# Case Study Workbook

## Problem Formulation Workshop:
## Use of Gene Drive Technology in Mosquitos

May 25-27, 2016
Sheraton Reston Hotel
Reston, Virginia

FNIH
Foundation for the National Institutes of Health

Center for Environmental Risk Assessment

ILSI Research Foundation

# Case Study

Center for Environmental Risk Assessment, ILSI Research Foundation
1156 Fifteenth Street N.W., Washington D.C. 20005-1743 USA

May 2016

## SECTION 1: BIOLOGY OF ANOPHELES GAMBIAE

### Species Complex, Taxonomy and Common Nomenclature

#### Taxonomy

Anopheles gambiae sensu lato (s.l.) Patton (Cellia)—mosquito (Kingdom: Animalia; Phylum: Arthropoda; Class: Insecta; Order: Diptera; Superfamily: Culicoidea; Family: Culicidae; Subfamily: Anophelinae; Genus: Anopheles

*Anopheles gambiae* was originally identified as a single species of mosquito within the genus Anopheles by Giles in 1902[1]. Following research through the first half of the 20th century, it has since been recognized that what was originally identified as a single species is, in reality, a complex of 6-8[2] morphologically indistinguishable species. One of these species retains the name *Anopheles gambiae* and is frequently referred to as *Anopheles gambiae s.s. (sensu stricto-* in the strict sense), while all species within the complex may be referred to as *Anopheles gambiae s.l. (sensu lato-* in the broad sense). The species themselves may be distinguished by microscopic examination of polytene chromosomes[3] in the salivary glands or through the use of molecular markers. Species may also be distinguished by the failure of hybrid pairings to produce fertile male offspring. Species in the *Anopheles gambiae s.l.* complex follow similar life cycle and life habits, although there is known variation in preferred breeding sites and other characteristics. Species also vary in their ability to transmit malaria parasites, with some serving as important vectors while others are unable to transmit the parasite at all.

#### Morphology

*Anopheles gambiae* has a gross morphology similar to most other mosquitos, featuring a slender body divided into the three prototypical insect sections: head, thorax and abdomen. The head is specialized for sensory functions and feeding, and contains two multi-segmented antennae as well as pairs of compound and simple eyes. Mouth parts include a long proboscis for feeding and two sensory palps. The thorax is thin and supports six pairs of legs, two pairs of which point back toward the abdomen and one pair that points forward towards the head. Like all insects in the order Diptera, An. gambiae have two fully developed wings and two small organs called halteres that are used for maintaining balance during flight. This arrangement makes dipterans agile flyers. The slender abdomen is specialized for digestion and in females the abdomen can expand greatly for the storage of a large blood meal.

*Figure 1: Mosquito egg with characteristic floats[4]*

[1]Giles, G.M. (1902). A handbook of the gnats or mosquitoes giving the anatomy and life history of the Culicidae together with descriptions of all species noticed up to the present date. John Bale, Sons & Danielsson, Limited. London, United Kingdom. 530pp

[2]Proposals for new species or species subgroup continue to be made, however 7 species may be generally recognized at the time of this writing: Anopheles arabiensis; Anopheles bwambae; Anopheles coluzzi; Anopheles merus; Anopheles melas; Anopheles quadriannulatus; Anopheles gambiae s.s.

[3]Polytene chromosomes are produced by endoreplication, in which chromosomal DNA undergoes mitotic replication, but the strands do not separate. Ten rounds of endoreplication produces 1,024 DNA strands, which when arranged alongside of each other produce distinctive banding patterns. Endoreplication occurs in cells of the larval salivary glands of many species of Diptera, and increases production of mRNA (see https://www.mun.ca/biology/scarr/Polytene_Chromosomes.html)

[4]Image obtained from http://www.cdc.gov/malaria/about/biology/mosquitoes/

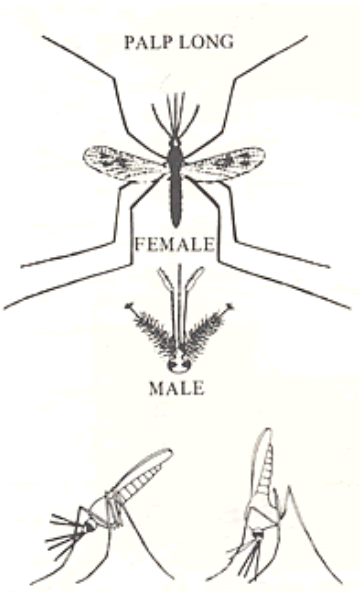
*Figure 2: General Morphology*

*(A) Adult mosquitos showing the distinguishing head structures of males as well as the distinct body positioning common to Anophelids.*

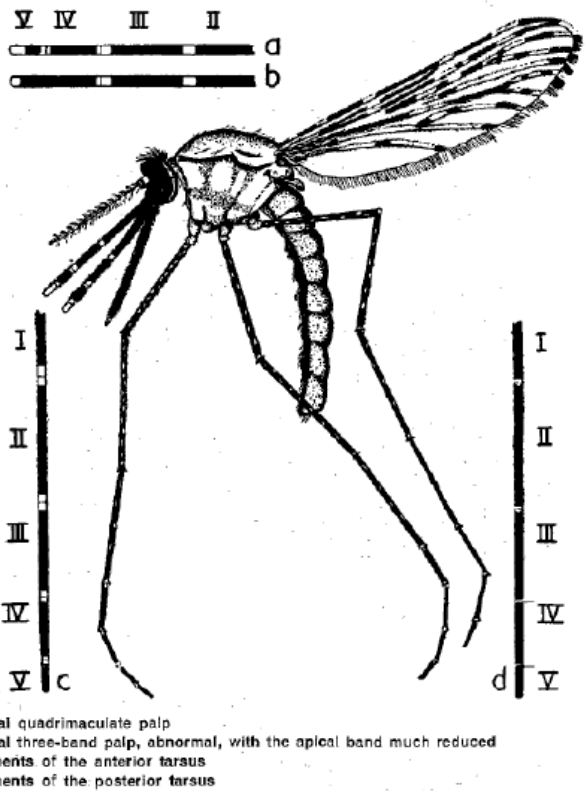
*(B) Typical Gambiae Female*

*(C) General Morphology of Gambiae*

*(A)*

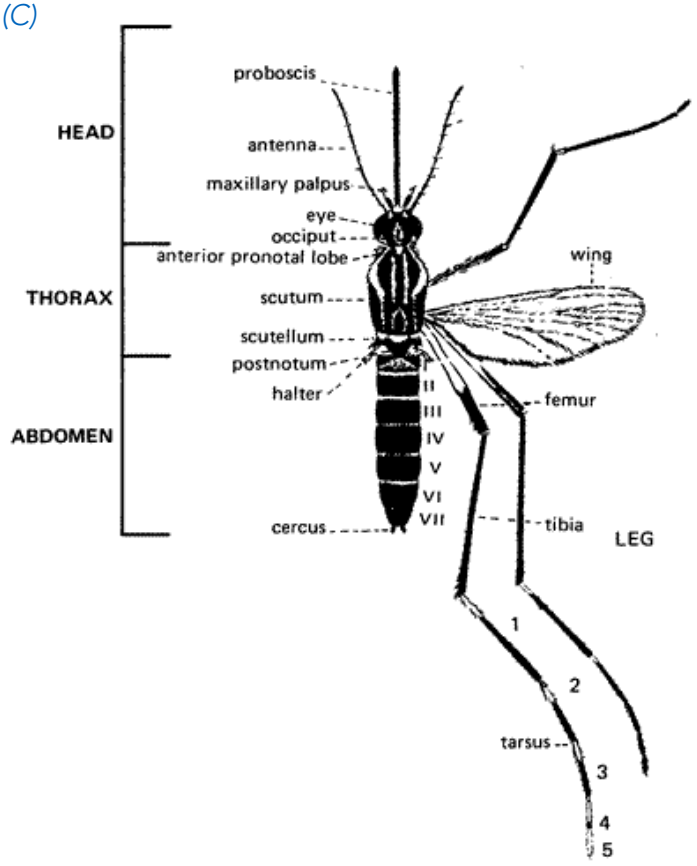



*(B)*




**a = typical quadrimaculate palp**
**b = typical three-band palp, abnormal, with the apical band much reduced**
**c = segments of the anterior tarsus**
**d = segments of the posterior tarsus**

*(C)*




## Reproductive Biology

### Life Cycle

An. gambiae undergo four distinct life stages: egg, larva, pupa, and adult. It is a freshwater insect and females lay eggs in shallow, fresh and non-polluted water exposed to sunlight. The eggs hatch 24-36 hours after they have been laid and give rise to L1 larvae. The L1 larvae undergo 3 major transformations (L2-L3-L4) before pupating. From L1 to L4, the larvae feed on microorganisms and decaying materials in their habitats. The L4 larva undergoes a last metamorphosis to reach a pupal stage before turning into an adult. It takes about 6-8 days for the egg to reach the adult stages but the length of development is strongly influenced by the availability of food and temperature in larval habitat.

The male-female ratio is 1:1 at adult emergence. After they have emerged, both males and females find a resting site to strengthen their exoskeleton, then they look for sugar meals, primarily nectar from flowers. Two to three days after emergence, both males and females are sexually mature and begin to look for mates. Mating in An. Gambiae mostly happens outdoors and at sunset. Males gather at specific mating stations waiting for females. Females visit these mating stations and depart

[5]Images obtained from http://www.cdc.gov/malaria/about/biology/mosquitoes/index.html

in copula with the most competitive males. While males can mate several times, females mate only once in their lifetime. The sperm that they receive from this mating is stored in their spermathecae and will be used in their lifetime to fertilize eggs that they lay.

After mating, a female looks for a blood meal and then rests nearby to digest the blood and mature her eggs. Three days after the blood meal, she looks for a water body where she will lay eggs and the cycle will start again. Females lay about 100-200 eggs per oviposition cycle.

An adult An. gambiae has an average lifespan of 20 days in captivity, but in wild settings populations experience significant mortality on a daily basis[6]. Males return to the mating sites every day. However, after females mate, they seldom look for another mating.

### Genetics

The exact center of origin of An. gambiae is unknown, but the current distribution of the species is throughout most of sub-Saharan Africa (Sinka et al., 2012).

Though An. gambiae is widely distributed in Africa, several studies have shown that a more complex genetic structure is found in West Africa with the existence of the two molecular forms recently reclassified as species (*Anopheles gambiae* and *Anopheles coluzzi*) (della Torre et al., 2001; della Torre et al., 2002; della Torre et al., 2005)

## Ecology

### Dispersion

An. gambiae disperses actively via winged flight (Costantini et al., 1996), and passively by wind. An. gambiae is capable of flying 700 m to 1.5 km per day, but can go beyond with the assistance of wind. However, its survival and spread in the environment is strictly constrained by the presence of resources, such as larval sites and blood meal sources, as well as the climate. It is well known that wild mosquito populations in many ecological settings in Africa crash after the rainy season due to the hard climatic conditions and the lack of water bodies for oviposition. Mosquito survival in a natural environment is known to be short.

Typically, An. gambiae mosquitoes do not aestivate, however, there are reports that under adverse conditions, An. gambiae s.l.females can be dormant (aestivate) for as long as 7 months, until favourable conditions return (Lehmann et al., 2010; Dao et al., 2014). Male anopheles mosquitoes do not survive unfavourable conditions (Eldridge, 2005). Egg viability is up to 12 days (Beier et al., 1990). While it is generally thought that there is no egg dormancy in An. gambiae, some cases of dormancy have been reported (Minakawa et al., 2001), and some eggs are able to withstand short-term desiccation, which enables the eggs to survive for several days (Beier et al., 1990).

At larval stages, An. gambiae rarely occupies water bodies such as cisterns or other containers that are preferentially colonized by more invasive species of mosquitoes (Aedes). Therefore, long distance dispersion of An. gambiae by humans appears to be less likely than species belonging to those taxa. However, two documented exceptions are the transport of An. gambiae from East Africa to Mauritius in the late 19th century, and the closely related An. arabiensis from Africa to northern Brazil in the 1930's (Lounibos, 2011).

An. gambiae has been reported to be invasive in habitats where the species has been introduced into Brazil and Mauritius (Juliano & Lounibos, 2005). However, it would not normally be thought of as invasive in its native range. There are a number of factors affecting the spread and persistence of An. gambiae. Temperature is an important factor in egg hatching. The optimal temperature is in the range of 24 to 30°C (Impoinvil et al., 2007). Mosquito populations will persist in the range of 17 to 33°C (Beck-Johnson et al., 2013). Since the larvae are aquatic, a water source is necessary. The chances of finding larvae in large bodies of water are low (>100m perimeter) (Fillinger et al., 2009; Service, 1971; Chase & Shulman 2009). Reduced sunlight also reduces the abundance of larvae (Fillinger et al., 2009; Minakawa et al., 2005; Kaufman et al., 2006; Munga et al., 2006; Animut et al., 2012). More turbid waters are also less preferred by larvae (Animut et al., 2012). Larvae must also have access to adequate algae, bacteria, or detritus as a food source (Gimnig et al., 2002; Kaufmann et al., 2006; Garros et al., 2008; Tuno et al., 2006; Merritt et al., 1992; Piyaratne et al., 2005).

[6]Charlwood et al., 1997, Survival And Infection Probabilities of Anthropophagic Anophelines From An Area of High Prevalence of *Plasmodium falciparum* in Humans, Bulletin of Entomological Research, 87, 445-453

## Important Environmental Interactions

Free-living populations of An. gambiae exist all over sub-Saharan Africa (Sinka et al., 2012). Habitats that support free-living populations of An. gambiae exist throughout the region (Sinka et al., 2012). These habitats are usually temporary bodies of water such as ground puddles, depressions, pools or hoof-prints; or irrigated areas such as rice fields or flooded areas (Sinka et al., 2010). A map of important malaria vector distribution, including for An. gambiae can be found in Figure 4.

During both aquatic and terrestrial life stages, mosquitoes serve as food for a variety of other organisms. However, there is no known obligate/specific natural predator of An. gambiae. It has often been held that mosquitos make up a substantial part of the diets of insectivorous bats, but studies suggest mosquitos constitute only a small percentage of bat diets (Gonsalves et al., 2013). There are at least two known spider species that show a preference for Anophelids (Jackson & Cross, 2015), and likely other examples could be found. However, this should be considered in the context of the approximately 430 species in the genus Anopheles, and more than 650 species of mosquitos in Africa.

Generalist vertebrate predators of An. gambiae include amphibians, reptiles and bats. Certain species of fish, including members of the genus Gambusia, are also known to be predators of mosquito larvae (Krumholz, 1948). Insect predators include insects from the orders Araneida, Odonata, Hemiptera, Hymenoptera, Diptera, and Coleoptera (Service, 1973, 1977; Schielke et al., 2007; Obha et al., 2010; Muiruri, 2013).

Although males and females are both known to visit flowers to feed on nectar, there is no evidence that An. gambiae is an important pollinator for any plant species (W. Foster, personal communication May 12, 2016).

## Human Interaction and Malaria Transmission

An. gambiae is a source of nuisance biting and can carry malaria parasites that inflict a huge burden on humans in terms of death and economical losses (della Torre et al., 2002; World Health Organization, 2012).

When a female takes a blood meal from a human infected with Plasmodium, she can transmit malaria to someone else once the parasite reaches the sporozoite stage in her salivary glands. It takes about 12 days from an infected blood meal for the parasite to reach an infectious stage in the saliva glands. Males do not bite; hence do not transmit the disease. They exclusively feed on sugar from spoiled fruits and plant nectar.

The effectiveness of An. gambiae as a vector is due in large part to its strong preference to feed on humans (US Centers for Disease Control http://www.cdc.gov/malaria/about/biology/mosquitoes). Current tools to control this mosquito species are based on insecticide application on indoor walls or through the use of insecticide treated bed nets, but the emergence and spread of insecticide resistance seriously limit the efficacy of these intervention tools.

An. gambiae can also transmit other diseases in addition to malaria, including the parasite causing lymphatic filariasis and the virus causing O'nyong-nyong fever (Gillies & De Meillon, 1968; Saxton-Shaw et al., 2013; US Centers for Disease Control http://www.cdc.gov/parasites/lymphaticfilariasis/gen_info/vectors.html)

Plasmodium spp., including *P. falciparum* the causal agent of the deadliest form of malaria, are the primary parasite of concern in An. gambiae. Some genetic variation exists in An. gambiae for susceptibility to Plasmodium infection (Niaré et al., 2002). Thus, different strains of An. gambiae may be more or less capable of transmitting malaria.

## Allergenicity

Allergic reactions to mosquito bites are common, and have been studied in another mosquito species, *Aedes aegypti* (Peng & Simons, 2007). We are unaware of specific allergenic proteins isolated from or studied in An. gambiae, however an immune (non-allergic) response to An. gambiae salivary antigens has been reported (King et al., 2011; Ali et al., 2012).

*Figure 3: Malaria Life Cycle*

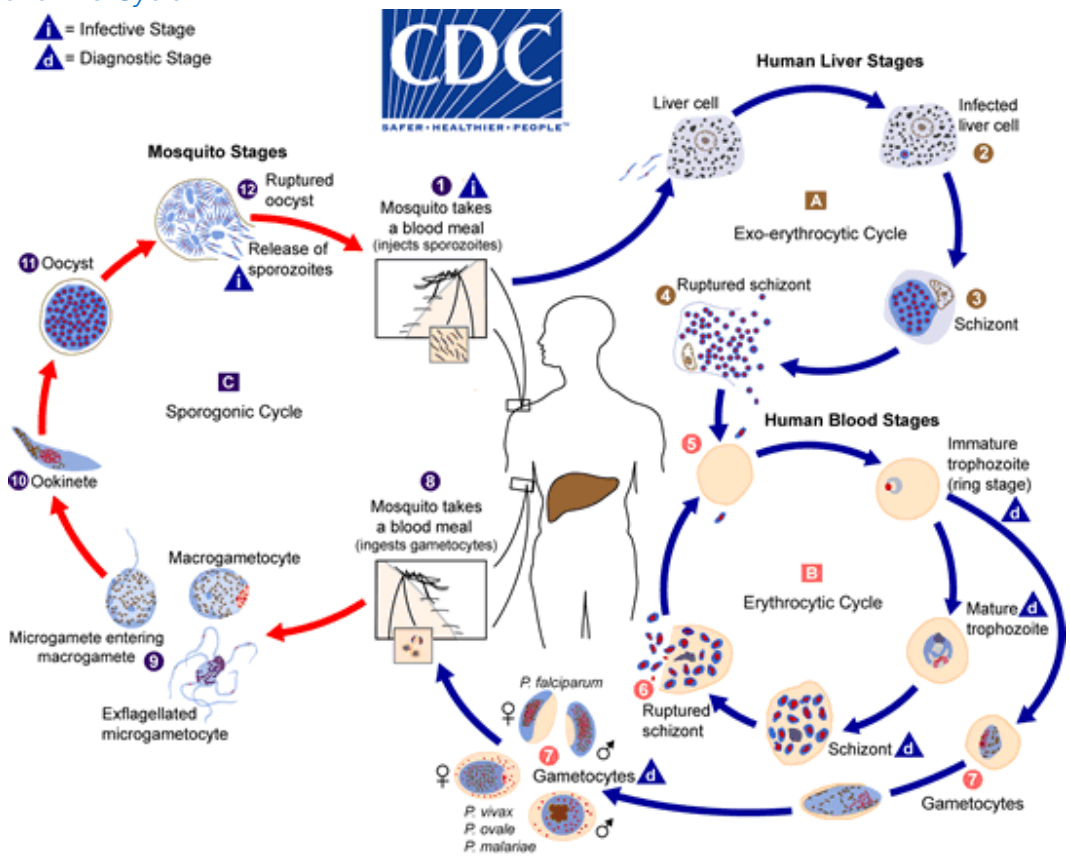
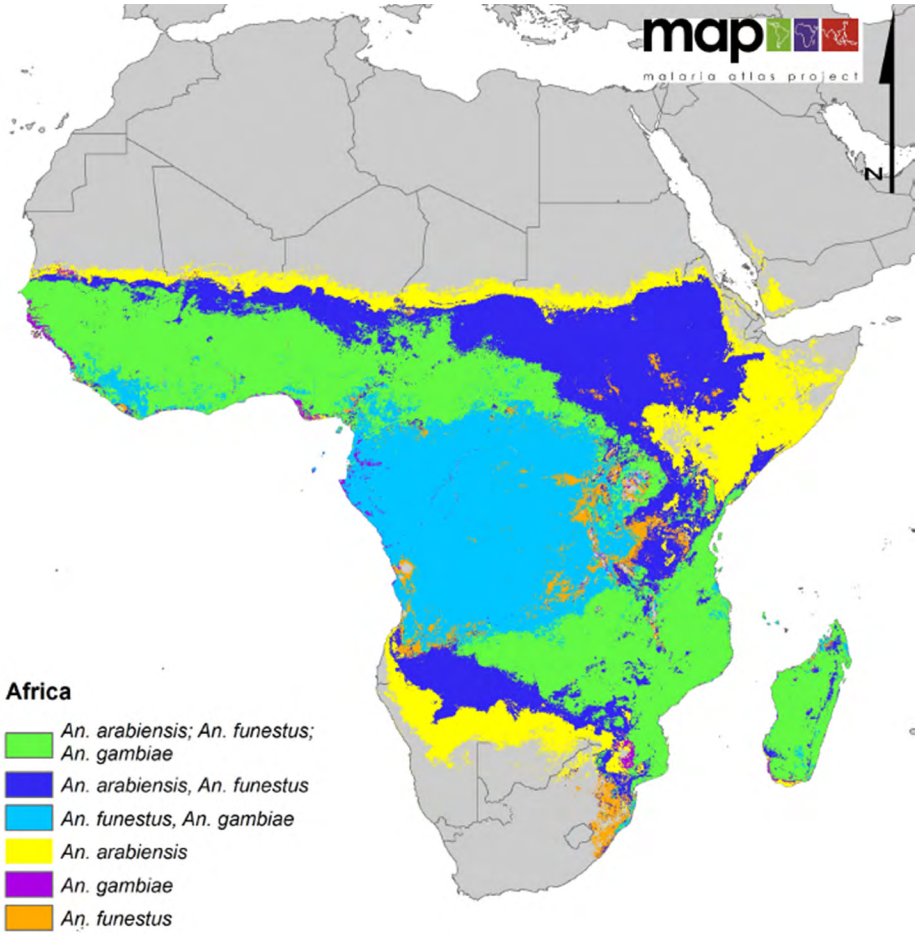



*Figure 4: Distribution of important malaria vectors in Africa by species*



# SECTION 2: SOURCE AND MECHANISM OF THE MODIFICATION

## CRISPR/Cas9 Gene Editing

The modifications presented for these case studies make use of the CRISPR/Cas9 gene editing system. CRISPR is an acronym for clustered, regularly interspaced, short, palindromic repeats, and Cas is an abbreviation of the CRISPR associated protein. This protein functions as an endonuclease. These are named for the genomic region and gene, respectively, in bacteria where the system functions to provide an innate immunity to infection by bacteriophage. The details of the mechanism are fascinating and elegant.However, for the purposes of its use in genetic engineering, the CRISPR/Cas9 system can be considered one example of a family of techniques that make use of site directed endonucleases to cause genomic mutations in a sequence specific manner. The critical components of a site directed nuclease gene editing system are the ability to recognize and bind to a specific DNA sequence, and then to catalyze a break in the DNA. Once the break is made, generally one of two different mechanisms may be employed to either knock out the gene function in the targeted gene, or to insert a novel DNA sequence encoding for a gene of interest (sometimes referred to as "knock-in"). Gene editing to knock out, or to disable a gene function, typically makes use of the error-prone non-homologous end-joining (NHEJ) machinery of the cell. In response to a double-stranded DNA break, the NHEJ mechanism repairs the broken DNA strand, but will frequently induce short insertions or deletion (indel) mutations at the site of repair. If the break has been targeted to an appropriate site within a coding gene, these random indel mutations can cause a frame shift, effectively inactivating the gene of interest.

"Knock-in" gene editing makes use of the cells homology directed repair (HDR) machinery. In order to use this mechanism, the system must provide a homologous template DNA that contains homology to the site surrounding the target site for the endonuclease. In response to a double stranded break, the cell may employ the HDR machinery, which has high fidelity, to copy the template onto the strand undergoing repair. In this way, if a homologous template containing a novel DNA sequence is available, that novel sequence will be copied into the genome at the site of repair. It is possible to introduce novel genetic material, including large sequences that may contain multiple genes, using this method.

## CRISPR/Cas9-Mediated Gene Drives

By definition, gene drives are mechanisms that allow for the spread of traits through populations faster than would be expected for Mendelian inheritance, and with the possibility to introgress a gene or genetic element without offering adaptive advantage within the population. Transposons and other "selfish" genetic elements make use of conceptually similar, naturally occurring mechanisms for self-replication within genomes. However, engineered gene drives are intended to be self-replicating and to induce spread of an engineered trait through a population without relying on any selective mechanism.

Although the details may differ, the essential component of a gene drive is the insertion of a construct within the genome that encodes all of the machinery necessary for copying the construct to homologous chromosomes containing the insertion site. In this case, the construct includes the Cas9 gene along with the appropriate regulatory elements and an accompanying template to target DNA binding, as well as any additional genetic "cargo" which may include a novel gene to introduce a desired phenotype if a "knock-in" mechanism is being used. Once inserted into the genome and expressed within a cell, the Cas9 protein will target the specific sequence in the homologous chromosome and induce a break. This break will be repaired using the HDR mechanisms described above, and therefore copy the construct including any genetic cargo. When this occurs in the germline, a single copy of the modification inherited from a parent will be effectively transmitted to 100% of the offspring, rather than the 50% expected through Mendelian genetics.

The CRISPR/Cas9 system is the subject of these case studies because the details of the molecular mechanism make it likely, at this time, to be the most convenient and effective way to reliably produce functioning gene drive mechanisms in practice.

Similar to the gene editing techniques described above, gene drives are intended to propagate a genetic change by either disrupting the function of a target gene, or else introducing novel genetic material that confers a desirable trait to the organism. In both cases, gene drives typically employ the homology directed repair mechanism. For gene drives intended to disrupt gene

function, most knock-out schemes call for the insertion of the construct coding for the gene drive machinery at a site within the targeted gene. This insertion disrupts gene function in a predictable way and couples the inheritance of the gene drive machinery directly to the intended genetic change.

*Figure 5: Homology Directed Repair (HDR)*

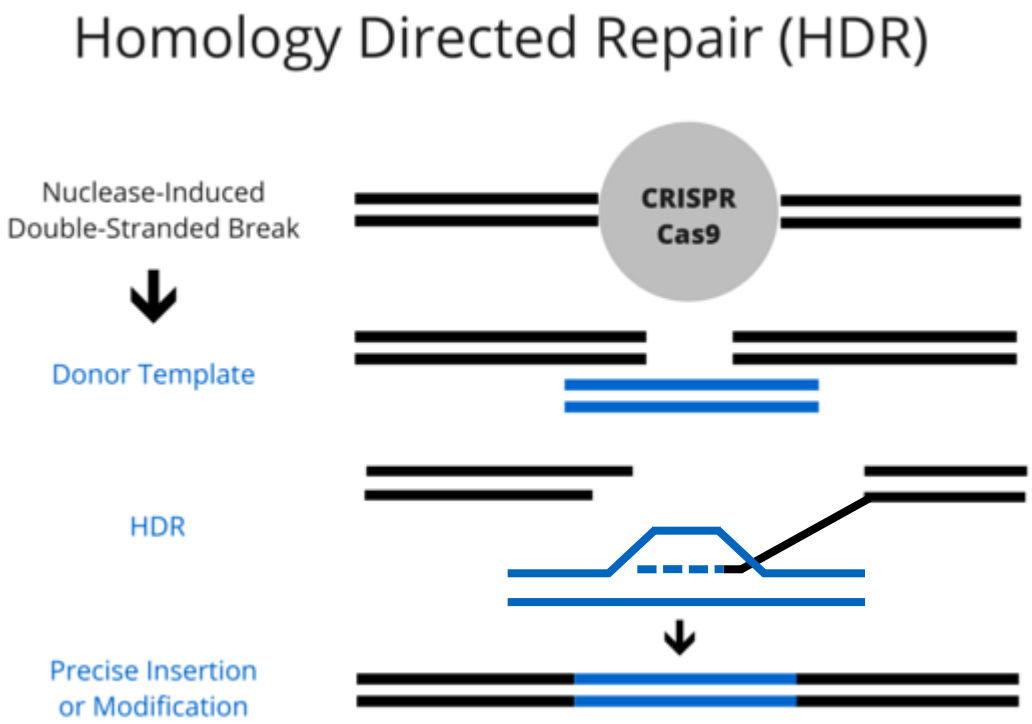
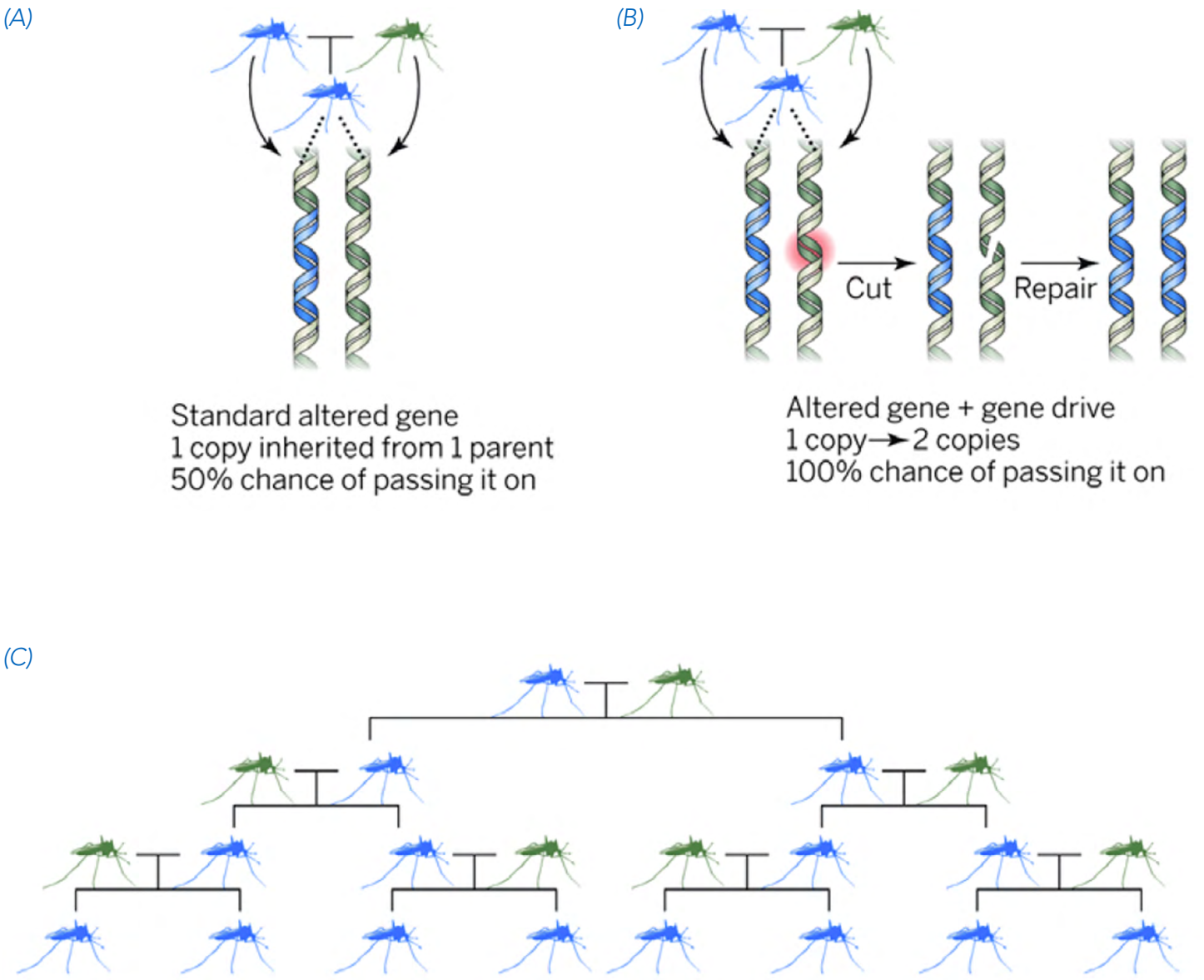


*Figure 6: How endonuclease gene drives spread altered genes through populations*[7]

*(A) Altered genes (blue) normally have a 50% chance of being inherited by offspring when crossed with a wild-type organism (gray).*

*(B) Gene drives can increase this chance to nearly 100% by cutting homologous chromosomes lacking the alteration, which can cause the cell to copy the altered gene and the drive when it fixes the damage.*

*(C) By ensuring that the gene is almost always inherited, the gene drive can spread the altered gene through a population over many generations, even if the associated trait reduces the reproductive fitness of each organism. The recently developed CRISPR nuclease Cas9, now widely used for genome engineering, may enable scientists to drive genomic changes that can be generated with Cas9 through sexually reproducing organisms.*




[7]Image obtained from Oye et al., 2014

# SECTION 3: DESCRIPTION OF THE GM ORGANISM

## Class 1: Modification for Population Replacement

Population replacement (also known as population modification) strategies target vector competence with the intent to reduce the inherent ability of individual mosquitoes to transmit a given pathogen. This most often is conceived as the introduction of engineered DNA or the manipulation of endogenous genes so as to inhibit pathogen replication within the mosquitoes, although other types of modification, such as those affecting critical mosquito behaviors, might also be envisioned. Upon release into the environment, these refractory mosquitoes will be expected to introduce, through mating, the genetic change into the local mosquito population, "replacing" their inherent ability to spread the targeted pathogen with a reduced or eliminated transmission capability. The intent is that the genetic modification will become fixed permanently and at high levels within the local mosquito population, thus remaining in the environment indefinitely.The details of population replacement will vary depending on the introduced gene and the intended goals of the replacement strategy.

Two hypothetical case examples are presented below. Participants are asked to consider the details attached to the specific case assigned to their group, however, discussions should also consider broad aspects of population replacement as a strategy when answering the questions assigned to each case.

### Case Example 1: Introduction of a novel substance to inhibit *Plasmodium falciparum* infection and development

A novel gene has been introduced to the mosquito genome using a CRISPR/Cas9 system, coupled with HDR to a provided template encoding a protein that inhibits maturation of *Plasmodium* ookinetes.

This modification is not primarily intended to alter the behavior, life cycle or population dynamics of the mosquito, though it may impose some fitness cost (i.e., quantitative reduction in survival or reproduction). But it blocks the successful completion of the malaria parasite life cycle in these mosquitoes, rendering them less likely to transmit the disease to humans.

### Case Example 2: Gene editing of a native gene to inhibit *Plasmodium falciparum* infection

A CRISPR/Cas9 gene drive has been engineered for insertion at a site that disrupts proper expression and translation of a gene encoding a cell surface receptor protein that is expressed in the An. gambiae midgut. This receptor is required for completion of the *P. falciparum* lifecycle.

Although the endogenous function of the receptor is not fully understood, mosquitoes harboring the mutation show only a modest impact on fitness, but are substantially less able to transmit the disease to humans.

## Class 2: Population Suppression

Population suppression strategies intend to reduce (suppress) the size of the targeted mosquito population in a manner analogous to sterile insect techniques. These generally involve methods to reduce the overall numbers of female mosquitoes, with or without a concomitant direct effect on males, which will result in decreased reproduction. This could be accomplished by mechanisms that bias against the development of female progeny (sex-ratio distortion), reduce female fertility, or incapacitate/kill young female mosquitoes sufficiently early to decrease their ability to transmit a pathogen from one person to the next and reproduce.

The intent of population suppression strategies is to reduce the numbers of malaria-transmitting mosquitoes to levels too low to sustain transmission for a time period sufficient to eliminate the parasite from the region. The gene responsible for population suppression will be introduced through mating with the local mosquito population. Depending on the conditions, the genetic modification could disappear from the environment together with the mosquito population, be lost from the population due to selection pressure, or persist in low numbers in the environment.

### Case Example 3: Gene editing to affect the sex ratio.

Sex determination in An. gambiae makes use of sex specific chromosomes, where an XX genotype produces a female phenotype and an XY genotype produces a male phenotype. Thus the sex of the offspring is determined by the chromosome contributed paternally and under natural conditions where the sex ratio is

approximately 50/50 male to female. A "knock-in" gene editing construct has been used to insert a novel gene into the An. gambiae genome. This novel gene is activated during spermatogenesis and triggers cell death in spermatocytes containing an X chromosome. As a result, male carriers of the gene drive can only sire male offspring. The resulting decrease in the number of females is expected to suppress the population.

## Case Example 4: Gene editing to reduce female fecundity

A CRISPR/Cas9 gene drive has been engineered to insert at a site that disrupts expression of a protein transporter that is necessary for proper egg provisioning (the transport of materials into developing oocytes from specialized somatic cells). As a result, females carrying the gene drive have greatly reduced fecundity. Males are unaffected and the mating of carrier males with wild-type females is expected to lead to population suppression.

# Case Study Workbook

## Problem Formulation Workshop:
## Use of Gene Drive Technology in Mosquitos

May 25-27, 2016
Sheraton Reston Hotel
Reston, Virginia

FNIH
Foundation for the National Institutes of Health

Center for Environmental Risk Assessment

ILSI Research Foundation

# Case Study Exercise #1

Your group should be assigned one of the following case study examples for this exercise:

### Population Modification

1. Introduction of a novel substance to inhibit *Plasmodium falciparum* infection and development
2. Gene editing of a native gene to inhibit *Plasmodium falciparum* infection

### Population Suppression

3. Gene editing to affect the sex ratio
4. Gene editing to reduce female fecundity

## PART 1: IDENTIFICATION OF RELEVANT PROTECTION GOALS
### (Brainstorming)

You have heard about high level and general environmental protection goals during the presentations yesterday. These generally include:

- Air quality
- Water quality
- Soil quality
- Biodiversity
    - Threatened species
    - Valued species
    - Ecosystem and habitat preservation
- Human Health Considerations (including related to air, water and soil quality)
    - Exposure to toxins and allergens
    - Pathogens
- Natural Resource Protection
    - Forests
    - Fisheries
- Agricultural protection (air, soil and water quality)
    - Plant and animal health

Spend some time with your group considering these broad protection goals and identify those which might be harmed by the use of gene drive technologies in mosquitos. For this step, don't worry about the likelihood of harms, but you should be sure that there is at least a conceivable mechanism for the gene drive carrying mosquito to affect a protected entity. At this stage of the process, when in doubt, list the protection goal and a brief description of how it might be harmed. The plausibility of these harms occurring will be addressed in Part 2. Because exposure is a necessary component of risk, it can often be useful to think about the life cycle and behavior of the organism in the environment. Where does it exist? What does it interact with? How important are these interactions in relation to environmental protection goals?

NOTE: Keep in mind that wild type An. gambiae are present in the environment where the release will take place. Although the organism itself may be harmful in the environment (e.g. as a disease vector), these risks are well understood and this exercise is not intended to consider the species as a whole. Please focus your efforts on identifying novel harms that will be posed by the proposed release of the modified organism.

# Case Study Exercise #1 Continued

## PART 2: REFINING PROTECTION GOALS AND IDENTIFYING PLAUSIBLE PATHWAYS TO HARM

A. You now have a list of broad or general protection goals that might conceivably be harmed by the use of gene drive technology in mosquitos. Spend some time considering whether or not those protection goals are sufficiently defined to be useful in risk assessment. If they are not, refine them to identify as many specific protection goals as you can. For example, from "Protection of Biodiversity," you might refine this to "protection of threatened species." You might refine this further to "protection of threatened mammals" or "protection of elephants"). Try to identify a protection goal that is sufficiently well defined that you might easily explain to someone how to assess the likelihood of harming that protection goal (e.g. The introduction of gene drives in mosquitos might reduce the population of elephants). Some refined protection goals may be very specific, while others might be more general. For example, if the mechanism of harm for both Elephants and Lions as valued species is the same, then it may be appropriate to lump them together under the protection goal "protection of wild mammal populations."

Be sure to consider the mosquito, the gene drive mechanism and the introduced trait when considering pathways to harm. Do this for each general protection goal until the group agrees that you have a reasonably comprehensive set of refined protection goals (three other groups will be doing the same exercise, so don't worry if you might miss one)

B. Once your group agrees that you have thoroughly identified a reasonably comprehensive set of refined protection goals, develop a stepwise "pathway to harm" for each one. Try to identify all the steps that would have to occur in order for the introduction of the gene drive carrying mosquito to achieve a harmful impact to the identified protection goal. Some protection goals may be harmed through multiple pathways. For example, if you have identified the protection goal "protection of lion populations" it might be possible to harm lion populations by introducing lion diseases, or by reducing available prey animals.

# Case Study Exercise #1 Continued

## PART 3: REVIEWING PATHWAYS TO HARM

A. Consider each pathway to harm that you have described. Considering the steps, is it plausible? It's possible for people to disagree on this, as an element of judgment is required. But in general, plausible pathways are those without multiple steps that are either unlikely or nearly impossible.

B. For each plausible pathway, consider the types of information that will be useful to inform future risk assessments about the probability of harm occurring. This information may already exist, or it may require future experimentation. It can also be useful to identify steps in the pathway which may be particularly amenable to investigation or critically informative for the assessment.

# Case Study Exercise #2

Your group should now be assigned a different case study example, representing the other general malaria control strategy:

### Population Modification

1. Introduction of a novel substance to inhibit *Plasmodium falciparum* infection and development
2. Gene editing of a native gene to inhibit *Plasmodium falciparum* infection

### Population Suppression

3. Gene editing to affect the sex ratio
4. Gene editing to reduce female fecundity

## PART 1: IDENTIFICATION OF RELEVANT PROTECTION GOALS
(Brainstorming)

Consider the protection goals that you identified during your work on the previous case study. Do any of these not apply to this case example? Are there other protection goals that are relevant to this case but not the previous ones?

# Case Study Exercise #2 Continued

## PART 2: REFINING PROTECTION GOALS AND IDENTIFYING PLAUSIBLE PATHWAYS TO HARM

A. Consider your finalized list of protection goals from the first case example. Do these refined protection goals still make sense in the context of the different control strategy presented in this case? Are there additional protection goals that have relevance to this control strategy?

B. Review the pathways to harm for relevant protection goals. If you have identified new protection goals, develop pathways to harm for these.

# Case Study Exercise #2 Continued

## PART 3: REVIEWING PATHWAYS TO HARM

A. Consider each pathway to harm that you have described. Considering the steps, is it plausible in association with this control method?

B. For each plausible pathway, consider the types of information that will be useful to inform future risk assessments about the probability of harm occurring. Are there different sources of information related to this control method?