Highlights

Advanced tools and resources are available to silence or overexpress *Anopheles* genes

Precise genome editing is broadly applicable to multiple *Anopheles* species

Proof of principle for Anopheles population suppression and modification are successful in laboratory settings

Opening the toolkit for genetic analysis and control of Anopheles mosquito vectors

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Abstract

Anopheles is the only genus of mosquitoes that transmit human malaria and consequently the focus of large scale genome and transcriptome-wide association studies. Genetic tools to define the function of the candidate genes arising from these analyses are vital. Moreover, genome editing offers the potential to modify *Anopheles* population structure at local and global scale to provide complementary tools towards the ultimate goal of malaria elimination. Major breakthroughs in *Anopheles* genetic analysis came with the development of germline transformation and RNA interference technology. Yet, the field has been revolutionized again by precise genome editing now possible through site-specific nucleases. Here we review the components of the current genetic toolkit available to study *Anopheles*, focusing particularly on how these technical advances are used to gain insight into malaria transmission and the design of genetic methods to control *Anopheles* vectors.

Introduction

Malaria, transmitted solely by *Anopheles* spp. mosquitoes, remains the most important global vector borne disease and a priority target for control, local eradication and global elimination. Despite the halving of global malaria deaths between 2000-2015, mainly due to enormous programs that successfully targeted *Anopheles* vectors with insecticide treated nets, 445,000 people still die annually from the disease and some indicators point to a stalling of progress [1]. There is therefore an urgent need to tackle malaria transmission using all the methods available, including improved insecticide, vaccine and drug development. To complement these public health approaches to disease control, advances in genetic methods have promised 'realizable' tools that will bolster the fight against *Anopheles* mosquitoes.

Large scale comparative sequencing projects are currently used to identify genes that potentially impact malaria transmission [2]. Understanding the physiological function of these candidate genes requires techniques that silence, mutate or overexpress them *in vivo* to characterize the resultant alteration in phenotype. Here we will review these genetic tools (summarised in Table1), illustrated with recent insights into how they may be used to study the mosquito biology influencing vectorial capacity, including the latest advances that are directed towards *Anopheles* population control.

Functional genetic analysis

RNA interference (RNAi)

Main current use: rapid validation of the effects of gene knockdown on vector phenotype

Perhaps the most widely used method for genetic analysis in *Anopheles* mosquitoes has been RNAi. The technique can be highly gene specific, since it relies on near complete homology to target specific mRNAs for degradation (reviewed in [3]). The technique is based on the natural cellular response to the detection of double stranded (ds) RNA. After introducing synthetic dsRNA homologous to a target gene, the cellular machinery processes this template to direct cleavage of complementary mRNA, thereby reducing the encoded protein level over time (Fig 1A).

A recent extensive example of this approach involved silencing *Anopheles* innate immunity genes using a phenotype readout of prevalence and intensity of *Plasmodium* infection [4]. These experiments demonstrated that the antagonistic and agonistic effectors [5,6] of *Plasmodium* development are differentially expressed in alternative *Anopheles* species in response to the intensity of parasite burden. This robust technique has also been applied to many other diverse aspects of *Anopheles* biology ranging from insecticide resistance [7] to heart rate [8]. Although most commonly used in adults, the technique can also be successfully applied to embryos [9] to study developmental gene function and, with some difficulty, to pupae [10]. Larvae do not survive the injection process well, and techniques have been recently modified to silence larval genes by feeding chitosan/dsRNA nanoparticles or bacteria/yeast expressing dsRNAs [11].

Overall, the technique provides rapid assessment of loss of gene function phenotypes, however in anophelines not all genes and/or tissues are targeted efficiently [12] and so care is needed to monitor protein (or mRNA) turnover. In addition, off-target silencing has the potential to complicate analysis, but has not been extensively studied in anophelines [3]. Nevertheless, the major advantage of this approach is that the effect of gene silencing can be monitored stage-specifically, which can often be more difficult to achieve with other methods.

By targeting vital genes, RNAi may play a role in anopheline and malaria control. Perhaps the closest to translation are RNAi-based larvicides delivered as dried pellets to defined larval breeding sources to provide *Anopheles* specific killing with limited toxicity to other organisms [11].

Transposable elements

Main current use: assaying phenotypes produced by tissue specific and ectopic expression of transgenes, promoter trapping, creating docking lines for comparative analysis

The RNAi methodology offers a one stage or one generation approach to loss of function analysis, and so needs to be continuously repeated to provide experimental material. By modification of germline chromosomes, stable mosquito lines are generated that inherit loss or gain of gene function phenotypes. Such transgenic anopheline lines were firstly created using processes adopted from *Drosophila* methodologies. These included embryo microinjection targeted to developing germline cells, permissive transposable element transformation vectors (Minos and PiggyBac) (Fig 1B) that catalyse integration of themselves (and linked DNA cargoes) into germline chromosomes, and promoter sequences to drive expression of selectable marker genes to visually detect the rare transgenic progeny [13,14]. Using these transposon-based methods and a range of cloned tissue-specific promoters [15] to regulate transgene expression, stable *Anopheles* mosquito lines can be generated that ectopically express endogenous/foreign genes or dsRNAs to examine phenotypic consequences of increased or

decreased expression respectively. For example, as discussed in greater detail below, *Plasmodium* refractory strains of *Anopheles* have been developed through midgut expression of a number of different gene families [16].
The integration sites of transposons are nearly-random in the genome, often leading to variability in expression between lines carrying the same transgene in different genomic locations (position effect), and to insertional mutagenesis if transposon integration disrupts a coding sequence. The end result is that multiple lines need to be generated to draw reliable conclusions on the phenotype.
Although their use is likely to be superseded by the flexibility of site-specific approaches, the random nature of transposon insertion makes them particularly useful for unbiased screening techniques such as promoter trapping, whereby DNA regulatory regions can be identified following the insertion of a promoter-less fluorescent reporter gene in their vicinity [17]. Transposon integration can also be used to screen for genomic sites that produce limited fitness cost and consistent transgene expression [18], suitable for docking lines described in the following section.

Site directed integration

Main current use: comparative functional analysis

To normalise position effect variation of expression, docking lines have been created that direct the insertion of genes into 'tagged' genomic sites through the site-directed recombinase phiC31 [19]. This is achieved by including a recombinase docking (attP) site in the integrated transgene to create a stable line tagged at a defined locus. Alternative transgenes can then be inserted into this locus by including a donor (attB) site in the new transformation vectors and a source of PhiC31 enzyme to catalyse the recombination reaction (Fig 1Ci). Each line should express the alternative transgenes at similar temporal and spatial patterns and levels allowing robust comparison of relative gene function.
The phiC31 recombinase can also be used to precisely exchange transgenes into defined genomic sites (Fig 1Cii) by placing an attP site on either side of the docking transgene [18]. New DNA constructs carrying flanking attB sequences will then swop into these sites by recombination to seamlessly replace the original transgene [20].
A number of docking anopheline lines are now available and further refinements are discussed in [21]. This review also introduces a flow cytometry method (COPAS^{TM}) which allows rapid fluorescence screening of live mosquito larvae that speeds the process of generating and characterising transgenic lines.

Bipartite expression systems

Main current use: to study toxic genes, comparative functional analysis

The scope of *Drosophila* genetic analysis has been based largely on the use of bipartite expression systems. By separating the transgene under analysis (responder) from the regulatory regions controlling its expression (driver) in distinct transgenic lines, genes causing extreme fitness or developmental phenotypes can be examined in the progeny of driver and responder line crosses (Fig 1D). As well as examining toxic genes, bipartite systems also provide a flexible platform for comparative tissue specific expression analysis whereby different effector genes can be assayed in multiple expression patterns simply by crossing appropriate responder and driver lines.
Different bipartite systems have been utilized in *Anopheles*. The tTA/tetO system has been developed for gut [22] and flight muscle [23] specific expression in *An. stephensi*. Similarly, the GAL4/UAS has been used for enhancer trapping in *An. stephensi* [17], and ubiquitous [18] and gut specific expression [24] in

An. gambiae. Finally, the Q system has been exploited to characterise *An. gambiae* olfactory receptor neurons [25].
In the latter case, piggyBac insertions created driver lines carrying the *An. gambiae odorant receptor co-receptor* (*orco*) promoter controlling QF2 transcription activator expression and responder lines carrying QF2 binding sites (QUAS) upstream of a membrane-localised green fluorescent protein (mGFP) gene. In the progeny of driver and responder crosses, the mGFP localisation recapitulated the endogenous *orco* expression pattern, and hence fluorescently tagged all neurons bearing olfactory receptors. In this way olfactory innervations that may be involved in host recognition were exquisitely mapped. The bipartite system generated opens one way to express reporter genes that can monitor real time activity in discreet neuronal families to define the olfactory 'code' of odour recognition during host seeking. Moreover, by adoption of the many other reporter based approaches used in *Drosophila*, it may be possible to modulate and disrupt host-seeking behaviour [25].

Site specific nucleases

Main current uses: site specific genome editing, gene knockout and knockin

The advent of relatively flexible and powerful genome editing tools based on a range of site-specific nucleases offers the potential for a vast array of precise genome modifications which is revolutionizing genetic analysis in *Anopheles*, as in other model and non-model organisms [26]. They all rely on site specific endonuclease cleavage of germline DNA that is repaired in the cell by either i) 'imperfect' non homologous end joining (NHEJ) that most often creates frameshift mutations, or ii) homology directed repair (HDR) whereby homologous sequences are used as a recombination template to repair the damage. HDR can be exploited by providing transgenes flanked by homologous sections around the known cut site, so that during repair the transgene cargo is copied into a precisely defined location. Although not published extensively yet in *Anopheles*, it is relatively straightforward to mutate or insert a DNA cargo into virtually any genomic location desired, particularly in the case of Cas9 modifications as described and illustrated in Fig 1E.
Such site specific genome editing has been performed in anophelines using homing nucleases [27], transcription activator-like effector nucleases (TALENs)[28-30], and Cas9 nucleases [20,31-33]. With homing endonucleases and TALENs, specific genomic sequences targeted for cleavage are recognized directly by the nuclease, and so altering the target site can be a fairly intensive protein/DNA engineering process. Nevertheless, a homing endonuclease was the first used in *Anopheles* for site specific modification [27] (see below), and more recently TALENs were created for both gain and loss of function analysis of the TEP1 complement-like protein in *An. gambiae* to examine its role in *Plasmodium* killing [29,30]. A TALEN kynurenine 3-monooxygenase gene knockout in *An. stephensi* [28] also answered a long-standing question on the role of mosquito-derived xanthurenic acid in stimulating *Plasmodium* development in the mosquito midgut.
The CRISPR/Cas9 methods are generally much simpler to perform, since the Cas9 endonuclease is directed to a specific DNA locus site by a unique, complementary short guide RNA which can be rapidly designed and synthesised. Highly efficient CRISPR/Cas9-mediated NHEJ mutagenesis of visible phenotypic markers [31] has been demonstrated in multiple *Anopheles* species, as well as efficient HDR in *An. stephensi* [32] and *An. gambiae* [20], supporting the broad potential applications across the genus. With continual refinements in targeting efficiency, CRISPR/Cas9-based analysis thus seems likely to dominate genome modification approaches for the foreseeable future.
Most of the recent applications of CRISPR/Cas9 in anophelines have been in the field of gene drives and will be discussed in more detail below. However, the potential applications of CRISPR/Cas9 in functional

genetic analysis of anophelines is very broad: base pair changes to introduce point mutations, insertion into coding regions to create gene knockouts, substitution of genes or fluorescent protein tagging, chromosome rearrangement, regulation of gene expression, and chromatin modification. The above list of potential applications is far from exhaustive and further developments will undoubtedly broaden this portfolio as technological improvements increase control, efficiency and specificity.
As neurobiological examples, it would be relatively straightforward to target different classes of sensory neurons (e.g. Fig 2 – *Anopheles* chordotonal organs) to co-express bipartite drivers with specific neuronal genes by Cas9-mediated HDR. By appropriate design and fusion with a 2A self-cleaving peptide [34], the driver coding sequence could be inserted into a known neuronal specific gene to achieve bicistronic expression regulated by the native promoter. Co-translational cleavage would be expected to produce equimolar amounts of the two proteins in active forms that would create a neuronal driver suitable to express a variety of reporters. These may include fluorescent tags for anatomical mapping [25], genetically encoded ion sensors to follow real time activation [35], and knockout constructs to examine loss of function phenotypes. More simply, gene knockout and mutagenesis could be used to validate neurological target sites of novel insecticidal compounds.

Applications for malaria control

Two general strategies are being pursued for control of malaria through the release of genetically modified (GM) mosquitoes. One involves reducing the effective population size of *Anopheles* mosquitoes (suppression), while the other aims at reducing the number of infectious bites people receive without affecting population size (modification).

Population suppression strategies

Generally, suppression strategies aim at reducing the vector population size by impairing their ability to successfully reproduce (reviewed in [36-38]). A combination of transposon and site directed genetic approaches contributed to create anopheline lines displaying population suppressive traits.
PiggyBac transformed lines were used to express homing nucleases that cleaved zygotic X chromosomes resulting in male sterility [39], which was further refined to bias production of male-only progeny [40]. Sex distortion was demonstrated again using transposon-mediated insertion of CRISPR/Cas9 components resulting in selective destruction of X chromosome-bearing sperms [41]. Flight, and thus mating capability, has also been targeted through tTA regulated expression of apoptotic effectors in female flight muscles [23]. More recently, female-targeted suppression was reported in phiC31 cassette exchange-derived lines carrying CRISPR/Cas9 alleles that knocked out three ovarian genes in *An. gambiae* [20] rendering females essentially sterile. In the near future, the increasing knowledge of *Anopheles* sex determination [9,42] and the improved capability of modifying the Y chromosome [43] will greatly contribute to genetic sex distorting technologies.

Population modification strategies

While suppression strategies are based on introducing a significant fitness load into the population, modification (or replacement) approaches rely on the introgression of traits that impair parasite development in the vector to limit malaria transmission.
Various strategies for the creation of GM anophelines that are refractory to infection have been exploited and are reviewed in [44]. Successful parasite blocking relies on tightly regulated promoters

driving precise temporal and spatial expression of antimalarial molecules in relevant tissues (usually midgut, haemolymph, and salivary glands) to coincide with parasite development following ingestion. To date, most strategies to modify transmission have relied on the use of transposon or integrase-mediated strategies to express different effector genes including: endogenous or exogenous antimicrobial molecules; interaction inhibitor molecules; or the sustained activation of antiparasitic signalling pathways [16,44]. RNAi and then later CRISPR/Cas9-based NHEJ approaches have also been exploited to silence the parasite agonist fibrinogen-related protein-1 in *An. gambiae* [33,45]. While these strategies result in a significant decrease of *Plasmodium* prevalence and/or intensity of infection, they most often impose fitness costs that may also be indirectly responsible for parasite load reductions. A more specific anti-*Plasmodium* approach involves supplementing mosquito innate immunity by expressing single chain antibodies (scFvs) that target antigens expressed on the parasite surface during development within the mosquito [46]. In view of their specific blocking capacity and the fact that these mosquitoes appear as fit as wild type under the conditions tested [47], expression of multiplexed scFvs is perhaps the most promising current approach for population modification.

Gene drives

While proof-of-principle experiments for population suppression and modification have been successful, strategies that would rely on reiterative mass release of mosquitoes to spread a trait are very costly, and a drive system for the rapid introgression of favourable traits into a wild population from relatively low threshold numbers is likely to be more readily achieved and sustained.

Gene drives are based on genetic elements that are inherited with a frequency greater than Mendelian segregation rates, which allows them to spread in a population in relatively few generations, theoretically even against a degree of fitness cost [37] (Fig 3). While gene drives are not a new concept [48], the advent of programmable site-specific nucleases made their application more tractable [37,49] The first proof of principle for a gene drive in anophelines was based on the expression of the homing nuclease I-SceI [27] targeting a modified transgenic GFP. Cage trials indicated high drive efficiency and opened the potential for similar systems to force desirable traits into populations. More recently, CRISPR/Cas9 was used to generate two further gene drives [50]. A population modification system was successfully developed in *An. stephensi* to drive scFvs directed against *Plasmodium* proteins into a caged population with >99% efficiency [32]. Shortly after, a population suppression gene drive was described in *An. gambiae* that targeted one of the aforementioned candidate ovary specific genes for knockout and showed around 75% drive efficiency in caged populations [20].

These initial studies showcase the immense potential for transforming populations, but a number of significant hurdles need to be overcome before they can be used in real world scenarios for disease control. These issues and potential proposed solutions are extensively discussed in the indicated articles and include: technical obstacles, such as the generation of resistance to the drive [51,52]; concerns about standards for laboratory confinement [53,54]; the feasibility of conducting safe and ethical field trials [55] and options for recalling, limiting spread or stopping the drive once released [56]. In this context, modelling of long term population dynamics will be critical to predict potential epidemiological outcomes, as well as longer term effects on biodiversity and ecosystems [57-59].

While still in their infancy, CRISPR/Cas9-based gene drives hold out a promise of very powerful, cost-effective and self-sustainable tools to be added to the malaria control toolbox. Moreover, the ongoing advances in genetic technologies enable the discovery of gene functions that will inform and direct improvements in current intervention strategies through greater knowledge of vector biology.

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Table 1. Comparison of genetic manipulation techniques in *Anopheles*

Basis of modification	**RNA Interference**		**Transposon**	**Integrase/recombinase**	**Bipartite expression systems**	**Site specific nucleases**
	Transient	**Stable**				
System used	Synthetic dsRNA injection/feeding	Transgenic expression of hairpin RNA	Minos PiggyBac	phiC31	tTA Gal4/UAS Q	HEGS TALENS CRISPR
***Anopheles* Species modified**	*An. gambiae/coluzzii [3]* *An. stephensi [60]* *An. arabiensis [9]* *An. dirus [61]* *An. sinensis [10]* *An. aquasalis [62]*	*An. stephensi [63]*	*An. gambiae/coluzzii [13]* *An. stephensi [14]* *An. albimanus [64]*	*An. gambiae/coluzzii [19]* *An. stephensi [47]*	*An. gambiae/coluzzii [24,25]* *An. stephensi [22]*	*An. gambiae/coluzzi[20,27,29,31]* *An. stephensi [32]* *An. funestus [31,32]* *An. albimanus [31,32]*
Site of integration	N/A	Dependant on integration system used.	Essentially random (TTAA for piggyBac, TA for Minos)	Site directed into pre-existing attP tagged sites in the genome.	Dependant on integration system used.	Predetermined, targeted genomic sites.
Advantages	Rapid; Relatively high throughput; Can be targeted to adult stages.	Tissue and temporal specific knockdown; Heritable knockdown.	Unbiased integration; Suitable for locus, fitness, and enhancer/exon screening; Can be remobilised for mutation screens.	Comparative analysis of genes expressed at same genomic locus. Greater cargo size than TEs.	Suitable for toxic and developmental genes; Flexible analysis of tissue specific phenotypes.	Vast array of mutations and expression regulated lines can be created. Can utilise endogenously located promoters and genes for mutagenesis. Potential drives are easily designed (CRISPR/Cas9).
Drawbacks	Transient; One generation only; Does not target all genes/tissues equally.	Longer time to generate through transgenesis; May be selected against during maintenance.	Positional variegation of expression; Limited number of promoters available; Limited cargo size 10-12 Kb.	Need to create docking lines initially; Limited number of promoters available.	Multiple lines need to be maintained. Limited number of promoters available.	Except CRISPR/Cas9, quite extensive engineering required. Normally creates null mutations, potentially inviable if dominant.

FIGURE CAPTIONS

Figure 1.

A) RNA interference (RNAi)
Synthetic double stranded RNA (dsRNA) homologous to the target gene transcript are typically injected into the mosquito haemocoel, where they are taken up by some tissue types (including fat body, oenocytes, haemocytes and midgut) and cleaved by the Dicer ribonuclease into small (~20 nt) interfering dsRNAs (siRNAs). These are incorporated into the RNA-induced silencing complex (RISC) which degrades one strand, while the other strand directs the silencing complex to the target mRNA. After strict complementarity-based binding, the RISC nuclease Argonaute (Argo) catalyzes the cleavage and thus inactivation of the mRNA. Several days are left to allow for protein turnover, after which phenotypic assays are performed versus control mosquitoes.

B) The PiggyBac Transposon
Embryos are injected at the posterior pole (germline localization) with a donor plasmid carrying the transgene flanked by piggyBac inverted terminal repeats (ITR) and a transient source of transposase (TR), either plasmid encoded, mRNA or purified protein. The TR recognizes the ITRs and catalyzes the "cut and paste" of the cassette into a "random" TTAA sequence in the germline chromosome. Insertion preserves the ITRs and if transposase is supplied in later generations, remobilization of the cassette can occur. Modification of the germline is passed onto the next generation and can be followed by monitoring expression of a fluorescent marker gene (such as dsRed) linked to the transgene. Transgenes will normally consist of a promoter to drive tissue specific expression of a downstream effector gene followed by a signal sequence to terminate transcription.

C) The PhiC31/att system.
Embryos of phiC31 docking lines carrying (i) one or (ii) two attP sites in a defined chromosomal locus are injected with a donor plasmid carrying a transgene and fluorescent protein gene flanked by one (i) or two (ii) attB sites and a transient source of integrase (INT). **i) Integration**. The integrase catalyzes the recombination at the att sites resulting in the integration of the entire donor plasmid DNA into the genomic docking site. **ii) Cassette exchange**. The integrase catalyzes double crossover recombination the att sites resulting in the swap of the originally integrated cassette and removal of the plasmid backbone. The alternative transgenes are generally marked with distinct fluorescent proteins to facilitate inheritance screening. Recombination results in the formation of hybrid sites attR and attL that are resistant to remobilization.

D) Binary expression systems.
In anophelines, three types of bipartite expression systems have been developed: the tTA/tetO, GAL4/UAS, and Q systems. All comprise of separate driver lines that express a transcriptional activator factor under regulation of a desired promoter, and responder lines carrying the gene of interest downstream of corresponding activation factor binding sites. When the two components are brought together in the progeny of driver and responder crosses the transgene will be expressed and potential phenotype observed. The driver construct dictates the temporal and spatial pattern of expression; the responder construct determines which protein is expressed. The lines are usually marked with different fluorophores to facilitate screening for co-inheritance. GAL4, QF and tTA are transcriptional activators from yeast, *Neurospora crassa,* and *E. coli* respectively. UAS, QUAS and tetO are their corresponding binding sites. The tTA systems are responsive to tetracycline, and so gene expression can be switched on/off by supplying mosquitoes with tetracycline analogues. The GAL4 and QF system can be switched off by co-expressing either Gal80 or Q-S suppressors respectively in the same tissues (although this has not yet been performed in anophelines).

E) CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 site-specific mutagenesis.
During embryo injection, a transient source of site-specific endonuclease Cas9 is provided alongside a short (-20 nt) guide RNA (gRNA), either *in vitro* transcribed, plasmid encoded or synthesised, which has perfect complementarity to the sequence targeted for modification. The target site is defined by and lies immediately adjacent to a protospacer adjacent motif (PAM) with sequence NGG. The Cas9 is directed to a desired genomic target by the gRNA and creates a double stranded DNA break that is precisely located 3 bp upstream of the PAM site. Chromosome integrity is restored by the cell DNA repairing machinery using either of two different mechanisms: Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR). NHEJ results from the imprecise ligation of the broken DNA extremities which in most cases causes frameshift indel mutations and thus gene knockout when occurring in coding regions. HDR occurs when donor DNA having significant homology surrounding the breakpoint is used by the cell as a template to repair the break. By including a desired cargo (e.g. promoters, markers, mutagenised genes, tags) flanked by sequences homologous to the surrounding breakpoint locus into the co-injected donor DNA template, these are precisely incorporated into the genomic site via homologous recombination.

Figure 2. Anopheles chordotonal organs
A, B, C: Piggybac transformed *An. gambiae* carrying an alpha-tubulin promoter fusion with eGFP and dsRed fluorescent marker driven by the synthetic neuronal promoter Pax3. Fluorescently tagged chordotonal (mechanosensory) organs (yellow arrows) are visible through the cuticle and hence readily available for real time vital imaging in **A)** larval abdomen and thorax, **B)** dorsal side of the adult thorax and **C)** the junction of thorax and femur [65]. **D)** Immuno-stained section of Johnston's organ (JO) or the male mosquito antennal 'ear'; phalloidin (actin - blue) and anti-HRP (neuronal membrane marker - red), courtesy of Marta Andres Miguel [66]. The JO is a complex chordotonal organ located at the base of the flagellum (distal part of antennae) and contains over 15,000 auditory neurons that transduce sound-induced mechanical vibrations from the flagellum into electrical signals.
The auditory neurons are bipolar and monodendritic, with ciliated dendritic outer segments pointing towards and indirectly coupled to the base of the flagellum. The auditory neurons are arranged in groups of 2-3 and form together with two accessory cell functional units called scolopidia. Mosquitoes mate in swarms, and males detect potential mates by tuning into the wing-beat frequency of conspecific females. Analysis of mosquito hearing has many avenues for mosquito control, including insecticides that target signal transduction, genetic control targeting mating success and acoustic traps.

Figure 3. CRISPR/Cas9-based gene drives.
To create a gene drive, the germline transformation is designed so that the gRNA and Cas9 are included in the knockin construct and so are incorporated into the cleaved target site on the chromosome or so called 'drive' allele by HDR. The chromosomally encoded Cas9 will then cut the wild type allele on the homologous chromosome, which by HDR is converted into another drive allele. By using promoter sequences to drive nuclease expression in early germline tissues, most or all of the gametes will thus contain a drive allele which will be inherited by up to 100% of the subsequent progeny. Other genes (cargo) can be included along with the nuclease to encode desired traits that will be also inherited at the same frequency as the drive, and/or if the target sequence is within a coding region then this gene will be knocked out simultaneously during HDR.
In the suppression drives designed to date, the inheritance of the drive allele disrupts the coding region of genes that regulate reproductive capacity. If Cas9 activity is restricted to the germline, then fertile somatic heterozygotes, will carry homozygous gene drives in the germline and pass on the drive at super-Mendelian rates. In time, the increase of drive allele frequency will produce a predominance of somatic infertile homozygotes that results in a crash of population size.
In modification drives, genes conferring refractoriness to malaria are inherited at super-Mendelian rates along with the drive. Depending on the degree of fitness costs due to modification, the antimalarial trait will introgress into the population while the overall size will not be affected.

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The study describes the use of transposon based analysis to identify an endogenous ubiquitous promoter in An. gambiae and to define genomic sites that are permissive to the expression of Gal4 drivers in multiple tissues.
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The first report of the use of CRISPR/Cas9 in An. gambiae, as well as its development as a population suppressive gene drive system. A mixture of TALEN and Cas9 based approaches were used to examine sterility phenotypes (whether haplo-sufficient) of knockout of three ovary specific genes and concomitant knockin of a GFP marker and phiC31 docking sites. Moreover, RMCE was then used to insert Cas9/gRNA alleles at the same loci to assess the efficiency of suppressive gene drives targeting these genes. One of the loci was modelled and proven in cage testing to show an increase in allele frequency over 4 generations. Brief mention is made of the problem of alleles to drive.

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23. Marinotti O, Jasinskiene N, Fazekas A, Scaife S, Fu G, Mattingly ST, Chow K, Brown DM, Alphey L, James AA: **Development of a population suppression strain of the human malaria vector mosquito, Anopheles stephensi**. *Malar J* 2013, **12**:142.
24. Lynd A, Lycett GJ: **Development of the bi-partite Gal4-UAS system in the African malaria mosquito, Anopheles gambiae**. *PLoS One* 2012, **7**:e31552.
25 •. Riabinina O, Task D, Marr E, Lin CC, Alford R, O'Brochta DA, Potter CJ: **Organization of olfactory centres in the malaria mosquito Anopheles gambiae**. *Nat Commun* 2016, **7**:13010.
The first paper describing the use of the Q bipartite system in Anopheles. PiggyBac insertions created 4 different sets of neuronal promoter QF driver lines and a responder line carrying QUAS-mCD8 membrane GFP. One set of the driver lines was confirmed to recapitulate the expression pattern expected, and was elegantly used to fluorescently tag the neurons expressing the odorant receptor co-receptor, orco. The lack of activity in the 3 other drivers illustrates the difficulty in defining functional promoter sequences in mosquitoes.
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First report of a gene drive system in Anopheles. This was based on the use of the male germline expressed homing endonuclease targeting a GFP transgene, and demonstrated the proof of principle for drive systems to invade cage populations
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This paper uses multiple genetic tools to address the functional activity of immune factor TEP1 in plasmodium infection. Most novel is the first use of a deactivated site specific nuclease (TALEN) to modulate expression of the native gene. Although only moderate enhancement of expression was achieved that didn't impact plasmodium development, this is a significant step on the way to regulate endogenous expression in anophelines.
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Application of CRISPR/Cas9 NHEJ knockouts to multiple Anopheles species targeting the X linked white gene, that allowed easily scorable phenotype and differentiation between biallelic and single allele mutagenesis. Provides standard protocol to inject in vitro synthesised gRNAs and commercial purified Cas9 protein, and suggests a very high frequency of mutation.
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First report of CRISPR/Cas9 HDR-based germline transformation and CRISPR/Cas9-based gene drive in Anopheles. The paper describes efficient gene drive of antimalarial single chain antibodies through the male line, with near Mendelian inheritance through female lines, suggesting that strict germline expression is required for effective drives.
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Following on from RNAi approaches published previously, this uses work CRISPR/Cas9 knockout to demonstrate the role of FREP1 as a positive regulator of Plasmodium infection in the midgut of An. gambiae. Fitness costs of the null mutation may contribute to changes of parasite burden seen in the knockouts.
34. Wang Y, Wang F, Wang R, Zhao P, Xia Q: **2A self-cleaving peptide-based multi-gene expression system in the silkworm Bombyx mori**. *Sci Rep* 2015, **5**:16273.
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38 ●●. Burt A, Coulibaly M, Crisanti A, Diabate A, Kayondo JK: **Gene drive to reduce malaria transmission in sub-Saharan Africa**. *Journal of Responsible Innovation* 2018, **5**:S66-S80.
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41 ●. Galizi R, Hammond A, Kyrou K, Taxiarchi C, Bernardini F, O'Loughlin SM, Papathanos PA, Nolan T, Windbichler N, Crisanti A: **A CRISPR-Cas9 sex-ratio distortion system for genetic control**. *Sci Rep* 2016, **6**:31139.

Describes the production of a CRISPR/Cas9-based version of the X-shredder strategy introduced in [40]. This population suppression approach is based on the selective disruption of the X chromosome during male spermatogenesis to create strongly biased male-only progeny. The strategy may also be directly adaptable to other species with the same sex determination.

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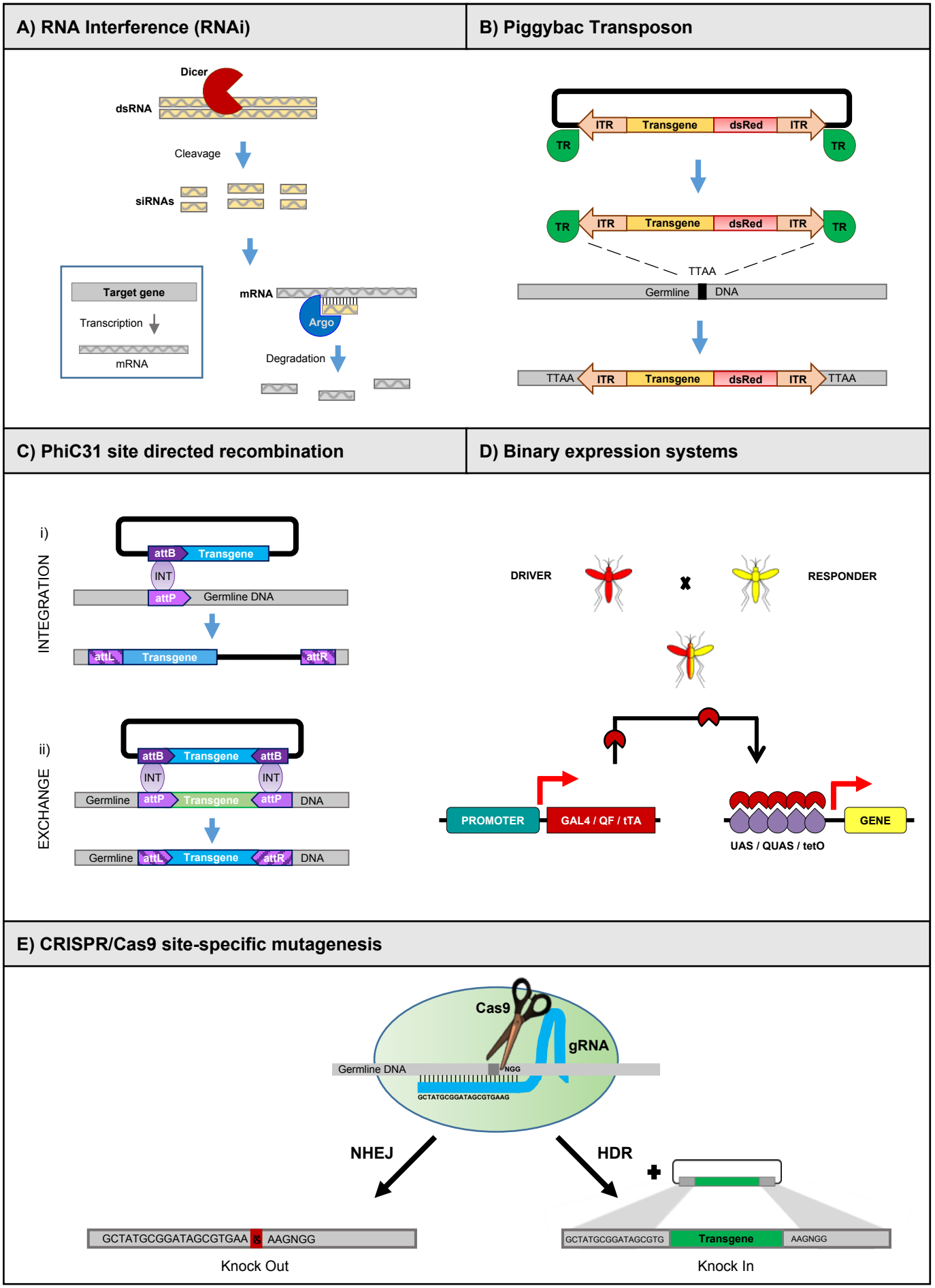
A) RNA Interference (RNAi)
Dicer
dsRNA
Cleavage
siRNAs
Target gene
Transcription
mRNA
mRNA
Argo
Degradation
B) Piggybac Transposon
ITR
Transgene
dsRed
ITR
TR
TR
TTAA
Germline
DNA
TTAA
C) PhiC31 site directed recombination
i)
INTEGRATION
attB
Transgene
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Germline DNA
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Transgene
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ii)
EXCHANGE
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Transgene
attR
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D) Binary expression systems
DRIVER
RESPONDER
PROMOTER
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E) CRISPR/Cas9 site-specific mutagenesis
Cas9
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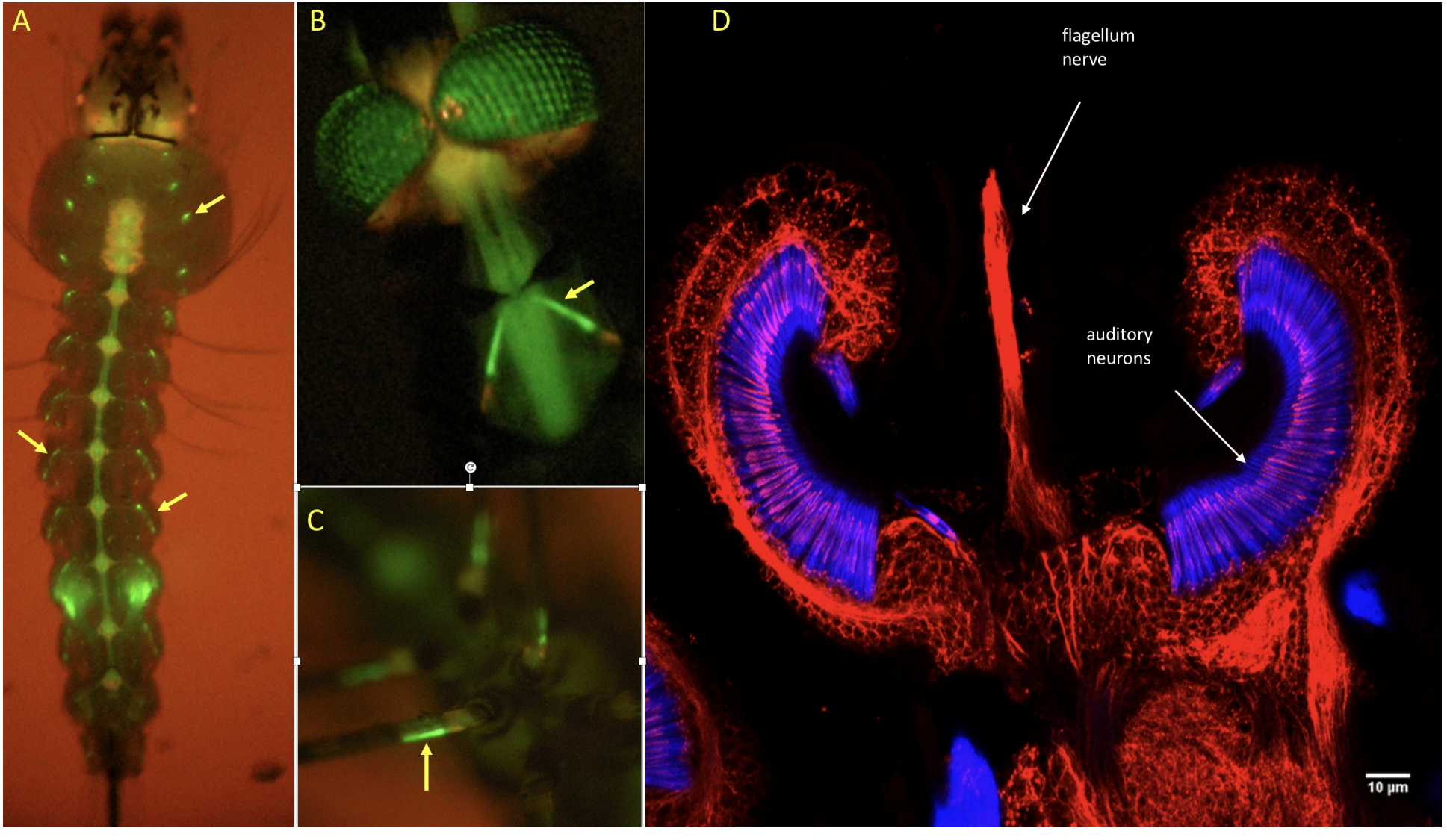
A
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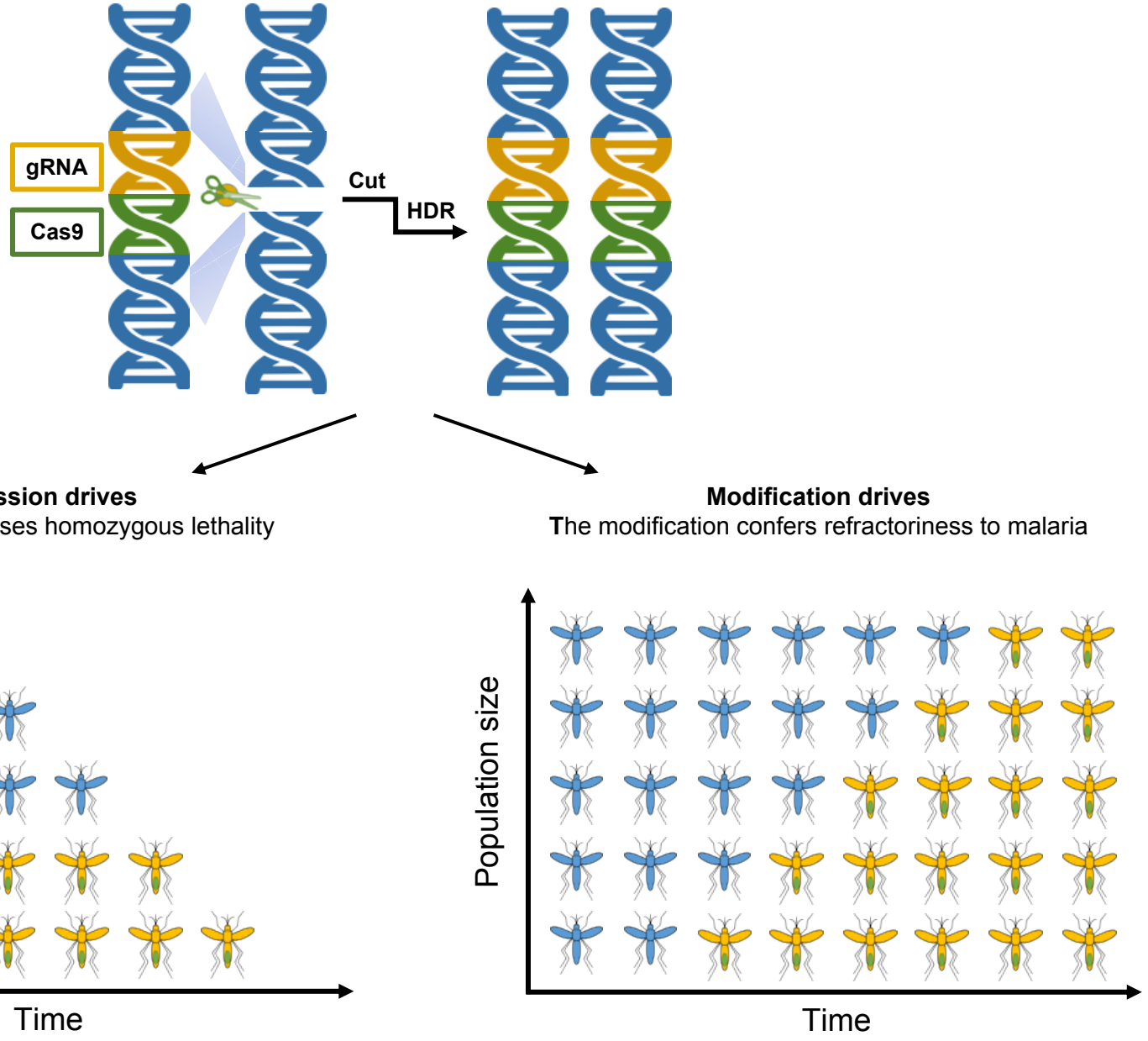
gRNA
Cas9
Cut
HDR
Suppression drives
The modification causes homozygous lethality
Modification drives
The modification confers refractoriness to malaria
Population size
Time
Population size
Time