infected *Aedes aegypti* did not vary in response to density or temperature as in uninfected mosquitoes.

Field evaluations with ***w*MelPop-PGYP** *Wolbachia* started in northern Australia (Machans Beach and Babinda) in 2012 (Nguyen et al., 2015). The studies demonstrate that a continuous effort is needed to sustain the population. Initially, the frequency of infected mosquitoes went up during releases, to 80% and 90% resp., but declined as soon as releases stopped. In contrast, subsequent releases of ***w*Mel** in Babinda and Machans Beach became stably established at a high frequency at all sites. The deleterious fitness effects of *w*MelPop as opposed to *w*Mel may explain the difference.

Starting early January 2011 during the wet season, adult female and male *Aedes aegypti* mosquitoes infected with the *Wolbachia* ***w*Mel** strain were released in north-eastern Australia, near Cairns, several times successively (Hoffmann et al., 2011). A total of 141,600 (Yorkeys Knob) and 157,300 (Gordonvale) adults were released. *Wolbachia* frequencies were monitored every 2 weeks. Frequencies in blocks across the release area pointed to significant spatial heterogeneity of *Wolbachia* prevalence. In some blocks release of additional mosquitoes was necessary. After releases have stopped, frequencies of *Wolbachia*-infected mosquitoes increased reaching near-fixation in a few months to 90% and more. The fitness costs was estimated on the order of 20%. *Aedes aegypti* in itself is a weak disperser, but occasionally *Wolbachia*-infected mosquitoes were detected 1-2 km from the release sites. Due to the fitness cost and the small number (below release threshold), they were not expected to establish. After 3 years the stability of the infection and changes in host fitness effects were investigated (Hoffmann et al., 2014b). The results suggest perfect or near perfect maternal transmission in the field. The reduction in egg numbers laid and larvae produced for the Gordonvale infected mosquitoes was similar to the figures pre-release. The Yorkeys Knob mosquitoes showed lower counts. Hence, the negative effects of the *w*Mel infection on fecundity and larval production have persisted in time. Also the cytoplasmic incompatibility remained complete. Nevertheless, a small amount of uninfected mosquitoes (3-6%) persisted in the release areas, probably due to immigration (Turelli and Barton, 2017).

In central Cairns, north-eastern Australia, another set of ***w*Mel**-infected *Aedes aegypti* were released at 3 sites in early 2013 (Schmidt et al., 2017). A slow but steady spatial spread, at about 100-200 m per year was observed for 2 sites. The third site failed, because it did not meet the minimum release area that is needed to achieve stable local establishment. The influx of uninfected mosquitoes pushed the prevalence below the threshold for invasion. The treshold level relates to

[6] http://www.eliminatedengue.com/vn/progress/view/news/378
[7] http://www.eliminatedengue.com/library/publication/document/july_2012_trial_update_-_cairns.pdf

the fitness cost due to the presence of *Wolbachia*. The size of this area depends on the distance that mosquitoes disperse each generation and the invasion threshold (Jiggins, 2017). Nevertheless, the study in Cairns demonstrates the feasibility of patchy releases across large cities, where population replacement occurs via introduction and local spread, provided that releases are conducted over sufficiently large areas. Populations can persist for at least 2 years (2 wet and 2 dry seasons) with fixation around 90%. Trap captures fluctuated with the seasons. The area in which *Wolbachia* persists at high frequency roughly doubled after 2 years. Local environmental factors (habitat quality) greatly influence the spread. The studies also show that areas of low mosquito density or barriers to dispersal, like roads, can slow or halt the spread. In the Gordonvale release, the invasion failed beyond the Bruce Highway for several years, despite persistent migration across the highway (Turelli and Barton, 2017). The threshold prevalence for invasion was never reached.

In comparison, the ***w*Ri** strain of *Wolbachia* in *Drosophila simulans* (natural infection) does not seem to result in fitness loss, but is likely even beneficial. *Wolbachia* advanced at 100 km/year in California (Turelli and Hoffmann, 1991) and Australia (Kriesner et al., 2013). Next to fitness, this also includes distance that flies disperse. *Aedes aegypti* tend to stay local.

In these *Wolbachia*-infected *Aedes aegypti* trials, populations fluctuate according to property attributes: housing (brick vs. wood; high vs. low), shading, availability of water containers, and therefore also the density of residing populations (Hoffmann et al., 2014a; Nguyen et al., 2015). These factors determine threshold frequencies that are needed to reach fixation. Models have been developed using data from the above mentioned releases to optimise the timing, spacing and intensity of future environmental releases (Turelli and Barton, 2017). Due to the diversity of urban landscapes many release areas are required for an area-wide control over just a few years, starting with areas that support a high mosquito density. For isolated populations the threshold frequency for *w*Mel-*Aedes aegypti* is about 20-30%.

#### 5.3.1.2 2013 *Aedes aegypti* - Vietnam

Field evaluations with ***w*MelPop-PGYP** *Wolbachia* were started in central Vietnam (Tri Nguyen, Hon Mieu Island) in 2013 (Nguyen et al., 2015). In Vietnam the residing population was reduced prior to release of the infected mosquitoes. As in Australia (Machans Beach and Babinda) initially, the frequency of infected mosquitoes increased during releases, to nearly 90% but decreased as soon as releases stopped. Releases with this *w*MelPop-infected *Aedes aegypti* therefore failed (Hoffmann et al., 2015).

Additional trials were conducted in Tri Nguyen village in 2014 and in Nha Trang city in 2016 with the ***w*Mel** strain[8].

#### 5.3.1.3 2014 *Aedes aegypti* – Indonesia, Colombia and Brazil

Further trials with *Wolbachia*-infected *Aedes aegypti* are carried out in Indonesia, Colombia and Brazil[9]. In Indonesia trials started in December 2014. Already in august 2016 the first *Wolbachia* mosquitoes were released in Yogyakarta City, Indonesia. Also in Brazil two pilot trials started in 2014 and further at larger scale in 2017. May 2015 the first trial in Colombia was launched. All trials are carefully monitored. No results are reported yet.

[8] http://www.eliminatedengue.com/vn/progress
[9] http://www.eliminatedengue.com/progress

### Insert 3
### Risk assessment for *Wolbachia*-infected *Aedes aegypti* releases

Before *Wolbachia*-infected *Aedes aegypti* were released in Australia, a risk assessment was conducted. This was the first time risks were formally assessed for organisms bearing a gene drive system. The Risk Analysis Framework developed by the Australian Office of the Gene Technology Regulator (OGTR) was followed (Murphy et al., 2010). Although this gene drive system is not a GMO, the methodology was found suitable for an organism with a novel trait. Because of the novelty of this specific gene drive, the lack of data on potential hazards and likelihoods was captured by expert opinions. A Bayesian Belief Net (BBN) was used as the tool to model the risk analysis.

Potential environmental hazards were evaluated including:

- horizontal *Wolbachia* transfer (via host biting, predation),
- ecosystem change (removal of ecosystem services),
- change in *Aedes aegypti* behaviour (e.g. increased biting, non-optimal host selection for blood meals),
- change in *Aedes aegypti* population, which may be induced due to a reduced fitness, followed by a population crash leading to a vacant niche which other mosquitoes may occupy.

As this species is exotic to Australia and totally human-dependent for breeding, the probability of ecosystem changes was found to be very unlikely. Also, because of its low biomass, *Aedes aegypti* was not considered to represent an important component of food webs. Moreover, the current control measures deliberately reduce or remove populations. The geographical range and ecological niche were estimated to remain the same. It was found to be very unlikely that *Wolbachia* would be transferred to invertebrata or vertebrata. Also, the density of mosquitoes is not expected to change given that the environmental holding capacity is a major limiting factor (*Aedes aegypti* has an anthropophilic behaviour).

The likelihood that *Wolbachia* would provide beneficial fitness characteristics to *Aedes aegypti* was also considered. Effects would be possible under intense selection not likely to occur in nature. *Wolbachia* infection rather induces a fitness cost. The hazard of dengue evolving to overcome transmission inhibition and the possibility of transmitting other pathogens was also included. The likelihood was estimated to be very low.

Together the risks regarding environmental hazards were estimated negligible to very low.

Also prior to releases in Vietnam of *Wolbachia*-infected *Aedes aegypti* (Hoc et al., 2011) a similar risk assessment was performed. The overall risk of the release of *Aedes aegypti* containing *Wolbachia* resulting in more harm than that currently caused by naturally occurring *Aedes aegypti* over a 30 year timeframe was estimated to be negligible.

### 5.3.2 Future initiatives

Other initiatives foresee the use of gene drive organisms, but have not yet resulted in their open release. Research is currently at the laboratory stage.

#### 5.3.2.1 New Zealand Predator Free 2050

In 2016, New Zealand's government formally launched "Predator Free 2050[10]", an ambitious plan to kill every rat, possum, and weasel across its 103,483-square-mile territory. Several approaches are envisioned among which the use of gene drives.

Actions are foreseen to achieve by 2025:

- Suppression of target predators on a further 1 million hectares,
- Eradication of predators from blocks of at least 20,000 hectares without the use of fences,
- Eradication of all predators from offshore island nature reserves, and
- Achieving a breakthrough scientific solution capable of eradicating at least one small mammalian predator from New Zealand.

The scientific component includes the use of gene drives as a pest management tool, when it can be made practically feasible, reducing potential risks and socially acceptable. Initially, gene drives may be researched for application to rat eradication.

#### 5.3.2.2 Genetic Biocontrol of Invasive Rodents

The Genetic Biocontrol of Invasive Rodents[11] (GBIRd) programme is a partnership of 7 expert groups investigating the feasibility and suitability of gene drive solutions to protect island communities and prevent island species extinctions. The research by Dr. P. Thomas and Dr. D. Threadgill on gene drive mice (see Table 3) is part of the programme.

## 5.4 Conclusions

This survey resulted in only a limited number of published reports on releases of gene drives. Except for two trials with the Australian sheep blowfly all releases concern mosquito species. The gene drive systems that are reported in this respect are either based on translocation effects, sometimes in combination with cytoplasmic incompatibility, or use the *Wolbachia* system. Engineered gene drive systems are not yet tested beyond the laboratory.

From the reports it is clear that gene drives not always result in the expected change in the target population (extinction or replacement). The reasons are manifold.

- Reduced fitness of the invading population (low mating competitivity, low fertility, long larval development time, low viability, genomic background not adapted to the target environment, limited dispersal ability);
- Immigration of wild-type individuals from neighbouring areas;
- Unlinking of the cytoplasmic incompatibility and the translocation;
- Inadequate introduction methods (below invasion threshold; delivery conditions relative to survival; minimum release area to allow stable local establishment).

[10] http://www.doc.govt.nz/predator-free-2050
[11] http://www.geneticbiocontrol.org/

# 6 Informing the risk assessment

Application of gene drive systems can offer important benefits *e.g.* for the elimination of pathogens and insect-vectored diseases. Nevertheless, the concern for irreversible changes of the introduction of certain traits inspired the need to assess risks and possible side-effects as demonstrated by the risk assessment performed in the World Mosquito Program. GM techniques enable more powerful and tractable gene drives. At the same time in most countries, GM-associated regulatory constraints impose a mandatory risk assessment.

This risk assessment of insects with gene drive systems can build on existing knowledge and experience with vector control programs using insects that do not contain gene drives (*e.g.* sterile insect technique (SIT); incompatible insect technique (IIT)). Experience with other techniques that are applied to achieve the same goal, such as biocontrol, removal of oviposition sites and the use of insecticides, may provide insight in possible effects on the environment (Carter and Friedman, 2016). Furthermore, the use of gene drives must be placed in the context of existing pathogen mitigation strategies (Roberts et al., 2017).

As described in previous sections, success or failure of a gene drive depends on several factors: gene drive type, invasion threshold, fitness cost, host species genetic background, resistance mechanisms, gametic vs. embryonic drive, etc. (de Jong, 2017). On top of that the effect of the payload gene is important. These factors have to be taken in consideration in assessing the risk of an organisms containing a gene drive that is released from containment, either on purpose for research, development or large scale deployment or accidentally. In a risk assessment, because of many determining factors (species, genomic change, location, …), candidate gene drives should be evaluated on a case-by-case basis (Oye et al., 2014).

In this overview, we were mandated to focus on those aspects of the risk assessment that are specific for the gene drives, since other elements may be covered in more general terms independent of gene drives. *E.g.* we will not discuss containment practices for insects as they are species-specific and unrelated to whether the insect population harbours a gene drive or not. Indeed, for general risk assessments guidelines exist describing the various aspects for GM plants and animals including mosquitoes (EFSA GMO Panel, 2010, 2013).

In a recent publication the basic features of a risk assessment were applied to classify gene drive systems to be used in a contained environment (van der Vlugt et al., 2018). For field trials no specific guidance has been developed, although WHO's work on GM mosquitoes is a good basis in case of insects (WHO, 2014).

## 6.1 Effect on the gene drive-bearing organism

### 6.1.1 Off-target modifications

Off-target effects within the recipient organism is a concern for gene drive systems that are based on a gene editing technique and RNAi methods. Off-target cutting by a HEG gene drive may lead to the loss or modification of native traits with potentially effects on the survival, behaviour and breeding success of the organism. This concern is linked to possible unspecific recognition of target sites in the genome and is not specific for gene drives. Off-target effects are often mentioned, but no data are available on their frequency. They are difficult to detect and quantify.

Rather than assessing the possible impact of off-target effects of homing endonucleases, methods are pursued to minimise them including optimisation of guide RNA design and of endonuclease cutting efficiency (Champer et al., 2016; Esvelt et al., 2014; Macias et al., 2017a; National Academies of Sciences Engineering and Medicine, 2016). The former makes use of predictive software to identify other sequences that guide RNA may target (Bae et al., 2014; Tsai et al., 2015; Xie et al., 2014). The latter makes use of mutant versions of the endonucleases to address the efficacy and specificity (Davis et al., 2015; Slaymaker et al., 2016). A prerequisite is the availability

of genome data sets from wild-caught mosquitoes or other target organisms (Macias et al., 2017a). The Ag1000G international collaboration aims to provide a high-resolution view of genetic variation in natural populations of *Anopheles gambiae*[12]. Once a homing gene drive is introduced, off-target effects may be measured in several ways (Koo et al., 2015). The potential impact of off-target mutations is bigger in replacement gene drives compared to suppression gene drives, since gene drives aimed at eradicating a population will also eliminate unintended mutations.

### 6.1.2 Interaction with host genome

Introduction of *Wolbachia* in a new species may induce evolutionary changes in the host genome (Hoffmann et al., 2015). Evidence was given for nuclear-based attenuation of *w*MelPop effects on longevity in *Drosophila melanogaster* hosts and in the novel host *Drosophila simulans* (references in (Hoffmann et al., 2015).

The release of *w*Mel into uninfected *Aedes aegypti* populations in 2011 has, 3 years later, not shown any attenuation of the host genome on the fitness effects of *Wolbachia* or the dengue virus interference (Hoffmann et al., 2015). For changes in the *Wolbachia* genome it is probably too early to be detected in the released mosquitoes. Changes have been detected earlier and the fact that multiple strains exist, shows that genomes evolve. Changes in the virus genome in response to *Wolbachia* blocking mechanisms may be expected, as evidenced by the existence of distinct dengue serotypes.

### 6.1.3 Modified susceptibility

Another concern, theoretically, may be the ability of the vector organism to have modified competency for pathogen transmission (Benedict et al., 2008; David et al., 2013). A mosquito that is modified so that it could not host the pathogenic virus (population replacement), may in theory become a more susceptible host to another existing or new virus that harms human health (Dodson et al., 2014; Hughes et al., 2012; National Academies of Sciences Engineering and Medicine, 2016; Zélé et al., 2014).

### 6.1.4 Stability of the gene drive system

Gene driver-cargo systems risk to unlink the payload gene, *e.g.* that targets a pathogen, and the elements of the drive system (Alphey, 2014). The driver may then continue to spread without having the desired effect on the population.

Modelling may help in determining the likelihood of adverse effects occurring including the probable spread of the transgene, mutation rates, and the effects on the phenotypic profile of the local insect population (Benedict et al., 2008). However, models are as precise as the designer is able to mimic natural situations. Nevertheless, models may be interesting in visualising the effect of changing parameters.

A model was constructed were male mosquitoes are introduced in a population that have a meiotic drive gene located on the Y-chromosome and a drive-insensitive response allele coupled to an antipathogen factor on the X-chromosome (Huang et al., 2007). When the cost for the drive gene and drive-insensitive response allele are weak the latter will go to fixation according to the model. Soon after the frequency of the drive gene in the population will diminish due to its fitness disadvantages over the non-drive gene. With high fitness cost the drive gene will quickly disappear after reaching its maximum. In the long run the population experiences an oscillation in frequencies of drive gene and a drive-insensitive response allele with the antipathogen gene. Still according to this model, in case more alleles exist for the response gene, the antipathogen factor will either disappear or find an equilibrium or exhibit stable periodic oscillations, depending on the sensitiveness of the other response alleles. Another complicating factor is the potential existence of a modifier gene that diminishes the response of the X-linked response allele to the drive gene. Again, depending on the fitness cost there will be a weak or strong selection for the modifier gene

[12] https://www.malariagen.net/projects/ag1000g

and the antipathogen gene will go extinct after a transient increase in frequency, or simply does not spread.

A recombination that might uncouple the drive-insensitive response allele and the antipathogen gene will reduce the effect of this strategy for disease control.

### 6.1.5 Horizontal gene transfer

Horizontal gene transfer is the movement of genetic material between unicellular and/or multicellular organisms other than by the ("vertical") transmission of DNA from parent to offspring.

Horizontal transfer of *Wolbachia* genome fragments to their insect and nematode hosts have been discovered (Klasson et al., 2009). These may have originated in the course of co-evolution of the endosymbiont and its host. Earlier it was found that most of these fragments are non-functional (not transcribed, except for background transcriptional noise). They were suggested to be on an evolutionary trajectory to degradation. However, the authors found in uninfected *Aedes aegypti Wolbachia* fragments that are transcribed. They presumably are from an ancient horizontal gene transfer event and probably have an evolutionary significance. Also, in *Drosophila ananassae* an integrated whole genome of *Wolbachia* has been described (Choi et al., 2015). In the common pill-bug *Armadillidium vulgare* researchers identified a 3-Mb insert of a feminising *Wolbachia* genome that was recently transferred into the nuclear genome (Leclercq et al., 2016). Horizontal transmission can be inferred from non-congruity of host and *Wolbachia* phylogenies, but is typically extremely rare.

As *Wolbachia* is an endosymbiont, it can be transferred as an organism between individuals and species; not being limited by sexual transmission barriers. Irrespective if this is considered a horizontal gene transfer *sensu stricto*, potential transfer of the *Wolbachia* system to humans and other species like predators was studied (Popovici et al., 2010). Notwithstanding its wide prevalence in arthropods, *Wolbachia* have never been found in humans or other mammals, neither in birds, reptiles or fish. The bacteria are present in the salivary glands, but absent in the saliva of insects (Moreira et al., 2009b). Humans that have been exposed to many thousands of bites from *Wolbachia*-infected mosquitoes over prolonged periods of time have antibody responses to mosquito saliva, but show no IgG antibodies specific to *Wolbachia* (Western blot, ELISA) (Popovici et al., 2010). The difficulties that were encountered in transferring *Wolbachia* from fruit flies to *Aedes aegypti* (McMeniman et al., 2009) indicates that the risk of transfer of *Wolbachia* to other lower flies is low. The presence of *Aedes aegypti* (not naturally infected) in the same habitat as the naturally infected *Aedes notoscriptus* in Australia, or *Aedes albopictus* in south east Asia, demonstrate that transfer between species is very unlikely, despite of the observation that one species may ingest smaller instars of the other species (Popovici et al., 2010). PCR screening in an experiment with mosquitoes and predator spiders did not result in a positive outcome (Popovici et al., 2010). Studies with 6 naturally occurring predator species confirmed that transfer of *Wolbachia* between prey and predator, if possible at all, is a very rare event (Hurst et al., 2012). However, transmission between arthropods does occur in nature (Ahmed et al., 2015; Stouthamer et al., 1999). These transfers have been detected between host and parasitoid, and between different parasitoids superinfecting the same insect host.

Concerning other gene drive systems, no data on horizontal gene transfer are available.

## 6.2 Effect on biodiversity

Regarding the potential effects on the environment a distinction should be made between population suppression and population replacement drives. They may have the same ultimate goal, *e.g.* eradication of an insect-borne pathogen, but they have different implications for potential environmental interactions. In contrast to earlier GM applications (*e.g.* SIT), in a population replacement drive the GM trait is intended to persist in the environment.

The extent of the effects on the ecosystem depends on whether the target organism is a “keystone” species in the environment, or whether there are ecological equivalents present. Moreover, pathogen-

host systems and predator-prey systems are co-evolving systems, *e.g.* removing a noxious weed may endanger pollinators depending on the plant considered to be a weed. Moreover, proteins introduced into organisms (including gene-drive components or markers) should be tested for toxicity to other species such as predators (Roberts et al., 2017).

A gene drive may be used to eradicate an invasive species. Although the intent may be the re-establishment of the original species diversity, the elimination of an invasive species may not restore the original ecosystem. Indeed, damage by the invasive species may have gone too far, inducing irreversible changes.

### 6.2.1 Target organism

Here "target organism" is used as a synonym for the gene drive host organism (as opposed to *e.g.* the pathogen that is targeted to be eliminated). Suppression gene drives may result in the extinction of the target organism. Although the target species may be locally affected (even eradicated), this is not different from other control techniques, but may be expanded and more effective with powerful gene drives.

### 6.2.2 Non-target organisms

While potential non-target effects are often raised as concern, there is hardly (field) information available. Also, since gene drives are based on mating potential, the potential for exchange with related species is very species specific (Alphey, 2014).

Effects on non-targets organisms may act directly, *e.g.* due to hybridisation between related species, or act indirectly *e.g.* due to trophic relationships. Transfer of a gene drive into a beneficial, threatened, endangered, neutral, or valued species could lead to its extinction. *E.g.* a gene drive intended for eliminating/controlling a noxious weed could be transferred to a related food crop via vertical gene flow. Practices of farm-saved seed may be affected.

Another aspect is the reduction of the target organism that may increase the population of other species (niche replacement). Elimination of a pest species may clear the way for another pest to fill in the niche. Removing one vector could allow another potentially harmful species to take its place. Again, these effects are not different from effects resulting from techniques that do not make use of gene drives, but are aiming as well at the eradication of a species. Technologies for population replacement instead of population suppression are therefore likely to induce less ecological harm. As the species is still present, no empty niche is created. Vertical gene transfer is still possible but is not likely to cause species elimination, since the gene drive is not lethal. However, in self-limiting strategies the gene drive is expected to disappear over time, in this way also reducing the potential for *e.g.* vertical gene transfer.

Nonetheless, data are not available to check these considerations.

### 6.2.3 Other trophic levels

Extinction (or reduction of abundance) of the gene drive-carrying species can have consequences for *e.g.* predators, competitors, prey, due to its ecological role, such as resource, consumer, competitor, or disease vector. These links create dynamic feedbacks that affect the relative abundances of different species.

Experiments were performed in *Wolbachia*-infected *Aedes aegypti* to investigate the influence of the presence of the bacteria on behaviour towards predators (Hurst et al., 2012). Larval and adult survival were tested against six predator species in laboratory conditions. For none of the predator species a significant difference was noticed between the consumption of *w*MelPop-infected and non-infected mosquitoes. Therefore, it was concluded that no behavioural change in predator avoidance occurred.

The potential of *Wolbachia* to be pathogenic to humans and other species was studied (reviewed in (Popovici et al., 2010)). Experiments injecting *Wolbachia* in chicken and mice, and humans exposed to *Wolbachia*-infected mosquitoes demonstrated that *Wolbachia* are not harmful.

### 6.2.4 Alternative protection mechanisms and herd immunity

If the gene drive is only partially successful in suppressing *e.g.* an insect vector, the result could be loss of immunity, *i.e.* individuals within the population may become more susceptible to the disease as the vector recovers from the initial suppression (David et al., 2013; James, 2005). In an area with high malaria incidence people acquire immunity after several attacks of malaria. These people remain infectious, but may lose their acquired immunity when they stop contracting malaria.

Although a replacement drive may successfully eradicate a certain pathogen, it leaves the vector in place. If a resistant pathogen emerges it could spread back rapidly. Especially, if the temporary absence of that pathogen resulted in less strict use of other control measures, *i.e.* pesticides and bed nets. Population suppression of the vector may be safer in this regard.

## 6.3 Resistance development

### 6.3.1 Resistance to the gene drive system

The presence or development of resistance against a gene drive system, will reduce its efficiency in the host population and limit the potential impact. It is therefore relevant for the risk assessment to acknowledge that gene drive systems may be particularly susceptible to resistance development.

The CRISPR/Cas9 gene drive systems – and HEGs in general - are prone to the development of resistance alleles that are immune to conversion by the drive system (Hammond et al., 2017). Most often this is caused by non-homologous end joining, resulting in disruption of the target site of the endonucleases (see 4.7). However, developers of gene drive organisms will employ strategies to reduce the probability of resistance occurring.

Already in the first publication on CRISPR/Cas9 gene drive resistance was mentioned (Gantz and Bier, 2015). A single-nucleotide change at the guide RNA cut site and an in-frame insertion-deletion (indel) most likely resulted from non-homologous end joining repair. These mutations appeared in the first generation after crossing.

An experiment with a CRISPR/Cas9 drive targeting the X-linked *yellow* gene in *Drosophila melanogaster*, revealed that 29% of wild-type alleles were converted to resistance alleles in the germline of heterozygous females as noticed in the first generation after the cross (Champer et al., 2017b). In this experiment a *nanos* promoter determined the timing of *Cas9* expression. Using the *vasa* promoter the proportion of resistant alleles was 48%. Also, resistance alleles were formed post-fertilisation in a fraction of female embryos, indicating carryover of the Cas9 protein in the embryo. Moreover, the genetic background of the insects was found to be a factor in resistance allele formation rates (Champer et al., 2017a; Champer et al., 2017b).

Also, in the first experiments in *Anopheles stephensi* resistant alleles appeared (Gantz et al., 2015). Although a very high rate (~98%) of successful conversion of wild type alleles into drive alleles was found in the germline of heterozygotes, also a high rate (>77%) of alleles became resistant post-fertilisation in embryos produced by females with the drive. The likely explanation is the persistence of maternally deposited Cas9 enzyme in the embryo (Champer et al., 2017a). From these experiments it follows that an ideal promoter would offer the high rate of germline drive conversion, but is germline-restricted to express Cas9 (no leaky expression) with low persistence of Cas9 to the embryo (Champer et al., 2017b). However, the ideal promoter is yet to be found. Leaky expression from *vasa* in somatic tissue produces resistance alleles in somatic cells, which is not observed in the drive using the *nanos* promoter (Champer et al., 2017a). Yet another strategy to overcome resistance development is the use of a male-only promoter that would avoid the issue of maternally deposited Cas9 (Champer et al., 2017a).

In *Anopheles gambiae* a CRISPR-based gene drive system was designed to target, in both sexes of the mosquito, haplo-sufficient, somatically expressed female-fertility genes (Hammond et al., 2016). Homing was again temporally and spatially confined (*vasa* promoter) to the germline during, or before the process of gamete formation to allow the normal development of heterozygotes. In the progeny of heterozygous parents crossed to wild-type mosquitoes events were observed where the targeted gene was modified either by non-homologous end joining, micro-homology-mediated end joining or incomplete homing, although rarely.

In *Anopheles gambiae* the homing endonuclease I-PpoI targeting the conserved sequence within the ribosomal rDNA repeats located in a single cluster on the X-chromosome and modified to be active only in spermatogenesis resulted in up to 97,4% male offspring (X-shredder) (Galizi et al., 2014). Resistance was observed in the low amount of female survivors due to misrepair and copy number variation of the ribosomal gene cluster.

Modelling several CRISPR/Cas9-based strategies to eliminate exotic mice from islands show that multiplex guide RNAs are needed to overcome resistance development due to non-homologous end joining to be successful (Marshall et al., 2017; Prowse et al., 2017). However, multiplexing guide RNAs in gene drives has only been experimentally studied with 2 guide RNAs (Champer et al., 2017a). The drive conversion efficiency increased, but to a lower degree than theoretically expected. Possible causes may be the saturation of the Cas9 enzyme, the distance between the target sites and the simultaneous cutting of the 2 sites.

Natural sequence polymorphisms in the population and *de novo* mutation of wild-type alleles could also prevent cutting (Unckless et al., 2017). Drury and co-authors modelled the effect of existing polymorphism in *Tribolium castaneum* (Drury et al., 2017). Even a non-cutting polymorphism at a low frequency can severely limit the spread of a very deleterious gene drive, such as one causing infertility. For a drive with low fitness cost it will take longer, but eventually the drive will disappear from the population.

Furthermore, any HEG that reduces the fitness of its host will face the potential evolution of resistance (de Jong, 2017; Godfray et al., 2017; Unckless et al., 2017). When resistant alleles are still functional, they will replace the costly drive allele. Thus, even though a driver may initially spread to high frequency in the population, its ultimate fate will depend on whether resistant alleles have emerged during this process. To prevent the spread of resistant alleles, it will be necessary to target genomic sites that cannot tolerate changes, *e.g.* active sites of proteins, conserved regions in genes (Deredec et al., 2011; Godfray et al., 2017).

Another path to gene drive resistance would be that the gene drive containing organism develops a method of specifically inhibiting the drive endonuclease (Bull, 2015; Esvelt et al., 2014). However, Esvelt et al. hypothesise that inhibitors of Cas9 are less likely to arise given the historical absence of RNA-guided nucleases from eukaryotes (Esvelt et al., 2014). Other mechanisms may be at play, *e.g.* overexpression of an RNA that competes with the guide RNA. Also, the driver construct itself may mutate preventing it from driving (Unckless et al., 2017). The zinc-finger nuclease and TALEN-based gene drives in *Drosophila* underwent recombination between repetitive sequences (Simoni et al., 2014). As a result only 75% and 40% of each respective drive was sufficiently intact after one copying event to catalyse a second round of copying. Because RNA-guided gene drives will not include such highly repetitive elements, they are likely to be more stable (Esvelt et al., 2014).

These findings suggest that in general there exist gene expression suppression mechanisms that are selected for, also in gametogenesis where a HEG would be active (Bull, 2015).

Resistance may be part of a scheme to confine the gene drive to a smaller geographical area or a certain time period (a number of generations), when short-term population transformations are aimed at (Champer et al., 2016; Esvelt et al., 2014; Unckless et al., 2017).

Also, with other drive mechanisms resistance is observed.

A synthetically engineered MEDEA gene drive system based on RNAi was introduced in *Drosophila suzukii* (Buchman et al., 2018). In the 5$^{th}$ and 6$^{th}$ generation some heterozygous females crossed with wild-type males produced a small number (4.3%) of wild-type offspring instead of the expected 100% MEDEA-bearing progeny. In later experiments mutations were found in the miRNA target sites. Sequencing individuals from 8 geographically distinct populations showed a similar trend. It was postulated that the efficiency of the miRNAs is influenced by naturally occurring genetic variation. The effect on the drive efficiency may be circumvented by increasing the number of miRNAs.

A resistance mechanism against transposon-based gene drives is an RNA interference pathway, called the piRNA pathway (Macias et al., 2014). First described in *Drosophila melanogaster*, it is also identified in *Anopheles stephensi*. It is responsible for inhibiting the movement of transposons by targeting transposon-derived RNAs for degradation in the germ-line tissue in *Drosophila*. In *Anopheles* transcripts of the orthologous genes of the piRNA pathway are found in germ-line tissue of adult mosquitoes, in the ovaries with egg development and are found in the embryos. Transcript abundance increases after a blood meal. It may explain the observation that transposons not easily remobilise in the germ line of mosquitoes, as opposed to somatic cells (O'Brochta et al., 2003). It is further suggested that this mechanism may inactivate other transgenic constructs (Macias et al., 2017a).

In *Drosophila melanogaster* a Y chromosome linked to an autosome showed segregation distortion towards males (Lyttle, 1977). After 7 generations a cage population initially containing 10% of these meiotic drive males collapsed. However, in some cages the distorting chromosome was imperfect, went to fixation but the distortion decreased over time due to the accumulation of polygenic, recessive modifiers (Lyttle, 1979). In one cage sex-chromosome aneuploids were formed where XXY individuals are female (Lyttle, 1981). This population did not go to extinction.

The mouse t-allele and the *Drosophila Segregation Distorter* are still found in the wild, but their impact on the species abundances is rather moderate although they are sterile/lethal in homozygotes. The SD complex in the *Drosophila melanogaster* population occurs only at a frequency of 1-5% (National Academies of Sciences Engineering and Medicine, 2016). This suggests that there are factors working against the drive that are not fully understood today (Bull, 2015; Sandler and Novitski, 1957).

Suguna and colleagues studied crosses of females from an Indian wild population of *Aedes aegypti* with males carrying the sex ratio distorter factor M$^{D}$ which shows meiotic drive (Suguna et al., 1977). It turned out that the wild population shows variation in resistance and sensitivity of the X-chromosome to the drive. Sex ratios ranged from 50% females (resistant) to less than 1.25% females (sensitive). Also in *Drosophila simulans* autosomal suppressors to an X-linked meiotic drive were discovered and further investigated (Atlan et al., 2003). The suppressors in wild populations in East Africa turned out to be polymorphic and can restore an equal or nearly equal sex-ratio.

Another factor that may counteract a meiotic drive is polyandry (Price et al., 2010; Wade, 2010). In a population where females are favoured, polyandry can rescue a population from extinction.

Also, behavioural resistance, *i.e.* females that reject mating attempts by the released males, may hinder the gene drive to act (David et al., 2013).

In the synthetic *MEDEA* construct described in 4.5, the toxin encoding miRNAs, or the promoter driving their expression, can mutate to inactivity, resulting in a non-functional drive (Akbari et al., 2014).
The same is true for maternal-effect lethal underdominance systems (Akbari et al., 2013) (Reeves et al., 2014). Genomic duplication or up-regulation of the targeted haplo-insufficient gene may render the drive inactive.

In summary (Bull, 2015):

- Single elements as opposed to stacking are more prone to resistance evolution.
- Genetic engineering that imposes a fitness cost will be more prone to resistance evolution if expressed in both sexes than if expressed in just one sex.
- Inbreeding (*e.g.* asexuality, sibling mating and selfing) slow the spread of harmful selfish elements and enhance evolution of resistance.
- In suppressing populations, if the proportion of introduced gene drive containing insects to the total population is small, gene drives advance slowly and will develop resistance. Small population sizes are least prone to evolve resistance. Migration inward from unaffected populations may retard resistance evolution.
- Sterility created prior to release of the insect is less prone to resistance evolution than is sterility or death resulting from processes expressed in the progeny of wild parents.
- Sequence-specific mechanisms of population suppression are prone to changes in the target sequence.
- Vector genes least prone to evolution of pathogen resistance are those conserved between related vectors and those required by related pathogens.
- Gene knockouts are less prone to evolutionary reversal than are introductions of functional genes.

### 6.3.2 Resistance to an effector

Resistance development to an effector is a common phenomenon that can occur in each species, *e.g.* a pest developing resistance against a management strategy. It is undesired and, under certain legislative frameworks, resistance development is also considered as an environmental concern. It was therefore included in this study.

Resistance can occur by selection of resistance of the target pathogen to the effector gene, or the selection of increased virulence of the target pathogen (Alphey, 2014; Franz et al., 2009; James, 2005). Even in the *Wolbachia*-mediated viral resistance in vectors it is not known whether this could trigger the emergence of potentially more virulent strains of arboviruses (Kamtchum-Tatuene et al., 2017).

*Wolbachia*-infected mosquitoes target rapidly evolving viruses, which could mutate to avoid the mechanism causing refractoriness. Some data suggests that mosquito immune responses to *Wolbachia* prime the mosquito against viruses, but this response could weaken over time as the virus and mosquito co-evolve, diminishing refractoriness to RNA viruses (Macias et al., 2017b). The same is true for GM vectors that bear a gene to block the pathogen. Another obstacle is the possibility that a transgenic drive mechanism could become unlinked from the effector genes, so that the drive continues, but the pathogen blocking alleles could be lost from the population.

The *Wolbachia* *w*MelPop strain that is found in *Drosophila* shortens the life-time by 50%, but still allows several reproductive cycles before death, limiting costs to reproductive output. The selection pressure for resistance to life-shortening is therefore rather low. In laboratory stocks no resistance development has been observed in 10 years (McMeniman et al., 2009).

Although not a matter of resistance, incomplete cytoplasmic incompatibility may render the suppression programme by *Wolbachia*-infected mosquitoes ineffective: instead of causing population suppression it leads to population replacement. This may be due to low *Wolbachia* density in males as was shown in *Aedes albopictus* population suppression trials (Calvitti et al., 2015). Crosses between *Aedes albopictus* males having a low *w*AlbA strain density and incompatible strain *w*Pip-infected females may be partially fertile. Also, incorrect sexing to separate male and female pupae in preparing the *Wolbachia*-infected males to be released, allows for fertile crosses.

Also the dynamics of resistance development have been modelled (Unckless et al., 2017).

## 6.4 Malicious intent

The potential for misuse (dual use) may cause economic damage or even bioterrorism (Gurwitz, 2014).

Although misuse of gene drives is theoretically possible, the technology would already be inapplicable to bacteria and viruses because they do not reproduce sexually. Because of the long generation time, humans will not likely be a target. The effect on crops and livestock will be limited again because of the generation time, but also because todays practice of commercial seed production and artificial insemination would hinder propagation of a gene drive (National Academies of Sciences Engineering and Medicine, 2016; Oye et al., 2014). Only developing countries that do not work according to these practices could be more vulnerable. More sophisticated misuse can be imagined but would be technically very difficult.

## 6.5 Effects beyond the target geographical area

The spread of a gene drive outside the expected geographical area could potentially change the environmental landscape well beyond the site of its introduction. A drive to eradicate an invasive species may be accidently introduced in its original environment, *i.e.* where it is endemic, where it has a function in the local ecosystem or is important as a human food source (*e.g.* eradication of exotic species from an island). Gene flow to other populations of the same species depends on the mode of dispersal between populations: human assisted, as a result of a disruptive event (*e.g.* fire, hurricane), normal movement of organisms, or as a result of habitat unsuitability (crowding, no nesting sites, …) (National Academies of Sciences Engineering and Medicine, 2016).

### 6.5.1 Dispersal

The potential for *Wolbachia* to invade in local populations not only depends on the threshold frequency (related to the fitness cost), but also on the ecological conditions at the site of introduction (Hoffmann et al., 2015). *w*MelPop develops well in humid and under low-density conditions of resident *Aedes aegypti* populations. In the dry season the threshold frequency will be much higher. Establishment also depends on the mosquito density outside the release area, being a source for influx. The potential spread of *Aedes aegypti* outside the study area is very low as shown in the field releases. *w*Mel did not spread outside areas where they were released, even though *Wolbachia* were occasionally detected in other areas (Hoffmann et al., 2014a; Hoffmann et al., 2011). Physical barriers and high-density areas occupied by uninfected mosquitoes stop the invasion. The fitness cost is a benefit in that respect: it will contain the population in a certain area.

In most of the above described releases, the release location was rather isolated. Models were developed in case the *Wolbachia*-infected mosquitoes do not remain geographically isolated (Crain et al., 2013). The results show a re-replacement by wild-type mosquitoes without incompatibility.

North and colleagues modelled the spatial spread of a HEG in *Anopheles gambiae* depending on the landscape characteristics (North et al., 2013). Landscapes were generated that differed in their densities of mosquito feeding and breeding sites. Where these mosquito resources are sparsely distributed (disconnected population structure), the HEG can drive the local population to extinction. But, wild-type mosquitoes can recolonise afterwards. Denser resources may lead to either extinction or population suppression depending on the HEG load. Seasonal variation, active or passive dispersal are not included and would make the model even more complex.

### 6.5.2 Options for limiting dispersal

Especially low threshold drives may have widespread consequences across national borders. Concerns arise from the accidental release of just a few organisms containing gene drive systems. Also, dispersal may create political tensions with bordering countries that may not have approved the technology (Macias et al., 2017a).

Using high threshold drives help confine the spread of a gene drive to a local breeding population (Australian Academy of Science, 2017). Marshall and Hay studied the possibility of replacement drives becoming established at their release site without spreading into neighbouring populations

using a simple model where a drive is introduced in a population with exchanges with a neighbouring population (Marshall and Hay, 2012). Several gene drive types were examined. The invasive MEDEA gene drive could not be confined to an isolated population unless it is associated with a very large fitness cost. The same is true for the highly invasive HEGs and *Wolbachia*. Transposable elements, when capable of spreading, show the same picture. Migration thresholds (below which no spread into the neighbouring population occurs) for these systems are unrealistically low. Underdominance systems display higher migration thresholds, next to a high release requirement.

A 'daisy chain' CRISPR/Cas gene drive where each genetic element drives the next is a gene drive that would stop after a few generations (Noble et al., 2016). This would limit the capacity of the gene drive to spread. It would be a way to temporarily and locally replace a population.

Modelling may help to understand population dynamic effects (*e.g.* seasonal fluctuations, density dependency) that can directly influence control strategies (Alphey, 2014). Comparing one-locus underdominance, two-locus underdominance, and daisy-chain drive, modelling reveals that the daisy-chain drive is the least capable of remaining localised due to the low threshold frequency (Dhole et al., 2018).

An RNA-guided gene drive could also be used to block the spread of other gene drives by recoding sequences targeted by the unwanted drive ('immunising' drive) (Esvelt et al., 2014). A proof-of-principle was delivered for a Cas9-ablated chain termination system that functions as a brake (Wu et al., 2016). The *cas9* sequence itself contains the target site for the guide RNA that becomes inserted in the gene and as a consequence expression of the endonuclease is lost. Any population containing *cas9* may be stopped from driving.
A daisy quorum drive that combines daisy chain drive characteristics with underdominance could theoretically be used to limit the drive locally and allow for reversal by introducing wild-type individuals (Min et al., 2017).

Vella et al. modelled the effect of some proposed countermeasures for CRISPR/Cas drives (Vella et al., 2017). Replacement drives with synthetic resistance alleles and reversal drives are not guaranteed to eliminate a homing drive from a population due to the existence, in general, of a stable polymorphic equilibrium where both systems co-exist. An immunising reversal drive that targets both the homing drive and wild-type alleles has the best chance to remove the drive. However, *cas9* gene and guide RNAs will remain in the population.

A strategy to confine a gene drive to suppress an invasive species might be to first introduce a standard drive to insert a unique sequence followed by a suppression drive that is targeted to that unique sequence (Esvelt et al., 2014). Alternatively, the invasive population may be made sensitive to pesticide using a gene drive. The application of the pesticide would then only affect the invasive species. Prowse et al. modelled four realistic CRISPR/Cas9 gene-drive strategies to eradicate exotic vertebrates (Prowse et al., 2017). They used heterozygotic XX sterility, heterozygotic XX sex reversal, homozygotic embryonic non-viability and homozygotic XX sterility in combination with multiplexed guide RNAs modelled in mice. Simulations reveal that only the latter two approaches lead to eradication in 4 to 5 years provided that 3 or more guide RNAs are used.

## Insert 4
## Recommendations for testing gene drive systems

Important in testing and applying gene drives is the step-wise introduction of the techniques as emphasised in EU legislation with regard to GMOs (Directive 2001/18/EC[1]). WHO guidelines on phased testing of GM mosquitoes are relevant for gene drives in insects in general (WHO, 2014). This phased testing approach includes:

- Preparation for Research (phase 0),
- Laboratory-Based Research (phase 1),
- Field-Based Research (phase 2),
- Staged Environmental Release (phase 3), and
- Post-Release Surveillance (phase 4).

Each of the phases allows for knowledge to be gained that will either force to return to the previous phase (feedback loops) or allow to advance to the next phase. Safeguards are in particular of concern for replacement gene drives as suppression gene drives are self-limiting.

Most organisms have been studied in laboratory conditions, and population dynamics of some insects were examined in cages. Noble and colleagues remark that, especially concerning CRISPR/Cas gene drives, laboratory research (and field trials) should not be conducted in regions where the studied host is present as an escape of only a small number of drive-bearing organisms would affect the local population (Noble et al., 2017). Modelling based on existing empirical data (from lab experiments and species biology), taking into account homing efficiency, fitness cost, resistance development, existing genetic variation, breeding specifics, exchange with neighbouring populations, shows that these systems are highly invasive. These modelled outcomes for CRISPR/Cas gene drives are clearly different from the field experiences with the *Wolbachia* and underdominance systems in mosquitoes in particular.

In doing basic research in gene drives, field trials may be performed safely using geographical, ecological, molecular and/or genetic containment (Figure 10) (Esvelt et al., 2014).

- Geographically field trials may be contained on *e.g.* an island (National Academies of Sciences Engineering and Medicine, 2016).
- Ecological containment means field trialling in an area that is either not fit for the species to survive (*e.g.* a tropical mosquito species tested in a temperate area) and/or where no wild type mates are present (outside the natural habitat) (Akbari et al., 2015; Benedict et al., 2008; Esvelt et al., 2014).
- Molecular containment ensures that only a non-self-sufficient drive, where the drive elements are separated, can be passed to the progeny after mating with wild-type. If on top of that the gene drive cuts engineered sequences that are not present in wild-type individuals, no drive will be passed on (Akbari et al., 2015; Esvelt et al., 2014).
- Genetic containment using unique target sequences that are present in a single genetically distinct target species but not in related species or wild populations, may prevent effects on these organisms. Whereas the occurrence of polymorphism in a population may hinder the efficacy of a gene drive (Drury et al., 2017), making use of polymorphisms, a gene drive may even target subpopulations only (Esvelt et al., 2014).

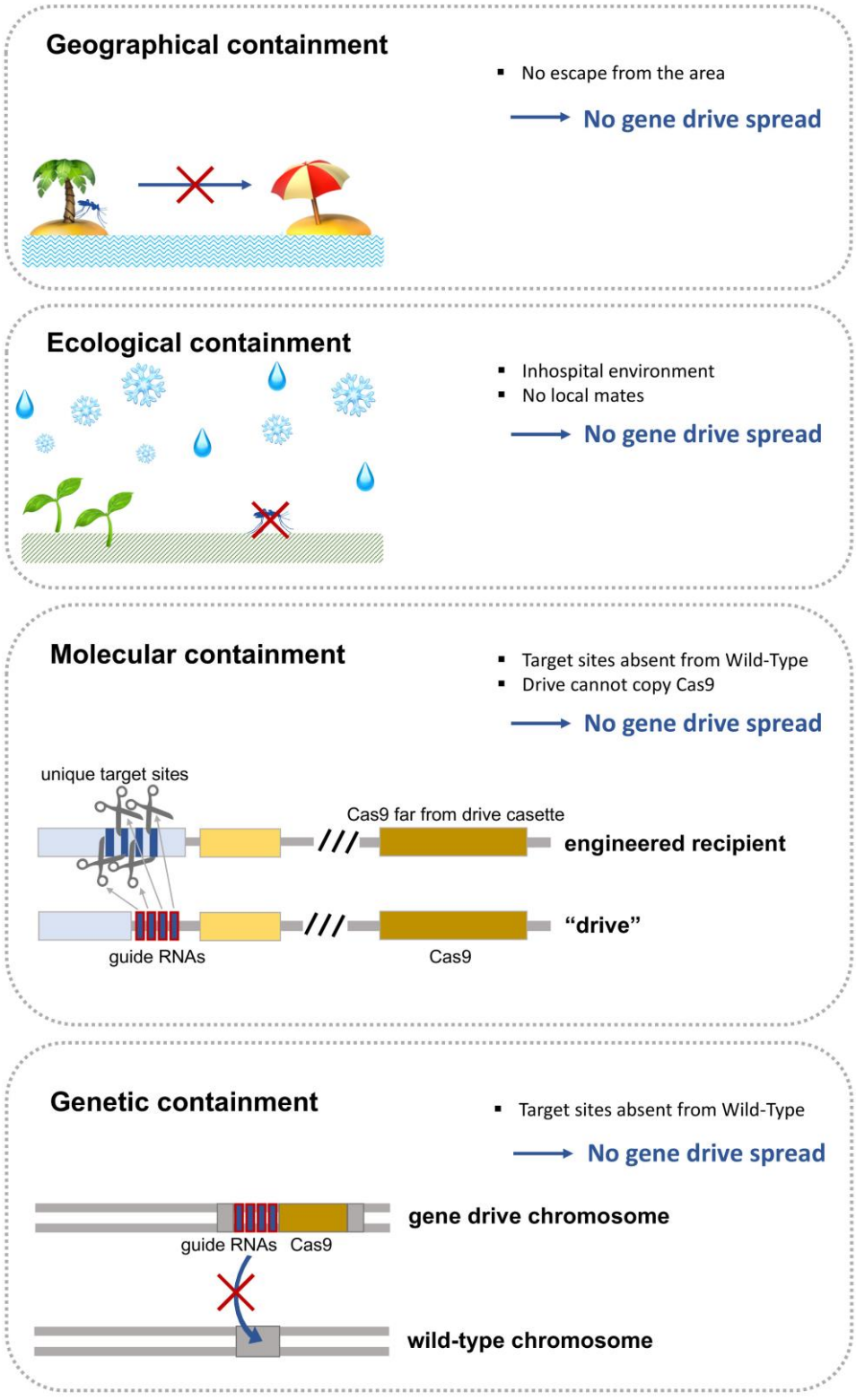


**Figure 10:** Examples of geographical, ecological, molecular and genetic containment of gene drives in basic research (adapted from Esvelt et al., 2014)

Guidance for selecting field trial sites has been composed for GM mosquitoes (Brown et al., 2014; Lavery et al., 2008). Although a lot can be learned from releases of other GM organisms, the focus of this report is on the specifics of gene drive systems. It is important to note that whereas with other genetic modification applications efforts are made to minimise dissemination of altered genetic elements, the goal of a gene drive is to rapidly spread genetic information throughout a population. Unintended releases are therefore to be prevented using geographical, ecological, molecular or genetic isolation. Before starting trials it is advised to consider strategies to remove, replace, or restrict the activity of the gene drive constructs.

Removability of a gene drive means the ability to completely remove the system from a population via the release of large numbers of wild-type organisms (Champer et al., 2016), although possibly with a different genetic background. Reversibility is the ability to replace an existing gene drive system with another system. It must be noted that a reversal gene drive does not restore the original modification to the wild-type.

The possibility to remove or replace depends on the characteristics of the drive system (Table 2). Strategies have been proposed to build a reversal drive for RNA-guided gene drives (DiCarlo et al., 2015; Esvelt et al., 2014; Gantz and Bier, 2016). A synthetic MEDEA replacement drive was also proposed (Akbari et al., 2014). However, ecological effects induced by the first gene drive would not necessarily be reversed (Esvelt et al., 2014; Oye et al., 2014). *Wolbachia*-based approaches may be replaced by a different *Wolbachia* strain via bidirectional incompatibility (Alphey, 2014).

Finally, monitoring post-release for non-target effects is essential for safe conduct of trials and releases (National Academies of Sciences Engineering and Medicine, 2016).

[1] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal L 106, 17/04/2001, p.1-39.

# 7 Discussion & Recommendations

The use of a gene drive mechanism offers opportunities for spreading desired traits (*e.g.* genes conferring refractoriness to malaria or dengue in mosquitoes) in populations. Whereas breeding and selection have been used for increasing the occurrence of desired traits in domesticated species, influencing the genetic make-up of natural populations is more challenging since the possibilities for man-controlled selection remain limited. Gene drives offer a potential mechanism for introducing and increasing the prevalence of a desired trait, even in wild populations, without continued human intervention. They have the potential to replace/modify or even suppress wild populations. Applications include the eradication of pests and diseases of humans, animals and plants; conservation of endangered species; the introduction of beneficial traits in crop plants and livestock.

Naturally occurring gene drives have been described for diverse organisms: microorganisms, plants and animals including insects. Their prevalence in populations is often lower than theoretically expected due to mechanisms that counteract the gene drive. Transposable element-based drives have so far turned out to be not efficient enough to be applied. Moreover, they cannot be directed. Translocation drives are hard to establish and suffer from a high fitness cost. Meiotic drives are widely present in nature. Synthetic versions, especially if sex-linked, promise to be useful in suppressing populations. Synthetic MEDEA systems may be suitable to replace a population if they can be developed in relevant species with a low fitness cost. For programmes to act only locally and/or temporarily synthetic underdominance gene drives may be one of the methods of choice.

Since the discovery of the gene editing capabilities of the CRISPR/Cas9 system and its use as a gene drive system, interest in gene drives has increased as the technology dramatically enhances the abilities to engineer gene drives. CRISPR/Cas-based drives turn out to be very efficient, at least in the laboratory. At the same time concerns were raised regarding the safety of their use. Further developments of *e.g.* daisy drives may result in efficient methods for acting locally and/or temporarily.

Many researchers have studied gene drive mechanisms with the goal to develop systems for application in the field. Until now research on synthetic gene drives in most of the cases was limited to lab experiments and modelling. Field experience comes from field cage experiments and releases almost exclusively with mosquitoes (except for the Australian sheep blowfly (*Lucilia cuprina*). Also, the current knowledge on risk management issues associated with gene drives is largely based on work in insects, especially mosquitoes. The behaviour of synthetic gene drives in other species than insects is hardly studied. The focus on mosquitoes can be explained by the goal to control/eradicate mosquito-borne infection diseases such as malaria, dengue, Chikungunya, Zika, and yellow fever.

Moreover, the most extensive research has been conducted on the *Wolbachia* system that does not involve the use of GMOs as commonly defined. Besides, this system is not always considered to be a true gene drive system. The developments on CRISPR/Cas are still too young to have experience beyond the lab.

While field experience remains limited, most reports on gene drive applications focus on the efficacy of the system and influencing factors, not on ecological effects.

This report aimed at collecting experiences on the behaviour of organisms with gene drives, both natural and synthetic, especially their effect on human health and the environment. In this report we organised the environmental issues as reported by different sources with the aim to identify those that are specific for gene drive systems. This leads to the following conclusions and recommendations:

**Separating concerns**

Likely inspired by the theoretical power of gene drive systems and in the absence of actual data, worst-case scenarios have been developed highlighting different areas of concern. Nevertheless, it would be inaccurate to consider that each of these concerns is relevant and realistic for each application of a specific gene drive system.

As for any environmental risk assessment, the specifics of each case must be evaluated (case-by-case risk assessment). Some aspects may be determined by the biology and population dynamics of the species, and the impact of a gene drive system can consequently be very different depending on the species. The quality of the risk assessment will in this case be largely determined by the information on the species.

Similarly, certain concerns may be related to the way that the gene drive system is introduced. Using X-rays to introduce translocations may introduce undesired side-effects. Concerns for off-target modifications have been raised for gene editing techniques. Depending on the techniques these may need to be considered.

Finally, some concerns relate to the strategy independent of the method to achieve it. *E.g.* suppressing and even eradicating an insect disease-vector can lead to concerns on impact on higher trophic levels, on possible replacement by other vector-organisms, replacement by non-indigenous populations, etc. These concerns are not unique for the gene drive, rather they are applicable to any approach with the same aim (*e.g.* use of chemical control agents).

The fact that these concerns are not directly linked to the gene drive does not mean that they should not be addressed. It merely indicates that the concern is not specific for gene drives and that consequently a broader reference framework can be used for the assessment: *e.g.* suppression drives have much in common with IIT, SIT or other eradication methods in terms of environmental effects, when applied locally.

**Recognising that different gene drive mechanisms have different features**

While by definition all gene drives result in an inheritance pattern different from standard Mendelian rules, the modes of action are very different. Also whether the gene drive is uni- or bidirectional makes a difference in terms of invasion thresholds and reversibility/removability. In consequence, not all possible concerns are relevant for each and every gene drive system. A thorough understanding on the mode of action is therefore the basis for the risk assessment.

**Envisage two separate factors: gene drive and effector**

The environmental impact of an organism carrying a gene drive system is determined by the type of gene drive system on the one hand and the effector, the trait or payload gene(s) that it carries, on the other hand. In some cases, these two factors are combined: if the effect of a CRISPR/Cas-based gene drive is induced by the location where the insertion has occurred, the gene drive directly induces the effector. However, in other cases, the CRISPR/Cas-based gene drive will be linked with a payload gene(s) that induces the effect. The endosymbiont *Wolbachia* steers the inheritance mechanism of its host and induces resistance against dengue virus, though presumably *via* two distinct mechanisms, so in principle likely separable.

*Sensu stricto*, the gene drive will only take care that a certain genetic element is inherited in a particular way. In itself, it may have no other discernible phenotypic effect on the host organism. Such 'invisible' drives may be self-sustaining or self-limiting; stable or transient. For most drives the environmental effect beyond simple presence of the drive depends on the other properties of gene drive and cargo (*e.g.* traits relevant to replacement or suppression). However, mutant derivatives of artificial drives where the gene drive and cargo have separated may have little evident phenotypic effect beyond their presence; this is also the case for many natural gene drives.

The type of the effector will be determining the environmental effect as well as the fate of gene drive and effector. In fact, if the effector has a high fitness cost, selection against the combination will occur in favour of individuals without the effector and associated gene drive, limiting and perhaps preventing spread of the combined element. In this respect using a gene drive might not differ that much from introducing GM organisms without gene drive into the environment, though the possibility of mutant elements arising that lack the high-fitness-cost element would need to be considered.

Different models were designed to assess the behaviour of gene drive-hosting organisms in terms of efficacy, stability, reversibility, etc. Most of them could not be validated with real world observations. Only in the case of *Wolbachia*-infected *Aedes aegypti* refining the models could be started. Models are, however, useful in visualising the effect of changing parameters.

**Existence and design of reversibility**

A major concern when releasing an organism carrying a gene drive is that a process is initiated that irreversibly results in the replacement of all wild-type alleles and/or individuals. As discussed above, there are different indications that suggest that this is unlikely or that this can be prevented:

- Many factors determine the "success" of a drive: the biology of the host organism, population dynamics, the drive's efficacy, its fitness cost to the host. Each factor may function as a brake;
- Development/presence of resistance against the gene drive can reduce or reverse the effect (in particular when the payload gene has a high fitness cost);
- Some gene drives can be removed by natural inflow and/or re-introducing wild-type individuals (in particular high threshold drives);
- Strategies for reversing gene drives by synthetic reversal drives to limit their activity have been proposed.

This is also relevant for potential adverse effects on other (non-target) species after sexual or horizontal transfer of the gene drive, *e.g.* eradication of beneficial or valuable species. However, the prospect of (horizontal) transfer of a functional gene drive to another species seems extremely remote. CRISPR/Cas9 drives are multi-component systems and extremely sensitive to sequence variation in their recognition sequences. One exception is *Wolbachia*, for which there is clear evidence of horizontal transfer over evolutionary time.

Although unlikely, the potential impact can be high and should therefore be properly covered in the risk assessment.

**Local deployment vs. uncontrollable, species-wide spread**

The type of gene drive and the accompanying fitness cost will determine how large the invading population needs to be relative to the target population (low level vs. high level invasion threshold). In the experiments carried out so far the threshold is rather high leading to the hypothesis that an escape of only a few individuals will not result in a successful invasion. However, this may be an artefact of relatively high fitness costs of initial attempts. Although, no real life data are available to confirm this, Noble and colleagues state that this is not true for the efficient CRISPR/Cas-based drives following modelling. Releasing a small number of organisms carrying a CRISPR/Cas gene drive may already invade a population. This is modelling; no real life data are available. The power of a gene drive must not be overestimated. Even if the drive is performing for 100%, its frequency in a population only doubles at each generation. Depending on the host species, this may take several years to reach a substantial frequency in a large population. And gene drives do not always go to fixation, as seen in models, lab and field experiments.

Also genes may be transferred to non-intended populations (compatible species, populations outside the target area). Depending on the threshold frequencies, fitness cost, evolutionary robustness, breeding behaviour and environmental circumstances the gene drive might enter and be active in other populations. The risk assessment is then comparable to that for GM crops that can outcross with wild relatives. In the limited amount of field releases that were performed, no such "escape" was evidenced. In this respect the advantage of an efficient suppression drive is that it is self-limiting: if a suppression drive works well, then the affected population will be reduced, thereby reducing the emigration risk.

**Resistance against gene drive**

Resistance towards the gene drive is an important issue especially for homing endonuclease and miRNA/shRNA-based methods. Both are extremely sensitive to mutations or genetic variability in

their recognition sites; this will likely affect the spread of drives based on such mechanisms, at least for simple designs. Already in the first laboratory experiments these phenomena were observed, and already in the first cross of a gene drive-bearing with a wild-type individual. Moreover, gene drives that bring a fitness cost are expected to accelerate resistance development. The speed and size of this happening is hard to predict. The concern that potent gene drives, once released, would potentially act globally, must therefore be nuanced; they might spread through different species but not necessarily achieving very high frequencies in each. Irrespective, researchers are designing systems to prevent or retard resistance development.

**Distinct strategies: replacement vs. suppression**

The effect of an environmental release of organisms carrying a gene drive may be the largest with a population suppression drive. Depending on the ecological function of the organism, eliminating a population can be expected to have effects on other species with trophic bonds, pollination requirements, host-pathogen relations, etc. The disappearance may leave a gap for other species to fill (niche replacement). However, effects of suppression by gene drives are not different from other eradication methods.

The effects may be smaller in replacement drive applications, but this will largely depend on the payload gene.

**Targeting wild populations**

A distinction can be made between gene drives targeted to domesticated species, like crops and animal breeds, and to wild/natural populations, such as pests and disease vectors and invasive species. In the former category the aim is to enhance a desired function (replacement), in the latter a certain characteristic is to be eliminated if not the entire organism (replacement or suppression). Although gene drives would allow the introduction of desired traits in wild/natural populations (*e.g.* in pollinators), we have not identified any research on such applications.

The modification and release in domesticated species is entirely human-controlled: the gene drive-hosting individual has not to compete with non-modified counterparts and will remain within certain human-managed boundaries. Given this high level of control, gene drives may not even be required since humans can select the desired traits and “drive” the traits.

On the other hand, gene drive technology makes it possible to radically intervene in wild/natural populations. This automatically leads to perceived loss of human control. In wild/natural populations only the moment of introduction is human-controlled. Yet the fate of the individuals with the gene drive is determined by population dynamics and ecology beyond human control.

Laboratory experiments testing efficacy almost inevitably use organisms which are highly uniform and different from wild populations. The gene drive systems are unlikely to perform in the same way in natural conditions that are much more variable and unpredictable; the likely effect of this is that the drives will not spread as readily as simple models suggest.

Also, the process may be slowed down or even stopped because of the fitness costs the insertion of a gene drive may bring to the host organisms. If fitness relative to the wild-type individuals is low, the introduced organisms will fail to replace or suppress the target population. This was clearly shown in the *Wolbachia*-infected *Aedes aegypti* field trials where two strains were used. *w*MelPop-bearing mosquitoes were less fit than mosquitoes infected with *w*Mel and as a result the invasion with the former was unsuccessful. Also, the genetic background of the host organism relative to the target population may be important as was illustrated by the *Aedes aegypti* trial in Kenya.

**Focus so far on safety design & management rather than risk assessment**

Only in two cases a formal risk assessment was conducted prior to the release of mosquitoes equipped with a gene drive: *Wolbachia*-infected *Aedes aegypti* in Australia and in Vietnam. These replacement drives were evaluated to represent a negligible risk.

While safety concerns are formulated, most attention has gone to identify design and management solutions for addressing them. The step-by-step approach embedded in the European Directives and phased approach proposed by WHO, should create opportunities for collecting relevant information.

This study focused on information relevant for the assessment of risks related to an introduction into the environment of an organism carrying a gene drive. Such an assessment is usually performed when an intentional introduction (an R&D trial or a large scale deployment) is anticipated. However, it may also be relevant when determining the risk level of an unintentional or accidental release from a contained facility such as a laboratory or insect rearing facility. The information included in this report suggest that many factors would need to be realised (*e.g.* presence of local mating population in the area of the release, amount released above threshold) before an impact can be realised.

In addition, the information on the gene drive systems illustrated that it is extremely unlikely that applications of the CRISPR/Cas in gene editing result in the inadvertent creation of a gene drive system.

To summarise the main conclusions:

- Natural as well as synthetic gene drive mechanisms have been explored for population suppression or replacement;
- Different gene drive mechanisms have different features warranting a case-by-case approach for the risk assessment;
- Two factors need to be considered: the gene drive itself and the effector;
- The power of a gene drive depends on the drive's efficacy, the biology and population dynamics of the host organism, the fitness cost of the drive to the host, environmental circumstances, the potential for resistance development/presence in the target population. These elements determine the speed and limits of dispersal (local vs. species-wide);
- Mitigating measures are being proposed/developed to limit and/or reverse the impact of gene drives;
- The stepwise approach intrinsic to research and development of gene drive applications allows gradually collecting information to verify the prediction models of population dynamics;
- Field (cage) trial experience is limited to insects, mainly mosquitoes. Releases showed a varying degree of success of the applied gene drives;
- Most of the applied research on gene drives focuses on insects for their role in disease development and as a pest. The World Mosquito Program is the most advanced, operating in 12 countries and passing the stage of experimental field releases;
- No harmful effects to human health or the environment have been observed so far;
- Overall, given the many constraints, gene drives are probably not as powerful as often assumed in worst-case scenarios.

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