PNAS COMMENTARY

Precision-guided tools for malaria control

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Malaria is the most important vector-borne disease affecting human populations, with almost 2 billion cases and over 10 million deaths recorded between the years 2000 and 2020, the majority of which are children under the age of five (1). The disease is caused by five different species of *Plasmodium* parasites which are transmitted to people exclusively by the bite of female *Anopheles* mosquitoes (1). While many species of *Anopheles* are capable of transmitting malaria parasites, one of the most important vectors is the species *Anopheles gambiae* (and the very closely related species *Anopheles coluzzii*) (1). Malaria suppression strategies are usually based on a combination of anti-parasitic drugs and vector control, the latter of which relies on the application of chemical insecticides. Due to the evolution of drug resistance by the malaria parasites, and insecticide resistance by the mosquito vectors, novel technologies are desperately needed for the control of this devastating disease. In PNAS, Apte et al. describe an important new CRISPR-based system for control of the major malaria vector *A. gambiae* (2).

Genetic technologies have shown great promise to control vector-borne diseases. These include gene drive strategies (based on *Wolbachia* symbionts or CRISPR) to modify mosquito populations to be unable to transmit pathogens, or to crash population numbers through sex ratio distortion. The application of *Wolbachia* to malaria control is problematic as artificial *Wolbachia* transinfections are limited to a single species (*Anopheles stephensi*) with serious fitness costs (3), some *Wolbachia* infections may actually enhance *Plasmodium* infections (4), and confirmed natural *Anopheles Wolbachia* infections are rare (5) and do not necessarily block *Plasmodium* in the wild (6). CRISPR-based gene drive strategies for malaria control are still in the laboratory stage and can have fitness issues that may make their deployment in the field problematic (7). In addition, it is not clear whether there will be sufficient public and political acceptance for the deployment of transgenic gene drives into natural populations (8).

Control strategies based on the release of nonbiting male mosquitoes have the potential to be not only effective, but more acceptable than those based on gene drive, as biting females are not released. The Sterile Insect Technique ("SIT") has a long history of use in diverse pest insect systems (9). In SIT, sterile male insects are released into natural populations, where they mate with wild females and reduce or eliminate their ability to have viable progeny. Traditional SIT relies on radiation exposure to sterilize males, which has the side effect of imposing severe fitness costs on released insects, significantly reducing the efficacy of the intervention (10). In addition, the sexes must be separated in the rearing facility (usually mechanically), as release of biting females is counterindicated (11).

Transgenic technologies have been developed to deal with these limitations to SIT. The first developed transgenic SIT strategy was termed Release of Insects carrying a Dominant

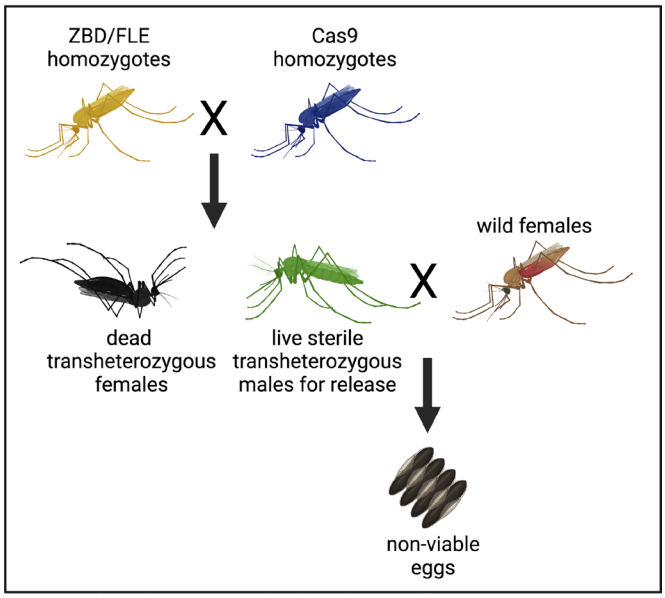

Fig. 1. Schematic of the pgSIT control method. Homozygous guide RNA and Cas9 *Anopheles* lines are maintained separately, and crossed when a release is planned. Transheterozygous female offspring die, while transheterozygous male offspring are viable for release but are sterile when mating with wild females, leading to population suppression.

Lethal ("RIDL") (12–14). With RIDL, insects are engineered to carry a chemically repressible lethal gene. The lethal gene is suppressed by drug application in the rearing facility, allowing normal rearing of the transgenic insects. When released, transgenic insects mate with their wild counterparts, generating heterozygous offspring. In the absence of the suppression drug, these offspring are rendered flightless (which is an evolutionary dead-end equivalent to death, as these insects cannot bite or mate) (13). Early versions of RIDL affected both sexes (12, 13); later refinements made the phenotype female-specific, allowing multiple generations of control from a single release through the production of heterozygous viable male offspring, the frequency of which is reduced by half each generation until the transgene is eliminated (14). Transgenic SIT strategies such as RIDL have significant advantages; they are

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highly targeted and species-specific, they rely on the release of nonbiting males, and no transgenes ultimately persist in the population (12). RIDL has been used successfully for the control of dengue virus through its application to the mosquito *Aedes aegypti* (13, 14). RIDL strains have also been developed for the mosquito *A. stephensi* (15). Other techniques, such as spermless males (16) or "X-shredder" mosquitoes (a genetically encoded sex ratio distortion system) (17), are also potentially useful for malaria control, although they are not as operationally advanced as RIDL. While RIDL and related methods are impressive technologies, they have issues that can make their adoption difficult. While RIDL has been developed for the Asian malaria vector *A. stephensi*, no RIDL strains have been built for *A. gambiae*. Technologies such as spermless males or X-shredder mosquitoes are resource-intensive to rear en masse because they are not repressible and there are issues with penetrance of the phenotypes.

In PNAS, Apte et al. describe an important new CRISPR-based system for control of the major malaria vector *Anopheles gambiae*.

A new technology, termed Precision-guided SIT ("pgSIT") has been developed to solve these issues. First developed as a proof of principle in *Drosophila* (18), then later extended to *A. aegypti* (19), pgSIT is a binary system where two different strains of transgenic mosquitoes are conventionally reared as homozygous lines. However, when crossed, the transheterozygous female offspring die, while the transheterozygous male offspring are viable and can be released. When these transheterozygous males mate with wild females, the mating is sterile. pgSIT has all the desirable characteristics of RIDL-based SIT while combining genetic sexing (where females are killed) with male sterility, in a scalable system that does not rely on chemical treatment (2, 18, 19).

In PNAS (2), Apte et al. develop a new pgSIT system for *A. gambiae*. Their initial attempt to create the strain (which carried a transgene expressing guide RNAs against the genes Zero population growth, doublesexF, and β2-tubulin; "gZBD") was a bit of a failure; while the produced transheterozygous males were 100% sterile when mated to wild-type females, the female lethal phenotype was not completely penetrant and resulted in approximately 5% viable females, a number below the acceptable threshold for control applications. However, the group had an ace up their sleeve; their previous development of a CRISPR-based sexing system called "Ifegenia", which carries a transgene expressing a guide RNA which disrupts the female essential gene femaleless ("FLE") (20), and results in nonviable females but which does not by itself sterilize males. The authors theorized that by combining both the gZBD and Ifegenia transgenes into a single mosquito strain, they could generate a pgSIT line that would exhibit both robust male sterility and female killing (Fig. 1). While one of the crosses was highly lethal in a non-sex-specific manner, the reciprocal cross was viable and had the required characteristics.

When crossed to wild-type mosquitoes, the new pgSIT strain exhibited almost 100% male sterility and female lethality. Survival experiments did not observe any detectable fitness costs in the transgenic lines compared to wild-type mosquitoes. In cage experiments, where pgSIT males were released in different ratios with wild-type counterparts, the cage populations were suppressed in a frequency-dependent manner; the more transgenic mosquitoes placed in the cage, the greater the suppression. Finally, mathematical modeling suggested that pgSIT could be used to suppress *A. gambiae* populations across a wide range of release sizes and frequencies.

The work by Apte et al. is the first scalable genetic SIT strategy to control the major malaria vector *A. gambiae*. It also has the potential to be extended to other *Anopheles* species. The target sites for the guide RNAs that drive the system are highly conserved across *Anopheles* species, suggesting that the system could be ported over to other vectors of interest either by generating transgenics in these species using the same constructs, or, in some cases where fertile hybrids can be produced, by mating introgression. Such portability is required for widespread malaria control, as although *A. gambiae* is one of the major vectors of malaria, it is far from the only vector of importance (1). Ultimately, the development of pgSIT for control of malaria vector mosquitoes opens a new chapter for the control and elimination of this devastating disease.

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