SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 14 October 2020

doi: 10.2903/j.efsa.2020.6297

Adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives

EFSA Panel on Genetically Modified Organisms (GMO),
Hanspeter Naegeli, Jean-Louis Bresson, Tamas Dalmay, Ian C Dewhurst,
Michelle M Epstein, Philippe Guerche, Jan Hejatko, Francisco J Moreno, Ewen Mullins,
Fabien Nogué, Nils Rostoks, Jose J Sánchez Serrano, Giovanni Savoini, Eve Veromann,
Fabio Veronesi, Michael B Bonsall, John Mumford, Ernst A Wimmer, Yann Devos,
Konstantinos Paraskevopoulos and Leslie G Firbank

Abstract

Advances in molecular and synthetic biology are enabling the engineering of gene drives in insects for disease vector/pest control. Engineered gene drives (that bias their own inheritance) can be designed either to suppress interbreeding target populations or modify them with a new genotype. Depending on the engineered gene drive system, theoretically, a genetic modification of interest could spread through target populations and persist indefinitely, or be restricted in its spread or persistence. While research on engineered gene drives and their applications in insects is advancing at a fast pace, it will take several years for technological developments to move to practical applications for deliberate release into the environment. Some gene drive modified insects (GDMIs) have been tested experimentally in the laboratory, but none has been assessed in small-scale confined field trials or in open release trials as yet. There is concern that the deliberate release of GDMIs in the environment may have possible irreversible and unintended consequences. As a proactive measure, the European Food Safety Authority (EFSA) has been requested by the European Commission to review whether its previously published guidelines for the risk assessment of genetically modified animals (EFSA, 2012 and 2013), including insects (GMIs), are adequate and sufficient for GDMIs, primarily disease vectors, agricultural pests and invasive species, for deliberate release into the environment. Under this mandate, EFSA was not requested to develop risk assessment guidelines for GDMIs. In this Scientific Opinion, the Panel on Genetically Modified Organisms (GMO) concludes that EFSA's guidelines are adequate, but insufficient for the molecular characterisation (MC), environmental risk assessment (ERA) and post-market environmental monitoring (PMEM) of GDMIs. While the MC, ERA and PMEM of GDMIs can build on the existing risk assessment framework for GMIs that do not contain engineered gene drives, there are specific areas where further guidance is needed for GDMIs.

Keywords: disease vector control, gene drive, modelling, monitoring, pest management, problem formulation, risk assessment

Requestor: European Commission

Question number: EFSA-Q-2018-00619

Correspondence: GMO@efsa.europa.eu

Panel members: Hanspeter Naegeli, Jean-Louis Bresson, Tamas Dalmay, Ian C Dewhurst, Michelle M Epstein, Leslie G Firbank, Philippe Guerche, Jan Hejatko, Francisco J Moreno, Ewen Mullins, Fabien Nogué, Nils Rostoks, Jose J Sánchez Serrano, Giovanni Savoini, Eve Veromann and Fabio Veronesioini.

Acknowledgements: EFSA wishes to acknowledge the contribution of the Gene Drive ERA Working Group experts (Michael B Bonsall, Leslie G Firbank, John Mumford, Fabien Nogué and Ernst A Wimmer) to this opinion, as well as that of Yann Devos (project manager and lead author), Konstantinos Paraskevopoulos and Ana M Camargo. John Mumford resigned as ad hoc expert from the Gene Drive ERA Working Group on 4 February 2020.

Suggested citation: EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli H, Bresson J-L, Dalmay T, Dewhurst IC, Epstein MM, Guerche P, Hejatko J, Moreno FJ, Mullins E, Nogué F, Rostoks N, Sánchez Serrano JJ, Savoini G, Veromann E, Veronesi F, Bonsall MB, Mumford J, Wimmer EA, Devos Y, Paraskevopoulos K and Firbank LG, 2020. Scientific Opinion on the adequacy and sufficiency evaluation of existing EFSA guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of genetically modified insects containing engineered gene drives. EFSA Journal 2020;18(11):6297, 90 pp. https://doi.org/10.2903/j.efsa.2020.6297

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Summary

The European Commission has requested the European Food Safety Authority (EFSA) to assess, through a problem formulation exercise, whether: (1) the deliberate release of genetically modified organisms (GMOs) containing engineered gene drives (termed hereafter as gene drive modified organisms [GDMOs]) could pose risks and potential novel hazards to human/animal health and the environment, considering relevant comparators; (2) the scientific considerations/requirements given in its previously published guidelines for the risk assessment of genetically modified animals (GMAs) (EFSA, 2012, 2013) are adequate and sufficient for GDMOs; and (3) there is a need for updated guidance in relation to previous documents (EFSA, 2012, 2013). Under this mandate, EFSA was not requested to develop risk assessment guidelines for GDMOs.

In agreement with the European Commission, this GMO Panel Scientific Opinion focuses on disease-transmitting insects, primarily mosquitoes, as they represent the most likely cases of GDMOs moving to practical applications for deliberate release into the environment, but it also covers agricultural insect pests and non-native invasive insects.

Genetic elements capable of biasing their own inheritance can be referred to as gene drives. The idea of harnessing naturally occurring gene drives to address challenges related to disease vectors, agricultural pests and invasive species is not new. However, the genetic approaches attempted have until recently either not been sufficiently flexible to construct efficient gene drive systems, or proved difficult to engineer. Recent advances in molecular and synthetic biology are enabling the engineering of gene drives in a wide range of organisms, with most initial focus on insects. While research on engineered gene drives and their applications in insects is advancing at a fast pace, it is generally accepted that it will take several years for technological developments to move to practical applications for deliberate release into the environment. Some gene drive modified insects (GDMIs) have been tested experimentally in the laboratory, but none has been assessed in small-scale confined field trials, or in open release trials as yet. Theoretically, GDMIs may complement and expand the range of genetic methods for disease vector/pest control that involve the release of living insects; they may be used as part of an integrated approach in conjunction with other disease vector/pest control methods.

As is the case for any other genetic disease vector/pest control strategy involving the release of insects, strategies for GDMIs can be differentiated based on: (1) the intended outcome; and (2) potential for the genetic modification to spread in target populations by mating and persist in the environment after release. Engineered gene drive systems can be designed either to suppress target populations or modify them with a new genotype. Moreover, depending on the engineered gene drive system (whose design and mode of action are diverse), theoretically, the genetic modification of interest could spread through interbreeding target populations (non-localised) and persist indefinitely (self-sustaining), or be restricted in its spread (localised) or persistence (self-limiting). The potential of engineered gene drives to spread and persist in the field will be affected by ecological factors. It is therefore important that such factors are taken into account when predicting the performance and fate of GDMIs in the field.

While engineered gene drives could be used to control disease vectors, agricultural pests and invasive species, or rescue endangered species, there is concern that they may lead to undesired side effects and uncontrolled spread, and alter organisms, populations and ecosystems in unanticipated and irreversible ways. Risks and potential novel hazards associated with the deliberate release into the environment of GDMIs previously proposed and reported in the scientific literature are briefly summarised in this GMO Panel Scientific Opinion, as mandated by the European Commission. Similarly, challenges related to the risk assessment and monitoring of GDMOs, including insects, are reported. This identification of risks, potential novel hazards and potential challenges for the risk assessment and post-market environmental monitoring (PMEM) of GDMIs for deliberate release into the environment is inevitably hypothetical to some extent, as to our knowledge, no GDMI application has been submitted for regulatory approval in any jurisdiction globally, and no direct regulatory, environmental risk assessment (ERA) and PMEM experience has been gained with GDMI deliberate releases at the time of writing. However, much relevant experience has been gained from deliberate releases of other GMOs and other disease vector/pest control strategies that involve the release of insects.

The preferential inheritance of a transgenic construct, the intended spatial and temporal scale of spread of the genetic modification(s) of interest, and population modification strategies can be considered as novel aspects of GDMIs when compared with naturally occurring gene drives and disease vector/pest control strategies that involve the release of genetically modified insects (GMIs) that do not contain an engineered gene drive (primarily the release of male insects carrying a dominant lethal gene [RIDL] or a dominant female lethal gene [fsRIDL]) and the release of non-GMIs

(sterile insect technique [SIT], *Wolbachia*-mediated incompatible insect technique [IIT] and pathogen interference [PI], and classical biological control [CBC]). Aspects that are not considered novel include: the scale of population suppression; the target populations and environments; and the lack of spatio-temporal controllability, because: (1) SIT and CBC have been used at a local and area-wide scale to suppress target populations, involving repeated releases over time to reach and maintain suppression; (2) current and emerging disease vector/pest control strategies can target non-domesticated or wild species in non-managed environments; and (3) *Wolbachia*-mediated PI and CBC often lack spatio-temporal controllability. Whether the novel aspects of GDMIs present potential novel hazards, and may introduce additional factors into the risk assessment of some GDMIs, needs to be assessed on a case-by-case basis as part of a specific problem formulation.

Previously proposed risks and potential risk assessment and PMEM challenges cannot be generalised, as they may not apply to all types of GDMIs considered in this GMO Panel Scientific Opinion.

There is substantial experience with releasing insects for genetic and biological disease vector/pest control, including their ERA and post-release monitoring (where applicable). This experience is useful to identify potential hazards, exposures and risks for GDMIs. Thus, it is appropriate to draw on the experience from current insect disease vector/pest control strategies involving the release of insects, seek relevant precedence from more or less similar situations and use this experience to inform the ERA and PMEM of GDMIs. However, caution is required as the systems compared differ in various aspects.

EFSA's GMO Panel reviewed relevant information reported in the scientific literature and progress in developing GDMIs, and followed a section-by-section approach to examine whether the considerations/requirements given in EFSA (2012, 2013) are adequate and sufficient for the molecular characterisation (MC), ERA and PMEM of the GDMIs considered in this GMO Panel Scientific Opinion.

As is the case for any GMO, the ERA of GDMIs should begin with an explicit problem formulation, which is based on the case-by-case approach, is framed by relevant protection goals and draws on the risk assessment experience gained with current insect disease vector/pest control strategies that involve the release of insects. As is the case for any other GMO, the information required for the ERA of GDMIs must be case specific; it will vary dependent on the biology and ecology of the insect species under consideration, the gene drive design and strategy, the introduced traits, the intended uses of the GDMI, the scale and frequency of the deliberate release, the receiving environments (covering the receiving environments where the GDMIs will be released and spread) and the interactions among these variables. Enhanced dialogue between risk assessors, risk managers and stakeholders is advocated to define specific protection goals and decision-making criteria for the ERA of GDMIs.

All considerations/requirements given in EFSA (2012, 2013) are adequate (except those pertaining to general surveillance), but not necessarily sufficient for the MC, ERA and PMEM of the GDMIs considered in this Scientific Opinion.

The EFSA (2012, 2013) guidelines follow the comparative risk assessment paradigm for GMOs, which uses the case-by-case principle and an iterative, stepwise/staged/tiered testing approach, and which considers different lines of evidence, including modelling, in a weight of evidence approach. This approach may leave some uncertainty before open field testing or field implementation of some GDMIs, as it may be challenging to collect data from experimental systems that would be fully applicable to field conditions. Modelling may help to fill this gap in data. This makes the use of mathematical modelling and the design and conduct of PMEM particularly important.

Gathering relevant data for self-sustaining and low threshold (independent) gene drives in open release trials may be challenging due to their spatially and temporally unrestricted nature and current inability for recall. Since self-sustaining engineered gene drives are designed for widespread and long-standing control, spatially and/or temporally restricting their spread would not be in keeping with the intended outcome of their deliberate release. Therefore, the utility of prior field testing of a related self-limiting strain could be considered as an intermediate step to reduce uncertainties in risk assessment. Theoretically, self-limiting engineered gene drive systems may enable localised and temporally restricted spread of the genetic modification of interest, resembling other self-limiting approaches for disease vector/pest control.

While the MC, ERA and PMEM of GDMIs can build on the existing risk assessment framework for GMIs that do not contain engineered gene drives (EFSA, 2012, 2013), there are specific areas where further guidance is needed for GDMIs. These include cross-cutting considerations of EFSA (2013) [i.e. receiving environments, comparators, non-GM surrogates, experimental design and statistics, long-term effects and modelling], specific areas of risk of EFSA (2013) [i.e. persistence and invasiveness including vertical gene flow, horizontal gene transfer, pathogens, infections and diseases, and interactions with target organisms], PMEM of EFSA (2013), and MC-related aspects of EFSA (2012).

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1. Introduction

In the European Union (EU), including its outermost regions, the placing on the market of genetically modified organisms (GMOs) is subject to risk assessment and regulatory approval according to Part C of Directive 2001/18/EC on the deliberate release of GMOs into the environment. In this process, the role of the European Food Safety Authority (EFSA) is to assess and provide scientific advice to risk managers on any plausible risk that the deployment of a GMO may pose to human health, animal health, and the environment. The decision on the level of acceptable risk, given the potential for appropriate risk management, and thus whether the use of a GMO should be permitted, is taken by risk managers (the European Commission and EU Member States). Directive 2001/18/EC follows the precautionary principle,[1] whose application has been clarified in detail by the European Commission in its 'Communication on the Precautionary Principle' (European Commission, 2000).

GMOs are officially defined in EU legislation as organisms in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. Examples of techniques of genetic modification yielding GMOs are described in Annex IA of Directive 2001/18/EC. On 25 July 2018, the judgement of the Court of Justice of the European Union in Case C-528/16 on mutagenesis has clarified that Directive 2001/18/EC is applicable to GMOs obtained by mutagenesis techniques that have emerged since its adoption in 2001 ('new mutagenesis techniques').[2] New mutagenesis techniques include gene editing using the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system (e.g. EASAC, 2017; SAM, 2017; GCSA, 2018).[3] To date, organisms containing engineered gene drives are considered GMOs within the scope of EU legislation, and thus are subject to risk assessment and regulatory approval.

Potential future applications for the placement of GMOs on the EU market, including public or non-commercial use, may include the deliberate release into the environment of GMOs that contain engineered gene drives (referred to hereafter as gene drive modified organisms [GDMOs]). As a proactive measure, EFSA has been requested by the European Commission to assess, through a problem formulation exercise, whether: (1) the deliberate release of GDMOs could pose risks and potential novel hazards to human and animal health and the environment, considering relevant comparators; (2) the scientific considerations/requirements given in its previously published guidelines for the risk assessment of genetically modified animals (GMAs) (EFSA, 2012, 2013) are adequate and sufficient for GDMOs; and (3) there is a need for updated guidance in relation to the previous published guidelines (EFSA, 2012, 2013; see Section 1.1). Under this mandate, EFSA has not been requested to develop risk assessment guidelines for GDMOs.

In agreement with the European Commission, the primary focus of the mandate is disease-transmitting insects, mainly mosquitoes, as they represent the most likely cases of GDMOs moving to practical applications for deliberate release into the environment, but it also covers agricultural insect pests and non-native invasive insects.

Since organisms containing engineered gene drives are living modified organisms (LMOs) within the scope of the Cartagena Protocol on Biosafety, EFSA's scientific advice is expected to support the EU in its work under the Convention on Biological Diversity[4] and the Cartagena Protocol on Biosafety, where the development of further risk assessment guidance on engineered gene drives is under discussion (AHTEG, 2020; Keiper and Atanassova, 2020).[5] The Cartagena Protocol aims to ensure the safe

[1] Recital 8 of Directive 2001/18/EC requires that the precautionary principle [...] must be taken into account when implementing [this Directive]. Furthermore, this principle is explicitly expressed in the objectives of the Directive as stated in its Article 1: "In accordance with the precautionary principle, the objective of this Directive is to approximate the laws, regulations and administrative provisions of the Member States and to protect human health and the environment when [...] placing on the market GMOs as or in products within the Community".

[2] In November 2019, the Council of the European Union requested the European Commission (Council Decision (EU) 2019/1904) to submit, by 30 April 2021, a study in light of the Court of Justice's judgment in Case C-528/16 regarding the status of new genomic techniques under Union law.

[3] CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) DNA sequences and associated Cas9 (CRISPR associated protein 9) constitute an adaptive immunity system in certain bacteria. Cas9 enzymes compose a family of RNA-guided endonucleases that use the CRISPR sequences as a guide to recognise and cleave DNA from viruses. The Cas9 endonuclease, when associated with a single guide RNA (sgRNA), can be used as a genetic engineering tool to edit a specific locus in a given genome (Doudna and Charpentier, 2014; Sternberg and Doudna, 2015).

[4] The Convention on Biological Diversity is a multilateral treaty under the auspices of the United Nations Environment Program. Its major goals are the conservation of biodiversity, sustainable use of the components of biodiversity, and fair and equitable sharing of benefits arising from genetic resources stemming from biodiversity.

[5] The Cartagena Protocol on Biosafety to the Convention on Biological Diversity was adopted on 29 January 2000, and entered into force on 11 September 2003. The Cartagena Protocol presently has 171 contracting parties, excluding Argentina, Canada and the United States.

handling, transport, and use of LMOs resulting from modern biotechnology that may have adverse effects on biodiversity, also taking into account risks to human health.

Any genetic elements[6] capable of biasing their own inheritance can be referred to as gene drives. The idea of harnessing naturally occurring gene drives to address challenges related to disease vectors (e.g. mosquitoes, ticks), agricultural pests (e.g. pigweed, screwworm, desert locust) and invasive species (e.g. mice, rats, other mammals, cane toads, some invasive plant species) is not new (e.g. Curtis, 1968; Esvelt et al., 2014; Ledford, 2015; Webber et al., 2015; Dearden et al., 2017; Harvey-Samuel et al., 2017; Raban and Akbari, 2017; Min et al., 2018; Scott et al., 2018; Lester and Beggs, 2019; Rode et al., 2019; Inwood et al., 2020; Lester et al., 2020; Li et al., 2020b; Serr et al., 2020; Teem et al., 2020). However, the classical genetic approaches attempted have until recently either not been sufficiently flexible to construct efficient gene drive systems, or have proven difficult to engineer (Rasgon and Gould, 2005; Champer et al., 2016; NASEM, 2016; Burt and Crisanti, 2018; James et al., 2018; Min et al., 2018). Advances in molecular and synthetic biology, including the discovery of the CRISPR-Cas9 system, have delivered molecular and computational tools that enable the design and development of a wide range of engineered gene drive systems in diverse organisms (Burt, 2003, 2014; Champer et al., 2016; NASEM, 2016; Godfray et al., 2017; Raban et al., 2020). The CRISPR-Cas9 system enables the insertion, deletion, or replacement of specific genes in many species (e.g. Terns, 2018), but also provides a molecular tool to engineer novel homing endonuclease genes (HEGs). Preliminary evidence, from laboratory studies, indicates that CRISPR-Cas9-mediated homing-based gene drives have the potential to spread a genetic modification of interest through a given population of yeast, fruit flies and mosquitoes (NASEM, 2016). These developments suggest that a practical application of engineered gene drive systems for disease vector/pest control could be more readily achievable than previously believed in insects (Esvelt et al., 2014; Burt et al., 2018). Recently, engineered gene drives have also been proposed as a technology to complement current efforts to enhance biodiversity conservation, for instance by modifying genes to increase the ability of organisms to resist climate change impacts (Goldman, 2016; NASEM, 2016; Esvelt and Gemmell, 2017; Simon et al., 2018; Redford et al., 2019; Rode et al., 2019; Inwood et al., 2020; Sandler, 2020). To date, no registration application for the deliberate release of gene drive modified insects (GDMIs) has been submitted for regulatory approval in any jurisdiction globally, but the technology could in principle be ready for use in mosquitoes in the near future (Scudellari, 2019).

While engineered gene drives could be used to control disease vectors, agricultural pests and invasive species, or rescue endangered species, there is concern that they may lead to undesired side effects and uncontrolled spread, and alter organisms, populations and ecosystems in unanticipated and irreversible ways (e.g. Simon et al., 2018; CSS–ENSSER–VDW, 2019; Cotter et al., 2020; Dolezel et al., 2020a,b; Then et al., 2020). These concerns have prompted some non-governmental organisations (internationally and Europe-wide), parliamentarians (including the European Parliament),[7] scientists and scientific bodies to call for either a moratorium or the strict application of the precautionary principle on gene drive research, including field tests (Callaway, 2016, 2018; NASEM, 2016; HCB, 2017; CSS–ENSSER–VDW, 2019; Cotter et al., 2020). Calls are also made for a better understanding of the potential ecological and evolutionary impacts associated with the deliberate release of GDMOs (e.g. Scott et al., 2002; Esvelt et al., 2014; NASEM, 2016; Courtier-Orgogozo et al., 2017; Esvelt and Gemmell, 2017; HCB, 2017; CSS–ENSSER–VDW, 2019; Giese et al., 2019; Rode et al., 2019; Snow, 2019; Dolezel et al., 2020a,b). This has led to the establishment of recommendations on phased testing (e.g. WHO, 2014; NASEM, 2016; Hayes et al., 2018; James et al., 2018, 2020), the responsible and sustainable deployment of the technology (James et al., 2018, 2020; Warmbrod et al., 2020), and effective engagement of all concerned parties/stakeholders (Oye et al., 2014; Caplan et al., 2015; NASEM, 2016; Adelman et al., 2017a,b; Emerson et al., 2017; Najjar et al., 2017; James et al., 2018; Barnhill-Dilling et al., 2019; Bartumeus et al., 2019; Brossard et al., 2019; Buchthal et al., 2019; CSS–ENSSER–VDW, 2019; George et al., 2019; Hartley et al., 2019; Kofler, 2019; Kuzma, 2019; Rabitz, 2019; Singh, 2019; Thizy et al., 2019; Kelsey et al., 2020; Palmer et al., 2020; Serr et al., 2020; Warmbrod et al., 2020; WHO, 2020). Since some engineered gene drives may eventually spread across jurisdictional boundaries, regional approaches that would facilitate multi-country/international

[6] Also termed: Selfish genes, ultra-selfish genes, selfish DNA, self-promoting elements, parasitic DNA and genomic outlaws.

[7] In early 2020, the European Parliament voted on a resolution calling on the European Commission and EU Member States to support a decision of the 15th Conference of the Parties serving as the meeting of the Parties to the Cartagena Protocol on Biosafety to the Convention on Biological Diversity for a global moratorium on releases of organisms containing engineered gene drives into the environment, including in experimental field trials (reviewed by Keiper and Atanassova, 2020).

regulatory oversight and governance have been suggested (Marshall, 2010; Brown, 2017; James et al., 2018; Rabitz, 2019; Kelsey et al., 2020; Reynolds, 2020; Warmbrod et al., 2020).

1.1. Background and Terms of Reference as provided by the requestor

In accordance with Article 29(1) of Regulation (EC) No 178/2002, the European Commission has mandated EFSA to deliver "*an opinion on genetically modified organisms engineered with gene drives (gene drive modified organisms) and their implications for risk assessment methodologies*".[8]

In particular, "*through a problem formulation exercise providing the foundation for the environmental risk assessment*", EFSA is requested:

- "*To identify potential risks in terms of impact on human and animal health and the environment that gene drive modified organisms could pose. In this respect EFSA is also asked to identify potential novel hazards of gene drive modified organisms, considering relevant comparators, where appropriate*";
- "*To determine whether the existing guidelines for risk assessment are adequate and sufficient for gene drive modified organisms or whether there is a need for updated guidance*";
- "*To identify the specific areas where such updated guidance is needed*".

EFSA is also requested "*to provide technical and scientific expertise on risk assessment of gene drive modified organisms to support the EU in the work under the Convention on Biological Diversity and the Cartagena Protocol on Biosafety*".

Under this mandate, EFSA is not requested "*to develop guidelines for the risk assessment of gene drive modified organisms*".

1.2. Interpretation of the Terms of Reference

Following discussions with the European Commission (Directorate-General for Health and Food Safety), it was agreed to limit the scope of the mandate (see Section 1.1) to insects, as they represent the most likely cases of GDMOs moving to practical applications for deliberate release into the environment. Although the use of engineered gene drive systems is under consideration in mammals (Leitschuh et al., 2018; Conklin, 2019; Godwin et al., 2019; Grunwald et al., 2019; Manser et al., 2019; Faber et al., 2020) and plants (Neve, 2018; Barrett et al., 2019; Gardiner et al., 2020), basic technical challenges need to be overcome before an engineered gene drive will be possible in these taxa (NASEM, 2016; Godwin et al., 2019; Pixley et al., 2019; Scudellari, 2019). In the future, however, EFSA could be mandated by the European Commission to evaluate whether its guidelines for the risk assessment of genetically modified (GM) mammals and plants are adequate and sufficient for the risk assessment of GM mammals and plants containing engineered gene drives.

In insects, the most likely engineered gene drive cases for deliberate release into the environment are expected to be those that are directed at human, livestock and wildlife disease vectors, followed by agricultural and horticultural pests in highly managed ecosystems and non-native invasive insect species. To reduce their threat to human or animal health, agricultural production and biodiversity, humans have aimed at controlling insect disease vectors (such as mosquitoes), agricultural pests and invasive species through a variety of methods, including the use of biological or chemical insecticides, resistant crop varieties, biological control and genetic control methods[9] such as the sterile insect technique [SIT] and the incompatible insect technique [IIT] (e.g. Ritchie and Staunton, 2019; Caragata et al., 2020; Romeis et al., 2020). Controlling disease transmission by mosquitoes, for instance, is a long-standing public health goal (Feachem et al., 2019; Masterson, 2019). While effective on a local/regional scale and despite diligent application, current control methods (e.g. removal of standing water for mosquito breeding and resting sites, use of insecticides delivered via bed-nets and indoor residual spraying, outdoor insecticide fogging, applications of chemical larvicides, mass release of sterile males, IIT) have not prevented the proliferation of mosquito-vectored diseases,[10] in part due to evolution of resistance to commonly used insecticides, difficulty in reaching all mosquito breeding and resting sites and global climate change that facilitates mosquito spread (e.g. Ritchie and Staunton, 2019; WHO, 2019; Fouet et al., 2020). This has prompted the development of new genetic

[8] registerofquestions.efsa.europa.eu/roqFrontend/ListOfQuestionsNoLogin (EFSA-Q-2018-00619).
[9] Genetic control can be defined as the dissemination, by mating or inheritance, of factors that reduce pest damage (Alphey, 2014).
[10] E.g. malaria, dengue, Zika, chikungunya, yellow fever and West Nile.

approaches to combat the spread of mosquito and other vector-borne diseases worldwide. One of these approaches utilises GM insects (GMIs) with engineered gene drives (e.g. Windbichler et al., 2007, 2008, 2011; Gantz et al., 2015; Hammond et al., 2016; Flores and O'Neill, 2018; Kyrou et al., 2018; Buchman et al., 2019). Likewise, increasing challenges associated with insecticide resistance in agricultural insect pests and the invasion of non-native insect species are driving the exploration of novel insect genetic control approaches, including engineered gene drives (Alphey, 2014; Dearden et al., 2017; Alphey and Bonsall, 2018; Buchman et al., 2018a; Lester and Beggs, 2019; Lester et al., 2020).

Although uses of engineered gene drives have been proposed for pest control to support biodiversity conservation (e.g. Goldman, 2016), this GMO Panel Scientific Opinion does not address such uses, nor uses of GDMIs for the direct enhancement of agricultural production systems, as no concrete applications have been reported at the time of writing (e.g. Goldman, 2016; NASEM, 2016; Esvelt and Gemmell, 2017; Redford et al., 2019; Rode et al., 2019; Sandler, 2020). Consequently, this GMO Panel Scientific Opinion focuses on harmful insect species, in particular disease vectors,[11] agricultural pests and invasive species.

The scope of the mandate focuses on the molecular characterisation (MC), environmental risk assessment (ERA) and post-market environmental monitoring (PMEM) of GDMIs for deliberate release into the environment (part C of Directive 2001/18/EC); such releases are non-confined[12] and not intended for food/feed uses,[13] because engineered gene drives are intended to spread autonomously through interbreeding wild type/target populations occurring in the environment. Consequently, the mandate excludes GDMI releases that are physically confined, and the deliberate release of GDMIs for food/feed uses (if any).

In summary, the scope of the mandate covers:

- The non-confined release of GDMIs into the environment for non-food/feed uses;
- The MC, ERA, including the problem formulation process and its function in ERA, and PMEM of GDMIs for deliberate release into the environment;
- The use of engineered gene drives to control harmful insects such as disease-transmitting insects, agricultural insect pests and invasive insects.

EFSA has not mandated to provide advice on ethical and socio-economic aspects and possible benefits associated with gene drive technology. Some of the latter aspects are expected to be addressed by the European Group on Ethics that has been requested by the European Commission to deliver an advice on GDMOs.[14] Moreover, EFSA is not responsible for providing scientific advice on the contained use of GM microorganisms (Directive 2009/41/EC) and other GMOs, the deliberate release of GMOs into the environment for experimental purposes (part B of Directive 2001/18/EC), the accidental release of GMOs and the transboundary movement of GMOs under Regulation (EC) 1946/2003, which transposes the Cartagena Protocol on Biosafety into EU law.

2. Data and methodologies

The section on data and methodologies reports on: (1) expertise (Section 2.1); (2) information/ data (Section 2.2); and (3) methodologies (Section 2.3) used for the completion of the mandate of the European Commission.

2.1. Expertise

EFSA established an ad hoc expert Working Group of the GMO Panel on the MC, ERA and PMEM of GDMIs that met 22 times to address the mandate of the European Commission.[15] The Working Group consisted of two scientific experts from the GMO Panel and three external ones (four until 4 February 2020) with expertise in arthropod genetics, insect biotechnology, disease vector/pest control strategies, ecological modelling, community ecology, MC of GMOs, ERA of GMIs and risk assessment of invasive species. In order to further support its work, EFSA also invited other scientists

[11] The mosquito species responsible for transmitting diseases in the EU, including its outermost regions, are mostly *Aedes albopictus*, *Aedes aegypti* and various species of Culex and Anopheles (as reviewed by HCB, 2017). Ticks represent another insect disease vector relevant for the EU.
[12] The terms 'confined', 'semi-confined' and 'non-confined' are defined in EFSA (2013).
[13] In line with EFSA (2012), food and feed refer to food and feed containing, consisting of or produced from GMAs.
[14] https://ec.europa.eu/info/sites/info/files/research_and_innovation/ege/letter_chair_of_the_ege_group.pdf
[15] http://www.efsa.europa.eu/sites/default/files/wgs/gmo/wg-gene-drive-era.pdf

(i.e. hearing experts) with particular and relevant knowledge to contribute to one or more meetings of the Working Group by providing additional data, reports and publications and answering questions. The composition of the Working Group is reported on EFSA's website.[16]

2.2. Information/data

In delivering its Scientific Opinion, the GMO Panel, along with its Gene Drive expert Working Group and EFSA's scientific officers (together referred to hereafter as GMO Panel), took into account the considerations/requirements given in the GMO Panel Scientific Opinions that provide guidance for the risk assessment of GMAs, including GMIs (EFSA, 2012, 2013), Directive 2001/18/EC on the deliberate release into the environment of GMOs and the Commission Directive (EU) 2018/350 amending Directive 2001/18/EC, where appropriate, and relevant information/data reported in the scientific literature, including unrefereed manuscripts available in bioRxiv.

Points raised by the participants of EFSA's stakeholder workshop have been considered by the GMO Panel during its deliberations (see Section 2.3.2.1; EFSA, 2020a). Moreover, the public consultation comments received on the draft GMO Panel Scientific Opinion through the online public consultation have been analysed and taken into consideration by the GMO Panel during the revision and finalisation of its Scientific Opinion, where appropriate (see Section 2.3.2.2; EFSA, 2020b).

The EFSA (2012, 2013) guidelines serve as the reference documents for the MC, ERA and PMEM of GMAs, respectively. These guidelines assist applicants in the preparation and presentation of their registration applications by describing the elements and information requirements for a structured risk assessment of GMAs.

- *EFSA (2012)*: EFSA (2012) covers the risk assessment of food/feed containing, consisting of, or produced from GMAs, as well as the health and welfare assessment of these animals, within the framework of Regulation (EC) No 1829/2003 on GM food/feed. EFSA (2012) focuses on husbandry animals, fish, crustaceans and molluscs, and does not consider insects and other arthropods. EFSA (2012) addresses the MC, which provides information on the structure and expression of the insert(s) and on the stability of the intended trait(s); the toxicological assessment, which addresses the possible impact of biologically relevant change(s) in the GMA and/or derived food/feed; the allergenicity assessment of the novel protein(s), as well as of the whole food derived from the GMA; and the nutritional assessment to evaluate whether food/feed derived from a GMA is as nutritious to humans and/or animals as food/feed derived from traditionally bred animals. EFSA (2012) also addresses the scientific requirements for the assessment of health and welfare of GMAs bred for food/feed use. EFSA (2012) does not cover the ERA of GMAs, which is addressed in EFSA (2013);
- *EFSA (2013)*: EFSA (2013) provides guidance for the ERA and PMEM of living GMAs, namely fish, insects and mammals and birds, to be placed on the EU market in accordance with Regulation (EC) No 1829/2003 or Directive 2001/18/EC. EFSA (2013) provides guidance for assessing potential effects of GMAs on animal and human health and the environment and the rationales for data requirements for a comprehensive ERA. EFSA (2013) follows Annex II of Directive 2001/18/EC, considering specific areas of risk to be addressed by applicants and risk assessors during the ERA of GM fish, GMIs and GM mammals and birds. Each specific area of risk must be considered in a structured and systematic way following the six successive steps for ERA: (1) problem formulation including hazard and exposure identification; (2) hazard characterisation; (3) exposure characterisation; (4) risk characterisation; (5) risk management strategies; and (6) an overall risk evaluation. In addition, EFSA (2013) describes several generic cross-cutting considerations (e.g. choice of comparators, use of non-GM surrogates, experimental design and statistics, long-term effects, quantification of uncertainty) that need to be accounted for throughout the whole ERA.

The GMO Panel notes that the development of EFSA (2012, 2013) called for a general approach, as the European Commission mandated EFSA to develop guidelines for the risk assessment of GMAs that would address both the food/feed safety assessment and ERA, including animal health and welfare aspects, and cover the ERA of broad range of taxa ranging from GM fish to GMIs, GM mammals and GM birds. Consequently, EFSA (2013) provides a non-exhaustive list of potential issues to consider, but

[16] https://ess.efsa.europa.eu/doi/doiweb/wg/685350

without necessarily clarifying how these issues should be addressed concretely for the ERA of GMAs, including GMIs. Although GDMIs are mentioned in EFSA (2013), little emphasis is given to them.

2.3. Methodologies

The section pertaining to methodologies focuses on: (1) the assessment performed by EFSA's GMO Panel (Section 2.3.1); and (2) consultations (Section 2.3.2).

2.3.1. Assessment

For the identification of risks and potential novel hazards associated with the deliberate release into the environment of GDMIs (see Section 4), relevant scientific literature has been reviewed to report previously proposed risks and potential novel hazards. This analysis is inevitably hypothetical to some extent, as no GDMI application has been submitted for regulatory approval in any jurisdiction globally at the time of writing.

A section-by-section approach was followed to examine whether the considerations/requirements given in EFSA (2012, 2013) are adequate and sufficient for the MC, and ERA and PMEM of GDMIs, respectively. The term 'adequate' means that the existing guidance documents can be used, but that additional qualifications would be appropriate, whereas 'sufficient' means that the guidance documents are fully fit for purpose. Thus, 'adequate' gives a lower acceptable bound below which quality or quantity would be unacceptable. 'Sufficient' gives an upper acceptable bound in terms of quality or quantity above which one needs not strive; more would be excessive. This evaluation is reported in Section 5 for each of the relevant headings and subheadings of EFSA (2012, 2013). The adequacy and sufficiency evaluation of EFSA (2012, 2013) has been performed on the basis of relevant information reported in the scientific literature and practical developments of GDMIs (see Section 3.3).

The 'applicability' of particular considerations/requirements of EFSA (2012, 2013) for individual risk assessments will vary between cases and do not apply in a generic way for all engineered gene drive applications. Since the GMO Panel has not been mandated by the European Commission to conduct a risk assessment, the practical applicability of the considerations/requirements given in EFSA (2012, 2013) has not been tested for a specific GDMI for regulatory approval. Based on the identification of previously proposed risks, potential novel hazards, risk assessment and monitoring challenges and the adequacy evaluation of EFSA (2012, 2013), specific areas potentially requiring updated/revised guidance were identified (see Section 6).

2.3.2. Consultations

Considering the current societal debate on the potential applications of engineered gene drives, given the need for greater dialogue, and in line with its policy on openness and transparency, EFSA has organised two consultations at different development stages of the GMO Panel Scientific Opinion to collect input from its stakeholders (including EU Member States) and other interested parties/ persons. One, in the shape of a stakeholder workshop, took place early in the development process; and the other, in the shape of an online public consultation, was carried out at a later stage in the development of this GMO Panel Scientific Opinion.

2.3.2.1. Stakeholder workshop 'Problem formulation for the environmental risk assessment of gene drive modified insects' (15 May 2019, Brussels)

Through an open workshop, EFSA aimed to engage with stakeholders to discuss potential environmental risks associated with the deliberate release into the environment of GDMIs. To focus the discussions, participants were invited to contribute to an example problem formulation (see Section 4.1). The problem formulation exercise was run for two hypothetical case studies in two separate discussion groups. The outcomes of the two discussion groups were presented and further developed in a final plenary session, during which the conclusions of the workshop were drawn.

The two hypothetical case studies were selected to represent harmful insect species relevant for the EU, but do not represent the most likely cases of GDMIs moving to practical applications for deliberate release into the environment.

1) Self-sustaining low threshold gene drives (see Section 3.2) to control disease-transmitting mosquitoes (i.e. the Asian tiger mosquito (*Aedes albopictus*)). *Ae. albopictus* is an aggressive biting mosquito native to Asia that has colonised all continents, except Antarctica, during the last ~ 30–40 years. The species is of great public health concern as it

can transmit several arboviruses, including dengue, chikungunya and Zika viruses (Lounibos, 2002). With climate change, the potential for transmission of these viruses through *Ae. albopictus* is likely to increase substantially for most of Europe even in the short term (Ryan et al., 2019);

2) Self-sustaining low threshold gene drives to control agricultural pests (i.e. the spotted-wing *Drosophila* (*Drosophila suzukii*)). *D. suzukii* is a highly invasive pest that has recently and rapidly expanded out of its native range, in Southeast Asia, to Europe and both North and South America, where it causes significant economic damage to the fruit sector (Ørsted and Ørsted, 2019). Females lay eggs inside ripening soft-skinned fruits, and larvae feed inside the fruit, which becomes soft and rots (e.g. Schetelig et al., 2018; Romeis et al., 2020).

The goal of the workshop was not to produce a comprehensive and detailed problem formulation of the two hypothetical case studies, but rather to familiarise the participants with the problem formulation process and its function in ERA, and gather feedback on this approach.

Points raised by the workshop participants, on defining protection goals, formulating specific pathways to harm and on structuring risks have been considered by the GMO Panel during its deliberations, and are reported in EFSA (2020a).

The workshop materials supplied by EFSA and speakers (i.e. agenda and briefing notes for participants, list of participating stakeholders and presentations) are available on EFSA's website.[17]

2.3.2.2. Online public consultation

EFSA also consulted interested parties/persons via an online public consultation. Between 17 February and 24 April 2020 (included), interested parties/persons were invited to submit their comments on each section of the draft GMO Panel Scientific Opinion through an online EU Survey.[18] Thirty-six interested parties/persons from 11 different geographical areas submitted comments: 13 from universities/public research institutes, either in personal capacity or on behalf of the organisation; nine from GMO risk assessment bodies or national competent authorities; eight from non-governmental organisations; four from the private sector (e.g. industry, consultancy); and two from other categories (i.e. network, foundation). Comments received through the EU Survey have been analysed and taken into account by the GMO Panel during the revision and finalisation of its Scientific Opinion, where appropriate.

The outcome of the public consultation is reported in EFSA (2020b). This report contains the comments received and explains how they have been considered for the revision and finalisation of the GMO Panel Scientific Opinion.

3. Explaining engineered gene drives

Gene drives can be described as any genetic elements that are capable of biasing their own inheritance (referred to hereafter as preferential inheritance)[19] to gain a transmission advantage over the rest of the genome (e.g. Burt and Trivers, 2006; Schenkel and Leggewie, 2015; NASEM, 2016; ZKBS, 2016; AAS, 2017; EASAC, 2017; HCB, 2017; SAM, 2017; High-Level African Panel on Emerging Technologies, 2018; Leftwich et al., 2018; Royal Society, 2018; Ethics Council of the Max-Max-Planck-Gesellschaft, 2019; Hurst, 2019; North et al., 2019; Redford et al., 2019; Wedell et al., 2019; Deplazes-Zemp et al., 2020; Hammond et al., 2020; Warmbrod et al., 2020). During sexual reproduction of diploid organisms, each of the two copies of a gene present in each parent has a 50% chance of being inherited by offspring according to the Mendelian laws of inheritance. Gene drives increase this probability and are transmitted to subsequent generations at a frequency greater than the 50% expected by Mendelian inheritance. This preferential inheritance may allow gene drive systems to rapidly spread in sexually reproducing populations, increasing their prevalence and that of any genetically linked cargo/payload genes,[20] even if they incur some fitness costs on their host.

Since gene drives occur naturally in a broad array of organisms (Burt and Trivers, 2006; Ågren and Clark, 2018; Cosby et al., 2019), Hurst (2019) has suggested that preferential inheritance may be the rule rather than the exception.

[17] https://www.efsa.europa.eu/en/events/event/190515
[18] Published at http://www.efsa.europa.eu/en/consultations/call/public-consultation-gmo-panel-scientific-opinion-evaluation
[19] Also termed: Biased or super-Mendelian inheritance.
[20] Also termed: Effector genes.

NASEM (2016) reported differences in the use of gene drive terminology and definitions, with terms often having overlapping definitions depending on the historical period and the scientific context in which they are used. Since research on engineered gene drives is evolving very quickly, it may potentially result in differences in definitions and terminology, and in the way each may conceptualise and interpret gene drive strategies among stakeholders (see Sections 3.2 and 3.3). Although the nuances of different definitions, interpretations and classifications can be valuable, there may be a need to address the existing ambiguity to improve comparability. This will promote consistency, transparency and transferability. The development of a common set of definitions and terminology – a 'standard lexicon' – if generally accepted, would help to frame gene drive-related discussions. Activities are ongoing to review the current published gene drive technical definitions; work with an international panel of scientific stakeholders towards developing consensus definitions for scientific use of terms; and make the definitions publicly available (see Alphey et al., 2020).[21]

The section on explaining engineered gene drives addresses: (1) mechanisms for preferential inheritance (Section 3.1); (2) strategies for engineered gene drives (Section 3.2); (3) approaches for GDMIs (Section 3.3); (4) ecological factors affecting engineered gene drive spread and persistence in the field (Section 3.4); (5) current and emerging genetic disease vector/pest control strategies (Section 3.5); and (6) a state of the art (Section 3.6).

3.1. Mechanisms for preferential inheritance

First reported in the 1920s, natural gene drives have been observed in a variety of organisms, and encompass a variety of different mechanisms: transposable elements[22] that insert copies of themselves at other places in the genome; homing endonuclease genes that copy themselves at targeted genomic sites; segregation distorters[23] that destroy competing chromosomes during meiosis; gamete killers that eliminate gametes not carrying the gene drive; the Medea (maternal-effect dominant embryonic arrest) system that confers maternal-effect lethality to all offspring that does not have a copy of the Medea element; and *Wolbachia* endosymbionts that favour offspring of infected females (e.g. Beeman et al., 1992; Burt and Trivers, 2006; Sinkins and Gould, 2006; Champer et al., 2016; Hammond and Galizi, 2017; Ågren and Clark, 2018; Collins, 2018; Rüdelsheim and Smets, 2018; Cash et al., 2019, 2020; Frieβ et al., 2019). The study of natural gene drive systems has provided considerable theoretical and empirical insights into how natural gene drives work, how they spread and how simple model predictions on engineered gene drives may fail (Courret et al., 2019; Dyer and Hall, 2019; Finnegan et al., 2019; Larner et al., 2019; Lea and Unckless, 2019; Price et al., 2019; Wedell et al., 2019; Dhole et al., 2020). These insights can provide baseline information for the design of engineered gene drives, and in some cases, for the risk assessment of GDMIs. The main limitation of this is that the early events in introduction and spread of natural gene drive systems are rarely seen – by the time they are observed, they have generally established beyond that initial phase.

Gene drives use the following three main/primary mechanisms to bias their own inheritance (Burt and Trivers, 2006):

1) *Over-replication*: Over-replicating genetic elements (such as transposable elements and homing elements) increase their copy number in the genome by replicating more often than other genes in the genome. For example, homing endonucleases use over-replication by copying themselves onto the homologous target sequence (a process termed homing), resulting in most or all offspring inheriting the gene drive allele;
2) *Interference*: Interfering genetic elements (such as meiotic gene drives, chromosomal translocations and maternal-effect killers) disrupt the transmission of other gene variants through the distortion of meiosis or gamete development,[24] or interference with offspring survival over generations. Pre-gametic gene drives distort transmission ratios during meiosis, so that gametes carrying the gene drive allele have a higher probability of being produced. Post-gametic gene drives accomplish segregation distortion via mechanisms that render gametes inviable after meiosis has taken place. Reducing the viability of gametes that inherit the wild-type allele gives the wild-type allele a fitness disadvantage compared to the gene drive allele;

[21] https://fnih.org/sites/default/files/final/pdf/SUMMARY%20-%204th%20Gene%20Drive%20Research%20Forum%20FINAL.pdf
[22] Also termed: Jumping genes.
[23] Also termed: Meiotic drive elements.
[24] Also termed: Transmission distorters.

3) *Gonotaxis*: Gonotaxis refers to selfish genetic elements that bias Mendelian segregation by moving away from dead-end polar bodies into the functional egg during oogenesis (e.g. some plant B-chromosomes or heterochromatic knobs of A-chromosomes). Since polar bodies do not become functional gametes, the selfish gene is transmitted to more than 50% of the offspring. The process is not well understood molecularly, and currently, there are no engineered gene drives proposed based on gonotaxis.

Engineered gene drive systems currently under investigation generally exhibit drive due either to over-replication mechanisms (e.g. homing-based gene drives), or interference mechanisms (e.g. Medea, cleave and rescue systems) (see Section 3.3 for examples).

3.2. Strategies for engineered gene drives

Scientists are working to harness gene drives, either by repurposing naturally occurring systems or by engineering (redesigning) them, so that they could be used to spread a genetic modification of interest through target populations over many generations (e.g. Champer et al., 2016; Marshall and Akbari, 2018; Raban et al., 2020). As is the case for any other genetic control strategy, strategies for engineered gene drives can be differentiated based on the following dimensions (see Table 1, below):

1) The intended outcome;
2) Potential for the genetic modification to spread[25] in target populations by mating and persist[26] in the environment after release.

3.2.1. Intended outcome

Depending on the intended outcome, engineered gene drives and their associated cargo/payload genes (if any) can be designed either to suppress target populations (termed hereafter as population suppression), or modify them with a new genotype (termed hereafter as population modification). This can be achieved either through the inactivation of an endogenous gene, or by the introduction of a new (engineered) genetic trait in a target population. Dhole et al. (2020) noted that the separation of engineered gene drives into population suppression or modification categories is not absolute, because some engineered modification drives could also cause some amount of population suppression.

3.2.1.1. Population suppression[27]

Population suppression strategies aim to reduce a target population by imposing a substantial fitness cost via the inactivation of important genes involved in the survival (non-developing offspring) or reproduction of the target population (e.g. reducing fertility of offspring, bias of the sex ratio towards males), or through the introduction of a new gene or genes that reduce(s) lifespan or bias(es) sex ratios (Galizi et al., 2014, 2016; Buchman et al., 2018b; Hammond and Galizi, 2017; James et al., 2018; Kyrou et al., 2018; Leitschuh et al., 2018). These suppression strategies are expected to result in population decline/reduction or even collapse (local elimination) over the period of a few generations, and may in some cases aim for (global) eradication of a disease vector species (HCB, 2017). In the case of disease-transmitting mosquitoes, model predictions suggest that it is unlikely that population suppression strategies would completely eliminate a species in the field (North et al., 2019). Engineered gene drives are being developed in insects to suppress populations of disease vectors, agricultural pests and invasive species.

Strategies aiming for population suppression from a single release would require the genetic modification of interest to persist, despite the fact that GDMIs are expected to decrease to low numbers as the overall target population is reduced. Alternatively, repeated releases over time would be required to reach and maintain suppression (see Section 3.2.2, below).

3.2.1.2. Population modification[28]

Population modification strategies, primarily for disease vector control, are used to modify a current genotype with one that is less able to transmit disease (impaired vector competence), or that is more

[25] Spread refers to the transmission of the engineered gene drive to other individuals within an interbreeding population through mating and inheritance, and is distinct from dispersal, which refers to movement of individuals to a different habitat.
[26] Persistence refers to the ability of the engineered gene drive to remain present in a population in the long-term.
[27] Also termed: Population reduction.
[28] Also termed: Population replacement, population alteration, population transformation or population conversion.

resistant to pathogen infection (disease refractory) (Franz et al., 2006; Mathur et al., 2010; Hegde and Hughes, 2017; Jupatanakul et al., 2017; Carballar-Lejarazú and James, 2017; Buchman et al., 2019, 2020a; Pham et al., 2019; Carballar-Lejarazú et al., 2020). These strategies can be based on the inactivation of a gene or genes that are required for the target organism to transmit the pathogen (e.g. a tendency to feed on humans in the case of mosquitoes), or that are involved in pathogen survival in the insect (see Section 3.3 for examples). They can also involve the introduction of a new gene or genes, such as those that produce molecules that block pathogen development, or that kill the pathogen in the insect (Gantz et al., 2015; Carballar-Lejarazú and James, 2017; James et al., 2018; Buchman et al., 2019, 2020a; Hoermann et al., 2020).

In order to be spread by an engineered gene drive, cargo/payload genes must be co-inherited with the gene drive, i.e. genetically linked to it. Strategies aiming for population modification require the genetic modification of interest to persist (James et al., 2018) (see Section 3.2.2, below).

3.2.2. Potential for an engineered gene drive to spread and persist in target populations

Engineered gene drives are anticipated to differ in their performance characteristics regarding their potential to spread and persist in target populations.[29] Based on these characteristics, engineered gene drives can fall into different categories:

1) Self-sustaining[30] vs self-limiting[31] systems (for the *temporal* characteristics);
2) Low[32] vs high[33] threshold systems (for the *spatial* characteristics).

While the binary divides between self-sustaining/self-limiting and low/high threshold systems are informative, it is important to take into account that there is a spectrum of spreading and persistence potential for engineered gene drives within and between each category (Alphey, 2014), which can be affected by ecological factors (Dhole et al., 2018, 2020; Backus and Delborne, 2019; see also Section 3.4, below).

3.2.2.1. Self-sustaining vs. self-limiting systems

Self-sustaining genetic control systems can be described as those in which the genetic modification is intended to become stably established in target populations. In the case of engineered gene drives, they can be designed to spread a genetic modification of interest in target populations rapidly, widely and for an indeterminate time, perhaps many generations or until the target population is eliminated (Alphey, 2014). Since self-sustaining gene drives can be engineered to be spatially and temporally unrestricted (non-localised and persistent, respectively), they could move to any interbreeding target population that has vertical gene flow with the target population where the gene drive modified individuals are released, within a relevant timeframe (Noble et al., 2018). Once established, such self-sustaining approaches are intended to be relatively stable and require only smaller and infrequent secondary releases.

Self-limiting genetic control systems can be described as those in which the genetic modification of interest is expected to be temporally limited (transient), and disappears from the target population in the absence of additional periodic releases. The number of generations over which the genetic modification of interest will remain apparent will vary according to the genetic control system employed. Conceptually, gene drives could be engineered to increase the frequency of the genetic modification of interest in a population for a limited number of generations, after which the frequency of the genetic modification of interest in the population decreases and is then lost from

[29] Alphey (2014), Backus and Delborne (2019) and Dhole et al. (2020) consider spread in terms of the potential for a genetic modification of interest to occur throughout a target interbreeding population. There are several dimensions to this: the spatial distribution of the target population, the target population densities at various locations within that space and the degree of interbreeding within the target population. Persistence relates to occurrence within a target interbreeding population over time. It implies that there is some continuous occurrence of a genetic modification of interest within a target population, but that could allow for discontinuities in specific portions of the target population, e.g. where there is temporary spatial isolation, subthreshold densities in some areas or barriers to interbreeding.

[30] Also termed: Self-propagating, non-localised or global drives.

[31] Also termed: Self-exhausting or localised drives.

[32] Also termed: Threshold independent drives.

[33] Also termed: Threshold dependent drives.

the target population.[34] Genetic modifications of interest could either be those that change harmful population characteristics or suppress population density (Gould et al., 2008; Noble et al., 2019).

3.2.2.2. Low vs. high threshold systems

Inherent in many engineered gene drive systems, as is the case with any other genetic control system, is the requirement for individuals to be released above a certain threshold frequency before they will drive the genetic modification of interest through the target population (Alphey, 2014; Leftwich et al., 2018; Backus and Delborne, 2019; Dhole et al., 2020). This threshold refers to the proportion of gene drive modified individuals with respect to the total target population that will reliably initiate spread of the genetic modification of interest. This threshold is determined as a combination of the action of the engineered gene drive system and its fitness load (Alphey, 2014; Leftwich et al., 2018).

Threshold independent gene drives may spread from very low initial population frequencies, requiring only a small number of gene drive modified individuals to be released to spread (Noble et al., 2018). Such types of engineered gene drives have a higher potential to spread into neighbouring populations for an indeterminate time (Alphey, 2014; Champer et al., 2016). The lower the threshold, the more likely that dispersal[35] of low numbers of gene drive modified individuals could be sufficient to initiate spread of the genetic modification of interest in neighbouring target populations. Threshold-dependent gene drives instead only spread if the number of gene drive modified individuals reaches a high proportion in the target population, requiring a larger introduction (or proportion) of transgenic individuals to be successful, compared to threshold-independent gene drives. These types of engineered gene drives may enable local confinement. Simple population models predict spread to high frequency in areas connected to the target area (in which the gene drive modified individuals would be released broadly) by low levels of dispersal would be inhibited, as the genetic modification of interest fails to reach the threshold frequency needed for drive (Altrock et al., 2010; Marshall and Hay, 2012a,b). However, as dispersal to neighbouring populations increases, spatial restriction to the targeted population may not be assured (e.g. Marshall and Hay, 2012b; Dhole et al., 2018, 2020; Champer et al., 2020c).

Dhole et al. (2020) noted that environment-dependent changes in fitness can cause some engineered gene drives to exhibit a threshold in some environments and not in others. This may complicate the categorisation of specific engineered gene drives as low vs. high threshold drives. However, the authors concluded that a context-specific categorisation can be useful for discussing the performance and fate of different engineered gene drives (Dhole et al., 2020).

Table 1: Possible dimensions to categorise engineered gene drive strategies

Intended outcome	**Potential for engineered gene drive to spread and persist**			
	Self-limiting		**Self-sustaining**	
	High threshold	**Low threshold**	**High threshold**	**Low threshold**
Population suppression or modification	Spatially restricted (*localised*) and temporally restricted (*transient*) drives	Spatially unrestricted (*non-localised*) and temporally restricted (*transient*) drives	Spatially restricted (*localised*) and temporally unrestricted (*persistent*) drives	Spatially and temporally unrestricted (*non-localised* and *persistent*) drives

3.3. Approaches for gene drive modified insects

While research on engineered gene drives and their applications in insects is advancing at a fast pace, it is generally accepted that it will take several years for technological developments to move to practical applications for deliberate release into the environment. Drawing inspiration from systems that exist naturally, a variety of engineered gene drives have been progressed or theorised in recent years (reviewed by Sinkins and Gould, 2006; Champer et al., 2016; NASEM, 2016; Hammond and Galizi, 2017; Macias et al., 2017; Burt and Crisanti, 2018; Marshall and Akbari, 2018; Rüdelsheim and Smets, 2018; CSS–ENSSER–VDW, 2019; Frieβ et al., 2019; Raban et al., 2020). They encompass (see Table 2, below):

[34] Assuming no residual fitness benefit.

[35] Dispersal is the ecological process of individuals moving between different habitats, but not necessarily returning to a natal habitat patch (as opposed to migration which is movement back and forth between two different habitats).

1) Self-sustaining low threshold (non-localised) gene drives
    a) Homing-based gene drives for either population suppression or modification
    b) Meiotic interference gene drives for population suppression
    c) Medea and other rescue (Medea-like) gene drives for population modification
2) Self-sustaining high threshold (localised) gene drives
    a) Underdominance gene drives for either population suppression or modification
    b) Tethered homing-based gene drives for either population suppression or modification
3) Self-limiting low threshold (non-localised) gene drives
    a) Daisy-chain gene drives for either population suppression or modification
4) Self-limiting high threshold (localised) gene drives
    a) Split homing-based gene drives for either population suppression or modification
    b) Split rescue gene drive systems for either population suppression or modification
5) Reversal gene drives

Examples of engineered gene drives are briefly described below to illustrate the different approaches followed for GDMIs and their characteristics. For some engineered gene drive systems, it must be recognised that there may be a spectrum of spread, persistence and dispersal characteristics depending on the specific design, fitness costs and context in which the drives will be used. Moreover, some types of engineered gene drives are not clearly distinct, and they can be used alone or in combination with other types of gene drives.

GDMI approaches and applications will likely continue to expand as gene editing tools become more refined (NASEM, 2016; Guichard et al., 2019; Holman, 2019). Consequently, the initial 'prototype' gene drives reported in the scientific literature may not necessarily be representative of the engineered gene drive systems that are currently under development, which aim to be more specific, stable and controllable systems (NASEM, 2016; Friedman et al., 2020; Raban et al., 2020; Hay et al., 2021). In this respect, several approaches have been proposed to restrict the spread of engineered gene drives within a single target population or geographic region, or reduce their persistence in the target population over the course of several generations (e.g. Huang et al., 2007; Gokhale et al., 2014; Burt and Deredec, 2018; Dhole et al., 2018; Leftwich et al., 2018; Marshall and Akbari, 2018; Noble et al., 2019; Champer et al., 2020c; Li et al., 2020a; Maselko et al., 2020; Terradas et al., 2020; Webster et al., 2020). Theoretically, localised gene drives are not expected to establish themselves at high frequency in neighbouring target populations when dispersal is low (Marshall and Hay, 2012a; Akbari et al., 2014; Buchman et al., 2018b; Dhole et al., 2018, 2019; Champer et al., 2020c; Sánchez et al., 2020a,b). Engineered localised gene drives may constitute a form of biological or molecular confinement that could supplement physical and ecological confinement (James et al., 2018). However, the engineering of localised or transient gene drive systems (alone or in combination with other types of engineered gene drives) is mostly at the theoretical level, though a few have been tested under laboratory settings (e.g. Akbari et al., 2013, 2014; Reeves et al., 2014; Buchman et al., 2018a; Champer et al., 2020c; Terradas et al., 2020; Webster et al., 2020). While these approaches are reasonable for population modification strategies, modelling suggests that they may be less effective to suppress local target populations (Dhole et al., 2019). Mathematical modelling also indicates that developing engineered gene drives that can achieve the balance between the ability to spread and remain locally confined is a challenge, as it can sometimes be difficult to achieve any spatial spread for threshold-dependent gene drives, especially those with high release thresholds (Dhole et al., 2020).

Conceptually, localisation may also be achieved by limiting the suppression effect of the engineered gene drive to a local subpopulation of the target species based on geographically restricted genetic polymorphisms (Sudweeks et al., 2019).[36] A CRISPR-Cas9-mediated homing-based gene drive could disrupt an allele that is fixed in the target population, but not other alleles at the same locus that are found at least at low frequencies in neighbouring populations (Sudweeks et al., 2019). This approach would be specifically appropriate for small populations on oceanic islands where genetic drift is expected to be strong (Dhole et al., 2019).

[36] Also termed: Precision drives.

Another approach currently being explored is the engineering of strains that are reproductively isolated from wild-type individuals through the insertion of reproductive barriers (e.g. Moreno, 2012; Maselko et al., 2017, 2020; Waters et al., 2018; Buchman et al., 2020b).[37] Recently, Maselko et al. (2020) demonstrated the ability to rationally engineer barriers to sexual reproduction in *D. melanogaster* strains. Moreover, Buchman et al. (2020b) engineered SPECIES (Synthetic Postzygotic barriers Exploiting CRISPR-based Incompatibilities for Engineering Species) to generate postzygotic reproductive barriers.

At present, some GMIs containing engineered gene drives are either in development or have been tested experimentally in the laboratory; however, none has been assessed in small-scale physically and/or ecologically confined field trials, or in open release trials (e.g. Rüdelsheim and Smets, 2018).[38]

Table 2: Examples of engineered gene drive approaches in insects

Intended outcome	**Potential for engineered gene drive to spread and persist in target populations[(a)]**			
	Self-limiting (*transient*)		**Self-sustaining (*persistent*)[(b)]**	
	High threshold (*localised*)	**Low threshold (*non-localised*)[(c)]**	**High threshold (*localised*)**	**Low threshold (*non-localised*)**
Population suppression	Split homing-based drives*	Daisy-chain drives*	Underdominance drives [e.g. maternal-effect lethal underdominance*; Medusa*]	Homing-based drives***
	Split rescue drives [e.g. killer and rescue*]		Tethered homing-based drives*	Meiotic interference drives [e.g. X-shredding sex-distorter***]
Population modification	Split homing-based drives [e.g. home and rescue*]	Daisy-chain drives*	Underdominance drives [e.g. reciprocal chromosome translocation***; maternal-effect lethal underdominance***]	Homing-based drives [e.g. home and rescue***]
	Split rescue drives [e.g. killer and rescue***; toxin-antidote recessive embryo*; 2-locus cleave and rescue***]		Tethered homing-based drives*	Engineered Medea and rescue (Medea-like) drives [e.g. cleave and rescue***; toxin-antidote recessive embryo***]

Development status: * Theoretical/conceptual; ** Laboratory proof-of-principle; *** Laboratory proof-of-principle with (some) multigenerational data.
(a): Depending on their design and specificities (e.g. split vs. non-split, same locus vs. distant site, DNA target sequence), and fitness costs, some engineered gene drive systems can vary in threshold, and thus fit into different categories.
(b): In the absence of mutation or heritable resistance, and assortative mating.
(c): Likely hypothetical only because temporal restriction will constrain the engineered gene drive to the vicinity of the release area.

3.3.1. Self-sustaining low threshold (non-localised) gene drives

Various engineered gene drive systems have been proposed in the scientific literature that are more likely to be non-localised and that may promote prolonged persistence of the genetic modification of interest in target populations.[39]

3.3.1.1. Homing-based gene drives

Homing-based gene drive systems are being engineered either to spread cargo/payload gene(s) in interbreeding populations, or disrupt a target gene by homing into it, which may lead to recessive lethality or sterility. Such engineered gene drives may be designed to manipulate populations by

[37] Also termed: Synthetic species, artificial hybrid incompatibility or artificial reproductive isolation.
[38] According to the WHO (2014) testing phases.
[39] In the absence of mutation or heritable resistance, and/or assortative mating.

targeting genes to reduce lifespan, bias sex ratios, impede host seeking, block pathogen development or block the ability of the modified organism to act as a vector for pathogens (Champer et al., 2016).

In recent years, CRISPR-Cas9 has revived the interest in homing-based gene drives owing to its ease of use compared with other genome-editing techniques (such as transcription activator-like nucleases [TALENs] and zinc finger nucleases [ZFNs]) and its adaptability to a wide range of organisms (Esvelt et al., 2014; Raban et al., 2020). Once a CRISPR-Cas9-mediated homing-based gene drive is engineered into the genome of an organism, the organism's offspring inherits one allele containing the gene drive element from the GM parent and one wild-type allele from its other parent. The Cas9 endonuclease cuts at the corresponding wild-type allele – its target prescribed by a guide RNA (gRNA) – producing a double-strand break (DSB; Jinek et al., 2012). This break is then repaired either through homology-directed repair (HDR), producing a second copy of the gene drive construct, or through a non-homologous repair pathway (non-homologous end joining, NHEJ, or microhomology-mediated end joining, MMEJ), which typically generates insertions or deletions (indels) at the target site (Cong et al., 2013; Mali et al., 2013). The HDR mechanism leaves the offspring with two copies of the modification. Thus, CRISPR-Cas9-mediated homing-based gene drive systems function by converting heterozygotes for the gene drive allele into homozygotes in the late germline or early embryo (Esvelt et al., 2014; Gantz and Bier, 2015, 2016; Gantz and Akbari, 2018; Scudellari, 2019). A CRISPR-Cas9 gene drive cassette typically comprises: (1) a gene encoding a gRNA that can recognise a specific target DNA sequence; (2) a *Cas9* gene encoding a Cas9 endonuclease that can cut DNA at the site specified by the gRNA; (3) sequences at the extremities that are homologous to sequences flanking the target site, so that the cassette can copy itself at the cleavage site via HDR; and (4) optional cargo/payload genes conferring trait(s) of interest.

Several proof-of-concept studies have demonstrated the feasibility of using engineered homing-based gene drive systems under laboratory settings for either population suppression or modification strategies. Substantial research investments have been made in mosquitoes for malaria control (*Anopheles stephensi* and *Anopheles gambiae*). The most advanced engineered gene drive systems for *Anopheles* vectors have been tested under laboratory settings, and prevent reproduction in *An. gambiae* [I-SceI: Windbichler et al. (2011); CRISPR-Cas9: Hammond et al. (2016) and Kyrou et al. (2018)]. A second proof of concept prevents *Plasmodium falciparum* malaria infection in *An. stephensi* [CRISPR-Cas9: Gantz et al. (2015)].

Homing-based gene drive systems for *An. gambiae* and *An. stephensi* based on CRISPR-Cas9 might become available for roll-out by 2030 (Feachem et al., 2019). However, further research is required before the engineered gene drive systems mentioned above can be tested under small-scale physically and/or ecologically confined field settings (NASEM, 2016; Scudellari, 2019). Such self-sustaining gene drives are expected to have prolonged persistence and interbreeding target populations, provided that the evolution of resistance alleles can be minimised (Hammond and Galizi, 2017; Unckless et al., 2017). Resistant mutants to the gene drive have been identified in engineered homing-based drives over short timescales (1–2 generations) (Gantz et al., 2015; Hammond et al., 2016; Champer et al., 2017; KaramiNejadRanjbar et al., 2018; Kyrou et al., 2018; Kandul et al., 2020a; Li et al., 2020a) and resistant alleles may proliferate over longer time periods and spatial scales (Raban et al., 2020). For engineered homing-based gene drives, the mechanism of resistance is determined in large part by the DNA repair pathway involved in the repair of the induced DSB (Basu et al., 2015; Hammond et al., 2016, 2017; Champer et al., 2017, 2018; Marshall et al., 2017; Noble et al., 2017; Unckless et al., 2017; KaramiNejadRanjbar et al., 2018; Kyrou et al., 2018; Oberhofer et al., 2018; Raban et al., 2020). Such engineered gene drives inherently rely on HDR pathways. However, alternative repair pathways such as NHEJ can generate insertions or deletions (indels) at the target site (Cong et al., 2013; Mali et al., 2013). If the NHEJ-generated indels affect the recognition of the genomic sequence targeted by the gene drive, then the drive may no longer cut at the site. Over time, with accumulation of NHEJ repair events that alter the gene drive cleavage sites, coupled with any fitness advantage for resistance to the drive, the population will become resistant to the drive, impeding its spread in a population (Hammond et al., 2016; Marshall et al., 2017; Raban et al., 2020). Moreover, gene drive-resistant alleles are expected to exist in wild populations simply due to standing genetic variation (Drury et al., 2017; Unckless et al., 2017).

Other research efforts have focused on developing engineered homing-based gene drive systems in the common fruit fly, *Drosophila melanogaster* [I-SceI: Chan et al. (2011, 2013a); I-OnuI: Chan et al. (2013b); CRISPR-Cas9: Gantz and Bier (2015) and López del Amo et al. (2020a); TALENs and ZFNs: Simoni et al. (2014)]. Raban et al. (2020) indicated that immediate further developments for homing-based gene drives should focus on the development of future cargo/payload genes to link to the drive

and next generation drives with reduced resistance allele generation, and the testing in large-scale confined field trials to determine drive efficacy.

Carballar-Lejarazú et al. (2020) developed and tested the engineered gene drive strain, AgNosCd-1, that could be used to deliver antiparasite cargo/effector genes for population modification in mosquitoes. The strain AgNosCd-1 carries a CRISPR-Cas9-mediated homing-based gene drive and targets the mosquito's *cardinal* gene, which encodes an enzyme involved in synthesising eye colour pigment. The authors reported that the engineered gene drive has an average efficiency of 96.7% in both sexes of *An. gambiae*, minimal off-target effects *in vitro*, few adverse effects on the fitness of gene drive modified mosquitoes and a low frequency of potentially resistant alleles that could counteract it. Single releases of AgNosCd-1 males at ratios of 1:1 transgenic:wild-type achieved full introduction (every mosquito containing at least one copy of the gene drive construct) in small caged experiments within six generations.

Kandul et al. (2020b) developed and tested a home and rescue (HomeR) gene drive system targeting an ultraconserved, haplosufficient gene required for insect viability in *D. melanogaster*. Multigenerational population cage experiments demonstrated the persistence of the genetic modification of interest in the presence of Cas9 under several release thresholds. However, many of the engineered home and rescue gene drives did not reach, nor maintain complete fixation. The design of the HomeR gene drive systems enables to limit the accumulation of resistance alleles.

3.3.1.2. Meiotic interference gene drives[40]

Meiotic interference gene drives bias the transmission of certain alleles during meiosis, resulting in increased frequencies of those alleles in the gametes, and hence in the offspring. Many types of meiotic interference gene drive systems are found in nature that function by altering the sex ratio of offspring of affected individuals (Cha et al., 2006; Champer et al., 2016; Lindholm et al., 2016; Courret et al., 2019).

X-chromosome shredding gene drives (X-shredding sex-distorter) have been proposed as possible tools to suppress insect populations by biasing the sex ratio of the wild population towards males (e.g. Windbichler et al., 2007, 2008; Deredec et al., 2008; Klein et al., 2012; Simoni et al., 2020). X-shredders would rely on the expression of a sequence-specific endonuclease during male spermatogenesis that recognises and cleaves sequences that are both specific and abundant on the X-chromosome. As a result, X-chromosome-bearing gametes would be excluded from the fertilising-sperm population, biasing offspring sex ratios towards males (Deredec et al., 2011; Alcalay et al., 2019; Simoni et al., 2020). X-shredding could be used for self-sustaining genetic control applications, in the form of Y-chromosome gene drives; by linking a functional X-shredder to the Y-chromosome, both the Y-chromosome and the X-shredder would gain a transmission advantage through preferential inheritance of male-forming gametes (Alcalay et al., 2019; Simoni et al., 2020). The first steps have been taken to engineer a Y-linked X-shredder in *An. gambiae*. Galizi et al. (2014) developed an autosomal X-shredding sex-distorter, using variants of the I-PpoI endonuclease that specifically cut a specific DNA target sequence within the 28S ribosomal DNA locus that is located exclusively on the X chromosome in *An. gambiae*. This approach enabled to shred the X-chromosome during male meiosis, resulting in a majority of the sperm in the transgenic males carrying a Y chromosome and fertile males producing > 95% male offspring. In caged experiments, multiple releases of the transgenic males led to a reduction in frequency of females and egg productivity of the population over successive generations, suppressing mosquito populations (Galizi et al., 2014; Facchinelli et al., 2019). Given that these autosomal linked X-shredders are non-driving in their current form (i.e. not linked to a Y-chromosome) and are not preferentially inherited (i.e. not able to home into targeted sequences), and do not display any fitness advantage over wild-type mosquitoes, the genetic modification of interest is expected to disappear over time when releases are discontinued (Burt and Deredec, 2018; Alcalay et al., 2019). Attempts to convert the engineered sex ratio distorters into a Y-chromosome gene drive have been unsuccessful so far (Simoni et al., 2020). Moreover, Alcalay et al. (2019) concluded that a functioning Y-linked X-shredding gene drive resulting from a naturally induced transposition or translocation of the transgene onto the Y-chromosome is unlikely, even if the I-PpoI was translocated to the Y-chromosome.

While the use of I-PpoI as an X-shredder in *An. gambiae* holds much promise, it only functions in the few organisms that have an X-chromosome with repeated I-PpoI target sequences and thus may not be portable across species (Champer et al., 2016). In the same species, Galizi et al. (2016)

[40] Also termed: Sex ratio distorters or sex-distorters.

developed a CRISPR-Cas9 sex distortion system, using a CRISPR-Cas9 nuclease that targets an X-linked rDNA sequence that is different from the previously utilised I-PpoI target site and that is conserved among the *An. gambiae* complex, yet absent from more distantly related species. This CRISPR-Cas9-mediated system achieved a male bias of between 86% and 95% (Galizi et al., 2016). Engineering X-shredders based on CRISPR-Cas9, the selection of gRNA targets and drive resistance mitigation have proven challenging, since such repeats are not accurately resolved in genome assemblies and cannot be assigned to chromosomes with confidence (Papathanos and Windbichler, 2018). Nonetheless, future efforts to exploit randomly generated Y-chromosome docking strains (Bernardini et al., 2014) or even generate new site-specific docking strains on the Y-chromosome using CRISPR-Cas9 (Buchman and Akbari, 2019) may improve functionality of Y-chromosome nucleases and thus the development of engineered X-shredders (Raban et al., 2020). Moreover, X-shredder systems may be adaptable to other organisms (Bernardini et al., 2019; Fasulo et al., 2020), though the development of such technology has been hampered by the inability to express transgenes, under the control of meiotic promoters, from the sexual chromosomes (Bernardini et al., 2019).

Recently, Simoni et al. (2020) coupled the I-PpoI-based autosomal X-shredding sex distorter to a CRISPR-Cas9-based gene drive inserted into a conserved sequence of the *doublesex* gene of *An. gambiae*, and showed that this engineered gene drive system progressively biased the sex ratio towards males in 10–14 generations in caged target populations, with no selection of resistance.

3.3.1.3. Engineered Medea and other rescue (Medea-like) gene drives

Although the molecular underpinnings of natural Medea systems remain unknown, they confer maternal-effect lethality to all offspring that fail to inherit the Medea system. Engineered Medea systems rely on the tight linkage of a maternally expressed toxin,[41] targeting essential genes and a linked zygotically or embryonically active antidote gene. Females with a Medea gene drive deposit a toxin into all their eggs that must be counteracted by an antidote that is expressed early in development, or the offspring dies (Raban et al., 2020). The developmental defect is rescued only in those embryos that inherit the Medea elements and thus carry an early embryogenesis-expressed miRNA-insensitive version of the target gene. These two components are placed adjacent to each other in the genome and can rapidly drive a linked cargo/payload gene through a population (Huang et al., 2009; Hay et al., 2010; Guevara-Souza and Vallejo, 2011; Ward et al., 2011). In successive generations, this system results in a disadvantage for wild-type alleles.

Multiple versions of the Medea inheritance pattern have been reverse engineered and shown to act as engineered gene drives in *D. melanogaster* (Chen et al., 2007; Akbari et al., 2014a) and *D. suzukii* (Buchman et al., 2018b). These engineered Medea systems in the *Drosophila* spp. utilise an RNA interference (RNAi)-based toxin-antidote combination. Modelling suggests that engineered Medea systems can spread from low frequencies (Ward et al., 2011).

Engineered Medea gene drives could serve as self-sustaining drives when released over a certain threshold (Raban et al., 2020). Compared with homing-based gene drives, the rate of resistance to evolve to the gene drive is expected to be lower with Medea systems, as offspring that do not receive the full drive are removed from the population (Raban et al., 2020). Although engineered Medea gene drives are typically designed for population modification strategies, they could possibly be used to spread conditional lethal cargo/payload genes (e.g. temperature sensitivity or sensitivity to a small molecule) and thus be used to modify then suppress populations (Akbari et al., 2014a; Raban et al., 2020). Improvements in gene drive components are needed to move this technology forward in development (Raban et al., 2020). Medea has so far used elements that are specific to Drosophila. To date, attempts to develop mosquitoes and other species with functional engineered Medea elements have been unsuccessful (Champer et al., 2016; Raban et al., 2020).

Other conceptual designs for Medea systems have been proposed: the inverse Medea system, which relies on a toxin that takes effect in the zygote unless it receives a maternally delivered antidote (Marshall and Hay, 2011); the Merea system, which functions similar to Medea, but the antidote to the maternal toxin is recessive (Marshall, 2011); and the Semele system, which uses a paternal semen-based toxin and a maternally delivered antidote (Marshall, 2011; Marshall et al., 2011).

More recently, Medea-like 'rescue' (toxin and antidote) gene drives, such as cleave and rescue (ClvR) drives (Oberhofer et al., 2019, 2020a; Adolfi et al., 2020),[42] and toxin-antidote recessive embryo (TARE) drives (Champer et al., 2020b), have been developed and demonstrated in

[41] To date, microRNAs.
[42] Also termed: *CleaveR* drives.

D. melanogaster and *An. stephensi*. Such engineered gene drive systems typically consist of a DNA sequence-modifying enzyme such as Cas9/gRNAs that disrupts endogenous versions of an essential gene (cleaver/toxin) and a recoded version of that gene resistant to cleavage (rescue/antidote). These systems positively bias their transmission, and that of linked cargo/payload genes, as they are lethal to offspring that do not inherit the rescue/antidote.

Oberhofer et al. (2019) developed an engineered cleave and rescue gene drive system for population modification in *D. melanogaster*, and demonstrated through modelling that the system can spread to fixation under diverse conditions (Oberhofer et al., 2019, 2020a). This system is independent of HDR copying of the gene drive, and thus less subject to NHEJ-associated drive resistance (Adolfi et al., 2020; Raban et al., 2020). Adolfi et al. (2020) engineered a cleave and rescue gene drive system for population modification in *An. stephensi*, that relieves fitness costs in females caused by integration of the drive into the *kynurenine hydroxylase* (*kh*) gene by rescuing its function (see also Pham et al., 2019). The recoded *kh* sequence carried by the engineered gene drive construct supports normal survival and reproductive capacity in females. Females failing to inherit the recoded *kh* construct from their mothers and carrying non-functional mutated copies of *kh* are eliminated from the populations. In caged experiments, single releases of gene drive modified males resulted in population modification with $\geq$ 95% of mosquitoes carrying the drive within 5–11 generations over a range of initial release ratios. As is the case for engineered Medea gene drives, cleave and rescue ones could possibly be used to spread conditional lethal cargo/payload genes to suppress populations (Raban et al., 2020).

Champer et al. (2020b) developed a TARE gene drive, which limits resistance by targeting a recessive lethal gene, while providing a recoded sequence to rescue only drive-carrying individuals. This CRISPR-Cas9-based gene drive converts wild-type targ et al leles to disrupted alleles, at which point they would be removed from the population in embryos where no drive or wild-type allele is present to provide rescue. The TARE gene drive described by Champer et al. (2020b) has been shown to spread to fixation when gene drive modified individuals are introduced into a caged population at a frequency of 24% or above.

3.3.2. Self-sustaining high threshold (localised) gene drives

Several engineered gene drive systems have been proposed in the scientific literature that are intended to persist locally, but may have limited ability to spread the genetic modification of interest beyond the target population into which they were initially introduced.

3.3.2.1. Underdominance gene drives[43]

Underdominance occurs when heterozygotes (or their offspring) have a lower fitness than parental homozygotes and thus are selected against within a population. Engineered underdominance gene drive systems are threshold dependent, requiring a high introduction threshold to spread through a target population. Modelling suggests that such gene drives are likely to be spatially restricted and reversible (Davis et al., 2001; Altrock et al., 2010, 2011; Marshall and Hay, 2012a,b; Alphey, 2016; Champer et al., 2016, 2020c; Edgington and Alphey, 2017, 2018; Dhole et al., 2018; Leftwich et al., 2018; Sánchez et al., 2020a,b). Underdominance can be achieved using reciprocal chromosomal translocations, or toxin and antidote mechanisms (known as maternal-effect lethal underdominance) (Burt and Crisanti, 2018).

Reciprocal chromosomal translocations

Translocation gene drives generate a reciprocal chromosomal rearrangement or inversion that gives a disadvantage to individuals heterozygous for the drive. When mated to wild-type individuals, translocation heterozygotes produce a large proportion of inviable offspring, as 50% of the offspring do not inherit a balanced set of chromosomes. Homozygotes for the translocation contain a balanced set of chromosomes, so their offspring are viable and have higher fitness than the heterozygotes (Gantz and Akbari, 2018; Raban et al., 2020). The fitness advantage of homozygotes allows the translocation to spread into the population along with any cargo/payload gene linked to translocation breakpoints.

Translocation gene drives are threshold dependent; they need to be introduced into a new population at a frequency exceeding its threshold to be maintained in that population; otherwise, they are actively driven out (Marshall and Akbari, 2018). Their introduction is also reversible, as releasing

[43] Also known as heterozygote inferiority.

large numbers of wild-type individuals can push the gene drive below its threshold where over time it will become extinct in the population (Raban et al., 2020).

Buchman et al. (2018a) created an engineered reciprocal chromosome translocations gene drive in *D. melanogaster*, using HEGs that carried a cargo/payload gene, and tested them under laboratory settings. The strains showed frequency-dependent spread in laboratory populations. The spread of such drives can be hindered by fitness costs and resistance due to naturally occurring genetic variation (Buchman et al., 2018a). According to Raban et al. (2020), immediate needs for further development should focus on developing translocation gene drives in a disease vector/pest species and demonstrating their efficacy in those species.

Maternal-effect lethal underdominance[44]

Maternal-effect lethal underdominance gene drives rely on a dual toxin and dual antidote system. The genetics of this system result in heterozygous females generating mostly inviable offspring, while homozygous females are fully fertile and viable. This disparity in fitness can be exploited to drive cargo/payload genes into populations (Davis et al., 2001; Wimmer, 2013; Raban et al., 2020).

Strategies to engineer underdominant gene drives using combinations of toxins and antidotes have been proposed (Gould and Schliekelman, 2004) and implemented in *D. melanogaster*, as a proof-of-concept system (Akbari et al., 2013; Reeves et al., 2014) with some multigenerational data gathered under laboratory settings (Akbari et al., 2013). Akbari et al. (2013, 2014) used two constructs, each consisting of a maternally expressed toxin (multimers of miRNAs that act to suppress the corresponding gene via a mechanism of RNAi in the embryo) and a zygotic antidote gene, which is capable of neutralising the maternal toxin expressed by the other toxin and antidote construct (resistant mRNAs). Another design in *D. melanogaster* introduced gene constructs on different chromosomes (Reeves et al., 2014). Both approaches were successfully tested under laboratory settings, but underdominance gene drives remain to be developed in pest species and their efficacy is to be demonstrated in those species (Raban et al., 2020).

Marshall and Hay (2012a, 2014) have also proposed additional variants utilising toxin and antidote combinations, including the Medusa system[45] that could suppress populations by using a pair of sex-linked toxins and antidotes (Marshall and Hay, 2014).

3.3.2.2. Tethered homing-based gene drives

Dhole et al. (2019) proposed the concept of tethered homing-based gene drives, which include a split homing component that does not drive on its own, but that would be 'tethered' or anchored to a localised gene drive (see Leftwich et al., 2018). The localisation level of a tethered gene drive would be highly dependent on the drive used as the anchor. These systems may have the potential to be used for population suppression or modification strategies.

3.3.3. Self-limiting low threshold (non-localised) gene drives

Self-limiting non-localised gene drives contain a transient drive mechanism that can spread the genetic modification of interest within the target population as long as the drive persists.

3.3.3.1. Daisy-chain gene drives

Daisy-chain gene drives are theoretical systems envisioned to consist of multiple unlinked transgenic components in which each exhibits drive only in the presence of the previous component in the sequence (Noble et al., 2019).[46] When configured appropriately, the system is predicted to exhibit drive for a temporary period, after which the system decays due to loss of the earlier components in the sequence. Limitation of the persistence of the complete system is expected to result in some degree of spatial restriction, and therefore, they have sometimes been characterised as localised

[44] Also termed: Underdominance drives based on double Medea or UD^{MEL}.

[45] Also known as sex chromosome-associated Medea underdominance.

[46] In daisy-chain gene drives, the CRISPR components would be split up in a way that none of them can be effective on its own, and they are distributed throughout the genome. The components would be functionally arranged in a linear daisy-chain consisting of several components, and act similar to the booster stages of a rocket: components at the base promote the drive of the next component, which promotes the drive of the next higher component. Since the components cannot promote their own drive and probably carry some cargo/payload, they are expected to be successively lost again. Therefore, after a certain amount of time, the gene drive is expected to stop operating, and the drive components may be lost from the population. The spread of the cargo/payload gene(s) will depend both on the release ratio and the number of links to the daisy chain.

(Raban et al., 2020). The extent to which daisy-chain gene drives are non-localised is predicted to depend on both the potential for migration exchange with neighbouring populations of the target species (dispersal) and the fitness cost associated with the transgenic construct (Dhole et al., 2018). These theoretical systems have the potential to be used for replacement or suppression. To date, daisy-chain gene drives remain conceptual and need to be developed and tested in an insect model organism to demonstrate their potential efficacy (Raban et al., 2020).

3.3.4. Self-limiting high threshold (localised) gene drives

Self-limiting localised gene drives contain a transient drive mechanism that can spread the genetic modification of interest within the target population as long as the drive persists, but may have limited ability to spread the genetic modification of interest beyond the target population into which they were initially introduced. A self-limiting gene drive that persists through many generations could spread the genetic modification of interest substantially throughout the local target population.

3.3.4.1. Split homing-based gene drives

Engineered split homing-based gene drives separate the homing endonuclease and gRNA components into separate lines, rendering the cleavage and homing inactive until the lines are genetically crossed (Raban et al., 2020). Such gene drives have been recently developed in *D. melanogaster* and have shown comparable gene conversion efficiencies to a standard homing-based gene drive (Champer et al., 2019a; Kandul et al., 2020a; López del Amo et al., 2020a; Terradas et al., 2020).

Li et al. (2020a) have developed a split homing-based gene drive in *Ae. aegypti* for population modification that could enable local restriction of the drive and persist in target populations for several years but not indefinitely. Owing to fitness costs associated with the gene drive and cargo/payload genes, they could be eliminated from the target population on a relevant timescale according to modelling predictions. In this engineered gene drive system, resistance allele formation was also seen. Modelling shows that to become established in an idealised case, the split homing-based gene drive described by Li et al. (2020a) would require multiple releases each equivalent to the total size of the population being modified (50% frequency).

3.3.4.2. Split rescue gene drive systems

Localised split rescue gene drive systems, such as killer and rescue drives, have been developed and tested in *D. melanogaster* (Gould et al., 2008; Oberhofer et al., 2020b; Webster et al., 2020). Such engineered gene drive systems use independent toxin (killer) and rescue/antidote genes that are at different genomic positions (split system) to spread cargo/payload genes associated with the rescue/antidote. They drive through the suppression of the organisms that do not inherit the rescue/antidote and linked cargo/payload gene. In each generation, only insects that inherit the rescue/antidote will survive and all other offspring that inherit only the toxin (killer) die. So after a number of generations, while the killer gene is present at a significant frequency, it will drive the rescue/antidote gene through the population, killing any offspring that do not inherit the rescue/antidote gene and associated cargo/payload gene(s). It is predicted that both killer and rescue/antidote genes will be lost over time, if either: (1) there are any fitness costs associated with the rescue/antidote; or (2) the release ratio is low (Webster et al., 2020).

Webster et al. (2020) engineered and tested killer and rescue systems in *D. melanogaster* for locally restricted gene drive strategies. The authors reported that the engineered killer and rescue gene drives spread to fixation when gene drive modified individuals are introduced into a caged population at a 2:1 ratio of engineered to wild-type individuals, respectively. This particular killer and rescue gene drive system may be transferable to a number of insect pests such as mosquito disease vectors, and may also be effective for population suppression according to modelling predictions.

Modelling and multigenerational experiments suggests that the 2-locus cleave and rescue gene drive developed and implemented by Oberhofer et al. (2020b) may operate as a self-limiting system in *Drosophila*. Likewise, the threshold-dependent dynamics exhibited by split TARE and split home and rescue gene drives may enable such drives to be spatially restricted to certain regions, as the establishment in neighbouring populations through a small number of migrating individuals would be prevented, theoretically. However, if fitness costs are caused by the cargo/payload gene, these engineered gene drive may become less confined if it loses the cargo/payload (potentially resulting in

the spread of the drive to a larger area than the cargo/payload) (Champer et al., 2020a,b; Kandul et al., 2020b).

3.3.5. Reversal gene drives

Conceptually, reversal gene drives have been proposed as genetic remediation or neutralising systems that could remove previously introduced GDMOs in the event of unintended consequences. They could be designed to mitigate potential unintended consequences of another engineered gene drive by removing or preventing the spread of the original organism. The development of reversal gene drives is proceeding in flies and mosquitoes, and their potential use in the environment is being explored with population genetic models (Gantz and Bier, 2016; Vella et al., 2017; Friedman et al., 2020; Xu et al., 2020). However, it has been noted that reversal gene drives may induce further changes that may undo a phenotypic alteration caused by the initial drive, so they may not restore the original modification to the wild type or redress fully ecological effects from the original engineered gene drive (e.g. Champer et al., 2016; NASEM, 2016; Vella et al., 2017; CSS–ENSSER–VDW, 2019; Rode et al., 2020; Xu et al., 2020).

Systems have also been designed to either turn on or turn off gene drive activity in the presence or absence of small organic molecules that can easily enter cells (Heffel and Finnigan, 2019; López del Amo et al., 2020b). The concept is that the engineered gene drive would be activated only in the presence of a very specific small molecule so that the GDMI could not spread without the presence of that chemical. Alternatively, the GDMI could be designed so that gene drive activity is terminated when a specific small molecule is present. While this may increase the safety of open release trials of non-localised GDMIs, it is not clear how and when the small organic molecules should be delivered and at which doses outside confined settings. Proof of concepts have been demonstrated, and development is proceeding in flies and mosquitoes (Friedman et al., 2020).

Neutralising genetic elements such as e-CHACR (erasing Constructs Hitchhiking on the Autocatalytic Chain Reaction) and ERACR (Element Reversing the Autocatalytic Chain Reaction) have been proposed (Gantz and Bier, 2016), and subsequently developed and tested in *D. melanogaster* to halt the spread of an engineered gene drive by inactivating Cas9 carried by an engineered gene drive, or delete/ eliminate and replace a drive, respectively (Xu et al., 2020). While Xu et al. (2020) provide encouraging evidence for neutralising an engineered gene drive with e-CHACRs or ERACRs, the authors caution for unexpected outcomes. Using cleave and rescue gene drive systems, Oberhofer et al. (2020a) showed that engineered gene drive-mediated population modification in *Drosophila* can be overwritten with new content while eliminating old elements of an initial drive that has failed or lost efficacy.

3.4. Ecological factors affecting engineered gene drive spread and persistence in the field

The potential of engineered gene drives to spread and persist in the field will be affected by ecological factors such as the genetic diversity of target populations, density-dependent population dynamics, dispersal to neighbouring populations, intraspecific competition,[47] spatial heterogeneity, mating behaviour and sexual selection and heterogeneity of receiving environments (e.g. Yakob et al., 2009; Bonsall et al., 2010; North et al., 2013, 2019, 2020; Beaghton et al., 2016; NASEM, 2016; Godfray et al., 2017; Dhole et al., 2018, 2020; Edgington and Alphey, 2018; Backus and Delborne, 2019; Paton and Bonsall, 2019). It is therefore important that such factors are taken into account when predicting the performance and fate of GDMIs in the field (reviewed by Dhole et al., 2020). Moreover, this knowledge may also enable an understanding of the potential risks associated with unintended spread (NASEM, 2016).

Experiments carried out inside small-scale physically and/or ecologically confined field trials or semi-field testing may not reflect future field performance accurately. Moreover, long-standing laboratory colonies may not replicate the behaviour of populations as would be seen in the field (Boëte, 2009; Baeshen et al., 2014; Ross et al., 2019). While the spread and persistence of engineered gene drives have been typically described with simple deterministic models, their spread and persistence in target populations in the field are more complex, requiring more robust models. Improved modelling

[47] Intraspecific competition is the ecological process that determines how individuals within a species compete for limited resources. Interspecific competition is where two species which potentially share the same ecological niche compete for limiting resources.

capabilities and more empirical evidence are critical to support more realistic risk assessments, as they would enhance the ability to understand the sensitivity of the spread and persistence of engineered gene drives to important ecological factors and their associated uncertainties, and predict how gene drives might spread through target populations in the field (NASEM, 2016; Dhole et al., 2020).

Four critical ecological processes (i.e. seasonality, intraspecific competition, spatial heterogeneity and dispersal), which are briefly described in the following sections, shape the spread and persistence of engineered gene drives in the field.

3.4.1. Seasonality

Seasonality is a critical ecological factor that can affect the spread and persistence of engineered gene drives in target populations. For instance, for mosquitoes, the necessary requirement for aquatic habitats for larval development and the seasonal availability of water has important consequences for mosquito abundance and dynamics.

3.4.2. Intraspecific competition

Intraspecific competition is the ecological process that determines how individuals within a species compete for limited resources. Precise details on the magnitude of this competition is often lacking for many ecological systems. However, from different mathematical modelling approaches for different vector species, intraspecific competition is known to be a critical process for the efficacy of any integrated disease vector/pest control programme (Rogers and Randolph, 1984; Yakob et al., 2008a,b; Alphey and Bonsall, 2014). The timing of the genetic control with respect to the intraspecific competition can influence the outcome. For example, some control interventions, such as SIT, act early in the life cycle of an insect by disrupting egg production. If this mortality affects and weakens the strength of intraspecific competition, then it has consequences for the efficacy of genetics-based control.

3.4.3. Spatial heterogeneity

Spatial heterogeneity is the variation in the habitat that affects the demographic characteristics (e.g. birth, death, dispersal) and dynamics of species. It can be defined at a landscape or population level. Spatial heterogeneity introduces unevenness into landscapes and is an essential ecological criterion in understanding the spatial and temporal spread of engineered gene drives. Without consideration of spatial heterogeneity at different scales of organisation, it is impossible to define the spread and persistence of engineered gene drives in target populations in the field. For example, Tanaka et al. (2017), using a mathematical framework, investigated the spread of engineered gene drives under 'pushed-wave' (where the genetic spread proceeds from accentuated growth from populations somewhat behind the wave front that spills over the leading edge) compared to 'pulled-waves' (driven by growth and dispersal at the leading edge of the wave) scenarios. Understanding variation in space (the spatial heterogeneity) in demographic parameters (such as the selective disadvantage of the engineered gene drive compared to the wild type) will determine the ecological capacity of the genetic modification of interest to spread and persist. Understanding the characteristics of the engineered gene drive (its propensity to spread and persist) and suitability features of the landscape (e.g. the heterogeneity) will be important to characterise its spread and persistence potential.

3.4.4. Dispersal

The most critical ecological process in the spread and persistence of engineered gene drives in target populations in the field is dispersal. Dispersal is the ecological process of individuals moving between different habitats, but not necessarily returning to a natal patch (as opposed to migration which is movement back and forth between two different habitats). Dispersal will affect the outcome of engineered gene drives at different spatial scales. Within patches (which also need careful investigation), dispersal is critical to vector redistributions and dynamics. For instance, Manoranjan and van den Driessche (1986) modelled the efficacy of vector control under a self-limiting SIT control. They concluded that the number of mosquitoes required to eradicate a population was dependent on: (1) mosquito demography of births, deaths and movement; (2) the dimensions of the spatial; and most critically (3) the initial spatial population distribution. More recently, Ferreira et al. (2008) showed that in spatially heterogeneous environments, vector elimination under self-limiting control is difficult to achieve and can depend on the optimal timing of the genetic-based control (Yakob et al., 2008a,b).

At broader spatial scales, dispersal heterogeneity in relation to key environmental features (such as breeding sites) affects heterogeneity in the environmental impact effects (e.g. vectorial capacity). At these spatial scales, difference in species-specific dispersal is critical to the performance characteristics of an engineered gene drive. For example, *Aedes* are typically short-dispersing species (with a large proportion of species not necessarily moving large distances from the natal sites) (e.g. Harrington et al., 2005; Hemme et al., 2010). In contrast, *Anopheles* disperse much more widely (e.g. Thomson et al., 1995; Taylor et al., 2001; Dao et al., 2014; Huestis et al., 2019). For slowly dispersing species (like Aedes), local elimination and/or eradication may be achievable, but this may not be the case for fast dispersing species where repopulation of wild-type vectors may be strong. Spatial control depends critically on the combination of the genetics of control and the ecological aspects of dispersal.

At landscape scales, non-random distribution of insects can limit the performance of insect disease vector/pest control programmes (Yakob et al., 2008a,b; North et al., 2013, 2019, 2020) as higher density patches may not receive the critical threshold of genetically modified individuals necessary for control to be successful (Barclay, 1982). Connectivity networks and landscape structures and the coverage proportion (propensity of released modified insects to inhabit patches occupied by wild-type vectors) is crucial to vector outcomes. If patches are highly clustered, isolated patches or pockets of disease vector/pest insect persistence are likely to occur as they have reduced probability of colonisation and hence control (Yakob et al., 2008b).

3.5. Current and emerging genetic disease vector/pest control strategies

Engineered gene drives may complement and expand the range of current and emerging genetic disease vector/pest control methods. These methods are briefly described below to illustrate different approaches, and their spread and persistence characteristics.

3.5.1. Release of sterilised insects

SIT aims to suppress, eradicate or prevent establishment of target pest populations by the successive releases of large numbers of sterile insects of the same species, primarily males sterilised using ionising radiation, chemosterilants or genetic modification, over a defined area and time (Dyck et al., 2005; Alphey, 2014). The sterile insects are released in numbers in excess of the wild-type insects in the area. This ensures that the majority of mating by wild females is with released sterile males, producing non-viable offspring. If sufficient sterile males are released for a sufficient time, the target population in the release area will be suppressed and potentially eliminated. In the case of preventative SIT, sterile males are released in anticipation of a potential outbreak of an exotic pest, mating with any females of the target pest that may enter the area (Hendrichs et al., 2005). Effectiveness of SIT is associated with the fitness of the sterilised males as related to their dispersal ability, longevity and ability to compete with wild-type males for mating wild-type females (Dyck et al., 2005; Romeis et al., 2020). SIT is self-limiting, as the sterile factor disappears from the target population with each release generation (radiation or chemosterilisation) or its immediate progeny (inherited lethality), and is maintained only by periodic release of additional sterile males (Alphey, 2014).

SIT has enabled the suppression of populations of several insect pests of agricultural and veterinary importance such as the new world screwworm (*Cochliomyia hominivorax*) from North and Central America, the Mediterranean fruit fly (*Ceratitis capitata*) from various locations in the Americas and tsetse flies (*Glossina morsitans*) in Africa (Enkerlin et al., 2015; Vreysen et al., 2000; Wyss, 2000; Dyck et al., 2005; Dicko et al., 2014; Scott et al., 2017; Ciss et al., 2019). Some experience with SIT has also been gained in the EU, including outermost regions (WHO and IAEA, 2020). These examples include both native and exotic pests in different locations. SIT has been used against mosquitoes, with substantial progress on methods in recent years (Dame et al., 2009; Alphey, 2014; Soma et al., 2017; Bouyer and Vreysen, 2020; Bouyer et al., 2020). Significant advances have been made with the development of genetic sexing strains, mass-rearing, sex separation, handling, radiation, quality control and release technologies (Bourtzis et al., 2016). This enables production of highly competitive sterile males. Several countries are testing SIT against mosquitoes (mostly against *Aedes* species) alone (see Section 3.5.1) or in combination with *Wolbachia*-mediated IIT (see Section 3.5.3), which allows reducing the irradiation dose (Zhang et al., 2015). SIT is also being studied using genetic modification to produce males in mass rearing followed by radiation-induced sterilisation prior to

release for new world screwworm (Concha et al., 2016). SIT is most effective at low pest densities, so it is often employed in combination with other pest management measures that reduce target pest populations prior to release of sterile insects (Dyck et al., 2005).

An open release trial with genetically modified sterile male mosquitoes (*An. coluzzii*) carrying a non-driving I-PpoI construct designed to cause dominant male sterility was conducted in Bana (Burkina Faso) in 2019 (see also Simoni et al., 2020). The field trial consisted of a 'mark-release-recapture' experiment to test dispersal.[48] At the time of writing, no open release trials with GMIs have been performed in the EU.

3.5.2. Release of genetically modified insects with a dominant (female) lethal transgene

The release of male insects carrying a dominant lethal gene (RIDL) or a dominant female lethal gene (fsRIDL) is a development of SIT that involves the release of GMIs homozygous for a dominant (female) lethal transgene, instead of being irradiated (Thomas et al., 2000; Alphey, 2014; Alphey and Alphey, 2014). These genetic systems use a lethal (female) gene to suppress insect pest populations (Thomas et al., 2000). RIDL largely results in non-viable offspring (Evans et al., 2019), thereby decreasing the reproductive potential of the wild-type population (Phuc et al., 2007; Alphey et al., 2010; Benedict et al., 2010; Beech et al., 2012; Slade and Morrison, 2014). If sufficient numbers of wild-type females mate with RIDL males over time, then the population can collapse. By contrast, fsRIDL only leads to female-specific lethality through the release of fertile males that are daughterless. Releasing males that have viable and fertile sons enables the genetic modification of interest to be temporarily maintained in the population for a few subsequent generations. After deliberate releases cease, the genetic modification of interest declines to extinction, decreasing each generation by half (Harvey-Samuel et al., 2015).

Both RIDL and fsRIDL are self-limiting as the genetic modification of interest is expected to be lost from the target population over time due to negative fitness effects (i.e. dominant (female) lethality), unless periodically replenished (Alphey, 2014). For example, Garziera et al. (2017) observed that the genetic modification of interest was lost from the target population within 5 months upon cessation of the deliberate releases of the RIDL GM mosquito *Ae. aegypti* in Brazil. RIDL and fsRIDL typically require inundative releases of large numbers of individuals (Beech et al., 2009, 2012; Mumford, 2012; Reeves and Phillipson, 2017).

RIDL has been tested since 2009 in open release trials with the GM mosquito *Ae. aegypti* (strain OX513A) to suppress wild-type populations in Brazil, Cayman islands, Malaysia and Panama (Alphey and Beech,2012; Harris et al., 2012; Lacroix et al., 2012; Neira et al., 2014; Carvalho et al., 2015; Gorman et al., 2015; GeneWatch-TWN-ACB, 2019; Brooks, 2020; Williams et al., 2020), while activities have been planned in Florida (USA) and India (Slade and Morrison, 2014; Romeis et al., 2020). These studies confirmed sustained suppression of treated target populations, disappearance of the genetic modification of interest soon after releases stopped, survival of a small proportion (3–5%) of the RIDL mosquitoes, no increased hybrid vigour, no increased capacity to transmit disease, no increased resistance to commonly used insecticides and slow rebound in local target populations after the releases of the RIDL mosquitoes stopped.[49]

Field cage studies were performed with the fsRIDL GM mosquito *Ae. aegypti* (strain OX3604C) (Facchinelli et al., 2013). In 2018, open release trials with the fsRIDL GM mosquito *Ae. aegypti* (strain OX5034) were started in Brazil (GeneWatch-TWN-ACB, 2019) to test a number of performance features of the mosquitoes. In these open release trials with the fsRIDL GM mosquitoes, up to 96% suppression of the target populations was reached.[50] Further activities have been planned in Brazil,[51] and the USA (Florida and Texas).[52]

[48] https://targetmalaria.org/target-malaria-proceeded-with-a-small-scale-release-of-genetically-modified-sterile-male-mosquitoes-in-bana-a-village-in-burkina-faso/

[49] https://www.oxitec.com/en/news/oxitec-response-scientific-reports-article

[50] https://www.oxitec.com/en/news/oxitec-successfully-completes-first-field-deployment-of-2nd-generation-friendly-aedes-aegypti-technology

[51] https://www.oxitec.com/en/news/oxitecs-new-friendly-aedes-aegypti-mosquito-technology-receives-full-biosafety-approval-in-brazil

[52] https://www.oxitec.com/en/news/oxitecs-epa-approval-published-in-the-federal-register-permit-allows-friendly-mosquito-pilot-projects-in-us and https://www.oxitec.com/en/news/oxitecs-friendly-mosquito-technology-receives-us-epa-approval-for-pilot-projects-in-us

fsRIDL is under development/test to suppress wild-type *Ae. aegypti*, *Ae. albopictus*, *Anopheles albimanus* and *An. stephensi* (Fu et al., 2010; Wise de Valdez et al., 2011; Labbé et al., 2012; Slade and Morrison, 2014) and agricultural pests such as the diamondback moth (*Plutella xylostella*; strain OX4319L; Harvey-Samuel et al., 2015; Bolton et al., 2019), fall armyworm (*Spodoptera frugiperda*; strain OX4319; Jin et al., 2013), pink bollworm (*Pectinophora gossypiella*; strains OX3402C, OX4135 and OX4319; Simmons et al., 2011; Morrison et al., 2012; Jin et al., 2013), Mediterranean fruit fly (*C. capitata*; strain OX3864A; Leftwich et al., 2014; Asadi et al., 2019) and olive fly (*Bactrocera oleae*; strain OX3097D; Ant et al., 2012; Turner et al., 2018). These strains also express the fluorescent protein marker, DsRed, to permit the effective monitoring of the presence of such strains in the field. Recently, a series of open release trials took place in Geneva (NY, USA) with adult male fsRIDL GM diamondback moths (strain OX4319L) and wild-type counterparts to test dispersal, persistence and field survival of the local diamondback moth population in a cabbage field (Shelton et al., 2020). Further open release trials are recommended by Shelton et al. (2020) to assess suppression efficacy. Previous glasshouse experiments demonstrated the effectiveness of this approach (Harvey-Samuel et al., 2015).

3.5.3. Release of *Wolbachia*-infected individuals

Wolbachia are intracellular, maternally inherited endosymbionts that manipulate the reproduction of their host in various ways to favour their own maternal transmission (e.g. male killing, feminisation, parthenogenesis induction and cytoplasmic incompatibility (CI); reviewed by Iturbe-Ormaetxe et al., 2011; Nikolouli et al., 2018). This can result in an increase of the frequency of infected females in the host population, either by inducing a female-biased sex ratio in the offspring of infected females or by reducing viable egg production in uninfected females. *Wolbachia* occur naturally in many insects, and have been introduced experimentally into others. The possibility of transferring *Wolbachia* mechanically into novel hosts (transinfection) to create associations not restricted by mating barriers has significantly increased the possibilities to deploy *Wolbachia*-mediated control (Hughes and Rasgon, 2014).

Wolbachia have been used to: (1) suppress disease vector populations through the release of *Wolbachia*-infected males that are incompatible with the wild-type (uninfected) females (also known as self-limiting, sterile-male incompatible insect technique [IIT]; Turelli and Hoffmann, 1991; Sinkins et al., 1995; O'Connor et al., 2012; Alphey et al., 2013; Mains et al., 2016; Flores and O'Neill, 2018; Zheng et al., 2019; Crawford et al., 2020); and (2) modify a population of the target species with *Wolbachia*-infected disease-refractory individuals (females and males) (also known as self-sustaining pathogen interference [PI]; Hoffmann et al., 2011, 2014; Bourtzis et al., 2014; Shaw et al., 2016; Flores and O'Neill, 2018; Callaway, 2019, 2020; Servick, 2019). Artificially acquired strains of *Wolbachia* have been shown to be effective in suppressing populations of different species of mosquitoes, or replacing them with disease-refractory strains, when tested under small-scale physically and/or ecologically confined field settings, and/or in open release trials (e.g. De Barro et al., 2011; Hoffmann et al., 2011; Walker et al., 2011a; O'Connor et al., 2012; Atyame et al., 2015; Mains et al., 2016; HCB, 2017; Schmidt et al., 2017; Waltz, 2017; Flores and O'Neill, 2018; Nazni et al., 2019; O'Neill et al., 2019; Zheng et al., 2019; Ryan et al., 2020; Williams et al., 2020). Moreover, successful population suppression has been observed in physically confined laboratory experiments with *Wolbachia*-infected strains of the Mediterranean fruit fly *C. capitata* (Zabalou et al., 2004, 2013), and the transmission of the bacterial endosymbiont has been studied in ants (Pontieri et al., 2017). In the USA, an IIT product (ZAP Males®) to control *Ae. albopictus* using the *w*PIP strain of *Wolbachia* has received regulatory approval (Waltz, 2017).

3.5.3.1. *Wolbachia*-mediated incompatible insect technique

IIT population suppression strategies are based on *Wolbachia* infections that cause CI if the target population is uninfected (unidirectional CI, when only one *Wolbachia* strain is involved). CI commonly leads to embryonic lethality in crosses between infected males with uninfected females, all other crosses being fertile. In bi-directional CI (when two *Wolbachia* strains are involved), crosses between individuals infected with different (incompatible) strains are sterile. In this case, only matings between females and males carrying the same *Wolbachia* strain will result in offspring (Alphey, 2014; Bourtzis et al., 2014).

In this strategy, large numbers of infected males are repeatedly introduced into a target population (Armbruster, 2019; Buchman et al., 2019), because the introduced *Wolbachia* type does not establish

within the target population. Due to the similarity with the classical SIT, the self-limiting CI strategy is often referred to as IIT. *Wolbachia*-based sterilisation has little or no effect on male mating competitiveness or survival (Chambers et al., 2011; Zhang et al., 2015; Atyame et al., 2016).

The accidental release of females infected with the same *Wolbachia* strain as the released males may neutralise the sterility effect and thus undermine the strategy, because *Wolbachia* would tend to spread through the target population (O'Connor et al., 2012; Alphey, 2014). Developments are ongoing to combine *Wolbachia*-mediated IIT and SIT, so that any residual females that are not separated from the released males are sterilised using low dose irradiation (Zheng et al., 2019).

3.5.3.2. *Wolbachia*-mediated pathogen interference

PI population modification strategies are based on the release of *Wolbachia*-infected females, as well as males, that spread specific *Wolbachia* strains into target populations that themselves reduce vector competence (Walker et al., 2011). Examples for such strains are *w*Mel from *D. melanogaster* and *w*MelPop (a pathogenic mutant of *w*Mel). However, not all *Wolbachia* strains induce significant disease-refractoriness, as many disease vector species already carry one or more *Wolbachia* strains (Alphey, 2014; HCB, 2017). While this refractoriness can suppress a wide range of pathogens (Moreira et al., 2009; Blagrove et al., 2012), it can also potentially increase susceptibility to others (Hughes et al., 2012).

Since infected females can mate successfully with infected and uninfected males, they have a reproductive advantage. Consequently, the *Wolbachia* infection is likely to spread through the target population (Alphey, 2014). The molecular basis of *Wolbachia*-mediated pathogen-blocking is not well understood (Marshall et al., 2019). Nonetheless, such *Wolbachia* strains might operate as a cytoplasmically inherited gene drive like system for population modification in mosquitoes (Hoffmann et al., 2011, 2014, 2015; Walker et al., 2011a; Schmidt et al., 2017; O'Neill, 2018; Nazni et al., 2019; O'Neill et al., 2019). Above a critical threshold, the introduced infection can spread and persist. In this scenario, the *Wolbachia* infection, directly or indirectly, reduces pathogen transmission, and the outcome is a disease vector population less able to cause disease. *Wolbachia* have not proved amenable to transformation (Champer et al., 2016; Macias et al., 2017). However, the use of new genome editing tools in a growing number of species may change this, potentially enabling the development of improved strains of *Wolbachia* with enhanced disease-refractory properties and a reduced fitness impact on their host, allowing them to propagate more rapidly throughout target populations (Champer et al., 2016).

3.5.4. Integrated disease vector/pest control

Currently applied genetic methods for insect disease vector/pest control (SIT, RIDL, fsRIDL, *Wolbachia*-mediated IIT) are mostly self-limiting, and they are used to suppress target populations, except *Wolbachia*-mediated PI that is self-sustaining and used for population modification (Table 3). To suppress target populations effectively, these self-limiting approaches require inundative releases of large numbers of individuals, as the genetic modification of interest is not intended to persist in target populations in the absence of continued releases; the genetic modification of interest will not pass to progeny or if it does, it will only be inherited for a very limited number of generations. Consequently, their effect will be transient and limited to areas of release.

Depending on the engineered gene drive, theoretically, the genetic modification of interest could spread through target populations (non-localised) and persist indefinitely (self-sustaining), or be restricted in its spread (localised) or persistence (self-limiting) (see Sections 3.2 and 3.3). Thus, engineered gene drives may complement and expand the range of genetic methods for disease vector/pest control. Theoretically, they may be used as part of an integrated approach in conjunction with other disease vector/pest control methods.

Table 3: Overview of current and emerging genetic disease vector/pest control strategies in insects

Intended outcome	Potential to spread and persist in target populations			
	Self-limiting		Self-sustaining	
	High threshold (localised)	Low threshold (non-localised)	High threshold (localised)	Low threshold (non-localised)
Population suppression	– *Wolbachia*-mediated IIT – SIT – RIDL – fsRIDL – Engineered gene drives	Engineered gene drives	Engineered gene drives	Engineered gene drives
Population modification	Engineered gene drives	Engineered gene drives	Engineered gene drives	– *Wolbachia*-mediated PI – Engineered gene drives

fsRIDL: release of insects carrying a dominant female lethal transgene; GDMIs: gene drive modified insects; IIT: incompatible insect technique; PI: pathogen interference; RIDL: release of insects carrying a dominant lethal transgene; SIT: sterile insect technique.

3.6. State of the art

To summarise, gene drive research is currently focused on the following main areas:

1) Identifying, developing and testing cargo/payload genes of interest that may be spread by gene drive systems (e.g. Franz et al., 2006; Khoo et al., 2010; Mathur et al., 2010; Criscione et al., 2016; Jupatanakul et al., 2017; Buchman et al., 2019, 2020a; Duvall et al., 2019);
2) Developing and testing engineered gene drives and pairing them with cargo/payload genes of interest, if any (e.g. Chen et al., 2007; Akbari et al., 2014a; Simoni et al., 2014; Gantz et al., 2015; Hammond et al., 2016; Galizi et al., 2016; Buchman et al., 2018a,b; Oberhofer et al., 2019, 2020b; Carballar-Lejarazú et al., 2020; Adolfi et al., 2020; Champer et al., 2020c; Kandul et al., 2020a; López del Amo et al., 2020a);
3) Designing, developing and testing more specific, stable and spatially and temporally restricted (localised/transient) engineered gene drives (e.g. Gould et al., 2008; Altrock et al., 2010; Marshall, 2011; Marshall and Hay, 2011, 2012a, 2014; Marshall et al., 2011; Akbari et al., 2013, 2014; Champer et al., 2016, 2019b, 2020a,b,c; Esvelt and Gemmell, 2017; Tanaka et al., 2017; Buchman et al., 2018a,b, 2020b; Burt and Deredec, 2018; Dhole et al., 2018; Leftwich et al., 2018; Marshall and Akbari, 2018; Noble et al., 2019; Kandul et al., 2020b; Li et al., 2020a; Maselko et al., 2020; Oberhofer et al., 2020b; Terradas et al., 2020; Webster et al., 2020; Hay et al., 2021);
4) Designing, developing and testing reversal gene drives or other potential remediation strategies (e.g. Vella et al., 2017; Heffel and Finnigan, 2019; Friedman et al., 2020; López Del Amo et al., 2020b; Oberhofer et al., 2020a; Xu et al., 2020);
5) Studying the nature of target site resistance to mitigate its eventual occurrence (e.g. Basu et al., 2015; Beaghton et al., 2017a,b, 2019; Champer et al., 2017, 2018, 2019a, 2020d; Hammond et al., 2017, 2020; Marshall et al., 2017; Noble et al., 2017; Unckless et al., 2017; KaramiNejadRanjbar et al., 2018; Kyrou et al., 2018; Oberhofer et al., 2018; Bull et al., 2019; Champer et al., 2020a–e; Guichard et al., 2019; Marshall et al., 2019; Kandul et al. 2020b; Terradas et al., 2020);
6) Determining ideal gene drive characteristics, forecasting their behaviour at population and landscape level in real ecosystems and understanding associated uncertainties through mathematical modelling (e.g. Davis et al., 2001; Rasgon and Gould, 2005; Deredec et al., 2008, 2011; Altrock et al., 2010; Huang et al., 2011; Marshall and Hay, 2012a,b; Unckless et al., 2015; de Jong, 2017; Eckhoff et al., 2017; Godfray et al., 2017; Haller and Messer, 2017; Lambert et al., 2018; Noble et al., 2017, 2018; Dhole et al., 2018, 2019; Khamis et al., 2018; Beaghton et al., 2019; Edgington and Alphey, 2019; Girardin et al., 2019; Nash

et al., 2019; North et al., 2019, 2020; Golnar et al., 2020; Rode et al., 2020; Sánchez et al., 2020a,b; Verma et al., 2020);

7) Identifying and assessing potential adverse environmental consequences of GDMOs for deliberate release into the environment, and associated issues for ERA (e.g. NASEM, 2016; HCB, 2017; Roberts et al., 2017; Hayes et al., 2018; Rüdelsheim and Smets, 2018; Simon et al., 2018; CSS–ENSSER–V, 2019; Teem et al., 2019; Warner et al., 2019; Courtier-Orgogozo et al., 2020; Dolezel et al., 2020a,b; James et al., 2020; Mitchell and Bartsch, 2020; Sirinathsinghji, 2020; Smets and Rüdelsheim, 2020; Then et al., 2020);
8) Assessing the applicability of existing risk assessment frameworks and in which areas of such frameworks refinements may be needed for GDMOs (e.g. WHO, 2014; NASEM, 2016; Adelman et al., 2017a; Krishnan and Gillum, 2017; Lunshof and Birnbaum, 2017; Benedict et al., 2018; Meghani and Kuzma, 2018; Rüdelsheim and Smets, 2018; van der Vlugt et al., 2018; Kuzma, 2019; James et al., 2020; Smets and Rüdelsheim, 2020);
9) Assessing the applicability of existing regulatory frameworks and in which areas of such frameworks refinements may be needed for GDMOs (e.g. HCB, 2017; Rabitz, 2019; Brooks, 2020);
10) Developing pathways/recommendations to/for responsible and sustainable deployment of the technology (e.g. Oye et al., 2014; WHO, 2014; Akbari et al., 2015; NASEM, 2016; Adelman et al., 2017a,b; Emerson et al., 2017; Esvelt and Gemmell, 2017; Lunshof and Birnbaum, 2017; Baltzegar et al., 2018; James et al., 2018; Thompson, 2018; Backus and Delborne, 2019; Bartumeus et al., 2019; Kuzma, 2019; Cisnetto and Barlow, 2020; Warmbrod et al., 2020);
11) Assessing the desirability and ethics of engineered gene drives (Pugh, 2016; Thompson, 2018; Jones et al., 2019; Thomas et al., 2019; ECNH, 2019; Sandler, 2020; WHO, 2020);
12) Developing guidance/best practices on societal/stakeholder engagement and communication (e.g. Bartumeus et al., 2019; Brossard et al., 2019; Buchthal et al., 2019; George et al., 2019; Hartley et al., 2019; Schairer et al., 2019; Singh, 2019; Thizy et al., 2019; MacDonald et al., 2020; Palmer et al., 2020; Serr et al., 2020);
13) Developing effective management and implementation of disease vector control programmes (e.g. Feachem et al., 2019).

4. Risk assessment considerations for the deliberate release of gene drive modified insects into the environment

In line with the mandate of the European Commission, previously proposed risks and potential novel hazards associated with the deliberate release into the environment of GDMIs reported in the scientific literature are briefly summarised in the following sections (Sections 4.2 and 4.3, respectively). Similarly, previously proposed challenges related to the risk assessment and monitoring of GDMOs, including insects, are addressed briefly (Section 4.4). The identification of risks, potential new hazards and potential challenges for the risk assessment and PMEM of GDMIs for deliberate release into the environment is inevitably hypothetical to some extent, as no GDMI application has been submitted for regulatory approval in any jurisdiction globally to our knowledge. While some GMIs containing engineered gene drives are either in development or have been tested experimentally in the laboratory, none has been assessed in small-scale physically and/or ecologically confined field trials, or in open release trials (see Section 3). This indicates that no direct regulatory, ERA and PMEM experience has been gained with the deliberate release of specific GDMIs into the environment at the time of writing.

In the following sections, the summary of the previously proposed risks, potential novel hazards and challenges is preceded by an introduction of the problem formulation (Section 4.1), which serves as a starting point for conducting ERA.

Finally, similarities and differences between engineered gene drives and current and emerging disease vector/pest control strategies that involve the release of GMIs (SIT, RIDL and fsRIDL) and non-GMIs (SIT, *Wolbachia*-mediated IIT and PI, and biological control) are reported to investigate the risk assessment, post-release monitoring and regulatory experience that has been gained with those strategies, and clarify the extent to which lessons can be learned for the ERA and PMEM of GDMIs (Section 4.5).

4.1. Problem formulation

Robust ERAs begin with an explicit problem formulation, which involves among other steps: (1) identifying protection goals and making them operational for use in ERA; (2) devising plausible pathways to harm that describe how the deliberate release of a GMO could be harmful; (3) formulating risk hypotheses about the likelihood and severity of such events; (4) identifying the information that would be useful to test the risk hypotheses; and (5) developing a plan to acquire new data for hypothesis testing should tests with existing information be insufficient for decision-making (e.g. US EPA, 1998, Raybould, 2006, 2007, 2010; Wolt et al., 2010; Gray, 2012; Tepfer et al., 2013; Raybould and Macdonald, 2018; Devos et al., 2019a).[53] The problem formulation process helps to organise existing knowledge and identify relevant new knowledge to support decision-making.

As is the case for any other GMO, the information required for the ERA of GDMIs will be case specific; it will vary dependent on the biology and ecology of the insect species under consideration, the gene drive design and strategy, the introduced traits, the intended uses of the GDMI, the scale and frequency of the deliberate release, the receiving environments (covering the receiving environments where the GDMIs will be released and spread) and the interactions among these variables. Therefore, a case-by-case approach is taken for ERA.

4.1.1. Identifying protection goals and making them operational

A crucial step in the problem formulation is to identify protection goals,[54] and more specifically those that could possibly be harmed as the result of the deployment of a GDMI. Protection goals can vary among jurisdictions, but their overall aim is to reduce or avoid potential harm to the environment (including species, biodiversity, ecosystem functions and services, habitats) caused by human activity.

Legislative frameworks generally define protection goals broadly. Consequently, refinement is required to make them operational for use in ERA – they must be translated into specific, operational goals (also termed specific protection goals or assessment endpoints) (Nienstedt et al., 2012; Sanvido et al., 2012; Devos et al., 2014, 2015, 2016, 2019b; Garcia-Alonso and Raybould, 2014; Van den Brink et al., 2018). This process requires the delineation of what must be protected, where and over what time period, and setting limits of concern.[55] Three sequential steps can be followed to define operation protection goals: (1) identify relevant ecosystem services that could be at risk from the deliberate release of GDMIs; (2) identify service-providing units – structural and functional components of biodiversity – that provide or support these ecosystem services; and (3) specify the level of protection for these service-providing units. The level of protection is then defined by the ecological entity of the service-providing unit and its attributes, as well as limits of concern (EFSA, 2010a,b, 2016; Nienstedt et al., 2012; Sanvido et al., 2012; Devos et al., 2015, 2019b; Maltby et al., 2017a,b, 2018). This approach is not product-specific, and thus can be applied to GDMIs.

Since some currently used insect disease vector/pest control strategies are known to cause some harm (e.g. Golstein et al., 2019), an important consideration when setting specific protection goals is whether the proposed activity may lead to more or less harm, or new harms, compared with current practices. Some of the risks anticipated from the deliberate release into the environment of GDMIs may have been encountered before, from the use of GMIs that do not contain an engineered gene drive and from other current insect disease vector/pest control strategies. These are important considerations as part of any control effort, which are not linked exclusively to any particular technology or disease vector/pest control strategy (Fang, 2010; NASEM, 2016; Roberts et al., 2017; James et al., 2018; Golstein et al., 2019; Romeis et al., 2020).

4.1.2. Devising plausible pathways to harm

To further frame the ERA, plausible pathways to harm[56] are constructed in the problem formulation process to describe how the deliberate release of a GDMI could lead to possible harm to protection

[53] See also section C3.1 of the Annex of the Commission Directive (EU) 2018/350 of 8 March 2018 amending Directive 2001/18/EC of the European Parliament and of the Council as regards the environmental risk assessment of genetically modified organisms (C/2018/1371).

[54] Protection goals correspond to entities of value within the environment that must be protected.

[55] Limits of concern are defined in EFSA (2013) as the minimum ecological effects that are deemed biologically relevant and that are deemed of sufficient magnitude to cause harm. These limits of concern are set for each assessment endpoint in the problem formulation (see also Dolezel et al., 2017, 2018).

[56] A pathway to harm is a causal chain of events that need to occur for a harm to be realised.

goals. Such a pathway can be the function of a simple linear chain of events, or a complex one that is branched. An ERA typically includes many pathways, because the proposed activity could lead to different harms, or because a particular harm could arise in different ways, or both. Moreover, there may be multiple interconnected pathways to consider that may share some of the same steps.

Different techniques may be used to postulate pathways to harm (e.g. Wolt et al., 2010; Gray, 2012; Roberts et al., 2017; Hayes et al., 2018; Teem et al., 2019). The nature and formality of this exercise may reflect preferences and approaches of the responsible authority. When devising pathways to harm, potential pathways to harm should be systematically explored, and then prioritised based on their validity and consequences. In principle, only those pathways to harm that are valid according to existing knowledge and at least potentially consequential must be carried forward into the analysis. However, if the validity or consequences of a pathway to harm cannot be defined, one can expand efforts to consider existing knowledge and/or carry that pathway forward into the analysis.

Since it can be challenging to adequately devise multiple, complex pathways to harm over long time period, a wide area and/or a heterogeneous environment, it is important that all potential pathways are reported transparently. Moreover, a rationale justifying why potential pathways to harm are not considered sufficiently valid and/or consequential should be reported transparently for each potential pathway rejected.

Roberts et al. (2017), Teem et al. (2019) and Romeis et al. (2020) reported several relevant pathways to harm associated with the deliberate release of gene drive modified mosquitoes for malaria control and gene drive modified *D. suzukii* carrying a suppression drive, respectively, that can be considered further when devising such pathways.

4.1.3. Formulating risk hypotheses

The steps in a pathway to harm enable the formulation of risk hypotheses that can then be tested to characterise risk. If the testing of a risk hypothesis concludes that a step in a pathway is unlikely to occur, then the likelihood of that particular harm occurring through that particular pathway is also unlikely. A careful first scrutiny of the pathway to harm can usually help identify which of the risk hypotheses may be the most decisive or easiest to test in attempting to disrupt the pathway with a high degree of certainty. A particularly useful feature of this strategic analysis is that it decisively determines with sufficient confidence that a single (critical) step is highly unlikely, and so concludes that the likelihood that harm will result via the pathway is negligible and negates the need to analyse the other steps.

4.1.4. Identifying relevant information to test risk hypotheses and developing a plan to acquire new data

Risk hypotheses can be tested (evaluated) in a number of ways that include, but are not limited to, using existing information, which can come from many sources, or empirical data. In practice, some hypotheses may be difficult to test or testing using available information may not produce definitive conclusions regarding the likelihood of a particular step in a pathway to harm. As part of the ERA, such uncertainty may be addressed through an iterative, stepwise/staged/tiered testing approach,[57] by consideration of multiple lines of evidence including modelling (i.e. weight of evidence approach), and/or by new studies being undertaken (WHO, 2014; NASEM, 2016; Hayes et al., 2018; James et al., 2018; Romeis et al., 2020). Corroboration of risk hypotheses following a rigorous test gives greater

[57] Stepwise/staged testing approach: As a GDMI progresses through the phased testing and deliberate release pathway, the spatial and temporal scales of the concomitant risk assessment studies increase, and the suite of tools used to identify hazards and their potential associated adverse effects changes. Relevant data gathered under controlled, contained conditions provide confidence that the GDMI can safely progress to the next testing phase (NASEM, 2016; Hayes et al., 2018; James et al., 2018). Tiered testing approach: According to the tiered approach, information collected in lower tiers directs the extent and nature of any experimentation conducted in higher tiers: hazards are evaluated within different tiers that progress from worst-case exposure scenario conditions, framed in highly controlled laboratory environments, to more realistic scenarios under semi-field or field conditions. Progression to larger-scale experiments in higher tiers aims to provide increasingly refined estimates of exposure. Within each tier, all relevant data are gathered to determine whether there is enough information to conclude the risk assessment at that tier. The conclusion can only be made if any residual uncertainty has been defined; otherwise, additional investigations to generate further data at a higher tier(s) are conducted. Should potential hazards be detected in early tier tests or if unacceptable uncertainties concerning possible hazards remain, additional information is required to confirm whether the observed effect might still be detected at more realistic rates and routes of exposure. In the case that risk cannot be ruled out with enough certainty, risk management measures can be implemented (Devos et al., 2019a).

confidence than does a weak test. However, in some cases, uncertainties may remain that must be addressed by risk managers and decision makers.

Enabling the testing of risk hypotheses makes the pathway to harm approach very powerful for ERA, because harm is defined explicitly from the start, existing information is used effectively, new data are collected with a clear purpose and risk is characterised against well-defined criteria of hypothesis corroboration or falsification.

4.1.5. Additional considerations

Transparency in how a problem formulation is conducted is important to all stakeholders. Thus, sufficient detail about the methods, data, assumptions and uncertainties must be reported to promote transparency, facilitate an appropriate assessment of the quality of the problem formulation, ensure relevance and enable reproducibility.

While problem formulation is conceptually straightforward, its implementation is often challenging (Raybould and Macdonald, 2018; Devos et al., 2019a; Raybould and Burns, 2020). As is the case for any other ERA for a new technology, it is important for risk managers to define clear operational protection goals and decision-making criteria (e.g. what constitutes harm, limits or thresholds of concern, trigger values for action or acceptability of risk, judging the sufficiency of scientific knowledge and the extent to which uncertainty should be reduced for decision-making) that are needed to guide the interpretation of scientific information (Devos et al., 2019a,c). Hence, reaching agreement on protection goals and decision-making criteria is a prerequisite for producing ERAs that address them. Data collection and interpretation can then be directed towards evaluating the impact of any observed effect on what is desirable to protect. Consequently, enhanced dialogue between risk assessors and risk managers is advocated to clarify how ERA can address specific protection goals and decision-making criteria.

In addition, active stakeholder engagement on problem formulation (including the setting of protection goals and assessment endpoints) can improve the value of ERA, as it may help to ensure that ERA are meaningful and informative to the environmental decisions that affect them (e.g. Nelson et al., 2009; NASEM, 2016; Kuzma, 2019; Burgess et al., 2018). In the context of the potential deployment of engineered gene drives as part of malaria eradication strategies, researchers, donor organisations, ethicists, health professionals, regulators as well as government policymakers have embarked on consultations, workshops and public engagements aimed at problem formulation for the use of gene drive modified mosquitoes (e.g. Roberts et al., 2017; James et al., 2018; Teem et al., 2019). These types of consultation provide a helpful format to identify relevant protection goals (Craig et al., 2017; Hokanson et al., 2018) and frame ERA (Murphy et al., 2010; Kolopack et al., 2015; Murray et al., 2016). If risk managers consider that such engagement is useful to define and agree on protection goals, they may want to explore how it should be best designed, and whether it should be performed on single applications, groups of applications or on the technology per se. Experience gained from jurisdictions and domains where pre-submission exchange between applicants and risk assessment bodies is a well-established process shows that such an exchange can be helpful to frame the problem formulation by clarifying policy goals (including protection goals), decision-making criteria and information requirements, advise on study designs and navigate the regulatory process.

Since some GDMIs may eventually spread across jurisdictional boundaries, a point requiring further consideration is whether ERA should be framed only by the specific protection goals established by the jurisdictions that would host the deliberate release, or address those of the entire area of potential spread to cover the potential for transboundary movements.

4.2. Potential novel hazards

In some publications, the novel aspects[58] of engineered gene drives have been analysed (e.g. HCB, 2017; Simon et al., 2018; Dolezel et al., 2020a,b; Then et al., 2020). In line with the mandate of the European Commission, this section identifies specific aspects of GDMIs that are potentially novel compared to naturally occurring gene drives and disease vector/pest control strategies that involve the release of GMIs that do not contain an engineered gene drive (primarily RIDL and fsRIDL) and the release of non-GMIs (SIT, *Wolbachia*-mediated IIT and PI, and classical biological control [CBC]). This analysis focuses on: (1) the preferential inheritance of a transgenic construct; (2) the intended spatial and temporal scale of spread of the genetic modification of interest; (3) the scale of population

[58] Also termed: Features.

suppression; (4) population modification strategies; (5) target populations and environments; and (6) lack of spatio-temporal controllability (see Table 4).

1) *Preferential inheritance of a transgenic construct*: Engineered gene drives are designed to spread the genetic modification of interest preferentially from parent to progeny to achieve the intended outcomes in terms of population suppression or modification of target populations.[59] Preferential inheritance of the transgenic construct is repeated in subsequent generations. Such inheritance does not occur in GMIs that do not contain engineered gene drives. While preferential inheritance is observed in many natural gene drives and *Wolbachia*-mediated PI, such systems are not tailored to spread a transgenic construct to achieve intended outcomes. With engineered gene drives instead, natural gene drives are repurposed or re-engineered (redesigned) to achieve preferential inheritance of a transgenic construct (see Section 3.2). Therefore, preferential inheritance of a transgenic construct can be considered as a novel aspect of GDMIs;
2) *Intended spatial and temporal scale of spread of the genetic modification(s) of interest*: Depending on the GDMI, they may enable rapid, non-localised spread of the genetic modification of interest in target populations from low initial introductions, and its persistence in target populations, even if they incur some fitness costs on their host. The potential to spread the genetic modification of interest widely and for an indeterminate time is considered different from disease vector/pest control strategies that involve the release of GMIs (SIT, RIDL and fsRIDL) and non-GMIs (SIT, *Wolbachia*-mediated IIT), as they are generally intended to be self-limiting. In contrast, engineered gene drive systems with localised and temporally restricted spread of the genetic modification of interest would be more similar to other self-limiting approaches for disease vector/pest control (see Section 4.5);
3) *Scale of population suppression*: Depending on the engineered gene drive, theoretically, they could be designed for local or area-wide suppression. In some cases, they may aim for local elimination. While RIDL, fsRIDL and *Wolbachia*-mediated IIT have mainly been deployed at local scale, SIT and CBC have been used at a local and area-wide scale to suppress target populations, involving repeated releases over time to reach and maintain suppression. The potential area-wide scale of population suppression can therefore not be considered novel. Moreover, in the case of disease-transmitting mosquitoes, model predictions suggest that it is unlikely that suppressive engineered gene drive strategies would completely eliminate a species in the field (North et al., 2019, 2020);
4) *Population modification strategies*: *Wolbachia*-mediated PI is currently the only strategy applied for population modification. Theoretically, engineered gene drives may enable modifying target populations in the field, and expand the means to achieve population modification (including the spectrum and nature of novel cargo/payload genes) compared to *Wolbachia*-mediated PI (see Section 3.5.3). For engineered gene drives, a wide range of potential cargo/payload genes have been proposed, many of which have been shown to give a greater reduction in vector competence than the *w*Mel strain of *Wolbachia* in laboratory studies, but none has been tested in open field trials yet. Consequently, population modification achieved through engineered gene drives could be considered a novel aspect of GDMIs;
5) *Target populations and environments (primarily non-domesticated or wild species in non-managed environments)*: While some GDMIs can target non-domesticated or wild species in non-managed environments, this is also the case for disease vector/pest control strategies that involve the release of GMIs (SIT, RIDL and fsRIDL) and non-GMIs (SIT, *Wolbachia*-mediated IIT and PI, and CBC) (see Section 4.5). Therefore, these aspects are not specific for the GDMIs considered in this GMO Panel Scientific Opinion, and cannot be considered novel;
6) *Lack of spatio-temporal controllability*: The current inability to control the spread and persistence of the genetic modification of interest or to recall it after release has been previously proposed and reported as novel aspects of engineered gene drives (AHTEG, 2020; Dolezel et al., 2020; Then et al., 2020). At the time of writing, such considerations

[59] Unlike the transgenic construct, the genetic background of the gene drive modified individuals deliberately released into the environment is not inherited in a preferential manner.

apply to both self-sustaining and self-limiting engineered gene drive systems, as self-limiting systems have not been tested in open release trials. However, theoretically, self-limiting systems may enable localised and temporally restricted spread of the genetic modification of interest. Consequently, they would be similar to other self-limiting disease vector/pest control strategies involving the release of insects (such as SIT, RIDL, fsRIDL and *Wolbachia*-mediated IIT) (see Section 4.5). Due to the lack of spatio-temporal controllability associated with *Wolbachia*-mediated PI and CBC, this aspect cannot be considered novel for engineered gene drives.

In conclusion, the preferential inheritance of a transgenic construct, along with the intended spatial and temporal scale of spread of the genetic modification(s) of interest can be considered novel aspects of GDMIs when compared with disease vector/pest control strategies that involve the release of insects, which in turn may lead to potential adverse effects across large spatial and/or temporal scales in specific cases. Moreover, the means to achieve population modification options can be expanded with engineered gene drives, compared to *Wolbachia*-mediated PI. Theoretically, engineered gene drives may enable modifying target populations in the field, and expand the means to achieve population modification (including the spectrum and nature of novel cargo/payload genes, along with the diversity of target organisms).

Further consideration in any future ERA is required to scrutinise whether the aspects mentioned above (or others) are potential novel hazards, and whether they may introduce additional factors into the risk assessment of some GDMIs. The hazardous potential of any novel aspect identified will need to be assessed on a case-by-case basis using the problem formulation approach (see Section 4.1).

Table 4: Potential novel aspects of gene drive modified insects compared to genetically modified insects (GMIs) that do not contain an engineered gene drive and other disease vector/pest control strategies that involve the release of non-GMIs (in the scope of this GMO Panel Scientific Opinion)

Potential novel aspects	Comparators				
	GMIs that do not contain an engineered gene drive (primarily RIDL, fsRIDL)[(a)]	**Natural gene drives and *Wolbachia*-mediated pathogen interference (PI)**	**Sterile insect technique (SIT)**	***Wolbachia*-mediated incompatible insect technique (IIT)**	**Classical biological control (CBC)**
Preferential inheritance [of a transgenic construct]	No	Yes [No]	No	No	No
Intended spatial and temporal scale of spread of the genetic modification(s) of interest	Spatially temporally restricted	Spatially temporally unrestricted (case-specific)	Spatially temporally restricted	Spatially temporally restricted	Spatially temporally unrestricted (case specific)
Scale of population suppression	Local (at present)	NA	Local and area-wide	Local (at present)	Case specific
Population modification strategies [involving transgenes]	No [No]	Yes [No]	NA	NA	NA
Target non-domesticated or wild species in non-managed environments	Yes (case specific)	Yes (case specific)	Yes (case specific)	Yes (case specific)	Yes (case specific)
Spatio-temporal controllability	Yes	No (case specific)	Yes	Yes	No (case specific)

fsRIDL: release of insects carrying a dominant female lethal transgene; RIDL: release of insects carrying a dominant lethal transgene.
(a): GMIs contributing to the direct enhancement of production systems through enhanced stress tolerance, performance or fitness characteristics are not covered.

4.3. Risks

Several publications have previously proposed risks on broad protection goals (such as human and animal health, and the environment) associated with the deliberate release of GDMIs (e.g. NASEM, 2016; Roberts et al., 2017; James et al., 2018, 2020; Collins et al., 2019; CSS–ENSSER–VDW, 2019; Rode et al., 2019; Teem et al., 2019; Dolezel et al., 2020a,b; Romeis et al., 2020; Smets and Rüdelsheim, 2020; Then et al., 2020). Some of these previously proposed risks are listed below in Table 5. They represent areas of concern for further consideration in the ERA, especially the problem formulation, of potential GDMI applications, and fit into the specific areas of risk for GMIs outlined in EFSA (2012, 2013), which build on those laid down in Annex II of Directive 2001/18/EC.

It is important not to generalise the previously proposed risks reported in Table 5, as they may not apply to all the GDMIs considered in this GMO Panel Scientific Opinion. Any risk will need to be identified on a case-by-case basis using the problem formulation approach, and assessed as part of the ERA process (see Section 4.1).

Table 5: Previously proposed risks to human and animal health and the environment associated with the deliberate release of gene drive modified insects considered in this GMO Panel Scientific Opinion

Potential to cause harm to:	Previously proposed risks[(a)]
Human health and animal health	• Increased disease transmission – Increased abundance of disease-transmitting insects – Increased competence for transmission of the pathogen or other insect-borne pathogens and thus the prevalence of other insect-transmitted diseases – Altered mating, host seeking, or feeding behaviours, or geographic range (broader temperature tolerance) of disease-transmitting insects – Reduced control capability due insecticide resistance • Increased potential for resistance to evolve in the target organism – Reduced efficacy of the gene drive modified insect (GDMI) in the target population(s) • Increased toxicity and/or allergenicity – Transmission of toxic or allergenic substances (related to the components of an engineered gene drive) either directly by biting or indirectly by exposure from such substances released into the environment (e.g. incidental exposure through inhalation or ingestion) – Increased pathogen virulence in case of population modification
The environment (biodiversity, food webs, ecosystems and ecosystem services)	• Increased persistence and invasiveness potential – A competitive advantage of GDMIs as compared to the wild type, causing increased persistence and invasiveness and leading to the displacement of other insect pest species • Increased potential for resistance to evolve in the target organism – Management responses to reduced efficacy of the GDMI • Increased potential for vertical and horizontal gene transfer – Spread of the genetic modification of interest to non-target organisms through vertical and horizontal gene transfer • Increased toxicity – Transmission of substances (related to the components of an engineered gene drive) that are toxic to non-target organisms that consume the GDMI • Adverse effects associated with the suppression of the target organism – Suppression of the target organism that serves as food source (e.g. prey) for non-target organisms (e.g. predator)

Potential to cause harm to:	Previously proposed risks[(a)]
	– Suppression of the target organism may harm non-target organisms that rely on the species for the delivery of ecosystem services (such as pollination, biological control, decomposition) – Invasion of the ecological niche vacated by suppression of the target organism of another insect pest (e.g. other mosquito species in aquatic habitats during larval stages) (niche replacement) • Decreased water quality – Suppression of the target organism (e.g. mosquito larvae in aquatic habitats) which results in reduced larval consumption of algae causing levels of algae to increase and their associated toxins produced from algal bloom. This is in turn could lead to adverse effects on non-target organisms in the aquatic habitat, and negative effects on water quality

GDMI: gene drive modified insect.
(a): Based on NASEM (2016), Roberts et al. (2017), James et al. (2018, 2020), Collins et al. (2019), CSS–ENSSER–VDW (2019), Rode et al. (2019), Teem et al. (2019), Dolezel et al. (2020a,b), Romeis et al. (2020), Smets and Rüdelsheim (2020) and Then et al. (2020).

4.4. Potential risk assessment and monitoring challenges

Several publications have previously proposed potential challenges related to the risk assessment and PMEM of GDMOs (covering more organisms than insects only) (e.g. NASEM, 2016; CSS–ENSSER–VDW, 2019; ATHEG, 2020; Dolezel et al., 2020a,b; Then et al., 2020). Some of these previously proposed potential challenges are summarised in Table 6.

It is important not to generalise the previously proposed potential risk assessment and PMEM challenges reported in Table 6, as they may not apply to all types of GDMIs considered in this GMO Panel Scientific Opinion.

Table 6: Previously proposed risk assessment and monitoring challenges associated with the deliberate release of gene drive modified organisms into the environment

Related to	Previously proposed risk assessment and monitoring challenges[(a)]
Engineered gene drive system	• Prediction of all relevant genomic effects that could emerge in the next and subsequent generations, and from interactions with the receiving environments • Evaluation of off-target changes and their consequences over time in different genetic backgrounds and their potential accumulation in populations • The potential for the engineered gene drive to evolve after release, including through unexpected genetic drift • Controllability of engineered gene drive systems after release
Target organism	• Need for information on the potential genetic diversity of the target species • Need for information on the functional role of the target organism and potential cross-compatible species in the various ecosystems that may be encountered • Consideration of the reproductive strategies, population dynamics and life cycle of the target organism • Consideration of possible evolution of resistance in pathogens regarding disease vector control
Receiving environment	• Need for information on the potential for hybridisation with non-target organisms • Diversity of potential receiving environments, and limited information on the potential interactions with natural receiving environments • Limited information on long-term evolutionary processes occurring in ecosystems
Risk assessment methodologies	• Difficulties of applying the stepwise approach for risk assessment • Challenges to the comparative risk assessment framework • Assessing and taking into consideration uncertainty • Need to address the broader temporal and spatial scale

Related to	Previously proposed risk assessment and monitoring challenges[(a)]
	• Higher dependency on model-based predictions (e.g. to address the long temporal and wide spatial scale of some engineered gene drive applications and to anticipate the range of scenarios for the possible evolution of the engineered gene drive in the environment) • Difficulty to predict the non-linear, exponential effects of engineered gene drives • Difficulties in assessing next generation effects of organisms containing engineered gene drives • The need to develop knowledge and procedures for assessing the engineered gene-drive's long-term effects on ecosystems • Difficulty to comprehensively assess risks prior to release
Data collection and analysis	• Additional information needed on the molecular characterisation of both the engineered gene drive mechanism and the engineered gene drive-bearing organism • Information to predict off-target effects and potential consequences in the target organism • Advances in conceptual approaches are required to understanding the novel evolutionary and ecological couplings and feedbacks that gene drive modified organisms generate • Lack of environmental and ecological data • Difficulties with obtaining data for relevant modelling • Difficulties with validation and calibration of modelling data before the occurrence of an environmental release
Risk mitigation and monitoring	• Challenges pertaining to post-release environmental monitoring • Evaluation of impacts over long periods of time • Need for monitoring plans at supranational level to follow the spread of the engineered gene drive • Proven strategies for controlling the spread of an engineered gene drive, should monitoring data show that it has adverse effects on human, animal and plant health or the environment • Unavailability of management plans for possible reversion

(a): Based on NASEM (2016), CSS–ENSSER–VDW (2019), ATHEG (2020), Dolezel et al. (2020a,b) and Then et al. (2020).

4.5. Experience from current and emerging insect disease vector/pest control strategies

While GDMIs have not been released into the environment at the time of writing, there is substantial experience of releasing insects for genetic and biological disease vector/pest control (SIT, RIDL, fsRIDL, *Wolbachia*-mediated IIT and PI, and biological control). This experience is further analysed below to clarify the extent to which lessons can be learned for the ERA and PMEM of GDMIs (Table 7).

4.5.1. Release of radiation-sterilised insects

The release of radiation-sterilised insects is a widely accepted form of insect pest control (Dyck et al., 2005; HSCP, 2018; Romeis et al., 2020; WHO and IAEA, 2020). Formal ERA procedures are dependent on each country's general environmental regulatory framework. In the USA, where SIT has been used extensively, environmental assessments are required for SIT-based pest management under law, such as the National Environmental Policy Act. This is not specific to SIT, but covers requirements for management actions against specific pest species and affected areas with relatively narrow ecological scope. Similar requirements exist in many other countries. Bouyer et al. (2020) recommend a phased conditional approach to developing SIT, which includes explicit identification of relevant national regulatory approval. Recently, the World Health Organization (WHO) and the International Atomic Energy Agency (IAEA) have issued guidance about testing SIT as a vector control tool against *Aedes*-borne diseases (WHO and IAEA, 2020), and the general principles would apply to other uses of SIT for vector control. The guidance describes elements for the assessment of environmental and health risks related to pest control programmes using the technology, how environmental monitoring may be applicable, and some examples of relevant national regulatory processes. The objectives of a control programme are important in setting the implementation scenarios for any risk assessment. Eradication of the target pest is frequently an objective in SIT and releases may continue to occur for

some time after the elimination of the wild target pest population, until monitoring has demonstrated the regulatory defined criteria for pest freedom in the area (IPPC, 2016).

SIT suppression has spatial and temporal limits associated with the release area and period. The level of suppression is proportional to the effective release rate of the sterilised insects, determined by their number, distribution and mating success (Hendrichs et al., 2005).

Preventative release SIT is intended to prevent establishment of the target organism and is a unique example of an area-wide control strategy in which the target pest is not present in the release area most of the time (Hendrichs et al., 2005). Preventative SIT is used when there is a relatively high, regular challenge of pest invasion, so it acts as a constant anti-establishment measure. Preventative release may not stop all establishment, so it is usually coupled with capacity for a short, localised SIT or alternative eradication campaign if an outbreak occasionally occurs.

For SIT, the matching of target pest and released (mass-reared) sterile insects through characterisation is primarily to ensure efficacy. An environmental impact assessment (EIA), when required, would focus on risks to non-target organisms arising from reduction/elimination of the target organism, and from environmental/health effects related to ancillary actions within the control system (vehicles, noise, traps, pesticide, etc.) (WHO and IAEA, 2020). While the target organism is often an exotic pest species, some valued non-target organisms may acquire dependence on a long-established target organism or have close relationships with native target organisms.

Monitoring and data gathering in SIT are designed primarily for operational decisions. There would be preliminary estimates for overflooding ratios based on field population studies, and some pre-release mating competitiveness checks, designed for efficacy and logistics planning. In a large-scale programme that required an EIA, there would be an effort to find any non-target organism (especially any valued non-target organism) with dependence on the target organism, or sensitivity to ancillary operations. Sensitivity to associated operations (like a pre-release chemical suppression of the target organism in the receiving environment) is often the greatest effect on non-target organisms.

Quality assurance, including factors such as sterility and sex sorting, is essential to ensure efficacy and mitigate risk of unintended establishment (which is particularly important in preventative release SIT) (Calkins and Parker, 2005; Culbert et al., 2020)). Some similar quality indicators have been proposed for GMI systems (Mumford et al., 2018). Population monitoring in the field during releases gives feedback on whether more active control is required, or if there are faults in the rearing and release quality assurance (e.g. low sterility or low distribution density).

For SIT, marking of released individuals is important for operational efficacy (Vreysen, 2005). It is also related to legal proof of success, often in terms of reopening suspended high value trade (IPPC, 2016). Authorities need to be able to show that any monitoring evidence is from the presence of sterile released insects, as proof there is no viable outbreak population present so that trade can resume. For preventative release, it is important to demonstrate any pest insects found in surveillance are from sterile releases and not incipient outbreaks.

4.5.2. Release of genetically modified insects with a dominant (female) lethal transgene

As is the case with any other GMO, the deliberate release into the environment of GMIs is regulated in almost all jurisdictions under specific GMO legislation. They are subject to ERA before GMIs can be deliberately released into the environment. Consequently, regulatory and ERA experience has been gained in jurisdictions where actual deliberate releases have taken place. In all cases, potential adverse effects on the environment, including effects on human and animal health, have been assessed as part of the ERA. Such assessments involve the characterisation of the organism and product, which is based on: data on the recipient/parental organism (in terms of identity, source, strain, host range, geographical distribution); the method of modification and sequences introduced or deleted; the molecular characterisation; expression data; life-cycle parameters and differences between GMI and non-GMI; and mating competitiveness. In addition, the ERA considers: survival and dispersal; responses to abiotic factors; stability of the genetic modification of interest; the spread and persistence of the genetic modification of interest; the potential of hybridisation with related species; potential adverse effects on non-target organisms based on the genetic modification of interest and new metabolites; the toxicity, allergenicity of the genetic modification of interest and new metabolites for humans and animals; data of other deliberate releases with the same GMIs; details of receiving environment; and impact of change in management. Moreover, post-release monitoring activities are

typically conducted for such releases. Depending on the jurisdiction where deliberate releases take place, these include Cayman Islands, Brazil, Panama and most recently Florida.

Over the last few years, different guidelines have been proposed for the risk assessment of GMIs (e.g. CBD, 2016; Glandorf, 2017; HCB, 2017; Romeis et al., 2020).

4.5.3. Release of *Wolbachia*-infected individuals

Regulatory and ERA experience with the release of *Wolbachia*-infected insects has so far only been gained with mosquitoes (Romeis et al., 2020). Currently deployed mosquito suppression and modification strategies based on the mass release of *Wolbachia*-transinfected individuals, which are not considered GMOs, have been subject to an ERA that evaluates potential risks to human and animal health and the environment resulting from their deliberate release (e.g. Murphy et al., 2010; Popovici et al., 2010; Murray et al., 2016; US EPA, 2017).[60] This assessment falls under different regulatory frameworks depending on the jurisdiction where the releases take place. For instance, in the USA, when the developer claims that *Wolbachia* will limit the population of mosquitoes infected with it, *Wolbachia*-transinfected strains are regulated as biopesticides. When the developer claims that *Wolbachia* will limit the load of a disease-causing virus in the mosquito or will lower the rate of disease transmission to humans, *Wolbachia* in the mosquitoes would be regulated as a new animal drug, similar to how it is regulated in Australia. In Australia, *Wolbachia*-transinfected strains are evaluated as veterinary chemical products, i.e. considering *Wolbachia* as a substance by the Pesticides and Veterinary Medicines Authority (De Barro et al., 2011). According to the Commission Implementing Decision (EU) 2018/1623, *Wolbachia*-transinfected strains are regulated as a microbial biocidal agent in the EU, whereas the mosquitoes artificially transinfected with *Wolbachia* for disease vector control are out of the scope of the biocides legislation. GMO legislation would only apply in case *Wolbachia* or the mosquitoes are genetically modified.[61]

4.5.4. Biological control

There is substantial experience with releasing organisms into new environments as biocontrol agents. Releasing predators, parasitoids and pathogens to control insect pests is referred to as biological control, and is a proven and important pest management tool. There are two principle applications of biological control that involve release of organisms into the environment (Hoeschle-Zeledon et al., 2013; Hajek et al., 2016): (1) augmentative biological control (ABC); and (2) CBC.

Of the two forms of biological control, CBC is more like a self-sustaining spatially and temporally unrestricted gene drive system for population suppression (Romeis et al., 2020). However, CBC has two major differences from engineered gene drive systems in that the agent and the target organism are different species and from different trophic levels. This creates significantly different ecological interactions, particularly interspecific density dependence relationships, which affect the relevance of some aspects of biocontrol ERAs as partial models for GDMIs (HCB, 2017).

In ABC, large numbers of natural enemies (which may be native or exotic species) of the target pest are mass-reared and repeatedly released in the field or the glasshouse. ABC is particularly relevant to artificial and highly disrupted environments where natural enemies have poor persistence. Wider dispersal and establishment are not intended, but there may be some local spread and persistence if environmental conditions are suitable and hosts are available. The aim is a short-term or season-long suppression of the target pest.

In the case of CBC, natural enemies of invasive arthropod pests are typically introduced from the area of origin of the target pest. They are released with the aim to establish and provide long-term suppression of the target pest. They should be self-sustaining at fairly low threshold densities because they need to survive low host/prey densities. Potential environmental effects caused by such releases are likely to be irreversible and Regnier et al. (2009) give an example of loss of biodiversity resulting from unintended non-target effects. CBC is generally used against exotic pests, that is harmful species that have not been historically present in the target area. Reducing the target organism density may help to restore the original balance of the ecosystem to a state similar to that without the new insect pest species. However, it is recognised that some ecologically significant non-target organisms can

[60] http://www.eliminatedengue.com/library/publication/document/yogyakarta/risk_assessment_on_the_release_of_wolbachia-infected_aedes_aegypti.pdf and http://www.eliminatedengue.com/library/publication/document/july_2011_ra_report_eng.pdf

[61] https://ec.europa.eu/food/sites/food/files/plant/docs/reg-com_2001-18-ec_20181018_sum.pdf

become partially dependent on exotic target species, giving the target organism some acquired value as it becomes integrated into its new environment (Zavaleta et al., 2001; Ehrenfeld, 2010).

A major consideration in risk assessment and regulatory approval for CBC is the host specificity of any biocontrol agent to ensure that it will not adversely affect any non-target hosts directly (Shaw et al., 2011; Marchante et al., 2017). Risks to non-target organisms is evaluated in three phases: (1) information on host/prey range in the native area; (2) information on host/prey range retrieved from other areas into which the species has already been introduced; and (3) host/prey specificity testing taking into account species in the area of introduction. For testing under (3) non-target organisms are selected that are phylogenetically related to the target pest; have ecological similarities (e.g. same ecological niche) and/or are particularly valued (e.g. threatened species). Another consideration in CBC is that the suppression effect on the target organism does not have a wider indirect impact on the food web, where non-target organisms with wider value have become dependent on the target pest (van Lenteren et al., 2006). The application of CBC could serve as a partial model for ERA of GDMIs; Romeis et al. (2020) describe some similarities in pathways to harm in the problem formulation for risk assessments for CBC and engineered gene drive systems.

Shaw et al. (2011) provide lessons on the application of EU and Member State plant health regulations and risk assessment procedures to license the field release of a CBC agent for *Fallopia japonica* in the United Kingdom. Marchante et al. (2017) note that CBC releases are rare in Europe and they outline a series of Portuguese and European level applications, reviews and approvals before their introduction to control invasive *Acacia longifolia* was allowed. CBC agents have also been released in France (Panigaj et al., 2014) and Italy (EFSA, 2010c; Gibbs et al., 2011). The Harlequin lady beetle, first released in France in 1982, was the last non-specific agent (a general predator on aphids) released in Europe, which has led to significant negative impacts on non-target competitors (Hajek et al., 2016). The release of *Torymus sinensis* in Italy led to concerns about hybridisation with local species (Hajek et al., 2016). Recent trends in CBC have aimed at better molecular characterisation of agents, narrower population sourcing, greater host specificity and closer host and climate matching (Hajek et al., 2016). Louda et al. (2003) highlighted ecological issues that should be considered in selection and risk assessment of biocontrol agents, some of which may be relevant to engineered gene drive systems. These include risks from hybridisation; prediction of ecological consequences requiring population data; environmental conditions affecting impacts; poorly documented non-target impacts; indirect non-target effects; agent dispersal from target ecosystems; perturbation of wider assemblages of species; and lack of evidence on adaptation in most biocontrol cases. Barratt et al. (2009) reviewed advances in risk assessment for biological control, noting improved agent selection, characterisation and host range prediction and the increased role of population modelling to estimate post-release impacts.

The regulatory system for biological control in Europe is not well harmonised, limiting the potential for biocontrol ERA and PMEM as a model system. For ABC in Europe, there is a preference for native agents to avoid concerns about persistence of exotic organisms (Hajek et al., 2016). Introductions of CBC agents have been declining over the past 50 years because of greater regulatory control and more rigorous selection criteria (Hajek et al., 2016). An EPPO/COST-SMARTER (2015) report noted the lack of uniform guidance on how the regulations, developed for other purposes, should be applied for biocontrol releases. That report recommended that a distinction should be made in regulating self-sustaining and self-limiting biocontrol agents. It also suggested that benefits should be assessed alongside risks, which is different from GMO assessments in Europe, but is considered in assessments for invasive alien species risks. In some jurisdictions (e.g. New Zealand), a risk/benefit analysis is conducted as part of the ERA, along with an assessment of the effects of alternative pest control methods (Romeis et al., 2020).

4.5.5. Use of the experience from current and emerging insect disease vector/pest control strategies involving the release of insects

There is substantial experience with releasing insects for genetic and biological disease vector/pest control, including their ERA and post-release monitoring (if applicable). This experience is useful to identify potential hazards, exposures and risks for GDMIs (EFSA, 2013; Webber et al., 2015; Murray et al., 2016; HCB, 2017; Roberts et al., 2017; Hayes et al., 2018; James et al., 2018; Ritchie and Staunton, 2019; Romeis et al., 2020). Thus, it is appropriate to draw on the experience from current insect disease vector/pest control strategies that involve the release of insects, seek relevant precedence from more or less similar situations and use this experience to inform the ERA of GDMIs. However, caution is required as the systems compared differ in various aspects.

Table 7: Similarities and differences across disease vector/pest control strategies that involve the release of genetically modified insects [GMIs] (carrying a dominant [female lethal] transgene or containing an engineered gene drive), and non-GMIs (sterile insect technique, *Wolbachia*-mediated incompatible insect technique and pathogen interference, and biological control)

Aspects	Disease vector/pest control strategies involving the release of living insects						
					Biological control		
	Sterile insect technique (SIT)	**Release of insects carrying a dominant lethal transgene (RIDL) or a dominant female lethal transgene (fsRIDL)**	***Wolbachia*-mediated incompatible insect technique (IIT)**	***Wolbachia*-mediated pathogen interference (PI)**	**Augmentative biological control (ABC)**	**Classical biological control (CBC)**	**Engineered gene drives**
Intended outcome	Population suppression	Population suppression	Population suppression	Population modification	Population suppression	Population suppression	Population suppression, or modification dependent on engineered gene drive system
Released insect	Species already present (or regularly invasive) in the receiving environment	Species already present in the receiving environment	Species already present in the receiving environment	Species already present in the receiving environment	Native (usually) or exotic species	Exotic species (e.g. predator, parasitoid) from the area of origin of the target organism	Species already present in the receiving environment
	Males	Males	Males	Females only, or both sexes	Both sexes	Both sexes	Depending on engineered gene drive system
Target organism	Within a species	Within a species	Within a species	Within a species	Another (exotic) species and another trophic level	Another (exotic) species and another trophic level	Within a species
Species-specificity	High (mating)	High (mating)	High (mating)	High (mating)	Depending on host and environment specificity	Depending on host and environment specificity	High (mating)
Potential to spread*	Low (localised)	Low (localised)	Low (localised)	High (non-localised)	Low (localised)	Intended (dependent on climatic, biological and ecological characteristics)	Depending on engineered gene drive system (localised or non-localised)

Aspects	Disease vector/pest control strategies involving the release of living insects						
	Sterile insect technique (SIT)	Release of insects carrying a dominant lethal transgene (RIDL) or a dominant female lethal transgene (fsRIDL)	*Wolbachia*-mediated incompatible insect technique (IIT)	*Wolbachia*-mediated pathogen interference (PI)	Biological control		Engineered gene drives
					Augmentative biological control (ABC)	Classical biological control (CBC)	
Potential to persist*	Low (self-limiting)	Low (self-limiting)	Low (self-limiting)	Intended (self-sustaining)	Low (self-limiting)	Intended (self-sustaining)	Depending on engineered gene drive system (self-limiting or self-sustaining)
Scale of open releases	Area-wide/decades	Local/years	Local/years	Local/years	Local/decades	Area-wide/decades	No open releases at the time of writing
Regulatory context	Technology is not regulated per se	Subject to pre-market ERA and regulatory approval in most jurisdictions. Regulated as GMO	Jurisdiction-specific	Jurisdiction-specific	Subject to pre-market ERA and regulatory approval in most jurisdictions	Subject to pre-market ERA and regulatory approval in most jurisdictions	Subject to pre-market ERA and regulatory approval in most jurisdictions. Regulated as GMO
Primary (risk) assessment focus	Characterisation of insect for release, and an EIA dependent on the scale of release	Molecular characterisation, characterisation of insect for release, and case-specific ERA (see EFSA, 2013)	Characterisation of insect for release, and assessment of non-target and food web effects	Characterisation of insect for release, and assessment of non-target and food web effects	For native species, characterisation of insect for release and quality assurance; for exotic species concerns are as is the case for CBC	Characterisation of insect for release and case-specific ERA (e.g. assessment of non-target and food web effects)	Molecular characterisation, characterisation of insect for release, and case-specific ERA (see Section 5.1)
(Risk) Mitigation	Sterility and sex sorting quality assurance, and field population monitoring during releases. Stop the release	Case-specific (susceptibility to common insecticides confirmed). Stop the deliberate release	Effective sexing technology is required to minimise the risk that *Wolbachia*-infected females are released. Susceptibility to common insecticides confirmed. Stop the release	Susceptibility to common insecticides confirmed	Quality assurance in rearing and release to prevent contamination	Quality assurance in rearing and release to prevent contamination	Case-specific

Aspects	Disease vector/pest control strategies involving the release of living insects						
	Sterile insect technique (SIT)	**Release of insects carrying a dominant lethal transgene (RIDL) or a dominant female lethal transgene (fsRIDL)**	***Wolbachia*-mediated incompatible insect technique (IIT)**	***Wolbachia*-mediated pathogen interference (PI)**	**Biological control**		**Engineered gene drives**
					Augmentative biological control (ABC)	**Classical biological control (CBC)**	
Post-release monitoring	Sterilised insects should be marked to monitor release and SIT efficacy evaluation	Mandatory in some jurisdictions (e.g. EU)	Not mandatory. If monitoring is conducted, it focuses on efficacy evaluation	Not mandatory. If monitoring is conducted, it focuses on efficacy evaluation	Not mandatory. If monitoring is conducted, it focuses on efficacy evaluation	Expected in the NAPPO region and recommended by EPPO. Focus is on species establishment and efficacy of target population suppression	Mandatory in some jurisdictions (e.g. EU)

EIA: environmental impact assessment; EPPO: European and Mediterranean Plant Protection Organization; ERA: environmental risk assessment; GMO: genetically modified organism; NAPO: North American Plant Protection Organization; NTO: non-target organism; TO: target organism.
*: Of the genetic modification of interest or biological control agent.

5. Adequacy and sufficiency evaluation of the EFSA guidelines (EFSA, 2012, 2013) for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of gene drive modified insects

The adequacy and sufficiency evaluation of the considerations/requirements given in EFSA (2012, 2013) for the MC, ERA and PMEM of GDMIs, respectively, is reported below for each of the relevant headings and subheadings of EFSA (2012, 2013).[62] Section 5.1 of this GMO Panel Scientific Opinion focuses on the adequacy and sufficiency evaluation of EFSA (2013), while Section 5.2 assesses the adequacy and sufficiency of EFSA (2012). A rationale justifying inadequacy or insufficiency is reported below. The rationale to justify adequacy or sufficiency is provided in EFSA (2012, 2013), and no longer reported in this GMO Panel Scientific Opinion. Sections 5.1 and 5.2 should be read in conjunction with EFSA (2012, 2013).

5.1. Adequacy and sufficiency of EFSA guidelines (EFSA, 2013) for the environmental risk assessment and post-market environmental monitoring of gene drive modified insects

5.1.1. Scope of EFSA (2013) [Section 1]

As indicated in Section 1.2 of this GMO Panel Scientific Opinion, the adequacy and sufficiency evaluation of EFSA (2013) is limited to the use of engineered gene drives to control harmful insect species, in particular disease-transmitting insects, agricultural insect pests and invasive insects. Such GDMIs are expected to be developed for deliberate release into the environment, and thus are not confined or semi-confined animals. Moreover, they are not intended for food/feed uses. The scope of EFSA (2013) does encompass the non-confined release of GMIs for non-food/feed uses, among other GMA applications, and can thus be used as a reference document for the adequacy and sufficiency evaluation of the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.2. Strategies for the environmental risk assessment of genetically modified animals [Section 2]

Section 2 of EFSA (2013) briefly describes the strategies for the ERA of GMAs (covering the science-based nature of ERA, the case-by-case approach, the step-by-step approach, the problem formulation, the comparative approach, the consideration of intended and unintended effects and the appropriateness to draw on previous knowledge and experience) and the specific areas of risks for GMIs (which build on those laid down in Annex II of Directive 2001/18/EC). The adequacy and sufficiency of these elements are assessed and reported in the following sections.

5.1.2.1. Different steps of the environmental risk assessment [Section 2.1]

Section 2.1 of EFSA (2013) describes the different steps followed to conduct the ERA of GMIs. The adequacy and sufficiency of these steps are assessed and reported below.

Step 1: Problem formulation including identification of hazard and exposure pathways [Section 2.1.1]

The problem formulation approach for the ERA of GMIs described in Section 2.1.1 of EFSA (2013) is adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion. More details about the problem formulation process are reported in Section 4.1 of this Scientific Opinion.

Step 2: Hazard characterisation [Section 2.1.2]

As indicated in the Section 4.1.4 of this GMO Panel Scientific Opinion, some risk hypotheses formulated in the problem formulation step of ERA may be challenging to test in practice, or testing using available information may not produce definitive conclusions to characterise a hazard. As part of the ERA, such uncertainty may be addressed by new studies being undertaken. However, in some cases, uncertainties may remain that must be addressed by risk managers and decision makers.

[62] The term "adequate" means that the existing guidance documents can be used, but that additional qualifications would be appropriate, whereas "sufficient" means that the guidance documents are fully fit-for-purpose. Thus, "adequate" gives a lower acceptable bound below which quality or quantity would be unacceptable. "Sufficient" gives an upper acceptable bound in terms of quality or quantity above which one needs not strive; more would be excessive.

The considerations on the hazard characterisation for the ERA of GMIs given in Section 2.1.2 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Step 3: Exposure characterisation [Section 2.1.3]

As indicated in the Section 4.1.4 of this GMO Panel Scientific Opinion, some risk hypotheses formulated in the problem formulation step of ERA may be challenging to test in practice, or testing using available information may not produce definitive conclusions to characterise an exposure. As part of the ERA, such uncertainty may be addressed by new studies being undertaken. However, in some cases, uncertainties may remain that must be addressed by risk managers and decision makers.

The considerations on the exposure characterisation for the ERA of GMIs given in Section 2.1.3 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Step 4: Risk characterisation [Section 2.1.4]

As indicated in the Section 4.1.4 of this GMO Panel Scientific Opinion, some risk hypotheses formulated in the problem formulation step of ERA may be challenging to test in practice, or testing using available information may not produce definitive conclusions to characterise a risk. As part of the ERA, such uncertainty may be addressed by new studies being undertaken. However, in some cases, uncertainties may remain that must be addressed by risk managers and decision makers.

Gathering relevant data for self-sustaining and low threshold (independent) gene drives in open release trials as part of the stepwise/staged testing approach[63] may be challenging due to their spatially and temporally unrestricted nature and the inability for recall at the time of writing (see Section 4). Therefore, the utility of prior field testing of a related self-limiting strain may be considered as an intermediate step to reduce uncertainties in risk assessment (James et al., 2018).

In conclusion, the considerations on the risk characterisation for the ERA of GMIs given in Section 2.1.4 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Step 5: Risk management strategies [Section 2.1.5]

Several risk management strategies have been proposed in the scientific literature to mitigate potential risks associated with: (1) the spread and persistence of engineered gene drives; and (2) evolution of resistance.

1) Theoretically, self-limiting engineered gene drives with substantial release thresholds or other localised approaches may constitute a form of biological or molecular confinement, along with physical and/or ecological confinement (e.g. geographic/spatial and/or climatic isolation). Moreover, effects of self-limiting and localised approaches could be reversed by discontinuing releases of gene drive modified individuals. Since self-sustaining engineered gene drives are designed for widespread and long-standing control, spatially and/or temporally restricting their spread would not necessarily be in keeping with the intended outcome of their deliberate release. Therefore, the utility of prior field testing of a related self-limiting strain may be considered as an intermediate step to reduce uncertainties in risk assessment (Benedict and Robinson, 2003; James et al., 2018);
2) Depending on the gene drive strategy, resistance evolution to an engineered gene drive and associated cargo/payload genes can be delayed by using multiplexed gRNA that target different target DNAs as resistance would require mutations at several target sites (e.g. Champer et al., 2018; Oberhofer et al., 2018), targeting ultra-conserved and functionally constrained genes essential for survival or fertility (e.g. Burt, 2003; Kyrou et al., 2018; Champer et al., 2020d; Schmidt et al., 2020), optimising/regulating gene drive expression through the promoter controlling nuclease activity (Hammond et al., 2020), stacking multiple cargo/payload (inhibitory) genes in the same host individual (e.g. Gantz et al., 2015), designing engineered gene drives that target conserved or haploinsufficient genes and that also carry a recoded cDNA restoring endogenous gene activities (e.g. Oberhofer et al., 2019; Adolfi et al., 2020; Champer et al., 2020b,d; Kandul et al., 2020b; Terradas

[63] This testing approach reflects the stepwise process GMOs go through, beginning with experiments under contained use (e.g., laboratory, greenhouse), through experimental release, up to the placing on the market. According to this approach, the containment of GMOs can be reduced and the scale of release increased gradually, if assessment of earlier steps indicated that the next step can be taken.

et al., 2020) and/or by minimising any fitness costs of the engineered gene driver (Beaghton et al., 2019). Combining multiple engineered gene drive approaches, e.g., a suppressive gene drive that also distorts the sex ratio (Simoni et al., 2020), could be another strategy to delay resistance evolution (Price et al., 2020).

Reversal gene drives may be designed to either turn on or turn off engineered gene drive activity in the presence or absence of small organic molecules (Heffel and Finnigan, 2019; López Del Amo et al., 2020b), or mitigate potential unintended consequences of another engineered gene drive by removing or preventing the spread of the original drive, or overwriting it (Friedman et al., 2020; Oberhofer et al., 2020a; Xu et al., 2020; see Section 3.3.5). However, it is noted that such engineered gene drive systems are highly theoretical at the time of writing, and may induce further changes that may undo a phenotypic alteration caused by the initial drive, so they may not restore the original modification to the wild type or redress fully ecological effects from the original engineered gene drive (Champer et al., 2016; Xu et al., 2020). It has therefore been recommended not to rely upon a reversal gene drive as the sole strategy for mitigating the effects of another engineered gene drive, and to carefully examine risks associated with each of the countermeasures' limitations prior to release (e.g. NASEM, 2016; Vella et al., 2017).

As indicated in EFSA (2013), applicants need to demonstrate that the proposed risk management measures are practicable and feasible to reduce exposure and risk, and that these measures would work efficiently and reliably in relevant receiving environments. Moreover, applicants also need to state the post-commercialisation measures they will put in place in order to monitor and verify the efficacy of the risk management measures, and to allow changes in risk management strategies if circumstances change or if new data indicating the need for changes to the risk management become available.

In conclusion, the considerations on the risk management strategies for the ERA of GMIs given in Section 2.1.5 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Step 6: Overall risk evaluation and conclusions [Section 2.1.6]

The considerations on the overall risk evaluation for the ERA of GMIs given in Section 2.1.6 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.2.2. Information to identify potential unintended effects [Section 2.2]

Section 2.2 of EFSA (2013) refers to the MC, the compositional analysis, phenotypic characteristics and interactions between the GMA and its receiving environment as relevant data sources for the identification of potential intended and unintended effects.[64]

In line with EFSA (2013), the extent of the compositional and phenotypic analysis of GDMIs (i.e. the type and number of components and phenotypic parameters to consider [see for example James et al., 2018, 2020; Aldersley et al., 2019; Su et al., 2020 for possible assessment parameters]), which are not intended for food/feed uses, is case-specific, and may vary with the nature of the insect, the genetic modification of interest, the intended outcome of the deliberate release and the level of environmental exposure. Moreover, the need for compositional and phenotypic data should be triggered by the need to test specific risk hypotheses formulated as part of the problem formulation (and which are framed by agreed protection goals). In this respect, it is key to specify precisely what differences between the GMI and non-GMI are important in the comparison through limits of concerns, so that their environmental consequences can be assessed (see Section 5.1.4).

In conclusion, the considerations given in Section 2.2 of EFSA (2013) to identify potential unintended effects through the molecular, phenotypic and compositional characterisation of the GDMIs and comparisons of biotic and abiotic interactions are adequate, but insufficient for the MC of the GDMIs considered in this GMO Panel Scientific Opinion, as they focus on the gene drive modified individuals intended for release and not necessarily their progeny after release (see Section 5.2.2). The adequacy and sufficiency of the MC considerations/requirements are assessed and reported in Section 5.2.2 of this Scientific Opinion.

[64] EFSA (2013) defines intended effects as effects that are designed to occur from the introduction of the genetic modification in question and which fulfil the original objective(s) of the genetic modification. Unintended effects of the genetic modification are considered to be biologically relevant differences between the GMA and the appropriate selected comparator(s) which go beyond the primary intended effect(s) of the genetic modification.

5.1.2.3. Structural overview of EFSA (2013) [Section 2.3]

Section 2.3 of EFSA (2013) provides the structural overview of EFSA (2013) and clarifies the interplay between the different parts of it, namely the principles of the ERA, the cross-cutting considerations, the specific areas of risk and the PMEM.

The structural overview of EFSA (2013) given in Section 2.3 is adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.3. Cross-cutting considerations [Section 3]

Section 3 of EFSA (2013) describes the generic considerations that applicants should take into account throughout the whole ERA process of GMA, focusing on: (1) receiving environments; (2) experimental environment; (3) the choice of comparators; (4) the use of non-GM surrogates; (5) experimental design and statistics; (6) long-term effects; (7) modelling; (8) uncertainty analysis; and (9) aspects of GMA health and welfare. The adequacy and sufficiency of each of these cross-cutting considerations are assessed and reported below.

5.1.3.1. Receiving environments [Section 3.1, including subheadings]

Section 3.1 of EFSA (2013) is appropriate in highlighting the need for evaluating risks of GMIs across receiving environments and that these risks may differ in different environments. As noted in EFSA (2013), the receiving environment will vary in spatial scale, even when the deliberate release is not intended.

Characteristics of receiving environments highlighted in EFSA (2013) [in Section 3.1.2] are adequate for GDMIs. The range of potential receiving environments will depend on an understanding of the insect biology, the trait and the environmental suitability [in Section 3.1.3]. However, given the expected spatial and temporal extent of some engineered gene drive systems, the scope of what is deemed an accessible ecosystem (i.e. the environment into which a GDMI is intended for release compared to where it might spread to) will require careful consideration as potential spread into further accessible environments following deliberate release into a known and intended environment might be an anticipated outcome (with different risk evaluation and mitigation).

Selection of relevant sites for deliberate releases into the receiving environment requires much more scrutiny and assessment than is described in EFSA (2013). The expectation in EFSA (2013) is that applicants need to consider the potential full geographic range of a GMA, which will depend on the context of the deliberate release. Yet, for some GDMIs, this may be more difficult. It will depend on the type of engineered gene drive system, the selection of sites for release and the potential for range expansion (limited by host availability, trait characteristics and environmental suitability). The emphasis on additional tools (such as mathematical modelling) to evaluate the specification of receiving environments and inform ERA is briefly mentioned in EFSA (2013). However, with some GDMI systems, these predictive tools may need to play a much more prominent role to ensure ERAs for GDMIs consider risks across the full range of the potential receiving environment. Substantial rethinking beyond that covered in EFSA (2013) is needed to ensure ERAs systematically cover distinct intended and unintended receiving environments relevant to specific engineered gene drive applications.

In conclusion, while aspects of Section 3.1 of EFSA (2013) are adequate, any future guidance on GDMIs may need to emphasise details on receiving environments more as this section of EFSA (2013) is insufficient for some of the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.3.2. Experimental environment [Section 3.2]

Section 3.2 of EFSA (2013) emphasises that an appropriate experimental environment for GMIs should focus on the appropriate spatial scale associated with the experimental units. This is broadly in line with that required for the deliberate release of a GDMI.

EFSA (2013) highlights that suitable confinement measures should be in place, but for unconstrained GDMIs, the ultimate aim is for spatial and temporal spread. The use of small-scale physically and/or ecologically confined field trials compared to open release trials will thus involve different experimental environments and confinement measures (NASEM, 2016; Hayes et al., 2018; James et al., 2018). Confinement measures, and the temporal extent of each phase will likely vary as a GDMI progresses through phased testing and deliberate release pathways, and they may need to be relaxed to increase the spatial and/or temporal scale and realism of the experimental environment, if a decision is made to proceed to the next phase of testing/implementation (Hayes et al., 2018).

EFSA (2013) highlights the need for evaluation of the potentially different receiving environments for GMAs intended for release into the environment. For GDMIs, ERAs across different environments,

particularly for experiments/trials, should focus on the extent to which variation in ecological and environmental conditions might influence the environmental risks associated with the spread and persistence of the engineered gene drive.

In conclusion, the considerations given in Section 3.2 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.3.3. Choice of comparators [Section 3.3, including subheading 3.3.2]

The selection of suitable comparators as outlined in Section 3.3 of EFSA (2013) should include the intended outcome of engineered gene drive applications in insects, and put more emphasis on the purpose of the risk assessment studies conducted and thus purpose of comparisons. As a GDMI progresses through the phased testing and release pathway, the range of risk assessment studies and their purpose change (Hayes et al., 2018). Consequently, there will often not be a single comparator for a given proposed deliberate release into the environment of a GDMI, but a range of comparators that can inform ERA and contextualise risks (HCB, 2017).

Depending on the purpose of comparisons, relevant comparators may not be limited to the non-GMI of the same species with a genetic background that is as close as possible to that of the GDMI, or to the target organism, but also include other disease vector/pest control systems (e.g. species-specific genetic control methods involving the release of insects, insecticides); some of which may be operating over large areas and long timescales. Some engineered gene drives have similarities with current disease vector/pest control strategies that involve the release of GMIs (primarily RIDL and fsRIDL) and non-GMIs (SIT, and *Wolbachia*-mediated IIT and PI) in some of their aspects (see Table 7). For example, localised engineered gene drives aimed at population suppression are similar to SIT, (fs)RIDL and *Wolbachia*-mediated IIT, while non-localised engineered gene drives for population modification are similar to *Wolbachia*-mediated PI.

As outlined in Section 3.3 of EFSA (2013), comparisons at both the organismal and (management) system level may be relevant. Given that some GDMI systems will operate at an ecosystem level, the definition of comparator needs to be broadened from endpoints that solely consider genetic and phenotypic changes to those that can be indicative of potentially harmful ecosystem impacts. Comparators need to reflect the intended outcome of the GDMI applications, such as population suppression or modification.

Depending on the intended outcome of the GDMI application and purpose of comparison, the selection of comparators may need to consider issues relevant to offspring of the GDMI, and include comparisons with heterozygotes and homozygotes of the GDMI, where relevant. At the population and system level, multiple comparators may be needed to allow robust comparisons across a range of factors that are not sufficiently matched by a single comparator.

In conclusion, the considerations/requirements given in Section 3.3 of EFSA (2013) for the choice of comparators are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion. Future guidance on the choice of suitable comparators may need to consider the intended outcomes of GDMI applications, and put more emphasis on the purpose of risk assessment studies conducted.

5.1.3.4. The use of non-genetically modified surrogates [Section 3.4]

Section 3.4 of EFSA (2013) provides considerations/requirements for the selection of suitable non-GM surrogates that could be used to replace the GMA. EFSA (2013) notes that non-GM surrogates are likely to be particularly useful as a source of historic or parallel data (e.g. literature) to inform risk assessment rather than as experimental models from which to derive new information that can be related to the specific trait of the GMA under consideration.

EFSA (2013) addresses non-GM surrogate organisms, but does not consider surrogate systems. Depending on the interaction of the GDMI being assessed, suitable surrogates may not necessarily be limited to current species-specific disease vector/pest control strategies that involve the release of non-GMIs (SIT, and *Wolbachia*-mediated IIT and PI). Natural gene drives and disease vector/pest control strategies involving the release of GMIs (primarily RIDL and fsRIDL) could also be instrumental as surrogates to inform the ERA of the GDMIs considered in this GMO Panel Scientific Opinion. Moreover, theoretically, self-limiting engineered gene drives may be envisaged as surrogates.

For some engineered gene drive strategies, the scale in space and time makes experimental study difficult, even with a non-GM surrogate. It depends on the nature of the potential harm of concern.

In conclusion, the considerations given in Section 3.4 of EFSA (2013) are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion. Any future guidance on GDMIs may need to clarify and expand the range of surrogate types and their suitability (such as natural gene

drives, GM surrogates that do or do not contain an engineered gene drive), and cover deliberate releases of GMIs at larger spatial and longer temporal scale.

5.1.3.5. Experimental design and statistics [Section 3.5, including subheadings]

The aim of designing experiments is to ascertain the potential environmental harms associated with the release of GMAs. This needs: (1) clear risk-based hypotheses; (2) appropriate experimental design; and (3) appropriate statistical tools.

However, with some GDMIs, short-term ecological experiments to compare different treatment effects (through the use of linear statistical models such as analysis of variance) might not be appropriate. As outlined in EFSA (2013), comparative analyses are required to assess similarities and differences between GMAs and non-GMAs. Details on the experimental design and analyses will depend on the risk hypothesis, and what the expected differences should be between the gene drive modified individuals and target organism.

The use of open release trials and experiments with some GDMIs will differ from those in EFSA (2013). Measurement endpoints set around thresholds or limits of concern (following EFSA, 2010a,b,c) should reflect plausible environmental harms from the deliberate release of GDMIs. Depending on the expected outcome of the deliberate release of a GDMI, limits of concern will differ if the goal is population suppression versus population modification. Further, given the expected increase of spatial and temporal extent of some GDMIs, the use of small-scale physically and/or ecologically confined field trials would be one approach. It is anticipated that relevant information can be obtained through appropriately designed PMEM.

The use of multiplicative effect sizes (as outlined in EFSA (2013)) may be of limited use when the control of target organisms is the goal of a deliberate GDMI release. This needs more scrutiny. EFSA (2013) adequately considers a range of statistical principles such as the importance of phenotypic similarities and differences for comparative analyses, the importance of differences (e.g. in terms of risk hypotheses that can be addressed, scale of experiments that can be undertaken) between laboratory, small-scale physically and/or ecologically confined field trials and open release trials. However, the limits of confined space and environmental responses might be context dependent and highly non-linear for some GDMIs. As such, the focus on ANOVA is probably an inappropriate statistical principle to base risk evaluation of GDMIs around and stratified sampling through time and across space. Developing temporal and spatial approaches (e.g. Cressie and Wikle, 2011), would be better approaches to the statistical methodologies required to evaluate the potential environmental risks associated with GDMIs.

The requirements pertaining to statistical analysis (Section 3.5.3 in EFSA (2013)) are too prescriptive to be of benefit in assessing the potential environmental harms of GDMIs. Appropriate statistical analyses should be reflected through the specific choices of experimental designs and data collected.

In conclusion, the considerations given in Section 3.5 of EFSA (2013) are adequate, but may require further scrutiny to ensure that they are sufficient for the deliberate release of some of the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.3.6. Long-term effects [Section 3.6, including subheadings]

Section 3.6 of EFSA (2013) does not provide sufficient consideration of multiple generations, over which the GDMI characteristics could evolve through mutations and selection pressures, and the potential of some engineered gene drive to spread the genetic modification(s) of interest into target populations in wider and unintended receiving environments. This section of EFSA (2013) implies long-term effects arising from exposure to an increasing presence of GMAs and provides examples of delayed effects of invasive species, in which there is an increase in density over time. Further examples could be provided that are more relevant to population suppression strategies with GDMIs, in which populations would be expected to decline, causing exposure over time to diminish. Also, for an engineered gene drive-based modification strategy, the long-term effect would be due to the proportion of the population with gene expression rather than the density of the population (which would be expected to remain similar). For a modification strategy, the density may increase if other control efforts aimed at suppression stop. Effects of interbreeding could occur quite quickly in engineered gene drive systems that have a high potential to spread and persist.

Such long-term effects could occur through a causal chain of events or through mechanisms such as interactions with other organisms, transfer of genetic material, or changes in use or management (Directive 2001/18/EC). All these mechanisms considered by Directive 2001/18/EC are highly relevant

for GDMIs and go beyond that covered in Section 3.6 of EFSA (2013). However, these long-term effects require identified protection goals, and from the problem formulation approach, relevant risk hypotheses to be formulated. With these risk hypotheses in place, appropriate risk assessment methodologies, potentially with greater emphasis on the use of mathematical modelling tools, can be developed to assess long-term effects of GDMIs. Without clearly defined protection goals within the problem formulation approach to ERA, any open-ended expectations on the consequences of the long-term effects of GDMIs will substantially decrease the robustness of risk assessment and can lead to inconclusive risk evaluations.

In conclusion, the considerations on potential long-term effects of GMAs given in Section 3.6 of EFSA (2013) are adequate, but insufficient. Any future guidance on GDMIs may need to provide further considerations on long-term effects for the specific risks within defined protection goals for some of the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.3.7. Further guidance on modelling [Section 3.7]

While it is not possible to simulate the whole complexity of an ecosystem and that this is an unrealistic expectation, mathematical modelling has an important role to play in each step of the phased testing and release pathway of GDMIs (James et al., 2018; Golnar et al., 2020). Mathematical modelling provides a valuable contribution to the weight of evidence (rather than final proof) of aspects associated with performance characteristics, potential environmental harm and effectiveness of risk mitigation measures. Mathematical modelling is likely to be more important with some GDMIs than other GMIs due to the complexity of empirical studies. As there may be difficulties in validating model predictions, greater emphasis should be placed on the identification of key parameters. Moreover, the sensitivity of mathematical model predictions to the sensitivity of parameters is critical.

Appropriate and clear definition of model goals and assumptions (e.g. risk hypothesis; the limited ecology, temporal scales and spatial scales; which systems the models are applicable to/for; use in PMEM) for GDMIs go beyond those covered in EFSA (2013). Ecological outputs (e.g. changes in population numbers of an insect) may be less relevant than other metrics such as its vectorial and economic capacity.

It is expected that there will be a greater reliance of mathematical modelling to cope with increased spatial and temporal scales of some GDMI releases. Case-specific monitoring (CSM) will need more validity than in EFSA (2013) for the evaluation of model assumptions/predictions. Mathematical models could be given more value in designing appropriate ERA and PMEM schemes for the deliberate release of GDMIs. Further ecological work will be essential to enhance model predictions for such purposes.

EFSA has published guidance on good modelling practices (EFSA, 2014) that is relevant for the risk assessment of GDMI applications.

In conclusion, the considerations on mathematical modelling given in Section 3.7 of EFSA (2013) are adequate, but insufficient for some of the GDMIs considered in this GMO Panel Scientific Opinion. Greater use of models may be needed to address the long temporal scale and wide spatial scale of specific GDMI applications. ERAs will need to rely on modelled systems to describe expected outcomes, and guidance may be needed on model design, quality assurance, validation and interpretation. Any future guidance may need to focus on the sufficiency, use and application (with examples) of mathematical modelling tools to the ERA and PMEM of GDMIs.

5.1.3.8. Uncertainty analysis [Section 3.8, including subheadings]

Section 3.8 of EFSA (2013) provides considerations on how applicants should conduct and report an uncertainty analysis as part of the risk assessment. Since then, EFSA has published additional guidance on how to analyse and communicate uncertainty in scientific assessments (EFSA, 2018, 2019) that is relevant for the risk assessment of GDMI applications.

The considerations given in Section 3.8 of EFSA (2013) and additional EFSA guidance are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.3.9. Health and welfare aspects of genetically modified insects [Section 3.9, including subheading 3.9.3]

The European legislation related to health and welfare aspects of animals focuses on farmed animals and, only in exceptional cases, on wild animals. Consequently, no additional welfare risk assessment is needed for the GDMIs considered in this GMO Panel Scientific Opinion.

The considerations given in Section 3.9 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.4. Specific areas of risk for the environmental risk assessment of genetically modified insects [Section 4.2]

As explained in Section 1.2 of this GMO Panel Scientific Opinion, the scope of the adequacy and sufficiency evaluation of EFSA (2013) is limited to the use of engineered gene drives to control harmful insect species, in particular disease-transmitting insects, agricultural insect pests and invasive insects, and does not include the use of such gene drives for biodiversity conservation purposes or the enhancement of production systems.

The seven specific areas of risk for GMIs outlined in EFSA (2013), which build on those laid down in Annex II of Directive 2001/18/EC, are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.4.1. Persistence and invasiveness of genetically modified insects, including vertical gene flow [Section 4.2.1, including subheadings]

Section 4.2.1 of EFSA (2013) is designed to assess whether a GMI can persist in the environment (question 1), and hybridise with compatible relatives to produce viable and fertile offspring (question 2), and whether the genetic modification of interest can alter the fitness of the GMI (question 3) or its habitat and/or geographic range (question 4) compared with the non-GM comparator. This assessment primarily focusses on the overall fitness of the GM individuals and how the genetic modification of interest may alter their fitness, persistence and invasiveness compared to non-GM individuals.

While Section 4.2.1 of EFSA (2013) mentions the spread of transgenes to other locations and individuals by mating, it does not explicitly account for the biased inheritance potential of engineered gene drives. The statement in EFSA (2013) that 'the ability of a transgene to disperse and introgress into wild populations will depend to a large extent on the fitness and adaptation characters conferred by the transgene in different environments and populations' is not adequate for engineered gene drives. Engineered gene drive systems can be designed to overcome fitness costs associated with the genetic modification of interest, and to increase rapidly the frequency of the transgenic construct from low initial levels to fixation, or near fixation. Consequently, it is key to assess the intended and unintended spread[65] and persistence of engineered gene drives (see Sections 3.2–3.4), along with an assessment of the fitness of the gene drive modified individuals.

While the potential of engineered gene drives to spread and persist in target populations will be case-specific, their spread is intended to achieve the intended outcomes in terms of population suppression or modification. Section 4.2.1 of EFSA (2013) covers the risk assessment of genetically modified individuals deliberately released into the environment and their progeny, but does not explicitly address the gene drive ability to cause the intended spread and persistence of an engineered gene drive. Understanding how engineered gene drive systems may spread and persist in target populations in the field, and their potential intended outcomes is crucial for the assessment of potential risks to humans, animals and the environment associated with the deliberate release of GDMIs in the environment. Prediction of spread and persistence of engineered gene drives in target populations in the field, which could be at larger spatial scale and longer temporal scale for some self-sustaining engineered gene drives, will require mathematical modelling and data analysis that can inform on these temporal and spatial scales of interest (James et al., 2020).

Regarding the potential of GMIs to hybridise with compatible relatives to produce viable and fertile offspring, it should be noted that cross-species fertilisation is rare in insects and hybrids are rarely fertile. Thus, only closely related species may have fertile offspring (Besansky et al., 1997; Oliveira et al., 2008; Fontaine et al., 2015; Miles et al., 2017) with often reduced fitness. While this aspect is not different between GMI and GDMIs, once hybridisation occurred the engineered gene drive might enhance its spread and of potential cargo/payload genes (Courtier-Orgogozo et al., 2020). Data analyses of genome sequences of species phylogenetically closely related to the target species, as well as laboratory experiments could be informative to assess whether an engineered gene drive construct can drive in related species. Such data can also provide insights into population structure, and rates of hybridisation among sibling species (James et al., 2020).

In conclusion, considerations/requirements on persistence and invasiveness, including vertical gene flow, given in Section 4.2.1 of EFSA (2013) are adequate, but insufficient for the GDMIs considered in

[65] Spreading of an engineered gene drive beyond the defined target organism(s) and associated populations should be considered unintended.

this GMO Panel Scientific Opinion. Any future guidance on GDMIs may need to address the spread and persistence of engineered gene drives in the field.

5.1.4.2. Horizontal gene transfer [Section 4.2.2, including subheadings]

EFSA (2013) defines horizontal gene transfer (HGT) as any process in which an organism incorporates genetic material from another organism into its genome without being the offspring of that organism. The evaluation of the impact of HGT from GMIs includes analysis of the potential of exposure and transfer of recombinant DNA from GMIs and further spread to other organisms. Furthermore, if HGT can occur, the consequences of such transfer events for human and animal health and the environment must be evaluated.

The considerations/requirements given in Section 4.2.2 of EFSA (2013) for the assessment of the probability and frequency of HGT from GMIs to other organisms such as insects or microorganisms are based on the assumption that the genetic modification of interest may increase the likelihood of rare HGT events becoming established in those organisms. While there is clear evidence of HGT between insects (involving primarily transposable elements; Peccoud et al., 2017), there is only little evidence of HGT from insects to bacteria to date (Woolfit et al., 2009).

EFSA (2013) does not explicitly address that some engineered gene drives, primarily homing-based engineered gene drives, may facilitate the potential for HGT. By definition, an engineered gene drive itself will affect the mobility of the associated transgenic construct from a chromosomal locus to another, and in this respect, it could be compared with transposable elements. Because mobile elements represent a major source of HGT between insects (Peccoud et al., 2017), GDMIs containing homing-based engineered gene drives may theoretically increase the potential for HGT compared with GMIs that do not contain such an engineered gene drive. When the target sequence of an engineered gene drive is present in a non-target organism, the potential for HGT could be increased via the induction of a double-stranded DNA break in the target sequence of the non-target genome facilitating the integration of the engineered gene drive construct in this locus (Yamamoto and Gerbi, 2018). In addition, if the target sequence is flanked by sequences homologous to the gene drive cassette in the non-target organism, this will facilitate the integration of the engineer gene drive construct and its spread and persistence. If, based on the above, the HGT potential increases, then the probability that it will exhibit gene drive characteristics (i.e. spread within the non-target organisms) needs further assessment, in accordance with EFSA (2013). Consequently, information on the molecular elements of the transgenic construct is needed to assess the altered HGT potential for homing-based engineered gene drives.

In conclusion, the considerations/requirements given in Section 4.2.2 of EFSA (2013) are adequate, but insufficient for some of the GDMIs considered in this GMO Panel Scientific Opinion. Any future guidance on GDMIs containing site-directed nuclease (SDN)-based gene drives (e.g. CRISPR-Cas9) may need to consider engineered gene drive characteristics for the assessment of HGT and possible consequences thereof.

5.1.4.3. Pathogens, infections and diseases [Section 4.2.3, including subheadings]

Section 4.2.3 of EFSA (2013) is relevant for disease vectors.

Since some GDMIs may operate at large scale and/or over a long term, the problem formulation should consider whether all diseases that can be transmitted by a disease vector should be taken into account, or only the ones circulating in the particular receiving environment, and when species relationship justify this possibility.

Different selection pressures are likely to be placed on the pathogen and its vector insect with some GDMIs; the selective pressure will be particularly high in modification strategies due to long-term exposure which may impact pathogen–insect interactions. Risks will thus differ between GDMIs and GMIs (for which there is no population modification at present).

In conclusion, the considerations/requirements given in Section 4.2.3 of EFSA (2013) are adequate, but insufficient for some of the GDMIs considered in this GMO Panel Scientific Opinion. Since EFSA (2013) focusses on short-term effects arising from rearing processes and genetic insertions, and the effects of these in the immediate generations after deliberate release, any future guidance on GDMIs may need to address the longer potential exposure arising with some GDMIs.

5.1.4.4. Interactions of genetically modified insects with target organisms [Section 4.2.4, including subheadings]

As part of the problem formulation, it is critical to specify intended uses of the GMI and mechanisms of the genetic modification of interest, as stated in Section 4.2.4 of EFSA (2013). Target

organisms may include an individual population, single species, species complex (covering all strains and sibling species where reasonable levels of hybridisation or introgression can occur in the field), or a set of partially reproductively connected species. The extent of the set of target organisms should be defined by the applicant, in relation to the intended outcomes of a GDMI. Depending on the definition of the target organism and populations, intended outcomes may differ across the spectrum of such a complex.

Target populations are expected to be genetically heterogeneous, and so interactions between transgene and genetic background may be complex and difficult to predict. For engineered gene drives that are intended to spread over wide areas and persist in target populations, the diversity of interactions with target populations and their diverse receiving environments is likely to be greater than anticipated in EFSA (2013), and this should be addressed explicitly. James et al. (2018, 2020) recommend gathering data on the efficacy of engineered gene drives from gene drive modified individuals with a genetic background as similar as possible to that of local target individuals found at the site(s) of the proposed deliberate release (see also HCB, 2017). If deliberate releases will occur at multiple sites, but those sites are connected geographically or otherwise not reproductively isolated, a single colony derived from locally collected individuals may suffice. If the deliberate release sites are distant, the authors recommend the use of models of engineered gene drive spread to determine whether additional local colonies might be needed (James et al., 2018, 2020).

As is the case with any genetic control system, engineered gene drives are expected to continue to be subject to evolutionary processes (Bull, 2015; Marshall et al., 2019). Resistance to an engineered gene drive or cargo/payload genes may evolve. Resurgence of an intrinsically harmful target organism due to failure of an engineered gene drive or resistance to either the drive or its cargo/payload genes (e.g. through assortative mating) could cause harm like any other disease vector/pest control strategy. Consequently, a consideration for the ERA could include the risk that the population developing from the released gene drive modified individuals at some point has different effects on the target population than intended, for example due to loss of efficacy.

Loss of efficacy could evolve due to assortative mating and mutations in: (1) the transgenic construct itself, including the cargo/payload genes in case of population modification; (2) strategies aimed at coupling a cargo/payload gene to the engineered gene drive; and (3) the target sequence of the target organism's genome that renders the site no longer recognisable by the gRNA (Burt, 2003; Sinkins and Gould, 2006; Ward et al., 2011; Beaghton et al., 2017a,b; Unckless et al., 2017; Bull et al., 2019; Price et al., 2020). This target site resistance can result from variation in the population or can be induced by the nuclease activity itself, where repair by end-joining or imprecise/incomplete HDR can produce variant, non-cleavable alleles (James et al., 2020). Consequently, the likelihood that resistance evolves in the target species in response to the engineered gene drive will vary between different types of gene drives. For example, resistance as a result of spontaneous mutation in target sequences is more likely to arise in population modification strategies because they will need to function over long periods of time at relatively high densities (James et al., 2020). It is relevant for the different mechanisms of resistance to be addressed, and whether resistance evolves due to mutations in the transgenic construct itself (including the linkage between the gene drive and cargo/payload genes) and/or target sequences. The latter may require knowledge of mutation/resistance allele generation rate, failure rate of an engineered gene drive and the stability of the engineered gene drive system and cargo/payload genes over time (Marshall et al., 2019). The frequency of natural variation at the target locus can be examined by genome sequencing of individuals from the target population collected from the field, acknowledging that: (1) rare genetic variants will be difficult to detect; and (2) genomic sequence data for engineered gene drives with a large intended geographic range may not be as comprehensive as desired (James et al., 2020).

There may be larger space and longer time issues in terms of efficacy. In some cases, defining efficacy, and hence its failure, may be difficult over the variable spatial and temporal dimensions that are relevant to the types of engineered gene drive.

For population suppression strategies based on engineered gene drives, measurement endpoints may need to address size, density, age structure and sex ratio of the target population, and also the spread and persistence of the engineered gene drive, in addition to the EFSA (2013) paragraph on endpoints.

In conclusion, the considerations/requirements given in Section 4.2.4 of EFSA (2013) are adequate, but insufficient for some of the GDMIs considered in this GMO Panel Scientific Opinion. Any future guidance may provide further considerations on the different possible mechanisms of resistance that can arise, and the release and subsequent self-sustaining generations, over increasing spatial range –

not just the release generation. Moreover, there may be a need to consider longer time periods and uncontrolled self-replication in the field.

5.1.4.5. Interactions of genetically modified insects with non-target organisms [Section 4.2.5, including subheadings]

EFSA (2013) details the many ways how GMIs may interact with non-target organisms, and how ERA can be developed: the approaches given remain valid. The challenge is to avoid disproportionate open-ended data collection exercises by focussing on potential harm to protection goals as defined within the ERA, which are likely to include levels of biodiversity and/or ecosystem services. For deliberate releases for population modification, the effects will depend on the intended traits that are being introduced. These may be different from any seen in GMIs to date.

In conclusion, the considerations given/requirements given in Section 4.2.5 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.4.6. Environmental impacts of the specific techniques used for the management of genetically modified insects [Section 4.2.6, including subheadings]

EFSA (2013) underlines the importance of comparing the impacts of management techniques associated with the release of the GMI, which again raises the importance of the selection of appropriate comparators. EFSA (2013) notes that the management techniques include the process of developing the GMI populations (e.g. the production of wastes) as well as management once released (e.g. changes to insecticide use). The importance of scale of the deliberate release is noted (Step 3). EFSA (2013) notes the value of analogous situations from insect disease vector/pest control for providing data and mathematical models for analysis of impacts on defined relevant protection goals. Some engineered gene drives may operate over larger space and longer time. Risk characterisation based on modelled scenarios may be particularly appropriate for GDMIs.

In conclusion, the considerations given/requirements given in Section 4.2.6 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.4.7. Impacts of genetically modified animals on human and animal health [Section 4.2.7, including subheadings]

The deliberate release into the environment of GDMIs considered in this GMO Panel Scientific Opinion is not intended for food/feed uses. Since ingestion or intake of GDMIs or parts of them by humans or livestock would be accidental, exposure is expected to be extremely low. Based on current knowledge, the GMO Panel is of the opinion that variations in the level of compound(s) in GMOs are generally not large enough to impact the nutritional or safety characteristics of an ingredient even under low exposure conditions (EFSA, 2017). Consequently, a compositional analysis is not considered necessary for the GDMIs considered in this GMO Panel Scientific Opinion. However, as outlined in Section 4.2.7 of EFSA (2013), there may be plausible pathways to harm for humans in particular cases (e.g. blood-feeding mosquitoes through biting) that may need consideration. This is particularly true for GDMIs designed to express antiparasitic or antiviral agents in the salivary glands. The need for specific data should be triggered by the need to test specific risk hypotheses formulated as part of the problem formulation (and which are framed by agreed protection goals). In this respect, it is key to specify precisely what differences between the GMI and non-GMI are important in the comparison through limits of concerns, so that their health consequences can be assessed.

In the case of population modification, the extended temporal dimension of GDMIs may need consideration.

In conclusion, the considerations/requirements given in Section 4.2.7 of EFSA (2013) are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion. Considerations/ requirements that are explicitly tailored to the food/feed safety assessment of GMAs are applicable only if they specifically address: the accidental ingestion or intake of GMAs or parts of them by humans or livestock, or exposure of persons to the GMA and derived material as part of their professional activities.

5.1.5. Post-market environmental monitoring [Section 5]

Directive 2001/18/EC introduces the obligation for applicants to implement monitoring plans to trace and identify any direct or indirect, immediate, delayed or unforeseen effects on human health or the environment of GMOs as or in products after they have been placed on the market. The objectives

of post-market environmental monitoring (PMEM) according to Annex VII of Directive 2001/18/EC are twofold: (1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO, or its use, in ERA are correct; and (2) to identify the occurrence of adverse effects of the GMO, or its use, on human or animal health or the environment which were not anticipated in ERA. PMEM is composed of case-specific monitoring (CSM) and general surveillance (GS). Because CSM and GS have different objectives, their underlying concepts differ. CSM is tailored to determine whether, and to what extent, anticipated adverse effects occur during the deployment of a GMO, and thus to relate changes observed to specific causes. It is mainly triggered by risks and/or significant levels of critical uncertainty linked to the GMO and its management that have been identified in the ERA. CSM is therefore hypothesis driven and should be targeted at the assessment endpoints and protection goals identified in the ERA conclusions as being at risk, or where levels of critical uncertainty were identified in relation to potential risks associated with the GMO. In GS, by contrast, the general status of the environment that is associated with the deployment of the GMO is monitored without any preconceived hypothesis to detect effects that were not anticipated in the ERA. Should any such effects be observed, they are studied in more detail to determine whether the effect is adverse and whether it is associated with the deployment of a GMO. Whereas the need for CSM depends upon the conclusions of the ERA, GS is mandatory for any placing on the market of a GMO.

PMEM should serve as an early warning system that could lead to the activation of additional risk management actions.

The adequacy and sufficiency of PMEM considerations/requirements are assessed and reported below.

5.1.5.1. Case-specific monitoring [Section 5.1]

CSM is used to confirm that assumptions regarding the occurrence and impact of potential adverse effects of a GMI or its use characterised in the ERA are correct (EFSA, 2013). In this respect, it is important that the operational protection goals identified for ERA, along with the ERA conclusions, frame CSM. CSM can also support the assessment of the outcome of the deliberate releases, and for any further management actions. This would apply to GDMIs as to other GMI applications.

EFSA (2013) explains the basis of CSM, but provides little specific information to guide the CSM of GMAs. More direction is needed to ensure that CSM is fit for purpose for GDMIs and provides evidence that can feed back into the ERAs of future deliberate releases. Clear description of CSM is even more important for GDMIs than for other GMIs, as the potential impacts of deliberate releases may not be spatially or temporally constrained and any changes to the transgenic construct may require rapid management intervention. Spatial and temporal scales will be greater with most GDMI applications than other GMI applications, and reversibility may depend on the nature of the engineered gene drive. The point about the large-scale and long-term impact is particularly relevant to self-sustaining engineered gene drives because temporal/spatial scales are increased. Consequently, engineered gene drives will require PMEM to be dynamic and spatially explicit, tracking spread and persistence over space and time, including areas beyond the expected range of the release, and possibly across jurisdictional boundaries. The dynamics of GDMIs take place in a dynamic context, with changes in (e.g.) climate, land use, immunity, pathogen load, pesticide resistance prevalence. Therefore, CSM must explain both the approach to data acquisition and data interpretation.

CSM is more important for GDMI applications than other GMI applications as the stepwise/staged/tiered testing approach, even if complemented by mathematical modelling, may still leave some uncertainty before open field testing or field implementation of a GDMI for some engineered gene drives. It is therefore important that CSM is scientifically designed and implemented. CSM of environmental effects may need to take place in representative areas where the GDMI is deliberately released. The spatial and temporal scale of CSM will need to be adapted according to the spatial and temporal distribution of the GDMI in the environment.

Mathematical modelling will be important as a design tool for sampling protocols to define expectations of intended outcomes, deviations and responses. There should be clear triggers for management responses, based on modelling, for particular monitoring results/events. There is also a need to monitor changes in the target organisms and populations over time and space – due to changing conditions of climate, land use, immunity, pathogen load, insecticide resistance prevalence, etc. For GDMIs (compared to other GMIs), there is a strong and compelling case for mathematical modelling approaches, scenarios and sensitivity analyses to evaluate such changes. CSM strategies may need to be organised in broad zones based on target organism challenges by location or season. The likely scale of management will determine the scale of monitoring, both in space and time. The

heterogeneity of spread and persistence of engineered gene drives could greatly affect the spatial scale of monitoring. Over time, patterns of population dynamics may indicate critical or less critical timing of monitoring.

CSM is likely to be adaptive in nature, focussing resources in the light of data. Evidence should be provided of the capacity to undertake adaptive, targeted CSM that may lead to additional management interventions, and where needed long-term monitoring.

Guidance should be practicable. In particular, appropriate monitoring tools are needed to distinguish between wild-type target individuals, gene drive modified individuals and hybrids (especially over many generations after GDMI deliberate releases).

In conclusion, considerations/requirements on CSM given in Section 5.1 of EFSA (2013) are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.1.5.2. General surveillance [Section 5.2]

In light of Directive 2001/18/EC on the deliberate release into the environment of GMOs and the Commission Directive (EU) 2018/350 amending Directive 2001/18/EC, EFSA (2013) identifies that GS is required, and that the ERA should list the GS tools to be applied, including monitoring networks, literature reviews and questionnaires. Inevitably such GS is not specifically targeted at particular indicators relevant to either assumptions in the ERA or to some particular harm to the environment. As such, GS is not useful in the PMEM of GDMIs. EFSA (2013) highlights challenges to GS, including the difficulty of detecting change, determining harm and associating change with the GMA. These issues are equally applicable to GDMIs. With engineered gene drive systems, the spatial and temporal scale of potential adverse environmental effects are likely to be much greater for self-sustaining non-localised systems than for self-limiting ones, and this may exacerbate the inherent challenges of GS in the longer term and at greater distances from a release.

In conclusion, considerations/requirements on GS given in Section 5.2 of EFSA (2013) are inadequate for PMEM and require more thorough consideration for GDMIs. As such, the details on GS are neither adequate nor sufficient for the GDMIs considered in this GMO Panel Scientific Opinion. Any future guidance on GMOs, including GDMIs, may require greater emphasis on the applicability of GS.

5.2. Adequacy and sufficiency of EFSA guidelines (EFSA, 2012) for the molecular characterisation of gene drive modified insects

The GDMIs considered in this GMO Panel Scientific Opinion are intended for deliberate release into the environment to control disease-transmitting insects, agricultural insect pests and invasive insects. The GDMIs considered in this Opinion are not intended for food/feed uses. Thus, the evaluation of EFSA (2012) for its adequacy and sufficiency for the MC of GDMIs is tailored towards ERA and PMEM needs. Besides the MC-related considerations/requirements given in Sections 2.1.1 and 2.1.2 of EFSA (2012), where appropriate, those laid down in Section II of Annex III A of Directive 2001/18/EC are considered.

5.2.1. Information relating to the recipient or (where appropriate) parental animals [Section 2.1.1]

The considerations/requirements given in Section 2.1.1 of EFSA (2012) and Section II A of Annex III A of Directive 2001/18/EC are intended to support the risk assessment of food/feed containing, consisting of, or produced from GMAs, instead of the ERA of GMAs. Therefore, specific aspects relevant for the ERA and PMEM of GMAs, including GDMIs, are not covered by EFSA (2012). These include: (1) the assessment of the persistence and invasiveness of GDMIs, including vertical gene flow; and (2) the potential for resistance to evolve.

1) For the assessment of the persistence and invasiveness of a GDMI, including vertical gene flow (see Section 5.1.4.1), a thorough description and understanding of the biology of the target species (e.g. potential for interbreeding with other species, polymorphism in the population, disease vector competence, etc.) may be required. This is consistent with the requirements outlined in Directive 2001/18/EC (e.g. organisms with which transfer of genetic material is known to occur under natural conditions, pathological, ecological and physiological traits, nature of indigenous disease vectors etc.);
2) For the assessment of the potential for resistance to evolve (see Section 5.1.4.4), the following aspects may need consideration, dependent on the engineered gene drive system:

- Possible occurrence of parthenogenetic individuals in target populations, which would escape sexual reproduction;
- Potential for assortative mating within and between target populations;
- Possible polyploidy in target populations;
- The occurrence of polymorphisms in terms of sequence for the target gene(s) in target populations, and the rate of occurrence of such 'resistant' individuals (see Section 5.1.4.4);
- Possible biased repair of the SDN-mediated DSBs via NHEJ rather than homologous recombination (HR). Relevant data on the general mechanism of repair of DSBs (NHEJ vs. HR ratio) in target populations could be informative (specific repair of the target sequence is addressed in Section 5.2.2.2).

Concerning the monitoring of target populations after release of the gene drive modified individuals, information on the genome sequences of GDMIs and target populations can be useful. Information on the genomes, in addition to other tools (see Section 5.1.5) can contribute to characterising the dynamics of target populations post-release. However, it is noted that despite recent advances, genome sequencing data are still subject to some limitations such as the presence of repetitive sequences, heterozygosity or polymorphism (especially concerning the target populations). In some cases, these limitations may hamper data interpretability.

In conclusion, the considerations/requirements given in Section 2.1.1 of EFSA (2012) and Section II A of Annex III A of Directive 2001/18/EC are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Considerations/requirements that are explicitly tailored to the food/feed safety assessment of GMAs are applicable only if they specifically address: the accidental ingestion or intake of GMAs or parts of them by humans or livestock, or exposure of persons to the GMA and derived material as part of their professional activities (see Section 5.1.2.2).

5.2.2. Molecular characterisation [Section 2.1.2]

EFSA (2013) states that sufficient information should be provided on the genetic modification to identify the nucleic acid intended for transformation and related vector sequences potentially delivered to the recipient animal, and to characterise the DNA actually inserted in the animal and expression and stability of the intended trait(s). Section 2.1.2 of EFSA (2012) describes the considerations/ requirements for the MC of GMAs. The adequacy and sufficiency of these considerations/requirements are assessed and reported below.

5.2.2.1. Information relating to the genetic modification [Section 2.1.2.1]

Description of the methods and vectors used for the genetic modification [Section 2.1.2.1.1]

The considerations/requirements given in Section 2.1.2.1.1 of EFSA (2012) and Section II C.1 of Annex III A of Directive 2001/18/EC are adequate and sufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Source and characterisation of nucleic acid intended to be inserted [Section 2.1.2.1.2]

Aspects of the characterisation of the GDMIs considered in this GMO Panel Scientific Opinion that may require further consideration for their ERA, include:

- The engineered gene drive and its design covering both the underlying mechanisms involved (e.g. CRISPR-Cas9) and their (multiple) components (e.g. Cas9 protein and sgRNA and targeted sequence). In particular, information on the potential functionality of the engineered gene drive system in non-target cells may be needed for the HGT assessment (see Section 5.1.4.2);
- The stability and specificity of expression of the engineered gene drive system;
- The characteristics of any cargo/payload gene(s) linked to the engineered gene drive, and its/ their function;
- The molecular approaches used to detect and follow the intended and unintended spread of the genetic modification(s) of interest and its persistence in target populations.

In conclusion, the considerations/requirements given in Section 2.1.2.1.2 of EFSA (2012) and Section II C.1 of Annex III A of Directive 2001/18/EC are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Considerations/requirements that are explicitly tailored to the food/feed safety assessment of GMAs (i.e. information on the history of consumption of the gene product(s) arising from the regions intended

for insertion, and data on the possible relationship of the gene products with known toxins, anti-nutrients, allergens and other compounds with potential adverse health effects) are applicable only in conjunction with the accidental ingestion or intake of GMAs or parts of them by humans or livestock, or exposure of persons to the GMA and derived material as part of their professional activities.

5.2.2.2. Information relating to the genetically modified animal [Section 2.1.2.2]

General description of the trait(s) and characteristics introduced or modified [Section 2.1.2.2.1]

Aspects of the characterisation of the GDMIs considered in this GMO Panel Scientific Opinion that may require further consideration for their ERA, include:

- The target sequence (including any available information on the polymorphism in the population targeted);
- The nature of the target sequence (e.g. within a conserved domain of a particular protein);
- The ratio of NHEJ vs. HR repair resulting from the cleavage of the targeted sequence(s);
- The characterisation of the NHEJ repair step following the cleavage of the targeted sequence including information about the consequences on the targeted sequence(s) (e.g. whether the targeted gene remains functional);
- The pre-existence of resistance alleles to the cargo/payload genes in the target population;
- The possible occurrence of resistance alleles to the engineered gene drive itself;
- The size of the homologous sequences used for homing;
- Single/multiple target sites (within the same gene or in multiple genes);
- Cleavage efficiency of the target sequence including information on any additional steps to increase efficiency (e.g. activation/repression of other genes);
- The characterisation of the protein(s) newly expressed in the GDMI or modified endogenous proteins including information on its/their biological role (e.g. protein structure/function);
- Possible interruption of molecular pathways, possible metabolites accumulation, altered substrate specificity in case of enzymes, etc.

In conclusion, the considerations/requirements given in Section 2.1.2.2 of EFSA (2012) and Section II C.2 of Annex III A of Directive 2001/18/EC are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Information on the sequences actually inserted/deleted or altered [Section 2.1.2.2.2]

Unlike SDN-based GMO approaches, the SDN complex of a SDN-based GDMI is intended to remain active in the target population. Consequently, besides the intended spread of the genetic modification of interest through predicted repair (HR) at the target site, GDMIs may give rise to intended and unintended 'on-target' and unintended 'off-target' sequence modifications in the genome of target populations and their progeny (e.g. Sander and Joung, 2014; Taning et al., 2017). Repair at the target site may potentially produce resistant alleles in the target population (see Section 3.3.1.1). In addition, off-target activity in individuals of target populations can produce mutations that may decrease fitness, influencing characteristics such as survival, mating success and fecundity (James et al., 2020). Off-target effects may be more important for population modification strategies where the genetic modification of interest must remain present and active at high frequency in target populations over long periods of time (James et al., 2020).[66] In some cases, *in silico* analysis could help the identification of potential off-target effects in target populations. However, care is required when interpreting such data, as they are subject to some limitations (i.e. natural population heterogeneity: presence of repetitive sequences, heterozygosity or polymorphism). Off-target activity can also be evaluated by following fitness and other phenotypic changes (James et al., 2020).

In conclusion, the considerations/requirements given in Section 2.1.2.2.2 of EFSA (2012) and Section II C.2 of Annex III A of Directive 2001/18/EC are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Considerations/requirements pertaining to the food/feed safety assessment of GMAs are only applicable in conjunction with the accidental ingestion or intake of GMAs or parts of them by humans or livestock, or exposure of persons to the GMA and derived material as part of their professional activities.

[66] Less off-target sequence modifications are expected to occur in the genome than those caused by most mutagenesis techniques (see Abrahamson et al., 1973 for irradiation, for example), but they would be additive to inevitable spontaneous mutations (Katju and Bergthorsson, 2019).

Information on the expression of the inserted/modified sequence [Section 2.1.2.2.3]

The use of information on the expression of the inserted/modified sequences to inform the ERA of GDMIs will depend on the intended outcome of the GDMI application. Information on the expression of the inserted sequences can inform the ERA as regards the potential impact on other organisms (e.g. toxicity to non-target organisms), or on the level of nuisance caused by the GDMI (e.g. allergenicity due to mosquito bites) (Sections 5.1.4.4, 5.1.4.5 and 5.1.4.7). Therefore, the level and site of expression of the engineered gene drive components (e.g. Cas9 and sgRNA(s)) and the cargo/payload genes linked to the gene drive (if any) can be informative. Information on the expression of any modified sequences resulting from the gene drive cassette insertion (i.e. gene(s) targeted by the engineered gene drive or gene(s) present in the flanking regions of the gene drive cassette insertion locus) can also inform the assessment of the potential impact on other organisms (e.g. non-target organisms). For engineered gene drives that are designed to achieve the expected phenotype through molecular interactions (e.g. through multiple gene targeting, see section below) additional information may be needed for the assessment of those GDMIs to assess those interactions.

In conclusion, the considerations/requirements given in Section 2.1.2.2.3 of EFSA (2012) and Section II C.2 of Annex III A of Directive 2001/18/EC are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Considerations/requirements pertaining to the food/feed safety assessment of GMIs are applicable only in conjunction with the accidental ingestion or intake of GMAs or parts of them by humans or livestock, or exposure of persons to the GMA and derived material as part of their professional activities.

Inheritance and genetic stability of the inserted/modified sequence and phenotypic stability of the genetically modified insect [Section 2.1.2.2.4]

The concepts of inheritance and genetic and phenotypic stability as outlined in Section 2.1.2.2.4 of EFSA (2012) may need further consideration to address the potential for preferential inheritance of engineered gene drives, and the broad array of possible GDMI applications and their intended outcomes. For example, phenotypic stability of an engineered gene drive for population suppression will lead to reduced fitness (leading to mortality) of the gene drive modified individuals. In case of population modification strategies, phenotypic stability will be driven by the intended outcome conferred by the cargo/payload gene(s). In addition, some engineered gene drive systems can be designed to target multiple genes and the products of those genes themselves may interact to yield the intended phenotype. In some cases, genetic elements can be segregated out intentionally as part of the gene drive strategy (e.g. daisy-chain drives). Furthermore, the possibility of unintended genetic modification(s) in target populations (e.g. NHEJ repair of the CRISPR-Cas-mediated DSB or 'non-perfect' HR events) will generate new genetic modifications as the gene drive spreads. Consequently, additional approaches may be needed to detect such events and evaluate their inheritance and stability. Depending on the engineered gene drive strategy, this may require continued monitoring of genetic and phenotypic stability over multiple generations under confined conditions as part of ERA, as well as in the field as part of PMEM.

The aspects mentioned above will complicate the definition of genetic and phenotypic stability as stated in EFSA (2012).[67]

In conclusion, considerations/requirements given in Section 2.1.2.2.4 of EFSA (2012) and Section II C.2 of Annex III A of Directive 2001/18/EC are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

5.2.2.3. Conclusions of the molecular characterisation [Section 2.1.2.3]

Aspects of the characterisation of the GDMIs that may require further consideration for the ERA of GDMIs, include:

- The MC of the engineered gene drive system, including the underlying mechanisms involved and intended outcome of the release;
- An assessment of possible interactions between the multiple engineered gene drive components, if the transgenic construct is composed of multiple elements that can segregate out intentionally as part of the gene drive strategy;

[67] The applicability of the concept of transformation event as currently implemented for GMOs may require further consideration for the progeny of GDMIs deliberately released into the environment.

- Proof of the efficiency, stability and inheritance of the engineered gene drive system before and during deliberate release;
- MC approaches for the continued monitoring of intended and unintended effects.

In conclusion, the considerations/requirements given in Sections 2.1.1 and 2.1.2 of EFSA (2012) and laid down in Section II of Annex III A of Directive 2001/18/EC are adequate, but insufficient for the GDMIs considered in this GMO Panel Scientific Opinion.

Considerations/requirements pertaining to the food/feed safety assessment of GMAs are applicable only in conjunction with the accidental ingestion or intake of GMAs or parts of them by humans or livestock, or exposure of persons to the GMA and derived material as part of their professional activities.

6. Conclusions

The GMO Panel considers it both timely and appropriate to evaluate its existing risk assessment guidelines for their adequacy and sufficiency for the MC, ERA and PMEM of GDMIs, primarily disease-transmitting insects, agricultural insect pests and invasive insects, for deliberate release into the environment.

It is timely because:

- Some GDMIs have been developed to the stage that proposals for deliberate release into the environment are now being anticipated for disease vector/pest control (e.g. Feachem et al., 2019; Scudellari, 2019), though not necessarily in the EU;
- International discussions on the development of further risk assessment guidance on GDMOs and their regulatory oversight are ongoing under the Cartagena Protocol on Biosafety and the Convention on Biological Diversity, respectively (e.g. AHTEG, 2020; Keiper and Atanassova, 2020).

It is appropriate because:

- The current EFSA (2012, 2013) guidelines are not specific to GDMOs, and guidance tailored to GDMIs can be more specific, making it more relevant, effective and efficient for risk assessors, risk managers and applicants to collect, assess or act on the required information/data in a timely and proportionate manner;
- The scientific understanding of engineered gene drives has advanced significantly in recent years, and the GMO Panel is, therefore, more able to provide considerations relevant to the MC, ERA and PMEM of the GDMIs addressed in this GMO Panel Scientific Opinion than in the past.

The conclusions below are organised according to the five main points of the mandate of the European Commission:

1) The role of problem formulation for the ERA of GDMIs for deliberate release into the environment (see Section 4.1, above);
2) The identification of risks and potential novel hazards on human and animal health and the environment (see Sections 4.2 and 4.3, above);
3) The consideration of relevant comparators (see Section 5.1.3.3, above);
4) The adequacy and sufficiency of the EFSA (2012, 2013) guidelines for the MC, ERA and PMEM of GDMIs (see Section 5, above); and
5) The need for updated guidance in specific areas.

6.1. Role of problem formulation for the environmental risk assessment of gene drive modified insects for deliberate release into the environment

- Robust ERAs require an explicit problem formulation, which should involve among other steps: (1) identifying protection goals and making them operational for use in ERA; (2) devising plausible pathways to harm that describe how the deliberate release of a GDMI could be harmful; (3) formulating risk hypotheses about the likelihood and severity of such events; (4) identifying the information that would be useful to test the risk hypotheses; and (5) developing a plan to acquire new data for hypothesis testing should tests with existing information be insufficient for decision-making;

- Since some currently used insect disease vector/pest control strategies are known to cause harm, an important consideration when setting specific protection goals for the deliberate release of GDMIs is whether the proposed activity may lead to more or less harm, or new harms, compared with current practices;
- When devising pathways to harm, potential pathways to harm should be systematically explored, and then prioritised based on their validity and consequences. If the validity or consequences of a pathway to harm cannot be defined, efforts to consider existing knowledge can be expanded, and/or that pathway can be carried forward into the analysis;
- All potential pathways to harm should be reported transparently. Moreover, a rationale justifying why potential pathways to harm are not considered sufficiently valid and/or consequential should be reported transparently for each potential pathway rejected;
- Some risk hypotheses may be difficult to test or testing using available information may not produce definitive conclusions regarding the likelihood of a particular step in a pathway to harm. Such uncertainty may be addressed through an iterative, stepwise/staged/tiered testing approach, by consideration of multiple lines of evidence including modelling, and/or by new studies being undertaken. However, in some cases, uncertainties may remain that must be addressed by risk managers and decision makers;
- Enhanced dialogue between risk assessors and risk managers along with stakeholder/societal engagement is advocated to define specific protection goals, decision-making criteria and the identification of potential pathways to harm for the ERA of GDMIs.

6.2. Identification of risks and potential novel hazards associated with the deliberate release of gene drive modified insects into the environment

- The identification of risks, potential novel hazards and potential challenges for the risk assessment and PMEM of GDMIs for deliberate release into the environment is inevitably hypothetical to some extent, as no GDMI application has been submitted for regulatory approval in any jurisdiction globally, and no direct regulatory, ERA and PMEM experience has been gained with GDMI deliberate releases at the time of writing;
- Specific aspects of GDMIs that are potentially novel compared to naturally occurring gene drives and disease vector/pest control strategies that involve the release of GMIs that do not contain an engineered gene drive (primarily RIDL and fsRIDL) and the release of non-GMIs (SIT, *Wolbachia*-mediated IIT and PI, and CBC) have been analysed, focusing on: (1) the preferential inheritance of a transgenic construct; (2) the intended spatial and temporal scale of spread of the genetic modification of interest; (3) the scale of population suppression; (4) population modification strategies; (5) target populations and environments; and (6) lack of spatio-temporal controllability;
  - The preferential inheritance of a transgenic construct, the intended spatial and temporal scale of spread of the genetic modification(s) of interest, and population modification strategies can be considered as novel aspects of GDMIs when compared with naturally occurring gene drives and current disease vector/pest control strategies that involve the release of insects;
  - Aspects that are not considered novel include: the scale of population suppression, target populations and environments, and lack of spatio-temporal controllability, because:
    - SIT and CBC have been used at a local and area-wide scale to suppress target populations, involving repeated releases over time to reach and maintain suppression;
    - Current and emerging disease vector/pest control strategies can target non-domesticated or wild species in non-managed environments;
    - *Wolbachia*-mediated PI and CBC often lack spatio-temporal controllability;
  - Whether the novel aspects of GDMIs represent potential novel hazards, and may introduce additional factors into the risk assessment of some GDMIs, needs to be assessed on a case-by-case basis as part of a specific problem formulation;
- Previously proposed risks on broad protection goals (such as human and animal health, and the environment) associated with the deliberate release of GDMIs, and potential challenges related to the risk assessment and PMEM of GDMIs have been summarised briefly, as reported

in the scientific literature. Such risks and potential risk assessment and PMEM challenges cannot be generalised, as they may not apply to all types of GDMIs considered in this GMO Panel Scientific Opinion;
- The identification of potential novel hazards, and previously proposed risks and potential challenges related to the risk assessment and PMEM of GDMIs informed the adequacy and sufficiency evaluation of EFSA (2012, 2013) for the MC, ERA and PMEM of GDMIs (see below).

6.3. Consideration of relevant comparators

- There will often not be a single comparator (i.e. the non-GMI with a genetic background as close as possible and relevant to that of the GMI) for a given proposed deliberate release into the environment of a GDMI, but a range of relevant comparators to inform ERA and contextualise risks, so the choice of comparators should put more emphasis on the purpose of the risk assessment studies conducted and thus the purpose of comparisons;
- Depending on the intended outcome of the GDMI application and purpose of the comparison, relevant comparators may include: (1) the non-GMI of the same species with a genetic background that is as close as possible to that of the GDMI; (2) the target organism; and (3) other disease vector/pest control systems (e.g. species-specific genetic control methods involving the release of insects, insecticides) to enable comparisons at both the organismal and (management) systems level;
- Given that some GDMI systems will operate at an ecosystem level, the definition of comparator needs to be broadened from endpoints that solely consider genetic and phenotypic changes to those that can be indicative of potentially harmful ecosystem impacts;
- The selection of comparators may need to consider issues relevant to offspring of the GDMI, and include comparisons with heterozygotes and homozygotes of the GDMI, where relevant;
- At the population and system level, multiple comparators may be needed to allow robust comparisons across a range of factors that are not sufficiently matched by a single comparator.

6.4. Adequacy and sufficiency of existing guidelines for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of gene drive modified insects

- All aspects of EFSA (2012, 2013) are considered adequate for the MC, ERA and PMEM of GDMIs, except those pertaining to GS (see Table 8);
- Aspects of EFSA (2012, 2013) considered to be adequate and sufficient are:
  - Strategies for ERA (Section 5.2.1 of EFSA (2013));
  - Identification of potential unintended effects through phenotypic and compositional endpoints (Section 5.1.2.2 of EFSA (2013));
  - Experimental environment (Section 5.1.3.2 of EFSA (2013);
  - Uncertainty analysis (Section 5.1.3.8 of EFSA (2013));
  - Interactions with non-target organisms (Section 5.1.4.5 of EFSA (2013);
  - Environmental impacts of specific techniques used for the management of GMIs (Section 5.1.4.6 of EFSA (2013));
  - Impacts of GMIs on human and animal health (Section 5.1.4.7 of EFSA (2013));
- Aspects of EFSA (2012, 2013) considered to be adequate, but insufficient are:
  - Receiving environments (Section 5.1.3.1 of EFSA (2013));
  - Comparators (Section 5.1.3.3 of EFSA (2013));
  - Non-GM surrogates (Section 5.1.3.4 of EFSA (2013));
  - Experimental design and statistics (Section 5.1.3.5 of EFSA (2013));
  - Long-term effects (Section 5.1.3.6 of EFSA (2013));
  - Modelling (Section 5.1.3.7 of EFSA (2013));
  - Persistence and invasiveness, including vertical gene flow (Section 5.1.4.1 of EFSA (2013));
  - HGT (Section 5.1.4.2 of EFSA (2013));
  - Pathogens, infections and diseases (Section 5.1.4.3 of EFSA (2013));
  - Interactions with target organisms (Section 5.1.4.4 of EFSA (2013));
  - CSM (Section 5.1.5.1 of EFSA (2013));

  - Information relating to the recipient or (where appropriate) parental animals (Section 5.2.1 of EFSA (2012));
  - MC (Section 5.2.2 of EFSA (2012));

- The EFSA (2012, 2013) guidelines follow the comparative risk assessment paradigm for GMOs, which uses the case-by-case principle and an iterative, stepwise/staged/tiered testing approach, and which considers different lines of evidence, including modelling, in a weight of evidence approach;
  - The stepwise/staged/tiered testing approach may leave some uncertainty before open field testing or field implementation of some GDMIs, as it may be challenging to collect data from experimental systems that would be fully applicable to field conditions. Mathematical modelling may help to fill this gap in data. Moreover, greater use of models to address the long temporal scale and wide spatial scale of specific GDMI applications, and PMEM may be needed;
  - Gathering relevant data for self-sustaining and low threshold (independent) gene drives in open release trials may be challenging due to their spatially and temporally unrestricted nature and the current inability for recall. Since self-sustaining engineered gene drives are designed for widespread and long-standing control, spatially and/or temporally restricting their spread would not necessarily be in keeping with the intended outcome of their deliberate release. Therefore, the utility of prior field testing of a related self-limiting strain may be considered as an intermediate step to reduce uncertainties in risk assessment (e.g. Benedict and Robinson, 2003; James et al., 2018). Theoretically, self-limiting engineered gene drive systems may enable localised and temporally restricted spread of the genetic modification of interest, resembling other self-limiting approaches for disease vector/pest control;

- Some of the MC aspects given in EFSA (2012) are designed to support the risk assessment of food/feed containing, consisting of, or produced from GMAs, and thus are not necessarily tailored to the ERA needs of GMIs, including GDMIs, that are not intended for food/feed uses. The applicability of these aspects should be assessed on a case-by-case basis as part of the problem formulation.

Table 8: Summary of the adequacy and sufficiency[62] evaluation of EFSA (2012, 2013) for the molecular characterisation, environmental risk assessment and post-market environmental monitoring of gene drive modified insects for deliberate release into the environment

	Sufficient	**Insufficient**
Adequate	Cross-cutting considerations (according to EFSA (2013))	
	• Strategies for environmental risk assessment (Section 5.2.1) • Identification of potential unintended effects through phenotypic and compositional endpoints (Section 5.1.2.2) • Experimental environment (Section 5.1.3.2) • Uncertainty analysis (Section 5.1.3.8)	• Receiving environments (Section 5.1.3.1) • Choice of comparators (Section 5.1.3.3) • The use of non-genetically modified surrogates (Section 5.1.3.4) • Experimental design and statistics (Section 5.1.3.5) • Long-term effects (Section 5.1.3.6) • Further guidance on modelling (Section 5.1.3.7)
	Specific areas of risk (according to EFSA (2013))	
	• Interactions with non-target organisms (Section 5.1.4.5) • Environmental impacts of specific techniques used for the management of GMIs (Section 5.1.4.6) • Impacts of genetically modified insects on human and animal health (Section 5.1.4.7)	• Persistence and invasiveness, including vertical gene flow (Section 5.1.4.1) • Horizontal gene transfer (Section 5.1.4.2) • Pathogens, infections and diseases (Section 5.1.4.3) • Interactions with target organisms (Section 5.1.4.4)
	Post-market environmental monitoring (according to EFSA (2013))	
		• Case-specific monitoring (Section 5.1.5.1)

	Sufficient	Insufficient
	Molecular characterisation (according to EFSA (2012))	
		• Information relating to the recipient or (where appropriate) parental animals (Section 5.2.1) • Molecular characterisation (Section 5.2.2)
Inadequate	Post-market environmental monitoring (according to EFSA (2013))	
		• General surveillance (Section 5.1.5.2)

6.5. Specific areas where updated guidance is needed

- While the MC, ERA and PMEM of GDMIs can build on the existing risk assessment framework for GMIs that do not contain engineered gene drives (EFSA, 2012, 2013), there are specific areas where further guidance is needed for GDMIs;
- Cross-cutting considerations of EFSA (2013) that may need further consideration in any future guidance on GDMIs are:
  - Receiving environments (Section 5.1.3.1 of EFSA (2013));
  - Comparators (Section 5.1.3.3 of EFSA (2013));
  - Non-GM surrogates (Section 5.1.3.4 of EFSA (2013));
  - Experimental design and statistics (Section 5.1.3.5 of EFSA (2013));
  - Long-term effects (Section 5.1.3.6 of EFSA (2013));
  - Modelling (Section 5.1.3.7 of EFSA (2013));
- Specific areas of risk of EFSA (2013) that may need further consideration in any future guidance on GDMIs are:
  - Persistence and invasiveness, including vertical gene flow (Section 5.1.4.1 of EFSA (2013));
  - HGT (Section 5.1.4.2 of EFSA (2013));
  - Pathogens, infections and diseases (Section 5.1.4.3 of EFSA (2013));
  - Interactions with target organisms (Section 5.1.4.4 of EFSA (2013));
- Monitoring of GDMIs will pose practical challenges and the design and interpretation of monitoring schemes will depend heavily on models of expected outcomes. Further guidance may be required on the design, conduct and interpretation of CSM to ensure that the data add to our understanding of large scale and long-term processes. Moreover, further consideration is needed for the design and implementation of GS to identify potential unanticipated adverse effects in a proportionate manner;
- Since some of the MC-related aspects given in EFSA (2012) are not necessarily tailored to the ERA needs of GDMIs, additional ones are required to account for the ERA and PMEM of GDMIs.

7. Documentation as provided to EFSA

- Request for an EFSA opinion on genetically modified organisms engineered with gene drives. June 2018. Submitted by the European Commission (Directorate-General for Health and Food Safety);
- Acknowledgement of receipt of the mandate. August 2018. Submitted by the European Food Safety Authority;
- Reception of the mandate. October 2018. Submitted by the European Food Safety Authority;
- Acknowledgement of receipt of EFSA's reception letter of the mandate. November 2018. Submitted by the European Commission (Directorate-General for Health and Food Safety).

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Abbreviations

Cas9	CRISPR associated protein 9
CBC	Classical biological control
CI	Cytoplasmic incompatibility
ClvR	Cleave and rescue
CRISPR	Clustered regularly interspaced short palindromic repeats
CSM	Case-specific monitoring
DSB	Double strand break
ES	Ecosystem service
EFSA	European Food Safety Authority
ERA	Environmental risk assessment
fsRIDL	Release of insects carrying a dominant female lethal transgene
GDMI	Gene drive modified insect
GDMO	Gene drive modified organism

GM	Genetically modified
GMA	Genetically modified animal
GMI	Genetically modified insect
GMO	Genetically modified organism
GS	General surveillance
gRNA	Guide RNA
HDR	Homology-directed repair
HEG	Homing endonuclease gene
HGT	Horizontal gene transfer
HR	Homologous recombination
IAEA	International Atomic Energy Agency
IIT	Incompatible insect technique
MC	Molecular characterisation
mRNA	Messenger RNA
miRNA	MicroRNA
MMEJ	Microhomology-mediated end joining
NASEM	National Academies of Sciences Engineering and Medicine
NHEJ	Non-homologous end joining
PI	Pathogen interference
PMEM	Post-market environmental monitoring
RIDL	Release of insects carrying a dominant lethal transgene RNAi: RNA interference
SDN	Site directed nuclease
sgRNA	Single guide RNA
SIT	Sterile insect technique
TALEN	Transcription activator-like effector nuclease
TARE	Toxin-antidote recessive embryo
WHO	World Health Organization
ZFN	Zinc finger nuclease