Gene drive systems have long been sought by the vector biology community in order to drive pathogen-resistance traits into mosquito populations to combat malaria and dengue [1]. Following successful demonstrations that some homing endonuclease genes (HEGs) can edit the genomes of Anopheles mosquitoes [2,3], an HEG (I-SceI) was found to be capable of driving into a cage population of Anopheles gambiae when its target site was engineered in the mosquito genome [4], confirming earlier predictions [5]. The rapid development of reagents for clustered regulatory interspaced palindromic repeat (CRISPR) gene editing [6–8] introduced a new programmable nuclease that was far simpler to target at new sites than HEGs. CRISPR was thus rapidly seen as an alternative programmable nuclease that could replace HEGs in gene drive [9], with successful CRISPR-based gene drive described in Drosophila [10] and Anopheles mosquitoes [11,12] not long after. The relative simplicity of the CRISPR/Cas9 homing-based gene drive approach elevated concerns about gene drive technology, triggering the rapid development of a report by the National Academy of Science [13]. While genetic resistance to gene drive approaches was observed in early proof-of-principle experiments [14–16], it is now clear that selection for genetic resistance can be avoided by using conserved target sequences. Indeed, a recent gene drive approach targeting a highly conserved region of the doublesex (dsx) gene critical for female mosquito development successfully eliminated caged populations of A. gambiae in fewer than 10 generations, with selection unable to act on resistance alleles [17].

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The development of self-perpetuating CRISPR-based gene drive approaches has led to calls for increased regulatory capacity [18], institutional oversight [19,20] and responsible governance [21,22], as such gene drive transgenes could become established in the wild through the accidental release of just a few individuals during testing [23,24]. As a result, gene drive technology has arrived at a seemingly impossible paradox: how to safely field-test a system that by its very nature may permanently alter a natural population. Strategies to split a gene drive transgene into multiple pieces are predicted to limit the spatial distribution of the invading gene [25,26], but do not prevent the long-term establishment of one or more transgenes in nature, even during field-testing when potential hazards are unknown. Remediation in the form of additional large-scale mosquito releases are a potential reversal mechanism for current gene drive approaches [25,27]. However, this is far from ideal, as a field trial to evaluate a gene drive transgene may be forced to conclude abruptly owing to factors outside the control of the research team (natural disaster, armed conflict, political change, etc.).

The unwanted persistence of transgenic material is not a unique problem to gene drive or vector biology, and efforts to ensure the removal of unwanted transgenes have been ongoing in both agricultural [28,29] and human gene therapy [30–32] applications. We reasoned that approaches developed in these disciplines but applied in the context of gene drive might make it possible to pre-programme the reversion of a gene drive transgene to a non-transgenic allele through selective excision of all transgene material in a manner than can be repressed during a trial. To evaluate whether such technologies are worth pursuing in the context of homing-based gene drive, we developed a series of deterministic models to calculate the dynamics of the spread and persistence of a gene drive transgene in a population in the presence of what we refer to as a ‘self-elimination mechanism’ (SEM).

Self-eliminating approaches were found to provide temporal control, rapidly reversing the invasion of a gene drive transgene even at very low rates of effectiveness (less than 10%) while tolerating substantial rates of failure. Stacking multiple self-elimination approaches together provided an additional layer of spatial control and could potentially serve as a form of biocontainment, preventing the invasion of gene drive transgenes into native populations during the evaluation phase. Based on these results, we suggest that homing-based gene drive transgenes can be engineered to be completely biodegradable in the environment, obviating any need for bioremediation and allowing extensive risk assessment prior to the consideration for widespread use.

2. Results

We identified at least three independent mechanisms that could be incorporated into a homing-based gene drive to limit its persistence in nature (figure 1). In case 1, the gene drive transgene, any associated marker(s), and cargo genes would be accompanied by a gene encoding a recombinase, and the entire cassette flanked with corresponding recombination sites. Expression of the recombinase would result in intramolecular recombination between the two flanking regions resulting in the excision of the intervening gene drive transgene, as well as all other transgenes, and restoration of the host allele (figure 1a). In case 2, the gene drive transgene and associated marker/cargo genes are accompanied by a gene cassette encoding an integration-deficient transposase [30], and flanked with corresponding inverted terminal repeats (ITRs, figure 1b). Expression of the transposase results in its binding to the ITRs and initiation of targeted double-stranded DNA breaks, resulting in the loss of all transgene sequences. Subsequent repair of the gap would result in the restoration of the host allele. In the final case, flanking of the gene drive and associated transgenes by a direct repeat corresponding to the wild-type host allele renders all transgene sequences susceptible to loss via a form of DNA break repair known as single-strand annealing (SSA, figure 1c). In this case, a site-specific nuclease can be directed to generate a targeted DNA break, not in the host gene, but in the transgenic construct itself. This second nuclease could be an independently coded gene from that involved in gene drive, or the DNA break could simply be generated from the insertion of the homing elements.
inclusion of an independent synthetic guide RNA, different from that required for a CRISPR-based gene drive. In either scenario, homology between the two repeated sequences promotes SSA-based repair following the double-stranded break, resulting in the loss of all transgene sequences and restoration of the host allele (figure 1c). In each of the three independent cases, the effect of the SEM is to trigger in cis removal of all transgene sequences while simultaneously generating a transgene-free allele that is resistant to future cleavage by the same CRISPR/Cas9-based gene drive. While the use of a recombinase would leave behind a scar that might perturb the activity of the host gene, silent nucleotide changes incorporated into either the transposon- or SSA-based approaches could preserve the wild-type amino acid sequence at the target gene and still provide resistance to further cleavage by the CRISPR/Cas9 gene drive.

To evaluate how such an SEM might affect the spread and persistence of a gene drive transgene in a randomly mating population, we modified previously developed deterministic models for homing-based gene drive [33] to incorporate a probability for both successful and failed transgene removal of all transgene sequences while simultaneously generating a transgene-free allele that is resistant to future cleavage by the same CRISPR/Cas9-based gene drive. While the use of a recombinase would leave behind a scar that might perturb the activity of the host gene, silent nucleotide changes incorporated into either the transposon- or SSA-based approaches could preserve the wild-type amino acid sequence at the target gene and still provide resistance to further cleavage by the CRISPR/Cas9 gene drive.

To better understand the underlying dynamics, we calculated individual allele frequencies in the absence (figure 3a) or presence (figure 3b) of an SEM when naturally occurring resistance alleles cannot be selected for owing to their high fitness costs. Without the SEM, gene drive alleles rapidly dominate the population, with a small percentage of high-cost resistance alleles making up a consistent low-level minority. By contrast, no-cost resistance alleles (v) generated by the SEM quickly overtook gene drive alleles, which were lost from the population (figure 3b; electronic supplementary material, figure S5). This was true for a broad range of rates for both SEM (0–80%) and SEM failure (0–20%), despite the absence of selection for natural resistance alleles (δ = 0), as the inclusion of an SEM led to the restoration of the population to a transgene-free status (figure 3c; electronic supplementary material, figure S5C). We conclude that incorporating an SEM approach into a homing-based gene drive transgene can potentially provide unprecedented control over the persistence of these invasive genetic elements while still allowing their temporary spread into a target population during field-based evaluation and risk assessment.

We next considered the potential for multiplexing to increase self-elimination efficiency and prevent gene drive invasion into sites outside of any potential trial area (spatial control), as currently proposed methods for spatial control

Figure 2. SEMs reverse potent gene drive systems. (a) Fitness penalties applied in the simulation for each genotype for a homing-based gene drive system targeting a gene critical for female fertility. (b) Proportion of transgene-free alleles after a single simulated release of gene drive containing individuals at 1% or 10% of a wild-type population when the selection for gene drive-resistant allele is not possible. Model outcomes for four SEM rates (α = 0, 0.1, 0.4, 0.8) are shown, all include an SEM failure rate of 1%.
of gene drive require multiple independently segregating transgenes, bioremediation or both [25–27]. In particular, SEMs based on a nuclease-induced double-stranded DNA break and SSA repair (figure 4a) could be multiplexed by simply increasing the number of nuclease recognition sites in the gene drive transgene (figure 4b). We again modelled a gene drive scenario based on the disruption of a gene critical for female fertility such as dsx (figure 4c), and this time allowed five independent attempts at transgene elimination. Multiplexing of the SEM substantially delayed, but never prevented, invasion of the gene drive transgene in the simulated population (figure 4d). As our model only allows allele frequencies to approach, but never actually reach zero, we considered that during the extended lag phase observed for even moderate values of SEM (0.4), the allele frequency of the gene drive transgene might fall so close to zero as to be considered practically zero. We plotted the maximum frequency of the gene drive transgene at any point during the simulation for arbitrary thresholds (not to be confused with the threshold for invasion of the gene drive transgene itself) down to 10\(^{-16}\), below each of which it was considered lost owing to a stochastic event (figure 4e). While a relatively crude method of introducing stochasticity, the inclusion of a multiplexed SEM reduced the frequency of the dsx gene drive transgene in the target population by up to 6–7 orders of magnitude below the initial release frequency. Altogether, these data suggest that at high rates (greater than 0.8), a multiplexed SEM may serve as a form of biocountermeasure for low-threshold gene drives (spatial control, figure 4f), while at lower rates (greater than 0–0.2), even a single SEM renders the gene drive essentially biodegradable (temporal control).

3. Discussion

The ability to develop powerful gene drive approaches not subject to genetic resistance selection reinforces the idea that it is essential to develop methods that provide precise control over them if these tools are to be successfully tested in field-based applications. Splitting a homing-based gene drive into two [9,26,35] or more [25] independently segregating gene cassettes is predicted to provide a layer of spatial control over the process of gene drive, as all fragments are required to catalyse transgene invasion. While advantageous, the use of multiple gene fragments to a single effect may complicate any risk assessment process, as any environmental impacts may need to be measured for all fragments not only on their own, but also in all possible combinations with every other fragment. Additionally, in such circumstances, while the act of gene drive is predicted to be temporally restricted, the transgene components themselves are not. Thus, to remove these transgenic sequences from a field population would require sustained inundative releases of wild-type individuals at the conclusion of any trial [27]. While not explicitly tested here, it may be possible to accelerate the removal of split drive components using the same SEM mechanisms as described here. The use of synthetic-resistance alleles released simultaneously with gene drive individuals has also been proposed as potential mitigation strategy [36]. However, such alleles could not control any gene drive transgenes that spread outside the trial site, and thus would also require a wave of remediation in the wake of an invading gene drive; logistically, this may not even be possible as gene drive-containing organisms can cross borders. The incorporation of an SEM allows the development of a single-component gene drive system...
(potentially simplifying risk assessment) while also providing a strong temporal limitation on the presence of the gene drive transgene in nature, without the need for remediative releases of non-transgenic individuals. While the use of a repressible lethal gene in combination with gene drive is conceptually similar [37], the strong selection pressure to lose such detrimental genes implies that when the safeguard fails, the gene drive could spread uninhibited. By contrast, the use of an SEM does not necessarily impose a strong fitness cost on the host, and the successful elimination of at least some gene drive transgenes creates a pool of resistance alleles in the target population that could prevent thespread of the gene drive as discussed below. We note that recombination systems such as Cre are highly efficient at catalysing the excision of transgenes in disease vector mosquitoes [38,39], piggyBac transposase can be excised and remodelized in malaria vectors [40], and SSA-based repair can result in transgene elimination in dengue vectors [41]. While the development and optimization of potential SEMs will no doubt require substantial effort, there is already substantial evidence that the technical aspects are tractable using existing technologies.

Just as CRISPR/Cas9 or other homing-based gene drive approaches that rely on homology-based repair can be thwarted by unwanted end-joining repair [14,16], SEMs that rely on DNA repair will eventually break down through the accumulation of end-joining based resistance alleles at the target site. Deleterious mutations in the nuclease or transposase can also be expected to arise, given sufficient time and a large enough population. Importantly, our modelling suggests that approaches based upon self-eliminating transgenes will be robust against permanent SEM failure at rates of up to 10%, much higher than the rate of spontaneous mutation in eukaryotic genomes. Likewise, whereas rates of homing may need to be greater than 75% for efficient gene drive [42], our models suggest that very low rates of transgene self-elimination (less than 10%) are more efficient than higher rates at controlling gene drive transgenes. While somewhat counterintuitive, we found that when the initial spread of the gene drive is efficient, the transgene effectively immunizes the target population against versions that have lost the SEM (figure 5).

The inclusion of an SEM and any associated repressible drive evaluated at all other sites, gene drive lost

Figure 4. Self-elimination may provide spatial control of gene drive transgenes at low, but not arbitrarily low thresholds. (a) A single SEM failure through imperfect NHEJ-based repair at the nuclease recognition site. (b) The inclusion of multiple nuclease recognition sites (red arrows, \( n = 5 \)) allows multiple independent attempts at self-elimination. Fitness parameters (c) used in simulated release (d) of gene drive-containing males at 1% of the population with five failures of the SEM required to create an SEM-resistant allele (s); the formation of no-cost resistant alleles (u) was not allowed (\( \delta = 0 \)). Model outcomes for four SEM rates (\( \alpha = 0, 0.1, 0.4, 0.8 \)) are shown, all include an SEM failure rate of 1%. Arrow indicates a lag phase where gene drive frequencies approach, but can never reach, zero. (e) If the proportion of gene drive alleles fell below the indicated threshold, it was considered lost, and the maximum proportion of transgenic individuals (from \( T_0 \) to \( T_{\text{lag}} \) or, if never reached, \( T_0 \) to \( T_{\text{end}} \)) was calculated. (f) Potential spatial control provided by a SEM that was repressed conditionally during a contained field trial.
for controlling homing-based gene drives also require the pre-programing of at least one no-cost resistance allele to prevent re-invasion of the gene drive transgene. Ideally, such a resistance allele would be sufficiently complex as to be unable to arise spontaneously (i.e. resistance to multiplexed guide RNAs), and with the added benefit of providing a diagnostic signal that self-elimination of the transgene had occurred. Given these limitations, it may be possible to adapt SEM approaches to other recently developed gene drive approaches [43,44]. The silent changes underlying the resistant allele could themselves become the target of a future gene drive approach, allowing reuse of the target gene while also providing strict spatial restrictions, as only those that had eliminated the first gene drive would be susceptible to the second. Importantly, the phenotypic characteristics of pre-determined resistance alleles can be evaluated empirically in laboratory and field-based settings independent of the gene drive transgene. Such experiments could determine any fitness costs associated with the resistance allele generated by the SEM, as the fitness of this allele must be higher than those possessing all possible gene drive alleles (with or without the SEM), so selection can act on the former at the expense of the latter. Inclusion of an SEM adds a substantial layer of predictability to gene drive experiments: rather than striving to develop gene drive approaches that are indestructible (and yet likely to fail in unpredictable ways), gene drive approaches can be designed to fail, but in a pre-evaluated and consistent manner.

**Figure 5. Rationale for why a weak SEM may outperform a strong SEM.** GD, gene drive allele (g); GD-resistance allele (v); SEM-resistant GD allele (s). Colour of icons indicates genotype: WT (w: black, plain), GD (g: green), GD-resistant (v: black, glow), SEM-resistant GD (s: purple).

4. Material and methods

(a) Model structure

For each of the gene drive mechanisms, we developed a system of delayed differential equations that predicted the number of offspring generated during each time step. Malthusian population growth was assumed with a daily time step through the models. Differential equations were concatenated and analysed using MATLAB 2017b. A single core with 8 GB of memory was sufficient for running MATLAB models to capture the proportions of wild-type individuals and allele progressions for all models. Parameter spaces for the remaining models used 112 cores with 392 GB of memory for up to 24 h from the Texas A&M University High Performance Research Computing (HPRC) Terra cluster for the computation of these parameter spaces. Model outputs were saved to a comma-separated values (.csv) file and plotted using Python 3.7.

The system dynamics models returned the number of adult and juvenile individuals of each genotype for every time
step throughout the simulation. Initial model parameters are provided in the electronic supplementary material, table S1.

Using the fitness costs (c) associated with each genotype and sex, adult and juvenile mortality rates (μ_A and μ_J, respectively) were adjusted such that the mortality rate could not be more than 1, giving

\[
\mu_A = \frac{\mu_A}{(1 - c)} \quad \text{for} \quad (1 - c) \geq \mu_A, \quad \text{otherwise} \quad \mu_A = 1,
\]

\[
\mu_J = \frac{\mu_J}{(1 - c)} \quad \text{for} \quad (1 - c) \geq \mu_J, \quad \text{otherwise} \quad \mu_J = 1.
\]

Mortality rates were applied at each time step, where the surviving number of adult individuals of each genotype A_i(T) was calculated by reducing the number of adult individuals of each genotype at the previous time step A_i(T - 1) by the mortality rate, such that

\[
A_i(T) = (1 - \mu_A_i)A_i(T - 1).
\]

Juvenile mortality was applied at the time the juveniles became adults, where the number of juvenile individuals surviving the development period (η) was defined as

\[
J_i(T - \eta)(1 - \mu_J)^\eta.
\]

Combining the surviving adults with the fully developed juveniles (also now adults), the number of adults with a particular genotype at time T can be defined as the number of adults surviving a single time increment (from time T - 1) and the number of surviving juveniles (from time T - η), such that

\[
A_i(T) = (1 - \mu_A_i)A_i(T - 1) + J_i(T - \eta)(1 - \mu_J)^\eta.
\]

The number of females with a particular genotype F_i was directly used in calculating the number of offspring produced. Because males do not directly produce offspring, the proportion of adult males with a particular genotype M_i was calculated such that

\[
M_i = A_M \sum_{j=1}^{n} \frac{1}{A_M}.
\]

Using the equations generated for the calculation of the number of offspring of each genotype, the fitness costs, initial input, self-elimination (α, β, γ), the probability of double-stranded break induction (q, 0.95) and the probability of homology-dependant repair (p, 0.95), the number of offspring created for each time step were calculated.

(b) Equation generation

A two-dimensional matrix was generated of all the possible genotypes of females (F_i) and males (M_i). A third dimension was added to capture every possible outcome of offspring (g_i). The value of each index within this three-dimensional index corresponded to the probability that the combination of the two parental genotypes would produce the respective offspring of the genotype. Iterating through all possible combinations of F_i, M_i and g_i, a matrix of probabilities was generated. Once the matrix was fully populated, a string was concatenated with the parental genotypes and probability of producing an offspring, resulting in the form

\[
F_i \times \Psi(g_i \mid F_i, M_i) \times M_i.
\]

This was used in the calculation of the number of offspring in the system dynamics model. All combinations of parental genotypes to create a particular offspring genotype k were concatenated in the form

\[
g_i = \sum_{j=1}^{n} \sum_{k=1}^{n} F_j \times \Psi(g_k \mid F_j, M_k) \times M_k.
\]

Equations were simplified using MATLAB’s str2sym function to reduce the additional calculations necessary when referencing and calculating equations from the system dynamics model. To calculate the daily number of offspring of genotype i that were being produced, daily reproduction rates, sex ratio and fitness costs were additionally concatenated into the equation following the simplification of the equations, for females giving

\[
\frac{\partial g_i}{\partial t} = \lambda \times \sigma \times (1 - c) \sum_{j=1}^{n} \sum_{k=1}^{n} \left[ F_j \times \Psi(g_k \mid F_j, M_k) \times M_k \right]
\]

and for males

\[
\frac{\partial g_i}{\partial t} = \lambda \times (1 - \sigma) \times (1 - c) \sum_{j=1}^{n} \sum_{k=1}^{n} \left[ F_j \times \Psi(g_k \mid F_j, M_k) \times M_k \right].
\]

Data accessibility. All data are available in the main text, electronic supplementary material or at: https://github.com/jozinzapletal/Making-gene-drives-biodegradable.

Authors’ contributions. K.M.M. and Z.N.A.: conceptualization; J.Z., N.N., M.A.L., M.E., Z.N.A. and K.M.M.: methodology; J.Z., N.N. and M.E.: software; Z.N.A.: writing—original draft; J.Z., Z.N.A., K.M.M., M.E. and M.A.L.: writing—review and editing.

Competing interest. Z.N.A. and K.M.M are listed as inventors on provisional patent TAMC:054USP2 related to self-eliminating transgenes.

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