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Focus on Applications of CRISPR

Forum

CRISPR-Based Gene Drives for Pest Control

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Clustered regularly interspaced short palindromic repeats (CRISPR)-based gene drives (GDs) could be used to spread desirable genetic elements through wild populations. With the imminent development of this technology in vertebrates, we believe that it is timely to highlight two forms of sex-ratio distorting GDs that show potential as pest management tools.

In agriculture and wildlife, invasive pests are well known: rabbits and cane toads in Australia, mink in the UK, and the omnipresent infestation of rodents around the globe. It is estimated that in the USA, introduced rats cost the economy more than US\$27 billion per year. [1]. To counter the impact of vertebrate pests, control measures are deployed that include shooting, poison baiting, trapping, and the release of biological agents (Figure 1A). These methods are costly and inadequate, and often lead to unwanted suffering in both target and non-target species [2]. GDs may offer a more cost-effective, humane, and species-specific alternative than current approaches.

Gene Drives for Sex-Ratio Distortion

The GD concept has emerged from observations that naturally occurring selfish genetic elements, such as homing-endonuclease genes and transposons, are preferentially inherited at frequencies greater than predicted by Mendelian inheritance. This ‘super-Mendelian’ inheritance allows these elements to drive through a population even if they reduce the fitness of an individual organism [3] (Figure 1B). The recent discovery and repurposing of RNA-guided CRISPR endonucleases into a set of gene-editing tools (Box 1) allows the development of synthetic GDs in a standard molecular biology laboratory [4].

One potential application of GD is to distort the sex ratio of a population. By skewing the sex ratio away from the favored Fisherian ratio of 1:1 male to female, it is possible to manipulate the reproductive performance of a population. In most pest species, female procreative capacity is responsible for maintaining the overall population size. Therefore, an efficient means of population suppression is to bias the sex ratio in favor of males [5]. A grossly male population will result in a population decline, while an all-male population will lead to eradication. Here, we describe two forms of CRISPR-based sex-ratio distorting GDs, homing GD and X-shredder (XS), both of which have the potential to drive maleness. To date,

these GD systems have only been engineered in proof-of-concept studies in mosquitoes [6,7], with the focus on controlling vector-borne diseases. After insects, invasive vertebrate pests are likely to be the next GD target.

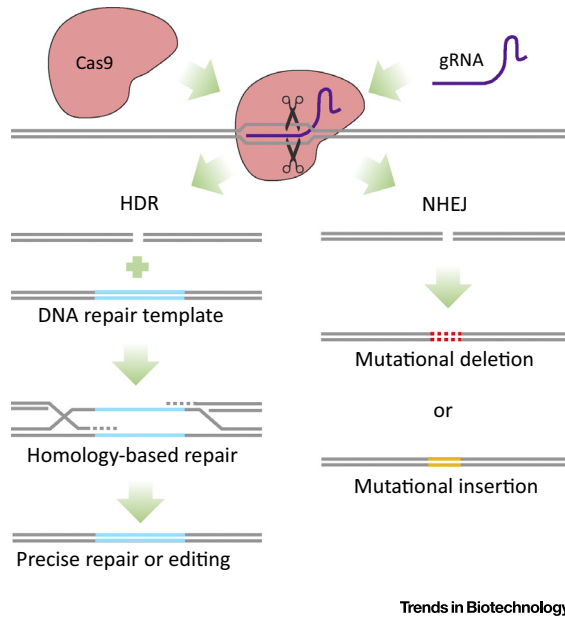
Homing Gene Drive Targeting Female Fertility

A homing GD works by copying or ‘homing’ itself into a target site in the genome. To build a CRISPR-based homing GD, an animal is engineered with a GD cassette that expresses a CRISPR endonuclease, such as Cas9, and one or more guide RNAs (gRNAs) (Box 1) from one allele that can cut at a conserved target site on the sister allele on the homologous chromosome (Figure 1C). After CRISPR-mediated cleavage, homology-directed repair (HDR) results in the CRISPR machinery and any additional payload included in the GD cassette being copied onto the homologous chromosome [4,8]. This process ensures homozygosity for the GD cassette.

Targeting a homing GD to a haplosufficient female-fertility gene (HFFG) can be used to disrupt the coding sequence of the gene, rendering homozygous female offspring infertile, while males and heterozygous females will retain normal fertility. Importantly, for this strategy to drive through a population, the homing event should occur only in germline cells that are precursors to sperm or eggs. This can

Box 1. CRISPR-Cas9

The CRISPR-Cas prokaryotic immune defence system, and its associated proteins (Cas), has been repurposed into a set of gene-editing tools. Currently, the CRISPR-Cas9 system from *Streptococcus pyogenes* is the most widely used CRISPR system for genetic manipulation. The system comprises two components: a guide RNA (gRNA) and a nonspecific endonuclease (Cas9; Figure 1). The gRNA includes an 89-nucleotide ‘scaffold’ sequence that Cas9 binds to and a 20-nucleotide user-defined ‘targeting’ sequence that delivers the endonuclease to the correct site in the genome by Watson–Crick base pairing with the target sequence. Provided that the genomic target is immediately adjacent to a protospacer adjacent motif (PAM; 5′-NGG-3′ for *S. pyogenes* Cas9), the endonuclease cuts the DNA generating a double-stranded DNA break (DSB). The natural DNA repair machinery of the cell then recognizes that the DNA is damaged and repairs the DSB by one of two pathways: (i) in the presence of a DNA repair template, such as the homologous chromosome or an exogenous DNA template, the DSB is repaired through high-fidelity homology-directed repair (HDR). HDR can be used to make accurate repairs or precisely edit the DNA sequence; or (ii) in the absence of a DNA repair template, a DSB is repaired by the error-prone nonhomologous end-joining (NHEJ) pathway. Repair by NHEJ yields deletion or insertion mutations (indels) [12].



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Figure 1. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 Gene Editing. The Cas9 endonuclease is guided to the target sequence in the genome by the guide RNA (gRNA). At the target site, Cas9 cleaves the DNA, creating a double-stranded DNA break (DSB). A DSB induced by Cas9 can be repaired by homology-directed repair (HDR) or nonhomologous end joining (NHEJ). HDR can precisely repair or edit the DNA sequence. NHEJ-mediated repair produces insertion or deletion mutations (indels).

be achieved by using a germline-specific promoter to express Cas9. By restricting homing to the germline, this will initially allow rapid spread of the GD and accumulation of fertile heterozygous GD animals that produce mostly GD gametes [7,9] (Figure 1C). As mating between heterozygous GD carriers becomes increasingly likely, the population will decline due to infertility of the homozygous GD female offspring, which are homozygous null for the HFFG [9]. With every generation, the sex ratio will become more biased towards males, eventually resulting in a population crash. Hammond and colleagues [7] developed this system in mosquitoes and achieved transmission rates of 91.4–99.6% in caged populations. In theory, homing GDs could be adapted to control most vertebrate pests and several groups are currently undertaking pilot studies in mouse models.

X-Shredder

In XY heterogametic species, an XS is a type of sex-ratio distorting GD that cuts

the X-chromosome at multiple sites during spermatogenesis, thus shredding the X-chromosome beyond repair [8]. To engineer a CRISPR-based XS, an XS cassette is inserted within a neutral intergenic region of the Y-chromosome. The cassette encodes Cas9, which is expressed under the control of a spermatogenesis-specific promoter, and one or more gRNAs that target conserved repetitive sequences unique to the X-chromosome. Given that most X-chromosomes are destroyed during spermatogenesis, most sperm that mature and reach the oocyte are Y-bearing, resulting in a biased sex ratio in favor of males. By placing the XS cassette on the Y-chromosome, all male offspring will inherit the cassette and continue transmitting the XS to subsequent generations.

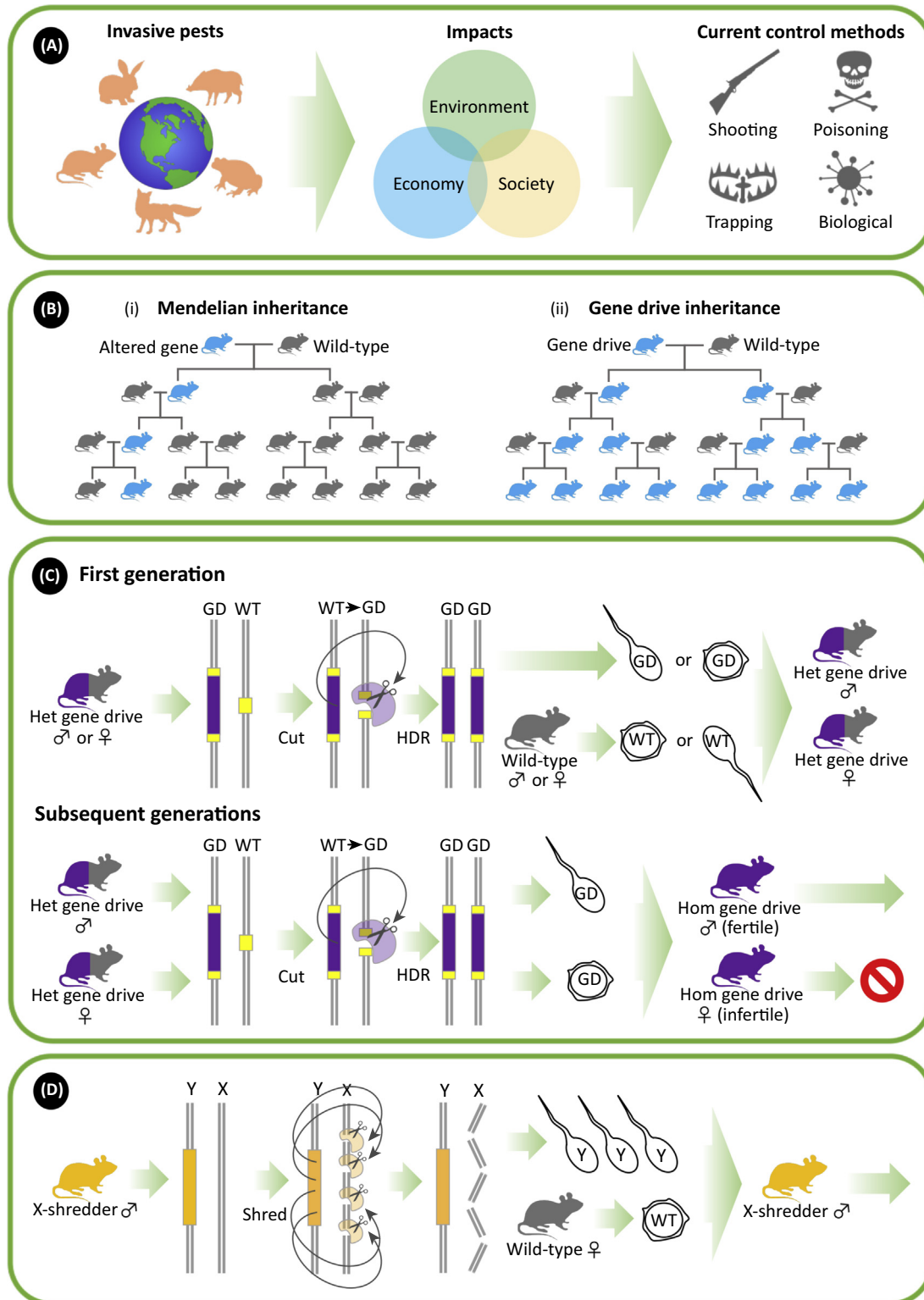
A CRISPR-based XS has been engineered in mosquitoes, although the system was commendably safeguarded by expressing the XS cassette from an autosome instead of the Y-chromosome. With

this approach, Galizi and colleagues [6] achieved male bias among progeny ranging from 86.1% to 94.8% in laboratory-contained mosquito populations. Although successful in mosquito, technical challenges facing the adaptation of an XS into vertebrates include identifying appropriate spermatogenesis-specific promoters in target species and the transcriptional silencing of mammalian sex chromosomes during meiosis. The latter may hinder expression of Cas9 from the Y-chromosome, as well as the accessibility of the endonuclease to shred the X-chromosome.

Drive Resistance and Inactivation Strategies

The two forms of GDs described above are self-perpetuating and, in theory, would only require the release of a small number of engineered animals to initiate drive. The duration and extent of spread would be limited by naturally arising resistant alleles that prevent CRISPR-mediated cleavage. Resistant alleles could exist in the population before release or originate from indels generated when CRISPR-mediated cleavage is repaired by the error-prone nonhomologous end-joining (NHEJ) pathway and alters the gRNA recognition sequence [8]. The rate of NHEJ-mediated repair will be dependent on the species, target site, and the stage of development that DNA cleavage occurs. Given that natural selection tends to favor equal sex ratios, resistant alleles that restore function would spread rapidly through the population [10].

Of the two strategies presented here, XS should be less prone to inactivation by resistant alleles because it targets multiple sites and, therefore, would require an animal to simultaneously acquire multiple resistant alleles to incapacitate the drive. Following a similar approach, it has been suggested that the evolutionary stability of homing GDs could be improved by using multiple gRNAs closely spaced along the target region [4]. To test this hypothesis,



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Figure 1. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Based Gene Drive (GD) Strategies for Controlling Vertebrate Pests.

(A) Invasive pests are a global concern. Current control strategies are inhumane, costly, and often inadequate. (B) (i) Mendelian inheritance of an altered gene. (ii)

(Figure legend continued on the bottom of the next page.)

Prowse and colleagues [9] used *in silico* modeling to demonstrate that multiple gRNAs are necessary for homing GDs to evade drive resistance and successfully suppress vertebrate pest populations. Even if drive resistance were to prevail, it would be possible to release a second GD targeting a different gene to continue suppressing the population.

Conversely, if a GD were not limited by naturally arising resistance, it would have the potential to spread indefinitely through a species. Therefore, it is essential to have strategies in place that could deliberately inactivate a GD that escaped containment or was causing unforeseen impacts. Fortunately, both homing GD and XS systems can be inactivated by the release of animals bearing engineered functionally resistant alleles or a reversal gene drive that immunizes the animal against the original drive [4,8]. However, it is important to recognize that, with the current technology, once either of these systems is released, complete reversion to a wild-type genotype would not be possible because residual Cas9 and gRNA would still be present.

Risks and Benefits

Genetically engineered animals normally come with few ecological risks. Most engineered traits are for human benefit and will not be favored by natural

selection. By contrast, GDs can spread through populations even if they reduce the fitness of each carrier animal [4]. This gives GDs more scope to escape the target population and unintentionally affect extraneous ecosystems. However, the potential benefits of GDs are equally as impactful as the risks. GDs could revolutionize public health, agriculture, and, as discussed here, could be applied for pest control and ecological restoration. In line with the recent decision at the United Nations Convention on Biodiversity, we believe that the potential benefits of GDs warrant further investigation.

Concluding Remarks

For the first time, we have the makings of a technology that could reduce or eliminate a pest population in a humane and species-specific manner. If proven effective, the decision to deploy a GD should be based on substantiated research and involve public engagement to ensure there is societal consensus. With the rapid progress in this space, the risks associated with current GD architectures are likely to be reduced with the realization of self-limiting GD strategies [11].

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Inheritance of a GD. (C) A homing GD targeting female fertility. In the first generation, heterozygous (Het) GD animals are released carrying a GD cassette (purple), which disrupts the coding sequence of a haplosufficient female-fertility gene (HFFG; yellow). Within the germ cells, the GD cassette expresses Cas9 and one or more guide RNAs (gRNAs) that cut the HFFG on the wild-type (WT) chromosome. The germ cells then repair the cut by homology-directed repair (HDR), using the GD chromosome as the repair template. This process copies the GD cassette onto the WT chromosome and ensures that most sperm or eggs carry the GD cassette. Matings between Het GD and WT animals will give rise to an increasing number Het GD animals. In subsequent generations, as mating between Het GD animals becomes increasingly likely, the population will decline through infertility of homozygous (Hom) GD female offspring, which are homozygous null for the HFFG. (D) X-Shredder (XS). During spermatogenesis, Cas9 and gRNA(s) are expressed from the XS cassette (orange) located on the Y-chromosome (Y) and shred the X-chromosome (X) beyond repair. Most sperm that mature and reach the oocyte are Y-bearing, resulting in most offspring being XS males.