Can CRISPR-Cas9 gene drives curb malaria?

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Gene drives in mosquitoes to reduce the spread of malaria move closer, though technical and regulatory hurdles remain.

Gene drives are synthetic selfish genetic elements capable of spreading through wild populations despite conferring no fitness benefit on individuals that carry them. Although their potential for controlling pest insect populations has long been recognized, they have not proven easy to implement. Now, following the recent demonstration of a CRISPR-Cas9 gene drive in *Drosophila*[1], a report in this issue by Hammond *et al*.[2] and a paper in PNAS by Gantz *et al*.[3] describe the development of CRISPR-Cas9 gene drives that function in the malaria mosquitoes *Anopheles gambiae* and *Anopheles stephensi*.

Gene drives are only one of several promising strategies for genetically modifying mosquitoes to decrease transmission of malaria parasites to humans. All of these approaches aim to reduce either the fitness of mosquitoes or their competence as malaria vectors. Transgenes conferring either of these traits could be spread into target mosquito populations without use of gene drives by releasing large numbers of modified mosquitoes (preferably males as they don't bite humans) to mate with the wild population. The effectiveness of such systems has recently been shown in a series of field trials in four countries. A strong reduced-fitness trait—dominant lethality—was successfully spread by mass-release of engineered males into wild populations of the dengue mosquito *Aedes aegypti*, resulting in >90% suppression of the target populations[4,5]. However, long-term rearing and release of the required number of mosquitoes is costly and requires an infrastructure that may not always be available. Thus, it would be helpful if transgenes conferring reduced fitness or reduced vector competence could spread on their own after an initial 'seeding' release.

Transgenes that do not confer a fitness advantage or that impose a fitness cost will tend to be eliminated through natural selection. However, some genetic elements can spread despite not conferring a fitness advantage; these "selfish" elements bias inheritance so that they are disproportionately inherited[6]. Many selfish genetic elements have been identified in diverse microorganisms and metazoans. These natural systems have inspired synthetic biologists to design gene drives—artificial selfish genetic elements with a strong propensity to spread through target populations or species.

A seminal paper[7] on using gene drives in wild pest populations proposed to use homing endonucleases—selfish genetic elements found in various microorganisms—for this purpose. After a homing endonuclease cuts wild-type DNA, the homing endonuclease gene is copied into the cut site by the cell's DNA repair machinery (**Fig. 1**). Propagation of these endonucleases can be very efficient in nature (**Fig 1**). These recent papers[1-3] substitute CRISPR-Cas9 nucleases for homing endonucleases – the principle is unchanged but CRISPR-Cas9 nucleases are much easier to retarget to specific sequences.

Gene drives based on CRISPR-Cas9 were first shown in *Drosophila*[1]. Hammond et al [2] and Gantz et al [3] have now demonstrated the approach in mosquitoes, achieving transmission rates from heterozygotes of more than 90%, and up to 99%—far higher than the 50% expected for normal Mendelian inheritance.

Hammond et al[2] devised a fitness-load approach. In their experiments the gene drive is inserted into a gene required for female fertility, thereby inactivating it. Mosquitoes with only one copy of the female-sterility gene drive are fertile, as are male carriers, and disproportionately transmit the gene drive to their offspring. The drive allele spreads, but as it becomes more abundant in the population, more females carry two copies and are sterile. Modeling indicates that nuclease-based drives could spread rapidly, e.g. from 0.05 to 0.9 allele frequency in less than two years, at which point >80% of females would be sterile, followed by further spread and the theoretical possibility of species extinction[7,8].

In contrast, Gantz et al [3] focused on the malaria-resistance trait. The pathogens that cause malaria, dengue and other mosquito borne diseases have little negative impact on their insect vector. Given that inserting an artificial gene is itself likely to have some associated fitness cost, and that not all mosquitoes become exposed to the pathogen, engineered malaria resistance is unlikely to confer a net fitness advantage and is therefore unlikely to spread by natural selection alone. Thus, malaria resistance must be linked to a gene drive if the trait is to spread. For experimental convenience, Gantz et al inserted their gene drive construct into an eye-color gene, *kynurenine hydroxylase*. This facilitated identification of construct-containing insects, however their data suggest that knocking out this gene has a fitness cost, so insertion into a more neutral location might be preferred for any future field use.

Although both papers describe functional CRISPR-Cas9 gene drives, successful deployment of these systems in the field would lead to very different outcomes. The population suppression system of Hammond et al.[2] would reduce the number of *A. gambiae* mosquitoes, perhaps to zero, as would another nuclease-based system from the same consortium that results in sex-ratio distortion and a deficit of females[9]. In contrast, the population replacement system of Gantz et al.[3] would leave the mosquito population intact—biting people and filling its ecological niche—but less able to transmit malaria.

Neither system could be implemented without further improvement. One key issue is genetic stability. Nuclease-based systems contains the seeds of their own destruction because the homologous DNA repair pathways in cells (repair by copying) compete with non-homologous end-joining, an error-prone process for ligating cut ends. Most mutations at the repair site make the sequence resistant to further cleavage and hence impervious to a nuclease-based gene drive. There are several ways to mitigate this issue, at least in principle; perhaps the simplest is to engineer the nuclease to cut at several sites in the target gene. For CRISPR-Cas9 this simply requires the expression of additional guide RNAs – this relative ease of multiplexing, together with the ease of adapting to new sequence targets (“re-programming”) are the major benefits of CRISPR-Cas9 over homing endonucleases as gene drives.

The studies have other, more specific limitations. The drive of Hammond et al[2] was designed such that female mosquitoes carrying only one copy would be fertile whereas females carrying two copies would be sterile. However, inheritance of even one gene drive copy reduced female fertility by 90-95%. This seemed to result from the element copying itself in somatic as well as germline cells, so that enough somatic cells carry two copies to render the females infertile. This greatly reduced the ability of the element to spread. In Gantz et al. [3], malaria-resistance is provided by two single-chain antibodies. *Plasmodium falciparum* would likely evolve resistance to these quite rapidly. The obvious solution is to include additional anti-parasite molecules, but how many are required is debatable. Although this would increase the size of the construct, this seems unlikely to be problematic. However, the 'linkage problem' of ensuring that the CRISPR-Cas9 gene stays attached to the anti-parasite gene has not yet been addressed. This is essential as the design must spread the parasite-resistance trait, not just CRISPR-Cas9.

Perhaps more problematic than these technical issues, the implementation of gene drives raises various societal and regulatory concerns. Highly invasive drives are expected to spread through an entire species or species complex. Proposed methods to remove or modify the drive post-release are untested. There is little precedent for this type of intervention, perhaps the closest being the release of parasitoids or predators in classical biological control. Even limited-scale field trials are challenging. Contained cage trials should be feasible, but for more realistic trials involving open releases it would be difficult to ensure that the drive cannot spread beyond the trial site. Approval for field testing of less invasive gene drive designs, such as those based on under-dominance, might be more straightforward and could inform testing of more invasive systems.

Even if CRISPR-Cas9 gene drives are approved for use, it is likely that other genetic engineering methods, including less invasive drives or the release of sterile-males, will still have a role in insect control**.** Invasive gene drives would affect an entire species. That might be desirable for some species, perhaps even these major malaria vectors, but we might prefer to affect only part of a species to avoid potential unanticipated ecological consequences. Targeting a single population in one geographic location is possible using release of sterile males and might be possible with

underdominance-based gene drives[4,10] but would not be feasible with highly invasive CRISPR-Cas9 gene drives. These reports illustrate how rapidly we are approaching the day when a full suite of genetic control methods is available to tackle malaria and other vector-borne diseases.

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Figure 1 Nuclease-based gene drives. For gene drives, a nuclease is designed to recognise and cut a specific site in the mosquito genome. (a) If the nuclease gene is inserted into this site, in a heterozygote (mosquito with one copy of the nuclease gene and one wild-type copy) the nuclease will cut the wild-type copy. The nuclease-containing copy does not cut itself as insertion of the nuclease gene disrupts the nuclease recognition sequence. The cell's DNA repair machinery repairs this damage, either by homology-based repair, copying across the undamaged – but nuclease-gene-containing – version, or simply by re-joining the cut ends (non-homologous end joining, not illustrated). If this effect is restricted to the germline, e.g. by expressing the nuclease only in the germline, mosquitoes inheriting one copy of the nuclease gene are heterozygous in somatic cells but homozygous in those germline cells where gene conversion takes place (b). This biases inheritance in favor of the nuclease; a fraction 0.5(1+*e*) of the offspring of such a heterozygote inherit the nuclease gene, where *e* is the rate of successful gene conversion, which can be 0.8-0.9 or more both for naturally-occurring homing endonucleases[7] and for CRISPR-Cas9 in insects [1-3] (c). At the population level (d), this leads to rapid spread of the nuclease gene from a small initial release, at least for high *e*[7,8].

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