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Fighting Malaria Using Gene Drives: Worthy Tool or Waste of Time?

A Safe-by-Design Assessment for Gene Drive Organisms

TU Delft

Fighting Malaria Using Gene Drives: Worthy Tool or Waste of Time?

A Safe-by-Design Assessment for Gene Drive Organisms

By

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Abstract

Malaria is both an economically and medically burdensome disease taunting people worldwide. Treatments for the disease – transmitted by malarial *Aedes* and *Anopheles* mosquitoes infected with *Plasmodium* – are either temporary or in developmental stages, while rising insecticidal resistance and mosquitoes' behavioral changes call for a lasting solution to responsibly fight malaria. The application of gene drive (GD) technology – biasedly propagating genetic material into a population using CRISPR/Cas9 – has been suggested. By introducing a sex ratio bias into malarial mosquito populations, or by targeting the mosquito's interaction with the *Plasmodium*-parasite, malaria could be eradicated. The design, testing and implementation phases of GDs must, however, be approached with caution, due to the invasive nature of and controversy around the technology. To prevent harmful consequences, the risk management strategy of Safe-by-Design (SbD) was used to compose a set of guidelines for selected SbD Risk Categories. Academic literature and scientist interviews were used to obtain insights of possible risks and to find balance between medical progress and technological threats. Stakeholder involvement was found to be an important part of the GD design process, with a multidisciplinary team of experts, appointed and enforced by international organizations. The team must be held co-responsible for compliance with the guidelines of all four SbD Risk Categories, covering (i) unintended effects on non-target organisms and ecosystems, (ii) horizontal gene transfer (HGT), (iii) pathogenicity and toxicity, and (iv) run-off risk and reversibility. Key findings include previously proposed models, including an inhibitory rock–paper–scissors and a confining split-drive model for GD regulation. After carefully considering the available knowledge and the guidelines necessary for responsible research, I concluded that further research into mosquitoes' ecosystems, target-specificity, HGT prevention and the theoretical GD models is required.

Acknowledgements

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1 Introduction

With over 240 million treatments performed and 627 thousand deaths reported in 2020 alone, malaria is one of the worst pests clutching our world (CDC, n.d.-a). The disease – caused by parasitic *Plasmodium* and transmitted by vector mosquitoes – leads to chills, high fevers, and sometimes death. Malaria cures have either been unsuccessful or impermanent, or are still in developmental stages (CDC, n.d.-b). Globalization, climate change, rising insecticide resistance and mosquitoes' observable behavioral changes require an urgent and lasting solution in the fight against malaria (Wang et al., 2021; Baik & Carlson, 2020).

Recent innovations regarding genetics might assist the development of a preventive malaria remedy. The invention of the gene drive (GD) – genetic material that biasedly propagates itself to offspring using CRISPR/Cas9 – allows traits to spread within a population (Jinek et al., 2012). Instead of Mendelian inheritance, characterized by fifty percent chance of inheritance, GDs allow for allele frequencies of over ninety percent, even with disadvantageous phenotypes harboring lower evolutionary fitness (Wang et al., 2022; Hammond et al., 2016). By genetically engineering vector mosquito genera – i.e. *Aedes* (*Ae.*) and *Anopheles* (*An.*) – to incorporate GDs in their DNA, malaria might be permanently eliminated.

Researchers and humanitarian organizations have long had – and still have – doubts regarding the ethics of testing and releasing invasive GD technology into the wild. Therefore, this thesis aims to answer the main question: How can we responsibly fight malaria using GD technology? Up until now, no GD organism field trials have been executed officially. Several field trials have taken place, however, with genetically engineered (GE) variants of *Ae. aegypti*. Controversy arose when local communities on the Cayman Islands and in Malaysia and Burkina Faso mentioned the absence of their informed consent prior to the trials occurring (Lacroix, 2012; Nading, 2014; Yao et al., 2022). The dissatisfaction regarding the executed field trials mainly emanates from substantial dangers harbored by GD technology.

Although uncertain risks and overall uncertainty subsist, several studies have proposed the utilization of GDs as a promising tool to eradicate malaria. Therefore, one of the sub-questions I aim to answer is: What are the technical options at our disposal in the development of GDs targeting malaria? Suggested techniques include altering the sex ratio of malarial mosquitoes to result in exclusively males, or sterile offspring (Hayes et al., 2016) – and ultimately extinction. Other studies have suggested targeting the culprit more directly, by focusing on the molecular interaction between *Plasmodium* and vector mosquitoes (Dong et al., 2022).

By targeting a living organism, i.e. parasite or vector mosquito, its ecosystem is targeted simultaneously. However, ecological threat makes up merely a fraction of all possible risks posed by the deployment of GD technology. Therefore, GD development must rely on an extensive risk management strategy, preferably prior to the design process; which is where Safe-by-Design (SbD) comes in. By combining existing SbD literature with values and expertise obtained from scientists, drawbacks of GD implementation can be limited significantly. This brings us to our last sub-questions: What series of anticipatory guidelines can we devise, based on SbD, to make safety an integral part of GD development? And how could we involve expert advice based on (empirical) knowledge to find a delicate balance between medical progress and technological threats?

2 Methods

2.1. Information Acquisition

Literature was searched and reviewed to collect a sufficient amount of diverse and reliable background information about malaria, GDs, and SbD. Additionally, information was gathered and theorized regarding (i) current and novel technological applications; (ii) the ethics of GD experimentation methodologies applied in the past; and (iii) well-grounded judgments and thoughts concerning the ethics and anticipations of scientific applications.

Electronic searches were conducted in search for relevant (and recent) scientific journal papers, books and other reliable sources; mostly using *Google Scholar*. The mainly applied search strategy used relevant combinations of keywords for gene drives (gene drive technology OR gene drive reversal OR gene drive inhibitor OR gene drive fitness OR gene drive field trial OR gene drive confinement OR gene drive ethics), malaria (malaria cure OR malaria infection OR malaria prevention OR human malaria), mosquito (malarial mosquito OR *Anopheles gambiae* OR anopheline mosquito OR *Aedes aegypti* OR mosquito extinction OR mosquito ecosystem OR mosquito ecology), malaria parasite (*Plasmodium*, *Plasmodium*–mosquito interaction OR *Plasmodium* extinction OR *Plasmodium* ecosystem OR *Plasmodium* ecology), and Safe-by-Design (SbD OR Safe-by-Design gene drives OR Safe-by-Design genetic engineering).

Some sources were provided by supervisors Dr. Ir. Bouchaut and Dr. Asveld. Additionally, sources were found in and cross-referenced with frequently cited articles and literature reviews. All search results were collated, checked for duplicates, and sorted in accordance with the American Psychological Association (APA) 7th referencing style by the thesis author.

2.2. Interviews

In order to form contemplative and nuanced guidelines for GD design and test processes, several scientists – *interviewees 1* through *3* – were consulted. Beforehand, they were given background information on malaria and GD technology, and definitions of relevant scientific concepts in order to minimize ambiguity, as shown in Appendix A. After providing and clarifying the background information, the interviewees were asked a range of questions as shown in Appendix B. The interviews were executed with the aim to determine which emerging risks they foresee and therefore which SbD guidelines must be established. Furthermore, interviewees illustrated their views upon possible solutions GDs might offer, out of which their professional perspectives regarding the fight against malaria were deduced. Lastly, all interviewees have given permission to share their views in this thesis.

2.3. Safe-by-Design Guidelines

SbD guidelines for branches within the biotechnological industry were used, along with information derived from the interviews and literature, to define boundary conditions for the design process of future GD applications.

3 Gene Drives Against Malaria

3.1. Malaria: The Problem

Besides malaria's onerous toll on human lives, the disease also badly affects economics. Malaria has a highly negative impact on the world's economic growth: direct yearly costs amount to approximately USD 12 billion in total (CDC, n.d.-a). Although the need for a malaria cure is pressing, developments stall due to various reasons. The "lack of a traditional market" – as there is no guaranteed profitability and demand is mainly concentrated in certain countries – and insufficient understanding of the immune response to the infection limit initiatives to further research the disease (CDC, n.d.-b; Santoso & Irawati, 2015).

Malaria is caused by a unicellular, blood-feeding parasite of the *Plasmodium* genus. Severe malaria cases arise from the infamous *Plasmodium falciparum* species, which can only complete its growth cycle above a temperature of twenty degrees Celsius (CDC, n.d.-c). The so-called obligate parasite is entirely dependent on a vertebrate or insect host, and infects mosquitoes by means of ingestion. As (infected) female mosquitoes feed on blood, bitten humans and other vertebrates may suffer an infection of *Plasmodium* (Koella, 1991). After *Plasmodium* starts feeding on human red blood cells, malarial symptoms start to show, such as anemia, fever and coma (Milner, 2017). Malaria treatment has proven to be difficult in some cases and symptoms might recur periodically for several years, making malaria prevention indispensable.

The International Committee of the Red Cross is one of the many organizations committed to aiding communities taunted by the pest (ReliefWeb, 2006) by providing preventive instruments. Current malaria prevention methods include time-consuming and expensive ones, like distribution of long-lasting insecticidal nets (LLINs), indoor residual spraying, and provision of housing and information to local communities in Sub-Saharan Africa and South-East Asia, among others (CDC, n.d.-c; Huijben & Paaijmans, 2017). Ongoing clinical trials of malaria vaccines might be promising (He et al., 2022), but the opposing force of rapid adaptation of *Plasmodium* and vector mosquitoes is still realistic. Furthermore, insecticide resistance is rising all around the world (Huijben & Paaijmans, 2017; Wang et al., 2021). Therefore, global health advancements call for cost-effective and more sustainable methods to fight malaria (Wang et al., 2022).

3.2. Genetic Engineering

Contending technologies in the prevention of malaria include genetic modification. Genetic modification can be used to specifically manipulate genetic traits, such as eye color, number of limbs, or even sex. Since genetically modified organisms (GMOs) were first created in the lab in the seventies (Cohen et al., 1973; Jaenisch & Mintz, 1974), novel methods have been suggested to fight disease. However, the Assembly of Life Sciences committee recommended a voluntary "moratorium on certain [recombinant DNA] experiments" until the technology has been "better evaluated" or "adequate [prevention] methods are developed for preventing their spread" prior to the Asilomar Conference of 1975 (Institute of Medicine (US) Committee to Study Decision Making, 1991).

3.3. Birth of the Gene Drive

Since then, a wide range of innovative discoveries have taken place. In 2012, CRISPR/Cas9 – an adaptive bacterial immunity system – was found to specifically target and edit DNA in a programmable manner (Jinek et al., 2012). The discovery led to unforeseen applications, opening doors for GD technology, which allows for biased chance of inheritance in organisms in which the tool has been incorporated. The mechanism makes use of the CRISPR/Cas9 system to propagate itself from GD organisms to their progeny, even disadvantageous traits – as opposed to traditional GMOs (Wang et al., 2021). Theoretical and laboratory-based research have led to believe that GDs could play an important role in pest and disease control (Curtis & Graves, 1988; James, 2005; Esvelt et al., 2014; Caragata et al., 2020). Seemingly, promising lab results have been published, although researchers acknowledge the presence of technological limitations along with ethical concerns (Hayes et al., 2018). Ideas have been proposed to fight the spread of malaria, dengue and Zika – diseases that have long held a firm grip on countries with socioeconomic burdens and even on developed countries – by releasing GD mosquitoes in the wild (Committee on Gene Drive Research in Non-Human Organisms et al., 2016).

3.4. Controversy

To this day, GDs have not been officially released into the wild, mainly due to uncertainties around how GDs will affect population fitness and its ecosystem. However, field trials have been carried out with other types of GE mosquitoes (non-GD). For instance, the British *Oxitec Ltd.* has carried out an open field test with a GE strain of *Ae. aegypti* – a mosquito species known to transmit *inter alia*, malaria (CDC, n.d.-b) – on the Cayman Islands in 2010. The mosquitoes were "genetically sterile" and had a maximum dispersal distance of 220 meters and a two-day life expectancy (Lacroix, 2012). The trial was highly controversial, as *Oxitec Ltd.* allegedly failed to sufficiently inform Caymanian communities about the potential consequences (Nading, 2014), although no punitive measures were imposed on the company. A similar, but self-dubbed "safe and successful" release of the *Ae. aegypti* strain was done in an uninhabited Malaysian forest later in 2010 with multilingual disclosure of information and approval from Malaysia's National Biosafety Board (Lacroix, 2012). Non-GD mosquito tests have been carried out in Burkina Faso as well, demonstrating "reduced fitness and dispersal of genetically modified sterile malaria mosquitoes" (Yao et al., 2022).

During the United Nations (UN) Convention on Biological Diversity in 2018, discussions have taken place about whether a moratorium on GD field tests are justified. Some argue, however, that a (temporary) moratorium would desist public debates which, in effect, might thwart the development of GD technology (Bouchaut et al., 2022). The moratorium was later elaborated to serve as a requirement to consult with potentially affected communities prior to the field trials occurring (Wedell et al., 2019), essentially allowing GD technology to be further researched with necessary precautions in place.

To responsibly conduct GD trials, researchers have proposed involvement of public opinion – i.e. of local communities and indigenous people – as well as multidisciplinary partnerships with relevant institutions and other stakeholders (Long et al., 2020; Lunshof & Birnbaum, 2017; Kormos et al., 2022; ETC Group et al., 2019; Manske et al., 2012). The World Health Organization (WHO) Expert Advisory Group has also predicted that supervisory parties will require a positive expectation regarding GDs' effect on epidemiology (Vector Ecology and Management Unit et al., 2017). As recently as July 2021, New Partnership for Africa's Development (NEPAD) was authorized to import and release "genetically modified male mosquitoes, known as *biased males*" into Burkina Faso for further experimentation in a "confined environment." Reportedly, the release occurred in absence of risk assessment and informed consent (Vekcha, 2022). It is unclear in what way the confinement took shape, and whether "biased males" refer to GD organisms or traditional GMOs.

3.5. Potential with Regard to Malaria

Organizations like NEPAD and Target Malaria – both funded by the Open Philanthropy Project (Vekcha, 2022) among others – develop and research new GD applications regarding malaria. One of the proposed GD methods describes genetically modifying *An. gambiae* – another malarial mosquito – to introduce a suppressive "sex ratio bias" and result in an exclusively male offspring. If the offspring of a mosquito population is exclusively male, sexual reproduction will become impossible, leading to extinction of the population. However, experimentation using this technique has led to completely sterile mosquitoes, rather than exclusively male offspring (Hayes et al., 2016). Sterility will ultimately have the same effect: the targeted mosquito population will go extinct (ETC Group et al., 2019; Hammond et al., 2016). A similar approach has been suggested, in which an evolutionary fitness cost is introduced into the population, with mosquito extinction as a result (Connolly et al., 2022).

Another proposed technique focuses on the interaction between mosquitoes and the malarial parasite. GDs might be used to obstruct the binding of – or increase the immune reaction against – the malaria-causing unicellular *Plasmodium* parasites in *An. gambiae* (Dong et al., 2022). By targeting the parasitic interaction on a molecular level, the mosquitoes themselves should survive, but they will lose their infectious trait. This way, mosquito extinction would be averted while still eradicating the host-dependent *Plasmodium* (Wikipedia contributors, 2022). With the parasite experiencing local extinctions, due to seasonality, any malaria intervention targeting *Plasmodium* must be optimally timed to target the point of highest extinction risk (McKenzie et al., 2001) for maximum effect.

Noted should be that GD applications mostly target *An. gambiae* exclusively, but GD systems for *Ae. aegypti* and *An. stephensi* have been theorized and developed as well (Li et al., 2020; Lambrechts et al., 2008).

4 Safe-by-Design

Along with the benefits of GDs, aforementioned applications might involve unforeseen drawbacks which must be assessed prior to implementation. Several approaches to scientific risk management have been applied in the past, such as Adaptive Risk Management, Site-Specific Risk Management, and SbD. By applying the latter, the concept of Responsible Research and Innovation is incorporated with safety as the anticipated outcome of the GD design process. The concept of SbD can be applied to a wide range of biotechnological fields, including industrial biotechnology (Robaey, 2018) and crop breeding innovation (van der Berg et al., 2020). As GDs are a recent advancement with little past experimentation, no specific SbD guidelines have been set up. Nonetheless, the invasiveness of GD technology calls for distinct criteria regarding its risks (Noble et al., 2018).

To prevent unforeseen, detrimental consequences, laboratory and (semi-)field tests must be performed. Said tests occur in a contained (i.e. laboratory) or semi-contained environment (e.g. cage experiments [Hammond et al., 2016]), or in a confined manner, such as preprogrammed mortality after several generations. Furthermore, most researchers attempt to recapture the released organisms after concluding their research (Vekcha, 2022; Yao et al., 2022; Lee et al., 2012). Such contained experiments are therefore not considered entirely representative of an actual GD organism release, as the latter is indefinitely released. In order to better understand the consequences of GD technology in the wild, experimentation involving mosquitoes' complex ecological networks is inevitable. However, risk management plans must be set up beforehand to ensure the safety of the applied technology and its corresponding risks. The UN has been involved in governing and asserting regulations regarding GD research, although "a significant amount of field research on genetically modified mosquitoes operates under guidelines established by [the WHO, and the research community]" (Committee on Gene Drive Research in Non-Human Organisms et al., 2016). If a GD design does not comply with the guidelines, use of said product would be irresponsible, and thus (further) testing and deployment should be prevented (Golnar et al., 2021) by, for instance, the UN or WHO.

4.1. Stakeholder Involvement

GD designs are specifically designed to serve a certain purpose, such as fighting malaria through malarial mosquitoes in Sub-Saharan Africa, and misuse of the technology may irreversibly inundate the charitable goal. As each GD is designed to target a specific organism within a predetermined environment and influences multiple parties, case-by-case analyses should be performed by an independent team of multidisciplinary experts (Lunshof & Birnbaum, 2017; Kormos et al., 2022; Oye et al., 2014). A similar approach was undertaken during the 1973 Gordon Conference on Nucleic Acids, where attendees demanded the National Academy of Sciences "to establish a committee to consider the problem of recombinant DNA research and recommend specific actions or guidelines" (Institute of Medicine (US) Committee to Study Decision Making, 1991).

Also, as GDs are specifically designed for a certain environment, the design process must occur concurrently – not consecutively – with informed consent and permission from the affected parties. To minimize threats posed by GDs, independently curated genetic engineers, immunologists and ecologists must be given responsibility to oversee the technical aspects of the design process, including SbD guidelines that will be recommended in Section 4.2. The GD elements in need of oversight include functionality, efficiency and organism-specificity. Additionally, jurists, social scientists and cross-cultural experts must be involved to ascertain that the GD conforms to local laws, and to address expressed concerns and expectations of the region's inhabitants and legislators.

The multidisciplinary team should have some co-responsibility for the design phase (Bouchaut & Asveld, 2021), as well as the consecutive testing and implementation phases, and provide the GD designers with valuable feedback in an iterative manner (Bouchaut & Asveld, 2020). By involving critics and volunteering locals in the feedback process, mutual learning and trust will likely follow (Radenbaugh & Sutter, 2005). If GD design milestones are published in the form of journal articles and public disclosure is made obligatory (Loewenstein et al., 2012; Oye et al., 2014), a transparent and reliable peer-review process takes place (Rubin et al., 1993), which the multidisciplinary team will be able to include in their judgment as well.

The team must be "challenged to develop scenarios on […] misuse" (Oye et al., 2014), after which detailed instructions are to be composed for extensive briefing of all relevant parties involved in the testing and releasing phase. Quantification and sensitivity analyses of risks must be carefully assessed (Connolly et al., 2022) to determine the effects of misuse and potential abuse of the GD – the former being accidental and the latter intentional. Stakeholders need to be aware of the degree of harm which misuse of the GD could entail (Bouchaut et al., 2022).

4.2. Risk Categories and Corresponding Guidelines

Risks are inevitably associated to any scientific development. However, both literature and *interviewees 1* through *3* (Appendix B) insist on anticipatory safeguards to minimize risk. To set boundary conditions for the acceptability of risks that may be involved alongside GD design, testing and implementation, four main SbD Risk Categories were composed from insights obtained from literature and interviewee recommendations:

1. Unintended effects on non-target organisms and ecosystems;
2. Horizontal gene transfer;
3. Pathogenicity and toxicity to other organisms (Robaey, 2018); and lastly,
4. Run-off risk (Bhutkar, 2005) and reversibility.

The SbD Risk Categories were (i) based on previous analyses concerning GDs (Oye et al., 2014), (ii) compared to industrial biotechnology (Robaey, 2018) and synthetic biology (Bhutkar, 2005), and (iii) altered specifically for GD organism design, testing and release. Each SbD Risk Category includes known risks affiliated with GD technology (Groot Kormelink, 2019), and strives to address and mitigate uncertain risks by generally delineating unwanted consequences and assigning specific guidelines to each SbD Risk Category. This way, unwanted drawbacks of both known and uncertain risks are ruled out to a great extent.

4.2.1. Unintended Effects on Non-Target Organisms and Ecosystems

One of the proposed GD targets is the direct culprit of malaria: *Plasmodium*. There is no commonly known ecological value of *Plasmodium* and research has shown that the parasite already experiences local, seasonal extinctions (McKenzie et al., 2001). Past research stated that *Plasmodium*'s infectious and manipulative nature appears to negatively affect anopheline mosquitoes' biting rate and fecundity (Schwartz & Koella, 2001). However, a recent study has shown using transcriptome analysis that infected *An. gambiae* demonstrate an increase in fitness. After infection by *Plasmodium*, the mosquito seems to have elevated levels of olfactory acuity, as well as anti-aging and reproductive abilities (Carr et al., 2021). Furthermore, a study has revealed that *An. stephensi* with genetically engineered *Plasmodium*-resistance harbor net fitness costs compared to non-transgenic mosquitoes (Lambrechts et al., 2008). This means that consequences to *Plasmodium* might negatively affect mosquitoes and, evidently, their ecosystem.

Although GDs have been theorized to have quantifiable and adjustable fitness levels (Girardin et al., 2019), in reality GD organisms often possess smaller fitness levels compared to their WT counterparts (Yao et al., 2022). However, studies have predicted that even less fit GD organisms are likely to be highly invasive. Keeping that in mind, GDs might invade and therefore alter an entire population, effectively lowering the population's fitness level. This fitness cost might bring about an unnatural and unfair competition between the GD population and rival species. In the worst case scenario, the rivalry will lead to extinction of the altered population, possibly leaving a gap in its ecosystem. A proposed recommendation for field testing includes a condition stating GD organisms must not pose "more harm to human health than wild-type mosquitoes of the same genetic background" and "no more harm to the ecosystem than other conventional vector control interventions" (James et al., 2018). Therefore, analyzing the importance of targeted organisms within their ecosystems is crucial.

The order of species extinction is of utmost importance to the ecosystem's vitality. Empirically, by disappearing essential functions within an ecosystem first – as opposed to a randomly ordered loss of function – severe extinction cascades follow (Larsen et al., 2005). As *An. gambiae* could play an ecological role as a pollinator, its extinction might affect pollination of several plant species (Gyimah, 2021). Some state that "mosquitoes have an enormous ecological impact" as both pollinator and food source (Baik & Carlson, 2020; Pugh, 2016), while others claim *An. gambiae* as a pollinator adds "minor (and redundant)" value to its ecosystem (Callies & Rohwer, 2022).

However, mosquitoes are also part of the dietary intakes of a multitude of predatory organisms, such as amphibians, bats, birds, insects, reptiles, and spiders (Baik & Carlson, 2020). These factors make that mosquitoes play roles in a wide range of distinct ecosystems. Furthermore, due to regional differences in ecology, no universal consensus exists regarding the consequences of mosquito species' extinction.

Therefore – after a theoretical prototype has been designed – extensive, long-term field testing must occur in ecologically representative, semi-contained environments, e.g. on an island with sibling and rival species released into the target species' habitat. Afterwards, *interviewee 3* recommends, determination of ecosystem dynamics should be fulfilled in order to understand the influence of the GD on other organisms, prior to definitively implementing the technology. Ideally, potentially affected plants should be involved in the experiment to determine mosquitoes' influence as potential pollinators, as well. Besides, literature and all three interviewees recommend that GDs are target-specific as a safeguard to prevent off-targets and ecological harm (Esvelt et al., 2014; Gantz et al., 2015; DiCarlo et al., 2015). In order to prevent unintended effects to non-target organisms and ecosystems, the following guidelines are to be adhered to:

- GDs must undergo extensive, long-term field experimentation within an ecologically representative, semi-contained environment prior to implementation; and
- GDs may only affect the target species' survivability; and
- GDs must not eradicate the target species if the species functions as an essential link within the target's ecosystem; and
- GDs must not cause rival species to go extinct if those species function as essential links within their ecosystem.

Ecological roles of GD candidate populations should be extensively analyzed for each particular target region. Further research is required, however, to gain sufficient understanding of the ecological roles of mosquitoes (Pugh, 2016); and current initiatives already seek to simulate, predict, and adapt to ecological advancements (Naeem et al., 2012). Despite *interviewee 1*'s medical point of view, both he and *interviewee 2* agree that off-targets must be avoided and more ecological research is required.

4.2.2. Horizontal Gene Transfer

Horizontal gene transfer (HGT) is the dispersion of genetic material between different organisms – as opposed to vertical gene transfer, in which genetic material is inherited by offspring. HGT of GDs to the target and sibling species would accelerate the dispersion process (Connolly et al., 2022), rather than solely relying on vertical gene transfer. This way, the target population could demonstrate the desired effects in an expedited manner. HGT even facilitates essential and ancient mutualistic insect–microbial relationships, i.e. that of *Wolbachia*-strains (Lees et al., 2015; Woolfit et al., 2008; Coolen et al., 2022).

Although HGT to other insect species (via microbial interactions) usually appears to be non-functional, some genes bestow novel functions upon the recipient organism (Nakabachi, 2015). The latter could entail undesirable consequences if the insect species is an integral part of an essential ecosystem. Besides, multitrophic insect–plant interactions via microbes seem to induce plant evolution and pathogen spread, resulting in "unexpected outcomes" (Coolen et al., 2022).

If, nevertheless, undesired HGT occurs, *interviewee 2* suggests to incorporate a phenotypical trait to facilitate traceability of any off-target GD organism. Additionally, *interviewee 3* recommends the establishment of an efficient quantification method to determine HGT occurrences upon which improvements are to be based during the iterative design process. Therefore, GD designs must adhere to the following restrictions:

- GDs may be horizontally transferred to (wild-types of) the target species; and
- GDs must not be horizontally transferred to non-target insects if substantial change in function arises; and
- GDs must include a phenotypical traceability trait; and
- An efficient quantification method must be established to determine the frequency and effects of, and to improve upon undesired HGT occurrences.

Studies researching transgenic organisms have been trying to tackle the naturally occurring, intra- and interspecies processes of HGT, as well as genetic mutation and recombination. Such traditional GMO experimentation requires precautionary biocontainment strategies, e.g. using heterologous nucleic acids (Zhu et al., 2022), as opposed to GD technology which must be dispersed to effectuate its employability (Connolly et al., 2022). To minimize HGT of GDs to non-target species (including insects and plants), a genetic target sequence could be selected that is characteristic of the target species and would cause infertility or lethality in non-target species. This way, the GD might be coincidentally horizontally transferred to non-target species, but would not be propagated further into the non-target population by means of reproduction. Inducing a stark immune reaction to GD acquired by non-target species via HGT may produce similar results, causing either ineffective HGT or lethality. However, causing infertility or lethality in off-targets should not become common practice, due to their potentially negative effects (Sections 4.2.1, 4.2.3). Frankly, a "genetic firewall" specifically preventing HGT to non-target organisms has yet to be developed.

4.2.3. Pathogenicity and Toxicity to Other Organisms

Any pathogenic or toxic threat to non-target species is generally unacceptable, since ecosystems are complex networks, which demand extensive studies to acquire sufficient knowledge to drastically interfere with. Also, built-in pathogenicity and toxicity pose a serious bioterrorism threat. For instance, organisms could be genetically engineered to resist common antibiotics administered to humans. Other concerns include acts of bioterrorism – intentional and malignant release of viruses or microbes. Possibilities regarding bioterrorism are incessant, and GDs allow for larger scale acts due to the rapid dispersion process. Possibilities include embedding the complete Polio genome into GDs (Bhutkar, 2005), or propagating GDs in a population through viral vectors (Walter & Verdin, 2020). Applications must therefore abide by the following design restrictions:

- GDs may only cause pathogenicity or toxicity to the target species if extinction of said species is intended; and
- GDs must not cause pathogenicity or toxicity to non-target species.

4.2.4. Run-off Risk and Reversibility

In case unforeseen threats do present themselves, anticipated action is necessary to battle GDs' overly invasive nature (Noble et al., 2018). SbD Risk Categories 1 through 3 mainly address GDs' influence on target organisms and coherent matters, as these are considered the most drastic in the long term. However, this one (SbD Risk Category 4) covers both short-term effects on the target organism, as well as anticipation to prevent irreparable damage in the long term. Novel biotechnological applications are commonly assigned reversibility or inhibitory mechanisms as a result of regulations and ethical analyses (Munthe, 2017).

These safeguards might be necessary if, for instance, a GD does not demonstrate the desired outcome within a predetermined timespan – resulting in the need to attenuate its effects. The risk of persistent GDs partially arises from the fact that GD organisms are known to retain less evolutionary fitness, compared to wild-type (WT) ones. In fact, recent mathematical predictions have shown that even inferior GDs "are likely to be highly invasive" (Noble et al., 2018), reinforcing the importance of such safeguards. GD designs have been suggested to suit reversibility or inhibitability options, allowing for a shift in GD organism representation, as opposed to WT organisms, within a population. A straightforward approach describes the introduction of GD-resistant individuals – with the targeted gene, but lacking a genetic recognition sequence – into the population (Rode et al., 2019).

Another approach is the theoretical brake-driven system, which has been presented to introduce GD organisms – as well as "brake" organisms – into existing WT populations. The technique "induces rock–paper–scissors dynamics" (Figure 1), in which chance of inheritance and natural selection are the driving mechanisms. In this model, GD mosquitoes have a biased chance of inheritance over WT ones. In turn, brake mosquitoes have a biased inheritance over GD ones, but WTs have a greater level of fitness than the others (Girardin et al., 2019). The model can therefore be used to shift the balance of WT to GD organisms, and vice versa via brake organisms. The technique serves as a technological safeguard – an inhibitory or reversal mechanism – in case unintended effects arise. Concurrently, the technique allows for a programmable balance between GD and WT organisms by precisely tweaking brake-organism frequencies.

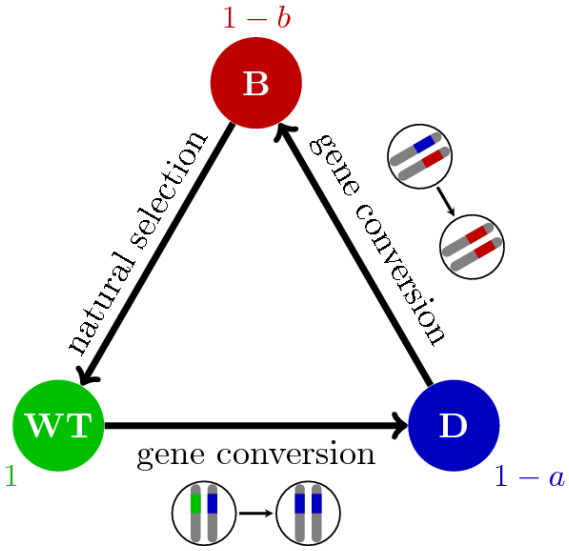

Figure 1. Gene Drive Model with Brake-Driven Rock–Paper–Scissors Dynamics.

The GD organism (D) wins over the WT organism (WT) by biased inheritance; the brake organism (B) wins over the GD organism by biased inheritance and due to its higher fitness level; and the WT organism wins over the brake organism due to its higher fitness level.

Adapted with explicit permission from "Catch Me If You Can: A Spatial Model for a Brake-Driven Gene Drive Reversal," by L. Girardin et al., 2019, *Bulletin of Mathematical Biology, 81*(12), 5054-5088. Copyright 2019 by Léo Girardin.

In addition to previously mentioned safeguards, a GD should have a confinement characteristic to limit dispersion within a targeted region. A hardwired "controlled lifespan outside the lab" (Bhutkar, 2005) has been recommended for laboratory-sourced biotechnological inventions. This effectively describes the assignment of an (expected) expiry date to GDs' persistence (Wang et al., 2021) – *inter alia*, to mitigate human design flaws.

Alternatively, "local" or confinable GDs have been proposed to restrict the GD's effects to the intended release site. The technique commences with a separate release of the CRISPR and Cas9 components into the mosquito population's territory in high frequencies, with either or both components inactive in absence of the other. This so-called split-drive system translates into the components' dependence of one another in order to be active, resulting in spatial confinement. Ultimately, long-term habitation within the release site leads to mosquitoes reaching high active GD frequencies, while migrators from and to non-target populations will not acquire threshold frequencies of active GD components (Wang et al., 2021; Oye et al., 2014). The following guidelines addressing run-off risks and reversibility were thus drawn up:

- GDs must be inhibitable or reversible. Additionally,
- GDs must be either temporally or spatially confined, or both.

Either reversibility or inhibitability was highly recommended by *interviewees 1* through *3* to be an integral safeguard of every GD design. Furthermore, as temporal confinement addresses the same problem as spatial confinement in a similar manner, either will suffice to achieve the same goal with minimum threat. Due to the split-drive system's requirement of site-specific, long-term habitation; this method is also characterized by temporal persistence (Wang et al., 2021), and is expected to be reversible (Li et al., 2020). Simultaneous compliance to all three criteria is therefore possible, and recommended.

5 Conclusion and Discussion

To find out how we can responsibly fight malaria using GD technology, a range of technological innovations and conceptual ideas were compiled by obtaining insights from academic literature and interviewees. Additionally, SbD guidelines were composed that are necessary to responsibly design, test and implement GDs in the fight against malaria.

5.1. Gene Drives with Regard to Malaria

Recent advancements have allowed for varying potential solutions with regard to malaria, including the introduction into the mosquito population of a sex ratio bias or an evolutionary fitness cost using GDs. Introducing a sex ratio bias GD into a population has resulted in sterile offspring, rather than the intended male offspring. All suggested methods – introducing a sex ratio bias, sterility or evolutionary fitness cost into the population – ultimately result in mosquito population suppression or even extinction.

Alternatively, GDs have been proposed to increase the mosquito's immune reaction against the parasite, although the *Plasmodium*-resistance trait has actually shown to negatively affect the mosquito's evolutionary fitness. Any intervention targeting *Plasmodium*'s presence in mosquitoes is likely to provide more fruitful results during *Plasmodium*'s natural seasonal extinction phases. However, additional research and field tests have yet to take place to definitively determine technical success rates of all proposed methodologies.

5.2. Safe-by-Design

The specificity necessary to safely and effectively utilize GD technology calls for case-by-case analyses, which need to be fulfilled by a group of independent and knowledgeable individuals of various scientific fields. The individuals must form a multidisciplinary team of genetic engineers, immunologists, ecologists, jurists, social scientists, and cross-cultural experts who are given some co-responsibility for the design, testing and implementation phases, and must publicly disclose their findings. Although disclosure agreements are common practice within medicine to combat conflicts of interest, bias and pressure have both been experimentally observed to increase when advisors were conflicted. However, "more comprehensive and uniform disclosure [increases likeliness to discourage] entering into problematic conflicts because of the threat of having to clearly disclose [the conflicts]" (Loewenstein et al., 2012). In addition, public disclosure of information combined with peer reviews and public discussion might prevent or counter any foul behavior inflicted (Oye et al., 2014).

The multidisciplinary team must also actively interact with local inhabitants and legislators to receive informed consent and feedback, and to establish mutual trust. The team must provide all stakeholders with detailed instructions to prevent misuse and abuse, including quantification and sensitivity analyses of the adverse effects of known risks to create risk awareness (Peccoud et al., 2018; Chen & Reniers, 2018; Braumann, 2018).

Importantly, I believe that asserting regulations and appointing the multidisciplinary team of experts to supervise the scientific community's advancements regarding GDs should be effectuated by the WHO, due to the organization's credibility and significant influence on researchers (see Chapter 4). In turn, national governments, the European Union, or ideally the UN, could enforce these regulations as asserted by the WHO. However, such decision-making processes regarding novel concepts are often time-consuming. Besides, it is likely that the majority of state officials would need to discuss about the guidelines prior to agreeing – let alone asserting and enforcing – them. All in all, I think responsible GD research and implementation complying with SbD should become possible in the long term.

Safety and fighting disease using GDs could be considered two weights on a delicate scale. *Interviewees 1* through *3* all stated that they prefer a cautious approach during GD research. However, especially *interviewee 1* emphasized his tendency to lean towards successfully fighting malaria using GDs, even if ecological stability would be slightly obstructed, illustrating that the topic is subject to bias.

To minimize harmful consequences of GD design, testing and implementation, four SbD Risk Categories were composed with distinct guidelines per category, as shown in Table 1. Limitations to technology and to knowledge regarding recommended guidelines are discussed and reflected upon below Table 1.

Table 1. Safe-by-Design Risk Categories and Corresponding Guidelines for Gene Drive Organisms.

Risk Categories	Guidelines
1. **Unintended Effects on Non-Target Organisms and Ecosystems**	GDs must undergo extensive, long-term field experimentation within an ecologically representative, semi-contained environment prior to implementation; and GDs may only affect the target species' survivability; and GDs must not eradicate the target species if the species functions as an essential link within the target's ecosystem; and GDs must not cause rival species to go extinct if those species function as essential links within their ecosystem.
2. **Horizontal Gene Transfer**	GDs may be horizontally transferred to (wild-types of) the target species; and GDs must not be horizontally transferred to non-target insects if substantial change in function arises; and GDs must include a phenotypical traceability trait; and An efficient quantification method must be established to determine the frequency and effects of, and to improve upon undesired HGT occurrences.
3. **Pathogenicity and Toxicity to Other Organisms**	GDs may only cause pathogenicity or toxicity to the target species if extinction of said species is intended; and GDs must not cause pathogenicity or toxicity to non-target species.
4. **Run-off Risk and Reversibility**	GDs must be inhibitable or reversible. Additionally, GDs must be either temporally or spatially confined, or both.

As mentioned in Section 4.2.1, eradication of *Plasmodium* might negatively affect mosquitoes' evolutionary fitness, which in turn could affect mosquitoes' ecological network (Risk Category 1). Due to great uncertainty around the essence of mosquitoes and *Plasmodium* within their ecosystems, the effect of mosquito eradication must not be neglected yet. Further research regarding reliable ecological simulations or predictions is thus required.

Target-specificity of CRISPR/Cas9 systems has been successfully demonstrated to "specifically [express Cas9] in germ cells" of fruit flies of the *Drosophila* genus (Kondo & Ueda, 2013) and to allow for "tissue-specific gene inactivation in zebrafish" (Ablain et al., 2015). Anopheline mosquitoes have been targeted specifically with low off-target frequencies (Garrood et al., 2021). Potentially reliable target-specificity in GDs has been demonstrated, and the effects of off-targets have been researched in anopheline mosquitoes (Garrood et al., 2021) and wasps (Lester et al., 2020). However, I believe GD target-specificity in both *Aedes* and *Anopheles* genera should be further researched to address limitations affecting Risk Categories 1 through 3.

Unfortunately, no "genetic firewall" technology is currently available to specifically prevent HGT to non-target organisms (Risk Category 2). In addition, further research is required to better understand both insect–insect and insect–plant interactions via microorganisms (Coolen et al., 2022). Hence, I believe GDs targeting mosquitoes in the fight against malaria is considered irresponsible for the time being, when taking the knowledge gap of HGT prevention and consequences into consideration.

Despite the presence of hardwired reversibility safeguards (Risk Category 4), literature has shown that suppression of inheritable effectors (Zhao et al., 2020) and formation of mutated escapees can occur. Thus, such reversibility mechanisms are sometimes in vain. However, recommendations include the "combination with other containment strategies" (Hirota et al., 2017). Besides inhibitability and reversibility, GDs are therefore recommended to be confined in either a temporal or spatial manner (or both) to possess a larger degree of control over the GD dispersion process and to mitigate any human design flaws.

5.3. Recommended Alternative and Complementary Techniques

If certain solutions are sought in which the GD design cannot abide by the provided restrictions, other disease control methodologies – or combinations thereof – may be considered as a safer alternative to the specific application. In many cases, however, GD technology must be combined with a complementary technique for optimal results. A small number of potential technologies to malaria have recently been suggested.

5.3.1. Traditional Genetic Modification

Firstly, traditional GMOs – GE organisms without biased hereditary traits – could still be considered. GMOs are oftentimes far less drastic alternatives to GDs, and are even a current candidate in the fight against malaria. Genetically modified mosquito control could be achieved by suppression of mosquito populations using several methods: sterile insect technique (SIT), and incompatible insect technique (IIT). SIT describes an irradiation method of male *An. gambiae* until they are genetically modified to be infertile. Alternatively, IIT has been proposed to combat population growth by introducing into the population male mosquitoes with symbiont *Wolbachia*-strains of a certain variant, which is incompatible with WT female mosquitoes, resulting in successful embryonic lethality (Lees et al., 2015; Caragata et al., 2020; Wang et al., 2021).

5.3.2. Phage Display

Also, bacteriophages might play an important role in the fight against malaria. Despite the fact that human malaria is not caused by a bacterium – but rather by a unicellular parasite residing inside a mosquito host – bacteriophages could still prove useful in therapy development (van Langen Rosón, 2020). Phage display is an alternative method to traditional biochemical analysis and can be used to identify molecular host–parasite interactions, including antibodies and epitopes (Lanzillotti & Coetzer, 2008). Besides, phages have been proven useful in the transduction process of (synthetic) DNA, such as GDs, into host genomes. Therefore, phages might not only possess the key to vaccine development, but may also address technical GD challenges scientists are currently facing.

5.3.3. Gustatory and Olfactory Aversion

More temporary novel approaches to fight malaria, utilizing mosquitoes' tasting and smelling capabilities, have also been suggested. Tastants – compounds influencing mosquitoes' behavior – have shown promising results as disease control effectors. Triggering the gustatory senses – especially with the bitter-sensing tastants and odorant *N*,*N*-diethyl-*meta*-toluamide (DEET) – is deemed highly effective in averting mosquitoes' biting behavior. Apparently, behavioral changes to the duration of both biting and resting on the skin are inducible (Baik & Carlson, 2020). In fact, the repelling effect has also been observed on olfactory senses of both *Aedes* and *Anopheles* genera (Syed & Leal, 2008; Afify et al., 2019; Dennis et al., 2019), making the component an excellent candidate for disease control. For instance, LLINs could be treated with DEET to (temporarily) slow down the consequences of growing insecticidal resistance.

5.4. Recommended Follow-Up Topics

Currently, I believe we are not able to abide by all SbD guidelines mentioned in Section 4.2, as we have insufficient knowledge about certain scientific concepts that are intertwined with responsible GD design. The following follow-up topics are therefore recommended to further research prior to applying the SbD guidelines necessary to responsibly design, test and implement GDs in the fight against malaria.

In general, theoretically suggested technologies regarding GDs should be further studied to find more and possibly better-fitting solutions to specific pests and diseases. My impression is that in-depth studies should be conducted regarding (i) reliable ecological simulations or predictions; (ii) GD target-specificity; and (iii) prevention of HGT to non-target organisms. These focal points include research into both *Plasmodium* and malarial mosquitoes of the *Aedes* and *Anopheles* genera to allow for the application of the SbD guidelines in Chapter 4 to responsibly fight malaria. Additional research into the theoretical rock–paper–scissors GD model and the split-drive system – especially its efficacy after several generations of progeny – is also recommended.

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7 Appendix

A. Definitions and Background Information

The following definitions and introductory background information were provided to interviewees prior to the interview taking place:

gene drive (GD)	a CRISPR/Cas9-based genetic element with a biased chance of inheritance
Safe-by-Design (SbD)	a risk management strategy aiming to incorporate safety into the designed application
malaria	a serious, and sometimes fatal, flu-like illness characterized by chills and high fever
Plasmodium	a genus of protozoan and obligate parasite known to cause malaria
Aedes (Ae.)	a genus of mosquito known to transmit human malaria, originally (sub)tropical
Anopheles (An.)	a genus of mosquito known to transmit human malaria

Malaria is one of the world's worst problems with well over a 600 thousand deaths and 240 million treatments in 2020 alone. The disease is caused by the parasitic *Plasmodium* and is transmitted to humans by vector mosquitoes. *Plasmodium*'s growth cycle can only be completed at or above 20 °C, burdening mainly Sub-Saharan Africa. Successful vaccinations and long-term cures have yet to be developed. Globalization, climate change, rising insecticide resistance and mosquitoes' changing behavior hastily call for a lasting solution.

Recent genetic engineering innovations include gene drives (GDs), which have been suggested to target malarial mosquitoes (*Aedes* and *Anopheles*) and/or *Plasmodium*. The CRISPR/Cas9-based application can be incorporated into a mosquito's DNA, after which it will biasedly influence the genetic material of its offspring to incorporate the same GD. Even GDs that reduce the mosquito's fitness will be likely to invade a population by releasing enough GD mosquitoes. GDs might offer solutions to cause mosquito extinction by influencing a population to produce only male offspring, which cannot reproduce. Other GD applications describe an increase of the mosquito's immune response to *Plasmodium*, although this might cause a reduction in mosquitoes' evolutionary fitness.

To anticipate risks posed by GD technology, the concept of Safe-by-Design (SbD) was selected. SbD is a risk management strategy that's applicable to a range of different industries, including the chemical industry, biotechnology, and crop breeding innovation. The goal of SbD is to make safety an integral part of the development process.

B. Interviews with Scientists

Scientists with substantive knowledge in the field of genetically engineered organisms (or related fields thereof) might bring technological insights to the table. Therefore, the undermentioned scientists – *interviewees 1* through *3* – have been interviewed to illustrate their perspectives on the ethics of their profession, as well as their thoughts on the consequences of GD technology implementation.

Interviewees

1 Prof. Dr. A. W. Langerak	Department of Immunology	Erasmus University Rotterdam
2 D. Calderón Franco	Environmental Biotechnology Department	Delft University of Technology
3 Dr. R. G. Boot	Department of Biochemistry	Leiden University

Questions

1. What do you think of the problem malaria poses?

2. To what extent were you previously aware of the aforementioned applications of GD technology?

3. What do you think of the (symbiotic/evolutionary) value of *Plasmodium* within its ecosystem?

4. How do you believe eradicating the *Plasmodium*-parasite using GD technology (for the sake of fighting malaria) will turn out?

5. What do you think of the value of the mosquito within its ecosystem?

6. How do you believe eradicating mosquitoes using GD technology (for the sake of fighting malaria) will turn out?

7. Using GD technology, scientists might be able to eradicate malarial mosquitoes and consequently disrupt the ecosystem these mosquitoes were involved in. What trade-offs do you think are acceptable to live with, as long as malarial mosquitoes are permanently eradicated?

8. Do you think GD technology to fight malaria should have some kind of reversibility option, in case mosquitoes' ecosystem get in harm's way, and what do you envision?

9. Do you believe that GDs could be a useful tool in the fight against malaria, and why?

10. Do you have anything to add to the criteria for developing a GD-based or alternative solution for malaria?