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Opinion

Gene drive in species complexes: defining target organisms

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Engineered gene drives, which bias their own inheritance to increase in frequency in target populations, are being developed to control mosquito malaria vectors. Such mosquitoes can belong to complexes of both vector and nonvector species that can produce fertile interspecific hybrids, making vertical gene drive transfer (VGDT) to sibling species biologically plausible. While VGDT to other vectors could positively impact human health protection goals, VGDT to nonvectors might challenge biodiversity ones. Therefore, environmental risk assessment of gene drive use in species complexes invites more nuanced considerations of target organisms and nontarget organisms than for transgenes not intended to increase in frequency in target populations. Incorporating the concept of target species complexes offers more flexibility when assessing potential impacts from VGDT.

Highlights

Engineered gene drives share many environmental risk assessment considerations with other transgenes in genetically modified organisms, but they can differ significantly in their potential to spread, increase in frequency, and persist in target populations.

Recently, introduction of mosquitoes with an engineered gene drive completely suppressed caged wild type laboratory populations of the malaria vector *Anopheles gambiae*, belonging to a species complex containing both vector and nonvector species that can produce fertile interspecific hybrids.

As target sequences of the gene drive are conserved amongst all species of this complex, vertical gene drive transfer to both vectors and nonvectors is plausible. This challenges the notion of a simple dichotomy between target organism and nontarget organism.

Using this gene drive as a specific case study, options on defining target organisms of engineered gene drives in species complexes are developed here, including proposal of the new concept of target species complex.

Environmental risk assessment of gene drive organisms

Gene drives (see Glossary) can allow genes, transgenes, or genetic traits to be transmitted to offspring at greater than Mendelian frequencies, a property that offers the potential for a genetic modification of interest to spread and increase in frequency through interbreeding target populations [1–3]. In particular, there is considerable interest in harnessing gene drives to control mosquito species that are **vectors** of human diseases such as malaria (Box 1) [4,5].

Before any environmental release of an engineered gene drive mosquito could be considered by regulators, decision-makers and stakeholders, **environmental risk assessment (ERA)**, whether **probabilistic**, **qualitative**, or a combination thereof, must be conducted to evaluate potential risks to human health, animal health and the environment [6–13]. A prerequisite for effective ERA of **genetically modified organisms (GMOs)** is to define intended and unintended effects of the intervention on **target organisms (TOs)** and **nontarget organisms (NTOs)** [9,10,14–17]. This paradigm is typically applied in the qualitative ERA of GM plants, where, in general, there is a clear distinction between the organisms to be targeted, the TOs, and those that are not intended to be targeted, the NTOs.

While engineered gene drives share many of the same considerations as other transgenes in GMOs, such as non-gene drive **genetically modified mosquitoes (GMMs),** they differ in that they are designed to spread, increase in frequency, and persist in target organisms of wild populations. In addition, the most significant malaria vectors belong to **species complexes** that contain both vector and nonvector species [18–22], some combinations of which are capable of **hybridisation** to produce fertile interspecific hybrids. Such semipermeable or porous species boundaries facilitate **introgression** [19,20,23] and could plausibly lead to **vertical gene drive transfer (VGDT)** amongst **sibling species**, including nonvectors. This represents a challenge to the notion of a binary choice between TO and NTO for engineered gene drives in

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Box 1. Malaria and engineered gene drives

The World Health Organization (WHO) has reported that in 2020 there were 241 million cases of malaria worldwide, associated with an estimated 627 000 deaths [68]. Countries from the WHO African Region continue to carry a disproportionately high share of the global malaria burden, being associated with 95% of malaria cases and 96% of malaria deaths. *Plasmodium falciparum* is the major pathogen responsible for causing malaria in humans and is spread via the bites of infected female *Anopheles* mosquitoes as they blood-feed on hosts to provide essential nutrients for development of their eggs. As a result of the use of insecticide-treated bed nets and indoor insecticide spraying, as well as both prophylactic and therapeutic pharmaceutical treatments, there has been a steady decline in malaria prevalence over the last decade. However, progress has recently stalled and remains under further threat from insecticide resistance and behavioural adaptations, such as increased **zoophilic** responses, in mosquitoes, as well as *Plasmodium* resistance to drugs, invoking the need for complementary approaches to reduce the burden of this disease, including via the use of novel vector control tools such as engineered gene drives [3–5,68].

Current gene drive strategies include the use of transgenes encoding both the CRISPR-Cas9 endonuclease that is expressed under the control of a germline promoter, along with ubiquitously and constitutively expressed gRNAs, that together can target and cleave specific sequences in the genome [2,69]. Once the transgene is introduced into its genomic target location on one of a pair of homologous chromosomes, the gRNA and Cas9 act in concert in germ cells to cause a double-stranded break in the target DNA site of the wild-type homologous chromosome. When this double-stranded lesion is repaired by homology directed repair using the transgenic homologous chromosome as a template, the entire transgene, along with flanking sequences either side of the transgene, is pasted into the target site of the homologous, formerly wild-type, chromosome. This process of **homing** can create mostly pairs of parental homologous chromosomes that are homozygous for the transgene, so that a greater proportion of parental germ cells are transgenic than would otherwise be the case, leading to super-Mendelian inheritance of the transgene in progeny. Thus, once introduced into a mating population, transgenes capable of homing will increase in frequency, or drive, within that population, assuming any fitness costs from the transgene do not outweigh its increased transmission from homing [2].

species complexes. Depending on how the TO and **protection goals** are defined, the potential impacts of VGDT could be evaluated in a number of different ways in the ERA.

Here, a case study involving a **population suppression gene drive** in *Anopheles gambiae* sensu lato (s.l.) [24,25] is used to illustrate these differing possibilities and their consequences (Box 2).

Engineered gene drives in mosquito species complexes

The dominant malaria vectors in Africa are *Anopheles coluzzii*, *Anopheles gambiae* sensu stricto (s.s.), *Anopheles arabiensis*, and *Anopheles funestus* (all Diptera: Culicidae) [26,27]. Engineered

Box 2. Case study: a population suppression gene drive in *Anopheles gambiae*

There are two principal strategies for use of engineered gene drives in malaria vector control. In **population replacement gene drives**, transgenes disrupt endogenous mosquito genes or contain cargo genes to prevent the development of pathogens in the mosquito or its transmission from mosquitoes to humans [69]. In population suppression gene drives, the transgene causes a decline in population density by introducing a fitness cost or sex bias. Here, for example, gRNAs can target haplosufficient female fertility genes. Transgenic heterozygous females are disrupted for one copy of the gene and so remain fertile. Via homing, the transgene is transmitted to offspring at super-Mendelian inheritance rates [2], so that heterozygous transgenic males and females increase in frequency in the population. They are therefore increasingly likely to mate with one another, generating increasing proportions of homozygous transgenics, females of which are sterile. Thus, the number of progeny decreases and the population is suppressed.

Early attempts to produce engineered gene drives to suppress populations of *A. gambiae* led to resistance to drive caused by mutations that simultaneously both disrupted the gRNA target site and retained functionality in the protein from the endogenous targeted gene [70]. Recently, the *doublesex* sex determination locus in *A. gambiae* s.l. has been investigated in the laboratory for a population suppression gene drive. The locus, which is highly conserved, encodes two transcripts, one of which is male specific (*AgdsxM*), the other female specific (*AgdsxF*) [24]. The gRNA of the engineered gene drive spans an intron–exon boundary, mutation of which selectively disrupts the *AgdsxF* isoform, causing homozygous transgenic females to be intersex, sterile and unable to blood feed. In the laboratory, introduction of mosquitoes with this engineered gene drive into small cages of wild type populations of *A. gambiae* caused population eliminations after 9–11 generations [24]. In recent large cage experiments that more closely mimic the feeding and reproductive environments of the field, introduction of the *doublesex* engineered gene drive into overlapping, age-structured generations of wild type *A. gambiae* caused population collapse within a year without any evidence for the development of resistance to drive [25].

gene drives targeting *A. coluzzii*, *A. gambiae* s.s., and *A. arabiensis* are currently under active development [28]. All three vectors are members of the *A. gambiae* s.l. complex (Box 3). However, *A. gambiae* s.l. also contains nonvector species. Most combinations of sibling species of the complex that have been examined in the laboratory are capable of producing fertile female hybrids [18]. Some combinations of these species hybrids have also been found in field populations, albeit typically at low frequencies [23,29–32], thus representing a potential VGDT route.

As outlined in Box 2, the *doublesex* locus in *A. gambiae* s.l. is highly conserved and has been used as a target for an engineered population suppression gene drive [24,25]. Because the guide RNA (gRNA) target sequence is conserved amongst all examined species of the complex [24], it would likely be capable of functional homing should it be transferred via hybridisation with other sibling species. Thus, a consequence of the use of such an engineered gene drive in this species complex is a plausible potential for VGDT to other sibling species. Some species of the complex are geographically separated [33,34] so that interspecific hybrids between them, and any consequential VGDT, would appear to be implausible in the wild (Figure 1). However, VGDT among all species of the complex would remain plausible via a combination of both direct and indirect routes between species.

To illustrate this point, consider West Africa as the potential location of the first investigational releases of the *doublesex* engineered gene drive [35,36]. *A. coluzzii* is largely restricted to this region, where it is one of the most common species of the *A. gambiae* s.l. complex [32–34,37]. Although the *doublesex* gene drive might first be introduced and increase in frequency in *A. coluzzii*, with successful hybridisation and functional homing, the transgene could transfer

Box 3. *Anopheles gambiae* s.l. species complex

The species complex of *A. gambiae* s.l. is typically recognised as containing at least nine morphologically indistinguishable sibling species that shared a common ancestor less than 2 million years ago [71]: *Anopheles amharicus*, *Anopheles arabiensis*, *Anopheles bwambae*, *Anopheles coluzzii*, *Anopheles fontenillei*; *Anopheles gambiae* s.s., *Anopheles melas*, *Anopheles merus*, and *Anopheles quadriannulatus*.

A. gambiae s.s., *A. coluzzii* and *A. arabiensis* are dominant regional vector species of malaria in large sections of Africa [26,27,72]. *A. melas*, *A. merus*, and *A. bwambae* are more geographically restricted, local vectors of the disease [48,72]. *A. quadriannulatus* is zoophilic and is not believed to contribute to significant transmission of *Plasmodium* [73]. *A. amharicus* is zoophilic and no sporozoite-positive samples have been identified in field samples to date suggesting no role in malaria transmission [74]. *A. fontenillei*, described in only one publication from 2019, may have a plastic status in blood feeding on humans and animals, although it may primarily be zoophilic and thus not contribute significantly to malaria transmission [75]. There is also some evidence for additional cryptic taxonomic units in the complex [37,76–78].

Successful hybridisation between all examined species of the complex has been observed in the laboratory, typically yielding fertile female hybrids and sterile male hybrids [18,79–81]. Although some species of the complex are geographically separated [33] and therefore could not produce hybrids directly in the wild, hybrids of a number of combinations of sympatric species of the complex have also been detected in the field, albeit at low levels [23,29–31,82,83]. Moreover, **vertical gene flow** between species of the complex is detected from genomic sequence analyses [71,84–87]. Such capacity for gene flow between species of the complex via hybridisation thus represents porosity or semipermeability of species barriers within the complex [19], which might otherwise be absent in species where there is more stringent reproductive isolation and represents an important avenue for the potential acquisition of adaptive characteristics such as insecticide resistance or altered **vectorial capacity**, as well as for speciation [53–55]. This gene flow could also be a potential source of resistance to gene drive.

Moreover, the specific target genomic sequence of the gRNA in the *doublesex* engineered gene drive is conserved between species of the complex [24], so that homing of this transgene in all species of the complex is mechanistically plausible. Even acknowledging potential interference from geographic barriers or assortative mating between sibling species in the field, an engineered gene drive could be transmitted from the species in which the gene drive is introduced to other species of the complex via rare hybridisation events, and if this was followed by efficient homing it could result in an increase in frequency of the transgene through populations of all species of the complex [36].

Glossary

Assortative mating: pattern of mating in which individuals with similar genotypes or phenotypes mate with each other more often than would be predicted via mating that was purely random.
Environmental risk assessment (ERA): process to identify potential environmental and health risks, estimate their magnitude and likelihood and define any risk management required.
Gene drive: (i) genetic elements that cause biased inheritance; (ii) process or phenomenon leading to biased inheritance; or (iii) management strategy to apply gene drive [66].
Genetically modified mosquito (GMM): mosquito with heritable traits derived through use of recombinant DNA technology, which alter the strain, line or colony in a manner usually intended to reduce transmission of human diseases [11].
Genetically modified organism (GMO): any organism that has in its genome novel DNA of endogenous, exogenous or mixed origin that was made using modern recombinant DNA technology [11].
Homing: process whereby copy of gene drive is inserted into specific genomic target, or homes, after endonuclease cleavage of target sequence and its repair using homologous chromosome containing gene drive [66].
Hybridisation: production of offspring (hybrids) from two different species by interbreeding.
Introgression: stable incorporation of genetic material from one species to another via hybridisation followed by backcrossing of the hybrid to members of that other species.
Nontarget organism (NTO): any organism that is not a direct target of the intended intervention [11].
Population replacement gene drive: gene drive that targets vector competence with intent to reduce inherent ability of individual vectors to transmit a given pathogen.
Population suppression gene drive: gene drive that reduces the size of a natural population to extent that it would not be able to sustain pathogen transmission.
Probabilistic ERA: based on quantitative modelling approaches to represent a probability distribution for a range of potential outcomes for a

and drive into the other species of the complex that are found in West Africa, namely *A. gambiae* s.s., *A. arabiensis*, and *Anopheles melas* [33]. From these species, further VGDT could occur into the other vector species of the complex that overlap geographically, *Anopheles merus* and *Anopheles bwambae*, as well as the remaining three species that are nonvectors, *Anopheles amharicus*, *Anopheles fontenillei*, and *Anopheles quadriannulatus* (Figure 1) [33]. Furthermore, in the case of the *doublesex* population suppression gene drive, such VGDT could eventually result in reduced population densities of all sibling species of the complex, both vectors and nonvectors.

Nevertheless, it remains feasible that hybridisation between GMMs and non-GMMs among some combinations of colocated sibling species could still be restricted by (i) geographical barriers such as the Congo Basin and the East African rift system [38]; (ii) **assortative mating** [39]; or (iii) viability of hybrid progeny in the wild [40]. Therefore, the likelihood of hybridisation in the field might be expected to vary considerably between different combinations of sibling species that are co-located in the same geographic regions (Box 3). It is also possible that an engineered gene drive could be transferred to another sibling species via hybridisation yet could still be incapable of functional homing in that species. Thus, VGDT to all members of the complex is biologically plausible but not inevitable. This makes it difficult to reliably predict ahead of potential environmental release of *doublesex* gene drive mosquitoes what the intended efficacy outcomes might be from VGDT to sibling species and therefore provides choices in how the TO might be defined.

Precedents in defining TOs in GMO applications

For ERA of gene drive applications, how the TO is defined might be considered on the basis of what precisely is meant by the term target, given that it could be interpreted from the viewpoints of both intention and mechanism. For example, the mechanism might be considered to consist of: (i) the introduction of the species containing the engineered gene drive into wild populations of the same species; (ii) transfer of the transgene to other sibling species via hybridisation; and (iii) functional homing in other sibling species due to the conserved nature of the gRNA target sites as described above. Thus, the mechanism of the engineered gene drive could plausibly allow VGDT to all species of the complex.

However, the intention of the gene drive intervention may only be to target the individual species responsible for malaria transmission. In that regard, the evidence supporting the roles in malaria transmission of each of the species in the complex varies widely (Box 3). Therefore, should the direct intention of the intervention be to target only species of the complex that were known to be capable of transmitting malaria, it would have to be acknowledged that three other nontarget species in the complex, *A. amharicus*, *A. fontenillei*, and *A. quadriannulatus*, may also unintendedly be impacted by the *mechanism* of the intervention.

Consideration of TOs and NTOs in other GM biocontrol applications does little to clarify the position. For example, Cry1 class insecticidal proteins from *Bacillus thuringiensis* expressed in GM plants can adversely affect lepidopterans other than the target pest species, including protected butterflies [41,42]. Yet, those species are not classified as TOs [43,44]. Instead, they have been considered NTOs and were the focus of the ERA to judge whether growing a particular *B. thuringiensis* plant causes harm to those valued entities.

Likewise, environmental releases of GMMs resulting in substantial population suppression of *Aedes aegypti* (Diptera: Culicidae) populations in the Cayman Islands, Brazil, and Panama provide potentially analogous examples to the population suppression gene drive described here [45–47]. However, this GMO is a self-limiting strain in which there is no engineered gene drive

particular event; the use of Bayesian networks can ensure that the input and output will be categorical so that risk can be defined both numerically and by a discrete number of categories [13].
Protection goals: policy, legislation, and stakeholder input defining environmental or health resources to be protected, the degree of protection they deserve, or the maximum impacts that should be tolerated.
Qualitative ERA: defines, in a structured and systematic way, the likelihood and consequences of outcomes into a limited number of ordered classes to yield categorical indications of relative risk, such as high, moderate, low, or negligible [13]; approach used in most jurisdictions to evaluate risks from environmental release of GMOs [67].
Sibling species: species that are members of the same species complex.
Species complex: group of closely related, but distinct, species that are similar in morphology with porous or semipermeable reproductive boundaries [19].
Target organism (TO): for GMMs, the direct target organism of the intended intervention is other mosquitoes of the same species in the wild population [11].
Target species complex (TSC): species complex in which target organism resides.
Target species complex organism (TSCO): organism which is member of TSC.
Vector: species that can transmit a particular disease pathogen.
Vectorial capacity: potential of a mosquito vector population to transmit a pathogen.
Vertical gene drive transfer (VGDT): transfer via hybridisation of gene drive from one sibling species to another leading to gene drive in latter.
Vertical gene flow: transfer of genes from parents to offspring [66].
Zoophilic: preferential seeking of bloodmeals by mosquitoes from animal hosts.

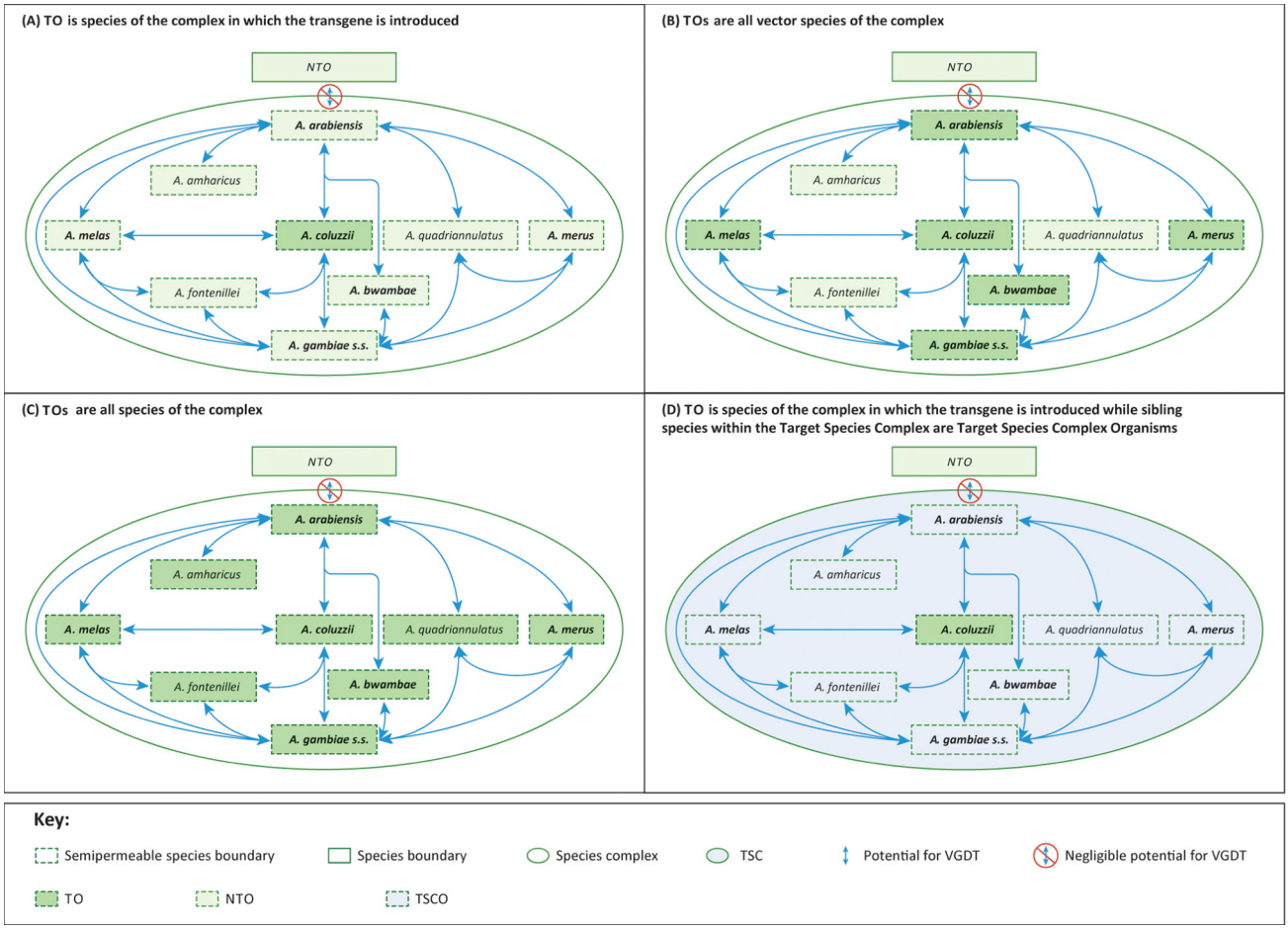

Figure 1. Target definition scenarios for engineered gene drives in species complexes. Potential routes of VGDT between different, geographically overlapping species of the complex, illustrated by double-headed arrows, result from incomplete reproductive isolation and semipermeable species boundaries. The double-headed arrows indicate that VGDT can be bidirectional so that the VGDT could occur from nonvectors to vector sibling species and *vice versa*. The likelihood of VGDT between sibling species could vary depending on rates of (i) hybridisation among and (ii) homing within species so that the uniform thickness of the arrows does not indicate the probability of VGDT. Vectors species shown in bold. (A) The TO is considered to be the species in which the transgene is introduced, but VGDT to other sibling species of the complex is plausible. (B) The engineered gene drive would be introduced in *Anopheles coluzzii* as a TO, but an intended efficacy outcome would be for VGDT to occur in other sibling species that are vectors, resulting suppression of their populations. Thus, vector species of the complex would also be considered TOs. (C) The engineered gene drive would be introduced and intended to spread via mating in *A. coluzzii* but would be expected to transfer and home in other sibling species that are either vectors or nonvectors via VGDT, resulting ultimately in suppression of their populations. Thus, all species of the complex would be considered TOs. (D) The engineered gene drive would be introduced in *A. coluzzii* which would be considered to be the TO. The other sibling species of the complex, whether vectors or nonvectors, would be considered as TSCOs within the TSC, where the potential for VGDT via hybridisation and homing would be considered as a plausible biological consequence of use of gene drive in the species complex. Abbreviations: NTO, nontarget organism; TO, target organism; TSCO, target species complex organism; VGDT, vertical gene drive transfer.

and so does not increase in frequency in target populations upon environmental release. This means that inundative environmental releases are required for population suppression. Therefore, even if the transgene were to be vertically transferred to another species, it would not subsequently be propagated through that population.

There also appears to be only limited clarity in guidance documents and the literature on how the TO might be defined in the case of gene drive mosquitoes belonging to species complexes

(Box 4). Pre-existing guidance and literature, therefore, leave room for interpretation, and thus flexibility, on how TOs of gene drive mosquitoes belonging to species complexes might be defined for ERA.

To develop further thinking on this issue, four target definition scenarios are outlined below that might be useful to developers, risk assessors and decision-makers in considering how best to define TOs of engineered gene drives in species complexes. Each scenario is illustrated using the specific case study of a simulated environmental release in West Africa of the *doublesex* engineered gene drive in a single species of *A. gambiae* s.l., *A. coluzzii* [24,35,36].

Target definition scenarios

The TO as the species in which the transgene is introduced

In the first scenario (Figure 1A), the TO would be considered to be the species in which the transgene is introduced and intended to increase in frequency, which would be *A. coluzzii* in the specific case study used here. However, it would also be recognised as plausible that VGDT could occur from the gene drive mosquito species released into the environment, the TO, to other species of the complex via hybridisation. This would subsequently lead to homing of the engineered gene drive at genomic target locations that are conserved amongst sibling species, potentially leading to their population suppression.

Adoption of this target definition scenario would mean that efficacy data would be required for the TO, the species in which the engineered gene drive is originally introduced and intended to

Box 4. Guidance on defining TOs of GMMs

The WHO [11], in its guidance framework for testing GMMs, does not define TO *per se,* but does provide a definition of NTO, as in James and colleagues [8], as 'any organism that is not a direct target of an intended intervention. For GMMs, the direct target organism is other mosquitoes of the same species in the wild population.' A strict reading of this definition would appear to support the consideration of TO as the individual species in which the transgene was introduced and disseminated via mating in the wild, but not to those species subsequently acquiring the engineered gene drive via interspecific hybridisation, even if they were competent vectors of disease and ultimately intended recipients of the transgene. In its guidance on risk assessment of GM animals including insects, the European Food Safety Authority (EFSA) [88] has previously defined the TO as 'an organism on which specifically designed characteristics of a GM animal are intended to act. A GM animal may have more than one TO…All other organisms (except the GM animal itself) should be considered as "NTOs".' EFSA [9] subsequently evaluated the adequacy and sufficiency of its existing guidance on GM insects in relation to engineered gene drives, recognising the potential challenge in defining TOs for gene drive insects by stating that they

> 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 [gene drive modified insect] GDMI. Depending on the definition of the target organism and populations, intended outcomes may differ across the spectrum of such a complex.

Moreover, James and colleagues [8] stated that a

> gene drive product in *Anopheles gambiae* s.s., presents an unusual case, in which it would be considered desirable for the genetic construct to move through cross-mating to other malaria vectors within the *A. gambiae* s.l. species complex. It should be made clear that movement of the construct to sibling species that also are malaria vectors is considered part of the intended effect, and in that case functionality with respect to population suppression or replacement should be monitored in those other species.

Indeed, in a recent series of recommendations on advancing ERA for gene drive applications, Connolly and colleagues recommended that the definition of the term TO for gene drive in species complexes required more nuanced consideration than for other GMO applications [13].

increase in frequency in wild populations, but not other sibling species. The overall impact on protection goals from VGDT between the gene drive mosquito species that was released into the environment, the TO, and NTOs both within and outside of the species complex would need to be assessed in the ERA. Importantly, a species which is a nonvector or NTO is not automatically considered to be a valued species.

The TOs as all vector species of the complex

In a second scenario (Figure 1B), the engineered gene drive would be introduced and increase in frequency in *A. coluzzii.* However, an intended efficacy outcome would be for VGDT to occur between that species and other geographically overlapping sibling species that vector the disease, namely *A. arabiensis*, *A. gambiae* s.s., and *A. melas* [33], potentially resulting in eventual suppression of those populations. Further VGDT would also be expected to occur from *A. arabiensis* and *A. gambiae s.s.* to two other vector species of the complex, *A. bwambae* and *A. merus* [33]. Thus, all vector species of the complex would be considered TOs. While the remaining sibling species of the complex are nonvectors and thus would be considered as NTOs, it would remain plausible for VGDT to occur between vector and nonvector sibling species, potentially also leading to eventual suppression of their populations.

Use of this target definition scenario would mean that an evaluation of efficacy of spread and increase in frequency of the engineered gene drive in all the vector species of the complex could be required. However, it may not be feasible to obtain these data in all vector species of the complex as there may be limited or no opportunity to establish and investigate laboratory colonies, due to: (i) difficulty in obtaining samples of some species from the wild owing to the restricted nature of their habitats [20,48]; (ii) limited success in stimulating laboratory matings [49,50]; (iii) the impact of genetic bottleneck and founder effects [51]; or (iv) requirements for highly specific environmental rearing conditions that are unconducive to routine laboratory maintenance [52]. Instead, data could be obtained in exemplar species in addition to the species in which the transgene is introduced, *A. coluzzii*, which in the specific case study illustrated here could be the dominant malaria vectors, *A. gambiae* s.s. and *A. arabiensis*. Assessment of the overall impact on protection goals of VGDT between the gene drive mosquitoes released into the environment, vector species of the complex and nonvector species within the same species complex would be required for ERA.

The TOs as all species of the complex

Here, the engineered gene drive would be introduced and increase in frequency in *A. coluzzii*, but VGDT would be expected to occur in geographically overlapping sibling species, namely *A. arabiensis*, *A. gambiae* s.s., and *A. melas* [33], potentially resulting ultimately in suppression of their populations (Figure 1C). Further VGDT would be expected between *A. arabiensis*, *A. gambiae* s.s., and *A. melas*, and the remaining species of the complex [33]. Thus, all species of the complex would be considered TOs, regardless of whether they are vectors or nonvectors, on the basis of the mechanism of the engineered gene drive.

Use of this target definition scenario would mean that an evaluation of efficacy of spread and increase in frequency of the engineered gene drive in all species of the complex, or at least in exemplar species of the complex as described above, would be required. Assessment of the overall impact on protection goals of VGDT between all species of the complex would also be required for ERA purposes.

Introducing the concept of the target species complex

A fourth target definition scenario involves the engineered gene drive being introduced and increasing in frequency in *A. coluzzii* (Figure 1D). This species alone would be considered as

the TO. However, the other sibling species of the complex, whether vectors or nonvectors, would be considered as members of the **target species complex (TSC)**, or **target species complex organisms (TSCOs)**, where the potential for VGDT amongst its member species would be considered a plausible biological consequence of use of gene drive technology to control vector species belonging to a complex. Therefore, both the intention and mechanism of the engineered gene drive would be accommodated in this target definition scenario.

Adoption of this target definition scenario would also mean that efficacy data would be required only in the species of gene drive mosquitoes that were released into the environment, *A. coluzzii*, as the TO. Additional efficacy data could be obtained in exemplar TSCOs, which in the specific case study here could be the dominant malaria vectors, *A. gambiae* s.s. and *A. arabiensis*. Evaluation of the overall impact on protection goals of potential VGDT between the gene drive mosquitoes released into the environment (the TO) and TSCOs would be required in the ERA. However, the impact on protection goals of potential VGDT from the gene drive mosquitoes released into the environment (the TO) to both vector and nonvector TSCOs would be recognised as a biological consequence of use of the gene drive intervention in a species complex. Moreover, the status of nonvector sibling species as TSCOs would also formally acknowledge the fact that the acquisition of the gene drive by nonvector sibling species could ultimately be beneficial to intended efficacy outcomes because nonvector species could serve as 'stepping stones' for the gene drive to transfer to other sibling species that are themselves vectors.

Concluding remarks

Owing to the inherent potential for hybridisation, increase in frequency and, thus, VGDT amongst species within complexes, more nuanced considerations and flexibility on the definition of TO is required for the ERA of gene drive mosquitoes belonging to species complexes than for other GMO applications [13]. In particular, defining the TO is complicated by the potential for VGDT of a gene drive from the original organisms released into the environment to other vector and nonvector species of the complex.

Four target definition scenarios have been developed here, including one proposing the new concept of TSC, to facilitate considerations and accommodate flexibilities on how TOs might be defined for gene drive mosquitoes belonging to species complexes. These differing approaches to defining TOs have the potential to impact on the nature and level of data required to support efficacy assessments and ERAs of gene drive mosquitoes in species complexes (see Outstanding questions). Additionally, it may not be feasible to obtain data in some species of the complex as there may limited or no opportunity to establish, or study pre-existing laboratory colonies. Instead, data could be obtained and evaluated in exemplar TOs of the complex, which in the specific case study here could be the dominant malaria vectors, *A. gambiae* s.s. and *A. arabiensis*. It is also important to accept that not all NTOs would be considered as valued species according to biodiversity protection goals, so a reduction in their abundance would not necessarily be considered an environmental concern.

Regardless of the target definition scenario chosen, the biological consequences of introduction of an engineered gene drive in a species complex would remain unchanged, as would the need for robust ERA. Whether a species would be defined as a TO, NTO, or TSCO, potential harms to protection goals would still have to be assessed in the ERA. Moreover, the choice of target definition scenario will most likely need to be made on a case-by-case basis, depending on the nature of the species complex, the extent of harm it causes, and its ratio of vector to nonvector species. For example, while six of the nine recognised species of the *A. gambiae* s.l. complex are malaria vectors, most of the seven recognised species of the *A. funestus* s.l. complex are zoophilic so that only one is considered to be a significant vector [20].

Outstanding questions

What are the criteria for choosing the appropriate target definition scenario for use of an engineered gene drive in a species complex?

How can developers and regulators deliberate and agree on defining TOs early in the development phase of engineered gene drives for use in a species complex, and which other stakeholders should be involved in this process?

In the context of VGDT, how are ERA considerations on whether a nonvector is a valued species affected by its membership of a species complex containing vector species?

What are the implications of choosing specific target definition scenarios on ERA requirements for pre-release data, modelling and post-release monitoring of organisms with engineered gene drives?

Use of an engineered gene drive could potentially lead to both reductions in local biodiversity, seemingly at odds with some high-level biodiversity policy goals, and reductions to harm to human health. In most jurisdictions worldwide, ERA of GMOs is focussed on risk so that reduced harm, or benefits, to protection goals are likely to be out of scope. How, therefore, do choices of target definition scenarios for use of an engineered gene drive in a species complex affect the trade-offs between different protection goals?

As well as environmental releases of engineered gene drives in single species, releases could be made simultaneously in all of the dominant vector species of the *A. gambiae* s.l. complex, namely *A. gambiae* s.s., *A. coluzzii*, and *A. arabiensis*. Would this approach alter choices around the target definition scenario?

The concept of TSC has been developed in the context of a population suppression gene drive in *A. gambiae* s.l. but might also be relevant for gene drive applications in the *A. funestus* s.l. species complex, which contains another dominant malaria vector in Africa. How far do the considerations here extend to gene drive applications in other species, including to those on other continents?

An alternative way to approach ERA for gene drive organisms in species complexes, is to consider that any impacts on species of the complex can only meaningfully be evaluated *en masse* and not on the individual species in isolation. Because of the existence of semi-permeable species boundaries in mosquito TSCs [19,20], introgression between sibling species could alter vectorial capacity amongst any component species of the TSC [53–55]. The TSC itself could therefore be considered as the functional biological entity or organism that is the vector of disease transmission and thus could be considered as both the intended and mechanistic target of the engineered gene drive. This approach becomes potentially even more relevant when one considers that the taxonomical understanding of species complexes is constantly evolving. For example, a new species of the *A. gambiae* s.l. was described only as recently as 2019 and several reports of cryptic taxa have also been reported in the literature in recent years (Box 3), so that further elaborations to descriptions of this complex can be anticipated. Furthermore, VGDT to a nonvector species should not simply been seen as an efficacy cul-de-sac. Rather, it could serve as a stepping stone for the engineered gene drive to transfer from that nonvector species to other sibling species that are themselves vectors, thus benefitting intended efficacy outcomes and underscoring the inseparable and fluid genetic relationships between species of the complex.

While a population suppression gene drive in the *A. gambiae* s.l. complex to control malaria transmission has been used here as a case specific study, considerations may differ for different gene drive applications targeting the same or different populations; for example, where the target site of the gene drive was not conserved between sibling species. Additionally, in the case of the environmental release of non-gene drive GMMs, although there could potentially be rare cases of the transgene transferring from the transgenic species to sibling species via hybridisation, the transgene would not home and therefore would not increase in frequency in sibling species into which it had introgressed. In this case, therefore, sibling species would be considered NTOs both from mechanistic and intentional points of view.

Finally, issues on defining the TO may extend beyond the potential use of gene drives in mosquito vector control to management of other pests. For example, the Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae), is a species complex composed on four species yet only one of these is considered to be a significant pest of fruit crops in Australia [56]. Potential control of this pest species using an engineered gene drive might therefore be expected to impact also on its sibling species. Because there are also numerous examples of introgression between a broad range of closely related animal species, including insects [57], chiclid fish [58], salamanders [59], frogs [60], lizards [61], Darwin's finches [62], and mammals [63–65], the considerations here may inform, on a case-by-case basis, ERA for the potential use of engineered gene drives in the biocontrol of a wide array of animal pest species.

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The views expressed in this publication are those of the authors and should not be interpreted as representing the official position of the European Food Safety Authority (EFSA). EFSA assumes no responsibility or liability for any errors or inaccuracies that may appear.

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